Faculty of Medicine and Health Sciences

Heymans Institute of Pharmacology

Promotor: Prof. Dr. R. A. Lefebvre

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Gastrointestinal effects of soluble guanylate cyclase activation by NO-independent compounds and by NO delivery via nitrite

Sarah M. R. Cosyns

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List of abbreviations

8-Br-cGMP	8-bromoguanosine 3', 5' cyclic monophosphate
AAC	area above the curve
ADHF	acute decompensated heart failure
ALT	alanine transaminase
ANOVA	analysis of variance
AST	aspartate transaminase
ataciguat	5-chloro-2-[[(5-chloro-2-thienyl)sulfonyl]amino]-N-[4-(4-morpholinyl sulfonyl)phenyl]benzamide (=HMR1766)
ATP	adenosine triphosphate
AU	arbitrary units
AUC	area under the curve
BAY 41-2272	3-(4-amino-5-cyclopropylpyrimidin-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b] pyridine
BK channel	large conductance Ca^{2+}-activated K^+ channel
cGK	cGMP-dependent protein kinase (= PKG)
cGMP	cyclic guanosine 3'-5'-monophosphate
CGRP	calcitonin gene-related peptide
cinaciguat	4-[((4-carboxybutyl)-(2-[(4-phenethylbenzyl)oxy]phenethyl)amino) methyl]benzoic acid (= BAY 58-2667)
CNS	central nervous system
CO	carbon monoxide
CORM-3	carbon monoxide-releasing molecule-3
COX-2	cyclo-oxygenase-2
CRF	corticotrophin-releasing factor
DAMPs	damage associated molecular patterns
DETA-NO	diethylenetriamine NONOate
DGME	diethylene glycol monoethyl ether
DMSO	dimethylsulfoxide
EC_{50}	half maximal effective concentration
EDTA	ethylene diaminetetraacetic acid
EFS	electrical field stimulation
EGTA	ethylene glycol tetraacetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
E_{max}	maximum effect
eNOS	endothelial nitric oxide synthase (= NOS3)
ENS	enteric nervous system
FD-70	fluorescein-labeled dextran 70 kDa

FDA	Food and Drug Administration
GALT	gut-associated lymphoid tissue
GC	geometric centre
GMC	giant migrating contractions
GTP	guanosine-5'-triphosphate
HNE	4-hydroxy-2-non-enal
H_2S	hydrogen sulfide
ICAM-1	intercellular adhesion molecule-1
ICC	interstitial cell of Cajal
IFNγ	interferon gamma
IL-6	interleukin-6
IL-12	interleukin-12
IM	intestinal manipulation
iNOS	inducible nitric oxide synthase (= NOS2)
i.p.	intraperitoneally
I/R	ischemia/reperfusion
i.v.	intravenously
K_{ATP} channel	ATP-sensitive potassium channel
L_o	optimal load
L-012	8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H)dione
LES	lower esophageal sphincter
L-NAME	N^{ω}-nitro-L-arginine methyl ester
MCP-1	monocyte-chemoattractant protein-1
MDA	malondialdehyde
MMC	migrating motor complex
MPO	myeloperoxidase
MRS2500	(1R*,2S*)-4-[2-iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy) bicycle[3.1.0]hexane-1-methanol dihydrogen phosphate ester
NANC	non-adrenergic non-cholinergic
NF-κB	nuclear factor-κB
nNOS	neuronal nitric oxide synthase (= NOS1)
NO	nitric oxide
NOS	nitric oxide synthase
NSAIDS	nonsteroidal anti-inflammatory drugs
ODQ	1H[1,2,4,]oxadiazolo[4,3-a]quinoxalin-1-one
PACAP	pituitary adenylate cyclase-activating polypeptide
PBS	phosphate buffered saline
PDE	phosphodiesterase
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PKG	protein kinase G (= cGK)
PMSF	phenylmethylsulfonyl fluoride

POI	postoperative ileus
PTIO	2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide
ROS	reactive oxygen species
sGC	soluble guanylate cyclase
S.E.M.	standard error of the mean
SK channel	small conductance Ca^{2+}-activated K^+ channel
SNAP	S-nitroso-N-acetyl-DL-penicillamine
TCA	trichloroacetic acid
TNFα	tumor necrosis factor alpha
UES	upper esophageal sphincter
VIP	vasoactive intestinal polypeptide
WT	wild-type
YC-1	3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole

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Chapter I

LITERATURE SURVEY

Chapter I   Literature survey

I.1   The gastrointestinal tract

I.1.1   Anatomy and functions

The human gastrointestinal tract consists of a 6-9 m long canal from mouth to anus, and the associated organs that empty their content into the canal (Fig. I.1). The gastrointestinal canal consists of the mouth, the upper esophageal sphincter (UES), the esophagus, the lower esophageal sphincter (LES), the stomach (composed of the cardia, fundus, corpus and antrum), the pyloric sphincter, the small intestine (composed of the duodenum, jejunum and ileum), the ileocecal valve, the large intestine (composed of the cecum, colon and rectum) and the internal and external anal sphincter (Fig. I.1).
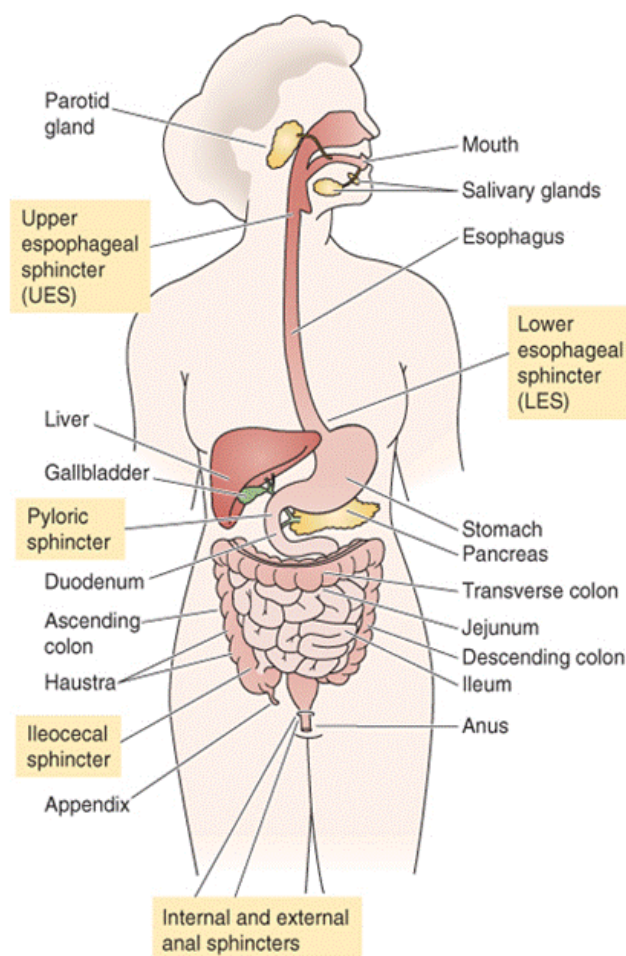
Fig. I.1   Schematic illustration of the anatomy of the gastrointestinal tract (adapted from Boron and Boulpaep, 2009).

The structural organization throughout the gastrointestinal canal is quite similar and consists of four layers (Fig. I.2):

- the inner layer is called the *mucosal layer* and can be divided into the epithelium, the lamina propria (a layer of loose connective tissue containing the mucosal capillaries) and a thin smooth muscle layer, the mucosal muscle layer;

- the second layer is the *submucosa*, also consisting of loose connective tissue and containing the plexus of Meissner or the submucosal nerve plexus;

- the third layer or *muscularis externa* consists of inner circular and outer longitudinal smooth muscle layers, with in between the plexus of Auerbach or the myenteric nerve plexus;

- finally, the adventitia is a thin layer of connective tissue surrounding the tract. Where the gut lies within the abdominal cavity, the adventitia is referred to as the *serosa* or visceral peritoneum.
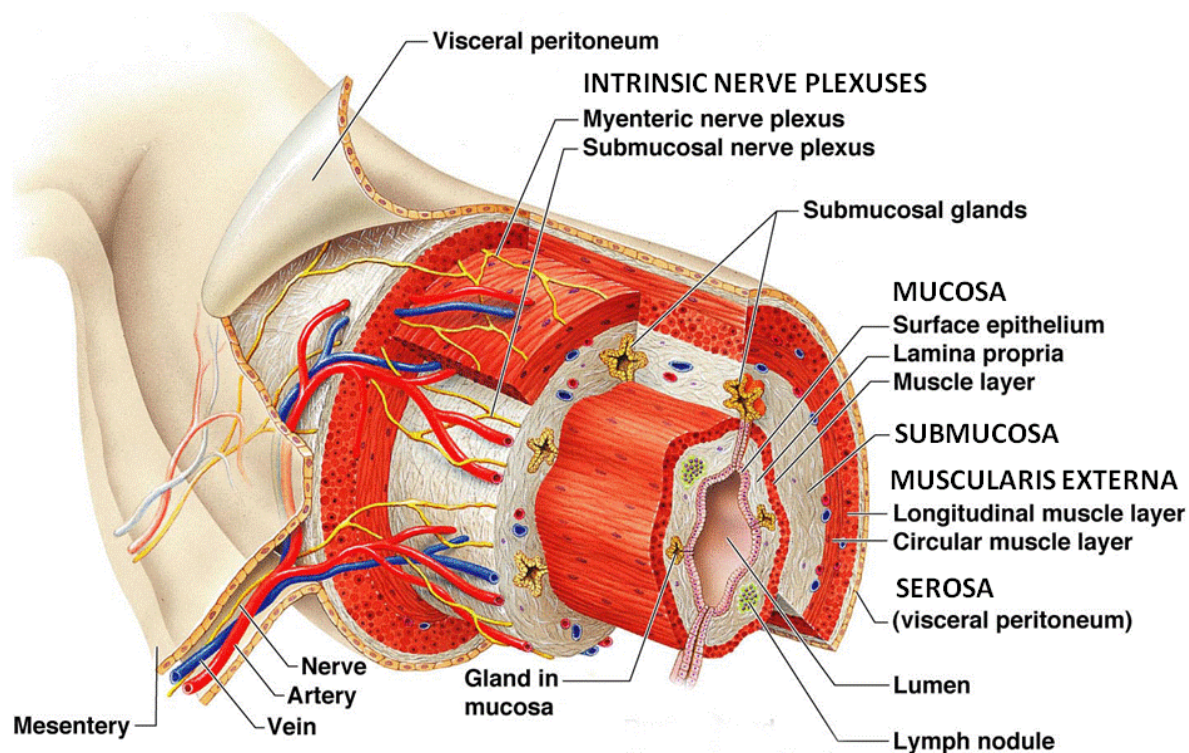
Fig. I.2 Schematic representation of the different layers of the gastrointestinal tract (adapted from Marieb *et al.*, 2004).

The major physiologic processes that occur in the gastrointestinal tract are motility, digestion, secretion, absorption and elimination (Fig. I.3). These processes help to fulfill the main functions of the gastrointestinal tract: to take in nutrients and to eliminate waste. Food enters the mouth where it will be reduced in size (*mechanical digestion*) before moving to the stomach via the esophagus. The stomach will store the food, mix it with its secretions containing enzymes, ions and water (*chemical digestion*) and grind it until the particles are small enough (*mechanical digestion*) to pass the pylorus and enter the small intestine. In the small intestine, enzymes secreted by the pancreas and the intestinal wall convert the macromolecules into absorbable material (*chemical digestion*), that will be absorbed across the epithelium of the small intestine to enter the blood or lymph (*absorption*). Finally, undigested rests will reach the colon where water will be resorbed before the contents leaves the body (*elimination*).
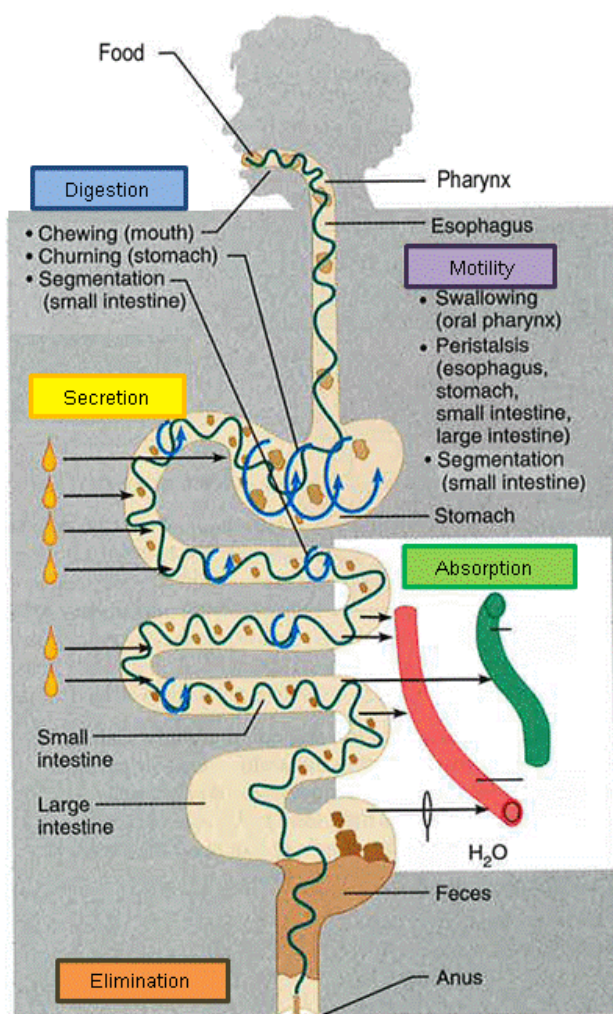
Fig. I.3 Schematic illustration of the physiological processes that take place in the gastrointestinal tract (adapted from Marieb *et al.*, 2004).

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The gastrointestinal tract also plays a prominent role in the *immune system*. The mucosal immune system, or gut-associated lymphoid tissue (GALT), consists of both organized (e.g. Peyer's patches) and diffuse populations (e.g. lymphocytes) of immune cells. The two primary functions of GALT are to protect against pathogens (bacteria, viruses, protozoans) and to ensure immunologic tolerance to food components and commensal bacteria. Other, non-immunologic, mechanisms are also important in protecting against pathogens and maintaining the intestinal flora and include gastric acid secretion, intestinal mucin, peristalsis and the epithelial cell permeability barrier (Boron & Boulpaep, 2009).

I.1.2   Gastrointestinal motility

Esophagus. *Swallowing* is the process by which food is transported from the mouth to the stomach via the esophagus. It is a complex process; its initiation can be voluntarily controlled, but it proceeds automatically once started. The striated muscles of the oral cavity, tongue, pharynx, UES, and the cervical esophagus are under conscious control, whereas the smooth muscles of the lower two thirds of the esophagus and the LES are not under conscious control. Under resting conditions (in between swallows), muscles of the swallowing passages are generally relaxed, except for those of the esophageal sphincters. Swallowing initiates relaxation of the UES and propagating contractions of the muscles along the esophagus; in the meantime, the LES has already relaxed. The result of the advancing contractile wave is propulsion of the food bolus toward the stomach.

Stomach. Upon the ingestion of a meal, the stomach accommodates to receive and store large amounts of food, without major changes in the intraluminal pressure. This accommodation capacity of the stomach is mainly mediated by its proximal part (i.e. the fundus and the proximal part of the corpus). The proximal stomach maintains a high basal muscle tone in between meals, but once food is swallowed, a reflex is initiated that decreases the proximal gastric tone, allowing for increased storage capacity. This mechanism, called *receptive relaxation,* thus prepares the stomach to receive food, that is not yet in the stomach. When the food bolus reaches the stomach, gastric relaxation is maintained by another reflex, called *adaptive relaxation*, and it is characterized by moment-to-moment adjustments of the stomach wall to its content. Food that entered the stomach

will leave to the small intestine by the process of *gastric emptying*. This is due to contractile waves progressing in the distal part of the stomach (i.e. the distal part of the corpus and antrum) towards the pylorus at a frequency of 3 waves/min. Gastric content pushed to the pylorus but still too large in particle size to be emptied via the pylorus will be retropulsed and further grinded before it can be emptied.

Small intestine. The two processes involved in small intestinal motility are called peristalsis and segmentation. *Peristalsis* ensures progression of the contents throughout the tract by coordinated waves of relaxation of the smooth muscle layers in front of the bolus and contraction of the smooth muscle layers distally of the bolus. In *segmentation*, non-adjacent segments of the intestine alternately contract and relax; because the active segments are separated by inactive regions, segmentation moves the food onward and then backward, mixing the food rather than propelling it forward, in this way allowing optimal digestion and absorption (Fig. I.4).

Peristalsis   Segmentation
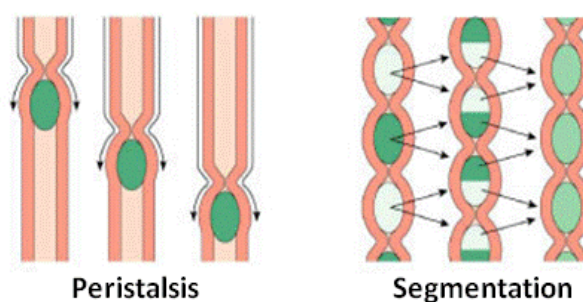
Fig. I.4   In peristalsis, adjacent segments of the alimentary canal alternately contract and relax, moving food distally along the gastrointestinal tract. In segmentation, non-adjacent segments of the intestine alternately contract and relax; because the active segments are separated by inactive regions, segmentation moves the food onward and then backward, mixing the food rather than propelling it forward (adapted from Marieb *et al.*, 2004).

Large intestine. About 2 l of small intestinal effluent reaches the colon daily, but the absorptive mechanisms of the large intestine reduce the volume to about 200 ml per day. The colonic motility plays a pivotal role in this process of water absorption. Indeed, when stool moves too quickly, not enough water is absorbed, resulting in diarrhea; however, when the motility of the colon is too low, the colon can absorb too much water, resulting in hard stool and constipation. As the fecal material moves along the colon and water is absorbed, it becomes progressively harder and thicker; the colonic motor activity must thus be able to propel and mix semisolid to solid contents, which may require contractions of large amplitude and long duration. The colonic motility is therefore regulated via 3 types of

contractions: (1) individual phasic contractions, (2) peristaltic reflexes and (3) giant migrating contractions (GMC). The *individual phasic contractions* are poorly coordinated and occur independently, resulting in back and forward movement of the content, in this way exposing it to the mucosa for optimal absorption. *Peristaltic* reflexes propel the colonic content over a short distance, but the major mass movement of colonic content occurs via *GMCs,* which are powerful peristaltic contractions that propel the content aborally over a large part of the colon. In this way, GMCs bring an important mass of feces into the rectum, where it will be stored until it is emptied by the defecation process.

In the period between meals (or fasting), a specific motor activity occurs, called the *migrating motor complex* (MMC). The MMC is a cyclic, recurring motility pattern, moving distally along the gastrointestinal tract from the corpus of the stomach to the ileocecal sphincter; feeding will disrupt this MMC cycle. The MMC can be divided in four phases: phase I is quiescent with virtually no contractions, phase II is characterized by intermittent, irregular low-amplitude contractions, phase III consists of short bursts of rhythmic high-amplitude contractions and phase IV represents a short transition period back to the quiescence of phase I. In the human body, phase III occurs with an interval of approximately 90 minutes; about half of all phase III onsets is located in the stomach and the other half in the duodenum. MMCs have an important role as "intestinal housekeeper" in the interdigestive period, and especially phase III contributes to mechanical cleaning of the gastrointestinal tract in preparation for the next food bolus (Sarna, 1991; Olsson & Holmgren, 2001; Marieb *et al.*, 2004; Mashimo & Goyal, 2006; Curro *et al.*, 2008; Boron & Boulpaep, 2009; Deloose *et al.*, 2012).

I.1.3 *Neuronal control of gastrointestinal motility*

The different functions of the gastrointestinal tract are controlled by two principle types of mechanisms: hormonal and neuronal. These control mechanisms allow for a continuous optimal adjustment in secretion, motility and absorption in response to a meal. This section will concentrate on the neuronal control of gastrointestinal motility.

The motility of the gastrointestinal tract is controlled via both extrinsic and intrinsic pathways. The extrinsic pathway, that receives input from the central nervous system (CNS),

can be subdivided into a parasympathetic and sympathetic branch. The intrinsic pathway, also known as the enteric nervous system (ENS), is located within the wall of the gastrointestinal tract and includes the submucosal nerve plexus (Meissner) and the myenteric nerve plexus (Auerbach).

Extrinsic innervation. The *parasympathetic nervous system* innervating the gastrointestinal tract consists of the vagus nerve and the pelvic nerves. The vagus nerve innervates the esophagus, stomach, gallbladder, pancreas, small intestine, cecum and the proximal part of the colon. The pelvic nerves innervate the distal part of the colon, rectum and the anal canal. Sensory nerve endings in the gut respond to a wide range of chemical and mechanical stimuli. Parasympathetic afferent fibers transmit information from these sensory nerve endings to the CNS, either through cell bodies in the nodose ganglia, which have a central projection terminating in the nucleus of the tractus solitarius in the brainstem (vagus), or through cell bodies in the dorsal root ganglia (pelvic). In the CNS, the sensory neurons connect to the parasympathetic efferent fibers. Originating from the preganglionic nerve cell bodies in the brainstem (vagus) or the sacral spinal cord (pelvic), these efferent fibers run within respectively the vagus and pelvic nerves to the wall of the gut (Fig. I.5), where they synapse with postganglionic, enteric neurons of the ENS. These enteric neurons can be excitatory cholinergic neurons, or inhibitory non-adrenergic non-cholinergic (NANC) neurons.

The *sympathetic nervous system* innervating the gastrointestinal tract transfers sensory information from the gut to the CNS by neurons that have their endings in the gut wall and their cell bodies in the dorsal root ganglia. These sensory neurons are nociceptors, sensing high-intensity mechanical, chemical and thermal stimuli that threaten or damage the tissue. Sympathetic efferent fibers have their preganglionic cell bodies in the thoraco-lumbar part of the spinal cord. These nerve fibers are cholinergic and make nicotinic synapses in the paravertebral and/or prevertebral ganglia, from where postganglionic noradrenergic neurons innervate the upper (celiac ganglion), middle (superior mesenteric ganglion) and lower (inferior mesenteric ganglion) part of the gastrointestinal tract (Fig. I.5). The regulation of motility is not mediated via a direct action of the postganglionic noradrenergic neurons on the gastrointestinal smooth muscle, except for the sphincter

regions, but via inhibition of the release of excitatory neurotransmitters from parasympathetic neurons or enteric neurons.
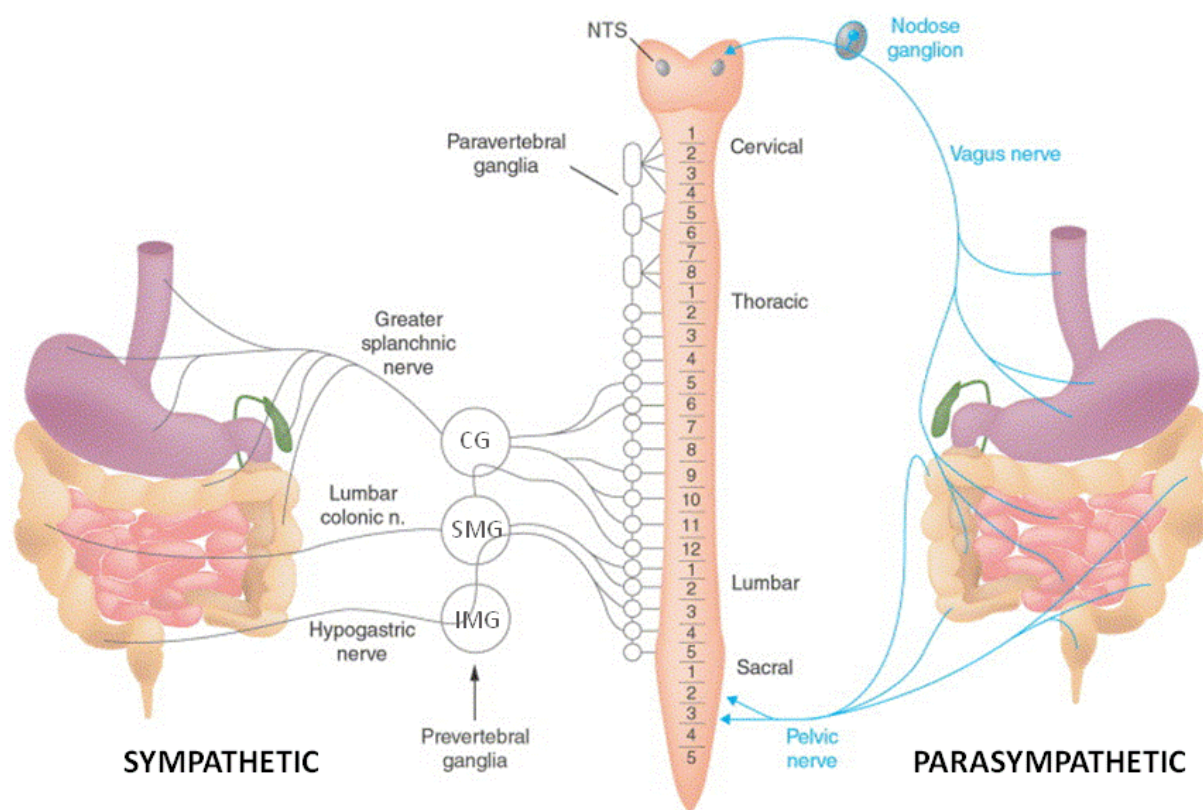
Fig. I.5   The extrinsic innervation of the gastrointestinal tract. The sympathetic nervous system is illustrated on the left, the parasympathetic nervous system on the right (NTS, nucleus of the tractus solitarius in the brainstem; CG, celiac ganglion; SMG, superior mesenteric ganglion; IMG, inferior mesenteric ganglion) (Blackshaw & Gebhart, 2002).

The parasympathetic nervous system generally results in the activation of physiological processes in the gut wall, like for example movement and secretion in response to a meal. In contrast, the sympathetic nervous system is more frequently activated in pathophysiological circumstances. Overall, sympathetic activation inhibits smooth muscle function; the exception to this is the sympathetic innervation of the sphincters, in which sympathetic activation tends to induce contraction of smooth muscle cells. The sympathetic nervous system is also particularly important in regulation of blood flow in the gastrointestinal tract (Blackshaw & Gebhart, 2002; Grundy *et al.*, 2006; Koeppen & Stanton, 2008; Furness *et al.*, 2011).

Intrinsic innervation. The enteric nervous system (ENS) can act autonomously from the extrinsic innervation. However, neurons in the ENS are innervated by extrinsic neurons, and their function can be modulated by the extrinsic nervous system. The ENS can be found within the wall of the gastrointestinal tract and is organized in two continuous chains of ganglia: the myenteric (Auerbach) and the submucosal (Meissner) nerve plexus (Fig. I.2). The *myenteric plexus* lies in between the longitudinal and circular muscle layers and forms a continuous network around the circumference of the tubular digestive tract from the upper esophagus to the internal anal sphincter. It primarily controls the contraction and relaxation of gastrointestinal smooth muscle cells and is thus mainly involved in gastrointestinal motility. The *submucosal plexus* lies in the submucosa; whereas in experimental animals (mouse, rat, guinea pig) it is only prominent in the small and large intestine (some isolated ganglia can be found in the esophagus and stomach), an extensive submucosal plexus is present in the human stomach. The submucosal plexus controls secretion and absorption, but also local blood flow and neuro-immune function.

Neurons in the ENS are characterized functionally as sensory neurons, interneurons and motor neurons; stimuli in the wall of the gut are detected by sensory neurons, which then activate interneurons, which in turn will activate motor neurons to regulate secretion or to alter smooth muscle tone. The motor neurons involved in *secretion* are called secretomotor neurons and two classes have been identified: one is a cholinergic and the other releases non-cholinergic neurotransmitters, particularly vasoactive intestinal peptide (VIP). The secretomotor neurons release acetylcholine and/or VIP at their junctions with the epithelium of the crypts, where they then stimulate intestinal secretion of water, electrolytes and mucus. Additionally, secretomotor neurons project to submucosal arterioles, dilating blood vessels to increase blood flow. Inhibitory input from sympathetic postganglionic fibres suppresses the activity of the secretomotor neurons and thereby inhibits secretion. The motor neurons involved in the regulation of *smooth muscle tone* innervate the longitudinal and circular smooth muscle layers, as well as the mucosal muscle layer. They are classified into excitatory and inhibitory neurons, depending on whether the neurotransmitters they release cause respectively contraction or relaxation of smooth muscle cells. *Excitatory* motor neurons primarily contain acetylcholine, which acts on smooth muscle muscarinic receptors, but also excitatory NANC neurotransmitters such as tachykinins (e.g. substance P) can be released from excitatory neurons. *Inhibitory* motor

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neurons release inhibitory NANC neurotransmitters such as nitric oxide (NO), VIP and adenosine triphosphate (ATP) (Koeppen & Stanton, 2008; Furness *et al.*, 2011; Wood, 2011).

Dense networks of interstitial cells of Cajal (ICC) are found in between the motor neurons of the ENS and the smooth muscle cells. ICC are referred to as the pacemaker cells of the gut. Although they are non-neural but mesenchymal in origin, they can generate a rhythmic pacemaker current, which manifests itself as slow waves in the membrane potential of smooth muscle cells (Sanders, 1996). Smooth muscle cells will contract when action potentials superpose on the slow waves. Next to their role as pacemaker, ICC also play a role in the transmission of excitatory and inhibitory signals from enteric neurons to smooth muscle cells (Ward *et al.*, 2004; Tanahashi *et al.*, 2013; Klein *et al.*, 2013).

I.1.4   Nitrergic neurotransmission

As the control of gastrointestinal motility was long believed to depend only on two neurotransmitters, noradrenaline and acetylcholine, all involved excitatory and inhibitory neurotransmitters discovered after 1970 were classified as non-adrenergic non-cholinergic (NANC) neurotransmitters. In the gastrointestinal tract, substance P is accepted as the primary excitatory NANC neurotransmitter (Shuttleworth & Keef, 1995). NO, ATP and VIP mediate the inhibitory NANC responses; NO being the primary inhibitory NANC neurotransmitter in the ENS. Additionally, some evidence has been reported for a role of carbon monoxide (CO), hydrogen sulfide (H_2S) and pituitary adenylate cyclase-activating polypeptide (PACAP) as inhibitory NANC neurotransmitter; these molecules indeed also have a relaxant effect on gastrointestinal smooth muscle (Matsuda & Miller, 2010). For this thesis, we will focus on nitrergic neurotransmission and its characteristics.
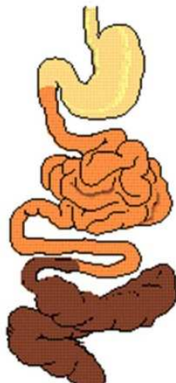
NO is enzymatically formed together with L-citrulline from the amino acid L-arginine by the catalytic activity of nitric oxide synthase (NOS) (Bruckdorfer, 2005). Unlike the classic neurotransmitters, NO is not stored in classic presynaptic vesicles, but rather it is synthesized and released on demand (Kasparek *et al.*, 2008). In the gastrointestinal tract, NO is produced primarily by the constitutively expressed neuronal isoform of NOS (nNOS or NOS1), and is released from inhibitory motor neurons of the ENS. Endothelial NOS (eNOS or NOS3), which is constitutively expressed mainly in endothelial cells, is primarily involved in the control of vascular perfusion of the gastrointestinal tract, whereas the inducible isoform

of NOS (iNOS or NOS2) is expressed mainly in inflammatory cells (e.g. macrophages) and is involved in inflammation and host defense (Kasparek *et al.*, 2008). It is thus the nNOS isoform localized in the myenteric neurons that accounts for the synthesis of NO as an inhibitory NANC neurotransmitter in the gastrointestinal tract.

NO released from nitrergic neurons within the NANC neuron population plays an important physiological role in various parts of the gastrointestinal tract: (1) NO regulates the muscle tone of the lower esophagus sphincter, pylorus, sphincter of Oddi (controls the flow of bile and pancreatic juice to the duodenum) and anus; (2) NO regulates the accommodation reflex of the fundus upon the receival of food; and (3) NO plays an important role in the peristaltic reflex of the intestine (Desai *et al.*, 1991; Takahashi, 2003). The important role of NO in NANC relaxation and hence gastrointestinal motility is evident from the delay in gastric emptying and intestinal transit upon NOS inhibition or in nNOS knockout mice (Table I.1).

Species	Model	Reference		
dog	NOS inhibition	Orihata *et al.* (1994) and Anvari *et al.* (1998)	**DELAYED**	
human	NOS inhibition	Konturek *et al.* (1999)	**GASTRIC EMPTYING**	
mouse	NOS inhibition	Mashimo *et al.* (2000)		
pig	NOS inhibition	Lefebvre *et al.* (2005)		
mouse	nNOS knockout	Mashimo *et al.* (2000)		
rat	NOS inhibition	Karmeli *et al.* (1997)	**DELAYED**	
dog	NOS inhibition	Chiba *et al.* (2002)	**SMALL INTESTINAL TRANSIT**	
human	NOS inhibition	Fraser *et al.* (2005)		
guinea pig	NOS inhibition	Foxx-Orenstein *et al.* (1996)	**DELAYED**	
rat	NOS inhibition	Mizuta *et al.* (1999)	**COLONIC TRANSIT**	
dog	NOS inhibition	Chiba *et al.* (2002)		

Table I.1 Overview of the studies dealing with the effects of NOS inhibition or knocking out nNOS on gastrointestinal motility. NOS inhibition was found to delay gastric emptying, slow down small intestinal transit and inhibit colonic propulsion. In addition, nNOS knockout mice showed delayed gastric emptying.

I.2 Soluble guanylate cyclase

I.2.1 Structure

On its release from nerves in the gut wall, NO diffuses through the cell membrane of the smooth muscle cell and binds to its principle target, soluble guanylate cyclase (sGC). sGC is a heterodimeric heme-containing protein consisting of an α and a β subunit (Fig. I.6A). Both α and β subunits exist in 2 isoforms: α_1 and α_2 (Harteneck *et al.*, 1991), and β_1 and β_2 (Yuen *et al.*, 1990). Theoretically, the association between the α and β isoforms could result in four different heterodimers, but only sGC$\alpha_1\beta_1$ and sGC$\alpha_2\beta_1$ seem to be physiologically active, with no differences in kinetic properties and sensitivity towards NO (Russwurm *et al.*, 1998). In the brain, the amounts of sGC$\alpha_1\beta_1$ and sGC$\alpha_2\beta_1$ are quantitatively similar, but in all other tissues, including the gastrointestinal tract, sGC$\alpha_1\beta_1$ is the dominant heterodimer (Mergia *et al.*, 2003).
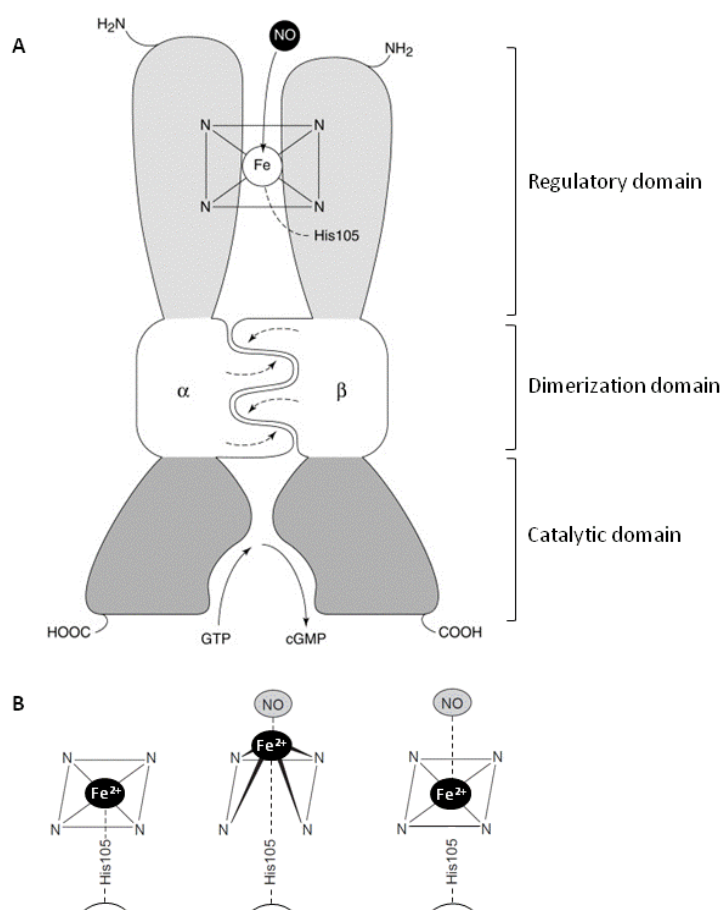
Fig. I.6   Schematic representation of the sGC enzyme (A) and the activation of sGC by NO (B) (Hobbs, 1997).

Each α and β subunit contains three functional domains: an N-terminal regulatory heme-binding domain, a central dimerization domain and a C-terminal catalytic domain (Lucas *et al.*, 2000) (Fig. I.6A). In the *N-terminal regulatory domain* of the β subunit, histidine at position 105 is the essential amino acid required for binding of the prosthetic heme moiety in the β subunit (Wedel *et al.*, 1994). The heme group contains either Fe^{2+} (ferrous or reduced form), or Fe^{3+} (ferric or oxidized form); this central iron is located between four nitrogen atoms and the axial ligand histidine 105, building a penta-coordinated histidine-heme complex. Binding of NO to Fe^{2+} results in the formation of a temporary hexa-coordinated histidine-heme-NO intermediate that rapidly decays into a penta-coordinated nitrosyl-heme complex (Fig. I.6B). This change in heme conformation is transduced to the catalytic domain, leading to a 200-400-fold increase in catalytic activity (Lucas *et al.*, 2000; Murad, 2011). The *central dimerization domain* is involved in the formation of the heterodimers, which is the first requirement for sGC to exhibit catalytic activity (Harteneck *et al.*, 1990). The *C-terminal catalytic domain* is the most conserved region between species and between guanylyl and adenylyl cyclases and activation of this domain will convert guanosine-5'-triphosphate (GTP) into the second messenger cyclic guanosine monophosphate (cGMP). Increased intracellular cGMP levels will exert physiological effects through (1) activation of cGMP-dependent protein kinases (known as protein kinases G or PKG), (2) altering the conductance of cGMP-gated ion channels, and (3) changing the activity of cGMP-regulated phosphodiesterases (PDEs; i.e. PDE-2 and PDE-5 are activated and PDE-3 is inhibited by cGMP). The most important mediators for gastrointestinal signaling are the PKGs and PDEs.

- PKGs exist in two subtypes: (1) cytosolic PKG I, which is present in high concentrations in smooth muscle cells and plays an important role in intestinal smooth muscle relaxation by lowering the intracellular Ca^{2+} concentration and/or by desensitization of the contractile apparatus to Ca^{2+} (Pfeifer *et al.*, 1998), and (2) membrane-bound PKG II, which is expressed in the intestinal mucosa and regulates intestinal secretion.

- PDEs can degrade cGMP by hydrolyzing cGMP towards 5'-GMP. In smooth muscle, the most important cGMP-specific PDE is PDE-5 and inhibition of this PDE induces relaxation by means of cGMP accumulation (Carvajal *et al.*, 2000; Lucas *et al.*, 2000; Pyriochou & Papapetropoulos, 2005; Toda & Herman, 2005).

I.2.2 sGC in oxidative stress conditions

A major requirement for the activation of sGC by NO and hence smooth muscle relaxation, is the presence of the *reduced Fe^{2+} heme group*. In experiments using purified sGC, Wedel *et al.* (1994) replaced histidine at position 105 of the β_1 subunit by a phenylalanine, leading to a mutant sGCβ_1 gene of which the gene product is no longer able to correctly incorporate the heme group. The absence of a heme group rendered sGC completely insensitive to NO. In addition, oxidation of sGC towards a Fe^{3+} state of the heme group by oxidizing compounds such as 1H[1,2,4,]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), also led to the formation of an NO-insensitive form of sGC (Schrammel *et al.*, 1996; Zhao *et al.*, 2000).

Experiments in vascular smooth muscle tissue corroborate that sGC exists in a *physiological* equilibrium between two redox states: a native reduced and NO-sensitive form and an oxidized/heme-free NO-insensitive form. Under *pathophysiological* conditions associated with oxidative stress (associated with an increase in reactive oxygen species or ROS), this redox equilibrium is shifted towards the oxidized NO-insensitive form (Fig. I.7). As heme oxidation leads to a highly unstable protein that easily loses its heme group, this will lead to an oxidation-induced loss of the redox-sensitive heme-group; the resulting heme-free protein is also NO-insensitive (Fritz *et al.*, 2011). In addition to rendering the enzyme insensitive to NO, oxidation of the heme group makes sGC more prone to ubiquitination and subsequent proteosomal degradation (Stasch *et al.*, 2006; Meurer *et al.*, 2009). Under pathological conditions associated with oxidative stress, one might thus expect ROS to interfere with the NO-sGC-cGMP pathway.
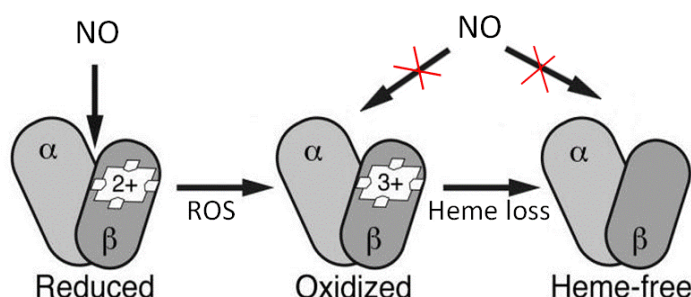
Fig. I.7 sGC exists in a physiological equilibrium between two redox states. The native reduced and NO-sensitive form, can be oxidized, leading to an oxidation-induced loss of the redox-sensitive heme-group. Both the oxidized state and the heme-free state are not sensitive to NO (adapted from Stasch *et al.*, 2006).

I.2.3 Role of sGC in gastrointestinal motility

As mentioned before, sGC$\alpha_1\beta_1$ is the dominant isoform in the gastrointestinal tract. Interestingly, although sGC$\alpha_1\beta_1$ indeed has the principal role in gastrointestinal nitrergic relaxation, studies in *sGCα_1 knockout mice* demonstrated that some degree of nitrergic relaxation can occur via sGC$\alpha_2\beta_1$ activation, in this way avoiding major in vivo changes in gastric emptying and intestinal motility in these mice (Vanneste *et al.*, 2007; Dhaese *et al.*, 2008; Dhaese *et al.*, 2009). *Full knockouts of sGC*, eliminating activation of both sGC isoforms by NO and also basal sGC activity, are associated with severely delayed gut transit, systemic hypertension and premature death (Friebe *et al.*, 2007). *Smooth muscle-specific deletion of sGC* is associated with hypertension and loss of vascular smooth muscle responsiveness to NO (Groneberg *et al.*, 2010); the responsiveness to NO of gastrointestinal muscle is however only mildly reduced, suggesting that sGC in gastrointestinal smooth muscle is dispensable for nitrergic relaxation (Groneberg *et al.*, 2011). However, also ICC express NO-sensitive sGC and expression levels appear to be higher than in smooth muscle cells (Iino *et al.*, 2008; Groneberg *et al.*, 2011). *Selective deletion of sGC in ICC* does not induce a decrease in NO responsiveness of gastrointestinal muscle, but *deletion of sGC in both smooth muscle and ICC* results in an impairment of nitrergic relaxation and an increase in gut transit time that is similar to that in mice lacking sGC ubiquitously (Groneberg *et al.*, 2013). Together, these results suggest a redundant action of sGC in both ICC and smooth muscle cells to induce gastrointestinal nitrergic relaxation. Recently, Thoonen *et al.* (2009) generated mice in which *sGC is heme-deficient (apo-sGC)*. Whereas Friebe *et al.* (2007) generated full knockouts of sGC by gene inactivation of the β_1 subunit, these apo-sGC mice were created -based on the experiments of Wedel *et al.* (1994)- by replacing histidine at position 105 of the β_1 subunit by a phenylalanine. In the resulting sGC$\beta_1^{His105Phe}$ knockin mice, sGC retains its basal catalytic activity, but it can no longer be activated by NO. These mice are characterized by a reduced live span, growth retardation and elevated blood pressure (Thoonen *et al.*, 2009); the consequences on gastrointestinal nitrergic signaling and motility of inducing heme-free status of sGC are discussed later in this thesis (chapter III). A major advantage of these apo-sGC mice over the full knockouts is that, despite the reduced life span, these mice are viable with a median survival of 30 weeks. This is in sharp contrast with the extreme short life span of the full knockout mice, where 60 % of the mice die

within the first two days after birth and less than 10 % survives longer than a month; it must be noted however that, when given a fiber-free diet, 60 % of the mice still die within the first two days after birth, but the survival of the remaining 40 % greatly improves (Friebe *et al.*, 2007; Thoonen, 2010). The reduced survival of the full knockouts points to the pivotal role of basal sGC activity; the produced low amounts of cGMP in apo-sGC mice seem vital for survival. The apo-sGC mouse model also allows to study heme-free sGC, as seen in oxidative stress conditions, and to investigate the effect of possible new therapies targeting sGC under oxidative stress conditions.

I.3 NO pharmacotherapy and alternatives

I.3.1 *Classic NO pharmacotherapy and its limitations*

In the cardiovascular system, nitrergic relaxation is therapeutically applied by use of NO donors, such as organic nitrates. Dysfunction of the endothelium is found in several cardiovascular diseases and will lead to NO deficiency, limiting NO-mediated signal transduction in normal physiological processes. Organic nitrates proved to be effective NO donors in a variety of cardiovascular disorders including heart failure and angina pectoris (Ignarro *et al.*, 2002) and inhaled NO proved to be effective in pulmonary hypertension, including neonatal pulmonary hypertension (Pepke-Zaba *et al.*, 1991; Kumar, 2013). However, the development of (pseudo-) tolerance limits the continuous clinical application of NO donors (Munzel *et al.*, 2011; Iachini *et al.*, 2012). *Pseudo-tolerance* is a term referring to the progressive attenuation of pharmacological effects of NO donors, not due to impairment of their biochemical properties, but rather due to increased secretion of substances exerting biologically opposing effects, such as catecholamines, angiotensin II and endothelin-1. *"True" tolerance* is associated with progressive impairment of the biochemical properties of the NO donors. The concept of "true" tolerance remains a complex phenomenon, but it was shown to be related to increased vascular ROS production upon prolonged use of NO donors. Pathological conditions such as heart failure and pulmonary hypertension are also associated with oxidative stress (Konduri *et al.*, 2007; Mitrovic *et al.*, 2009). The presence of oxidative stress, associated with the prolonged use of NO donors or with the pathological conditions itself, is a major problem in the treatment of NO deficiency

with classical NO pharmacotherapy. Oxidative stress may directly impair the biotransformation of NO donors, preventing them to release NO (Munzel *et al.*, 2011). In addition, ROS can interfere with the NO-sGC-cGMP pathway either (1) by reduced availability of NO through e.g. scavenging of NO, (2) by oxidation of sGC towards the heme-free status (Fritz *et al.*, 2011), making it unresponsive towards endogenous NO, but also NO donors, or (3) by irreversible loss of the enzymatic capacity by downregulation of sGC protein levels as oxidation of the heme group will make sGC more prone to degradation (Stasch *et al.*, 2006). NO donors will thus fail in obtaining the desired therapeutic effect.

During the last 15 years, two novel drug classes have been discovered that seem to address these problems: the heme-dependent sGC stimulators and the heme-independent sGC activators (Fig. I.8) (Evgenov *et al.*, 2006; Meurer *et al.*, 2009). These novel classes of compounds are capable of activating the reduced and/or oxidized/heme-free state of sGC in an NO-independent manner. *sGC stimulators* are capable of directly stimulating the reduced form of sGC, acting in synergy with NO, but they can also stimulate reduced sGC independently of NO, allowing to circumvent conditions with decreased endogenous generation of NO (Stasch & Hobbs, 2009). *sGC activators* preferably activate the oxidized/heme-free enzyme (Schmidt *et al.*, 2009).
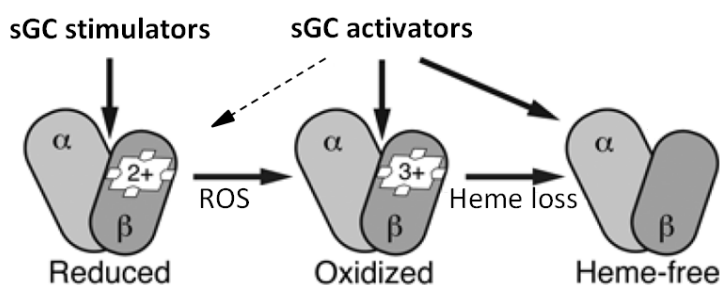
Fig. I.8 sGC stimulators are capable of directly stimulating the reduced form of sGC independently of NO and sGC activators are capable of activating the oxidized/heme-free enzyme (adapted from Stasch *et al.*, 2006).

I.3.2   *sGC stimulators*

YC-1. The first reported NO-independent sGC stimulator was 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole or YC-1 (Fig. I.9); it was originally introduced as an inhibitor of platelet aggregation by increasing cGMP levels via sGC in an NO-independent manner (Ko *et al.*, 1994; Wu *et al.*, 1995; Teng *et al.*, 1997). Different pharmacological studies now also show its smooth muscle relaxant properties. For example, *in vitro*, YC-1 induces relaxation in

vascular (Mulsch *et al.*, 1997; O'Reilly *et al.*, 2001), gastrointestinal (De Backer & Lefebvre, 2007), corpus cavernosum (Nakane *et al.*, 2002), urethra (Schroder *et al.*, 2002; Che *et al.*, 2003) and tracheal smooth muscle (Glaza *et al.*, 2011; Turgut *et al.*, 2013), while *in vivo*, YC-1 attenuates pulmonary hypertension (Huh *et al.*, 2011), decreases blood pressure (Rothermund *et al.*, 2000), and facilitates penile erection (Hsieh *et al.*, 2003). YC-1 was found to act in synergy with NO and its ability to stimulate sGC was shown to depend upon the presence of the reduced heme-group, as its removal or oxidation abolished any YC-1-induced sGC activation. Although YC-1 has many potential therapeutic properties, it has relatively weak sGC stimulating potency and a lack of specificity, as it was found to inhibit phosphodiesterases (Friebe *et al.*, 1998; Galle *et al.*, 1999) and induce many cGMP-independent effects (Wohlfart *et al.*, 1999; Garthwaite *et al.*, 2002; Slupski *et al.*, 2007). Based on the lead compound YC-1, high-throughput screening and identification of modulators of the NO-sGC-cGMP pathway was performed at Bayer to systematically optimize the structure of YC-1. A first breakthrough in terms of improved potency resulted from the replacement of the hydroxymethylfuran group of YC-1 by a 5-substituted 4-aminopyrimidine (Straub *et al.*, 2001). This 5-cyclopropyl-4-aminopyrimidine derivate was named BAY 41-2272.
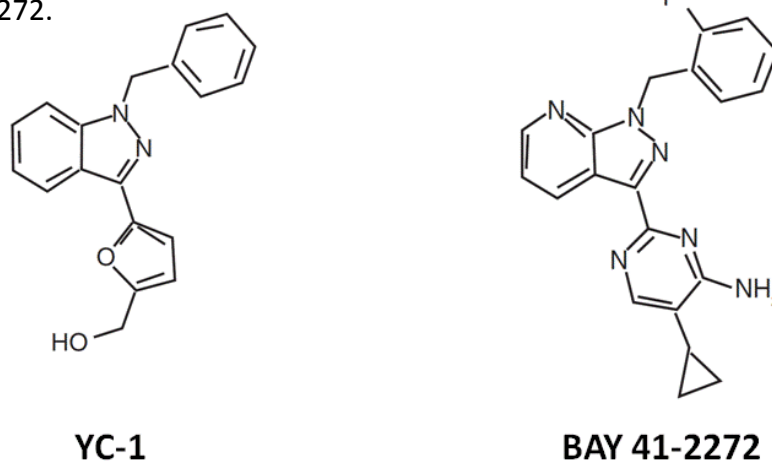
Fig. I.9 NO-independent, heme-dependent stimulators of sGC: YC-1 and BAY 41-2272 (Stasch & Hobbs, 2009).

BAY 41-2272. Similar to YC-1, 3-(4-amino-5-cyclopropylpyrimidin-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b] pyridine or BAY 41-2272 (Fig. I.9) directly stimulates sGC, increases the enzyme's sensitivity towards NO and fails to activate the enzyme after removal or oxidation of the prosthetic heme moiety (Stasch *et al.*, 2001). *In vitro*, BAY 41-2272 induces relaxation in arterial (Bawankule *et al.*, 2005; Teixeira *et al.*, 2006a; Teixeira *et al.*, 2006b), corpus cavernosum (Kalsi *et al.*, 2003), urethra (Toque *et al.*, 2008), detrusor

(Bau *et al.*, 2010) and tracheal smooth muscle (Toque *et al.*, 2010), while *in vivo,* the compound attenuates pulmonary hypertension (Evgenov *et al.*, 2004), decreases blood pressure, has anti-platelet activity (Stasch *et al.*, 2001; Hobbs & Moncada, 2003; Roger *et al.*, 2010) and unloads the heart in a model of congestive heart failure (Boerrigter *et al.*, 2003). Analogous to YC-1, BAY 41-2272 was found to induce cGMP-independent effects: in arterial, detrusor and tracheal smooth muscle (Teixeira *et al.*, 2006a; Bau *et al.*, 2010; Toque *et al.*, 2010), inhibition of extracellular calcium entry by BAY 41-2272 was reported, and in ovine pulmonary artery (Bawankule *et al.*, 2005), stimulation of Na^+/K^+-ATPase leading to membrane hyperpolarization was described. The effect of BAY 41-2272 on PDE-5 activity is controversial. Stasch *et al.* (2001) and Bischoff and Stasch (2004) reported BAY 41-2272 to be devoid of PDE-5 inhibitory activity, whereas Mullershausen *et al.* (2004) demonstrated this compound to inhibit PDE-5 in platelets. Still, as the concentration used by Mullershausen *et al.* (2004) is several orders of magnitude above that needed for sGC stimulation, the PDE-5 inhibitory activity by BAY 41-2272 is considered as irrelevant (Evgenov *et al.*, 2006).

Based on the promising pharmacological effects of sGC stimulators, Bayer and several other companies (Abbott, Astellas, Merck, Pfizer) started programs to identify new series of sGC stimulators (Follmann *et al.*, 2013). The most successful compound BAY 63-2521 (or riociguat, another YC-1 derivative), has just completed phase III clinical trials (Conole & Scott, 2013; Ghofrani *et al.*, 2013a; Ghofrani *et al.*, 2013b) and has been approved by the Food and Drug Administration for treatment of pulmonary hypertension (as Adempas®).

I.3.3 sGC activators

Cinaciguat. Following the discovery of the NO-independent, heme-dependent sGC stimulators, the high-throughput screening at Bayer identified compounds with surprising qualities: they can directly activate sGC (independent of NO) and preferably do so when sGC is in the oxidized/heme-free position. These drugs should thus target the enzyme more extensively in pathological conditions associated with oxidative stress (Schmidt *et al.*, 2009). Within this new class, cinaciguat (Fig. I.10; BAY 58-2667; 4-[((4-carboxybutyl)-(2-[4-phenethylbenzyl)oxy]phenethyl)amino)methyl] benzoic acid has been extensively studied,

especially in the cardiovascular system. *In vitro*, the effectiveness of cinaciguat is increased in blood vessels of animal models of vascular disease that are associated with endogenously induced oxidative stress and in blood vessels of controls, that are pretreated with the oxidant peroxynitrite (Stasch *et al.*, 2006; Korkmaz *et al.*, 2012). *In vivo*, cinaciguat is also more effective in conditions associated with oxidative stress or mimicking it. In spontaneously hypertensive rats and control rats, that are pretreated with ODQ, the decrease in blood pressure caused by cinaciguat lasts longer than in normotensive controls (Stasch *et al.*, 2002; Stasch *et al.*, 2006); similarly, pulmonary vasodilatation induced by inhaling cinaciguat microparticles is greatly enhanced after pretreatment with ODQ (Evgenov *et al.*, 2007). Furthermore, cinaciguat shows beneficial effects in models of heart failure (Boerrigter *et al.*, 2007; Erdmann *et al.*, 2012), pulmonary hypertension (Chester *et al.*, 2011) and cardiac ischemia/reperfusion models (Korkmaz *et al.*, 2009; Radovits *et al.*, 2011; Salloum *et al.*, 2012). Based on these promising results, a non-randomized, unblinded Phase IIa study in patients with acute decompensated heart failure (ADHF) was initiated. Continuous intravenous infusion of cinaciguat (ranging from 50 to 400 µg/h for a total of 6 h) was well-tolerated and resulted in improved cardiopulmonary hemodynamics in an uncontrolled proof-of-concept study (Lapp *et al.*, 2009). Subsequent randomized, double-blind, placebo-controlled Phase IIb studies investigated the effects of both high (≥200 µg/h) and low (< 200 µg/h) doses of cinaciguat in ADHF patients; the clinical development of cinaciguat was stopped as, even at low doses, cinaciguat decreased blood pressure, which might be unfavorable in patients with ADHF (Gheorghiade *et al.*, 2012; Erdmann *et al.*, 2012).

Cinaciguat
(BAY 58-2667)
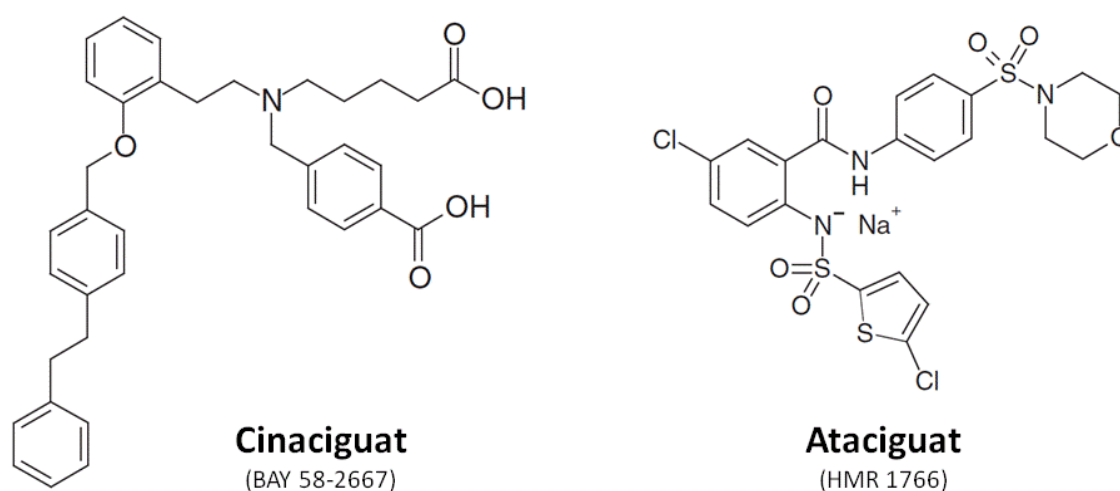
Ataciguat
(HMR 1766)

Fig. I.10 NO- and heme-independent activators of sGC: cinaciguat (BAY 58-2667) and ataciguat (HMR 1766) (Schmidt *et al.*, 2009).

The fact that oxidation or removal of the heme-group increases cinaciguat-induced enzyme activation, might be explained by the mechanistic model by which sGC is activated by cinaciguat. Cinaciguat is believed to activate the heme-deficient sGC enzyme via direct interaction with the unoccupied heme-binding pocket, or by displacing the weakly bound oxidized heme-group. Furthermore, cinaciguat is able to prevent oxidation-accelerated degradation of the sGC heterodimers, thereby stabilizing and conserving the enzyme protein levels in an active, cinaciguat-bound form, even under oxidizing conditions (Stasch *et al.*, 2006).

Ataciguat. Schindler and coworkers (2006) reported a novel structural class of compounds, anthranilic acid derivates, that were also reported to activate the oxidized/heme-free form of sGC. 5-chloro-2-[[(5-chloro-2-thienyl)sulfonyl]amino]-N-[4-(4-morpholinylsulfonyl)phenyl]benzamide or ataciguat (Fig. I.10; HMR1766) is the best described example. *In vitro*, ataciguat induces corpus cavernosum (Schindler *et al.*, 2006) and arterial relaxation (Schindler *et al.*, 2006; Schafer *et al.*, 2010); similar to cinaciguat, the effectiveness of ataciguat is increased in blood vessels of animal models of vascular disease that are associated with endogenously induced oxidative stress (Schafer *et al.*, 2010) and in blood vessels that are pretreated with ODQ (Schindler *et al.*, 2006). *In vivo*, ataciguat attenuates pulmonary hypertension (Weissmann *et al.*, 2009), decreases blood pressure (Schindler *et al.*, 2006) and shows anti-platelet activity (Schafer *et al.*, 2010). Ataciguat was studied in a phase II clinical trial for patients with peripheral arterial occlusive disease and in patients with neuropathic pain, but these studies were discontinued without further explanation (Follmann *et al.*, 2013).

Analogous to cinaciguat, ataciguat is believed to activate both the oxidized and the heme-free form of sGC (Evgenov *et al.*, 2006). However, different binding modes for cinaciguat and ataciguat to the heme pocket were reported (Hoffmann *et al.*, 2009). While cinaciguat stabilizes sGC under heme-oxidizing conditions and thereby protects it from ubiquitination and subsequent degradation, binding of ataciguat lacks these stabilizing properties. Cinaciguat's effect probably depends on high affinity binding to the heme-binding pocket in a manner that reassembles the native prosthetic group; this high affinity binding of cinaciguat stabilizes sGC. Binding of ataciguat cannot stabilize sGC, making it the prototype of a distinct class of sGC activators (Hoffmann *et al.*, 2009).

I.3.4   NO pharmacotherapy for gastrointestinal disorders

As mentioned before, NO synthetized from nNOS in NANC neurons and targeting sGC in smooth muscle cells, is the most important inhibitory NANC neurotransmitter in the gastrointestinal tract. It is therefore not surprising that impaired nitrergic innervation of the smooth muscle plays a crucial role in several disorders with gastrointestinal dysmotility, such as functional dyspepsia, esophageal achalasia, infantile hypertrophic pyloric stenosis, delayed gastric emptying after vagotomy and Hirschsprung's disease (Goyal & Hirano, 1996; Takahashi, 2003). This has led to therapeutic strategies targeting the NO-sGC-cGMP pathway. As early as the 1940s there were reports on the use of organic nitrates such as nitroglycerin in patients with achalasia (Ritvo & McDonald, 1940; Field & Lond, 1944). However, later studies aiming to enhance NO signaling with organic nitrates in patients with esophageal motor disorders have shown limited success (Robson & Wilkinson, 1946; Swamy, 1977; Gelfond *et al.*, 1982; Wen *et al.*, 2004). Organic nitrates have also been suggested as treatment for functional dyspepsia, diabetic gastroenteropathy and anismus (Whittle, 2005); topical nitrates are used for the therapy of anal fissure (Collins & Lund, 2007). However, with the exception of achalasia, organic nitrates have not been used much in gastrointestinal disorders associated with nitrergic neuronal dysfunction, also because of the well-known attenuation of their effect after long term usage due to the development of tolerance; this was indeed also reported in the treatment of achalasia with organic nitrates (Robson & Wilkinson, 1946).

Aging, colitis and diabetes can also lead to enteric nitrergic neuronal dysfunction and consequently motility disturbances (Mizuta *et al.*, 2000; Phillips & Powley, 2007; Zandecki *et al.*, 2008). These conditions are associated with oxidative stress (Kashyap & Farrugia, 2011; Cannizzo *et al.*, 2011; Zhu & Li, 2012). ROS were suggested to increase the likelihood of damage to enteric nitrergic neurons (Rivera *et al.*, 2011) and it can be expected that enteric sGC will be driven to the oxidized/heme-free status in these conditions, contributing to reduced effectiveness of the NO-sGC-cGMP pathway. Treatment with NO donors such as organic nitrites would thus be useless in these conditions.

sGC stimulators, capable of directly stimulating the reduced form of sGC independently of NO, or sGC activators, capable of activating the oxidized/heme-free

enzyme, showed effectiveness in the cardiovascular system, but they were not yet investigated in the gastrointestinal tract.

I.3.5 Nitrite as a source of NO

Until recently, the inorganic anions nitrate (NO_3^-) and nitrite (NO_2^-) were considered inert end products of NO. Benjamin *et al.* (1994) reported for the first time NOS-independent NO generation from inorganic nitrite in the stomach. From additional research performed during the last decade, it is now obvious that nitrate and nitrite are physiologically recycled in blood and tissue to form NO and other bioactive nitrogen oxides, representing an alternative for the 'classical' NOS pathway (Fig. I.11). This nitrogen oxide cycle can be fueled by the diet, e.g. green leafy vegetables, beetroot and fennel, as these contain high amounts of inorganic nitrate. Processed food, like e.g. cured meat, in which nitrite is added as a preservative to inhibit bacterial growth, can be a source of direct dietary nitrite intake. As mammals lack specific and effective nitrate reductase enzymes, the reduction of nitrate to nitrite is mainly carried out by commensal bacteria in the gastrointestinal tract and on body surfaces. Once nitrite is formed, there are numerous enzymatic and non-enzymatic pathways in the body for its further reduction to NO, and the generation of NO by these pathways is greatly enhanced during hypoxia and acidosis, ensuring NO production in situations where the oxygen-dependent NOS pathway is compromised (Fig. I.11). In the human body, it was shown that both dietary and endogenous nitrate reduction towards NO goes mainly through the *entero-salivary circulation*. Nitrate derived from the diet is swallowed, where it is rapidly absorbed by the gastrointestinal tract. Although much of the circulating nitrate is excreted in the urine, up to 25 % of both endogenous and dietary nitrate is extracted by the salivary glands and concentrated in saliva. In the mouth, commensal anaerobic bacteria will reduce nitrate to nitrite, after which the nitrite will be swallowed together with the saliva. In the acidic stomach, nitrite is reduced to NO; any remaining nitrite is absorbed by the gastrointestinal tract and can be converted to NO in blood and tissues where oxygen levels are low (Lundberg *et al.*, 2008; Lundberg *et al.*, 2009; Raat *et al.*, 2009a; Raat *et al.*, 2009b; Weitzberg *et al.*, 2010).
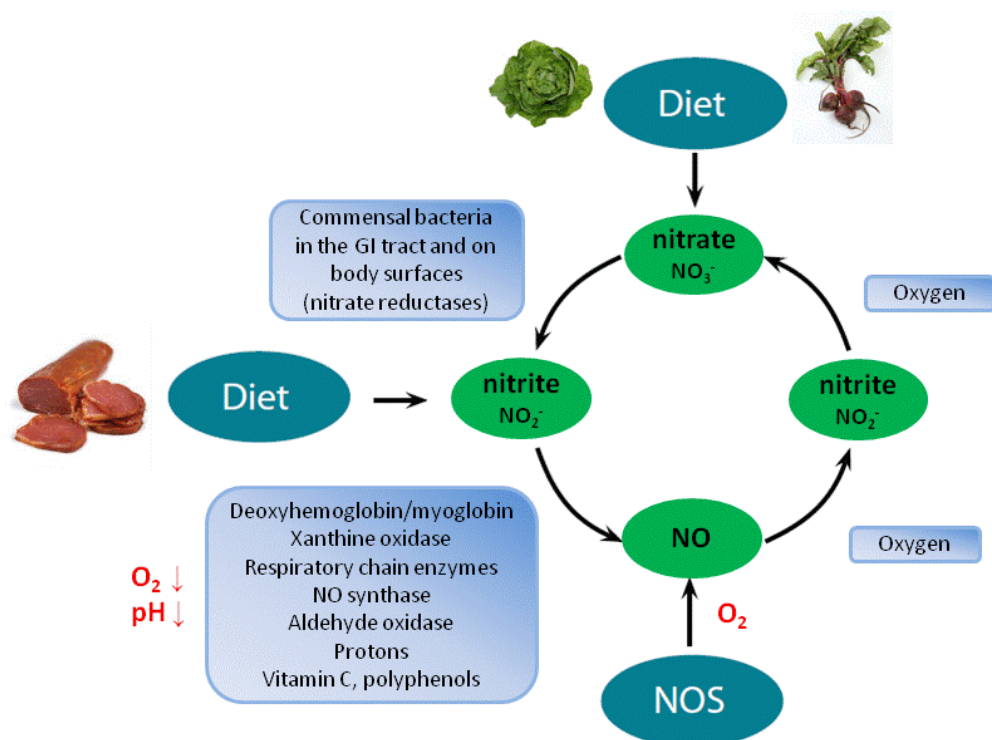
Fig. I.11 NO is generated by NO synthases (NOS) and is rapidly oxidized towards nitrite and nitrate. Nitrate can undergo reduction to nitrite, a process dependent on commensal bacteria in the gastrointestinal tract and on body surfaces. Nitrite can undergo further reduction to NO, a process catalyzed by various enzymatic and non-enzymatic pathways, which are greatly enhanced under hypoxic or acidic conditions. While the NOS-dependent pathway towards NO is oxygen dependent, this nitrate-nitrite-NO pathway is actually gradually activated when oxygen levels go down. This NOS-independent pathway can thus serve as a back-up system, to ensure that sufficient NO is formed when oxygen supply is limited. This nitrogen oxide cycle can be fueled by the diet (based on Lundberg *et al.*, 2009).

In pathological conditions associated with local or systemic oxygen shortage, it might thus be beneficial to support the nitrate and nitrite stores, either pharmacologically or by dietary intervention. Indeed, nitrite was shown to be protective in ischemia/reperfusion models of the liver, heart, brain and kidney. Duranski *et al.* (2005) showed in a mouse model of hepatic ischemia/reperfusion and in a mouse model of myocardial ischemia/reperfusion, that nitrite (48 nmol being the optimal dose in both models) was able to respectively reduce liver transaminase levels (indicators for liver injury) and reduce infarct size. Nitrite protection was dependent on NO and signaling via sGC, as administration of the NO-scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) or the sGC inhibitor ODQ completely abolished protection in the hepatic ischemia/reperfusion model. Jung *et al.* (2006) demonstrated the protective effect of nitrite in a cerebral

ischemia/reperfusion model in rats. Nitrite reduced infarct size and enhanced local cerebral blood flow; neuroprotective effects that were inhibited with the NO-scavenger PTIO. Tripatara *et al.* (2007) demonstrated the protective effect of nitrite in a renal ischemia/reperfusion model in rats; as in the other models, nitrite protection was shown to be dependent on NO as the NO scavenger PTIO completely abolished protection. Since the pioneers Duranski (2005), Jung (2006) and Tripatara (2007) established the effect of nitrite in respectively the liver and heart, the brain and the kidneys, many others explored this field of research (Dezfulian *et al.*, 2007; Raat *et al.*, 2009b; Weitzberg *et al.*, 2010).

The exact mechanism of the protective effect of nitrite in ischemia/reperfusion models is not completely understood, but it is clear that NO is an essential intermediate step. Shiva *et al.* (2007) showed in a hepatic ischemia/reperfusion model that nitrite can lead to *inhibition of mitochondrial complex I* by S-nitrosation (Fig. I.12). This inhibition can be reversed by the NO-scavenger PTIO, suggesting that this inhibition of complex I is NO dependent. Inhibition of mitochondrial complex I dampens the electron transfer and was shown to limit ROS (Lesnefsky *et al.*, 2004). Indeed, Shiva *et al.* (2007) found that nitrite decreased H_2O_2 production, preserved the activity of aconitase (a mitochondrial enzyme prone to oxidative damage) and decreased calcium induced mitochondrial permeability transition (MPT) pore opening and mitochondrial cytochrome c release. Reversible inhibition of mitochondrial complex I as a pathway for the nitrite-dependent NO protective effect was also described in a cardiac ischemia/reperfusion model (Dezfulian *et al.*, 2009). As mentioned before, in the hepatic ischemia/reperfusion model of Duranski *et al.* (2005), an *sGC-dependent* protective effect of nitrite was suggested, as pretreatment with the sGC inhibitor ODQ completely inhibited the protective effects of nitrite. An sGC-dependent protective effect of nitrite was also suggested in a model of TNF-induced sepsis, in which TNF is known to cause inflammation accompanied by oxidative stress; treatment with nitrite decreased oxidative stress, mitochondrial damage and mortality, and this protection by nitrite was largely abolished in $sGC\alpha_1$ knockout mice (Cauwels *et al.*, 2009). This NO-sGC-cGMP pathway may contribute to improved vasodilatation and inhibition of platelet aggregation, two processes which will help to maintain the microcirculation of the vital organs. In addition, activation of sGC can also lead to opening of the mitochondrial K_{ATP} channels on the mitochondrial inner membrane, as shown in NO-mediated protection from ischemia/reperfusion injury in isolated mouse hearts (Bell *et al.*, 2003) and in rabbit

cardiomyocytes (Sasaki *et al.*, 2000) with the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP). Opening of K_{ATP} channels has been associated with prevention of mitochondrial permeability transition pore opening and prevention of cytochrome c release from the mitochondrial intermembrane space, thereby preventing cell death (Korge *et al.*, 2002). A third possible mechanism suggested in the protective effect of nitrite is *modulation of the inflammatory response*. Administration of nitrite was shown to significantly attenuate the increase in infiltrating leukocytes in an ischemia/reperfusion model of the brain (Jung *et al.*, 2006) and in a renal ischemia/reperfusion model (Milsom *et al.*, 2010). Although Jung *et al.* (2006) and Milsom *et al.* (2010) did not investigate how nitrite was reducing inflammation, a possibility seems that nitrite-derived NO inhibits transcription factor NF-κB. NO donors indeed showed to inhibit transcription factor NF-κB (Matthews *et al.*, 1996; Shin *et al.*, 1996; Bogdan, 2001); this is associated with a variety of anti-inflammatory effects, minimizing tissue injury in different models of ischemia/reperfusion injury (Phillips *et al.*, 2009).
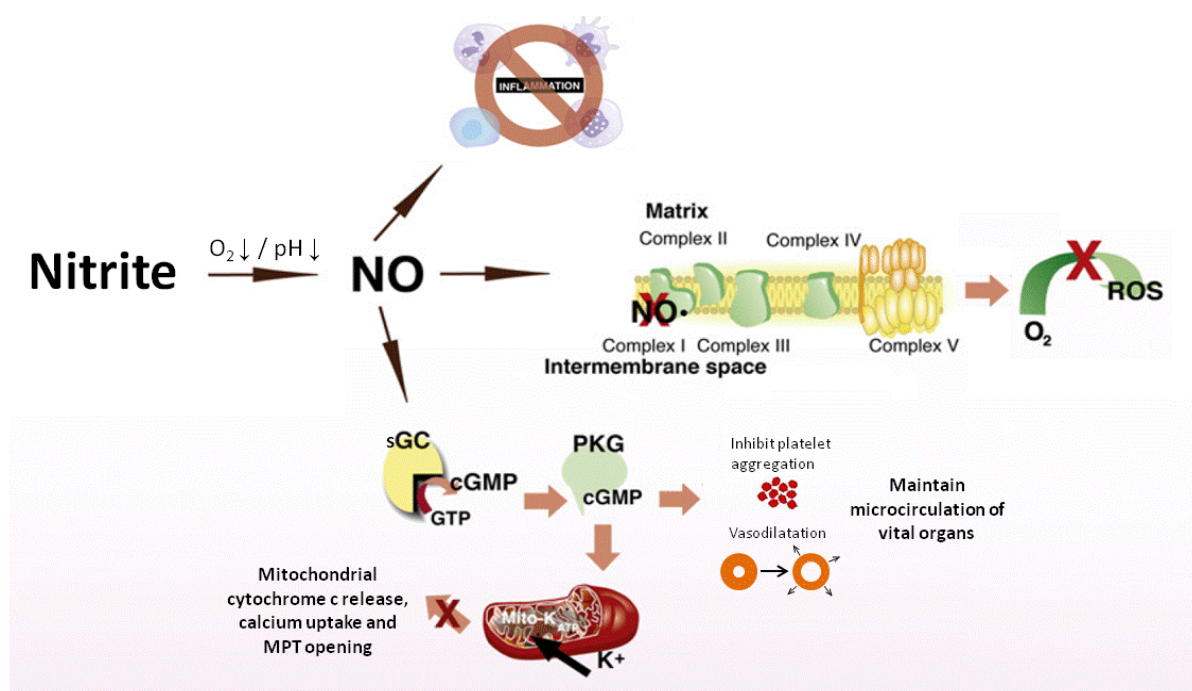
Fig. I.12 Nitrite-dependent NO generation might modulate inflammation, is suggested to inhibit mitochondrial respiration and mitochondrial derived ROS formation, and exerts cGMP-dependent effects. These cGMP-dependent effects consist of vasodilatation and inhibition of platelet aggregation, both maintaining the microcirculation in the vital organs, and opening of the mitochondrial inner membrane K_{ATP} channels, which will lead to the inhibition of MPT pore opening and cytochrome c release (adapted from Raat *et al*., 2009b).

I.4   Postoperative ileus

Postoperative ileus (POI) is defined as a transient impairment of gastrointestinal motility following abdominal surgery (Holte & Kehlet, 2000) and to a lesser extent also following extra-abdominal surgical procedures, such as major cardiothoracic or orthopedic procedures (Bederman *et al.*, 2001; Dong *et al.*, 2012). As a certain degree of POI develops in every patient undergoing abdominal surgery, it is usually considered a normal part of the postoperative course. Interest in this condition has nevertheless arisen from the observation that prolonged ileus can slow patient recovery, prolong hospital stay, increase postoperative morbidity and increase healthcare costs (Table. I.2); the economic impact of POI in the USA is estimated at $750 million per year (Person & Wexner, 2006; Doorly & Senagore, 2012).

Adverse effects of postoperative ileus

Increased incidence of nausea and vomiting
Delayed resumption of oral feeding
Delayed absorption of orally administered medications
Increased postoperative pain
Delayed wound healing
Delayed postoperative ambulation
Increased risk of postoperative complications:
- Atelectasis
- Aspiration pneumonia
- Deep venous thrombosis
- Pulmonary embolism
- Bacterial translocation and sepsis
- Nosocomial infections

Increased length of hospitalization
Increased patient discomfort and decreased satisfaction
Increased healthcare costs

Table I.2 Adverse effects of postoperative ileus (Person & Wexner, 2006).

POI can be classified into normal, physiologic POI and prolonged, paralytic POI. *Physiologic POI* is defined as the interval from surgery until passage of stool or flatus and tolerance of an oral diet, resolving spontaneously within 3 days. *Prolonged POI* is defined as ileus lasting longer than 3 days and is diagnosed if at least two of the following criteria are met, once 3 days have passed: nausea and vomiting, inability to tolerate an oral diet over 24 h, absence of flatus over 24 h, abdominal distension and radiologic confirmation (Vather *et al.*, 2013).

All parts of the gastrointestinal tract are affected during POI and the recovery of each section occurs at different rates, because the different anatomical parts of the gastrointestinal tract differ in mechanical and electrical activity. Immediately after the

operation, the electrical rhythm in the *stomach* becomes disorganized and the migrating motor complexes (MMC) of the fasting patient are usually absent (Clevers *et al.*, 1991). Within 24 hours, the electrical rhythm returns, but with disturbances in its coordination. Contractile waves in the distal part of the stomach can go in the oral direction and gastric emptying remains impaired; a phenomenon that may partially be explained by an increased pyloric tone. This may last up to 3 to 4 days, after which the stomach function resumes normal motor activity (Dauchel *et al.*, 1976). The *small intestine* shows contractile activity shortly after or even during the surgical procedure, but this early activity is irregular and rarely results in normal, coordinated MMC. Only after 3 to 4 days, the small bowel function is expected to turn back to normal (Benson *et al.*, 1994; Miedema *et al.*, 2002). The *colon* is usually the last section of the gastrointestinal tract to return to normal function. Initial electrical activity is characterized by disorganized bursts, which only lead to coordinated motor activity by the 4th day after operation, marking the resolution of ileus (Benson *et al.*, 1994).

I.4.1 Pathogenesis

The pathogenesis of POI is multifactorial and three main mechanisms are known to contribute: neurogenic inhibitory reflexes, inflammatory responses and pharmacological factors (Bauer & Boeckxstaens, 2004). Studies in experimental models showed that postoperative ileus after abdominal surgery is triggered in two phases: an acute neurogenic phase (starts during surgery and ends soon after) and a prolonged inflammatory phase (starts ± 3 h after surgery) (Fig. I.13). The importance of each mechanism varies over time, with considerable overlap and possible interactions (Boeckxstaens & de Jonge, 2009).
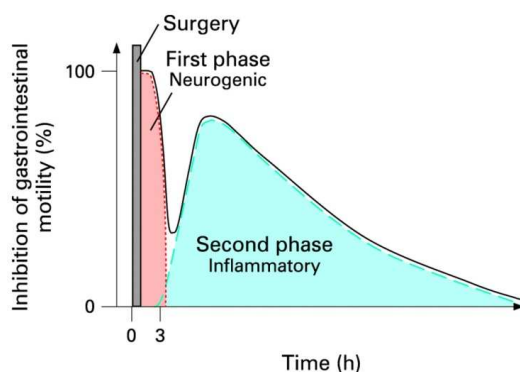
Fig. I.13 The two phases involved in postoperative ileus. The neurogenic phase starts during surgery and ends shortly after. The inflammatory phase starts approximately 3 h after surgery and lasts much longer (Boeckxstaens & de Jonge, 2009).

Neurogenic phase. The most important factor believed to cause the acute phase of POI is impairment of the normal function of the autonomic nervous system. The *sympathetic nervous system* plays a major role in neurogenic inhibitory reflexes: afferent neurons originate both in the site of skin incision (somatic fibers) and in the intestines (visceral fibers), and efferent neurons conduct impulses back towards the enteric nervous system, which will result in decreased motility (Person & Wexner, 2006). The *parasympathetic nervous system* also plays a role: vagally mediated pathways will contribute to inhibition of gastrointestinal motility by synapsing to inhibitory nitrergic (NO) and VIPergic (VIP) neurons (Boeckxstaens *et al.*, 1999).

The activation of various neural pathways depends on the intensity of the nociceptive stimulus and the part of the intestine studied. *Skin incision or laparotomy* alone results in a short reduction of gastrointestinal motility, probably due to activation of a low-threshold spinal reflex. The laparotomy activates spinal afferents, which synapse in the spinal cord where they activate an inhibitory pathway involving prevertebral noradrenergic neurons, abolishing the motility of the entire gastrointestinal tract (Fig I.14A). *More intense stimuli*, such as intestinal manipulation, result in prolonged inhibition of gastrointestinal motility by triggering an additional high-threshold supraspinal pathway involving hypothalamic neurons (Holzer *et al.*, 1992; Boeckxstaens *et al.*, 1999). Within this pathway, corticotrophin-releasing factor (CRF) seems to play a central role. Afferent signals are transmitted to the brainstem where they trigger the release of CRF, which stimulates neurons in the hypothalamus. From the hypothalamus, projections are sent to the spinal cord, from where sympathetic preganglionic neurons will synapse to prevertebral noradrenergic neurons. Activation of these nerves will lead to prolonged inhibition of the motility of the entire gastrointestinal tract (Taché *et al.*, 1993). In addition to this noradrenergic inhibitory pathway, the motor nucleus of the vagus nerve is activated, synapsing to inhibitory nitrergic (NO) and VIPergic (VIP) neurons (Fig I.14B) (De Winter *et al.*, 1997; De Winter *et al.*, 1998).

A   **Neural pathways induced by laparotomy**

B   **Neural pathways during intestinal manipulation**
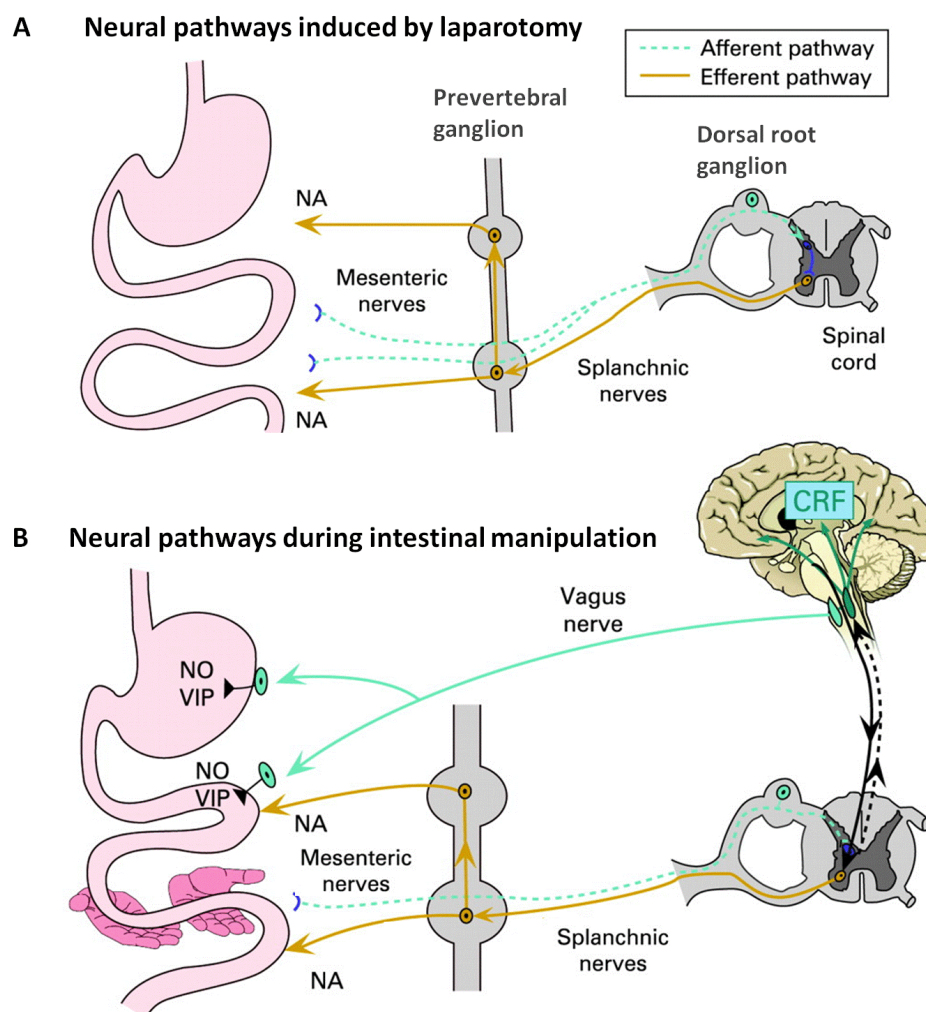
Fig. I.14   Representation of the neural pathways involved in the inhibition of gastrointestinal motility induced by laparotomy (A) and more intense intestinal manipulation (B). Laparotomy activates spinal afferents, which synapse in the spinal cord, leading to activation of an inhibitory pathway involving prevertebral noradrenergic neurons. The release of noradrenaline (NA) will abolish the motility of the entire gastrointestinal tract. More intense stimuli, such as intestinal manipulation, transmit afferent signals to the brainstem where they trigger the release of corticotrophin-releasing factor (CRF). CRF will stimulate neurons in the hypothalamus, which sends projections to the spinal cord. Spinal efferents will then synapse to prevertebral neurons releasing NA. Activation of these noradrenergic neurons will result in prolonged inhibition of gastrointestinal motility. In addition to this noradrenergic inhibitory pathway, the motor nucleus of the vagus nerve is activated, synapsing to inhibitory nitrergic (NO) and VIPergic (VIP) neurons (Boeckxstaens & de Jonge, 2009).

Inflammatory phase. The inflammatory phase lasts much longer than the neurogenic phase. It is characterized by the activation of resident macrophages present in the intestinal muscle layer (Kalff *et al.*, 1998; Wehner *et al.*, 2007). Activated macrophages will release inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6), chemokines such as monocyte-chemoattractant protein-1 (MCP-1) and adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1). MCP-1 and ICAM-1 will recruit more

circulatory leukocytes, and, together with the resident macrophages, these will enhance the release of NO and prostaglandin, through upregulation of inducible NO synthase (iNOS) and cyclo-oxygenase-2 (COX-2) respectively. Both NO and prostaglandin have potent inhibitory effects on the gastrointestinal tract and cause ileus (Kalff *et al.*, 1999b; Kalff *et al.*, 2000; Schwarz *et al.*, 2001; Turler *et al.*, 2006).

The mechanisms leading to activation of the resident macrophages are not fully elucidated. A *first* proposed mechanism suggests that intestinal CD11b$^+$CD103$^+$ dendritic cells are involved (Engel *et al.*, 2010). These dendritic cells are typically found in the lamina propria of the small intestine and can be activated by luminal antigens. The activated dendritic cells will release interleukine-12 (IL-12), which will promote memory T helper type 1 (T$_{H1}$) cells to secrete interferon-γ (IFN-γ). IFN-γ will then activate the resident macrophages. In addition, IL-12 can cause some T$_{H1}$ cells to migrate from a manipulated area to unmanipulated areas, which will contribute to impairment of motility of the entire gastrointestinal tract. A *second* possible route of resident macrophage activation, is through the release of damage-associated molecular patterns (DAMPs) in response to tissue damage evoked by intestinal manipulation (Bauer, 2008) (Fig. I.15). A *third* mechanism, proposed by the group of Guy Boeckxstaens (Translational Research in GastroIntestinal Disorders (TARGID), KU Leuven, Belgium), is degranulation of connective tissue mast cells in mesentery, lamina propria and serosa. Their results are based on mast cell-deficient mouse models with abnormal Kit signaling, the measurement of mast cell mediators (proteases and tryptases) and experiments with the mast cell stabilizers ketotifen and doxantrazole (de Jonge *et al.*, 2004; The *et al.*, 2008). In line with their theory, neuropeptides, such as substance P or calcitonin gene-related peptide (CGRP) released from afferent nerves, are believed to activate the mast cells (Bueno *et al.*, 1997). Once activated, mast cell mediators (such as histamine and proteases) will diffuse into the mesenteric blood vessels, after which they will cause increased mucosal permeability with translocation of intraluminal bacteria and activation of resident macrophages (Fig. I.15) (Snoek *et al.*, 2011); luminal bacteria and their products only start to appear in the muscularis externa 6 h after intestinal manipulation (Schwarz *et al.*, 2002), whereas the immune response is induced shortly after manipulation e.g. ICAM-1 mRNA is already expressed 15 min after manipulation (Kalff *et al.*, 1999a), implying that translocated bacteria are not the trigger of the immune response in the muscularis, but rather strengthen the immune response (Snoek *et al.*, 2011). A very

recent publication by the same group now questions 10 years of elaborated research on the involvement of mast cells in the pathogenesis of POI (Gomez-Pinilla *et al.*, 2014), claiming that the "mast cell" mediators measured can be released by other immune cells, that the "mast cell" stabilizers used are not specific for mast cells, and that the use of mast cell deficient mice based on Kit mutations have alterations in multiple cell types of both immune and non-immune origin in addition to the mast cell defect. Gomez-Pinilla *et al.* (2014) used a mouse model with specific mast cell depletion, and apart from a reduction in basophile numbers, other subpopulations of immune cells were intact. They demonstrated that the previously studied mast cell deficient mice based on Kit mutations had a clearly delayed intestinal transit per se and showed no further delay in intestinal transit after intestinal manipulation. However, the mice with specific mast cell depletion did show a delay in transit after intestinal manipulation, just as in controls. These recent results suggest that mast cells are not required for the development of POI. Further research into the extent to which mast cells are involved in the pathogenesis of POI is definitely required.
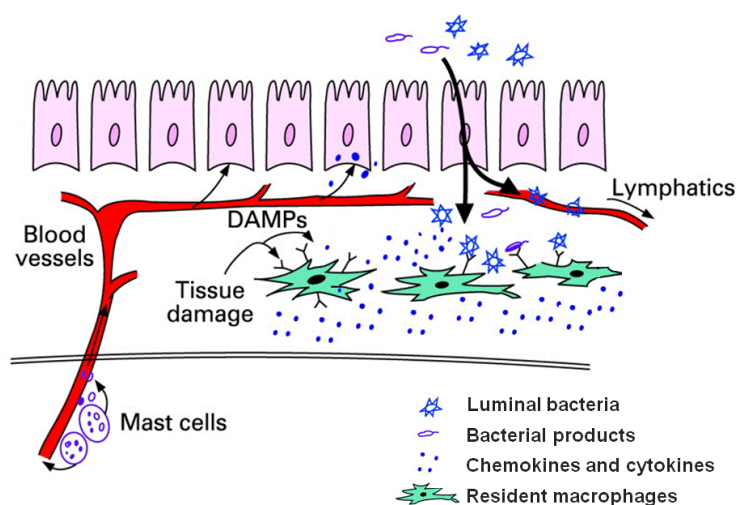
Fig. I.15 Mast cells located closely to the mesenteric vessels are activated after intestinal manipulation and release mast cell mediators, diffusing into the mesenteric blood vessels. These mediators will increase the mucosal permeability, allowing entrance of luminal bacteria and bacterial products to enter the lymphatic circulation (contributing to the pathogenesis of POI in unmanipulated areas) or to interact with the resident macrophages. In addition, the release of damage-associated molecular patterns (DAMPs) in response to tissue damage evoked by intestinal manipulation can also activate the resident macrophages (Boeckxstaens & de Jonge, 2009).

Interestingly, reactive oxygen species (ROS) might also contribute to POI. Anup *et al.* (1999) demonstrated that surgical manipulation of the rat intestine resulted in oxidative stress in the mucosa, as evidenced from an increase in xanthine oxidase activity in the enterocytes. This was associated with widened intercellular spaces and increased mucosal

permeability; changes which were prevented by pretreatment of the animals with xanthine oxidase inhibitors (Anup *et al.*, 2000). In addition, our group previously reported an increase in oxidative stress levels in both mucosa and muscularis of the mouse small intestine starting shortly after intestinal manipulation; reducing ROS generation (with the carbon monoxide-releasing molecule CORM-3) correlated with a positive effect on postoperative intestinal transit and might thus help to reduce ileus (De Backer *et al.*, 2009).

Pharmacological factors. Many agents that are commonly used in *general anesthesia* may impair gastrointestinal motility (Schurizek, 1991; Ogilvy & Smith, 1995). However, most new gaseous anesthetic agents (desflurane and sevoflurane) and intravenous anesthetics (propofol) have a relatively short half-life and they seldom cause the typical prolonged POI. The fact that the incidence and severity of POI in patients who undergo non-abdominal procedures under general anesthesia is very low, also supports the notion that the overall contribution of general anesthesia for the etiology of POI is small (Person & Wexner, 2006)

Opioids, administered for post-operative pain relief, are well known to interfere with normal gastrointestinal motility and can actually complicate and prolong POI. The main function of opioids is to suppress neuronal excitability. Three types of opioid receptors are believed to play a role in mediating the effect of opioids: μ, δ and κ receptors, with several subtypes in each class. By binding to the opioid receptors, opioids decrease the membrane potential which prevents the generation of action potentials necessary for release of neurotransmitters at synapses, neuromuscular junctions and neuroepithelial junctions. The effects of opioids on gastrointestinal motility are largely a reflection of the suppression of excitability and inhibition of neurotransmitter release from enteric excitatory and inhibitory neurones (Pasternak, 1993; Wood & Galligan, 2004). The receptor primarily involved in the adverse effects of opioids on gastrointestinal motility is the μ-receptor (De Schepper *et al.*, 2004). Therefore, the combination of peripherally acting μ-opioid receptor antagonists with opioid analgesics to improve motility but not altering the analgesic effect is currently an active area of research (see I.4.2).

I.4.2   Management of POI

Prevention or treatment of POI remains mainly supportive and no single standard treatment is currently available. Because of its multifactorial origin, treating POI generally consists of a multimodal approach, also referred to as fast-track surgery. The fast-track concept was first introduced by Kehlet *et al.* (1997). By targeting multiple factors that delay postoperative recovery, this approach has shown to reduce complications, accelerate recovery and reduce hospital stay. Fast-track protocols have been used successfully in patients undergoing several types of abdominal surgery, but most data are available for colorectal surgery (Ansari *et al.*, 2013). The multimodal approach for the prevention or treatment of POI includes early enteral feeding and mobilization, minimally invasive laparoscopy and epidural local anesthetics. Additional measures include peripheral opioid receptor antagonists, opioid-free analgesia (NSAIDs), laxatives, chewing gum and avoidance of routine nasogastric tubes and fluid excess (Bauer & Boeckxstaens, 2004; Kehlet, 2008; Vather & Bissett, 2013) (Table I.3).

Non-pharmacological treatment options	
Treatment	*Potential Mechanism*
Early enteral nutrition (or gum chewing)	Stimulates GI motility by eliciting reflex response and stimulating release of several hormonal factors
Early mobilization	Possible mechanical stimulation
Laparoscopic surgery	Decreased opiate requirements, decreased pain, less abdominal wall trauma

Pharmacological treatment options	
Treatment	*Potential Mechanism*
Laxatives	Stimulant, prokinetic effects
Opiate antagonists	Block peripheral opiate receptors
Epidural anesthesia	Inhibits sympathetic reflex at cord level, opioid-sparing analgesia
NSAIDs	Opiate-sparing analgesia, inhibits COX-mediated prostaglandin synthesis

Table I.3 Management of postoperative ileus by non-pharmacological and pharmacological treatment options (Behm & Stollman, 2003).

Avoid nasogastric tubes. Up to 2002, prophylactic nasogastric decompression following abdominal surgery was a routine procedure with the intention of hastening the return of bowel function, decreasing the risk of pulmonary aspiration, increasing the patient comfort by lessening abdominal distension and shortening hospital stay. However, recent re-evaluation of the use of nasogastric tubes has clearly demonstrated that none of these goals are met. Contrarily, it might contribute to pulmonary morbidity. Nasogastric intubation should therefore not be used routinely (Nelson *et al.*, 2005; Bauer, 2013).

Limit fluid therapy. The use of isotonic dextrose-saline crystalloid solution as a maintenance fluid is common practice postoperatively. However, several randomized studies have shown that fluid excess prolongs POI. Generous perioperative fluid administration can lead to intestinal edema, which might even contribute to the development of POI. Lobo *et al.* (2002) showed that restrictive postoperative fluid prescription following colonic resection was associated with a significantly faster return of gastrointestinal function when compared to standard fluid therapy. Therefore, administration of maintenance fluid within a restrictive regime should be considered (Holte *et al.*, 2002; Vather & Bissett, 2013).

Laparoscopy. In numerous studies, it was shown that the use of minimal invasive techniques produced less tissue trauma, resulting in reduced postoperative pain, less need for intense postoperative analgesia, a weaker immune response and consequently a reduction in time for ileus to resolve. Whether the reduced length of POI after laparoscopy is a result of the reduction of postoperative pain and opioid use, a reduction of the inflammatory response, or additional factors is not clear (Person & Wexner, 2006; Xu & Chi, 2012).

Epidural anesthesia and analgesia. As described earlier, activation of neurogenic inhibitory reflexes originating from incision and manipulation of the intestine have been proposed to play a role in POI. It has been hypothesized that epidural anesthesia and analgesia may decrease POI by blocking the afferent and efferent sympathetic inhibitory reflexes (Liu *et al.*, 1995). Indeed, thoracic epidural anesthesia and analgesia have shown beneficial effects on recovery of bowel function after major abdominal surgery (Steinbrook, 1998; Holte & Kehlet, 2000).

Opioid-sparing analgesia. NSAIDs will reduce the need for opioids. Additionally, because of the role of prostaglandins in the inflammatory response, it has been suggested

that NSAIDs, by inhibition of COX-2, may be used to increase gastrointestinal motility postoperatively. Indeed, NSAIDs were shown to be effective in managing postoperative ileus and preventing prolonged POI (Story & Chamberlain, 2009; Wattchow *et al.*, 2009).

Peripheral opioid receptor antagonists. The peripherally acting μ-opioid receptor antagonists alvimopan and methylnaltrexone (both FDA approved since 2008) were designed to reverse opioid-induced side-effects on gastrointestinal motility without compromising pain relief. Both compounds seem well tolerated and effective for the treatment of opioid-related gastrointestinal dysfunction. Further research should assess the safety and effectiveness of these drugs in clinical practice (Traut *et al.*, 2008; Becker & Blum, 2009; Wang *et al.*, 2012).

Prokinetic agents and laxatives. Although prokinetic agents were shown to be effective in treating POI in animal studies, currently approved prokinetics are generally not effective in human studies. Traut *et al.* (2008) analyzed 39 randomized control trials that included the use of 15 prokinetic agents (including cisapride, erythromycin, lidocaine and neostigmine) to evaluate their potential benefits in POI. For most prokinetic agents, there was no positive effect observed, or the evidence was not sufficiently conclusive to attribute a beneficial effect. Intravenous lidocaine and neostigmine may be beneficial in certain situations, though further studies are needed (Traut *et al.*, 2008; Story & Chamberlain, 2009). Laxatives, such as bisacodyl and magnesium oxide, have shown beneficial effects with respect to gastrointestinal recovery after abdominal surgery (Fanning & Yu-Brekke, 1999; Wiriyakosol *et al.*, 2007; Zingg *et al.*, 2008). Given the safety and low cost of laxatives, postoperative laxatives can be added as part of the multimodal approach for POI.

Ambulation. Early postoperative mobilization is advised, although there is actually no evidence that proved it to be beneficial for the duration of POI. However, as prolonged immobilization after surgery has never been proven to be beneficial (Allen *et al.*, 1999) and early ambulation is believed to reduce the risk of developing postoperative respiratory and thrombotic complications, early mobilization after abdominal surgery should be encouraged (Waldhausen & Schirmer, 1990; Kibler *et al.*, 2012).

Early postoperative oral feeding. The intake of food causes a reflex response that is propulsive in action. In addition, the presence of food stimulates the secretion of various intestinal hormones, with a general stimulating effect on gastrointestinal motility. One might thus expect early postoperative feeding to reduce the length of POI. Several clinical

trials have shown the institution of early enteral feeding to be safe, but not all of them found beneficial effects for the duration of ileus (Holte & Kehlet, 2000; Han-Geurts *et al.*, 2007). Nevertheless, since early feeding after gastrointestinal surgery did not show any deleterious effects and might even reduce the incidence of infectious complications, postoperative enteral feeding should be included as routine in perioperative care (Lewis *et al.*, 2001).

Gum chewing. Chewing gum following abdominal surgery showed significant benefits in reducing the duration of POI (Li *et al.*, 2013). It must be noted however that, for the subgroup of colectomies, inconsistent results were found. Well-designed, large-scale randomized trials are needed to answer the question whether gum chewing can significantly reduce POI after different abdominal surgeries. A possible explanation for the reported effect of chewing gum on POI is that it acts as sham feeding, stimulating the motility of the gastrointestinal tract and triggering the release of gastrointestinal hormones, saliva, pancreatic juice, gastrin and neurotensin. Since chewing gum is cheap, well tolerated and free of side effects, it may be added to the multimodal approach to deal with POI.

When some of these techniques are combined as part of the concept of multimodal postoperative rehabilitation (fast-track surgery), the duration of POI after abdominal surgery can be reduced to 24–48 h in most patients (Kehlet, 2008; Story & Chamberlain, 2009). Additional studies are needed to make specific recommendations regarding to which components of the fast-track protocols are most beneficial in the different kinds of abdominal surgeries. Though many of these combined strategies have proven to be beneficial, treatment of POI remains mostly supportive and no prevention currently exists. The inflammation-related dysmotility during POI has been addressed only recently and, with the exception of the NSAIDs targeting COX-2, its prevention and treatment have not yet been incorporated into clinical practice. It is believed that drugs interfering with the inflammatory response might have great potency to shorten POI and consequently hospitalization (Boeckxstaens & de Jonge, 2009). Of course these new drugs will have to prove their superiority against the fast-track approach, or they should be incorporated in it. In this sense, treating POI with nitrite can be considered. Although ischemia/reperfusion injury is associated with an upregulation of iNOS (Iadecola *et al.*, 1995; Wang *et al.*, 2003) and selective iNOS inhibitors can prevent ischemia/reperfusion injury (Barocelli *et al.*, 2006)

similar to POI (Kalff *et al.*, 2000; Turler *et al.*, 2006), administration of nitrite showed to be beneficial for ischemia/reperfusion injury. Hence our interest to test nitrite in a POI model (see Chapter VI)."

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Chapter II

AIMS

Chapter II Aims

The principal intracellular receptor for NO as smooth muscle cell relaxant, is sGC (Hobbs, 1997). In both physiologically functional isoforms of sGC (sGC$\alpha_1\beta_1$ and sGC$\alpha_2\beta_1$), NO binds to heme that is linked to histidine 105 in the β_1 subunit. This will generate cGMP, that mediates smooth muscle cell relaxation (Feil *et al.*, 2003; Toda & Herman, 2005). In the cardiovascular system, this nitrergic relaxation is therapeutically applied by use of NO donors such as organic nitrates. Dysfunction of the endothelium is found in several cardiovascular diseases and will lead to NO deficiency. Organic nitrates proved to be effective NO donors in a variety of cardiovascular disorders (Ignarro *et al.*, 2002). However, the development of tolerance limits the continuous clinical application of NO donors (Munzel *et al.*, 2011; Iachini *et al.*, 2012). Tolerance is associated with increased oxidative stress (Munzel *et al.*, 2011), as are some pathological conditions such as heart failure and pulmonary hypertension (Konduri *et al.*, 2007; Mitrovic *et al.*, 2009). Oxidative stress interferes with the NO-sGC-cGMP pathway through scavenging of NO and formation of ROS, that oxidize sGC towards an NO-insensitive heme-free status (Fritz *et al.*, 2011); treatment with NO donors becomes thus less effective. During the last 15 years, two novel drug classes have been discovered that seem to address these problems: the heme-dependent sGC stimulators and the heme-independent sGC activators (Evgenov *et al.*, 2006). These novel classes of compounds are capable of activating the reduced and/or oxidized/heme-free state of sGC in an NO-independent manner. sGC stimulators are capable of directly stimulating the reduced form of sGC, acting in synergy with NO, but they can also stimulate reduced sGC independently of NO, allowing to circumvent conditions with decreased endogenous generation of NO (Stasch & Hobbs, 2009). sGC activators preferably activate the oxidized/heme-free enzyme (Schmidt *et al.*, 2009); these drugs should thus target the enzyme more extensively in pathological conditions associated with oxidative stress.

In the gastrointestinal system, NO synthesized by nNOS and released from NANC neurons will target sGC and induce gastrointestinal smooth muscle relaxation. This contributes to the control of gastrointestinal motility, as evident from the delay in gastric emptying and intestinal transit upon NOS inhibition or in nNOS knockout mice (Huang *et al.*,

1993; Karmeli *et al.*, 1997; Mizuta *et al.*, 1999; Mashimo *et al.*, 2000; Chiba *et al.*, 2002; Fraser *et al.*, 2005). Impaired nitrergic innervation of gastrointestinal smooth muscle plays a crucial role in several disorders with gastrointestinal dysmotility, such as functional dyspepsia, esophageal achalasia, infantile hypertrophic pyloric stenosis, delayed gastric emptying after vagotomy and Hirschsprung's disease (Goyal & Hirano, 1996; Takahashi, 2003). NO donors, such as organic nitrates, have not been applied frequently in these gastrointestinal disorders, also because of the well-known attenuation of their effect after long term usage due to the development of tolerance (Robson & Wilkinson, 1946). Aging and diseases such as colitis and diabetes can lead to enteric nitrergic neuronal dysfunction and motility disturbances (Mizuta *et al.*, 2000; Phillips & Powley, 2007; Zandecki *et al.*, 2008). These conditions are associated with oxidative stress (Kashyap & Farrugia, 2011; Cannizzo *et al.*, 2011; Zhu & Li, 2012). ROS were suggested to increase the likelihood of damage to enteric nitrergic neurons (Rivera *et al.*, 2011); additionally, it can be expected that enteric sGC will be driven to the oxidized/heme-free status in these conditions, making it unresponsive towards endogenous NO but also NO donors. Direct sGC stimulation and/or activation might thus also be useful in gastrointestinal disorders. Reports on the gastrointestinal effects of sGC stimulators/activators are however limited.

Recently, sGC$\beta_1^{His105Phe}$ knockin (apo-sGC) mice were developed (Thoonen *et al.*, 2009). The histidine 105 residue of the β_1 subunit is a crucial amino acid for the binding of the heme group to sGC (Schmidt *et al.*, 2004); the resulting heme-deficient sGC isoforms retain their basal activity but can no longer be activated by NO (Wedel *et al.*, 1994). These apo-sGC mice can thus be considered as a model for oxidized/heme-free sGC. Apo-sGC mice are characterized by a reduced live span, growth retardation and elevated blood pressure (Thoonen *et al.*, 2009). Our first aim was to investigate the consequences of inducing a heme-free status of sGC on gastrointestinal nitrergic signaling and motility. These results are summarized in **chapter III**.

BAY 41-2272 is an NO-independent heme-dependent sGC stimulator, but its relaxant effect in vascular, respiratory and urogenital tissue is only partially dependent on sGC activation. As its effect and mechanism of action have not yet been studied in the gastrointestinal tract, it was investigated in mouse gastric fundus and colon. The results are summarized in **chapter IV**.

Cinaciguat, an NO- and heme-independent sGC activator, was shown to be more effective when sGC is oxidized in vascular tissue. In our third study, we compare the influence of cinaciguat on *in vitro* smooth muscle tone of gastrointestinal tissues and on gastric emptying in WT and apo-sGC mice. These results are summarized in **chapter V**.

Next to the sGC stimulators/activators as alternatives to classic NO pharmacotherapy, we also looked at the inorganic anion nitrite (NO_2^-), which has been reported to be a source of NO under hypoxic conditions (Lundberg *et al.*, 2008). Exogenous administration of nitrite showed to protect the heart, liver, kidney and brain from ischemia/reperfusion injury; a possible mechanism of action is activation of sGC by NO, produced from nitrite under hypoxic conditions (Duranski *et al.*, 2005; Dezfulian *et al.*, 2007; Raat *et al.*, 2009). An sGC-dependent protective effect of nitrite-derived NO was also suggested in a model of TNF-induced sepsis (Cauwels *et al.*, 2009). Postoperative ileus is a transient impairment of gastrointestinal motility commonly seen after abdominal surgery. The surgical handling of the bowel during abdominal surgery leads to muscular inflammation (Bauer & Boeckxstaens, 2004) and oxidative stress (De Backer *et al.*, 2009), two factors known to also play a major role in ischemia/reperfusion injury and sepsis. The aim of our last study was therefore to investigate whether nitrite also has a protective, possibly sGC dependent, effect in a model of postoperative ileus. These results are summarized in **chapter VI**.

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Chapter III

HEME DEFICIENCY OF SOLUBLE GUANYLATE CYCLASE INDUCES GASTROPARESIS

Sarah M. R. Cosyns [1], Ingeborg Dhaese [1], Robrecht Thoonen [2,3], Emmanuel S. Buys [4], Anne Vral [5], Peter Brouckaert [2], Romain A. Lefebvre [1]

[1] Heymans Institute of Pharmacology, Ghent University, Ghent, Belgium

[2] Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

[3] Tufts Medical Center, Molecular Cardiology Research Center, Boston, USA

[4] Anesthesia Center for Critical Care Research, Department of Anesthesia, Critical Care and Pain Medicine, Harvard Medical School, Boston, USA

[5] Department of Medical Basic Sciences, Ghent University, Ghent, Belgium

Chapter III

Heme deficiency of soluble guanylate cyclase induces gastroparesis

III.1 Abstract

Background. Soluble guanylate cyclase (sGC) is the principal target of nitric oxide to control gastrointestinal motility. The consequence on nitrergic signalling and gut motility of inducing a heme-free status of sGC, as induced by oxidative stress, was investigated.

Methods. $sGC\beta_1^{His105Phe}$ knockin (apo-sGC) mice, which express heme-free sGC that has basal activity but cannot be stimulated by NO, were generated.

Key Results. Diethylenetriamine NONOate did not increase sGC activity in gastrointestinal tissue of apo-sGC mice. Exogenous NO did not induce relaxation in fundic, jejunal and colonic strips, and pyloric rings of apo-sGC mice. The stomach was enlarged in apo-sGC mice with hypertrophy of the muscularis externa of the fundus and pylorus. In addition, gastric emptying and intestinal transit were delayed and whole gut transit time was increased in the apo-sGC mice, while distal colonic transit time was maintained. The nitrergic relaxant responses to electrical field stimulation at 1-4 Hz were abolished in fundic and jejunal strips from apo-sGC mice but in pyloric rings and colonic strips only the response at 1 Hz was abolished, indicating the contribution of other transmitters than NO.

Conclusions. The results indicate that the gastrointestinal consequences of switching from a native sGC to a heme-free sGC, that cannot be stimulated by NO, are most pronounced at the level of the stomach establishing a pivotal role of the activation of sGC by NO in normal gastric functioning. In addition, delayed intestinal transit was observed, indicating that nitrergic activation of sGC also plays a role in the lower gastrointestinal tract.

III.2 Introduction

Nitric oxide (NO), synthesized by neuronal NO synthase (nNOS, NOS-1) and released from non-adrenergic non-cholinergic (NANC) neurons, induces smooth muscle relaxation and contributes to the control of gastrointestinal motility, as evident from the delay in gastric emptying and intestinal transit upon NOS inhibition or in nNOS knockout mice (Huang *et al.*, 1993; Karmeli *et al.*, 1997; Mizuta *et al.*, 1999; Mashimo *et al.*, 2000; Chiba *et al.*, 2002; Fraser *et al.*, 2005). The principal intracellular target of NO is the heme-protein soluble guanylate cyclase (sGC), generating the second messenger cyclic guanosine 3'-5'-monophosphate (cGMP) to induce smooth muscle relaxation (Toda & Herman, 2005). However, sGC-independent relaxant effects of NO involving activation of small conductance Ca^{2+}-dependent K^+ channels have been described in duodenum (Martins *et al.*, 1995; Serio *et al.*, 2003) and colon (Van Crombruggen & Lefebvre, 2004). Additionally, sGC can be activated by other stimuli than NO such as carbon monoxide (CO). Even though CO is a very weak activator of purified sGC (3-fold) (Friebe *et al.*, 1996), CO-induced relaxation in gastrointestinal smooth muscle is inhibited by the sGC inhibitor ODQ (De Backer *et al.*, 2008b). CO has been proposed as a gastrointestinal inhibitory neurotransmitter (Hidaka *et al.*, 2010) or as an endogenous hyperpolarizing factor (Sha *et al.*, 2007).

The physiologically active isoforms of sGC are $sGC\alpha_1\beta_1$, the predominant isoform in the gastrointestinal tract, and $sGC\alpha_2\beta_1$ (Mergia *et al.*, 2003). We previously reported that in the gastrointestinal tract $sGC\alpha_2\beta_1$ can compensate at least partially for the absence of $sGC\alpha_1\beta_1$ as gastric emptying was only mildly impaired and small intestinal transit was not influenced in $sGC\alpha_1$ knockout mice (Vanneste *et al.*, 2007; Dhaese *et al.*, 2009). Full knockout of sGC, eliminating activation of both sGC isoforms by NO but also basal sGC activity, is associated with systemic hypertension, severely delayed gut transit and premature death (Friebe *et al.*, 2007). Smooth muscle-specific deletion of sGC was associated with hypertension and loss of vascular muscle responsiveness to NO (Groneberg *et al.*, 2010) but the responsiveness to NO of gastrointestinal muscle was only mildly reduced, suggesting that sGC in gastrointestinal muscle is dispensable for nitrergic relaxation (Groneberg *et al.*, 2011). Selective deletion of sGC in the interstitial cells of Cajal (ICC) did not induce a decrease in NO responsiveness of gastrointestinal muscle. Deletion of sGC in both smooth muscle and ICC resulted in an impairment of nitrergic relaxation and an

increase in gut transit time that was similar to that in mice lacking sGC ubiquitously (Lies *et al.*, 2011). Together, these results suggest a redundant action of sGC in ICC and smooth muscle cells to induce gastrointestinal nitrergic relaxation.

The prosthetic heme group that interacts with the β_1 subunit of sGC is essential for the activation of both sGC isoforms by NO. Oxidation of sGC by reactive oxygen species (ROS) results in an NO-insensitive heme-free enzyme (Fritz *et al.*, 2011); the latter status of sGC might contribute to disturbed vasodilatation under oxidative stress (Stasch *et al.*, 2006). Oxidative stress is also involved in diabetic gastroparesis leading to dysfunction of nitrergic nerves and ICCs (Kashyap & Farrugia, 2010); however it is unclear whether oxidation of sGC contributes to disturbed gastric nitrergic relaxation in this condition. The histidine 105 residue of the β_1 subunit is a crucial amino acid for the binding of the heme group to sGC (Schmidt *et al.*, 2004). Recently, sGC$\beta_1^{His105Phe}$ knockin (apo-sGC) mice were developed (Thoonen *et al.*, 2009)**;** the resulting heme-deficient sGC isoforms retain their basal activity but can no longer be activated by NO (Wedel *et al.*, 1994). The apo-sGC mice are characterized by a reduced life span, growth retardation and elevated blood pressure (Thoonen *et al.*, 2009)**.** In the present study, we investigated the consequence of switching native sGC to heme-free sGC, that cannot be stimulated by NO, in apo-sGC mice on gastrointestinal nitrergic signalling and motility.

III.3   Materials and methods

III.3.1 Ethical approval

All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

III.3.2 Animals

sGC$\beta_1^{His105Phe}$ knockin mice (apo-sGC) were generated by homologous recombination in which the targeting vector introduces a mutation of the histidine residue at position 105 (exon 5) to phenylalanine, as well as 5 silent mutations. Correct recombination and germline transmission was confirmed using PCR and Southern Blot.

Homozygous sGCβ$_1$^{His105Phe} knockin (apo-sGC) mice and wild type (WT) controls were derived from a heterozygous breeding on a mixed background (129/SvJ-C57Bl/6J). WT and apo-sGC mice of both sexes (male: n = 79 [WT] and 84 [apo-sGC], 7-15 weeks; female: n = 36 [WT] and 36 [apo-sGC], 7-16 weeks) had free access to regular drinking water and Transbreed Chow (SDS). However, when investigating transit using the phenol red, fluorescein-labelled dextran, or the colonic bead expulsion method (see below), food was withheld for 16 hours overnight with free access to water.

III.3.3 Muscle tension experiments

III.3.3.1 Tissue preparation

Animals were sacrificed by cervical dislocation and the gastrointestinal tract was put in aerated (5 % CO_2 in O_2) Krebs solution (composition in mM: NaCl 118.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1). The stomach was emptied from its contents and weighed. Two full wall thickness fundus strips (2 x 11 mm) were prepared by cutting in the direction of the circular muscle layer; one full wall thickness ring (width: 2 mm) was prepared from the pyloric region. A ~ 5 cm long fragment of small bowel, starting approximately 10 cm distal to the pylorus, and a ~ 4 cm long segment of distal colon, taken above the pelvic brim, were isolated and opened along the mesenteric border. The mucosa was removed by sharp dissection under a microscope and two full-thickness muscle strips (4 × 5 mm) were cut along the circular axis.

III.3.3.2 Isometric tension recording

After a cotton thread (fundus) or a silk thread (USP 4/0; jejunum and colon) was attached to both ends of the strips and two L-shaped tissue hooks were inserted into the pyloric ring, strips and rings were mounted in 5, 7 or 15 ml organ baths between 2 platinum plate electrodes (6 mm apart). The organ baths contained aerated (5 % CO_2 in O_2) Krebs solution, maintained at 37°C. Changes in isometric tension were measured using MLT 050/D force transducers (ADInstruments) and recorded on a Graphtec linearcorder F WR3701 (Graphtec, Yokohama, Japan) or on a PowerLab/8sp data recording system (ADInstruments) with Chart software. Electrical field stimulation (EFS) was performed by means of a Grass

S88 stimulator (fundus, jejunum and colon) or a 4 channel custom-made stimulator (pylorus).

After an equilibration period of 30 min with flushing every 10 min at a load of 0.75 g (fundus), 0.25 g (colon) or 0.125 g (jejunum), the length-tension relationship was determined. Muscle tissues were stretched by load increments of 0.25 g (fundus and colon) or 0.125 g (jejunum) and at each load level exposed to 0.1 (fundus and jejunum) or 1 (colon) μM carbachol to determine the optimal load (L_o; the load at which maximal response to the contractile agent occurred). The pyloric rings were equilibrated at a preliminarily determined L_o of 0.25 g and received carbachol (10 μM) once, in order to check the activity of the tissue. The medium was then switched to Krebs solution containing 1 μM atropine and 4 μM guanethidine to obtain NANC conditions and tissues were allowed to equilibrate for 60 min at L_o with flushing every 15 min in Krebs solution.

III.3.3.3 Protocol in fundic, jejunal and colonic strips

All relaxant stimuli were examined after pre-contraction of the strips with 300 nM (fundus and jejunum) or 3 μM (colon) prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$); relaxations were induced when the contractile response to PGF$_{2\alpha}$ was stable for at least 2 min (10 to 15 min after adding PGF$_{2\alpha}$). In a first series, relaxations were induced by application of EFS (40 V, 0.1 ms, 1-2-4-8 Hz for 10 s [jejunum], 30 s [colon] or 60 s [fundus] at 5 min interval) via the platinum plate electrodes, then by application of exogenous NO (1-10-100 μM with an interval of at least 5 min during which the effect of a given concentration of NO had disappeared) and finally by vasoactive intestinal polypeptide (VIP; 100 nM; 5 min contact time). Strips were washed for 30 min, and were subsequently incubated with the sGC inhibitor 1H[1,2,4,] oxadiazolo [4,3-a]quinoxalin-1-one (ODQ; 10 μM) for 30 min. PGF$_{2\alpha}$ was then applied again and the responses to EFS, NO and VIP were studied again in the presence of ODQ.

In a second series, cumulative contractile responses to carbachol (1 nM – 30 µM) or PGF$_{2\alpha}$ (1 nM - 30 µM) were first obtained in the absence of atropine and guanethidine. In jejunal and colonic strips, the contact time for each concentration of carbachol and PGF$_{2\alpha}$ was fixed at 2 min; in fundic strips, a higher concentration of carbachol and PGF$_{2\alpha}$ was given when the former concentration reached its maximal contractile effect. Then, the influence of ODQ (10 µM) versus 10 µM (fundus) or 100 µM (jejunum and colon) 8-bromoguanosine 3',5' cyclic monophosphate (8-Br-cGMP; 10 min contact time) was studied. In a third series, the influence of the NOS inhibitor N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME; 300 µM) was tested against the relaxation evoked by EFS. In colonic tissues, the PGF$_{2\alpha}$-EFS cycle was repeated a third time in order to test the combination of L-NAME (300 µM) plus the small conductance Ca^{2+}-dependent K^{+} channel blocker apamin (500 nM). In a fourth series of experiments, the relaxing effect of carbon monoxide (CO; 300 µM; 10 min contact time) was studied in fundus and colonic strips.

In all series, the reproducibility of the relaxant responses was evaluated by running time-control vehicle treated strips in parallel. At the end of each experiment, the tissue wet weight was determined (mg wet weight, see data analysis). The drug application protocol for fundus, jejunum and colon strips is shown in Fig. III.1.

III.3.3.4 Protocol in pyloric rings

In a first set of experiments, 6 min after adding PGF$_{2\alpha}$ (3 µM), when the contractile response was stable for at least 2 min, relaxations were induced by application of EFS (40 V, 0.1 ms, 1-2-4 Hz for 10 s at 5 min interval). Then, the pyloric rings were washed for 30 min and the relaxant responses were studied again in the presence of the NOS inhibitor L-NAME (300 µM) or its solvent. In a second set of experiments, the relaxation by exogenous NO (100 µM; 5 min contact time) was studied before and in the presence of the sGC inhibitor ODQ (10 µM) or its solvent. The drug application protocol for the pyloric rings is shown in Fig. III.2.

First series

PGF$_{2\alpha}$
Fundus: 300 nM
Jejunum: 300 nM
Colon: 3 µM

EFS
Fundus: 60 s
Jejunum: 10 s
Colon: 30 s
(5 min interval)

1 Hz 2 Hz 4 Hz 8 Hz

NO
(5 min interval)

1 µM 10 µM 100 µM 100 nM

VIP
(5 min contact time)

Rinsing

ODQ or solvent
10 µM
(30 min incubation)

PGF$_{2\alpha}$
Fundus: 300 nM
Jejunum: 300 nM
Colon: 3 µM

EFS
Fundus: 60 s
Jejunum: 10 s
Colon: 30 s
(5 min interval)

1 Hz 2 Hz 4 Hz 8 Hz

NO
(5 min interval)

1 µM 10 µM 100 µM 100 nM

VIP
(5 min contact time)

Second series

Rinsing

Carbachol or PGF$_{2\alpha}$
Cumulative administration

1 nM 3 nM 10 nM 30 nM 100 nM 300 nM 1 µM 3 µM 10 µM 30 µM

Rinsing

PGF$_{2\alpha}$
Fundus: 300 nM
Jejunum: 300 nM
Colon: 3 µM

8-Br-cGMP
Fundus: 10 µM
Jejunum: 100 µM
Colon: 100 µM
(10 min contact time)

Rinsing

ODQ or solvent
10 µM
(30 min incubation)

PGF$_{2\alpha}$
Fundus: 300 nM
Jejunum: 300 nM
Colon: 3 µM

8-Br-cGMP
Fundus: 10 µM
Jejunum: 100 µM
Colon: 100 µM
(10 min contact time)

Third series

PGF$_{2\alpha}$
Fundus: 300 nM
Jejunum: 300 nM
Colon: 3 µM

EFS
Fundus: 60 s
Jejunum: 10 s
Colon: 30 s
(5 min interval)

1 Hz 2 Hz 4 Hz 8 Hz

Rinsing

L-NAME or solvent
300 µM
(30 min incubation)

PGF$_{2\alpha}$
Fundus: 300 nM
Jejunum: 300 nM
Colon: 3 µM

EFS
Fundus: 60 s
Jejunum: 10 s
Colon: 30 s
(5 min interval)

1 Hz 2 Hz 4 Hz 8 Hz

only in colonic strips

Rinsing

L-NAME + apamin, or solvent
L-NAME: 300 µM
Apamin: 500 nM
(30 min incubation)

PGF$_{2\alpha}$
Colon: 3 µM

EFS
Colon: 30 s
(5 min interval)

1 Hz 2 Hz 4 Hz 8 Hz

Fourth series (only in fundus and colonic strips)

PGF$_{2\alpha}$
Fundus: 300 nM
Colon: 3 µM

300 µM

CO
(10 min contact time)

Fig. III.1   Drug application protocol for fundus, jejunum and colon strips.

First series
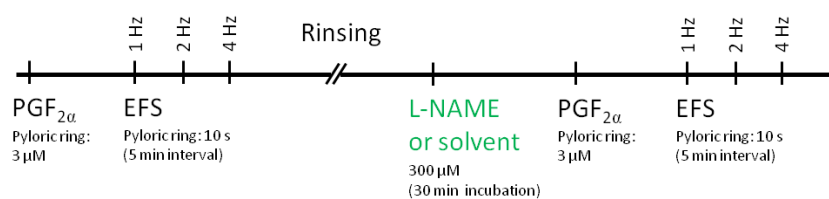
Second series
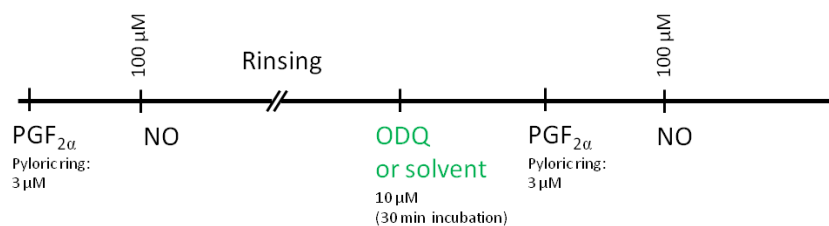
Fig. III.2 Drug application protocol for pyloric rings.

III.3.3.5 Data analysis

In the fundic strips and pyloric rings that show tonic responses, the amplitude of the contractile and relaxant responses can be determined. The amplitude of the responses to EFS was measured at the end of the stimulation train; the amplitude of the responses to carbachol, $PGF_{2\alpha}$, NO and 8-br-cGMP was measured at their maximal effect. The relaxant responses were then expressed as % of the contraction evoked by $PGF_{2\alpha}$. As jejunal and colonic strips show phasic activity, the area under the curve (AUC) above baseline was determined to measure the contractile responses to carbachol and $PGF_{2\alpha}$. To measure relaxant responses in jejunal and colonic strips, the AUC for a given response was determined and subtracted from the AUC of a corresponding period just before applying the relaxing drug or stimulus, yielding the area above the curve for the relaxant response. The duration of the relaxant responses was determined as 10 s (jejunum) or 30 s (colon) for EFS (i.e. the length of the stimulus train applied). VIP and 8-Br-cGMP induced a sustained response and the duration was fixed at 5 min for VIP and 10 min for 8-Br-cGMP. NO abolished phasic activity for a concentration-dependent period, after which phasic activity progressively reoccurred. The duration of the relaxant responses to NO was therefore determined as the time necessary for phasic activity to regain 50 % of the interval between the mean peak level of phasic activity during the 2 min before administration of NO and the

minimum tone level during the NO response. This calculation was performed during the 1st cycle of $PGF_{2\alpha}$-EFS-NO-VIP and the determined duration was further used for the NO-induced responses in the 2nd cycle of $PGF_{2\alpha}$-EFS-NO-VIP. The responses are expressed as (g.s)/mg wet weight. EC_{50} values of the concentration-response curves were calculated by linear interpolation.

III.3.4 Gastric emptying

As modified from de Rosalmeida *et al*. (2003), mice were fasted overnight and 250 µl of a phenol red meal (0.1 % w/v dissolved in water) was administered by gavage; in apo-sGC mice, gastric emptying was also measured with a test meal volume of 500 µl. 15 min later, mice were sacrificed by cervical dislocation and the stomach and small bowel were clamped at both sides. Both organs were cut into small fragments and placed into 20 ml of 0.1 N NaOH in a 50 ml Falcon tube. This mixture was homogenised for approximately 30 s and allowed to stand for 20 min at room temperature. 10 ml of supernatant was placed into a 15 ml Falcon tube and centrifuged for 10 min at 1600 g. Proteins in 5 ml supernatant were precipitated with 0.5 ml of 20 % (w/v) trichloroacetic acid and the solution was centrifuged for 20 min at 1600 g. 0.5 ml of supernatant was added to 0.667 ml of 0.5 N NaOH and the absorbance of 300 µl of this mixture was spectrophotometrically determined at 540 nm in a Biotrak II plate reader (Amersham Biosciences). Gastric emptying was calculated as the amount of phenol red that left the stomach as % of the total amount of phenol red recovered and the phenol red recovery was determined as the amount of phenol red recovered, expressed as % of the amount of phenol red administered. The phenol red recovery was 74 ± 5 % in WT mice (n = 10) and 77 ± 4 % in apo-sGC mice (n = 8).

III.3.5 Transit and small intestinal contractility

III.3.5.1 Intestinal transit (fluorescein-labelled dextran method)

Mice were, after food was withheld overnight, administered 200 µl of non-absorbable fluorescein-labelled dextran (FD70; 70 kDa, 2.5 % w/v dissolved in water) by gavage with a feeding needle. Ninety minutes later, mice were sacrificed by cervical dislocation. For a full description of the technical details of this method, we refer to De

Backer *et al.* (De Backer *et al.*, 2008a). Briefly, the entire gastrointestinal tract was excised and the mesenterium was removed. The gastrointestinal tract was then pinned down in a custom-made Petri dish filled with Krebs solution. Immediately after, FD70 was visualized using the Syngene Geneflash system (Syngene, Cambridge, UK). Two full-field images -one in normal illumination mode and another in fluorescent mode- were taken and matched for analysis. The fluorescent intensity throughout the intestinal tract was calculated and data were expressed as the percentage of fluorescence intensity per segment (sb, small bowel segments 1-10; caecum; col, colon segments 1-2). The geometric centre was calculated as (Σ (% FD70 per segment x segment number))/100.

III.3.5.2 Small intestinal contractility

Immediately after the evaluation of intestinal transit by fluorescence imaging, the spontaneous contractile activity in the jejunum was recorded. For a full description of the technical details of this method, we refer to De Backer *et al.* (2008a). Briefly, a 6 cm long segment of jejunum was recorded for 30 s and the video files were imported in ImageJ. After the contrast threshold value was set, the images were converted to black-and-white and the mean diameter of the jejunal segment was measured in the first frame. Next, all 750 frames were sequentially analysed using Amplitude Profiler software - written as an ImageJ plugin. The change in intestinal diameter within this 30 s period for every pixel (768 pixels) along this 6 cm long jejunal segment was calculated as % contraction amplitude by the following equation: [(maximal diameter − minimal diameter)/maximal diameter] * 100. Finally, the mean value of these 768 amplitude values was calculated. The oscillatory changes were also represented in a three-dimensional (3-D) plot using Spatiotemporal Motility Mapping software - written as an ImageJ plugin with a GnuPlot backend - allowing to see contractility in function of time.

III.3.5.3 Whole gut transit time (carmine method)

As adapted from Friebe *et al.* (2007), 200 µl carmine (6 % w/v dissolved in 0.5 % methylcellulose) was administered by gavage. Mice were then returned to individual cages,

without food deprivation. The time taken for excretion of the first red coloured faeces was determined at 30 min intervals.

III.3.5.4 Distal colonic transit.

Distal colonic transit was measured according to previously described methods (Jacoby & Lopez, 1984; Sibaev *et al.*, 2009). After overnight fasting, a single 2 mm plastic bead was inserted 2 cm into the distal colon of each mouse using a custom-made polished metal applicator. The applicator and bead were preheated to 37°C. After the bead insertion, mice were immediately placed in their cages with a white paper on the bottom, to help visualisation of bead expulsion. The time for expulsion of the bead was determined for each animal.

III.3.6   Histology

Specimens of fundic, pyloric, jejunal and colonic tissues were harvested from WT and apo-sGC mice. The tissues were fixed in 4 % neutral buffered formalin (NBF), dehydrated through a graded series of ethanol and embedded in paraffin wax. Serial transverse sections of 5 µm thickness were cut at 500 µm intervals, using a rotary microtome (SLEE CUT 4060) and stained with hematoxylin and eosin for morphological observation.

III.3.7   sGC enzyme activity

sGC enzyme activity was measured cfr. Buys *et al*. (2008). Fundus, jejunum and distal colon were harvested. The fundus was immediately snap frozen in liquid nitrogen; the jejunum and distal colon were snap frozen after removal of the mucosa. Tissues were homogenized in buffer containing 50 mM Tris-(hydroxymethyl)aminomethane hydrochloride (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol (DTT), and 2 mM phenylmethylsulfonyl fluoride. Extracts were centrifuged at 20 000 g for 20 min at 4°C. Supernatants (containing 40 µg protein) were incubated for 10 min at 37°C in a reaction mixture containing 50 mM Tris hydrochloride (pH 7.5), 4 mM $MgCl_2$, 0.5 mM 1-methyl-3-isobutylxanthine, 7.5 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 1 mM L-NAME, and 1 mM GTP with or without 1 mM diethylenetriamine NONOate (DETA-NO). The

reaction was terminated by the addition of 0.9 ml of 0.05 M HCl. cGMP in the reaction mixture was measured using a commercial radioimmunoassay (Biomedical Technologies, Stoughton, MA). sGC enzyme activity is expressed as pmol of cGMP produced per min per milligram of protein in gastrointestinal extract supernatant.

III.3.8   Drugs used

The following drugs were used: apamin (obtained from Alomone Labs), atropine sulphate, 8-Br-cGMP sodium salt, carmine, guanethidine sulphate, L-NAME, phenol red, $PGF_{2\alpha}$ tris salt, VIP (all obtained from Sigma-Aldrich), DETA-NO (from Alexis Biochemicals), carbachol (from Fluka AG), fluorescein-labelled dextran (70 kDa, FD70; from Invitrogen), ODQ (from Tocris Cookson). All drugs were dissolved in de-ionized water except for the following: ODQ, which was dissolved in 100 % ethanol and DETA-NO, which was dissolved in sGC enzyme activity buffer (see above). Saturated NO (2 mM) and CO (1mM) solutions were prepared by bubbling oxygenated Krebs solution with, respectively, 99.9 % NO or CO gas (Air Liquide, Belgium) as described by Kelm and Schrader (Kelm & Schrader, 1990). The CO-saturated Krebs solution contained $PGF_{2\alpha}$ (300 nM for the experiments in the fundus; 3 μM for the experiments in the colon) and was maintained at 37 °C.

III.3.9   Statistics

All results are expressed as means ± S.E.M. n refers to tissues obtained from different animals unless otherwise indicated. Comparison between apo-sGC and WT tissues was done with an unpaired Student's *t*-test. Comparison within tissues of either WT or apo-sGC was done by a paired Student's *t*-test. When more than 2 sets of results within the same tissue had to be compared, repeated measures ANOVA followed by a Bonferroni corrected *t*-test was applied. A *P*-value less than 0.05 was considered to be statistically significant (GRAPHPAD, San Diego, CA, USA).

III.4   Results

All in vivo and in vitro experiments were performed in mice of both sexes, except for the measurement of sGC activity, distal colonic transit, and the experiments with pyloric rings, which were investigated in male mice only. No systematic differences between the sexes were observed; results are therefore presented for male mice only.

III.4.1 General observations and histology

The body weight of apo-sGC mice was significantly smaller than that of WT mice. The stomach of apo-sGC mice was significantly enlarged. The mean empty stomach weight of apo-sGC mice was significantly larger than this of WT mice. The length of the small intestine and colon in apo-sGC was significantly higher than in WT mice (Table III.1). Smooth muscle layers of the muscularis externa were markedly thicker in the fundus of apo-sGC mice than in WT mice (Fig. III.3). Also the muscularis externa of the pylorus was thicker in apo-sGC mice, although the difference with WT mice was less pronounced than for the fundus. No histological differences between WT and apo-sGC mouse at the level of the jejunum and the colon were observed.

Table III.1   Body weight, small/large intestine length, stomach weight and weight of the gastrointestinal preparations.

	WT	**apo-sGC**
Body weight (g)	30 ± 1 (n = 23)	22 ± 1 (n = 25) ***
Small intestine length (cm)	30.0 ± 0.8 (n = 23)	33.7 ± 0.7 (n = 25) ***
Colon length (cm)	5.4 ± 0.2 (n = 23)	5.9 ± 0.2 (n = 25)*
Stomach weight (mg)	209 ± 10 (n = 23)	379 ± 28 (n = 25) ***
Fundus strips (mg)	5.83 ± 0.27 (n = 46)	15.73 ± 1.55 (n = 50) ***
Pyloric rings (mg)	7.86 ± 0.37 (n = 26)	11.38 ± 0.75 (n = 21) ***
Jejunum strips (mg)	0.54 ± 0.05 (n = 47)	0.45 ± 0.05 (n = 45)
Colon strips (mg)	0.53 ± 0.03 (n = 41)	0.44 ± 0.03 (n = 41) *

Values are means ± S.E.M. n refers to separate animals for body weight, small intestinal length, colon length, stomach weight and pyloric rings and to fundus, jejunum and colon strips taken per 2 from separate animals. * $P < 0.05$, *** $P < 0.001$: unpaired student t-test (apo-sGC vs. WT).
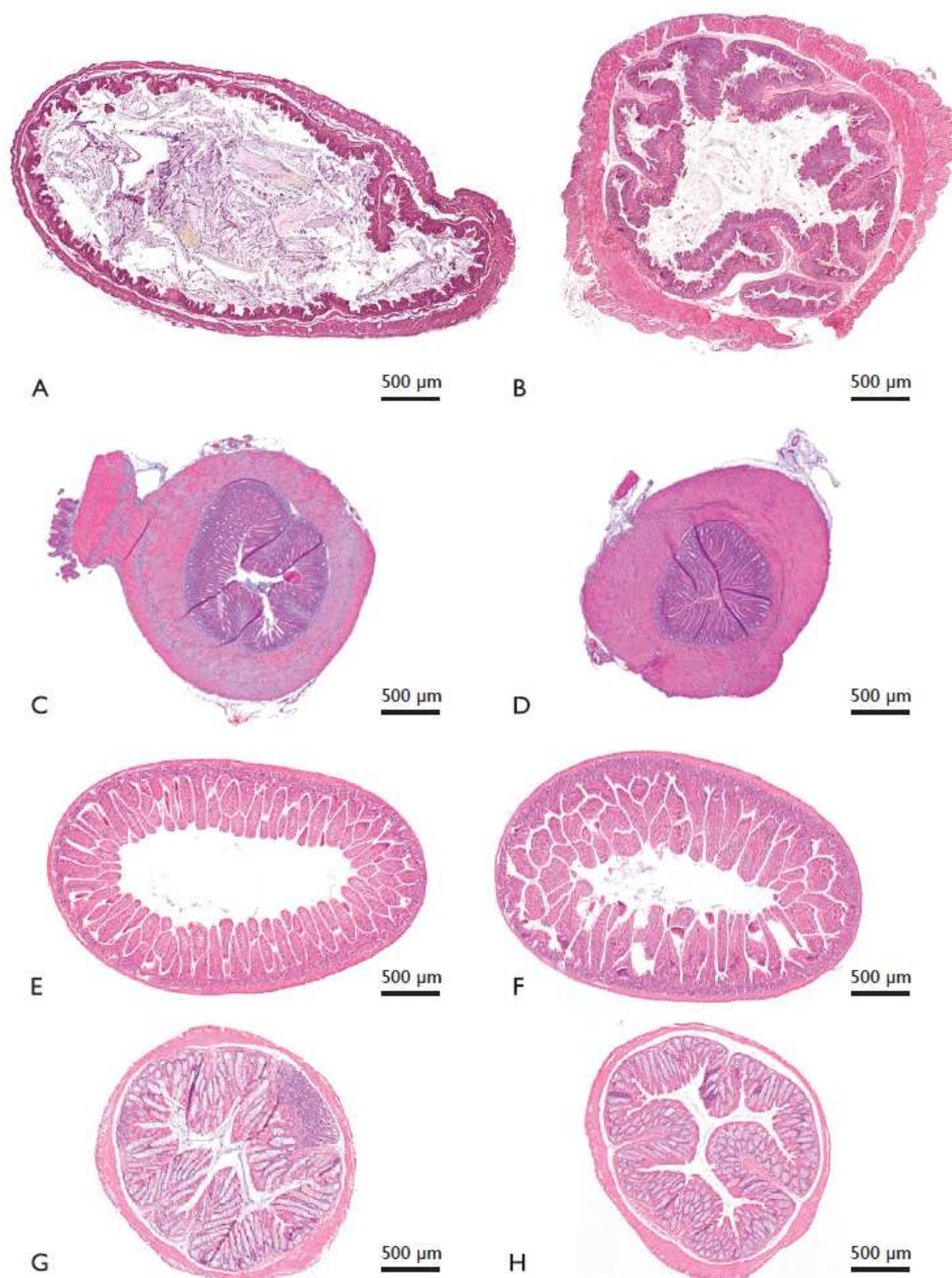
Fig. III.3 Comparison of fundic, pyloric, jejunal and colonic histologic transverse sections from a WT and an apo-sGC mouse. Microscopic view of a histologic transverse section of a WT (*left panel*) and an apo-sGC (*right panel*) mouse fundus (A, B), pylorus (C, D), jejunum (E, F) and colon (G, H). The transverse sections of 5 μm thickness were stained with hematoxylin and eosin for morphological observation.

III.4.2 sGC enzyme activity

Baseline sGC activity was slightly higher in the colon of apo-sGC mice than in WT mice; (Fig. III.4). DETA-NO significantly increased sGC activity in WT tissues, but not in apo-sGC tissues. The level of sGC activity in the presence of DETA-NO was as a result significantly lower in apo-sGC versus WT tissues.
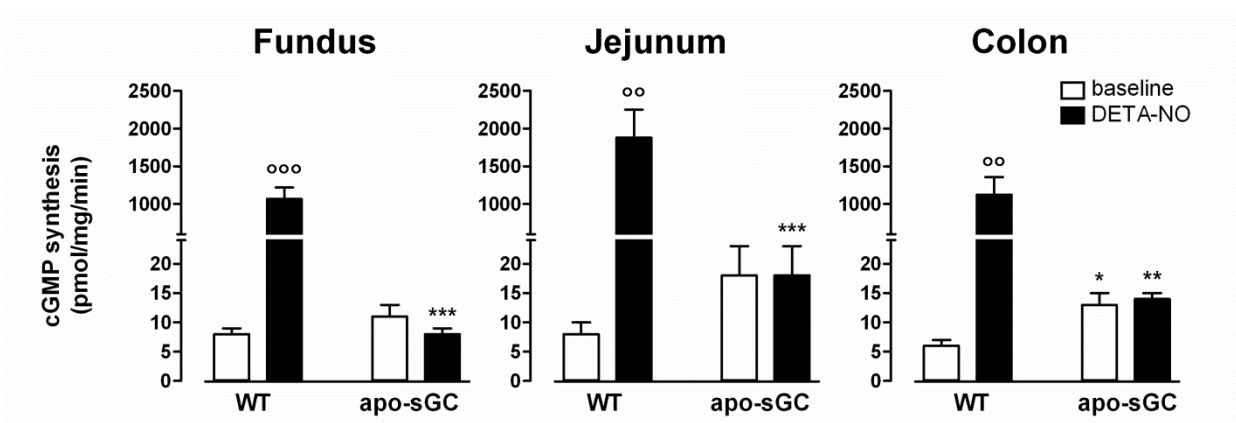
Fig. III.4 sGC enzyme activity.
Un-stimulated (baseline) and DETA-NO-stimulated sGC enzyme activity (expressed as picomoles cGMP produced per mg protein per minute) in fundic (A), jejunal (B) and colonic (C) extracts of WT and apo-sGC mice. Means ± S.E.M. of n = 5-6. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: apo-sGC versus WT (unpaired Student's t-test); ○○ $P < 0.01$, ○○○ $P < 0.001$: DETA-NO versus baseline (paired Student's t-test).

III.4.3 Muscle tension experiments

III.4.3.1 Tissue weight.

When comparing strips or rings of the same dimensions, the fundic strips (2 x 11 mm) and the pyloric rings (width: 2 mm) of apo-sGC mice weighed significantly more than these prepared from WT mice (Table III.1). On the other hand, the colonic strips (4 x 5 mm) of apo-sGC mice were slightly lighter than these of WT mice (Table III.1).

III.4.3.2 Contractile responses to carbachol and PGF$_{2\alpha}$.

The EC$_{50}$ and E$_{max}$ of the cumulative concentration-response curves of carbachol and PGF$_{2\alpha}$ (1 nM – 30 μM) did not significantly differ in fundic, jejunal or colonic strips from apo-sGC and WT mice (Table III.2). PGF$_{2\alpha}$ at a concentration of 300 nM was chosen to pre-contract fundic and jejunal strips in order to investigate the relaxant responses to EFS, NO, VIP and 8-br-cGMP (see below); in pylorus and colonic strips 3 μM PGF$_{2\alpha}$ was used.

Table III.2 EC$_{50}$ and E$_{max}$ of the contractions to carbachol and PGF$_{2\alpha}$.

	Carbachol				PGF$_{2\alpha}$			
	EC$_{50}$		E$_{max}$		EC$_{50}$		E$_{max}$	
	WT	apo-sGC	WT	apo-sGC	WT	apo-sGC	WT	apo-sGC
Fundus	0.30 ± 0.06	0.40 ± 0.07	400 ± 37	557 ± 110	1.68 ± 0.69	0.25 ± 0.13	267 ± 52	315 ± 78
Jejunum	0.27 ± 0.09	0.24 ± 0.07	55 ± 19	46 ± 7	0.03 ± 0.01	0.09 ± 0.05	27 ± 5	23 ± 5
Colon	0.43 ± 0.07	0.71 ± 0.14	170 ± 35	211 ± 77	7.68 ± 1.04	9.14 ± 1.86	53 ± 13	44 ± 9

EC$_{50}$ in µM; E$_{max}$ in (g.s)/mg wet weight for jejunum and colon and g/g wet weight for fundus. Values are means ± S.E.M. of n = 4-6.

III.4.3.3 Fundus

PGF$_{2\alpha}$ (300 nM) induced an increase in tone in fundus strips. In WT fundus strips, application of EFS (1-8 Hz) induced frequency-dependent relaxations, consisting of a progressive decline in tone which recovered upon ending stimulation (see Fig. III.5A for the response at 8 Hz and Fig. III.5C for mean responses); these relaxations were abolished by ODQ (10 µM; Fig. III.5C) and L-NAME (300 µM; data not shown). In apo-sGC fundus strips, the relaxant responses to EFS at 1-4 Hz were totally abolished; EFS at 8 Hz induced a variable effect: relaxation was abolished in 4 out of 6 strips (see Fig. III.5A for an example where EFS at 8 Hz even induced a small contractile response) but in 2 out of 6 strips, a relaxant response (10 and 53 %) was obtained, no longer occurring when EFS at 8 Hz was repeated in the presence of ODQ. In an additional series with fundic strips from 5 apo-sGC mice, EFS at 8 Hz again induced relaxation in 2 tissues (Fig. III.6A and B), that was abolished by ODQ. In the series where L-NAME was tested, 2 out of 7 fundic strips of apo-sGC mice showed a relaxation in response to EFS at 8 Hz; this relaxation was not influenced by L-NAME (Fig. III.6C). Exogenous applied NO (1-10-100 µM) induced concentration-dependent relaxations in WT strips, consisting of a quick and transient decline in tone (see Fig. III.5A for the response to 10 µM NO and Fig. III.5C for mean responses). ODQ abolished the relaxant response to 1 µM NO and reduced those to 10-100 µM NO (Fig 3C). In apo-sGC strips, the relaxant responses to NO were totally abolished (Fig. III.5A and C). In a small series, CO (300 µM) was tested. In WT fundus strips, CO induced a quick and transient decline in tone (n = 4), which was abolished in apo-sGC strips (n = 4; Fig. III.5B).

8-Br-cGMP (10 µM) and VIP (100 nM) induced a sustained decrease in tone. Mean responses to 8-Br-cGMP and VIP were not different between WT and apo-sGC strips (Fig. III.5D). ODQ (10 µM) did not significantly decrease the responses to 8-Br-cGMP and VIP in either WT strips or apo-sGC strips (results not shown).
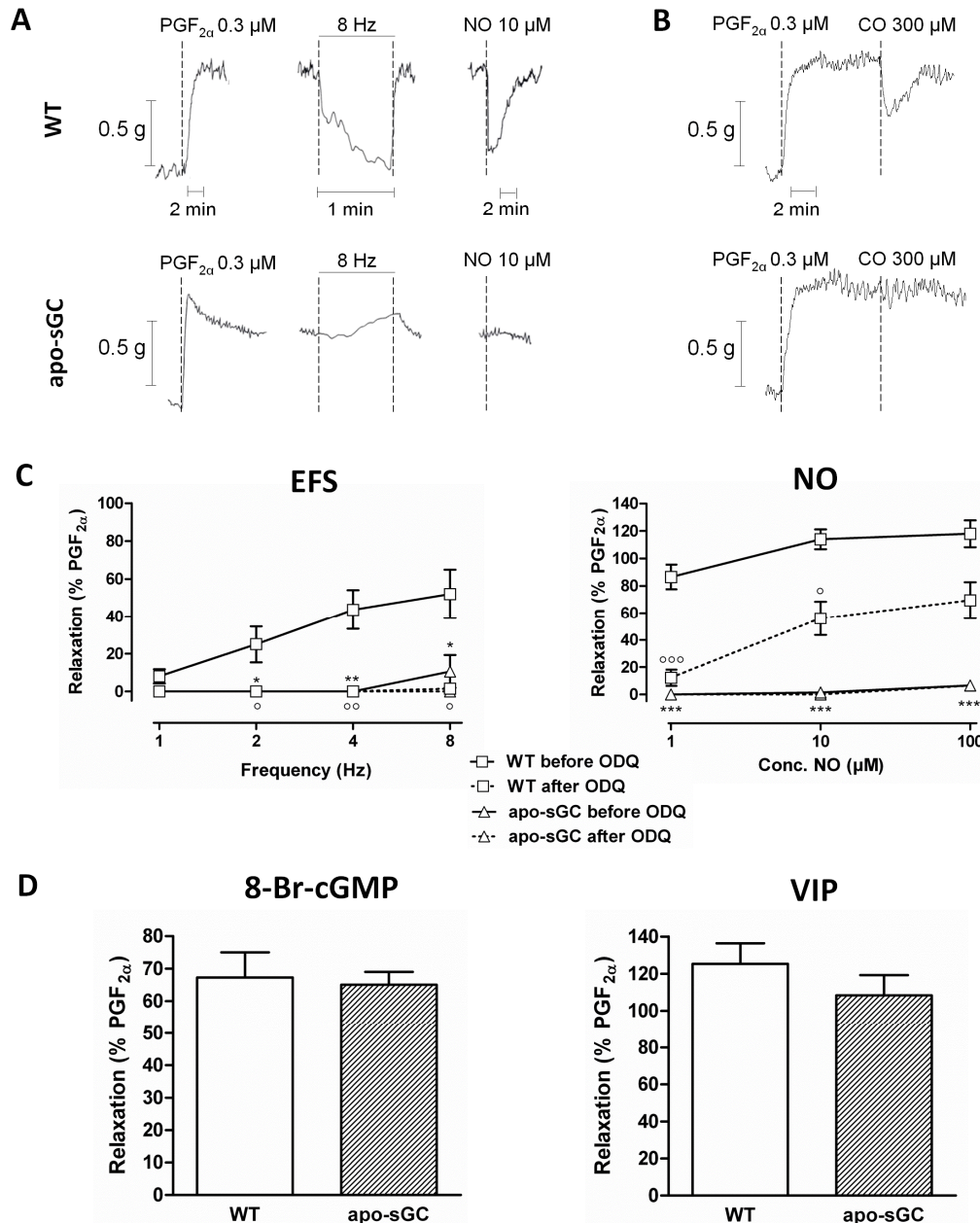
Fig. III.5 Responses to EFS, NO, CO, 8-Br-cGMP and VIP in fundus strips of WT and apo-sGC mice.
(A-B) Representative traces showing the inhibitory responses to EFS (40 V; 0.1 ms; 8 Hz) and exogenously applied NO (10 µM) or CO (300 µM) in $PGF_{2\alpha}$-pre-contracted circular muscle strips of gastric fundus from a WT mouse (*upper trace*) and an apo-sGC mouse (*lower trace*). (C) Frequency-response curves of EFS (40 V; 0.1 ms; 1-8 Hz) (*left*) and concentration-response curves of NO (1 µM – 100 µM) (*right*) in WT (□) and apo-sGC (Δ) strips of fundus before and after incubation with ODQ (10 µM). Means ± S.E.M. of n = 7 are shown. * P < 0.05, ** P < 0.01, *** P < 0.001: apo-sGC before incubation versus WT before incubation (unpaired Student's *t*-test); ○ P < 0.05, ○○ P < 0.01, ○○○ P < 0.001: WT after incubation with ODQ versus before (paired Student's *t*-test). (D) Relaxant responses to 8-Br-cGMP (*left*; 10 µM) and VIP (*right*; 100 nM) in fundus strips from WT and apo-sGC mice. Means ± S.E.M. of n = 12 out of 6-7 animals are shown. An unpaired Student's *t*-test was applied but no significance was found.
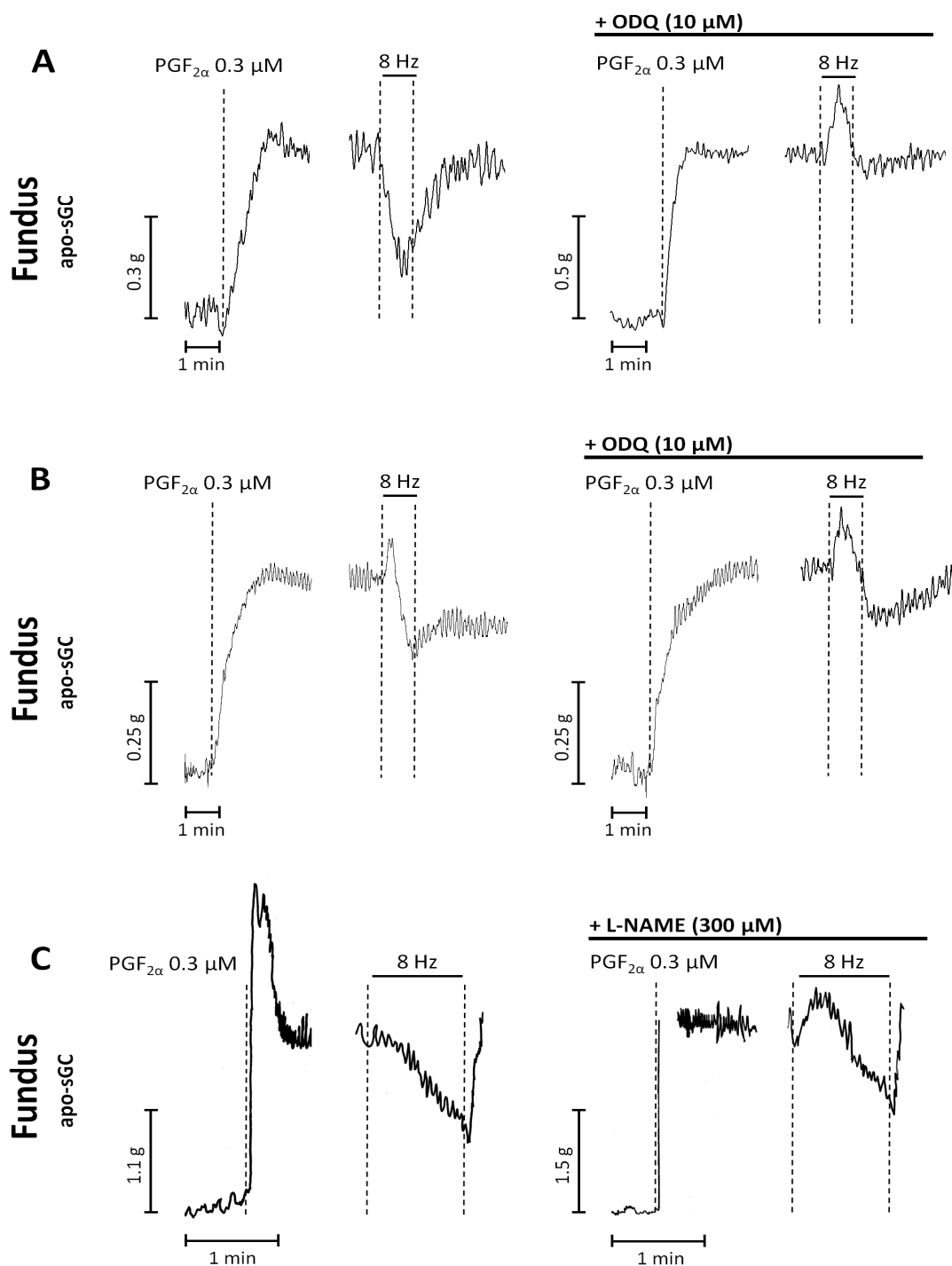
Fig. III.6 Influence of ODQ and L-NAME on response to EFS in fundic strips from apo-sGC mice.
Representative traces showing the inhibitory response to EFS (40 V; 0.1 ms; 1-8 Hz) occasionally occurring in PGF$_{2\alpha}$-pre-contracted circular muscle strips of the gastric fundus of apo-sGC mice before and after incubation with 10 µM ODQ (A, B) or 300 µM L-NAME (C). The initial part of the contractile response to PGF$_{2\alpha}$ after L-NAME was out of range for 10 seconds, therefore the trace is not visible during this time.

III.4.3.4 Pyloric rings

PGF$_{2\alpha}$ (3 µM) induced an increase in tone in pyloric rings. In WT pyloric rings, EFS (1-4 Hz) induced a quick decline in tone recovering immediately upon the end of stimulation followed by a rebound contraction (Fig. III.7A). The amplitude of relaxation increased by stimulation at 2 Hz compared to that at 1 Hz, but did not further increase by EFS at 4 Hz (Fig. III.7A and C). L-NAME (300 µM) abolished the EFS-induced responses at 1 Hz, and reduced the responses at 2 and 4 Hz (Fig. III.7C). In apo-sGC pyloric rings, the relaxant response to EFS was reduced at all stimulation frequencies, with the response at 1 Hz being nearly abolished; relaxation still occurring with EFS at 2 and 4 Hz was not influenced by L-NAME (Fig. III.7A and C).
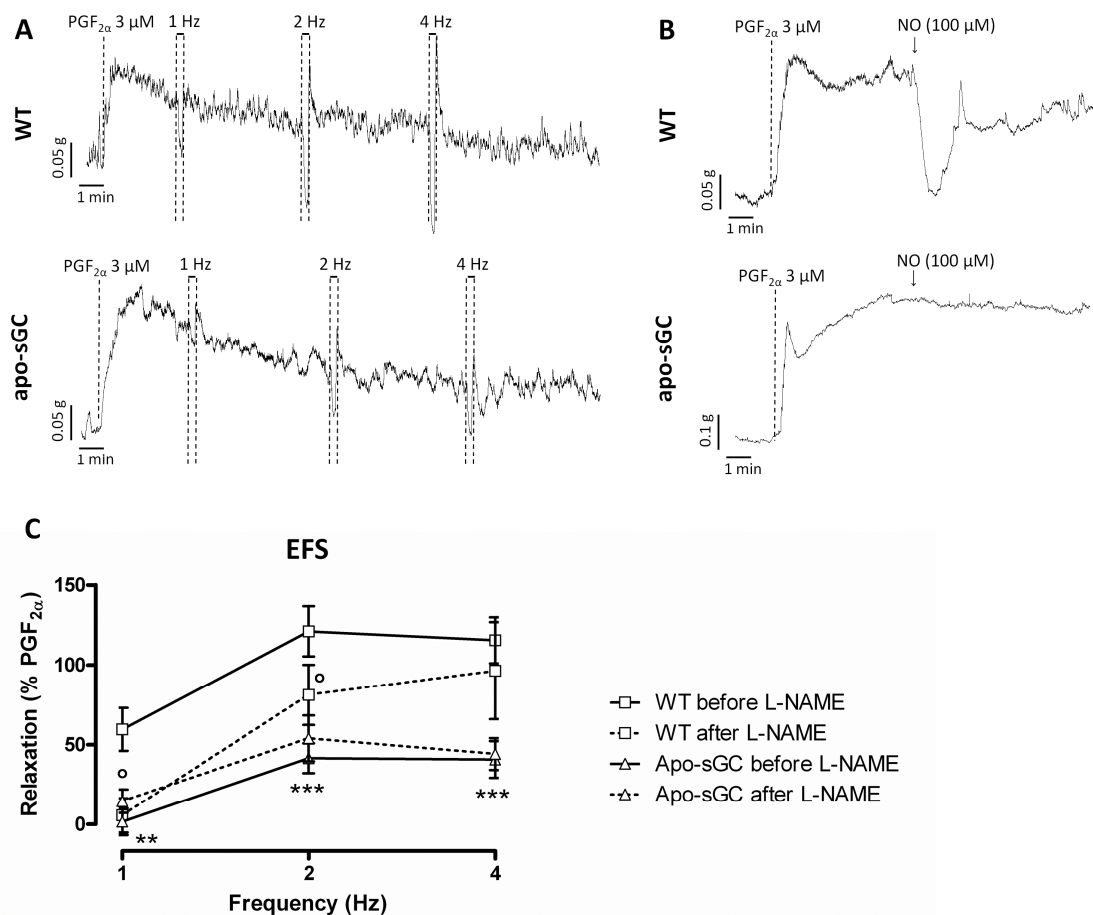
Fig. III.7 Responses to EFS and NO in pyloric rings of WT and apo-sGC mice.
(A) Representative traces showing the inhibitory responses to EFS (40 V; 0.1 ms; 1-2-4 Hz) in PGF$_{2\alpha}$-pre-contracted pyloric rings from a WT mouse (*upper trace*) and an apo-sGC mouse (*lower trace*). (B) Representative traces showing the inhibitory responses to exogenously applied NO (100 µM) in PGF$_{2\alpha}$-pre-contracted pyloric rings from a WT mouse (*upper trace*) and an apo-sGC mouse (*lower trace*). (C) Frequency-response curves of EFS (40 V; 0.1 ms; 1-2-4 Hz) in WT (□) and apo-sGC (Δ) pyloric rings before and after incubation with L-NAME (300 µM). Means ± S.E.M. of n = 6-9 are shown. ** $P < 0.01$, *** $P < 0.001$: apo-sGC before incubation versus WT before incubation (unpaired Student's *t*-test); ○ $P < 0.05$: WT after incubation with L-NAME versus before (paired Student's *t*-test).

The response to NO (100 µM) in WT pyloric rings consisted of a quick and transient decline in tone (Fig. III.7B). ODQ (10 µM) decreased the relaxant response to NO in WT pyloric rings (109.8 ± 10.9 %) to 33.3 ± 13.2 % (n = 5). In apo-sGC mice, the relaxant response to NO was totally abolished (Fig. III.7B).

III.4.3.5 Jejunum

In jejunal strips, $PGF_{2\alpha}$ (300 nM) induced a combined tonic and phasic response. The tone decreased back to baseline within maximally 3 minutes after addition of $PGF_{2\alpha}$, but the increase in phasic activity remained. In WT strips, EFS induced suppression of phasic activity; upon ending stimulation at 8 Hz, a rebound contraction was observed (Fig. III.8A). The EFS-induced relaxations had a similar size at the different stimulation frequencies (Fig. III.8A and B) and were abolished by ODQ (10 µM; Fig. III.8B) and L-NAME (300 µM; data not shown). In apo-sGC strips, no relaxant response to EFS was obtained at 1-4 Hz. At 8 Hz, the phasic activity was initially suppressed. However near the end of the stimulation train an increase in phasic activity was systematically observed, which further progressed when the stimulation train was ended (Fig. III.8A).

In jejunal strips of WT mice, NO (1-10-100 µM) suppressed pre-imposed phasic activity with a concentration-dependent duration (Fig. III.8A and B). ODQ abolished the relaxant response to 1 µM NO and reduced that to 10 and 100 µM NO (Fig. III.8B). In jejunal strips of apo-sGC mice, the relaxant responses to NO were totally abolished (Fig. III.5A and B).

In both WT and apo-sGC strips, 8-Br-cGMP (100 µM) and VIP (100 nM) induced a sustained suppression of phasic activity. Mean responses to 8-Br-cGMP and VIP were not significantly different between WT and apo-sGC strips (Fig. III.8C). ODQ (10 µM) did not significantly decrease the responses to 8-Br-cGMP and VIP in either WT strips or apo-sGC strips (results not shown).

Jejunum
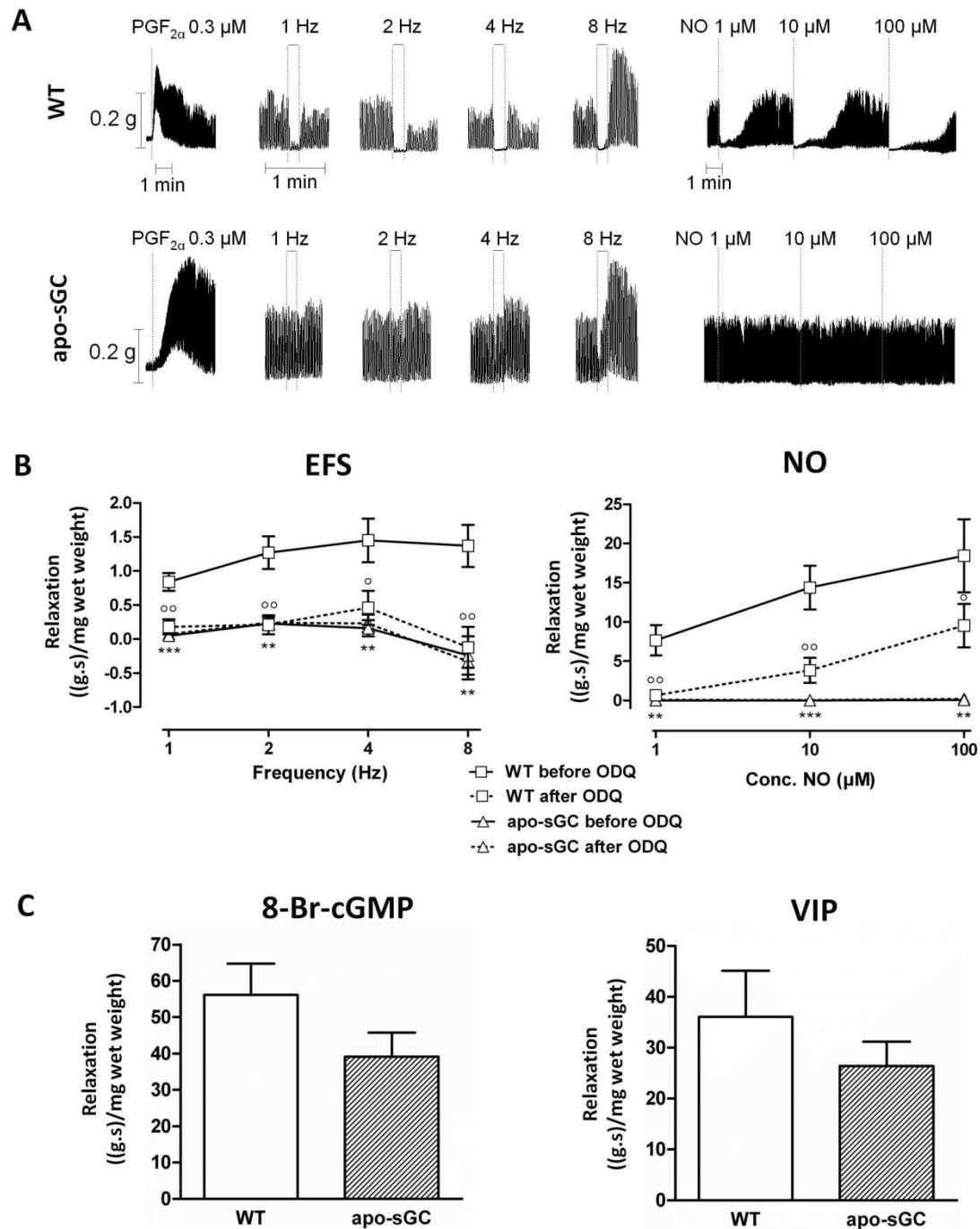
Fig. III.8 Responses to EFS, NO, 8-Br-cGMP and VIP in jejunal strips of WT and apo-sGC mice.
(A) Representative traces showing the inhibitory responses to EFS (40 V; 0.1 ms; 1-8 Hz) and exogenously applied NO (1-100 μM) in PGF$_{2\alpha}$-pre-contracted circular muscle strips of jejunum from a WT mouse (*upper trace*) and an apo-sGC mouse (*lower trace*). (B) Frequency-response curves of EFS (40 V; 0.1 ms; 1-8 Hz) (*left*) and concentration-response curves of NO (1 μM – 100 μM) (*right*) in WT (□) and apo-sGC (Δ) strips of jejunum before and after incubation with ODQ (10 μM). Negative values in the y-axis (*left*) indicate that a contractile response instead of a relaxation was obtained. Means ± S.E.M. of n = 6-8 are shown. ** P < 0.01, *** P < 0.001: apo-sGC before incubation versus WT before incubation (unpaired Student's *t*-test); ○ P < 0.05, ○○ P < 0.01: WT after incubation with ODQ versus before (paired Student's *t*-test). (C) Relaxant responses to 8-Br-cGMP (*left*; 100 μM) and VIP (*right*; 100 nM) in jejunal strips from WT and apo-sGC mice. Means ± S.E.M. of n = 12 out of 6-7 animals. An unpaired Student's *t*-test was applied but no significance was found.
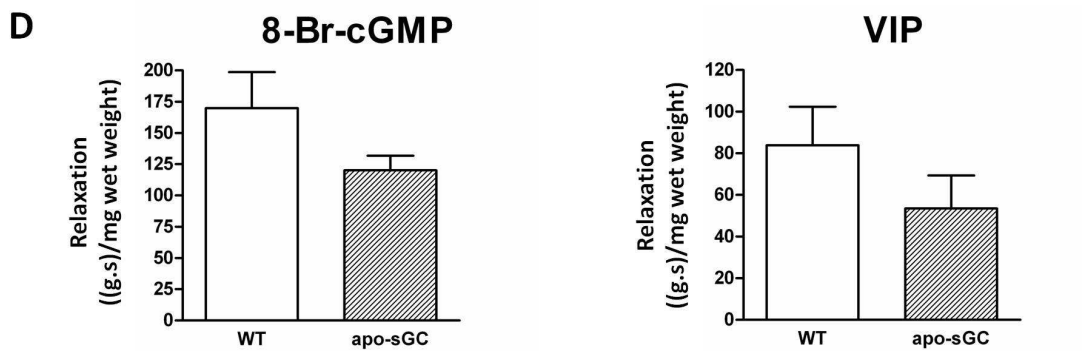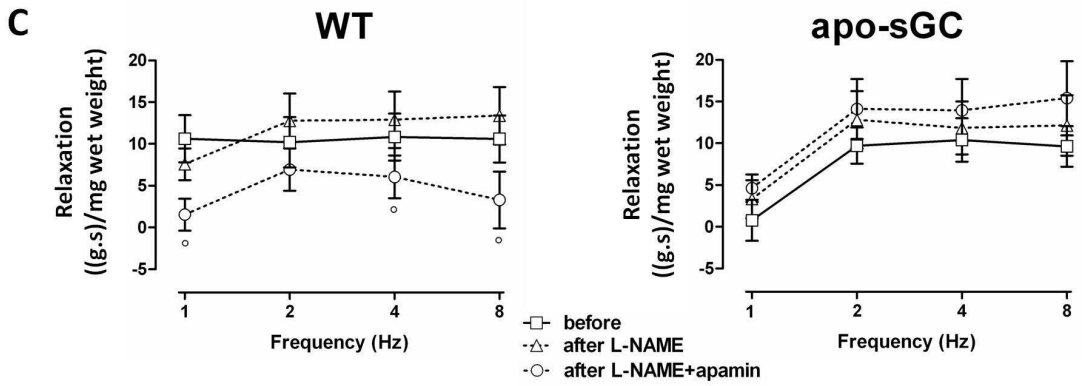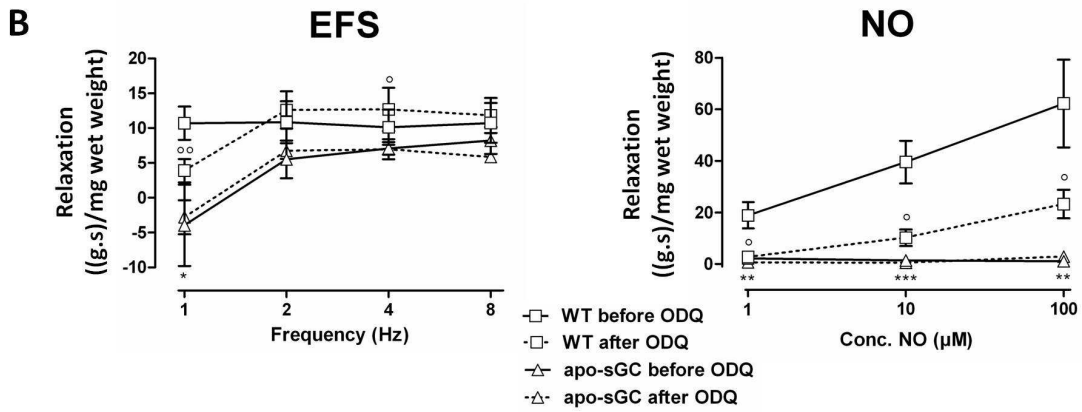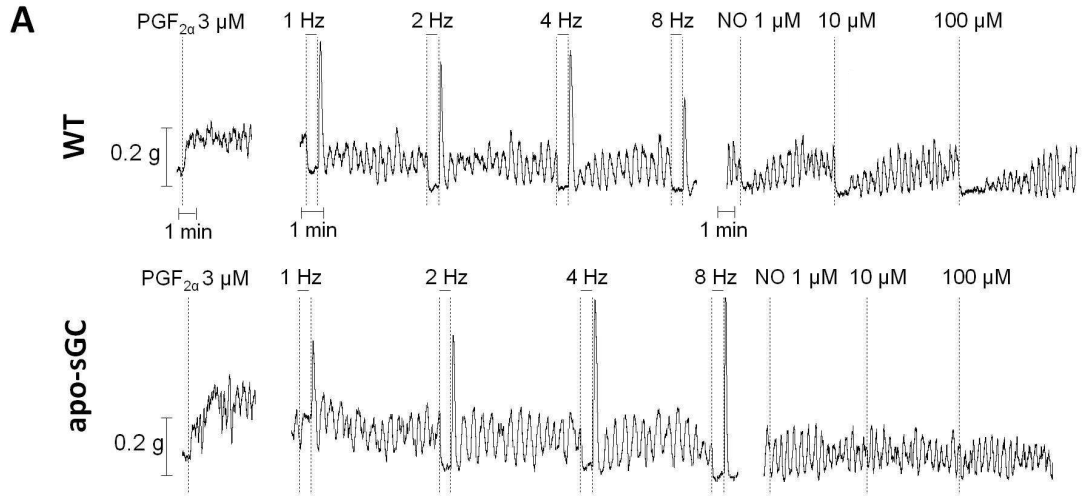
III.4.3.6 Colon

PGF$_{2\alpha}$ (3 µM) induced a combined tonic and phasic response. In WT strips, EFS induced suppression of phasic activity as well as a decrease in tone during the stimulation train, followed by a rebound contraction (Fig. III.9A). The size of the relaxant response was similar at all stimulation frequencies. Only the response to 1 Hz was reduced by ODQ (10 µM; Fig. III.9B); the combination of L-NAME (300 µM) plus apamin (500 nM) was able to decrease the response at all frequencies in these strips but did not abolish them (Fig. III.9C). In apo-sGC strips, EFS at 1 Hz only induced a short decrease in tone at the beginning of the stimulation train. At higher frequencies a pronounced relaxant response was present (Fig. III.9A). Neither ODQ (Fig. III.9B), nor L-NAME, or L-NAME plus apamin (see Fig. III.9C) influenced the remaining EFS-induced responses at 2 to 8 Hz in apo-sGC strips.

NO (1-10-100 µM) suppressed pre-imposed phasic activity in WT strips with a concentration-dependent duration (Fig. III.9A). ODQ abolished the relaxant response to 1 µM NO and reduced the response to 10 and 100 µM NO (Fig. III.9B). In apo-sGC mice, the relaxant responses to NO were totally abolished (Fig. III.9A and B). In a small series of experiments, CO (300 µM) was tested. In WT strips, CO induced a transient inhibition of phasic activity (n = 4); in apo-sGC strips, the ability of CO to inhibit phasic activity was abolished (n = 6; results not shown).

Fig. III.9   Responses to EFS, NO, 8-Br-cGMP and VIP in colonic strips of WT and apo-sGC mice.
(A) Representative traces showing the inhibitory responses to EFS (40 V; 0.1 ms; 1-8 Hz) and exogenously applied NO (1-100 µM) in PGF$_{2\alpha}$-pre-contracted circular muscle strips of distal colon from a WT mouse (*upper trace*) and an apo-sGC mouse (*lower trace*). (B) Frequency-response curves of EFS (40 V; 0.1 ms; 1-8 Hz) (*left*) and concentration-response curves of NO (1 µM – 100 µM) (*right*) in WT (□) and apo-sGC (Δ) strips of colon before and after incubation with ODQ (10 µM). The responses to EFS and NO in the apo-sGC colonic strips were multiplied with a factor "PGF$_{2\alpha}$ response in WT colonic strips / PGF$_{2\alpha}$ response in apo-sGC colonic strips" in order to correct for the significantly smaller PGF$_{2\alpha}$-induced pre-contraction in the apo-sGC versus the WT colonic strips in these series (apo-sGC: 32.00 ± 4.94 (g.s)/mg wet weight versus WT: 89.92 ± 17.94 (g.s)/mg wet weight, n = 6, P < 0.05). Means ± S.E.M. of n = 6-8 are shown. * P < 0.05, ** P < 0.01, *** P < 0.001: apo-sGC before incubation versus WT before incubation (unpaired Student's *t*-test); ○ P < 0.05, ○○ P < 0.01: WT after incubation with ODQ versus before (paired Student's *t*-test). (C) Frequency-response curves of EFS (40 V; 0.1 ms; 1-8 Hz) in WT (*left*) and apo-sGC (*right*) colonic strips before (□) and after incubation with L-NAME (300 µM; Δ), and L-NAME (300 µM) plus apamin (500 nM; ○). Means ± S.E.M. of n = 6-7 are shown. ○ P < 0.05: after incubation with L-NAME plus apamin versus after incubation with L-NAME (repeated measures ANOVA followed by a Bonferroni corrected *t*-test). (D) Relaxant responses to 8-Br-cGMP (*left*; 100 µM) and VIP (*right*; 100 nM) in colonic strips from WT and apo-sGC mice. Complementary to the EFS- and NO-induced relaxation, the responses to VIP in the apo-sGC colonic strips were multiplied with the correction factor "PGF$_{2\alpha}$ response in WT colonic strips / PGF$_{2\alpha}$ response in apo-sGC colonic strips". Means ± S.E.M. of n = 9-12 out of 6-7 animals are shown. An unpaired Student's *t*-test was applied but no significance was found.

Colon

A

B

EFS

NO

- WT before ODQ
- WT after ODQ
- apo-sGC before ODQ
- apo-sGC after ODQ

C

WT

apo-sGC

- before
- after L-NAME
- after L-NAME+apamin

D

8-Br-cGMP

VIP

In both WT and apo-sGC strips, 8-Br-cGMP (100 µM) and VIP (100 nM) induced a sustained suppression of phasic activity, sometimes accompanied by a decrease in tone. Mean responses to 8-Br-cGMP and VIP were not significantly different between WT and apo-sGC strips (Fig. III.9D). ODQ (10 µM) did not significantly decrease the responses to 8-Br-cGMP and VIP in either WT strips or apo-sGC strips (results not shown).

III.4.4 Gastric emptying, small intestinal transit and whole gut transit time

Fifteen min after gavage, gastric emptying of a phenol red solution was significantly lower in apo-sGC mice (51 ± 10 %, n = 8) than in WT mice (74 ± 3 %, n = 10) (Fig. III.10A). In 3 apo-sGC mice, gastric emptying was measured with a test meal volume of 500 µl, yielding 37 ± 8 % gastric emptying, excluding the possibility that the observed delay of gastric emptying in apo-sGC mice was related to using the same test meal volume for the clearly greater stomach in apo-sGC mice.

Ninety min after gavage, we observed a delayed intestinal transit of a fluorescein-labelled dextran solution in apo-sGC versus WT mice as manifested from the significant decrease in geometric center (Fig. III.10D). Small intestinal contractility at that time point was however not different between WT and apo-sGC mice (% contraction amplitude in apo-sGC: 23 ± 3 % versus WT: 22 ± 3 %, n = 5-6; Fig. III.10E). The whole gut transit time of a carmine solution was between 120 and 180 min in WT mice. In apo-sGC mice, the whole gut transit time was more variable and the mean value was significantly increased (apo-sGC: 320 ± 25 min versus WT: 146 ± 10 min, n = 6-7; P < 0.001; Fig. III.10B). Distal colonic transit time did not differ between apo-sGC (22 ± 3 min, n = 8) and WT mice (20 ± 3 min, n = 9; Fig. III.10C).
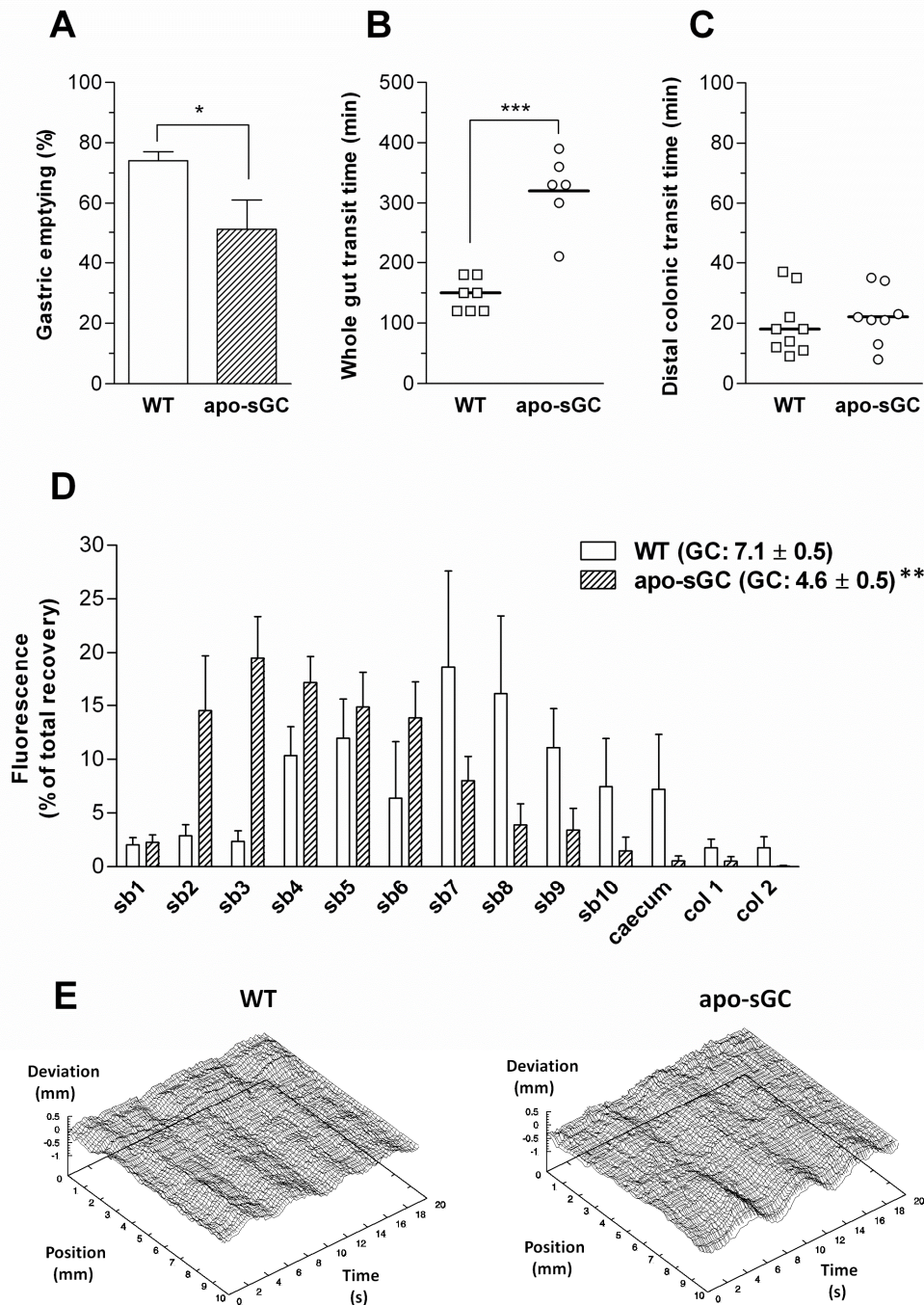
Fig. III.10 In vivo measurements in WT and apo-sGC mice.
(A) Gastric emptying 15 min after gavage of 250 μl of a phenol red meal (0.1 % w/v dissolved in water) in WT and apo-sGC mice. Values are means ± S.E.M. of n = 8-10 animals. (B) Scatter graph showing the whole gut transit time of a carmine solution (6 % w/v dissolved in 0.5 % methylcellulose) in WT and apo-sGC mice. The mean value is represented by a solid line (n = 6-7 animals). (C) Scatter graph showing the distal colon transit time in WT and apo-sGC mice. The mean value is represented by a solid line (n = 8-9 animals). (D) Distribution of fluorescein-labelled dextran in 10 equal small bowel (sb) segments, cecum, and 2 equal colon (col) segments 90 min after gavage of 200 μl fluorescein-labelled dextran (70 kDa; 2.5 % w/v dissolved in water) and geometric center (GC) in WT and apo-sGC mice. Values are means ± S.E.M. of n = 5-6 animals. (E) Representative contractility traces showing spontaneous oscillatory contractions in a 10 mm jejunal segment (*X-axis*) as deviations in mm (*Y-axis*) for a period of 20 s (*Z-axis*); the intestinal diameter measured at t = 20 s was used as reference value. * P < 0.05, ** P < 0.01, *** P < 0.001: unpaired Student's *t*-test (apo-sGC versus WT).

III.5   Discussion

The consequence of switching sGC to the heme-deficient state on nitrergic signalling and motility in the gut was studied in apo-sGC mice. The NO donor DETA-NO failed to increase sGC activity in the gastrointestinal tissues of apo-sGC mice. In addition, exogenous and endogenous NO was not able to induce in vitro relaxation in the fundic, jejunal and colonic smooth muscle strips of apo-sGC mice. In view of the redundant role of sGC in ICC and smooth muscle cells in inducing gastrointestinal nitrergic relaxation (Groneberg *et al.*, 2011; Lies *et al.*, 2011), the lack of responsiveness to NO in gastrointestinal smooth muscle strips of apo-sGC mice must also be related to the heme-deficient state of sGCβ$_1$ in both ICC and smooth muscle cells. The observation that the cGMP analogue 8-Br-cGMP relaxes gastrointestinal smooth muscle strips equally in WT and apo-sGC mice, indicates that the relaxant pathway downstream of sGC is intact. Furthermore, the similar relaxant response to VIP, that acts through adenylate cyclase coupled VIP receptors, in WT and apo-sGC mice argues against compensatory increase in cAMP-induced relaxation in apo-sGC mice. Also contractile mechanisms are not influenced by sGCβ$_1^{His105Phe}$ mutation as contractions to carbachol and PGF$_{2\alpha}$ did not consistently differ between apo-sGC and WT mice. In vivo, the observed gastrointestinal phenotype of the apo-sGC mice included delayed gastric emptying and intestinal transit and increased whole gut transit time.

Apo-sGC mice are hypertensive (Thoonen *et al.*, 2009). Although delayed gastric emptying was reported in spontaneously hypertensive rats (Hatanaka *et al.*, 1995), it is unlikely that hypertension per se delayed gastric emptying and intestinal transit in apo-sGC mice: hypertension in mice with smooth muscle specific deletion of sGC was not associated with abnormal gastrointestinal transit or impaired NO responsiveness in gastrointestinal smooth muscle (Groneberg *et al.*, 2011). The disturbed gastric emptying and intestinal transit in apo-sGC mice illustrate the importance of NO-sensitive sGC in gastrointestinal motility. Still, the gastrointestinal morphological consequences in apo-sGC mice, limited to the enlarged stomach, are less pronounced than in mice lacking sGC ubiquitously (Friebe *et al.*, 2007), suggesting that basal sGC activity, maintained in apo-sGC mice, plays a role in gastrointestinal motility. The disturbed gut motility in apo-sGC mice resembles that in mouse diabetic models (Yamamoto *et al.*, 2008; Choi *et al.*, 2010). Diabetic gastroparesis has been related to oxidative stress-induced dysfunction of nitrergic nerves and ICCs (Kashyap &

Farrugia, 2010), but oxidative stress will also turn sGC to the heme-free state. The results presented here illustrate that the heme-free state of sGC in the diabetic stomach will also lead to disturbed gastrointestinal nitrergic signalling and might thus contribute to the pathogenesis of diabetic gastroparesis.

EFS-induced relaxations are abolished by L-NAME and ODQ in WT fundic strips, indicating that NO is the principal relaxant neurotransmitter acting through activation of sGC in the mouse gastric fundus. Still, EFS at 8 Hz induced relaxation, not sensitive to L-NAME, in some apo-sGC fundic strips suggesting that another neurotransmitter than NO can be released at stimulation frequencies from 8 Hz on in the gastric fundus of apo-sGC mice. The identity of this neurotransmitter remains to be determined. As VIP is known to be released at higher stimulation frequencies in gastric fundus (D'Amato *et al.*, 1992; Boeckxstaens *et al.*, 1992; Tonini *et al.*, 2000; Mule & Serio, 2003), it might already be released in apo-sGC mice at stimulation frequencies where this does not yet occur in WT mice to compensate for the loss in responsiveness to NO. Surprisingly, the relaxation by EFS at 8 Hz in gastric fundus of apo-sGC mice was inhibited by ODQ. VIP receptors are coupled to Gs proteins and adenylyl cyclase, and ODQ is not expected to affect the activity of adenylyl cyclase (Garthwaite *et al.*, 1995). Non-specific effects of ODQ on excitation-contraction coupling with inhibition of contractile activity were previously described in canine colon (Franck *et al.*, 1997). The mechanism responsible for the ability of ODQ to inhibit EFS-induced relaxation of apo-sGC fundus preparations remains to be determined.

Fundic nitrergic relaxation is essential for gastric accommodation (Desai *et al.*, 1991) and its deficiency in apo-sGC mice might be expected to speed up liquid gastric emptying as fundic storage of the liquids is impaired. However, similar to what was observed in nNOS KO mice (Mashimo *et al.*, 2000) and cGMP-dependent protein kinase (cGKI) KO mice (Pfeifer *et al.*, 1998), liquid gastric emptying in apo-sGC mice was delayed. Also, the stomach of apo-sGC mice, nNOS (Huang *et al.*, 1993; Mashimo *et al.*, 2000) and cGKI (Pfeifer *et al.*, 1998) KO mice, is markedly enlarged and characterized by hypertrophy of the muscularis externa of the fundus. Mashimo *et al.* (2000) suggested this gastric smooth muscle thickening represents work hypertrophy secondary to functional pyloric obstruction. The muscular layer of the pylorus in apo-sGC mice was indeed enlarged as obvious from histology and from the higher pyloric weight of the pyloric rings, and the electrically induced relaxation in pyloric rings of apo-sGC mice was decreased. Impairment of pyloric relaxation will

counteract the accelerating effect of deficient fundic relaxation on gastric emptying leading to delayed gastric emptying (Anvari *et al.*, 1998; Mashimo *et al.*, 2000). Some electrically induced relaxation, not sensitive to L-NAME, was maintained in pyloric rings of apo-sGC mice. This illustrates that NO is not the sole inhibitory transmitter at the level of the pylorus, as was also reported for rat pylorus (Soediono & Burnstock, 1994; Ishiguchi *et al.*, 2000).

The disturbances in gastric emptying, delaying gavaged liquid solution to enter the small intestine in apo-sGC mice, may contribute to the observed delay in small intestinal transit in apo-sGC mice. The complete inhibition of EFS-induced relaxations in WT jejunal strips by L-NAME, together with the absence of EFS-induced nitrergic relaxation in apo-sGC jejunal strips, identifies NO-sGC signaling as the principal inhibitory pathway in mouse jejunal smooth muscle. It seems therefore likely that an imbalance between inhibitory (nitrergic) and excitatory (cholinergic) input during peristalsis can contribute to the delay in intestinal transit observed in apo-sGC mice. Similarly, intestinal motility was impaired in cGKI KO mice, characterized by spastic contractions of long intestinal segments followed by scarce and slow relaxations (Pfeifer *et al.*, 1998). Still, in apo-sGC mice, the spatiotemporal mapped contractility of a jejunal segment was not different from that in the WT mice; isolated small intestinal segments of total sGC knockout mice also maintained spontaneous rhythmic contractions (Groneberg *et al.*, 2011). These in vitro data appear to be in contrast to the observed delay in intestinal transit in apo-sGC mice and total sGC knockout mice. It is conceivable that the in vitro spontaneous oscillatory contractions are preserved in the jejunum of the apo-sGC mice but that the coordinated interplay between ascending contractions and descending relaxations, essential for peristaltic propagation (Waterman *et al.*, 1994), is disturbed in apo-sGC mice and contributes to the delay in small intestinal transit.

The delay in gastric emptying and intestinal transit most likely contributes to the increase in whole gut transit time in apo-sGC mice. NOS inhibition was found to inhibit colonic propulsion of pellets in guinea pig colon (Foxx-Orenstein & Grider, 1996) and to delay colonic transit in rats (Mizuta *et al.*, 1999). However, because in mouse distal colon, NO -acting via sGC- is only the principal neurotransmitter at a stimulation frequency of 1 Hz and not at higher frequencies, the extent of delay in colonic transit was expected to be limited in apo-sGC mice. Distal colon expulsion of beads was indeed not delayed in apo-sGC mice. We previously suggested that a redundant action of NO, acting at sGC, and another

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neurotransmitter, acting at small conductance Ca^{2+}-dependent K^+ channels, is responsible for the relaxant responses to EFS at 2 to 8 Hz in mouse distal colon. This hypothesis was based on the observations that L-NAME plus apamin, or ODQ plus apamin inhibited the relaxant responses to EFS at 2 to 8 Hz in mouse distal colon (Dhaese *et al.*, 2008). Gallego *et al.* (Gallego *et al.*, 2012) showed that the other neurotransmitter is ATP or a related purine. However, in apo-sGC mice, L-NAME plus apamin failed to influence the relaxant responses by EFS at 2 to 8 Hz. Also in WT colonic strips, L-NAME plus apamin only partially attenuated the relaxations to EFS at 2 to 8 Hz. Together, these findings indicate the contribution of another neurotransmitter than NO and ATP to the relaxations at 2 to 8 Hz. The presence of this unidentified neurotransmitter could depend on the genetic background of the mice: in the current study, mice on a mixed 129/SvJ-C57BL/6J background were used while the previous study focused on mice on mixed Swiss-129 background (Dhaese *et al.*, 2008). The presence of a yet to be defined third neurotransmitter was also reported in rat distal colon (Van Crombruggen & Lefebvre, 2004). Because the relaxant effect of CO in gastrointestinal smooth muscle was abolished in apo-sGC mice, it is unlikely that CO, proposed as an inhibitory neurotransmitter in longitudinal muscle of C57Bl/6J mouse distal colon (Hidaka *et al.*, 2010), is the third neurotransmitter in mouse colon circular muscle. This result also definitely establishes that CO, although being a very weak activator of purified sGC, has a signalling pathway requiring sGC activation to induce relaxation in gastrointestinal tissue.

In conclusion, the gastrointestinal consequences of switching native sGC to heme-free sGC, that cannot be stimulated by NO, were most pronounced at the level of the stomach; the observed enlargement of the stomach with hypertrophy of the smooth muscle layers of the muscularis externa of the fundus and the pylorus and the delayed gastric emptying establish a pivotal role of the activation of sGC by NO in normal gastric functioning. In addition, the inability to stimulate sGC with NO, induced delayed intestinal transit and increased whole gut transit time.

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Chapter IV

MECHANISM OF RELAXATION AND INTERACTION WITH NITRIC OXIDE OF THE SOLUBLE GUANYLATE CYCLASE STIMULATOR BAY 41-2272 IN MOUSE GASTRIC FUNDUS AND COLON

Sarah M.R. Cosyns, Romain A. Lefebvre

Heymans Institute of Pharmacology, Ghent University, Ghent, Belgium

Chapter IV

Mechanism of relaxation and interaction with nitric oxide of the soluble guanylate cyclase stimulator BAY 41-2272 in mouse gastric fundus and colon

IV.1   Abstract

Background. BAY 41-2272 is a heme-dependent nitric oxide-independent soluble guanylate cyclase (sGC) stimulator, but its relaxant effect in vascular, respiratory and urogenital tissue is only partially dependent on sGC activation. As its mechanism of action has not been studied in the gastrointestinal tract, it was investigated in mouse gastric fundus and colon.

Methods. Circular smooth muscle strips were mounted in organ baths under non-adrenergic non-cholinergic (NANC) conditions for isometric force recording and cGMP levels were determined using an enzyme immunoassay kit.

Key results. BAY 41-2272 induced concentration-dependent relaxation in both tissues and increased cGMP levels. The sGC inhibitor ODQ totally inhibited this BAY 41-2272-induced increase of cGMP, but only partially reduced the corresponding relaxation. The PDE-5 inhibitor sildenafil had no effect on BAY 41-2272-induced responses. The NO synthase inhibitor L-NAME caused a significant decrease in BAY 41-2272-induced responses in colonic strips. Electrical field stimulation in the presence of BAY 41-2272 induced increased NANC relaxation in fundus, while in colon, rebound contraction at the end of the stimulation train was no longer visible. This suggests synergy with endogenously released NO. Responses to BAY 41-2272 were not significantly influenced by apamin, charybdotoxin or ouabain, excluding interaction with small, intermediate and large conductance Ca^{2+}-activated K^+ channels and with Na^+-K^+-ATPase. Under depletion of intracellular calcium, $CaCl_2$-induced contractions were significantly reduced by BAY 41-2272 in an ODQ-insensitive way.

Conclusions. The present study demonstrates that BAY 41-2272 exerts its relaxing effect in mouse gastric fundus and colon partially through a cGMP-dependent mechanism and at least one additional cGMP-independent mechanism involving Ca^{2+} entry blockade.

IV.2   Introduction

The nitric oxide (NO) signaling pathway is well established. Released from endothelial cells and/or nitrergic neurons, NO is an important inhibitory regulator of smooth muscle tone in the cardiovascular system but also in other tracts such as the urogenital, respiratory and gastrointestinal. The principal target of NO in smooth muscle cells is soluble guanylate cyclase (sGC), which is regarded as the key enzyme in mediating relaxation through elevations in the intracellular cGMP concentration. Since long the vasodilatory effect of NO has been applied for the therapy of cardiovascular diseases such as angina pectoris and heart failure by use of NO-donating nitrates. Clinical problems with nitrate therapy such as the development of tolerance and cGMP-independent effects induced the interest in direct stimulators of sGC. The lead compound YC-1 led to the development of a novel class of compounds, capable of directly stimulating sGC in an NO-independent manner (Stasch & Hobbs, 2009).

Within this new class, BAY 41-2272 (3-(4-Amino-5-cyclopropylpyrimidin-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridine) has been extensively studied. In vitro, BAY 41-2272 induces arterial relaxation (Bawankule *et al.*, 2005; Teixeira *et al.*, 2006a; Teixeira *et al.*, 2006b) while in vivo, the compound attenuates pulmonary hypertension (Evgenov *et al.*, 2004), decreases blood pressure, has anti-platelet activity (Stasch *et al.*, 2001; Hobbs & Moncada, 2003; Roger *et al.*, 2010) and unloads the heart in a model of congestive heart failure (Boerrigter *et al.*, 2003). BAY 41-2272 may thus offer a new therapeutic strategy for cardiovascular diseases. The effect of BAY 41-2272 is not limited to cardiovascular tissue as it induces relaxation in corpus cavernosum (Kalsi *et al.*, 2003), urethra (Toque *et al.*, 2008), detrusor (Bau *et al.*, 2010) and tracheal smooth muscle (Toque *et al.*, 2010). BAY 41-2272 directly stimulates sGC and increases the enzyme's sensitivity towards NO (Stasch *et al.*, 2001) but additional cGMP-independent mechanisms, contributing to its relaxing effect, have been proposed in arterial, detrusor and tracheal smooth muscle (Teixeira *et al.*, 2006a; Bau *et al.*, 2010; Toque *et al.*, 2010).

NO is a very important non-adrenergic non-cholinergic inhibitory neurotransmitter in the gastrointestinal tract (Toda & Herman, 2005). At the level of the gastric fundus, which contains a smooth muscle layer with tonic activity, NO is involved in gastric adaptive relaxation (Desai *et al.*, 1991). In the colon, where the smooth muscle layer shows phasic

activity, NO contributes to transit regulation (Mizuta *et al.*, 1999). We have previously shown in mouse gastric fundus and colon that endogenous NO induces relaxation mainly through sGC activation (Vanneste *et al.*, 2007; Dhaese *et al.*, 2008). Several conditions such as aging, diabetes and colitis lead to enteric nitrergic dysfunction (Mizuta *et al.*, 2000; Phillips & Powley, 2007; Zandecki *et al.*, 2008). Direct sGC stimulation might thus also be useful in gastrointestinal disease. Reports on the gastrointestinal effects of BAY 41-2272 are limited. The aim of this study was therefore to investigate the effect of BAY 41-2272 in mouse gastric fundus and colon, with special attention for the role of sGC and possible additional mechanisms of action and for the interaction with NO.

IV.3   Materials and methods

IV.3.1 Animals

Male C57BL/6J mice (11-15 weeks, 22-30 g) were purchased from Janvier, Le Genest St-Isle, France and had free access to water and commercially available chow. All experimental protocols were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

IV.3.2   Muscle tension experiments

IV.3.2.1 Tissue preparation and isometric tension recording

Animals were sacrificed by cervical dislocation and the gastrointestinal tract was removed and put in aerated (5% CO_2 in O_2) Krebs solution (composition in mM: NaCl 118.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1). The stomach was emptied from its contents and the gastric fundus was isolated from the rest of the stomach. Two full wall thickness fundus strips (2 x 11 mm) were prepared by cutting in the direction of the circular muscle layer. For the distal colon, a ~ 4 cm long segment was taken above the pelvic brim. The segment was opened along the mesenteric border and pinned mucosa side up in Krebs solution. The mucosa was then removed by sharp dissection under a microscope and 2 full-thickness muscle strips (4 × 5 mm) were cut along the circular axis.

After a silk thread (USP 4/0) was attached to both ends of the strips, they were mounted in 7 ml (distal colon) or 10 ml (gastric fundus) organ baths between 2 platinum electrodes (6 mm apart). The organ baths contained aerated (5% CO_2 in O_2) Krebs solution, maintained at 37°C. Changes in isometric tension were measured using Grass force transducers (colon) or MLT 050/D force transducers (fundus) and recorded on a PowerLab/8sp data recording system (ADInstruments) with Chart software.

All experiments were performed at optimal load. Therefore, after an equilibration period of 30 min with flushing every 10 min at a load of 0.25 g (colon) or 0.75 g (fundus), the length-tension relationship was determined. Muscle tissues were stretched by load increments of 0.25 g and at each load level exposed to 1 µM (colon) or 0.1 µM (fundus) carbachol to determine the optimal load (L_o; the load at which maximal response to the contractile agent occurred). Once the optimal load was determined, tissues were allowed to equilibrate for 60 min at L_o with flushing every 15 min in Krebs solution. The optimal load varied between 0.25 and 0.75 g for colon strips and between 0.75 and 1.25 g for fundus strips.

IV.3.2.2 Protocols

All experiments were carried out after switching to Krebs solution containing 1 µM atropine and 4 µM guanethidine to block cholinergic and noradrenergic responses respectively (NANC conditions), except for the cumulative contractile responses to calcium chloride ($CaCl_2$). All relaxant stimuli were examined after a pre-contraction of the strips with 3 µM (colon) or 300 nM (fundus) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), once the contractile response was stabilized (approximately 6 min after administration of $PGF_{2\alpha}$). Interfering drugs were incubated for 30 min.

In a first set of experiments, the strips were pre-contracted with $PGF_{2\alpha}$ and BAY 41-2272 was cumulatively added (0.3-1-3 µM for the colon and 1-10 µM for the fundus, with an interval of 5 min between the administration of the increasing concentrations). Strips were studied in parallel in the absence and presence of the sGC inhibitor 1H[1,2,4,]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 µM), the NO synthase inhibitor N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME; 300 µM), the voltage-gated Na^+ channel blocker tetrodotoxin (3 µM), the N-type Ca^{2+} channel blocker ω-conotoxin (3 µM)

and the phosphodiesterase-5 inhibitor sildenafil (0.001-0.01-0.1-1-10 µM for the colon and 0.1-1-10 µM for the fundus). The influence of the low conductance Ca^{2+}-activated K^{+} channel blocker apamin (0.3 µM), the intermediate and high conductance Ca^{2+}-activated K^{+} channel blocker charybdotoxin (0.1 µM), the Na^{+}-K^{+}-ATPase inhibitor ouabain (10 µM), and a higher concentration of ODQ (30 µM) was tested versus one concentration of BAY 41-2272 (3 µM for the colon and 10 µM for the fundus, left in contact with the tissue for 5 min).

In a second set of experiments, after pre-contraction with $PGF_{2\alpha}$, relaxations were induced by application of exogenous NO (1-10-100 µM with an interval of at least 5 min during which the effect of a given concentration of NO had disappeared). Strips were then washed for 30 min, and were incubated with ODQ (10 µM). $PGF_{2\alpha}$ was then applied again and the response to NO was studied in the presence of ODQ. In an additional set of $PGF_{2\alpha}$ contracted strips, the responses to electrical field stimulation (EFS; 40V, 0.1 ms, 1-2-4-8 Hz for 30 s [colon] or 10 s [fundus] with a 5 min interval) were obtained. Strips were washed for 30 min, and were subsequently incubated with L-NAME (300 µM). $PGF_{2\alpha}$ was then applied again and the responses to EFS were studied in the presence of L-NAME. In a similar way, the influence of BAY 41-2272 (0.3 µM for the colon and 1 µM for the fundus) and of sildenafil (10 µM, only in the fundus) was studied on the relaxing responses to EFS and NO. In the experiments with BAY 41-2272 and sildenafil, the response to EFS and exogenous NO was studied in the same strips by starting the administration of the first NO concentration 5 min after the last EFS stimulation. Finally, the influence of BAY 41-2272 was studied on the relaxing response to low concentrations of NO (0.01-0.03-0.1-0.3-1 µM) in a similar set-up.

In the third set of experiments, cumulative concentration-response curves to $CaCl_2$ (0.01-100 mM, with an interval of 2 min for the colon and 4 min for the fundus between the administration of increasing concentrations) before and after the addition of BAY 41-2272 and/or ODQ were obtained in Ca^{2+}-free medium. This was done according to previous studies (Lagaud *et al*., 1999). Briefly, strips were pre-contracted with carbachol (10 µM). After washing, the Krebs solution was replaced with a Ca^{2+}-free Krebs solution containing the Ca^{2+}-chelator ethylene glycol bis tetraacetic acid (EGTA, 1 mM). Carbachol (10 µM) was given until the contractions were totally abolished, illustrating total depletion of the intracellular Ca^{2+}-stores. Next, the Krebs solution was replaced with Ca^{2+}-free KCl (80 mM) depolarizing Krebs solution, containing cyclopiazonic acid (10 µM) to block reuptake of Ca^{2+}

in the sarcoplasmic reticulum. Then, concentration-response curves of $CaCl_2$ were obtained before and after the addition of BAY 41-2272 (0.3-1-3 µM for the colon and 1-3-10 µM for the fundus) and/or ODQ (30 µM). Before adding BAY 41-2272 and/or ODQ for the second concentration-response curve of $CaCl_2$, strips were extensively washed with the Ca^{2+}-free KCl Krebs solution.

In all set ups, time-control strips that did not get the interfering drugs or that received the solvent of the interfering drugs were run in parallel. At the end of each experiment, the wet weight of the smooth muscle strips was determined (mg wet weight; see data analysis).

IV.3.2.3 Functional data analysis

In fundus strips, the amplitude of the tonic contractile and relaxant responses can be measured. Responses to EFS, NO and BAY 41-2272 were determined at their maximal level and were expressed as the percentage of contraction evoked by $PGF_{2\alpha}$. Additionally, in the series of experiments where the responses to EFS and NO were obtained before and in the presence of sildenafil, the duration of the responses was determined as time from 50 % relaxation to 50 % recovery of tone.

As colonic strips showed phasic activity, the area under the curve (AUC) above baseline was determined to measure the contractile responses to $PGF_{2\alpha}$. To express relaxant responses in colonic strips, the AUC for a given response was determined and subtracted from the AUC of a corresponding period just before applying the relaxing drug or stimulus, yielding the area above the curve (AAC) for the relaxant response. The duration of the relaxant responses was fixed at 5 min for BAY 41-2272 and NO and for EFS, it was determined as 30 s (colon) or 10 s (fundus), as this is the length of the stimulus train applied. Responses are expressed as (g.s)/mg wet weight.

In both colon and fundus, contractile responses to $CaCl_2$ were expressed as a percentage of the contraction by the first administration of carbachol (10 µM).

IV.3.3 cGMP analysis

Circular muscle strips of the distal colon and of the gastric fundus were prepared as for the muscle tension experiments. Before mounting them in the organ baths with aerated Krebs solution, they were weighed to determine the tissue wet weight. The optimal load L_o was determined and the Krebs solution was replaced with Krebs solution containing 1 µM atropine and 4 µM guanethidine, to have the same conditions as in the muscle tension experiments. After pre-contraction with $PGF_{2\alpha}$, BAY 41-2272 (3 µM for the colon and 10 µM for the fundus) or its solvent DMSO was added to the organ baths. Also, some strips were incubated during 30 min with ODQ (30 µM) before contraction with $PGF_{2\alpha}$ and addition of BAY 41-2272. Exactly 5 min after adding BAY 41-2272 or DMSO, strips were snap frozen in liquid nitrogen and they were stored at -80°C until further processing.

cGMP was extracted and quantified using an enzyme immunoassay kit (Cayman Chemical cyclic GMP EIA kit, Michigan, USA). Briefly, frozen tissues were pulverized by a Mikro-Dismembrator U (B. Braun Biotech International, Germany), homogenized in 5 % trichloroacetic acid (TCA) and centrifuged for 15 min at 4°C at 2000 g to collect the supernatant. The supernatant was washed three times with water-saturated ether to extract the TCA after which it was dried under nitrogen at 60°C. After drying, it was dissolved in a 10 times volume of assay buffer. Then, samples, controls and standards were acetylated and were added to the enzyme immunoassay plate to incubate for 18h at 4°C. Optical density was measured with a 96-well plate reader (Biotrak II, Amersham Biosciences) at 405 nm. The concentration of cGMP was expressed as pmol/g tissue wet weight.

IV.3.4 Drugs used

The following drugs were used: apamin (Alomone Laboratories, Israel), atropine (Sigma-Aldrich, Belgium), 3-(4-Amino-5-cyclopropylpyrimidin-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridine (BAY 41-2272; Alexis, Switzerland), calcium chloride-dihydrate ($CaCl_2$; Sigma-Aldrich), carbachol (Fluka AG, Switzerland), charybdotoxin (Alomone Laboratories, Israel), ω-conotoxin (Alomone Laboratories, Israel), cyclopiazonic acid (Tocris Cookson, UK), ethylene glycol bis tetraacetic acid (EGTA; Fluka AG, Switzerland),

guanethidine (Sigma-Aldrich), N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma-Aldrich), 1H[1,2,4,]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Tocris Cookson, UK), prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$; Sigma-Aldrich), sildenafil (Sigma-Aldrich), tetrodotoxin (Alomone Laboratories, Israel) and trichloroacetic acid (TCA; Merck Chemicals, Belgium). All drugs were dissolved in deionized water except for ODQ which was dissolved in 100 % ethanol, and BAY 41-2272 (1 and 10 mM stock solution), cyclopiazonic acid and sildenafil (10 mM stock solution) which were dissolved in 100% DMSO. NO solution was prepared from gas (Air Liquide, Belgium) as described before (Kelm & Schrader, 1990). The final concentration of ethanol and DMSO in the organ bath did not exceed 0.1 %.

IV.3.5 Statistics

All results are expressed as means ± S.E.M. n refers to tissues obtained from different animals. Comparison of data obtained in parallel strips in the presence of an interfering drug or solvent (aqua, DMSO or ethanol) was done by an unpaired Student's t-test (2 groups) or by a one-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparison t-test (more than 2 groups). Comparison of data obtained within the same tissues (before and after an interfering drug or solvent) was done by a paired Student's t-test. A P-value less than 0.05 was considered to be statistically significant (Graphpad, San Diego, CA, USA).

IV.4   Results

IV.4.1   Role of sGC-cGMP in the relaxant effect of BAY 41-2272 in gastric fundus and colon

IV.4.1.1 Influence of ODQ and L-NAME on BAY 41-2272-induced relaxations

$PGF_{2\alpha}$ induced an increase in tone in fundus strips, and a combined tonic and phasic response in colonic strips. The effective relaxant concentrations of BAY 41-2272 were determined in preliminary experiments. In this effective concentration range (0.3-1-3 µM [colon], 1-10 µM [fundus]), addition of BAY 41-2272 induced concentration-dependent relaxations in both fundus and colonic strips. The responses to BAY 41-2272 consisted of a sustained decline in tone; in colonic strips, a suppression of phasic activity was additionally observed. The contraction to $PGF_{2\alpha}$ was almost completely abolished by 10 µM BAY 41-2272 in the fundus; in the colon it was completely abrogated by 3 µM BAY 41-2272 (Fig. IV.1).
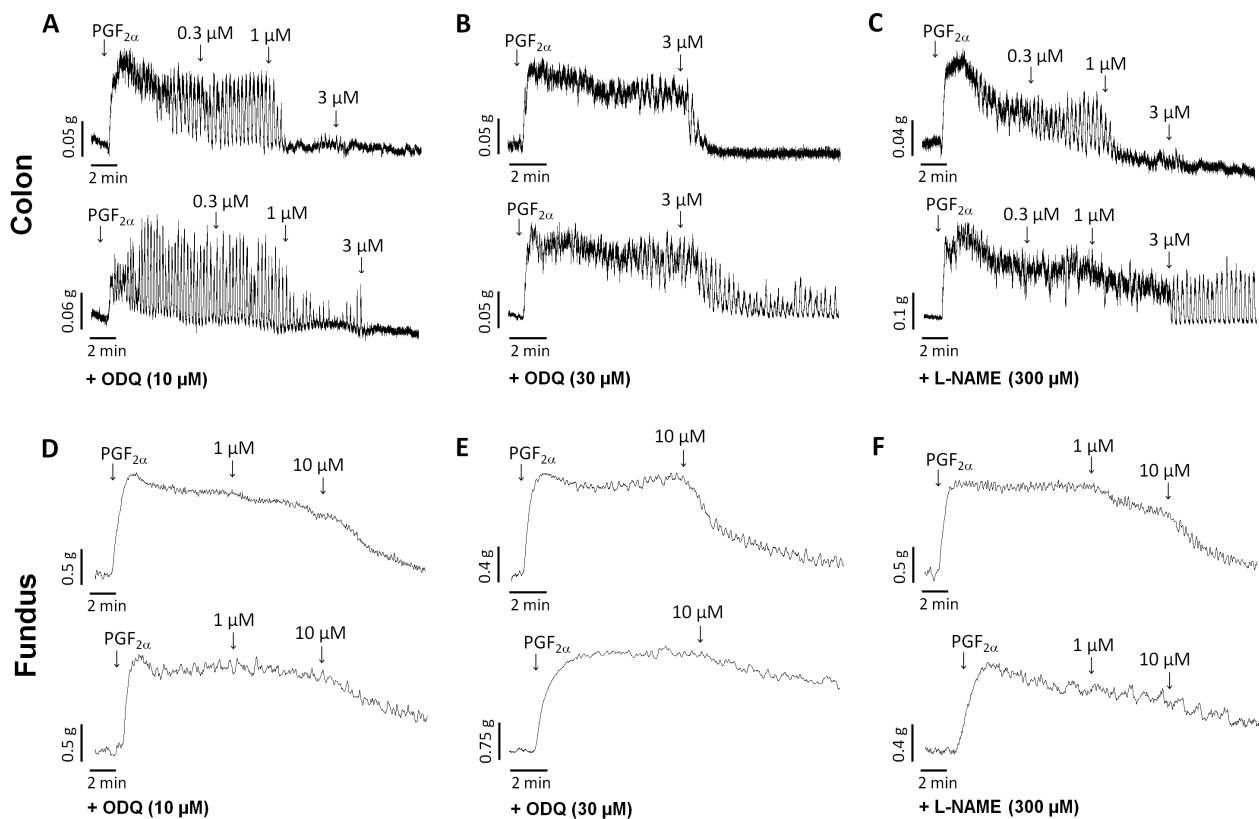
Fig. IV.1 Representative traces of smooth muscle responses to BAY 41-2272 in the absence or presence of ODQ (10 µM: A and D; 30 µM: B and E) or L-NAME (300 µM: C and F) in $PGF_{2\alpha}$ pre-contracted circular muscle strips of the distal colon (A, B and C) and gastric fundus (D, E and F).

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In both fundus and colonic strips, the sGC inhibitor ODQ (10 µM) did not significantly affect the BAY 41-2272-induced relaxations (Figs. IV.1A and D, IV.2A and D). Only in the presence of a higher concentration of ODQ (30 µM), the relaxing effect of BAY 41-2272 (10 µM) was significantly (P<0.01) reduced in fundus strips (Figs. IV.1E and IV.2E). In the colonic smooth muscle strips, the decrease of phasic activity by BAY 41-2272 (3 µM) seemed to some extent suppressed by the higher concentration of ODQ (30 µM; Fig. IV.1B) but the decrease in BAY 41-2272-induced relaxation, calculated as area above the curve, by ODQ did not reach significance (Fig. IV.2B). In the fundus, the NO synthesis inhibitor L-NAME (300 µM) did not have a significant effect on the relaxant effect of BAY 41-2272 (Figs. IV.1F and IV.2F). In the colon however, pre-treatment with L-NAME caused a significant decrease in the relaxing responses to BAY 41-2272 when compared to control strips (Figs. IV.1C and IV.2C).
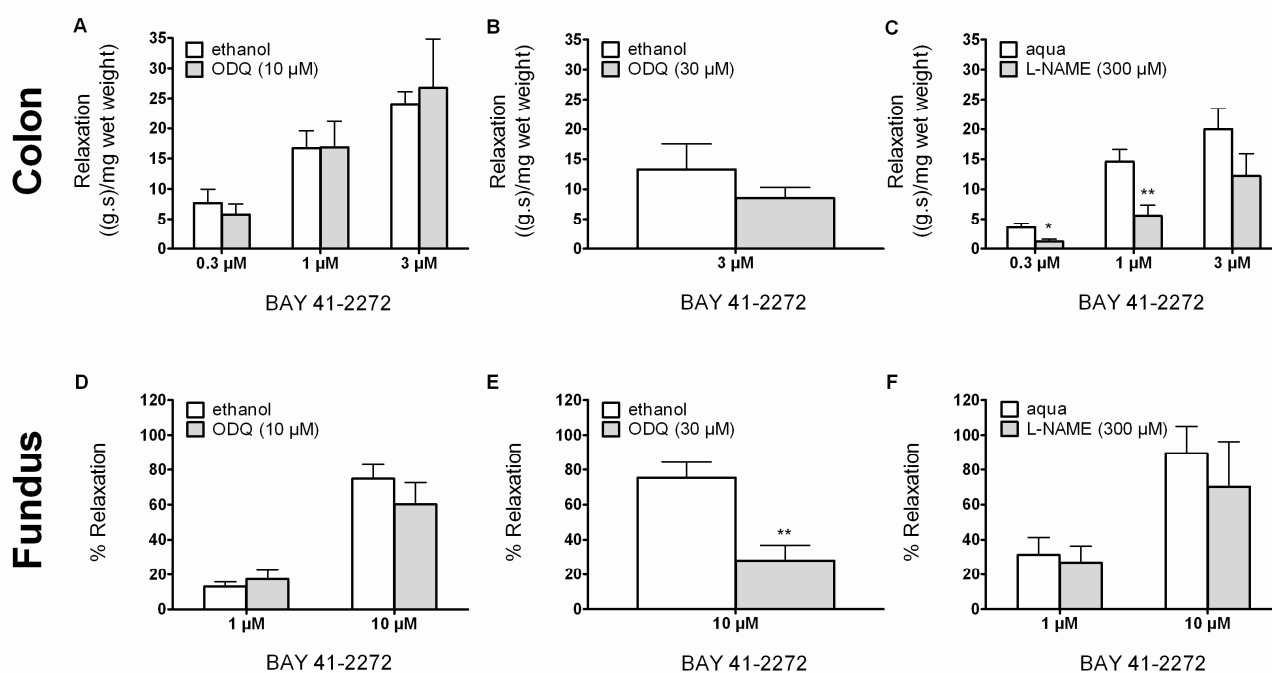
Fig. IV.2 Relaxant responses to BAY 41-2272 in the presence of ODQ (10 µM: A and D; 30 µM: B and E) or L-NAME (300 µM: C and F) or the solvent (ethanol, aqua) in colonic (upper part of the figure: A, B and C) and fundus (lower part of the figure: D, E and F) smooth muscle strips. Data represent the means ± S.E.M. of n = 6. *P<0.05, **P<0.01: significantly different versus ethanol or aqua control; unpaired Student's t-test. ODQ (30 µM) and L-NAME (300 µM) increased the contractile response to $PGF_{2\alpha}$ in the colonic strips (contraction to $PGF_{2\alpha}$ in (g.s)/mg wet weight: 4.58 ± 1.30 [ethanol control] versus 10.98 ± 2.21 [ODQ, 30 µM], n = 6; 5.90 ± 1.68 [aqua control] versus 11.80 ± 4.00 [L-NAME, 300 µM], n = 6), meaning that there is more contractile activity to suppress, which might per se increase the absolute relaxant responses to BAY 41-2272. The responses to BAY 41-2272 in the presence of ODQ (30 µM) and L-NAME in the colonic strips were therefore corrected by multiplying with a factor "$PGF_{2\alpha}$ response in control strips"/"$PGF_{2\alpha}$ response in strips that received ODQ (30 µM) or L-NAME".

IV.4.1.2 Influence of phosphodiesterase-5 inhibition on BAY 41-2272-induced relaxations

Pre-treatment of the fundus smooth muscle strips with the phosphodiesterase-5 inhibitor sildenafil (0.1-1-10 µM, n = 7) did not significantly affect the BAY 41-2272-induced (1-10 µM) relaxations when compared with untreated strips. After pre-treatment of the colonic smooth muscle strips with sildenafil (0.1-1-10 µM, n = 3), $PGF_{2\alpha}$ contraction was significantly decreased or abolished, so that BAY 41-2272 relaxations could not be compared in untreated and sildenafil-treated strips. Lower concentrations of sildenafil (1-10-100 nM, n = 6) were therefore used in colonic strips, but BAY 41-2272-induced relaxations were not significantly affected by these concentrations of sildenafil (Fig. IV.3).
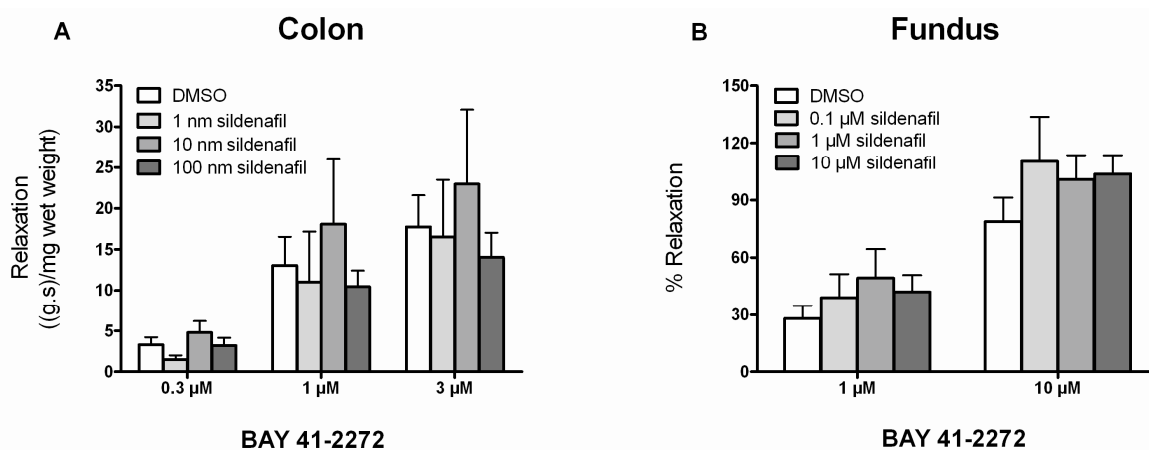
Fig. IV.3 Relaxant responses to BAY 41-2272 in the presence of different concentrations of sildenafil (1-10-100 nM for the colon (A) and 0.1-1-10 µM for the fundus (B)) or its solvent DMSO. Data represent the means ± S.E.M. of n = 6 for the colonic strips and n = 7 for the fundus strips. Data-analysis: ANOVA followed by a Bonferroni multiple comparison t-test.

IV.4.1.3 cGMP analysis

In the fundus smooth muscle strips, BAY 41-2272 (10 µM) significantly (P<0.01) increased basal cGMP levels by 3-fold. BAY 41-2272 (3 µM) also increased the level of cGMP in the colonic strips by 4.5-fold, though this increase is not significant due to one outlier (the cGMP levels in colonic strips incubated with BAY 41-2272 varied between 13.86 and 52.00 pmol/g tissue, except for one strip that contained 141.00 pmol cGMP/g tissue and highly increased the variation of the result). Without this outlier, the mean cGMP level in colonic strips exposed to BAY 41-2272 (28.18 ± 6.60 pmol/g tissue; n = 5) was significantly different from the levels in the presence of DMSO (10.65 ± 0.91; n = 6; P < 0.05). The sGC inhibitor

ODQ (30 µM) totally inhibited the BAY 41-2272-induced increase of cGMP in both fundus (P<0.01) and colonic strips (Fig. IV.4).
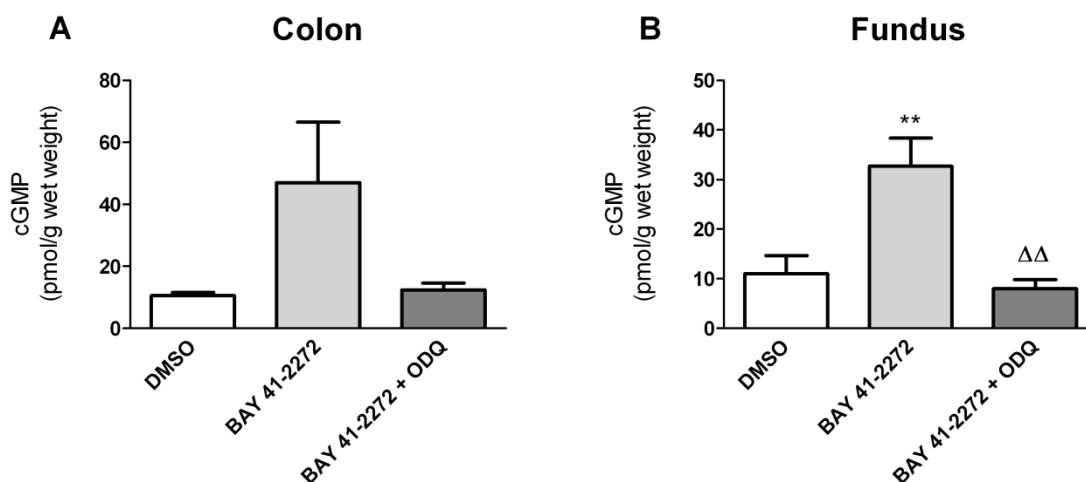
Fig. IV.4 cGMP levels in basal conditions (DMSO, solvent of BAY 41-2272) and when incubated with BAY 41-2272 (3 µM in colon and 10 µM in fundus) in the absence or presence of ODQ (30 µM) in colonic (A) and fundus (B) smooth muscle strips. Data represent the means ± S.E.M. of n = 6. **P<0.01: significantly different versus DMSO; ΔΔP<0.01: significantly different versus BAY 41-2272; ANOVA followed by a Bonferroni multiple comparison t-test.

IV.4.2 Interaction with endogenous and exogenous NO

IV.4.2.1 Influence of ODQ and L-NAME on EFS and exogenous NO

In colonic strips, EFS (1-2-4-8 Hz, 40 V, 0.1 ms, 30 s) induced a quick and transient decline in tone with suppression of the phasic activity (Fig. IV.5A). Upon ending stimulation, a fast rebound contraction occurred, followed by progressive recuperation of contractile activity. In the fundus (EFS; 1-2-4-8 Hz, 40 V, 0.1 ms, 10 s), a quick and transient decline in tone was also observed, but without rebound contraction (Fig. IV.5C). After adding L-NAME (300 µM) the EFS-induced relaxations were totally abolished in the fundus strips, but in the colonic strips, it was only at 1 Hz that L-NAME abrogated relaxations.

Application of exogenous NO (1-10-100 µM) induced relaxations with a concentration-dependent duration in fundus and colonic smooth muscle strips. In both tissues, the response to NO consisted of a quick decline in tone, and in colonic strips, a suppression of the phasic activity was observed. In colonic smooth muscle strips, ODQ (10 µM) totally abolished relaxant responses to 1 and 10 µM NO, and it reduced the relaxing response to 100 µM NO (Fig. IV.5B). After incubation of the fundus strips with ODQ (10 µM), a complete abrogation of the relaxant responses to NO was observed at 1 µM, and a clear-cut reduction of relaxation was seen at 10 and 100 µM (Fig. IV.5D).
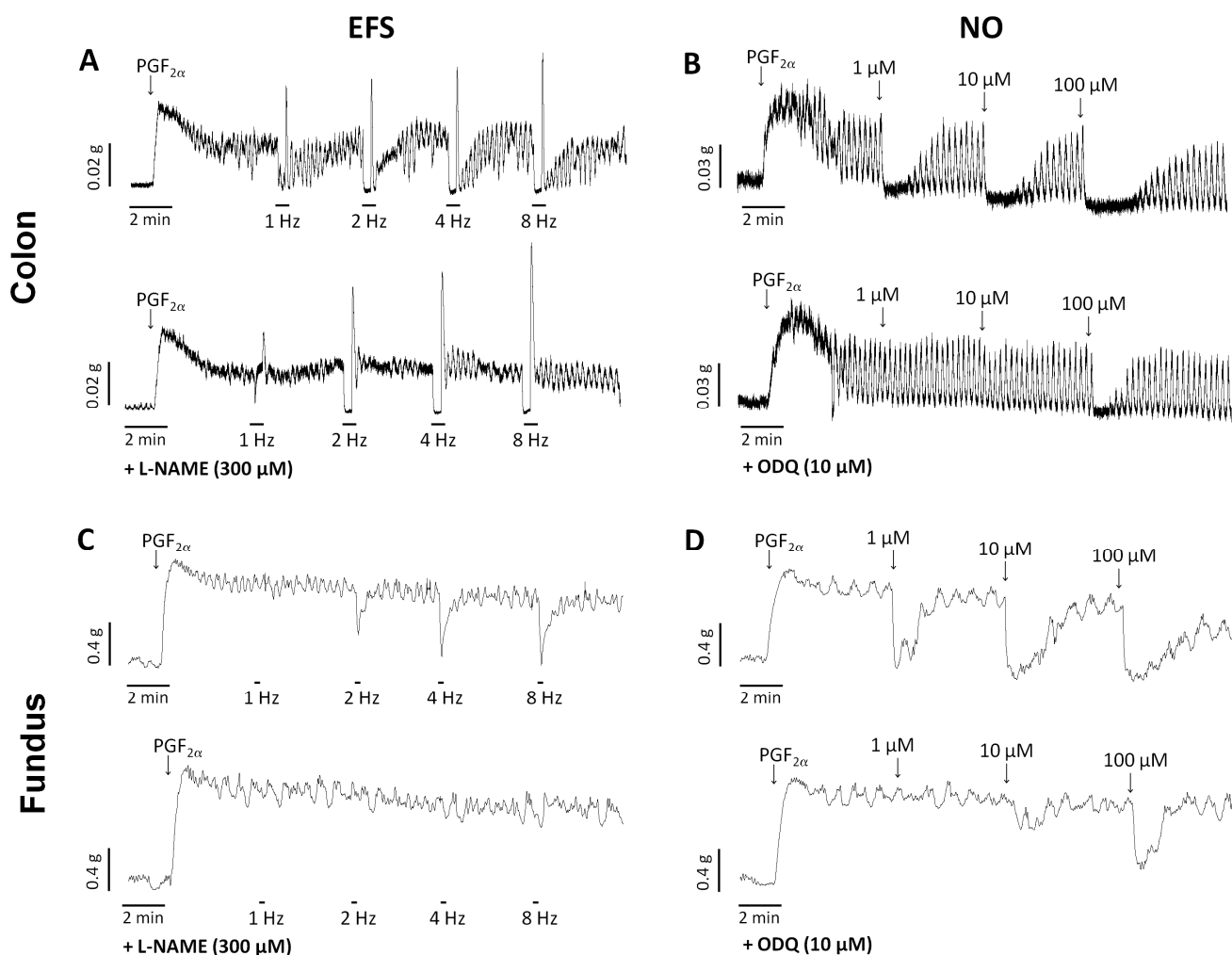
Fig. IV.5 Representative traces of smooth muscle responses to EFS (40V, 0.1 ms, 1-2-4-8 Hz; A and C) before and after addition of L-NAME (300 µM) and smooth muscle responses to NO (1-10-100 µM; B and D) before and after addition of ODQ (10 µM) in $PGF_{2\alpha}$ pre-contracted smooth muscle strips of the colon (A and B) and the fundus (C and D). The traces represent examples for n = 4 for each condition.

IV.4.2.2 Interaction of BAY 41-2272 with EFS and exogenous NO

In order to study the influence of BAY 41-2272 versus the responses to EFS (1-2-4-8 Hz, 40 V, 0.1 ms, 30 s [colon] or 10 s [fundus]) and exogenous NO (0.01-100 µM), BAY 41-2272 was used in the lowest concentration that had shown relaxant activity (0.3 µM in colon and 1 µM in fundus, see figure IV.2). In the presence of BAY 41-2272, EFS-induced relaxations tended to increase in colonic smooth muscle strips at 2, 4 and 8 Hz (Figs. IV.6A and IV.7A). Remarkably, the rebound contractions disappeared at all frequencies (Fig. IV.6A). In the fundus smooth muscle strips, incubation with BAY 41-2272 increased EFS-induced relaxations at all frequencies, reaching significance at 1 and 2 Hz (Figs. IV.6B and

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IV.7D). In both tissues, BAY 41-2272 did not significantly influence the relaxations by exogenous NO, as well in the concentration range of 1-100 µM (Fig. IV.7C and F) as of 0.01-1 µM (Fig. IV.7B and E).

Fig. IV.6 Representative traces of smooth muscle responses to EFS (40V, 0.1 ms, 1-2-4-8 Hz) and NO (1-10-100 µM) before and after addition of BAY 41-2272 (0.3 µM for the colon and 1 µM for the fundus) in PGF$_{2\alpha}$ pre-contracted smooth muscle strips of the colon (A) and the fundus (B). BAY 41-2272 (0.3 µM) significantly decreased the contractile response to PGF$_{2\alpha}$ in the colonic strips, in the series where the relaxing response to EFS and 1-100 µM of exogenous NO was investigated (contraction to PGF$_{2\alpha}$ in (g.s)/mg wet weight: 12.33 ± 2.71 [strips before giving BAY 41-2272] versus 9.35 ± 2.37 [strips after giving BAY 41-2272], n = 6, P < 0.05). The relaxing responses to EFS and NO in the presence of BAY 41-2272 in the colonic strips were thus multiplied with a factor "PGF$_{2\alpha}$ response before addition of BAY 41-2272"/ "PGF$_{2\alpha}$ response after addition of BAY 41-2272" in order to correct for the significantly lower PGF$_{2\alpha}$-induced contraction in the presence of BAY 41-2272.
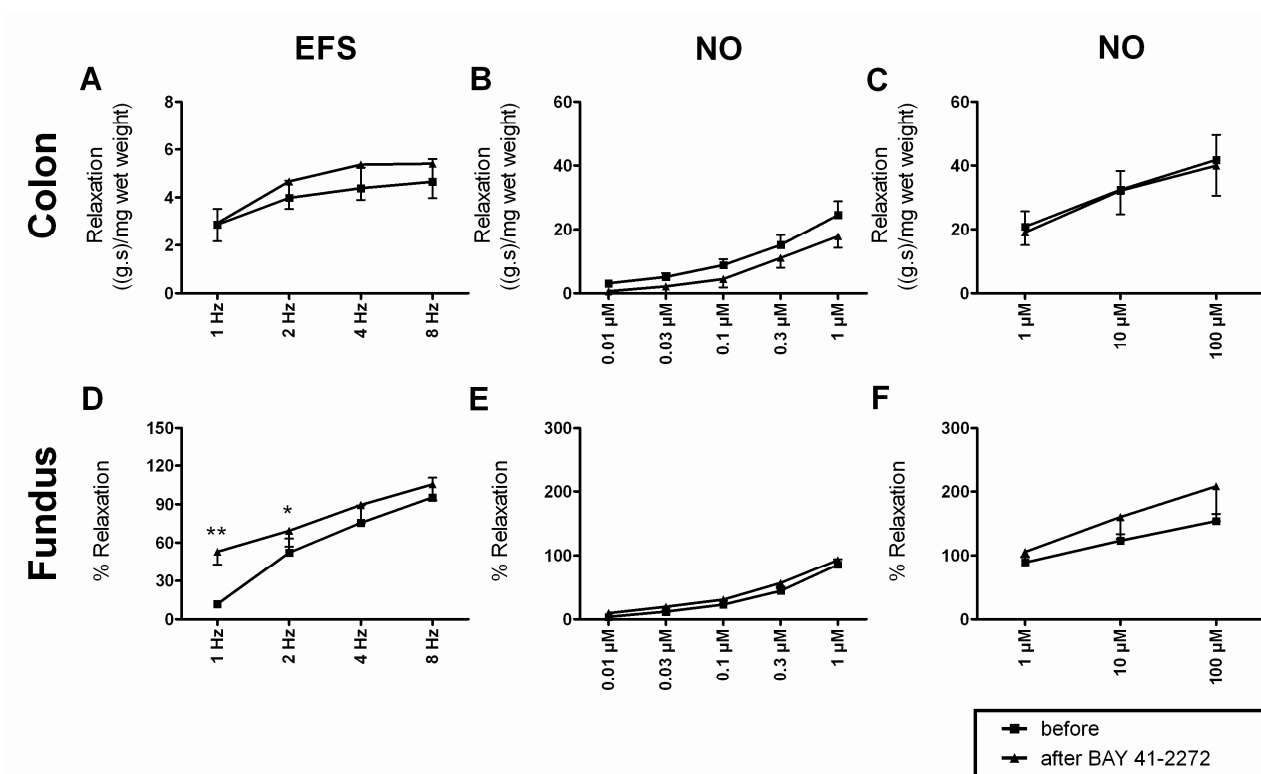
Fig. IV.7 Frequency-response curves of EFS (40V, 0.1 ms, 1-2-4-8 Hz; A and D) and concentration-response curves of NO (0.01-0.03-0.1-0.3-1 µM; B and E; 1-10-100 µM; C and F), in colonic (A, B and C) and fundus (D, E and F) smooth muscle strips before and after incubation with BAY 41-2272 (0.3 µM for the colon and 1 µM for the fundus). Data represent the means ± S.E.M. of n = 6-8. *P<0.05, **P<0.01: after significantly different versus before; paired Student's t-test.

IV.4.2.3 Influence of phosphodiesterase-5 inhibition on EFS and exogenous NO

To study the influence of sildenafil on the responses to EFS (1-2-4-8 Hz, 40 V, 0.1 ms, 10 s) and exogenous NO (1-10-100 µM) in the fundus, sildenafil was used in the highest concentration as tested versus BAY 41-2272 (10 µM, see IV.4.1.2.).

The amplitude of the relaxations induced by EFS and exogenous NO was not increased in the presence of sildenafil (Fig. IV.8A, B and C). However, sildenafil increased the duration of the relaxations by EFS at 2-8 Hz and of all concentrations of exogenous NO (Fig. IV.8A, D and E). At 100 µM NO, the relaxation in the presence of sildenafil was even maintained for the 10 min incubation period in 6 out of 8 tissues; duration was taken at 600 s for these tissues.
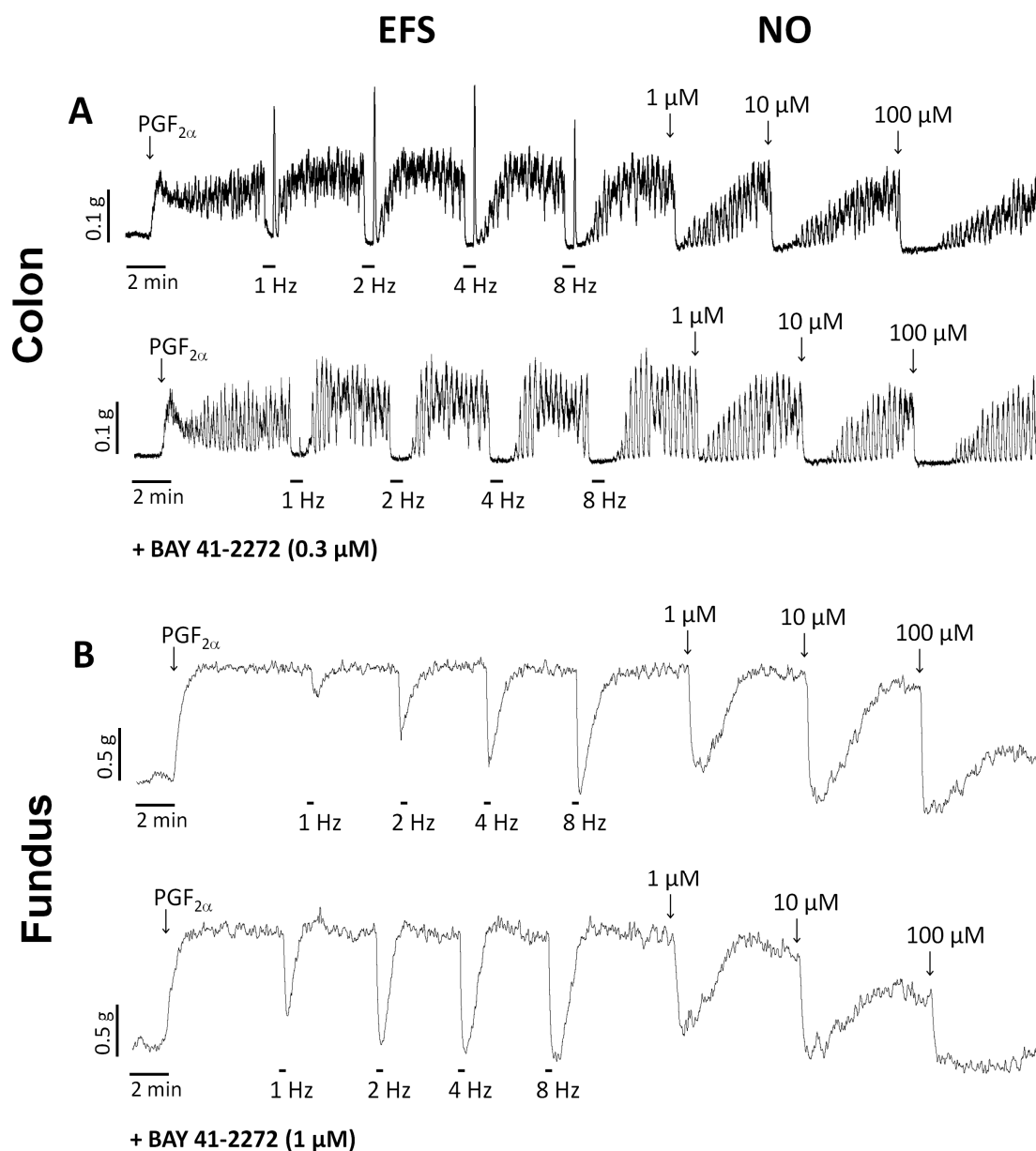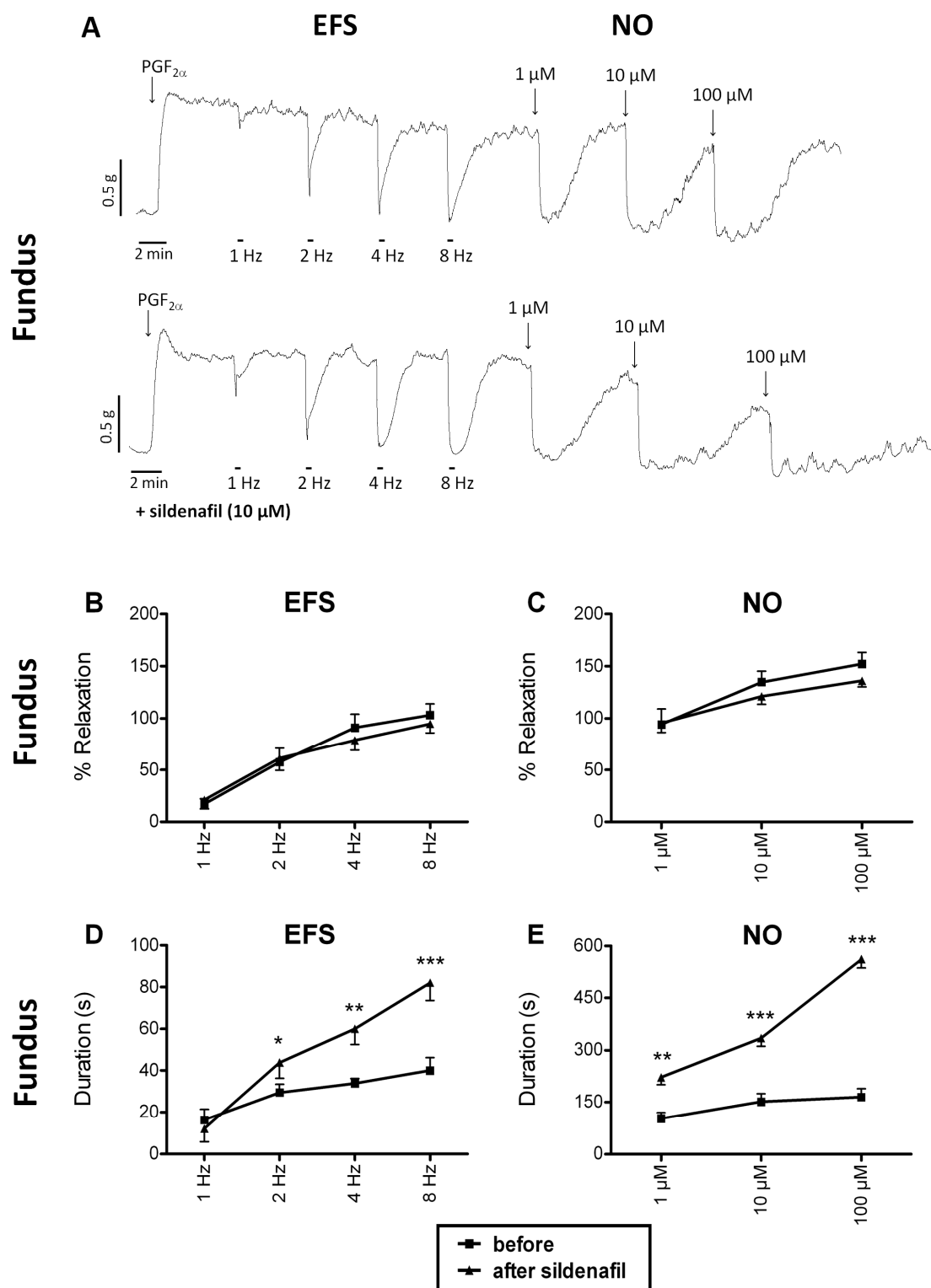
Fig. IV.8 (A) Representative traces of smooth muscle responses to EFS (40V, 0.1 ms, 1-2-4-8 Hz) and NO (1-10-100 µM) before and after addition of sildenafil (10 µM) in a PGF$_{2\alpha}$ pre-contracted smooth muscle strip of the fundus. (B-C) Amplitude of the relaxant responses to EFS (B) and NO (C) before and after addition of sildenafil. (D-E) Duration of the relaxant responses to EFS (D) and NO (E) before and after addition of sildenafil. In B-E, data represent the means ± S.E.M. of n = 7-8. * $P<0.05$, **$P<0.01$,***$P<0.001$: after significantly different versus before; paired Student's t-test.

IV.4.3 Role of K^+ channels, Na^+-K^+-ATPase, voltage-gated Na^+ channels, N-type Ca^{2+} channels and calcium entry in the relaxant effect of BAY 41-2272

The relaxant responses to BAY 41-2272 (3 µM [colon], 10 µM [fundus]) in strips that received apamin (0.3 µM, n = 4), charybdotoxin (0.1 µM, n = 4), ouabain (10 µM, n = 4), tetrodotoxin (3 µM, n = 4) or ω-conotoxin (3 µM, n = 4) were not significantly different from those in parallel untreated control strips (results not shown).

CaCl$_2$-induced contractions (0.01-100 mM) were concentration-dependent and incubation with BAY 41-2272 (0.3-1-3 µM [colon], 1-3-10 µM [fundus], 30 min) reduced these contractions significantly at a BAY 41-2272 concentration of 1 µM and 3 µM in the colonic strips and of 3 µM and 10 µM in the fundus strips (Fig. IV.9). In an additional series, the influence of ODQ was tested on the inhibitory effect of BAY 41-2272 (3 µM in the colon; 10 µM in the fundus) versus CaCl$_2$-induced contractions. In the colon, ODQ per se tended to decrease the CaCl$_2$-induced contractions although this did not reach significance. ODQ did not prevent the inhibitory effect of BAY 41-2272 on calcium-induced contraction. In the fundus, ODQ had no effect per se and did not influence the reduction of calcium-induced contractions by BAY 41-2272 (Fig. IV.10).
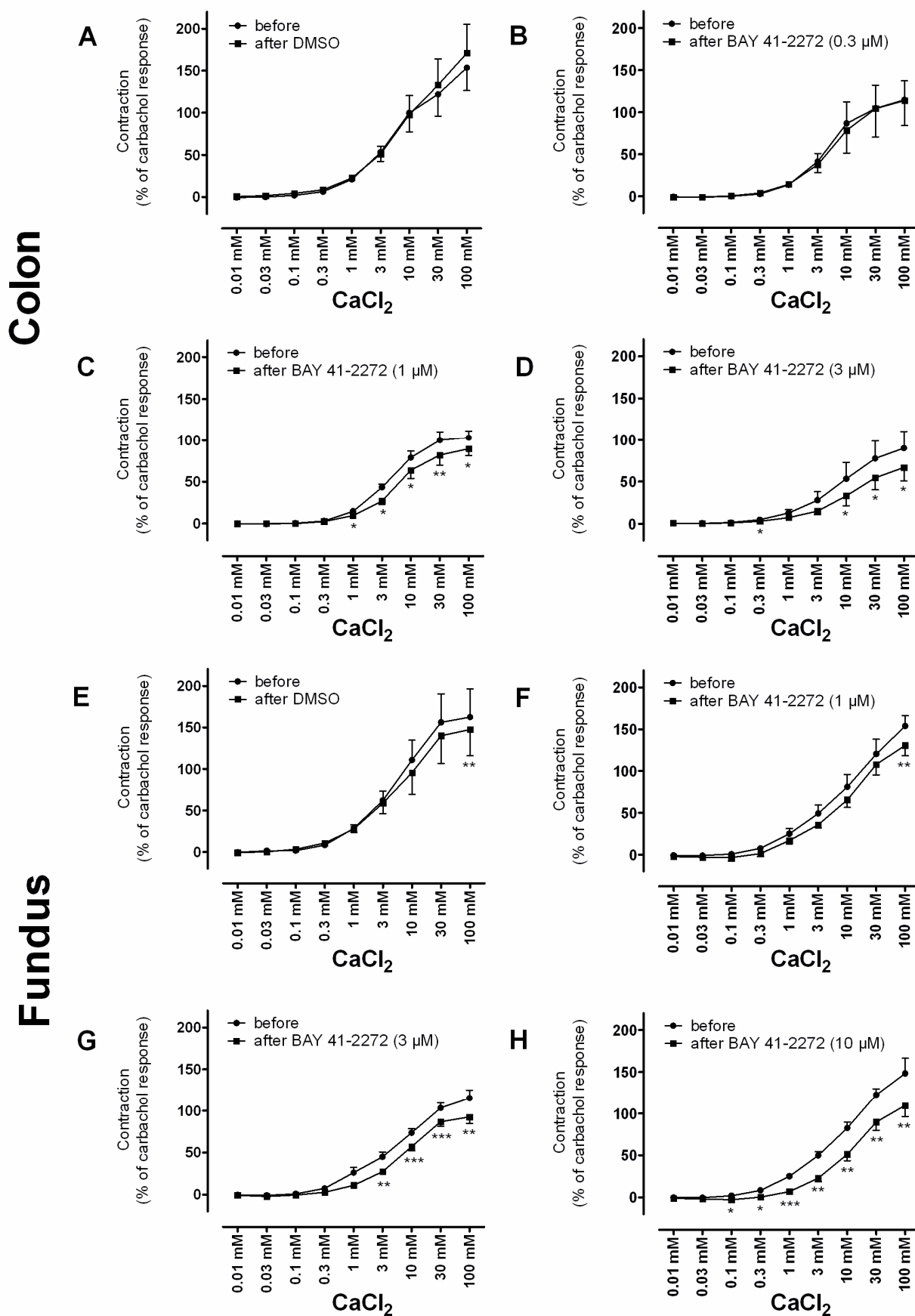
Fig. IV.9 Concentration-response curves of extracellular $CaCl_2$ before and after adding different concentrations of BAY 41-2272 (0.3-1-3 μM for the colon: B-D; and 1-3-10 μM for the fundus: F-H) or its solvent DMSO (A, E). Data represent the means ± S.E.M. of n = 6. *P<0.05, **P<0.01: after significantly different versus before; paired Student's t-test.
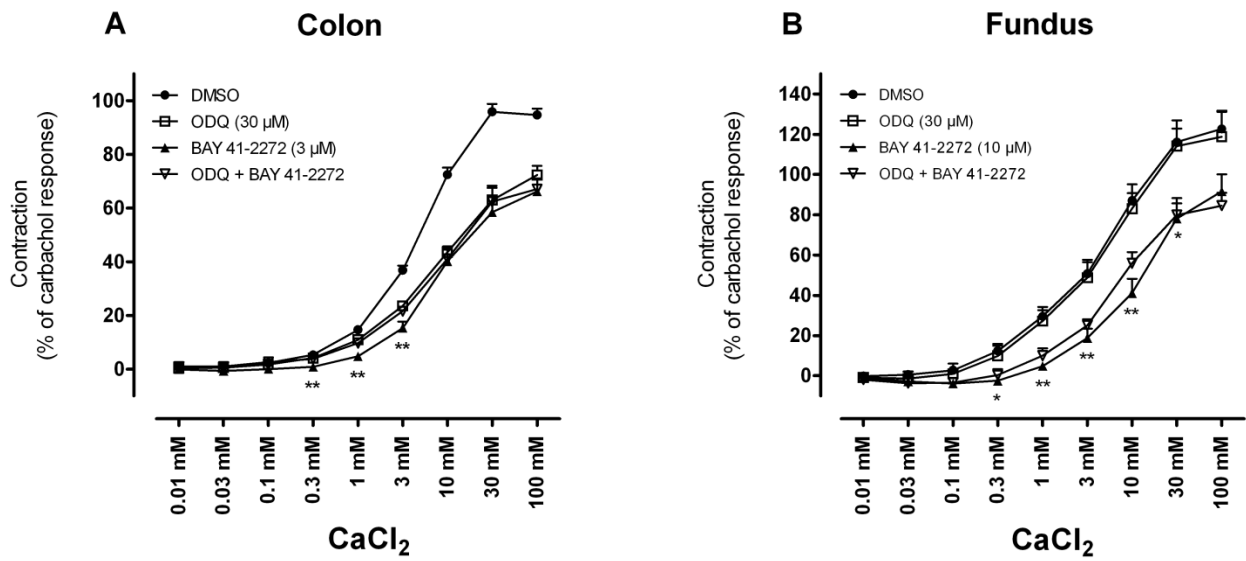
Fig. IV.10 Concentration-response curves of extracellular $CaCl_2$ in the presence of DMSO, ODQ (30 µM), BAY 41-2272 (3 µM for the colon (A) and 10 µM for the fundus (B)) or ODQ + BAY 41-2272. Data represent the means ± S.E.M. of n = 6. *P<0.05, **P<0.01, ***P<0.001: BAY 41-2272 versus DSMO; ANOVA followed by a Bonferroni multiple comparison t-test.

IV.5   Discussion

The aim of this study was to investigate the mechanism of relaxation and the interaction with NO of BAY 41-2272 in mouse gastric fundus and colon. The gastric fundus is involved in gastric receptive relaxation upon food intake and fundus smooth muscle shows sustained tonic responses. The colon is involved in colonic storage and transit and colonic smooth muscle develops phasic activity. The results obtained illustrate that in both tissues, BAY 41-2272 induces relaxation through cGMP-dependent and cGMP-independent mechanisms; the interaction with NO however differs to some extent between both tissues.

In both mouse gastric fundus and colon, BAY 41-2272 induced concentration-dependent relaxation, the maximal effect being reached at similar concentrations as previously reported in vascular and non-vascular tissue (Bawankule *et al.*, 2005; Priviero *et al.*, 2005; Teixeira *et al.*, 2007; Bau *et al.*, 2010). The sensitivity of the gastrointestinal tissues seems low as relaxation by BAY 41-2272 only started at 0.3 µM in the colon and 1 µM in the fundus, while relaxations already clearly occurred at 0.01 µM in vascular (Bawankule *et al.*, 2005) and tracheal (Toque *et al.*, 2010) tissues. BAY 41-2272 stimulates sGC in a heme-dependent way (Stasch & Hobbs, 2009) and evidence was now obtained that the BAY 41-2272-induced relaxation in mouse gastric fundus and colon is also partially due to sGC activation. The sGC inhibitor ODQ, in a concentration (10 µM) shown to at least partially reduce the relaxant effect of BAY 41-2272 in different types of tissues, did not influence the relaxant effect of BAY 41-2272 in gastric fundus and colon. But at 30 µM, it significantly reduced the response to BAY 41-2272 in gastric fundus, and showed a tendency to reduce that in colon. Based on the amount of sGC present and the percentage of sGC that is already in the heme-oxidized/heme-free condition, the concentration of ODQ required to show inhibition might differ between tissues. In detrusor muscle, 100 µM ODQ was required to reduce the relaxant effect of BAY 41-2272 (Bau *et al.*, 2010). Although only partially reducing the relaxant effect of BAY 41-2272, ODQ (30 µM) completely abolished the increase in cGMP levels by BAY 41-2272. On the one hand, this result corroborates that BAY 41-2272 acts by sGC activation in gastric fundus and colon; on the other hand, the discrepant effect of ODQ on the increase in cGMP (abolished) and relaxation (partially reduced) by BAY 41-2272 points to sGC-cGMP-independent mechanisms involved in relaxation by BAY 41-2272 in gastrointestinal tissue. This correlates with data we previously

obtained in the gastric fundus of sGCα_1 knockout mice, where the relaxant effect of BAY 41-2272 was reduced but not abolished, although sGC$\alpha_1\beta_1$ is the predominant form in the gastrointestinal tract (Vanneste *et al.*, 2007). The phosphodiesterase-5 (PDE-5) inhibitor sildenafil did not influence the relaxation by BAY 41-2272. This contrasts to observations in arterial (Priviero *et al.*, 2005) and corpus cavernosum (Teixeira *et al.*, 2007) tissue, where sildenafil enhanced BAY 41-2272-induced relaxation, but corresponds with the non-effect of sildenafil versus BAY 41-2272 in detrusor muscle (Bau *et al.*, 2010). The latter authors suggested that this might be related to PDE-5 inhibitory activity of BAY 41-2272 itself, as demonstrated in platelets (Mullershausen *et al.*, 2004). However, Stasch *et al.* (2001) and Bischoff and Stasch (2004) reported BAY 41-2272 to be devoid of PDE-5 inhibitory activity. In the gastric fundus and colon, the cGMP increase induced by BAY 41-2272 was completely prevented by sGC inhibition, also corroborating that inhibition of PDE-5 does not contribute to the effect of BAY 41-2272 in these tissues. The non-effect of sildenafil versus BAY 41-2272 in gastric fundus and colon might be related to the fact that the BAY 41-2272-induced relaxation is partially sGC-cGMP-independent and that BAY 41-2272 is able to induce a sustained relaxation suggesting that the sGC-dependent part is associated with maintained sGC activation. This might counteract cGMP breakdown by PDE-5. Correspondingly, the sharp and short lasting relaxations by endogenous (EFS) and exogenous NO showed a clearcut increase of duration in the presence of sildenafil but not in their amplitude as studied in the fundus. The influence of PDE-5 inhibition on nitrergic relaxations differs between tissues and conditions as discussed by O'Kane and Gibson (1999). In the latter paper, the relaxation by exogenous authentic NO in female anococcygeus shows an increase in duration but not in amplitude under PDE-5 inhibition, while EFS-induced nitrergic relaxations were enhanced in both amplitude and duration. In circular muscle strips of porcine gastric fundus, PDE-5 inhibition enhanced the duration but not the amplitude of EFS-induced nitrergic relaxations (Lefebvre *et al.*, 1995).

In gastrointestinal tissue, NO has been shown to be able to stimulate small conductance Ca^{2+}-dependent K^+ channels (SK channels) in a cGMP-dependent or direct way (Serio *et al.*, 2003; Van Crombruggen & Lefebvre, 2004) and in vascular and urogenital tissue, NO-cGMP was shown to induce relaxation involving large conductance Ca^{2+}-dependent K^+ channels (BK channels; Archer *et al.*, 1994; Gragasin *et al.,* 2004). In mouse gastric fundus and colon, the SK channel blocker apamin and the BK channel blocker

charybdotoxin did not influence the relaxant effect of BAY 41-2272, excluding SK channels and BK channels, both as a serial target of BAY 41-2272-sGC-cGMP, or as a cGMP-independent target of BAY 41-2272. In ovine pulmonary artery, the relaxant effect of BAY 41-2272 was shown to partially rely on cGMP-independent stimulation of Na^+-K^+-ATPase (Bawankule *et al.*, 2005). As stimulation of Na^+-K^+-ATPase can also induce relaxation in gastrointestinal smooth muscle (Yaktubay *et al.*, 2009), we tested the influence of the Na^+-K^+-ATPase inhibitor ouabain versus BAY 41-2272. Ouabain had no influence on the relaxation by BAY 41-2272, as was also reported in rat mesenteric artery (Teixeira *et al.*, 2006b), illustrating that activation of the sodium pump is not a general mechanism of BAY 41-2272. In contrast, a cGMP-independent mechanism that was proposed to contribute to relaxation by BAY 41-2272 in vascular, urinary and tracheal smooth muscle (Teixeira *et al.*, 2006a; Bau *et al.*, 2010; Toque *et al.*, 2010), i.e. inhibition of extracellular calcium entry, also contributes to the relaxant effect of BAY 41-2272 in mouse gastric fundus and colon. In these tissues, under conditions of depletion of the intracellular calcium stores and of high K^+ depolarization, BAY 41-2272 concentration-dependently inhibited contractions evoked with extracellular calcium, and this inhibitory effect of BAY 41-2272 was not prevented by the sGC inhibitor ODQ. Surprisingly, ODQ per se tended to reduce the calcium-induced contractions in the colonic tissues. A non-specific effect of ODQ on excitation-contraction coupling has been reported for canine proximal colon, where it reduced histamine-induced contractions (Franck *et al.*, 1997). A general depression of excitation-contraction coupling by ODQ in mouse colon seems excluded, as it did not reduce the contractions by $PGF_{2\alpha}$.

In assays with purified sGC, BAY 41-2272 and NO synergize to stimulate enzyme activity (Stasch *et al.*, 2001); a synergistic interaction of BAY 41-2272 with NO donors or endogenous NO released by EFS was reported in several tissues with measurement of smooth muscle relaxation (Teixeira *et al.*, 2006a; Teixeira *et al.*, 2006b; Teixeira *et al.*, 2007; Toque *et al.*, 2010). In mouse gastric fundus, EFS-induced relaxations, that were fully nitrergic as abolished by NO synthesis inhibition, were enhanced by BAY 41-2272, illustrating synergy between BAY 41-2272 and endogenous NO; relaxant responses to exogenous NO, that were sensitive to inhibition by ODQ, were not significantly influenced by BAY 41-2272. Our group has previously (Vanneste *et al.*, 2007) shown via experiments in sGCα_1 knockout mice that EFS with 10 s trains (1-8 Hz) relaxes gastric fundus strips mainly through sGCα_1 activation while sGCα_2 activation only occurs at high stimulation frequencies

and when stimulating for a sufficiently long time (8-16 Hz, 60 s); exogenous NO and BAY 41-2272 in contrast stimulated both sGCα_1 and sGCα_2 in the concentrations tested. In the actual study, BAY 41-2272 might thus enhance the relaxations by the 10 s trains of EFS in the gastric fundus by sensitizing sGCα_2 to react to endogenous NO released by these trains. The amount and still more the spatial distribution of endogenous NO released from nitrergic nerves can be expected to differ from that of exogenous NO, that reaches the smooth muscle cells over the whole surface and stimulates sGCα_1, as well as sGCα_2. This could also explain why BAY 41-2272 did not influence relaxations by exogenous NO in the colon. In addition to synergy between BAY 41-2272 and NO, endogenous NO seems also partially involved in the relaxant effect of BAY 41-2272 in vascular, cavernosal and tracheal tissue (Teixeira *et al.*, 2006a; Teixeira *et al.*, 2006b; Teixeira *et al.*, 2007; Toque *et al.*, 2010). This was not the case in mouse gastric fundus as L-NAME did not influence the effect of BAY 41-2272, corresponding to observations in detrusor muscle (Bau *et al.*, 2010). In contrast, L-NAME reduced the relaxation by BAY 41-2272 in mouse colon, suggesting a role of endogenous NO. It seems unlikely that BAY 41-2272 is capable of releasing endogenous NO from nitrergic neurons, as the N-type Ca^{2+} blocker ω-conotoxin and the voltage-gated Na^+ channel blocker tetrodotoxin did not influence BAY 41-2272-induced relaxations. Both toxins have been shown to reduce or abolish EFS-induced nitrergic relaxations (Kasakov *et al.*, 1995; Amato *et al.*, 2009). The effect of L-NAME versus BAY 41-2272 in the colon might be related to the fact that BAY 41-2272 sensitizes sGC to the effect of tonically released endogenous NO. The tonic release of NO in mouse colon is indeed illustrated by the increase in $PGF_{2\alpha}$-induced contraction by L-NAME and ODQ and the decrease in $PGF_{2\alpha}$-induced contraction by sildenafil. Although the area above the curve of the non-adrenergic non-cholinergic (NANC) relaxations induced by EFS in mouse colon was not significantly changed by BAY 41-2272, an effect of BAY 41-2272 was obvious as the rebound contractions occurring at the end of the stimulation train were abolished; these NANC rebound contractions are most probably tachykininergic in origin (Serio *et al.*, 1998). EFS-induced relaxations in mouse colon are only fully nitrergic at a stimulation frequency of 1 Hz, as shown by the abolishment with L-NAME. We previously showed that at higher stimulation frequencies (2-8 Hz), NO acts in a redundant way together with another transmitter, that stimulates SK channels (Dhaese *et al.*, 2008). As we are not aware of tachykinin receptor antagonist effects of BAY 41-2272, and as the SK channels blocker apamin did not influence

the relaxation by BAY 41-2272, excluding potentiation of a colonic transmitter acting at SK channels, the abolishment of the rebound contractions is most probably due to sustained enhancement of the effect of endogenous NO, thereby preventing the breakthrough of the rebound contractions.

In conclusion, in both mouse gastric fundus and colon, BAY 41-2272 induces relaxation partially by activation of sGC but also via a cGMP-independent mechanism involving inhibition of Ca^{2+} entry; additional cGMP-independent mechanisms cannot be excluded. In gastric fundus, endogenous NO does not contribute to the relaxation by BAY 41-2272, while it does in colon; in both tissues, BAY 41-2272 can enhance the effect of endogenous NO.

IV.6   References

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Chapter V

INFLUENCE OF CINACIGUAT ON GASTROINTESTINAL MOTILITY IN APO-sGC MICE

Sarah M. R. Cosyns [1], Leander Huyghe [2], Johannes-Peter Stasch [3], Peter Brouckaert [2], Romain A. Lefebvre [1]

[1] Heymans Institute of Pharmacology, Ghent University, Ghent, Belgium

[2] Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

[3] Institute of Cardiovascular Research, Bayer Healthcare, Wuppertal, Germany

Chapter V

Influence of cinaciguat on gastrointestinal motility in apo-sGC mice

V.1   Abstract

Background. Cinaciguat (BAY 58-2667), an NO- and heme-independent sGC activator, was shown to be more effective when sGC is oxidized in vascular tissue. In apo-sGC mice (sGC$\beta_1^{His105Phe}$ knockin) both sGC isoforms (sGC$\alpha_1\beta_1$ and sGC$\alpha_2\beta_1$) are heme-deficient and can no longer be activated by NO; these mice, showing decreased gastrointestinal nitrergic relaxation and decreased gastric emptying, can be considered as a model for oxidized sGC. Our aim was to compare the influence of cinaciguat, on in vitro muscle tone of gastrointestinal tissues, and on gastric emptying in WT and apo-sGC mice.

Methods. Gastrointestinal smooth muscle strips were mounted in organ baths for isometric force recording and cGMP levels were determined by enzyme immunoassay. Protein levels of sGC subunits were assessed by immunoblotting. Gastric emptying was determined by phenol red recovery.

Key results. Although protein levels of the sGC subunits were lower in gastrointestinal tissues of apo-sGC mice, cinaciguat induced concentration-dependent relaxations and increased cGMP levels in apo-sGC fundus and colon to a similar or greater extent than in WT mice. The sGC inhibitor ODQ increased cinaciguat-induced relaxations and cGMP levels in WT fundus and colon. In apo-sGC antrum, pylorus and jejunum, cinaciguat was not able to induce relaxations. Cinaciguat did not improve delayed gastric emptying in apo-sGC mice.

Conclusions. Cinaciguat relaxes the fundus and colon efficiently when sGC is in the heme-free condition; the non-effect of cinaciguat in pylorus explains its inability to improve the delayed gastric emptying in apo-sGC mice.

V.2   Introduction

The principal intracellular receptor for nitric oxide (NO) as smooth muscle cell relaxant, is soluble guanylate cyclase (sGC). In both physiologically functional isoforms of sGC (sGC$\alpha_1\beta_1$ and sGC$\alpha_2\beta_1$), NO binds to heme that is linked to His 105 in the β_1 subunit. This will generate cyclic guanosine 3'-5'-monophosphate (cGMP), that mediates smooth muscle cell relaxation (Feil *et al.*, 2003; Toda & Herman, 2005). In the cardiovascular system, nitrergic relaxation is therapeutically applied with NO donors, such as organic nitrates. In pathological conditions such as heart failure and pulmonary hypertension, oxidative stress interferes with the NO/sGC/cGMP pathway through scavenging of NO and formation of reactive oxygen species (ROS) (Konduri *et al.*, 2007; Mitrovic *et al.*, 2009), that oxidize sGC towards the NO-insensitive heme-free status (Fritz *et al.*, 2011); treatment with organic nitrates becomes less effective. Recently, a new class of drugs was developed, activating sGC directly, by preference in its oxidized/heme-free position. The sGC activator cinaciguat has been extensively studied in the cardiovascular system. Cinaciguat is more effective in isolated blood vessels of animal models of vascular disease, associated with endogenously induced oxidative stress, and in blood vessels of controls, pretreated with the oxidant peroxynitrite (Stasch *et al.*, 2006; Korkmaz *et al.*, 2012). In spontaneously hypertensive rats and control rats, that are pretreated with 1H[1,2,4,]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) -inhibiting sGC by oxidizing the heme group-, the decrease in blood pressure caused by cinaciguat lasts longer than in normotensive controls (Stasch *et al.*, 2002; Stasch *et al.*, 2006). Furthermore, cinaciguat shows beneficial effects in models of heart failure (Boerrigter *et al.*, 2007; Erdmann *et al.*, 2012), pulmonary hypertension (Chester *et al.*, 2011), and cardiac ischemia/reperfusion (Korkmaz *et al.*, 2009; Radovits *et al.*, 2011; Salloum *et al.*, 2012).

sGC activators may thus offer a new therapy for cardiovascular diseases; when applied for these diseases, possible effects on the gastrointestinal tract might have to be taken into account. Indeed, the NO/sGC/cGMP pathway is also essential for physiological gastrointestinal motility. Aging and diseases such as colitis and diabetes can lead to enteric nitrergic dysfunction and motility disturbances (Mizuta *et al.*, 2000; Phillips & Powley, 2007; Zandecki *et al.*, 2008). These conditions are associated with oxidative stress (Kashyap & Farrugia, 2011; Cannizzo *et al.*, 2011; Zhu & Li, 2012). Reactive oxygen species (ROS) were

suggested to increase the likelihood of damage to enteric nitrergic neurons (Rivera *et al.*, 2011). It can be expected that enteric sGC will be driven to the oxidized/heme-free status in these conditions, contributing to reduced effectiveness of the NO/sGC/cGMP pathway. Decreased sensitivity of sGC was reported in DSS-induced colitis in mice (Hamada *et al.*, 2012) and rats (Van Crombruggen *et al.*, 2008). Cinaciguat might thus be able to activate sGC in this type of gastrointestinal disorders, possibly improving gastrointestinal dysmotility. We recently showed that apo-sGC mice, where His at position 105 is mutated to Phe leading to heme-free NO-insensitive sGC (Thoonen *et al.*, 2009), can be considered as a model for gut dysmotility under oxidative stress (Cosyns *et al.*, 2013). The gastrointestinal consequences of lacking NO-sensitive sGC are most pronounced at the level of the stomach, as apo-sGC mice show an enlarged stomach, hypertrophy of the muscularis externa of fundus and pylorus, and disturbed gastric emptying (Cosyns *et al.*, 2013). Our aim was therefore to investigate the effect of cinaciguat on in vitro muscle tone of the different sections of the gastrointestinal tract, and on gastric emptying in wild-type and apo-sGC mice. As we had not yet investigated the consequence of the $sGC\beta_1^{His105Phe}$ shift on relaxation by exogenous NO and the endogenous inhibitory neurotransmitters in antrum -as done before for fundus, pylorus, jejunum and colon (Cosyns *et al.*, 2013)- this was first examined.

V.3 Materials and methods

V.3.1 Animals

All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University. Homozygous $sGC\beta_1^{His105Phe}$ knockin (apo-sGC; n = 80) mice and wild-type (WT; n = 94) controls were derived from a heterozygous breeding on a mixed background (129/SvJ-C57BL/6J) (Thoonen *et al.*, 2009). One set of experiments with cinaciguat was performed in gastric fundus tissue of $sGC\alpha_1$ knockout mice (C57BL/6J background; n = 6 for $sGC\alpha_1$ knockout mice and for C57BL/6J controls); homozygous $sGC\alpha_1$ knockout mice were generated by targeting exon 6 of the $sGC\alpha_1$ gene which codes for an essential part of the catalytic domain of sGC (Buys *et al.*, 2008). All mice were used in the age range of 11-16

weeks and had free access to water and Transbreed Chow (SDS, Essex, UK); when investigating gastric emptying using phenol red, food was withheld for 16 hours with free access to water.

V.3.2 Muscle tension experiments

V.3.2.1 Tissue preparation

Mice were sacrificed by cervical dislocation and the gastrointestinal tract was removed and put in aerated (5 % CO_2 in O_2) Krebs solution (composition in mM: NaCl 118.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1). The stomach was emptied from its contents and two full wall thickness fundus strips (2 x 11 mm) and four full wall thickness antrum strips (2 x 5 mm) were prepared by cutting in the direction of the circular muscle layer; one full wall thickness ring (width: 2 mm) was prepared from the pyloric region. A ~5 cm long fragment of small bowel, starting approximately 10 cm distal to the pylorus, and a ~4 cm long segment of colon, taken above the pelvic brim, were isolated. The fragments were opened along the mesenteric border and pinned mucosa side up in Krebs solution. The mucosa was then removed by sharp dissection under a microscope and 2 full-thickness muscle strips (4 × 5 mm) were cut along the circular axis in the jejunal as well as in the colonic fragment.

V.3.2.2 Isometric tension recording

After a silk thread (USP 4/0) was attached to both ends of the strips and two L-shaped tissue hooks were inserted into the pyloric ring, they were mounted in 7 or 15 ml organ baths between two platinum plate electrodes (allowing electrical field stimulation; EFS). The organ baths contained aerated (5 % CO_2 in O_2) Krebs solution, maintained at 37°C. Changes in isometric tension were measured using Grass force transducers (antrum, pylorus, jejunum, colon) or MLT 050/D force transducers (fundus) and were recorded on a PowerLab/8sp data recording system (ADInstruments) with Chart software. EFS was performed by means of a 4-channel custom-made stimulator.

After an equilibration period of 30 min with flushing every 10 min at a load of 0.75 g (fundus), 0.25 g (colon) or 0.125 g (jejunum), the length-tension relationship was

determined. Muscle tissues were stretched by load increments of 0.25 g (colon and fundus) or 0.125 g (jejunum) and at each load level exposed to 0.1 µM (fundus and jejunum) or 1 µM (colon) carbachol to determine the optimal load (L_o; the load at which maximal response to the contractile agent occurred). The antrum strips and the pyloric rings were equilibrated at a load of respectively 0.50 g and 0.25 g. The pyloric rings received carbachol (10 µM) once, to check the activity of the tissue. The medium was then switched to Krebs solution containing 1 µM atropine and 4 µM guanethidine to block cholinergic and noradrenergic responses respectively (NANC conditions) and tissues were allowed to equilibrate for 60 min with flushing every 15 min in this NANC Krebs solution (except for the experiments in antral strips and one set of experiments in jejunal strips, where we studied the effect of cinaciguat after pre-contracting the strips with carbachol).

V.3.2.3 Responses to EFS and NO in antrum

As prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 3 µM) induced only a very weak contractile response in antral strips (n = 2), all relaxant stimuli were examined after pre-contraction of the strips with 10 µM carbachol (Gil *et al*., 2013), once the contractile response was stabilized (~6 min after administration of carbachol). Relaxations were induced by application of exogenous NO (100 µM) and by application of EFS (40 V, 0.1 ms, 2-4-8 Hz for 60 s at 5 min interval). Strips were then washed for 30 min, and were subsequently incubated for 30 min with the sGC inhibitor ODQ (10 µM), the NOS inhibitor L-NAME (300 µM), the $P2Y_1$ receptor antagonist MRS2500 (1 µM) or the combination of L-NAME (300 µM) and MRS2500 (1 µM). Carbachol (10 µM) was then applied again and the responses to NO and EFS were studied again.

V.3.2.4 Responses to cinaciguat in fundus, antrum, pylorus, jejunum and colon

The responses to cinaciguat were studied after pre-contracting the tissues with $PGF_{2\alpha}$ (300 nM for fundus and jejunum, 3 µM for pylorus and colon), except for the antral strips which were precontracted with carbachol (10 µM). Once the contractile response was stabilized (~6 min after administration of $PGF_{2\alpha}$ or carbachol), cinaciguat was cumulatively added to the smooth muscle strips (1-10-100 nM in fundus, jejunum and colon and 1-10-

100-1000 nM in antrum, with an interval of 5 min between the administrations); strips were studied in parallel in the presence of the sGC inhibitor ODQ (10 µM; 30 min incubation) or in the presence of its solvent ethanol. In the pyloric rings, the effect of a single administration of cinaciguat (100 nM, left in contact with the tissue for 5 min) was studied.

In jejunal strips, additional experiments were performed studying the effect of cumulatively added cinaciguat (1-10-100-1000 nM) after pre-contracting the strips with carbachol (0.1 µM). Additional experiments were also performed in pyloric rings without inducing additional tone (no contractile agent was used): cinaciguat was added cumulatively (100-1000 nM, with an interval of 5 min between the administrations).

In fundus strips from sGCα_1 knockout mice and their corresponding control mice, the effect of cumulatively added cinaciguat (1-10-100 nM) and NO (1-10-100 µM) was studied.

In all set ups, time-control strips that did not get the interfering drugs or that received the solvent of the interfering drugs were run in parallel. At the end of each experiment, the wet weight of the smooth muscle strips was determined (mg wet weight).

V.3.2.5 Data analysis

In fundus strips and pyloric rings, the amplitude of the tonic contractile and relaxant responses can be measured. Responses to cinaciguat and NO were determined at their maximal level and were expressed as the percentage of contraction evoked by PGF$_{2\alpha}$ or, when no contracting agent was given (second set of experiments in the pyloric rings), as the percentage of the tone present in the pyloric ring at a load of 0.25 g.

As antral, jejunal and colonic strips showed phasic activity, the area under the curve (AUC) above baseline was determined to measure the contractile responses to PGF$_{2\alpha}$ or carbachol. To express relaxant responses, the AUC for a given response was determined and subtracted from the AUC of a corresponding period just before applying the relaxing drug or stimulus, yielding the area above the curve (AAC) for the relaxant response. The duration of the relaxant responses to cinaciguat was fixed at 5 min. The duration of the relaxant responses to EFS in the antrum was determined as 60 s (i.e. the length of the stimulus train applied). NO abolished phasic activity of antral strips for a concentration-dependent period, after which phasic activity progressively reoccurred. The duration of the relaxant responses to NO was therefore determined as the time necessary for phasic activity to regain 50 % of

the interval between the mean peak level of phasic activity during the 2 min before administration of NO and the minimum tone level during the NO response. This calculation was performed during the 1st cycle of carbachol-NO-EFS and the determined duration was again used for the NO-induced responses in the 2nd cycle of carbachol-NO-EFS. AAC responses are expressed as (g.s)/mg wet weight.

V.3.3 cGMP analysis, Western blot of sGC subunits and oxidative stress levels

V.3.3.1 cGMP analysis

Circular muscle strips from the gastric fundus and distal colon were prepared as for the muscle tension experiments. Before mounting them in the organ baths with aerated Krebs solution, they were weighed to determine the tissue wet weight. The optimal load L_o was determined and the Krebs solution was replaced with Krebs solution containing 1 µM atropine and 4 µM guanethidine, to have the same conditions as in the muscle tension experiments. After pre-contraction with $PGF_{2\alpha}$, cinaciguat (100 nM) or its solvent were added to the organ baths; cinaciguat was also added to strips, incubated for 30 min with ODQ (10 µM). Exactly 5 min after adding cinaciguat or its solvent, strips were snap frozen in liquid nitrogen and they were stored at -80°C until further processing.

cGMP was extracted and quantified using an enzyme immunoassay kit (Cayman Chemical cyclic GMP EIA kit, Michigan, USA). Briefly, frozen tissues were pulverized by a Mikro-Dismembrator (B. Braun Biotech International, Melsungen, Germany), homogenized in 5 % trichloroacetic acid (TCA) and centrifuged for 15 min at 4°C at 2000 g to collect the supernatant. The supernatant was washed three times with water-saturated ether to extract the TCA after which it was dried under nitrogen at 60°C. After drying, it was dissolved in a 10 times volume of assay buffer. Then, samples, controls and standards were acetylated and were added to the enzyme immunoassay plate to incubate for 18 h at 4°C. Optical density was measured with a 96-well plate reader (Biotrak II, Amersham Biosciences, Buckinghamshire, UK) at 405 nm. The concentration of cGMP was expressed as pmol/g wet weight.

V.3.3.2 Western blot analysis

After the mice were sacrificed by cervical dislocation, the whole fundus, antrum and pylorus, and 4 cm mucosa-free segments of jejunum and colon were snap frozen and stored at -80°C. Tissue samples were powdered at -70°C (Retsch MM301, Haan, Germany) and then further homogenized in 150 µl ice cold DBB buffer (0.5 mM EDTA, 20 % glycerol, 20 mM hepes, 350 mM NaCl, 0.5 % triton X-100) supplemented with 0.3 µM aprotinin, 25 mM β-glycerolphosphate, 1 mM leupeptin, 1 mM NaVO$_3$, 5 mM NaF, 1 mM pefabloc SC and 1 tablet 'complete' EDTA-free protease inhibitor cocktail per 50 ml (all from Roche Diagnostics), using a mechanical homogenizer (Polytron PT1600E, Fisher Scientific, Aalst, Belgium) for protein extraction. Samples were then centrifuged at 14000 g for 30 min at 4°C and the supernatant was collected. Protein extracts (30 µg) were separated on a 10 % SDS polyacrylamide gel and transferred to nitrocellulose membranes. After blocking of the membrane (5 % skim milk, 0.1 % Tween-20), blots were incubated with the following antibodies: rabbit polyclonal antibody specific for sGCα$_1$ (Sigma Aldrich G4280; dilution 1:10000), for sGCα$_2$ (Abcam ab42108; dilution 1:500), and for sGCβ$_1$ (Sigma Aldrich G4405; dilution 1:2000), and mouse monoclonal antibody specific for actin (MP Biomedicals; dilution 1:500), to correct for unequal loading. Detection and analysis of sGCα$_1$ and sGCβ$_1$ was performed with the Odyssey system (Odyssey 2.1.12, Li-Cor Biosciences, Cambridge, UK) using fluorophore-coupled secondary antibodies (goat anti-rabbit IRDye 800CW for sGCα$_1$ and sGCβ$_1$ (Li-Cor Biosciences; 1:5000 dilution), and goat anti-mouse IRDye 680 for actin (Li-Cor Biosciences; 1:15000 dilution)). Detection of sGCα$_2$ was done with Amersham Hyperfilm ECL (GE healthcare) and Pierce® ECL chemiluminescent substrate (Fisher Scientific) using the following secondary antibodies: goat-anti-rabbit IgG HPR for sGCα$_2$ (Cell signaling 7074; dilution 1:2000) and sheep anti-mouse IgG HRP for the loading control actin (GE Healthcare NA931; dilution 1:5000). Analysis of the bands was done by densitometry (ImageJ) and results were normalized to actin.

V.3.3.3 Oxidative stress levels

Samples were obtained and stored as for the western blot analysis. As an indicator of oxidative stress, the levels of malondialdehyde/4-hydroxy-2-non-enal (MDA/HNE) were

measured by the Lipid Peroxidation Assay (Oxford Biomedical Research, Michigan, USA) in whole fundus and antrum, and in 4 cm mucosa-free segments of colon and jejunum of both WT and apo-sGC mice, according to the manufacturer's protocol. MDA/HNE levels were normalized to total protein content and were expressed as nmol/mg protein.

The actual reactive oxygen species (ROS) levels in whole fundus and in colon segments were quantified using the luminol derivative L-012, a highly sensitive chemiluminescence probe. Intestinal tissue samples were homogenized in 10 volumes 50 mM Tris-HCl buffer (pH 7.5) and centrifuged at 10000 g for 15 min at 4°C. Supernatants were then incubated with L-012 (100 µM) and luminescence was counted (Victor Wallac, PerkinElmer, Massachusetts, USA) after a 10 min interval, allowing the plate to dark-adapt. ROS levels were normalized to total protein content and were expressed as arbitrary units/mg protein (AU/mg protein).

V.3.4 Gastric emptying

Mice were fasted overnight. Cinaciguat or the corresponding amount of solvent was administered intraperitoneally (IP; 300 µg/kg) or intravenously (IV; 300 µg/kg, 100 µg/kg or 30 µg/kg) and 15 min (IP injections) or 5 min (IV injections) later 250 µl of a phenol red meal (0.1 % w/v dissolved in water) was administered by gavage to measure gastric emptying as adapted from de Rosalmeida *et al.* (2003). Fifteen minutes after gavage, mice were sacrificed by cervical dislocation and the stomach and small bowel were clamped at both sides. Both organs were cut into small fragments and placed into 20 ml of 0.1 N NaOH in a 50 ml Falcon tube. The stomach and the small bowel were homogenized for approximately 30 s and allowed to stand for 20 min at room temperature. 10 ml of supernatant was placed into a 15 ml Falcon tube and centrifuged for 10 min at 1600 g. Proteins in 5 ml supernatant were precipitated with 0.5 ml of 20 % TCA and the solution was centrifuged for 20 min at 1600 g. 0.5 ml of supernatant was added to 0.667 ml of 0.5 N NaOH and the absorbance of 300 µl of this mixture was spectrophotometrically determined at 540 nm in a Biotrak II plate reader (Amersham Biosciences). Gastric emptying was calculated as the amount of phenol red that left the stomach as % of the total amount of phenol red recovered and the phenol red recovery was determined as the amount of phenol red recovered, expressed as % of the

amount of phenol red administered. The phenol red recovery was 58 ± 4 % in WT mice (n = 50) and 68 ± 3 % in apo-sGC mice (n = 36).

V.3.5 Drugs used

The following drugs were used: atropine sulphate, guanethidine sulphate, carbamoylcholine chloride (carbachol), N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), prostaglandin $F_{2\alpha}$ tris salt ($PGF_{2\alpha}$), phenol red (all obtained from Sigma-Aldrich, Diegem, Belgium), 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H)dione sodium salt (L-012; Wako Pure Chemical Industries Ltd., Osaka, Japan), (1R*,2S*)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS2500) and 1H[1,2,4,]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (both from Tocris Cookson, Bristol, UK) and 4-[((4-carboxybutyl)-(2-[(4-phenethylbenzyl)oxy]phenethyl)amino)methyl] benzoic acid (cinaciguat; kindly provided by Bayer Healthcare GmbH, Wuppertal, Germany). All drugs were dissolved in de-ionized water except for the following: cinaciguat, which was dissolved in 60 % PBS, 20 % DGME and 20 % Cremophor EL (Fluka AG, Diegem, Belgium) and ODQ, which was dissolved in 100 % ethanol. A saturated NO (2 mM) solution was prepared by bubbling oxygenated Krebs solution with 99.9 % NO gas (Air Liquide, Belgium) (Kelm & Schrader, 1990).

V.3.6 Statistics

All results are expressed as means ± S.E.M. n refers to tissues obtained from different animals unless otherwise indicated. Comparison between apo-sGC and WT tissues or tissues studied in parallel was done with an unpaired Student's *t*-test (2 groups) or by a one-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparison *t*-test (more than 2 groups). Comparison within tissues of either WT or apo-sGC was done by a paired Student's *t*-test (before and after an interfering drug or solvent). A P-value less than 0.05 was considered to be statistically significant (GRAPHPAD, California, USA).

V.4   Results

V.4.1   Response to EFS and NO in antrum

Carbachol (10 µM) induced a combined tonic and phasic response in antrum smooth muscle strips. The tone decreased back to baseline within 3 min after addition of carbachol, but the increase in phasic activity remained stable for at least 30 min. In both WT and apo-sGC strips, EFS suppressed phasic activity and, upon ending stimulation, a rebound contraction was observed (Fig. V.1A: representative traces for WT strips; not shown for the apo-sGC mice as these are similar to the traces for the WT mice). The EFS-induced relaxations were not influenced by ODQ (10 µM; n = 6 for WT and for apo-sGC; results not shown) or L-NAME (300 µM; Fig. V.1A, C and D). In both WT and apo-sGC strips, the selective P2Y$_1$ antagonist MRS2500 (1 µM) reduced the relaxant responses at all frequencies; in the apo-sGC strips the responses were nearly abolished for EFS at 2 and 4 Hz (Fig. V.1A, C and D). The combination of L-NAME (300 µM) plus MRS2500 (1 µM) abolished the relaxant responses completely in the WT strips at 2 and 4 Hz; at 8 Hz, there was still a very small response present (Fig. V.1A and C). In the apo-sGC strips, the combination of L-NAME and MRS25000 did not have more effect on EFS-induced relaxations than MRS2500 alone (Fig. V.1D).

Nitric oxide (100 µM) suppressed phasic activity in WT strips shortly: within one minute phasic activity was back to normal (Fig. V.1B). ODQ (10 µM) tended (p = 0.13) to reduce this response to NO (Fig. V.1B; respectively 0.81 ± 0.31 versus 0.30 ± 0.13 (g.s)/mg wet weight, n = 6). L-NAME (300 µM), MRS2500 (1 µM) or L-NAME plus MRS2500, had no influence on NO-induced relaxation (n = 6; results not shown). In apo-sGC strips, the relaxant response to NO was totally abolished (n = 6; results not shown).
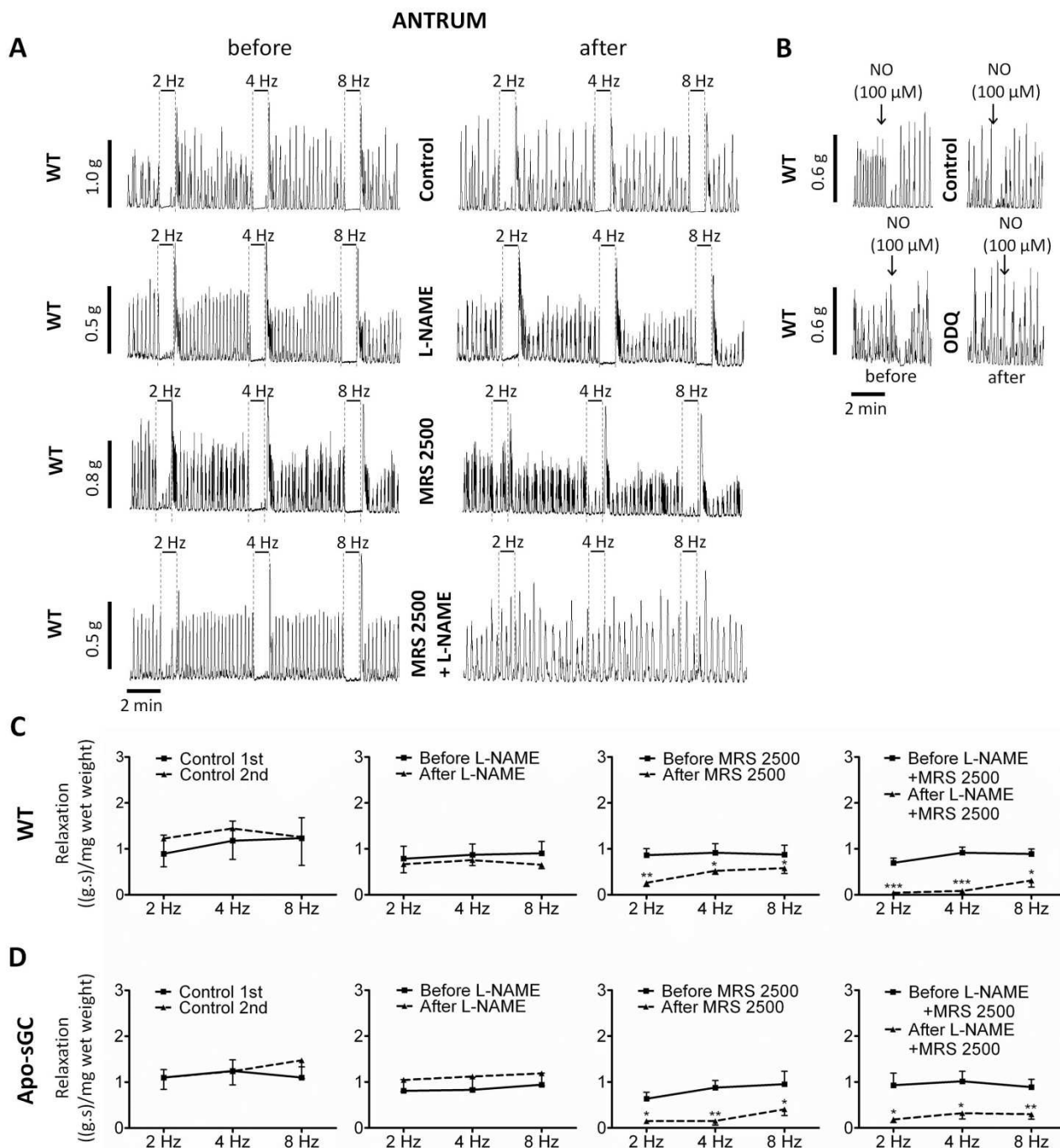
Fig. V.1   Relaxant responses to EFS and NO in antrum of WT and apo-sGC mice.
(A-B) Representative traces showing the inhibitory responses in carbachol pre-contracted circular muscle strips of the antrum from a WT mouse for EFS (A; 40 V, 0.1 ms, 2-4-8 Hz, 60 s) when EFS was repeated in control conditions (no interfering agent), and before and after L-NAME (300 μM), MRS2500 (1 μM) or the combination of L-NAME (300 μM) plus MRS2500 (1 μM) and for exogenous NO (B; 100 μM) when NO was repeated in control conditions (no interfering agent), and before and after ODQ (10 μM). (C-D) Frequency-response curves of EFS (40 V, 0.1 ms, 2-4-8 Hz, 60 s) in antrum strips of WT mice (C) and apo-sGC mice (D) when EFS was repeated in control conditions (no interfering agent), and before and after L-NAME (300 μM), MRS2500 (1 μM) or the combination of L-NAME (300 μM) plus MRS2500 (1 μM). Data represent the means ± S.E.M. of n = 6. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: after versus before (paired Student's t-test).

V.4.2 Responses to cinaciguat; sGC subunit levels

V.4.2.1 Fundus

PGF$_{2\alpha}$ (300 nM) induced an increase in tone in fundus strips; in preliminary experiments, it was shown that this increase in tone was stable for at least 30 min. The effective relaxant concentrations of cinaciguat were also determined in preliminary experiments. In the effective concentration range (1-10-100 nM), addition of cinaciguat induced concentration-dependent relaxations in strips from both WT and apo-sGC mice. The relaxing responses to cinaciguat consisted of a sustained decline in tone (Fig. V.2A). At the lowest given concentration of cinaciguat (1 nM), the response was significantly (P<0.05) greater in strips from apo-sGC mice than in WT mice (35 ± 8 % relaxation in apo-sGC versus 14 ± 5 % relaxation in WT), but the difference between apo-sGC and WT mice did not reach significance at higher concentrations of cinaciguat (for 10 nM cinaciguat: 76 ± 10 % in apo-sGC versus 54 ± 10 % in WT; for 100 nM cinaciguat: 102 ± 9 % in apo-sGC versus 92 ± 13 % in WT; Fig. V.2B). After incubation with cinaciguat (100 nM), no increase in cGMP levels was seen in strips from WT mice, whereas in strips from apo-sGC mice, cGMP levels were increased by 3-fold (P<0.01); compared to cGMP levels in strips of WT mice incubated with cinaciguat, cGMP levels in strips of apo-sGC mice incubated with cinaciguat were twice as high (Fig. V.2C).

Incubation with the sGC inhibitor ODQ (10 µM) increased the relaxing effect of cinaciguat at all concentrations in strips from WT mice, reaching significance (P<0.05) at 10 and 100 nM. In strips from apo-sGC mice, ODQ did not have any influence on the relaxing effect of cinaciguat (Fig. V.2A and B). Correspondingly, in strips from apo-sGC mice, cinaciguat did not induce a further increase in cGMP in the presence of ODQ, but a significant 8-fold increase (P<0.001) in cGMP levels by cinaciguat was seen in WT strips that were incubated with ODQ; the latter cGMP levels were significantly more pronounced than in strips of the apo-sGC mice (Fig. V.2C).

The relative protein expression of the sGC subunits, sGCα$_1$, sGCα$_2$ and sGCβ$_1$, was lower in the fundus of apo-sGC mice compared to the fundus of WT mice, reaching significance for sGCα$_1$ (P<0.001) and sGCβ$_1$ (P<0.05) (Fig. V.2D).
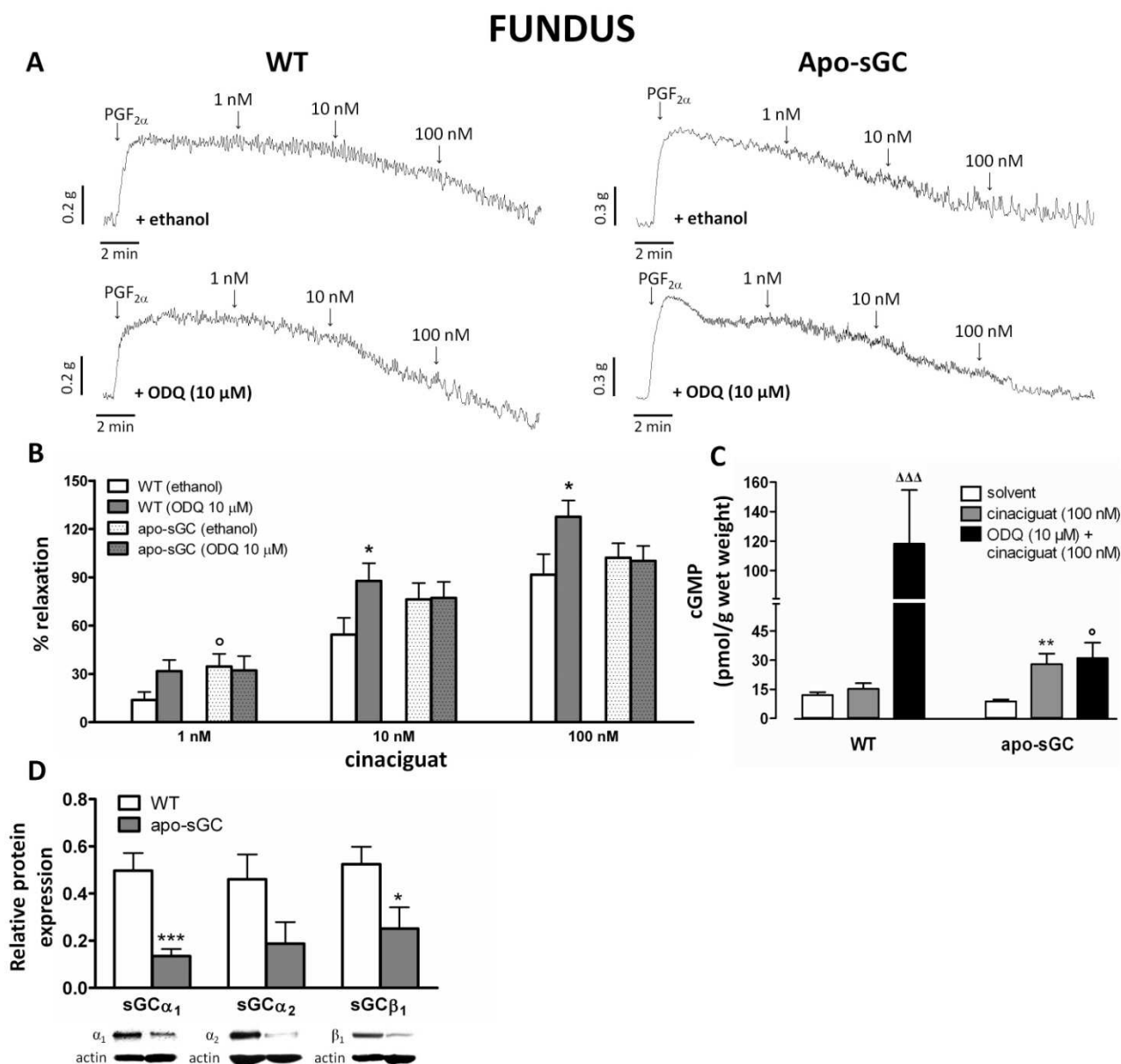
Fig. V.2 Relaxant and cGMP responses to cinaciguat and Western blot analysis of sGC subunits in gastric fundus of WT and apo-sGC mice. (A) Representative traces showing the responses to cinaciguat (1-10-100 nM) in $PGF_{2\alpha}$-pre-contracted circular muscle strips of the gastric fundus from a WT (left) and an apo-sGC mouse (right) in the presence of the sGC inhibitor ODQ (10 μM; lower trace) or its solvent ethanol (upper trace). (B) Relaxations to cinaciguat (1-10-100 nM) in fundus strips of WT and apo-sGC mice after incubation with ODQ (10 μM) or its solvent (ethanol). Data represent the means ± S.E.M. of n = 6-8. * $P < 0.05$: ODQ versus ethanol (unpaired Student's t-test); ° $P < 0.05$: apo-sGC versus WT (unpaired Student's t-test). (C) cGMP levels in fundus strips of WT and apo-sGC mice after incubation with the solvent of cinaciguat, cinaciguat (100 nM) itself and cinaciguat in the presence of ODQ (10 μM). Data represent the means ± S.E.M. of n = 12 for the solvent and cinaciguat group and of n = 6 for the ODQ + cinaciguat group. ** $P<0.01$: cinaciguat versus solvent (one-way ANOVA followed by a Bonferroni multiple comparison t-test); ΔΔΔ $P<0.001$: ODQ + cinaciguat versus cinaciguat (one-way ANOVA followed by a Bonferroni multiple comparison t-test); ° $P<0.05$: apo-sGC versus WT (unpaired Student's t-test). (D) Western blot analysis of the relative protein expression levels of $sGC\alpha_1$, $sGC\alpha_2$ and $sGC\beta_1$ in the fundus of WT and apo-sGC mice, expressed relative to the household protein actin. Data represent the means ± S.E.M. of n = 5-6. * $P<0.05$, *** $P<0.001$: apo-sGC versus WT (unpaired Student's t-test). Representative Western blot results were added showing the sGC subunits and the corresponding household protein actin, to correct for unequal loading.

In strips from sGCα$_1$ knockout mice, the relaxant responses to cinaciguat (1-10-100 nM) were nearly abolished compared to those in strips from control WT mice (Fig. V.3A and C). The relaxant response to NO (1-10-100 μM) tended to be lower in strips from sGCα$_1$ knockout mice only reaching significance (P<0.05) for 100 μM NO (Fig. V.3B and D).
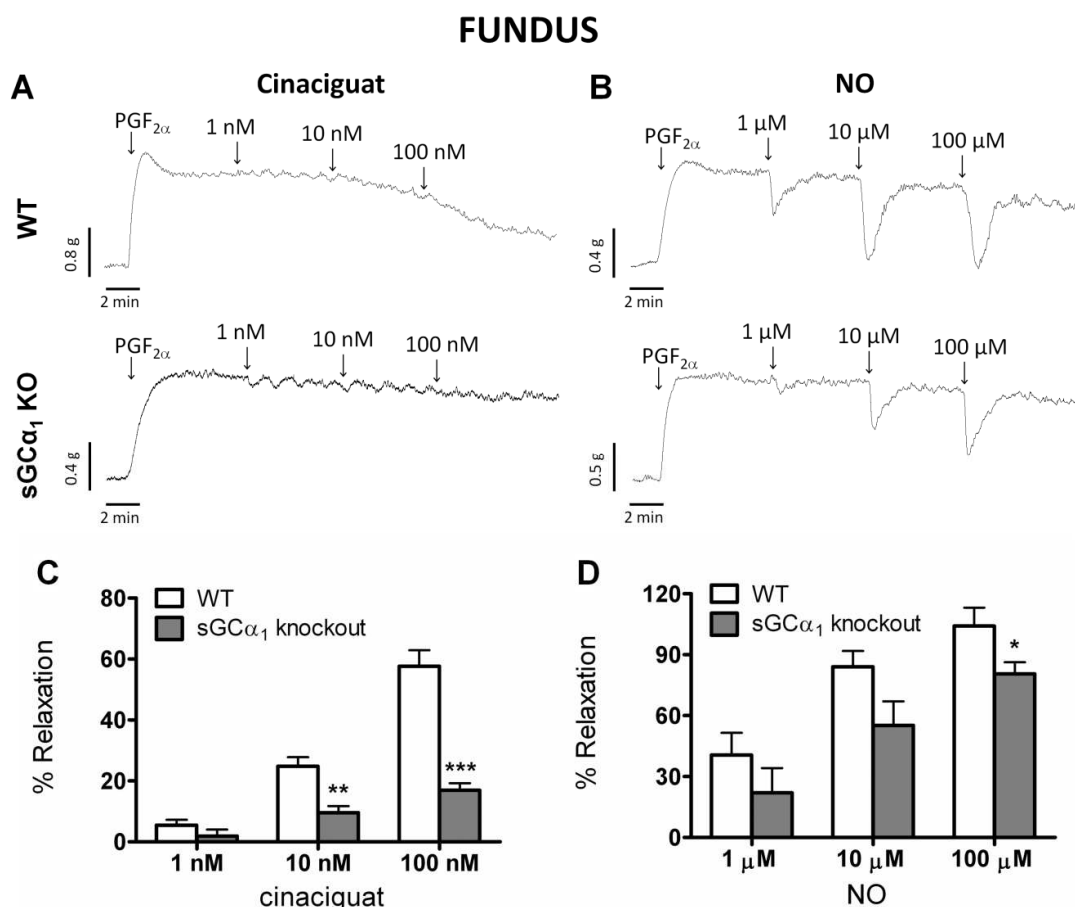
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Fig. V.3 Relaxant responses to cinaciguat and NO in gastric fundus of WT and sGCα$_1$ knockout mice. (A-B) Representative traces showing the responses to cinaciguat (A; 1-10-100 nM) and exogenously applied NO (B; 1-10-100 μM) in PGF$_{2α}$-pre-contracted circular muscle strips of the gastric fundus from a WT (upper trace) and an sGCα$_1$ knockout mouse (lower trace). (C-D) Relaxations by cinaciguat (C; 1-10-100 nM) and exogenously applied NO (D; 1-10-100 μM) in fundus strips of WT and sGCα$_1$ knockout mice. Data represent the means ± S.E.M. of n = 5-6. * P<0.05, ** P<0.01, *** P<0.001: sGCα$_1$ knockout versus WT (unpaired Student's *t*-test).

V.4.2.2 Antrum

In both WT and apo-sGC antrum strips, cinaciguat (1-10-100-1000 nM) did not have any effect on the phasic activity induced by carbachol, either in the absence (n = 2 for WT and for apo-sGC) or in the presence of ODQ (n = 2 for WT and for apo-sGC) (Fig. V.4A).

The relative protein expression of the sGC subunits, sGCα$_1$, sGCα$_2$ and sGCβ$_1$, was lower in the antrum of apo-sGC mice compared to the antrum of WT mice, reaching significance for sGCα$_1$ ($P<0.05$) (Fig. V.4B).
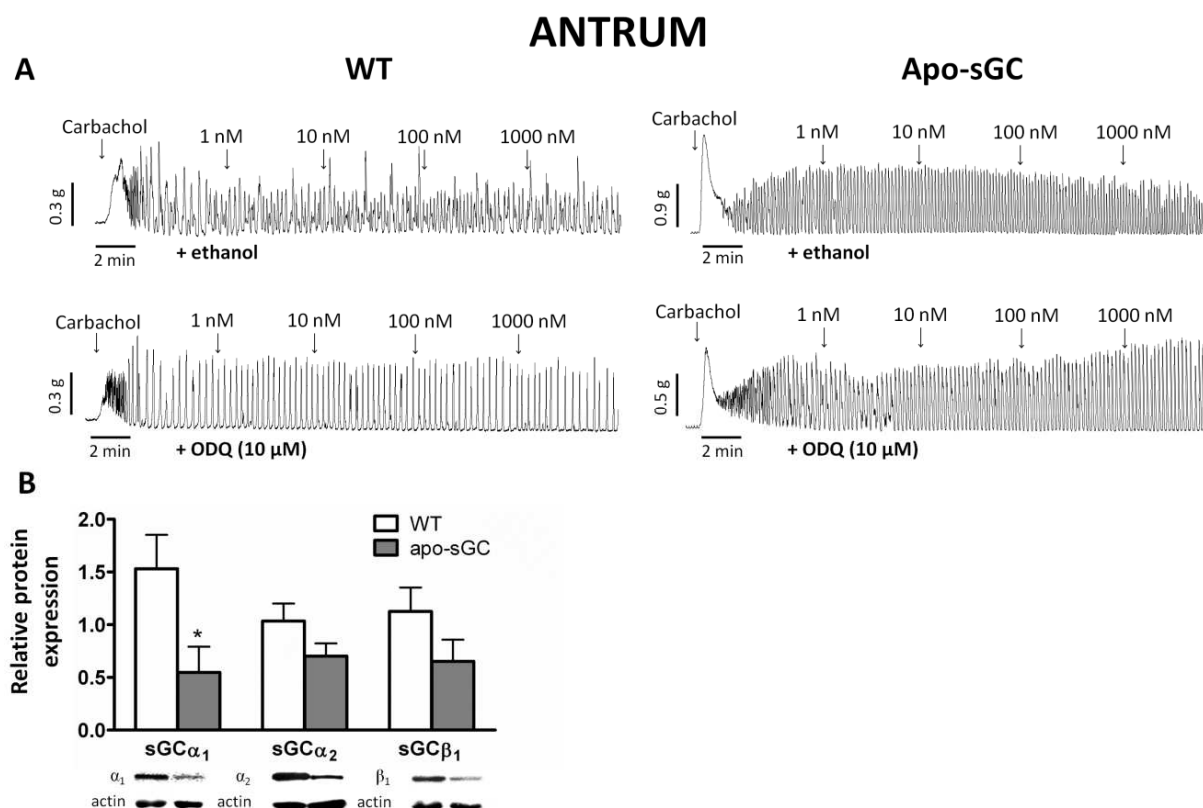
Fig. V.4 Lack of effect of cinaciguat and Western blot analysis of sGC subunits in antrum of WT and apo-sGC mice. (A) Representative traces showing the responses to cinaciguat (1-10-100-1000 nM) in carbachol pre-contracted circular muscle strips of the antrum from a WT (left) and an apo-sGC mouse (right) in the presence of the sGC inhibitor ODQ (10 μM; lower trace) or its solvent ethanol (upper trace). (B) Western blot analysis of the relative protein expression levels of sGCα$_1$, sGCα$_2$ and sGCβ$_1$ in the antrum of WT and apo-sGC mice, expressed relative to the household protein actin. Data represent the means ± S.E.M. of n = 6. * $P<0.05$: apo-sGC versus WT (unpaired Student's *t*-test). Representative Western blot results as for 2.

V.4.2.3 Pylorus

PGF$_{2\alpha}$ (3 μM)-induced responses in pyloric rings from WT and apo-sGC mice consisted of an increase in tone. Cinaciguat (100 nM) however did not have any effect on the tone induced by PGF$_{2\alpha}$ (n = 5 for WT and for apo-sGC; results not shown). The effect of cinaciguat (100-1000 nM) was then studied in pyloric rings that did not receive any contracting agent. 4 out of 7 rings from WT mice showed a sustained decline in tone when given 100 nM cinaciguat (Fig. V.5A). Subsequent addition of a higher concentration of cinaciguat (1000 nM) did not induce further relaxation (results not shown). 3 out of 7 rings

from WT mice did not show a clearcut effect to 100 nM (Fig. V.5A) or 1000 nM (results not shown) cinaciguat (mean relaxation to 100 nM cinaciguat in the 7 rings from WT mice: 12 ± 4 %). None of the pyloric rings from apo-sGC mice showed a clearcut relaxant effect of cinaciguat (100-1000 nM) as observed in 4 out of 7 WT pyloric rings (Fig. V.5B).

The relative protein expression of the sGC subunits, $sGC\alpha_1$ and $sGC\beta_1$, was lower in the pyloric rings of apo-sGC mice compared to the pyloric rings of WT mice ($P<0.05$) (Fig. V.5B).
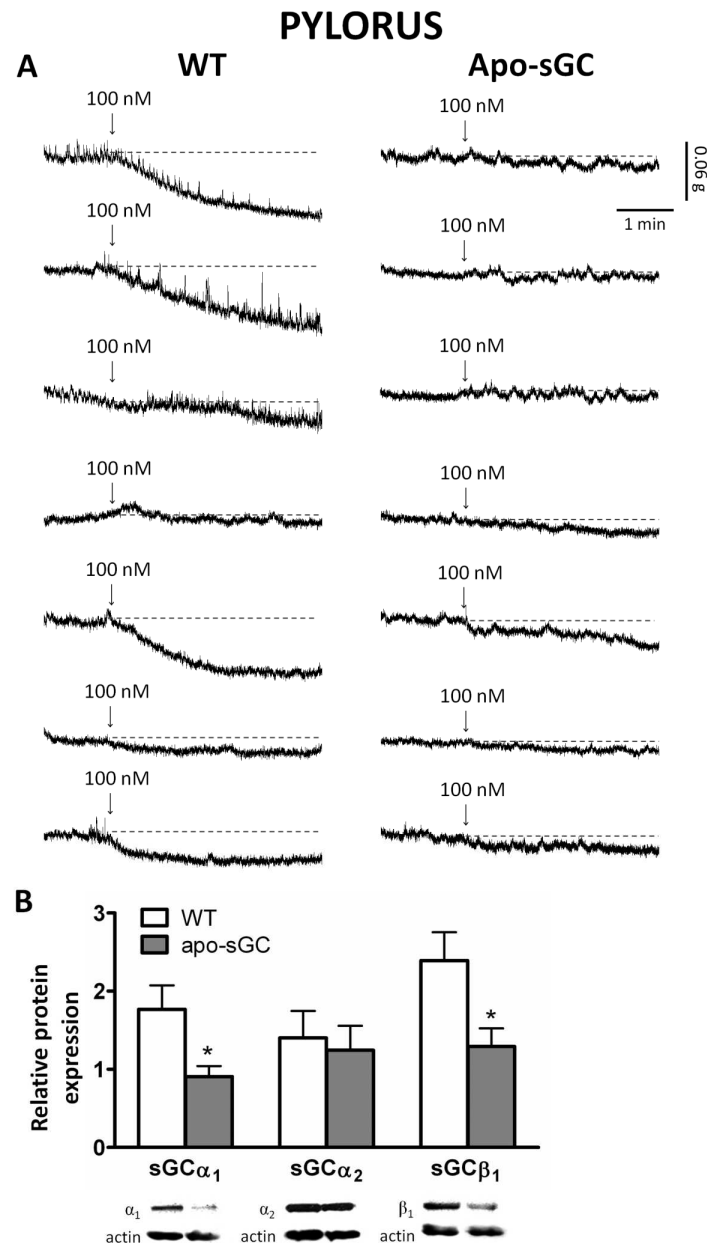
Fig. V.5 Relaxant responses to cinaciguat and Western blot analysis of sGC subunits in pylorus of WT and apo-sGC mice. (A) Traces showing the responses to cinaciguat (100 nM) in pyloric rings from WT mice (left), and from apo-sGC mice (right) when no contractile agent was used. (B) Western blot analysis of the relative protein expression levels of $sGC\alpha_1$, $sGC\alpha_2$ and $sGC\beta_1$ in pyloric rings of WT and apo-sGC mice, expressed relative to the household protein actin. Data represent the means ± S.E.M. of n = 5-6. * $P<0.05$: apo-sGC versus WT (unpaired Student's *t*-test). Representative Western blot results as for figure 2.

V.4.2.4 Jejunum

In both WT and apo-sGC jejunal smooth muscle strips, $PGF_{2\alpha}$ (300 nM) induced a combined tonic and phasic response. The tone decreased back to baseline within 3 min after addition of $PGF_{2\alpha}$, but the increase in phasic activity remained stable for at least 30 min. Cinaciguat (1-10-100 nM) did not have any effect on phasic activity induced by $PGF_{2\alpha}$ in WT (Fig. V.6A) or apo-sGC strips (n = 4 for WT and for apo-sGC). Incubation with the sGC inhibitor ODQ (10 μM) did not influence this non-effect of cinaciguat (Fig. V.6A; n = 4 for WT and for apo-sGC).

Also when jejunal strips were contracted with carbachol (0.1 μM), inducing a similar contractile response as $PGF_{2\alpha}$, cinaciguat (1-10-100-1000 nM) had no inhibitory effect (n = 4 for WT and for apo-sGC; results not shown).

Fig. V.6 Lack of effect of cinaciguat and Western blot analysis of sGC subunits in jejunum of WT and apo-sGC mice. (A) Representative traces showing the responses to cinaciguat (1-10-100 nM) in $PGF_{2\alpha}$-pre-contracted circular muscle strips of the jejunum from a WT (left) and an apo-sGC mouse (right) in the presence of the sGC inhibitor ODQ (10 μM; lower trace) or its solvent ethanol (upper trace). (B) Western blot analysis of the relative protein expression levels of $sGC\alpha_1$, $sGC\alpha_2$ and $sGC\beta_1$ in the jejunum of WT and apo-sGC mice, expressed relative to the household protein actin. Data represent the means ± S.E.M. of n = 6. ** $P<0.01$, *** $P<0.001$: apo-sGC versus WT (unpaired Student's *t*-test). Representative Western blot results as for figure 2.

The relative protein expression of the sGC subunits, $sGC\alpha_1$, $sGC\alpha_2$ and $sGC\beta_1$, was lower in the jejunum of apo-sGC mice compared to the jejunum of WT mice, reaching significance for $sGC\alpha_1$ ($P<0.01$) and $sGC\beta_1$ ($P<0.001$) (Fig. V.6B).

V.4.2.5 Colon

In colonic strips from WT and apo-sGC mice, $PGF_{2\alpha}$ (3 µM) induced an increase in tone with superimposed phasic activity. The effective relaxant concentrations of cinaciguat were determined in preliminary experiments. In this effective concentration range (1-10-100 nM), addition of cinaciguat induced concentration-dependent relaxations in strips from both WT and apo-sGC mice, that consisted of a sustained decline in tone combined with suppression of phasic activity (Fig. V.7A). The relaxant responses to cinaciguat did not significantly differ between WT and apo-sGC mice (Fig. V.7B); when 100 nM of cinaciguat was given, WT and apo-sGC strips completely lost $PGF_{2\alpha}$-induced tone and only limited phasic activity was still present (Fig. V.7A). cGMP levels were not increased in WT strips after incubation with cinaciguat (100 nM), but in apo-sGC strips, cGMP levels were increased by 2-fold ($P<0.05$); compared to cGMP levels in strips of WT mice incubated with cinaciguat, cGMP levels in strips of apo-sGC mice incubated with cinaciguat were 30 % higher (Fig. V.7C).

Incubation with the sGC inhibitor ODQ (10 µM) increased the relaxing effect of cinaciguat at all concentrations in strips from WT mice, reaching significance ($P<0.05$) at 1 nM of cinaciguat. In strips from apo-sGC mice, ODQ did not significantly influence the relaxing effect of cinaciguat, although at 10 and 100 nM of cinaciguat, the relaxation caused by cinaciguat tends to be increased after incubation with ODQ (Fig. V.7A and B). This corresponds with the results found for the cGMP levels: in WT strips, a significant 4.5-fold increase in cGMP levels by cinaciguat ($P<0.001$) was seen after incubation with ODQ; in apo-sGC strips, a small increase in cGMP levels ($P<0.05$) was seen after administration of cinaciguat in the presence of ODQ versus those induced by cinaciguat alone (Fig. V.7C).

The relative protein expression of the sGC subunits, $sGC\alpha_1$ and $sGC\beta_1$, was lower in the colon of apo-sGC mice compared to the colon of WT mice, reaching significance for $sGC\beta_1$ ($P<0.05$) (Fig. V.7D).
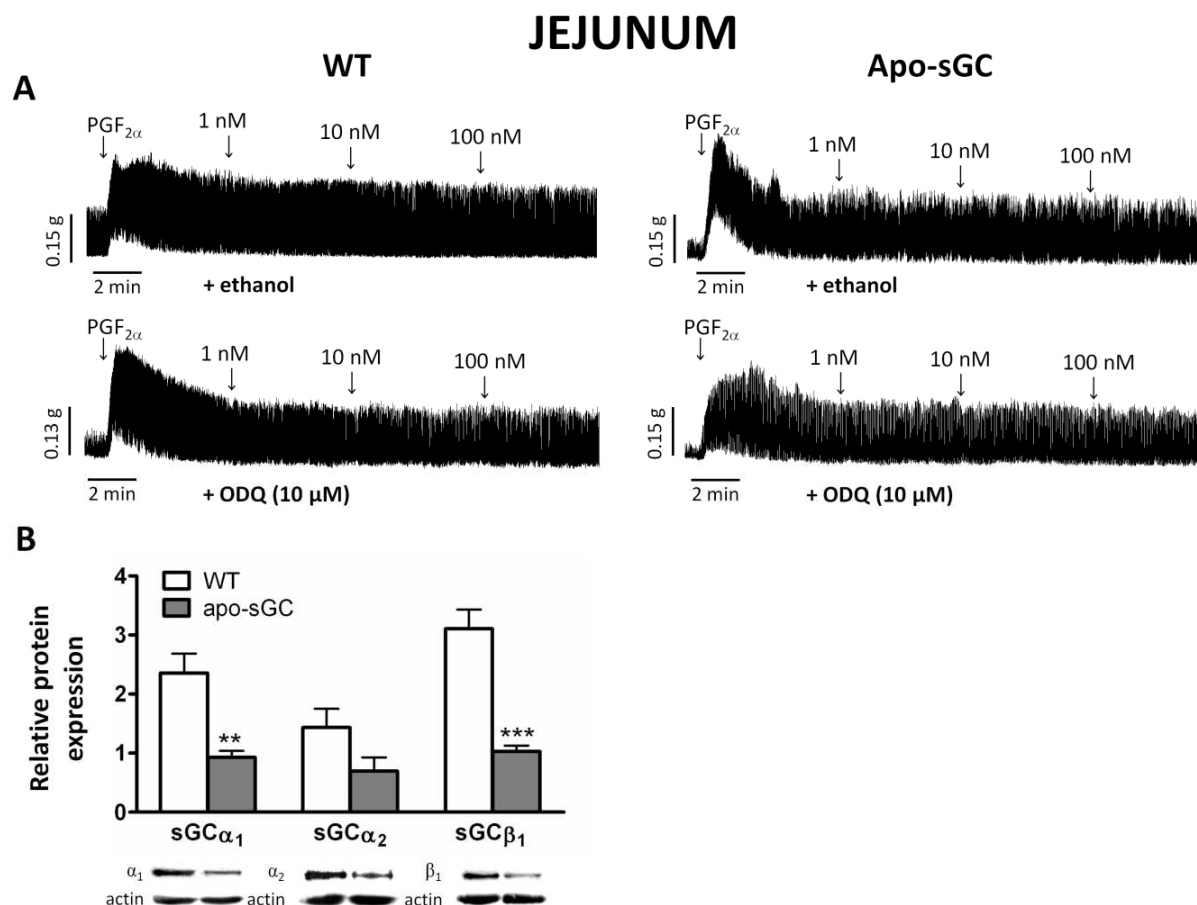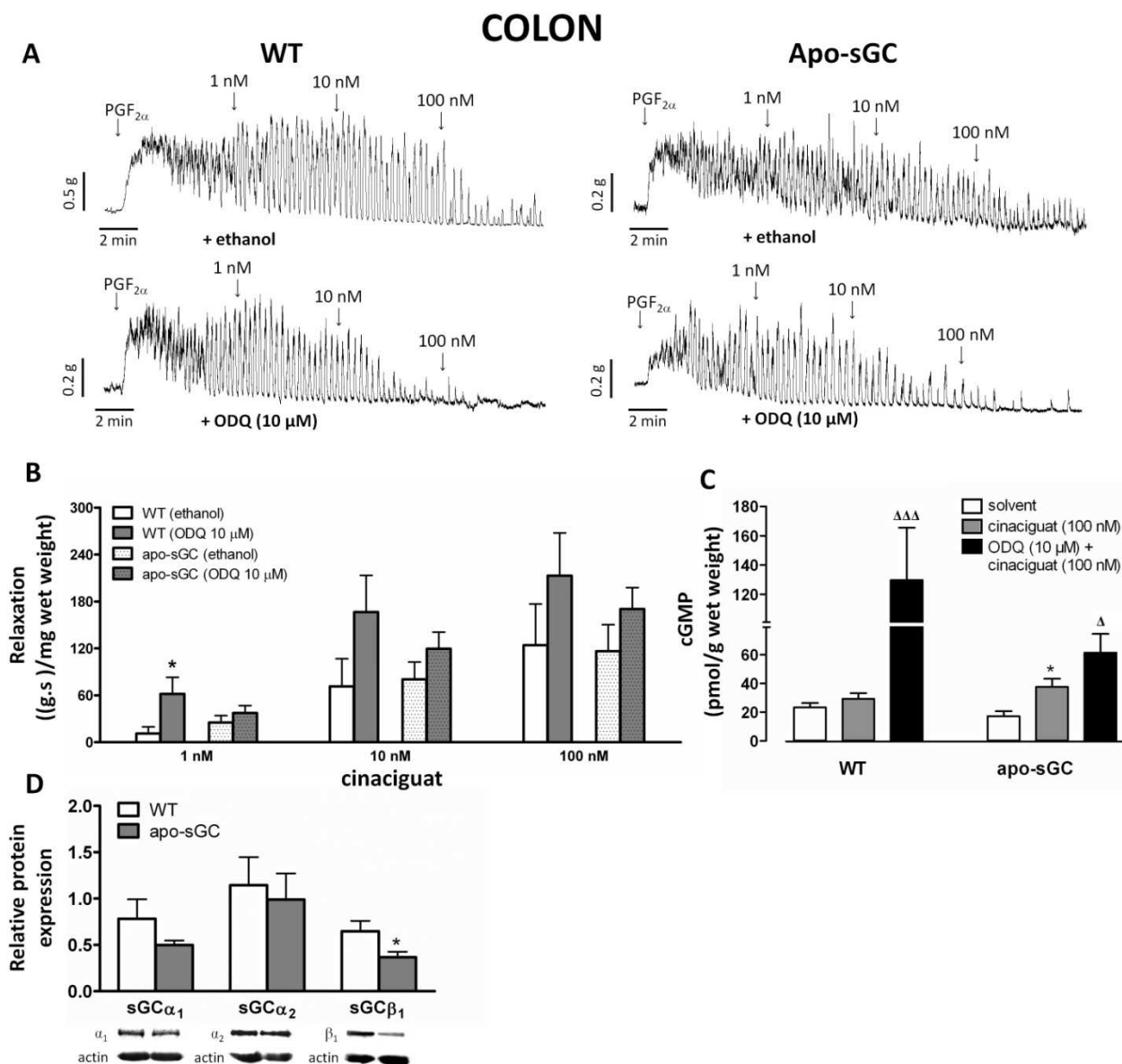
Fig. V.7 Relaxant and cGMP responses to cinaciguat and Western blot analysis of sGC subunits in colon of WT and apo-sGC mice. (A) Representative traces showing the responses to cinaciguat (1-10-100 nM) in $PGF_{2\alpha}$-pre-contracted circular muscle strips of the distal colon from a WT (left) and an apo-sGC mouse (right) in the presence of the sGC inhibitor ODQ (10 µM; lower trace) or its solvent ethanol (upper trace). (B) Relaxations to cinaciguat (1-10-100 nM) in distal colon strips of WT and apo-sGC mice after incubation with ODQ (10 µM) or its solvent (ethanol). Data represent the means ± S.E.M. of n = 7-8. * P < 0.05: ODQ versus ethanol (unpaired Student's *t*-test). In the presence of ODQ (10 µM), the contractile response to $PGF_{2\alpha}$ in WT colonic strips was more than 50 % less pronounced than in its absence (contraction to $PGF_{2\alpha}$ in (g.s)/mg wet weight: 18.8 ± 3.0 in the presence of ODQ versus 40.0 ± 16.5 in the presence of ethanol; n = 8). This means that there is less contractile activity to suppress, which will per se decrease the absolute relaxant responses to cinaciguat. The relaxant responses to cinaciguat of WT colonic strips in the presence of ODQ were therefore corrected by multiplication with the factor "Response to $PGF_{2\alpha}$ in WT strips receiving ethanol/response to $PGF_{2\alpha}$ in WT strips receiving ODQ". The contractile responses to $PGF_{2\alpha}$ in apo-sGC strips (contraction to $PGF_{2\alpha}$ in (g.s)/mg wet weight: 15.5 ± 5.5 in the presence of ODQ and 18.1 ± 8.3 in the presence of ethanol; n = 7-8) were also more than 50 % lower when compared to the contractile response to $PGF_{2\alpha}$ in WT control strips receiving ethanol. The relaxant responses to cinaciguat in apo-sGC strips (strips that received ethanol and strips that were incubated with ODQ) were therefore corrected by multiplying with, respectively, a factor "Response to $PGF_{2\alpha}$ in WT control strips/response to $PGF_{2\alpha}$ in apo-sGC control strips" and "Response to $PGF_{2\alpha}$ in WT control

strips/response to PGF$_{2\alpha}$ in apo-sGC strips that received ODQ". (C) cGMP levels in colon strips of WT and apo-sGC mice after incubation with the solvent of cinaciguat, cinaciguat (100 nM) itself and cinaciguat in the presence of ODQ (10 μM). Data represent the means ± S.E.M. of n = 12 for the solvent and cinaciguat group and of n = 5-6 for the ODQ + cinaciguat. * P<0.05: cinaciguat versus solvent (one-way ANOVA followed by a Bonferroni multiple comparison *t*-test); $^{\Delta}$ P<0.05, $^{\Delta\Delta\Delta}$ P<0.001: ODQ + cinaciguat versus cinaciguat (one-way ANOVA followed by a Bonferroni multiple comparison *t*-test).(D) Western blot analysis of the relative protein expression levels of sGCα$_1$, sGCα$_2$ and sGCβ$_1$ in the colon of WT and apo-sGC mice, expressed relative to the household protein actin. Data represent the means ± S.E.M. of n = 6. * P<0.05: apo-sGC versus WT (unpaired Student's *t*-test). Representative Western blot results as for figure 2.

V.4.3   Oxidative stress levels

MDA/HNE levels in fundus, antrum, jejunum and colon and ROS levels measured with L-012 in fundus and colon were not different in apo-sGC versus WT mice (Table V.1).

Table V.1 Levels of MDA/HNE as an indicator of oxidative stress and actual ROS levels measured with L-012.

	MDA/HNE nmol/mg protein				L-012 AU/mg protein	
	WT	**Apo-sGC**			**WT**	**Apo-sGC**
Fundus	1.3 ± 0.1	1.3 ± 0.2		**Fundus**	84.3 ± 5.1	84.2 ± 5.0
Antrum	0.82 ± 0.05	0.75 ± 0.05		**Colon**	194.9 ± 8.6	214.5 ± 47.4
Jejunum	1.5 ± 0.1	1.8 ± 0.1				
Colon	1.0 ± 0.1	0.9 ± 0.1				

Values are means ± S.E.M. of n = 6 (MDA/HNE) or n = 3 (L-012) .

V.4.4   Influence of cinaciguat on gastric emptying

After injection of the solvent of cinaciguat, gastric emptying was consistently significantly delayed in apo-sGC mice (Fig. V.8A-D). Intraperitoneal administration of cinaciguat (300 μg/kg) did not improve the delayed gastric emptying in apo-sGC mice nor did it influence that in WT mice. Intravenous injection of cinaciguat (300 μg/kg and 100 μg/kg) delayed gastric emptying significantly in WT mice (P<0.05), but had no influence on gastric emptying in apo-sGC mice. Intravenous injection of 30 μg/kg of cinaciguat did not have this inhibitory effect on gastric emptying in the WT mice, but it was also not able to improve the delayed gastric emptying in apo-sGC mice (Fig. V.8).
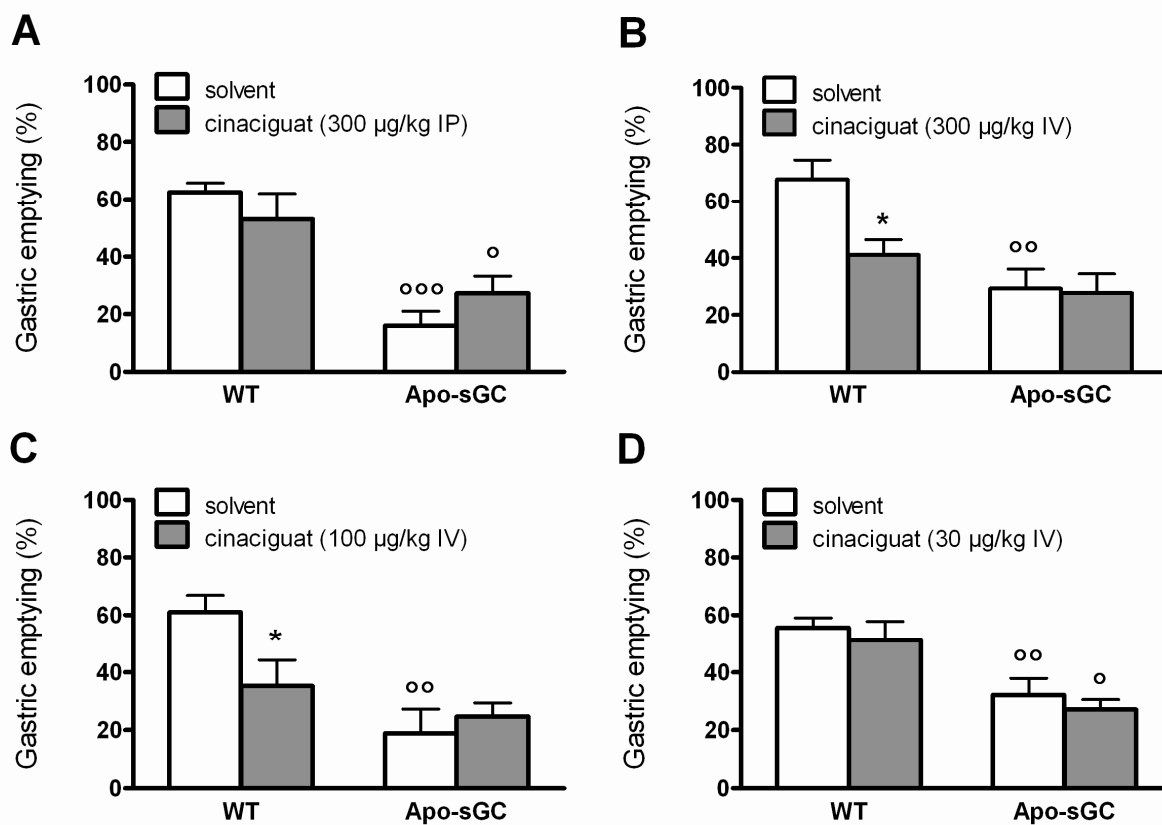
Fig. V.8 Gastric emptying in WT and apo-sGC mice after administering cinaciguat or its solvent.
Gastric emptying after intraperitoneal (A: 300 µg/kg) or intravenous injection (B: 300 µg/kg; C: 100 µg/kg; D: 30 µg/kg) of cinaciguat or its solvent. Data represent the means ± S.E.M. of n = 4-8. * $P<0.05$: cinaciguat versus solvent (unpaired Student's t-test); ^{o} $P<0.05$, ^{oo} $P<0.01$, ^{ooo} $P<0.001$: apo-sGC versus WT (unpaired Student's t-test).

V.5   Discussion

The disturbed gastric emptying and intestinal transit in apo-sGC mice illustrate the importance of NO-sensitive sGC in gastrointestinal motility (Cosyns *et al.*, 2013). The aim of the present study was to compare the effect of cinaciguat, an NO- and heme-independent sGC activator, on gastrointestinal motility in WT and apo-sGC mice.

Apo-sGC mice with their heme-free sGC can be considered as a model for gut dysmotility under oxidative stress as under disease conditions associated with oxidative stress, sGC will also be oxidized towards an NO-insensitive, heme-free status (Stasch *et al.*, 2006; Fritz *et al.*, 2011). This does not mean that oxidative stress levels should be increased in gastrointestinal tissues of apo-sGC mice; in gastrointestinal tissues of apo-sGC mice, MDA/HNE levels, that are an indicator of oxidative stress as MDA and HNE are key products of the peroxidative decomposition of lipids, were not different from those in WT mice; for fundus and colon, this was confirmed by direct measurement of ROS with L-012, which reacts with superoxide and hydrogen peroxide, but also with the reactive nitrogen species peroxynitrite (Daiber *et al.*, 2004). The protein levels of the sGC subunits (sGCα_1, sGCα_2 and sGCβ_1) however tended to be reduced or were even significantly reduced in the gastrointestinal tissues of apo-sGC mice. This is in agreement with previous reports suggesting that oxidation or loss of the heme group make sGC more prone for ubiquitination and subsequent proteosomal degradation (Stasch *et al.*, 2006; Hoffmann *et al.*, 2009; Meurer *et al.*, 2009).

At the level of the gastric fundus and distal colon, cinaciguat induced concentration-dependent relaxations in both WT and apo-sGC strips, the maximal effect being reached at similar concentrations as previously reported in vascular tissue (Stasch *et al.*, 2002; Stasch *et al.*, 2006). Although the protein levels of sGCα_1 and sGCβ_1 were lower in apo-sGC fundus and colon, the relaxant responses to cinaciguat in fundus and colon strips were similar or even higher in apo-sGC mice. This suggests that cinaciguat is more efficient when sGC is in the heme-free condition, as was previously reported for vascular tissue (Stasch *et al.*, 2006). The data obtained with the sGC inhibitor ODQ, that is thought to inhibit sGC by oxidation of its heme group (Schrammel *et al.*, 1996), corroborate this conclusion: after incubation with ODQ, the relaxant responses to cinaciguat were greatly increased in WT fundus and colon strips. The efficacy of cinaciguat to activate sGC in the oxidized/heme-free condition was

also confirmed by measurement of cGMP: in the absence of ODQ, cGMP levels were only increased by cinaciguat in fundus and colon strips of apo-sGC mice but not of WT mice, but in the presence of ODQ, cinaciguat produced a pronounced increased in cGMP levels in fundus and colon strips of WT mice. Unexpectedly, ODQ also significantly increased cinaciguat-induced cGMP levels in colon strips of apo-sGC mice, correlating with a clearcut tendency to increased relaxation by cinaciguat in the apo-sGC colon strips in the presence of ODQ. This is remarkable, as sGC is expected to be in the heme-free state already. We do not have an explanation for this. Although it has been reported that the degree of measurable cGMP increase in smooth muscle strips can differ enormously for a similar degree of nitrergic relaxation (Garcia-Pascual & Triguero, 1994; Smits & Lefebvre, 1996), depending upon the nitrergic stimulus, it was surprising to see no increase at all in cGMP levels in WT fundus and colon after the addition of 100 nM cinaciguat as this induced pronounced relaxation in both WT tissues. This might imply that other mechanisms, besides activation of sGC and generation of cGMP, are involved in the relaxation caused by cinaciguat in WT fundus and colon; however, no other mechanisms than sGC activation have been reported so far to explain smooth muscle relaxation by cinaciguat. An alternative possibility is that cinaciguat might induce moderate compartimentalized increases in cGMP, that cannot be found upon homogenization of the tissue. Correspondingly, maintained NO-induced relaxation, sensitive to the inhibitory effect of ODQ but without a significant increase in cGMP levels was reported for aortic rings of sGCα_1-KO mice, suggesting that local non-measurable cGMP increases via sGC$\alpha_2\beta_1$ are responsible for the induction of the nitrergic relaxation; the amount of sGCα_2 was indeed maintained in the sGCα_1-KO mice (Mergia *et al.*, 2006). We previously showed that the amount of sGCα_2 is also maintained (De Backer *et al.*, 2008) and that the relaxant response to exogenous NO is only moderately reduced (Vanneste *et al.*, 2007) in the gastric fundus of sGCα_1-KO mice. The latter was confirmed in the actual study; however, the relaxant effect of cinaciguat was practically non-existent in gastric fundus strips of sGCα_1-KO mice. This supports that the relaxation of WT strips by cinaciguat in the absence of ODQ is related to sGC activation, as it was reported that cinaciguat preferentially stimulates sGC$\alpha_1\beta_1$ (Haase *et al.*, 2010).

The evaluation of the response to exogenous NO in antrum illustrated the presence of an NO-sensitive cGMP-mediated relaxant pathway in antrum, as the ODQ-sensitive relaxation by NO in WT antrum was not present in antrum of apo-sGC mice. The study of

EFS under NANC conditions confirmed that NANC inhibitory neurotransmission in mouse antrum is largely purinergic (Gil *et al.*, 2013) with a supporting redundant role of NO. Indeed, EFS-induced relaxations were of similar size in WT and apo-sGC antrum; while the relaxant responses by EFS at 2 and 4 Hz were abolished by the $P2Y_1$ receptor antagonist MRS2500 plus the NO synthesis inhibitor L-NAME in WT antrum, MRS2500 alone abolished them in apo-sGC antrum, the contribution of NO through cGMP already being excluded by the $sGC\beta_1^{His105Phe}$ mutation. The small relaxation seen by EFS at 8 Hz under combined nitrergic plus purinergic blockade, might indicate contribution of an additional transmitter. The release of another unidentified neurotransmitter, besides NO and ATP, was also suggested for the pylorus and colon (Soediono & Burnstock, 1994; Ishiguchi *et al.*, 2000; Van Crombruggen & Lefebvre, 2004; Cosyns *et al.*, 2013). Cinaciguat was not able to induce relaxations in WT and apo-sGC antrum strips. As endogenous NANC relaxation in the antrum is largely dependent upon purinergic signaling, lower amounts of sGC might be present to be activated. However, sGC levels in antrum were similar to those in other gastrointestinal tissues. Additionally, also for the colon a large part of the inhibitory response is attributed to purinergic signaling (Van Crombruggen & Lefebvre, 2004; Dhaese *et al.*, 2008; Gil *et al.*, 2013), and cinaciguat causes relaxation in this tissue. Still more intriguing, cinaciguat was also not able to induce a relaxation in WT and apo-sGC jejunum strips, either when contracted by $PGF_{2\alpha}$ or by carbachol, to exclude the possibility that the used contractile agent determines the non-effect of cinaciguat, as it was reported before that the agents by which contraction is induced can affect the ability to induce smooth muscle relaxation (Gibson *et al.*, 1994). Still, endogenous NANC inhibitory responses in the jejunum are sGC-dependent and fully nitrergic in nature (Dhaese *et al.*, 2009). Cinaciguat is expected to be more efficient when sGC is in the oxidized condition (Stasch *et al.*, 2006), but MDA/HNE levels in antrum and jejunum were similar to those in colon and fundus; the MDA/HNE values were also comparable to those reported in the literature for mouse small intestine (0.97 nmol/mg protein; Diao *et al.*, 2012), rat small intestine (2.09 nmol/mg protein; Liu *et al.*, 2013) and rat colon (1.17 nmol/mg protein; Larrosa *et al.*, 2009). We have thus no explanation for the lack of effect of cinaciguat in WT and apo-sGC antrum and jejunum, nor for its ineffectiveness in the pylorus of apo-sGC mice.

Liquid gastric emptying was decreased in apo-sGC mice, as we reported previously (Cosyns *et al.*, 2013), probably related to impaired pyloric relaxation. Mashimo *et al.* (2000)

similarly suggested that functional pyloric obstruction, due to a loss in nitrergic neurotransmission, contributes to a great extent to the delay in gastric emptying seen in nNOS-KO mice. The inability of cinaciguat to relax the pylorus of apo-sGC mice might thus explain the inability of cinaciguat to improve delayed gastric emptying in apo-sGC mice. Surprisingly, when giving 300 or 100 µg/kg intravenously to WT mice, gastric empting was delayed. Injection of the same doses of cinaciguat has shown to cause a sudden drop in mean arterial blood pressure of 20-25 mmHg in mice for about 15-25 min (Vandendriessche *et al.*, 2013). This drop in blood pressure will decrease tissue perfusion and thus gastric blood flow, which might induce gastric dysmotility and delayed gastric emptying. In the apo-sGC mice, cinaciguat also causes a pronounced decrease in blood pressure (Thoonen, 2010), but as gastric emptying is already severely delayed in these mice, the hypotension will not lead to additional delay.

In conclusion, the NO- and heme-dependent sGC activator cinaciguat relaxes the fundus and colon efficiently when sGC is in the heme-free condition corresponding to its preferential activation of heme-free sGC in vascular tissue. But it is unable to relax the antrum, pylorus and jejunum of NO-insensitive, heme-free apo-sGC mice. This non-effect of cinaciguat in pylorus explains its inability to improve the delayed gastric emptying in apo-sGC mice.

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Chapter VI

PROTECTIVE EFFECT OF EXOGENOUS NITRITE IN POSTOPERATIVE ILEUS

Sarah M.R. Cosyns, Romain A. Lefebvre

Heymans Institute of Pharmacology, Ghent University, Ghent, Belgium

Chapter VI

Protective effect of exogenous nitrite in postoperative ileus

VI.1   Abstract

Background. As the pathogenesis of postoperative ileus (POI) involves inflammation and oxidative stress, similar to ischemia/reperfusion injury that can be counteracted with nitrite, we investigated whether nitrite can protect against POI and intended to elucidate the mechanisms involved.

Methods. POI was induced in C75BL/6J mice by small intestinal manipulation (IM); sodium nitrite (48 nmol) was administered intravenously just before IM. Intestinal transit was assessed using fluorescent imaging. Bethanechol-stimulated jejunal circular muscle contractions were measured in organ baths. Inflammatory parameters, neutrophil infiltration, inducible nitric oxide synthase (iNOS) activity, oxidative stress, mitochondrial complex I activity and cyclic guanosine monophosphate (cGMP) were measured in the intestinal muscularis.

Key results. Pre-treatment with nitrite markedly improved the delay in intestinal transit and restored the reduced intestinal contractility observed 24 h following IM. This was associated with reduced protein levels of tumor necrosis factor alpha (TNFα), interleukine-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1), as well as reduced iNOS activity and oxidative stress; the associated neutrophil influx was not influenced by nitrite. Treatment with nitrite did not influence the observed reduction in mitochondrial complex I activity following IM, but it did increase cGMP levels. Pre-treatment with the NO scavenger carboxy-PTIO or the soluble guanylate cyclase (sGC) inhibitor ODQ abolished nitrite-induced protective effects.

Conclusions. An intervention with exogenous nitrite can be a valuable tool in the treatment of POI. Nitrite-induced protection, shown to be dependent on NO, is not related to inhibition of mitochondrial complex I, but the activation of sGC does play a role.

VI.2   Introduction

Postoperative ileus (POI) is a transient impairment of gastrointestinal motility, commonly seen after abdominal surgery. It usually resolves within 3 days, but when prolonged, it can lead to increased morbidity, prolonged hospitalization and increased healthcare cost (Kehlet & Holte, 2001). The pathophysiology of POI is marked by an acute neurogenic phase followed by a prolonged inflammatory phase (Boeckxstaens & de Jonge, 2009). The inflammatory phase is characterised by the activation of resident macrophages in the muscular layer, which release inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6), chemokines such as monocyte-chemoattractant protein-1 (MCP-1) and adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1). MCP-1 and ICAM-1 will recruit circulatory leukocytes, that together with the activated resident macrophages will enhance release of nitric oxide (NO) through inducible NO synthase (iNOS). NO has potent inhibitory effects on the gastrointestinal motility and causes ileus (Bauer & Boeckxstaens, 2004; Turler *et al.*, 2006). Additionally, reactive oxygen species (ROS) might contribute to POI; our group previously reported an increase in intestinal oxidative stress levels starting shortly after intestinal manipulation (IM) (De Backer *et al.*, 2009).

Exogenous administration of nitrite was shown to protect heart, liver, kidney and brain from ischemia/reperfusion (I/R) injury (Duranski *et al.*, 2005; Jung *et al.*, 2006; Shiva *et al.*, 2007; Tripatara *et al.*, 2007). The main mechanisms underlying I/R injury include the generation of ROS and the activation of an inflammatory cascade; both mechanisms make cells more susceptible to cell death (Sanada *et al.*, 2011). The exact mechanism of the protective effect of nitrite in I/R models is not completely understood. Although iNOS-derived NO contributes to inflammatory damage in I/R injury, evidence suggests that exogenous nitrite needs to be reduced to NO to become effective as the NO-scavengers 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) and carboxy-PTIO prevent the beneficial effect of nitrite (Duranski *et al.*, 2005; Shiva *et al.*, 2007). Beneficial effects might be dependent on providing sufficient NO at areas with a shortage due to deficiency of the two constitutive NO synthases (endothelial and neuronal), which in case of hypoxia cannot produce NO anymore and might even produce ROS. Nitrite is unique in that it will be reduced to NO preferentially under hypoxic conditions, and might thus provide NO where

needed (Raat *et al.*, 2009). This can less systematically be obtained with NO donors, that were shown to induce beneficial, no or even detrimental effects in I/R models (Hoshida *et al.*, 1996; Zhu *et al.*, 1996; Mori *et al.*, 1998; Lozano *et al.*, 2005; Li *et al.*, 2009).

Two possible mechanisms of action have been suggested in the protective effect of nitrite-derived NO against I/R injury. Shiva *et al.* (2007) showed in a hepatic I/R model that nitrite can lead to reversible inhibition of mitochondrial complex I by S-nitrosation; inhibition of mitochondrial complex I dampens the electron transfer and was shown to limit mitochondrial ROS production (Lesnefsky *et al.*, 2004; Shiva *et al.*, 2007). Inhibition of mitochondrial complex I as a pathway for the nitrite-induced protective effect was also described in a cardiac ischemia/reperfusion model (Dezfulian *et al.*, 2009). In contrast, Duranski *et al.* (2005) showed in a model of hepatic I/R that nitrite protection was dependent on signalling via soluble guanylate cyclase (sGC), as it was completely abolished by the sGC inhibitor 1H[1,2,4,]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). An sGC-dependent protective effect of nitrite was also suggested in a model of TNF-induced sepsis, in which TNF is known to cause inflammation accompanied by oxidative stress; treatment with nitrite decreased oxidative stress, mitochondrial damage and mortality, and this protection by nitrite was largely abolished in sGCα_1 knockout mice (Cauwels *et al.*, 2009).

Treatment of POI remains mostly supportive and no real treatment or prevention currently exists. As the pathogenesis of POI also involves inflammation and oxidative stress, similar to I/R that can be counteracted with nitrite, the aim of this study was to investigate whether nitrite can protect against POI and to elucidate the mechanisms involved.

VI.3   Materials and methods

VI.3.1 Animals

Male C57BL/6J mice (20-25 g, n = 153) were purchased from Janvier, Le Genest St-Isle, France and had free access to water and commercially available chow. All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

VI.3.2 Hepatic I/R model

The hepatic I/R protocol has been described previously (Duranski *et al.*, 2005). Mice were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), dissolved in 0.9 % normal saline and administered intraperitoneally (i.p.). Body temperature of the mice was kept constant during the entire period of surgery and ischemia by use of a heat lamp. A midline laparotomy was performed and the intestine was gently lifted from the body cavity to access the liver. A microaneurysm clamp was applied for 45 min to the left branch of the portal vein and hepatic artery, resulting in ischemia of the left lateral and median lobes of the liver. This leads to segmental (70%) ischemia, preventing mesenteric venous congestion by allowing portal decompression through the caudate and right lobe of the liver. Just after placing the clamp, mice were injected with heparin (100 mg/kg; i.p.) to prevent blood clotting. During the period of ischemia, the intestine was placed back in the abdominal cavity and the liver was kept moist using gauze soaked in 0.9 % normal saline. Halfway ischemia, sodium nitrite (48 nmol) or its solvent (phosphate buffered saline; PBS) was administered into the vena cava inferior. After 45 min, the microaneurysm clamp was removed and the abdomen was closed with 2 layers of continuous sutures. Non-operated mice served as controls. The liver was reperfused for 5 h, and following this period, serum was collected and frozen at -80°C for determination of liver transaminase levels; these enzymes are liver specific and are released from the liver during injury. Liver aspartate aminotransferase/alanine aminotransferase (AST/ALT) levels were spectrophotometrically analyzed (Cobas 8000C; Roche, Basel, Switzerland) and expressed as U/l.

VI.3.3 POI model

Mice were anesthetized with inhaled isoflurane (5 % induction, 2 % maintenance) and the abdomen was opened by midline laparotomy. POI was induced by compressing the eventrated small intestine by using sterile moist cotton applicators for 5 min. Sodium nitrite (48 nmol) or its solvent (PBS) was administered into the inferior vena cava just before IM. After IM, the bowel was repositioned in the abdominal cavity and the incision was closed by two layers of continuous sutures. Mice were sacrificed 6 or 24 h after surgery and the gastrointestinal tract was removed. Non-operated mice served as controls.

In an additional set of experiments, we studied the influence of the NO scavenger carboxy-PTIO (1 mg/kg in PBS, i.p. 30 min before IM) and the sGC inhibitor ODQ (20 mg/kg in DMSO, i.p. 30 min before IM) and its solvent DMSO on nitrite-mediated effects in manipulated mice. Mice were sacrificed 24 h after surgery and the gastrointestinal tract was removed.

After measuring transit, the small intestine was flushed with aerated (5 % CO_2 in O_2) ice cold Krebs solution (composition in mM: NaCl 118.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1) containing 1 mM PMSF and divided into 6 segments. In the mice sacrificed 24 h after surgery, one segment was used to test the contractile response to bethanechol (see below); in the rest of the segments, the mucosa was removed by using a glass slide and the muscularis was stored at -80°C until further processing.

VI.3.4 Evaluation of intestinal motility

Intestinal transit was assessed by evaluating the distribution of a nonabsorbable tracer, i.e. fluorescein-labeled dextran (FD70; 70 kDa) in the gastrointestinal tract 24 h postoperatively, as described previously (De Backer *et al.*, 2008). Mice were gavaged 200 µl of FD70 (25 mg/ml in distilled water) 22h30 postoperatively. Ninety minutes later, the animals were sacrificed, the abdomen was cut open, a ligature was placed around the lower oesophagus (just above the cardia) and rectum, and the entire gastrointestinal tract was excised. Next, the mesenterium was removed and the gastrointestinal tract was pinned down in a custom-made Petri dish (5 x 30 cm) filled with aerated (5% CO_2 in O_2) Krebs solution containing 1 mM PMSF (Sigma Aldrich). Immediately after, FD70 was visualized using the Syngene GeneFlash system (Syngene, Cambridge, UK), consisting of a UV-light source, an excitation filter (410-510 nm conversion screen), a 8-bit monochrome CCD camera equipped with an 8-48 mm f/1.2 zoom lens, and an emission bandpass filter (550-600 nm emission) to detect fluorescence. Two full-field images -one in normal illumination mode and another in fluorescent mode- were taken and subsequently matched for analysis; the fluorescent intensity throughout the entire gastrointestinal tract was analyzed and calculated using Intestinal Transit Software (written as an ImageJ plugin; can be downloaded at www.heymans.ugent.be/En/DownloadsEn.htm). Data were expressed as the percentage

of fluorescence intensity per segment (stom, stomach; sb, small bowel segments 1-10; caec, caecum; col, colon segments 1-2) and plotted in a histogram. The geometric center (GC) was calculated by the following formula: Σ (% FD70 per segment x segment number)/100.

Contractile activity was evaluated using the muscarinic agonist bethanechol. Briefly, a mid-jejunal segment of the small intestine was opened along the mesenteric border and pinned mucosa side up in Krebs solution. The mucosa was removed by sharp dissection under a microscope and a full-thickness muscle strip (4 × 5 mm) was cut along the circular axis. After a silk thread (USP 4/0) was attached to both ends of the strip, it was mounted in a 7 ml organ bath, which contained aerated (5 % CO_2 in O_2) Krebs solution, maintained at 37°C. Changes in isometric tension were measured using Grass force transducers and recorded on a PowerLab/8sp data recording system (ADInstruments) with Chart software. After an equilibration period of 60 min with flushing every 15 min at a load of 0.25 g, carbachol (10 μM) was added once to check the functional viability of the muscle preparations. The tissues were then allowed to equilibrate for 30 min at 0.25 g with flushing every 10 min in Krebs solution, after which they were exposed to increasing concentrations of bethanechol (cumulative 0.3-300 μM; 2 min interval). As jejunal strips show phasic activity, the area under the curve (AUC) above baseline was determined to measure the contractile responses to bethanechol. Responses are expressed as (g.s)/mg wet weight.

VI.3.5 cGMP analysis

cGMP was extracted and quantified using an enzyme immunoassay kit (Cayman Chemical, Michigan, USA). Briefly, frozen tissues were pulverized by a Mikro-Dismembrator U (B-Braun, Melsungen, Germany), homogenized in 5 % trichloroacetic acid (TCA) and centrifuged for 15 min at 4°C at 2000 *g* to collect the supernatant. The supernatant was washed three times with water-saturated ether to extract the TCA after which it was dried under nitrogen at 60°C. After drying, it was dissolved in a 10 times volume of assay buffer. Then, samples, controls and standards were acetylated and were added to the enzyme immunoassay plate to incubate for 18 h at 4°C. Optical density was measured with a 96-well plate reader (Biotrak II, Amersham Biosciences, Buckinghamshire, UK) at 405 nm. The concentration of cGMP was expressed as pmol/g wet weight.

VI.3.6 *Mitochondrial isolation and complex I activity*

Mitochondria were isolated as described by Gadicherla *et al.* (2012). Briefly, intestinal muscularis segments were placed in 1 ml isolation buffer (200 mM mannitol, 50 mM sucrose, 5 mM KH_2PO_4, 5 mM MOPS, 1 mM EGTA, and 0.1 % BSA; pH adjusted to 7.15 with KOH), and minced into fine pieces. The suspension was homogenized with a teflon pestle homogenizer and was subsequently centrifuged at 8000 *g* for 10 min. The pellet was re-suspended in 1 ml isolation buffer and then centrifuged at 750 *g* for 10 min; the supernatant was centrifuged again at 8000 *g* for 10 min, and the final pellet, enriched in mitochondria, was re-suspended in 200 μl isolation buffer. Mitochondria isolated as described above were then centrifuged at 10000 *g* and the pellet was re-suspended in hypotonic buffer (25 mM potassium phosphate buffer with 5 mM $MgCl_2$; pH 7.2). After dilution to the appropriate concentration (0.5 mg/ml), mitochondria were subjected to three rounds of freeze-thaw cycles. The fractured mitochondria were used to measure complex I activity.

Complex I activity was determined by monitoring the change in transmittance from oxidation of NADH to NAD^+ at 340 nm (FLUOstar, BMG Labtech, Ortenberg, Germany). To 20 μl of mitochondria protein, hypotonic buffer containing 1 mM KCN, 0.1 mM NADH and 0.25 % BSA was added. The reaction was initiated by the addition of 160 μM ubiquinone (CoQ_1; Sigma Aldrich) and the decrease in optical density due to oxidation of NADH was measured for 10 minutes. Complex I activity was determined in the absence and presence of rotenone (mitochondrial complex I inhibitor; 50 μM; Sigma Aldrich); the rate of transmittance in the presence of rotenone was subtracted from the rate of transmittance without rotenone, to obtain rotenone sensitive activity. Rate of activity was calculated using the extinction coefficient of NADH, 6.18 mM^{-1} cm^{-1} with slopes derived over the 10 min period. Results were normalized to total protein content (Pierce BCA Protein Assay Kit, ThermoScientific, Illinois, USA) and expressed as μmol/min/mg protein.

VI.3.7 Protein expression levels of MCP-1, IL-6 and TNFα

Protein expression levels of IL-6, MCP-1 and TNFα were determined by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol (Invitrogen, Merelbeke, Belgium). Briefly, intestinal tissue samples were homogenized with a Mikro-Dismembrator, dissolved in 10 (MCP-1 and IL-6) or 5 (TNFα) volumes of 20 mM PBS buffer (pH 7.4) containing protease inhibitors (Complete Mini Protease Inhibitor EDTA-free tablets, Roche, Basel, Switzerland), and centrifuged at 10000 *g* for 15 min at 4°C. The supernatant (100 µl) was added to the appropriate microtiter wells, after which the plate was covered and incubated at room temperature. After 2 hours, the solution was thoroughly aspirated from the wells and wells were washed with wash buffer. 100 µl of IL-6 or MCP-1 Biotin Conjugate Solution or 50 µl of TNFα Biotin Conjugate Solution was added to each well, after which the plate was covered and incubated at room temperature. After 30 (IL-6), 45 (MCP-1) or 90 min (TNFα), the solution was aspirated from wells and wells were washed with wash buffer. Streptavidin-HRP Working Solution (100 µl) was then added to each well, after which the plate was covered and incubated at room temperature. After 30 (IL-6 and TNFα) or 45 min (MCP-1), the solution was thoroughly aspirated from wells and wells were washed with wash buffer. Stabilized Chromogen (100 µl) was then added to each well and incubated at room temperature in the dark. After 30 (IL-6 and TNFα) or 20 min (MCP-1), Stop Solution (100 µl) was added to each well and the absorbance was measured at 450 nm. Results were normalized to total protein content (Pierce BCA Protein Assay Kit) and expressed as pg/mg protein.

VI.3.8 Neutrophil infiltration

Myeloperoxidase (MPO) activity was measured as an index of neutrophil infiltration, and was based on a previously described protocol (de Jonge *et al.*, 2003). Frozen tissue samples were homogenized with a Mikro-Dismembrator and dissolved in 10 volumes of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5 % hexadecyl-trimethylammonium bromide (HETAB). The homogenate was sonicated on ice (15 pulses of 0.7 s at full power) and subsequently subjected to freeze/thaw. The suspension was centrifuged (14000 *g*, 20 min, 4 °C) and 10 µl of the supernatant was added to 200 µl of assay mixture, containing

ready-to-use TMB substrate, 0.5% HETAB, and 10 mM EDTA (on ice). The optical density was immediately read at 620 nm (Biotrak II). The reaction was then allowed to proceed for 3 min at 37 °C. The reaction was stopped by placing the 96-well plate on ice, and the optical density was measured again. One unit of MPO activity was defined as the amount of enzyme that produces a change in optical density of 1.0 per minute at 37 °C. Results were normalized to total protein content (Pierce BCA Protein Assay Kit) and expressed as U/mg protein.

VI.3.9 iNOS activity

Inducible NO synthase (iNOS) enzyme activity was assayed by measuring the conversion of [3H]-arginine to [3H]-citrulline using a NOS activity assay kit (Cayman Chemical, Michigan, USA), according to the manufacturer's recommended protocol. Briefly, frozen tissues were pulverized with a Mikro-Dismembrator and dissolved in 5 volumes of ice-cold homogenization buffer (250 mM Tris-HCl (pH 7.4), 10 mM EDTA and 10 mM EGTA). The homogenates were then centrifuged (10000 g, 15 min, 4°C) and 10 µl of the supernatant was added to 40 µl reaction mix (25 µl of Reaction Buffer (50 mM Tris-HCl (pH 7.4), 6 µM tetrahydrobiopterin, 2 µM flavin adenine dinucleotide, 2 µM flavin adenine mononucleotide), 5 µl of 10 mM NADPH (prepared in 10 mM Tris-HCl), 1 µl of [3H]-arginine (1 µCi/µl), 5 µl 8 mM MgAcetate, 4 µl calmoduline and 4 µl H_2O). The reaction samples were then incubated for 1 hour at room temperature, and the reaction was stopped by adding 400 µl of Stop Buffer (50 mM HEPES (pH 5.5), 5 mM EDTA) to the reaction sample. 100 µl of the equilibrated resin was then added into each reaction sample and the reaction samples were then transferred in the provided spin cups, which were centrifuged for 30 s in a microcentrifuge at full speed. The eluate was then transferred to scintillation vials, and, after adding 2 ml scintillation solution (Ultima Gold, Canberra Packard, USA) to each vial, the radioactivity was quantified in a liquid scintillation counter (Packard Tri-Carb 2100 TR, Canberra Packard, USA). Results were normalized to total protein content (Pierce BCA Protein Assay Kit) and iNOS activity was expressed as % of [3H]-citrulline in tissue from control non-manipulated mice.

VI.3.10 Oxidative stress levels

Tissue ROS levels were quantified using 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H)dione sodium salt (L-012), as described previously (Castier *et al.*, 2005). Intestinal tissue samples were homogenized with a Mikro-Dismembrator (B-Braun, Melsungen, Germany), dissolved in 10 volumes of 50 mM Tris-HCl buffer (pH 7.5) containing protease inhibitors (Complete Mini Protease Inhibitor EDTA-free tablets, Roche, Basel, Switzerland), and centrifuged at 10000 *g* for 15 min at 4°C. 195 µl of supernatant was incubated with 5 µl of L-012 (100 µM) and luminescence was measured (VictorWallac, PerkinElmer, Massachusetts, USA) after a 10 min interval, allowing the plate to dark-adapt. ROS levels were normalized to total protein content (Pierce BCA Protein Assay Kit) and expressed as Arbitrary Units/mg protein.

VI.3.11 Drugs used

The following drugs were used: carbamyl-β-methylcholine chloride (bethanechol), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), rotenone, sodium nitrite, ubiquinone (CoQ_1) (all obtained from Sigma-Aldrich, Diegem, Belgium), carbachol (Fluka AG, Diegem, Belgium), fluorescein-labeled dextran (70 kDa; Invitrogen, Merelbeke, Belgium), 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H)dione sodium salt (L-012; Wako Pure chemical Industries Ltd., Osaka, Japan) and 1H[1,2,4,]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Tocris Cookson, Bristol, UK). All drugs were dissolved in de-ionized water except for the following: sodium nitrite and carboxy-PTIO in 10 mM PBS (pH 7.4) and ODQ in DMSO.

VI.3.12 Data analysis

All results are expressed as means ± S.E.M. n refers to tissues obtained from different animals. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison t-test. A P-value less than 0.05 was considered to be statistically significant (Graphpad, San Diego, CA, USA).

VI.4   Results

VI.4.1   Confirmation of the protective effect of nitrite in hepatic I/R injury

In a first series of experiments we investigated whether the protective effect of nitrite in an I/R model of the liver, as previously reported by Duranski *et al*. (2005), could be reproduced. Both ALT and AST levels (markers for liver injury) were significantly (P<0.001) increased after I/R of the liver (ALT: 628.8 ± 88.9 U/l after I/R versus 92.0 ± 13.8 U/l in controls; AST: 458.8 ± 44.1 U/l after I/R versus 162.1 ± 13.8 U/l in controls; n = 6-7). Administration of 48 nmol (optimal dose reported by Duranski *et al*. (2005)) of nitrite during ischemia limited this increase (P<0.001; ALT: 193.9 ± 17.5 U/l; AST: 257.1 ± 24.6 U/l; n = 6-7), showing its protective effect in hepatic I/R injury. Having confirmed the efficacy of 48 nmol of nitrite, this dose was selected for testing in our model of POI.

VI.4.2   Effect of nitrite on manipulation-induced intestinal dysmotility

In non-operated control mice, fluorescein-labelled dextran (70 kDa) moved to the distal part of the small bowel, whereas in mice that had been intestinally manipulated, fluorescein-labelled dextran was retained in the proximal part of the small bowel (Fig. VI.1A); this delay in intestinal transit was reflected by a significant reduction in geometric centre (GC; Fig. VI.1B). Pre-treatment with nitrite reduced the manipulation-induced delay in transit, as indicated by a significant increase in GC (Fig. VI.1A and B).

The inhibition of intestinal transit after IM reflects inhibited smooth muscle contractile activity of the small intestine; compared to controls, IM caused a reduction in cholinergic contractile activity, indicated by a significantly reduced E_{max} of the cumulative concentration-response curve of bethanechol in jejunal smooth muscle strips. The contractile activity of smooth muscle strips of manipulated mice, that were pre-treated with nitrite, was restored to that of non-manipulated control mice (Fig. VI.1C).
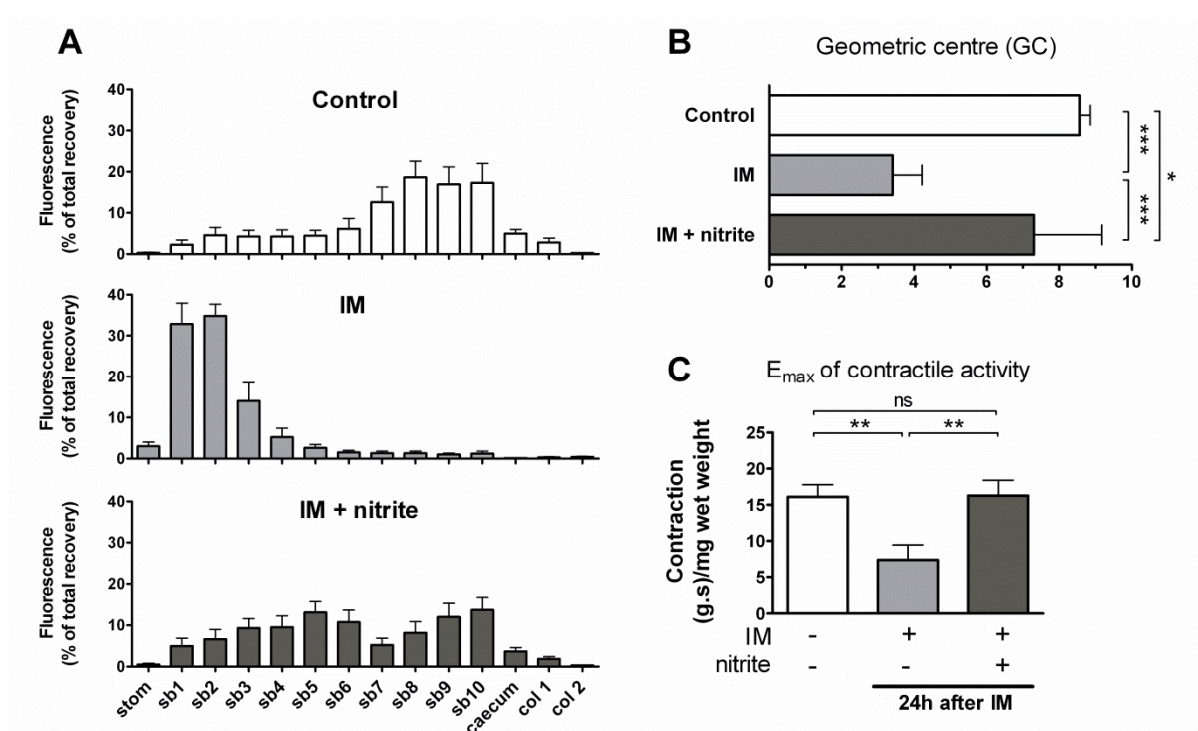
Fig. VI.1 Effect of nitrite on manipulation-induced intestinal dysmotility.
Transit histograms (A) and geometric centre (B) for the distribution of fluorescein-labelled dextran (70 kDa) along the gastrointestinal tract (stom, stomach; sb, small bowel segments; col, colon segments), measured 24 h after intestinal manipulation (IM). E_{max} of bethanechol-stimulated (cumulative 0.3-300 µM; 2 min interval) concentration-response curves of jejunal circular muscle contractile activity (C). Data represent the means ± S.E.M. of n = 14-15. *P<0.05, **P<0.01, ***P<0.001: one-way ANOVA followed by a Bonferroni multiple comparison test.

VI.4.3 Effect of nitrite on manipulation-induced inflammation and oxidative stress

The inflammatory cytokines TNFα and IL-6 and chemokine MCP-1 were significantly increased 24 h after manipulation of the intestine. Pre-treatment with nitrite reduced the IM-induced increase in cytokine/chemokine release, although not significantly for TNFα; note however that TNFα levels in nitrite-treated manipulated mice were no longer significantly different from those in non-operated control mice (Fig. VI.2A-C). MCP-1 protein levels were also assayed 6 h after IM: protein levels were 40 % higher than when measured 24 h after IM, but the IM-induced increase in MCP-1 levels could not be reduced by nitrite (Fig. VI.2C).

Neutrophil recruitment (MPO) into the muscularis was significantly increased 6 and 24 h after IM; compared to 6 h after IM, the influx of neutrophils was doubled at 24 h. Surprisingly, pre-treatment with nitrite markedly reduced the neutrophil infiltration at 6 h, but not at 24 h after IM (Fig. VI.2D).

iNOS activity increased non-significantly at 6 h and significantly at 24 h after IM. Pre-treatment with nitrite reduced this IM-induced elevation in iNOS activity significantly at 6 h and non-significantly at 24 h; note that iNOS activity in nitrite-treated manipulated mice was no longer significantly different from those in non-operated control mice at both 6h and 24 h after IM (Fig. VI.2E).

Oxidative stress, as measured with the chemiluminescent dye L-012, was not increased at 6 h after IM, but was markedly increased 24 h after surgery. Pre-treatment with nitrite reduced this IM-induced increase, though non-significantly; note however that oxidative stress levels of nitrite-treated manipulated mice were no longer significantly different from those in non-operated controls (Fig. VI.2F).
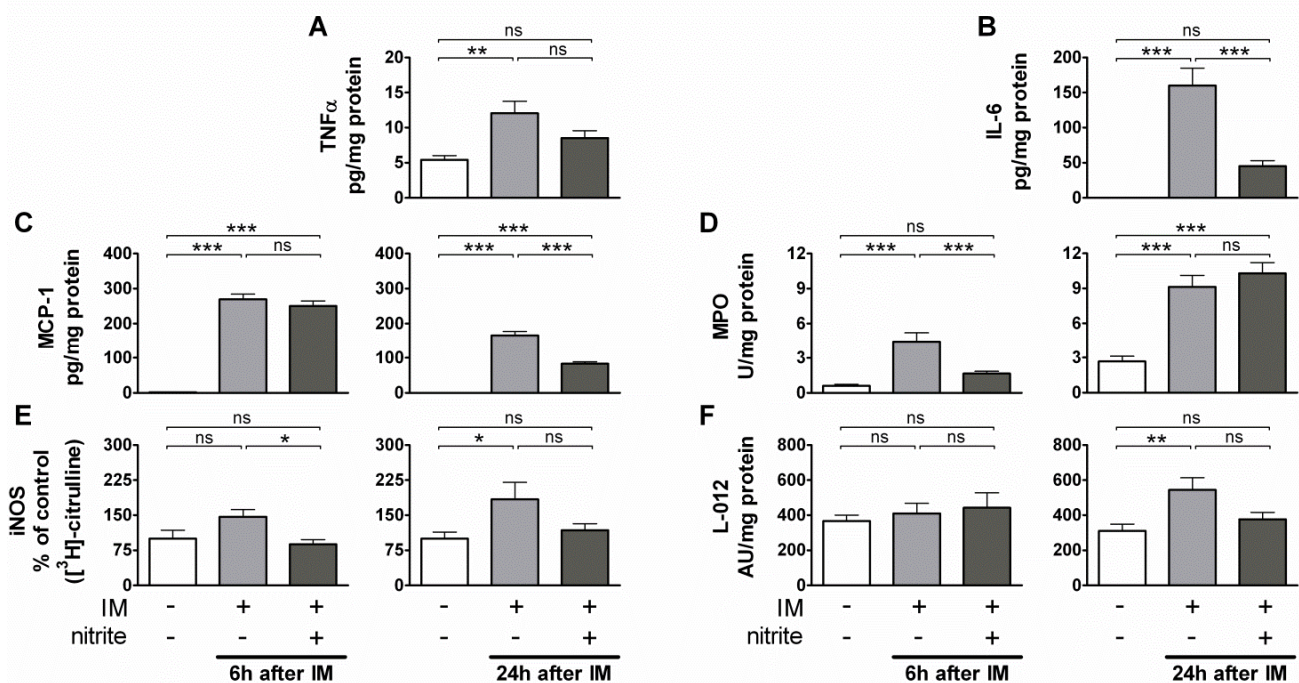
Fig. VI.2 Effect of nitrite on manipulation-induced inflammatory responses and oxidative stress.
Effect of nitrite on intestinal manipulation (IM)-induced changes in TNFα (A), IL-6 (B) and MCP-1 (C) protein levels, in neutrophil infiltration (D; myeloperoxidase, MPO), in iNOS enzyme activity (E), and in oxidative stress (F; assessed with the luminol derivate L-012), measured 6 h (except for TNFα and IL-6) and 24 h after IM. Values at 6 h were obtained in a separate series of experiments with its own non-manipulated control group. Data represent the means ± S.E.M. of n = 7-10. *P<0.05, **P<0.01, ***P<0.001: one-way ANOVA followed by a Bonferroni multiple comparison test.

VI.4.4 Investigation of the possible role of mitochondrial complex I and sGC in the effect of nitrite

Mitochondrial complex I activity was significantly reduced 6 and 24 h after IM. Pre-treatment with nitrite did not influence this reduction in enzyme activity after IM (Fig. VI.3A).

cGMP levels in the intestinal muscularis were significantly reduced 6 and 24 h after IM. Pre-treatment with nitrite increased these reduced cGMP levels after IM, although non-significantly at 24 h; but cGMP levels at 24 h of manipulated mice that were pre-treated with nitrite were no longer significantly lower than those of non-operated controls (Fig. VI.3B).
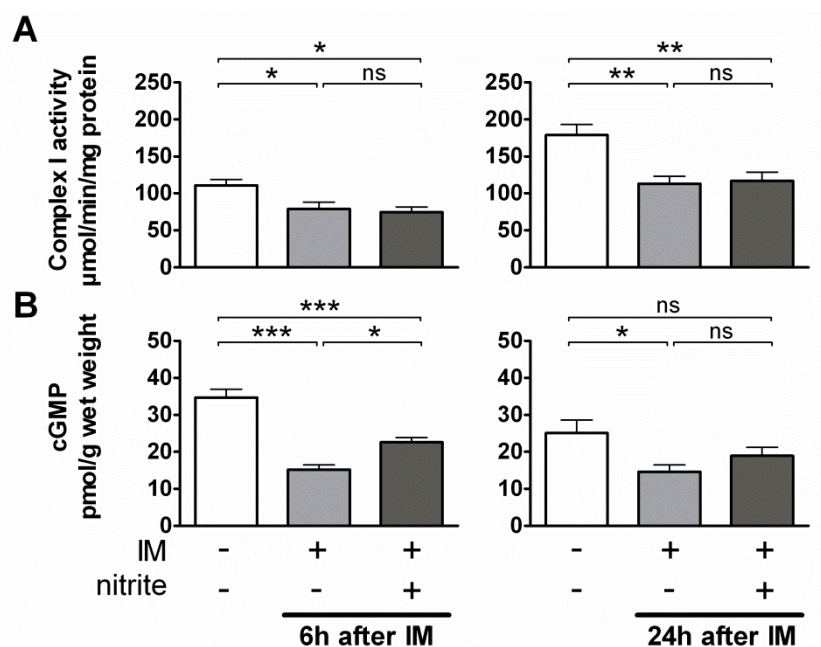
Fig. VI.3   Effect of nitrite on manipulation-induced changes in mitochondrial complex I activity and cGMP. Effect of nitrite on intestinal manipulation (IM)-induced changes in mitochondrial complex I activity (A) and cGMP levels (B), measured 6 h and 24 h after IM. Values at 6 h were obtained in a separate series of experiments with its own non-manipulated control group. Data represent the means ± S.E.M. of n = 8. *P<0.05, **P<0.01, ***P<0.001: one-way ANOVA followed by a Bonferroni multiple comparison test.

These results suggest that the nitrite-induced protective effect in POI might be dependent on sGC activation. This was further elaborated by exploring the influence of the sGC inhibitor ODQ on nitrite-mediated protective effects. Administration of ODQ completely prevented the accelerating effect of nitrite on delayed transit in manipulated mice, as evident from the reduction in GC to a level comparable to that in non-treated manipulated mice (Fig. VI.4A); correspondingly, the cholinergic contractile activity of jejunal smooth

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muscle strips was significantly reduced back to the level of non-treated manipulated mice (Fig. VI.4A and B). Inflammatory parameters (Fig. VI.5A-C) and oxidative stress levels (Fig. VI.5D) were increased or had a clear tendency to be increased after pre-treating nitrite-treated manipulated mice with ODQ. In addition, pre-treatment with ODQ prevented the nitrite-induced increase in cGMP levels in manipulated mice (Fig. VI.5E).

DMSO, the solvent of ODQ, was tested in parallel in nitrite-treated manipulated mice, to exclude the possibility that DMSO alone would have an influence per se on the protective effects of nitrite, but it was without influence (results not shown; n = 6).
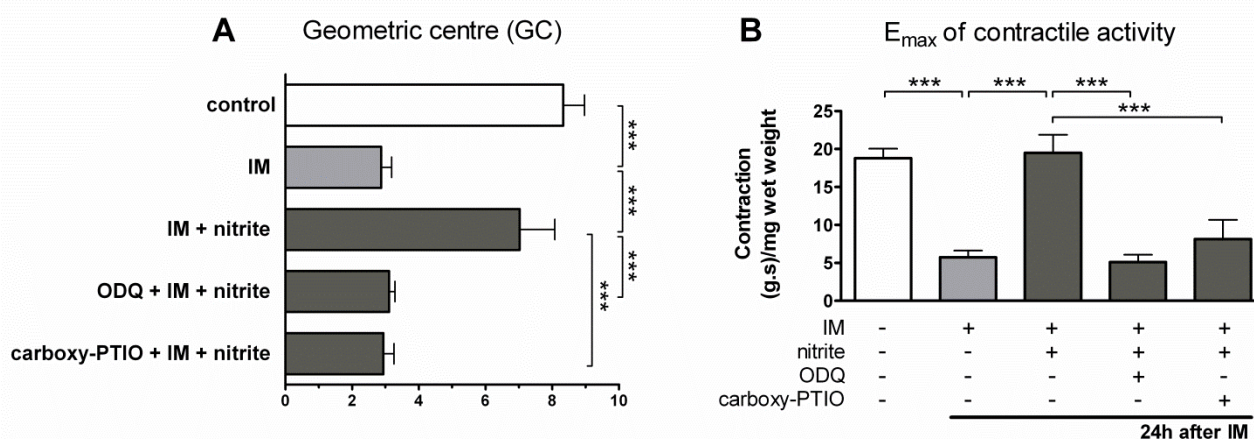
Fig. VI.4   Influence of the sGC inhibitor ODQ and the NO scavenger carboxy-PTIO on nitrite-induced protection against manipulation-induced intestinal dysmotility.
Geometric centre for the distribution of fluorescein-labelled dextran (70 kDa) along the gastrointestinal tract, measured 24 h after intestinal manipulation (IM; A). E_{max} of bethanechol-stimulated (cumulative 0.3-300 µM; 2 min interval) concentration-response curves of jejunal circular muscle contractile activity (B). Data represent the means ± S.E.M. of n = 6-8. ***P<0.001: one-way ANOVA followed by a Bonferroni multiple comparison test.

VI.4.5   Influence of the NO scavenger carboxy-PTIO on nitrite-induced protection

The protective effects of nitrite in POI appear to be NO dependent, as the NO scavenger carboxy-PTIO completely inhibited the nitrite-induced protection on gastrointestinal motility in manipulated mice (Fig. VI.4). Similar to ODQ, pre-treatment with carboxy-PTIO increased or at least had a tendency to increase inflammatory parameters (Fig. VI.5A-C) and oxidative stress levels (Fig. VI.5D), when comparing them to those of nitrite-treated manipulated mice. The nitrite-induced increase in cGMP levels of manipulated mice was also prevented by carboxy-PTIO.
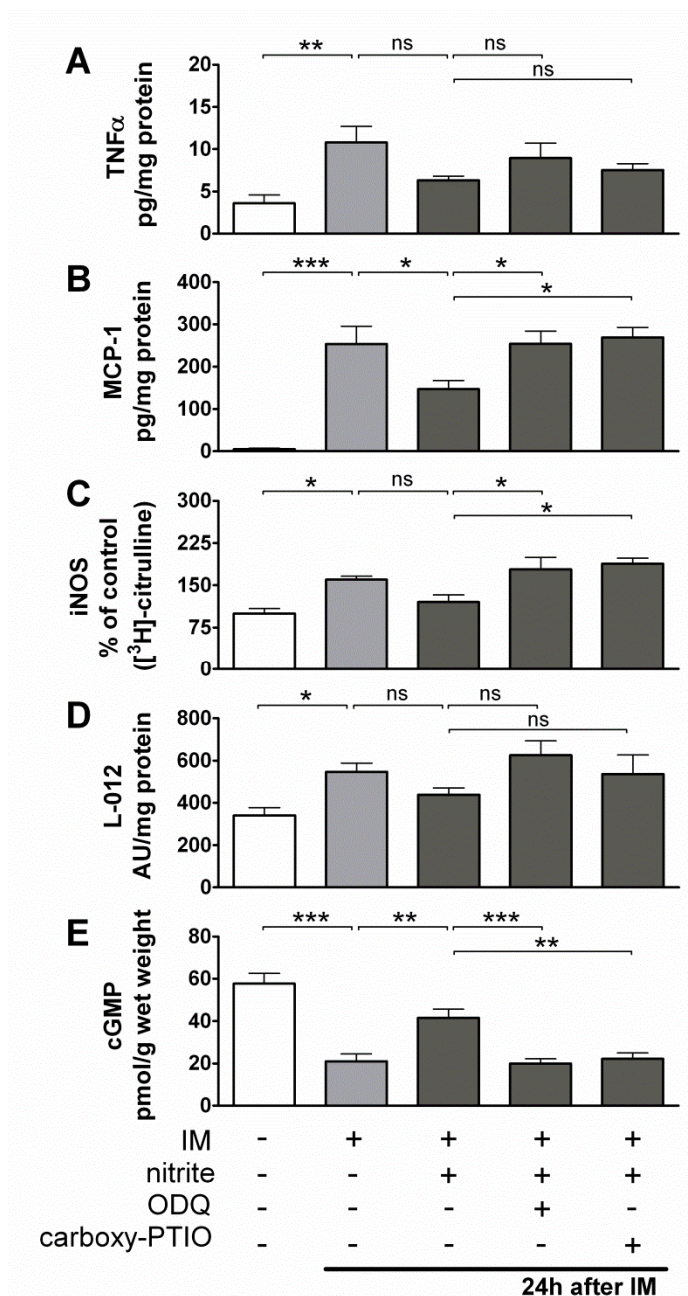
Fig. VI.5   Influence of ODQ and carboxy-PTIO on the effect of nitrite versus manipulation-induced changes in inflammatory markers, oxidative stress and cGMP.
TNFα (A) and MCP-1 protein levels (B), iNOS enzyme activity (C), oxidative stress (D; assessed with the luminol derivate L-012), and cGMP levels (E), measured 24 h after IM. Data represent the means ± S.E.M. of n = 5-8. *P<0.05, **P<0.01, ***P<0.001: one-way ANOVA followed by a Bonferroni multiple comparison test.

VI.5   Discussion

Ileus, a transient impairment of gastrointestinal motility, is a common complication seen after abdominal surgery for which no single preventive means exists. As the pathogenesis of POI involves inflammation and oxidative stress, similar to I/R injury that can be counteracted with nitrite, we investigated whether nitrite can protect against POI.

The inflammatory response triggered by handling of the intestine is now generally accepted as a key event in POI (Bauer & Boeckxstaens, 2004; Boeckxstaens & de Jonge, 2009). Previous studies have shown that surgical manipulation of the small intestine activates the resident macrophages in the muscularis externa, resulting in the release of macrophage-derived cytokines, chemokines and adhesion molecules (Wehner *et al.*, 2007). This local molecular inflammatory response is followed by a cellular inflammatory response with extravasation of circulatory leukocytes -mainly neutrophils and monocytes- into the intestinal muscularis (Kalff *et al.*, 1998; Kalff *et al.*, 1999). iNOS expressed in recruited and resident leukocytes will then lead to enhanced release of NO, which directly modulates the contractile activity of the muscularis, contributing to inhibition of gastrointestinal transit and POI (Kalff *et al.*, 2000; Turler *et al.*, 2006). Similarly, when manipulating the murine intestine in this study, this was followed by (1) an increase in inflammatory cytokines and chemokines, (2) an influx of neutrophils and (3) an increase in iNOS activity in the intestinal muscularis. For the increased levels of the chemokine MCP-1 and of MPO, a marker of neutrophil influx, a certain trend was observed over time: compared with the levels observed at 6 h after IM, MCP-1 was reduced with 40 % and neutrophil influx was doubled at 24 after IM; this corresponds to the time course for MCP-1 and MPO levels reported in previous rodent studies (de Jonge *et al.*, 2003; Wehner *et al.*, 2007; Schmidt *et al.*, 2012).

The extent of intestinal dysmotility was demonstrated to be proportional to the level of intestinal inflammation (Kalff *et al.*, 1998), and prevention or reduction of the manipulation-induced inflammatory response by e.g. inhibition of macrophage function or inhibition of leukocyte infiltration by ICAM-1 blockade attenuated dysmotility (The *et al.*, 2005; Wehner *et al.*, 2007). In accordance, we showed that administration of nitrite effectively accelerated the manipulation-induced delay in gastrointestinal transit corresponding with suppression of the inflammatory response, as evidenced by a reduction in the inflammatory cytokines TNFα and IL-6 and in MCP-1 chemokine levels 24 h after IM.

Surprisingly, at 6 h after manipulation, increased levels of the chemokine MCP-1, known to play an essential role in the recruitment of monocytes to sites of injury in several inflammatory models (Lu *et al.*, 1998), were not reduced by nitrite. This is in contrast with the results for MPO, a marker for neutrophil influx: nitrite reduced the influx of neutrophils at 6 h but not at 24 h after IM although the manipulation-induced decrease of gastrointestinal transit and the associated reduced contractile activity were almost completely restored by nitrite 24 h after IM. We do not have an explanation for the time-differential influence of nitrite on monocytes and neutrophils, but some degree of reduced monocyte infiltration and of delayed neutrophil infiltration seems involved in the protective effect of nitrite.

Reactive oxygen species (ROS) might also contribute to POI. Anup *et al.* (1999) reported that surgical manipulation of the rat intestine resulted in an increase of activity of one of the few ROS generating enzyme systems, xanthine oxidase, in the enterocytes. This was associated with widened intercellular spaces and increased mucosal permeability; changes which were prevented by pretreatment of the animals with xanthine oxidase inhibitors (Anup *et al.*, 2000). In addition, our group previously reported an increase in oxidative stress in mouse small intestine after IM; reducing ROS generation (with the CO-releasing molecule CORM-3) correlated with a positive effect on postoperative intestinal transit (De Backer *et al.*, 2009). In the present study, we measured an increase in oxidative stress in the intestinal muscularis 24 h after IM; nitrite attenuated this increase, thereby helping to reduce ileus. In line with our results, antioxidant effects of nitrite were also demonstrated in an I/R model of the brain and in an ischemic model of the heart, in this way providing protection against I/R injury (Jung *et al.*, 2006; Singh *et al.*, 2012).

Nitrite will be reduced to NO under hypoxic conditions (Raat *et al.*, 2009). This concept led to studies testing nitrite as a NO donor in experimental I/R models of the heart, liver, kidney and brain (Duranski *et al.*, 2005; Jung *et al.*, 2006; Shiva *et al.*, 2007; Tripatara *et al.*, 2007); nitrite will provide NO at the time and location needed, showing its superiority against the use of classical NO donors that have yielded conflicting results in previous I/R studies, probably due to their lack of 'specificity' (Hoshida *et al.*, 1996; Zhu *et al.*, 1996; Mori *et al.*, 1998; Lozano *et al.*, 2005; Li *et al.*, 2009). The critical role for nitrite-derived NO in I/R models was apparent from the fact that the protective effects of nitrite were abolished in the presence of an NO scavenger (Duranski *et al.*, 2005; Jung *et al.*, 2006; Shiva *et al.*, 2007;

Tripatara *et al.*, 2007). In the actual study, administration of the NO scavenger carboxy-PTIO completely inhibited the nitrite-induced protection of gastrointestinal dysmotility after IM and increased or at least had a tendency to increase the associated inflammatory parameters and oxidative stress levels; this supports the idea of a mechanism requiring the reduction of nitrite to NO to protect against POI. This might be related to temporarily decreased oxygen levels in the intestine, due to repetitive momentary ischemia by IM.

Two possible mechanisms of action have been suggested in the protective effect of nitrite-derived NO against I/R injury, namely reversible inhibition of mitochondrial complex I by S-nitrosation (Shiva *et al.*, 2007; Dezfulian *et al.*, 2009) and activation of sGC by NO (Duranski *et al.*, 2005; Jung *et al.*, 2006). In correspondence with I/R studies, mitochondrial complex I activity was significantly decreased after IM, probably due to temporally decreased oxygen levels during manipulation, necessary for oxidative phosphorylation. Although nitrite-induced protection by inhibition of the electron transport might seem counterintuitive, the continuation of mitochondrial oxidative phosphorylation in the context of low O_2 generates ROS, mitochondrial calcium overload, and the release of cytochrome c (Shiva *et al.*, 2007; Chen *et al.*, 2007). Consequences to the cell include oxidative damage, opening of the mitochondrial permeability transition pore, and activation of apoptotic cascades, all favouring cell death. Pre-treatment with nitrite did not influence complex I activity in mice upon IM, indicating that nitrite protection in our POI model is not mediated via reversible inhibition of mitochondrial complex I. We therefore focused on a possible mechanism via the NO-sGC-cGMP pathway, as was before suggested in I/R models of liver and brain, in an ischemic heart model, and in a model of TNF-induced sepsis (Duranski *et al.*, 2005; Jung *et al.*, 2006; Cauwels *et al.*, 2009; Singh *et al.*, 2012). In correspondence with the findings in the ischemic heart model where cGMP levels were also measured (Singh *et al.*, 2012), IM significantly decreased cGMP levels in the intestinal muscularis but pre-treatment with nitrite increased these cGMP levels again, supporting the idea that the protective effect of nitrite in POI might be dependent on sGC, generating cGMP upon activation. The fact that both the NO-scavenger carboxy-PTIO and the sGC inhibitor ODQ brought intestinal cGMP levels in nitrite-treated manipulated mice back to those of non-treated manipulated mice, and that they prevented nitrite-induced protection on IM-induced intestinal dysmotility and nitrite-induced reduction of IM-induced inflammation and oxidative stress, corroborates that the nitrite-induced protection in the POI model must be mediated via

sGC. The exact mechanism by which the nitrite-NO-sGC-cGMP pathway exerts its protective effects in POI is still to be elucidated. In an I/R model of the brain it was demonstrated that the protective effect of nitrite-derived NO via sGC activation was dependent upon its vasodilatory effects (Jung *et al.*, 2006), while in a model of I/R injury in isolated mouse heart (Bell *et al.*, 2003), activation of sGC by an NO donor led to opening of the mitochondrial K_{ATP} channels, thereby preserving mitochondrial function by preventing mitochondrial permeability transition pore opening and cytochrome c release, normally leading to cell death (Korge *et al.*, 2002). The latter might play a role in the effect of nitrite in POI, as enterocyte mitochondrial dysfunction was shown to be associated with surgical manipulation of the intestine; this dysfunction was prevented in the presence of the NOS substrate L-arginine (Thomas *et al.*, 2001; Anup *et al.*, 2001).

In conclusion, these data indicate that an intervention with exogenous nitrite can be a valuable tool in the prevention of POI. We demonstrated that nitrite attenuates POI in mice, corresponding with a reduction in manipulation-induced inflammation and oxidative stress in the intestinal smooth muscle. Mechanistically, nitrite-induced protection is dependent on the reduction of nitrite towards NO; it is not associated with inhibition of mitochondrial complex I, but it is clearly dependent on activation of sGC.

VI.6   References

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Chapter VII

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GENERAL DISCUSSION AND CONCLUSIONS

Chapter VII   General discussion and conclusions

In this thesis, we investigated the gastrointestinal effects of sGC activation by NO-independent compounds and by NO delivery via nitrite. For this, we first assessed in genetically engineered mice what happens to gastrointestinal nitrergic signaling and motility when NO cannot activate sGC anymore. Then, we investigated the gastrointestinal effects of both an sGC stimulator and an sGC activator that were reported to stimulate/activate sGC independently of NO. Finally, we investigated the possible protective effects of nitrite-derived NO in a model of POI.

VII.1   Induction of a heme-free, NO-insensitive state of sGC has important consequences on gastric motility

In the gastrointestinal system, NO synthesized by nNOS and released from NANC neurons will target sGC and induce smooth muscle relaxation. NO contributes to the control of gastrointestinal motility, as evident from the delay in gastric emptying and intestinal transit upon NOS inhibition or in nNOS knockout mice (Huang *et al.*, 1993; Karmeli *et al.*, 1997; Mizuta *et al.*, 1999; Mashimo *et al.*, 2000; Chiba *et al.*, 2002; Fraser *et al.*, 2005). Two physiological active isoforms of sGC have been described in the gastrointestinal tract: the predominantly expressed heterodimer $sGC\alpha_1\beta_1$ and the less abundantly expressed $sGC\alpha_2\beta_1$ (Mergia *et al.*, 2003); our laboratory previously reported that, with regard to the regulation of gastrointestinal motility, $sGC\alpha_2\beta_1$ can compensate at least partially for the absence of $sGC\alpha_1\beta_1$ as gastric emptying was only mildly impaired and small intestinal transit was not influenced in $sGC\alpha_1$ knockout mice (Vanneste *et al.*, 2007; Dhaese *et al.*, 2009); these $sGC\alpha_1$ knockout mice do thus not allow to fully answer to what extent NO contributes to the control of gastrointestinal motility via the sGC pathway. In both physiologically functional isoforms of sGC, NO binds to heme that is linked to histidine 105 in the β_1 subunit (Schmidt *et al.*, 2004). Recently, $sGC\beta_1^{His105Phe}$ knockin (apo-sGC) mice were developed (Thoonen *et al.*, 2009); the resulting heme-deficient sGC isoforms retain their basal activity but can no longer be activated by NO (Wedel *et al.*, 1994). As a first goal of this thesis, the consequence

of switching sGC to the heme-deficient state on nitrergic signalling and motility in the gut was studied.

Loss of responsiveness to exogenous NO in apo-sGC mice. Due to the heme-deficient state of sGC, the relaxant responses to exogenous NO were abolished in fundic, antral, jejunal and colonic smooth muscle strips and in pyloric rings isolated from the apo-sGC mice. Correspondingly, the NO donor DETA-NO failed to increase the sGC activity in gastrointestinal tissues of apo-sGC mice. These results indicate that exogenous NO-induced relaxation is completely dependent upon activation of sGC, which contrasts to sGC-independent actions -such as activation of the small conductance Ca^{2+}-dependent K^+ channels (SK channels)- reported in previous gastrointestinal studies (Lang & Watson, 1998; Serio *et al.*, 2003). The maintained responses to respectively the cell-permeable cGMP analogue 8-Br-cGMP and VIP, that acts through adenylate cyclase coupled VIP receptors, in the apo-sGC fundic, jejunal and colonic strips, indicate that there are no compensatory mechanisms in the relaxant pathway downstream of sGC nor in the cAMP-induced relaxation of apo-sGC mice.

NANC inhibitory neurotransmission in fundus, antrum, pylorus, jejunum and colon. In accordance with previous findings from our group (Vanneste *et al.*, 2007; Dhaese *et al.*, 2009), endogenous NANC inhibitory responses in the *fundus and jejunum* showed to be sGC-dependent and fully nitrergic in nature as ODQ- and L-NAME-sensitive EFS-induced relaxations observed in WT strips were virtually abolished in apo-sGC strips. Still, EFS at 8 Hz induced relaxation -not sensitive to L-NAME- in some apo-sGC fundic strips, suggesting the emergence of a compensatory mechanism involving another neurotransmitter than NO at higher stimulation frequencies in the gastric fundus of apo-sGC mice. We speculate this neurotransmitter to be VIP, as VIP is known to be released at higher stimulation frequencies in gastric fundus (D'Amato *et al.*, 1992; Boeckxstaens *et al.*, 1992; Tonini *et al.*, 2000; Mule & Serio, 2003); in compensation for the loss of the nitrergic contribution to NANC inhibitory neurotransmission, it might already be released in apo-sGC mice at stimulation frequencies where this does not yet occur in WT mice. In the *antrum, pylorus and colon*, NANC inhibitory neurotransmission showed to be only partially nitrergic. In mouse antrum, the study of EFS under NANC conditions confirmed that NANC inhibitory neurotransmission is largely

purinergic (Gil *et al.*, 2013) with a supporting role of NO; the small relaxation seen by EFS at 8 Hz under combined nitrergic and purinergic blockade in both WT and apo-sGC antrum strips suggests contribution of an additional transmitter, besides NO and ATP. This neurotransmitter is still to be determined. In apo-sGC pylorus, EFS-induced relaxations were only decreased compared to those in WT pyloric rings and in the presence of L-NAME, some EFS-induced relaxation was maintained in WT pyloric rings, indicating that NO is not the sole inhibitory transmitter at the level of the pylorus. Correspondingly, purinergic and nitrergic co-transmission was suggested in rat pylorus (Soediono & Burnstock, 1994; Ishiguchi *et al.*, 2000). In mouse colon, it was confirmed that NO -acting via sGC- is only the principal neurotransmitter at a stimulation frequency of 1 Hz and not at higher frequencies. Our laboratory previously suggested that a redundant action of NO, acting at sGC, and another neurotransmitter, acting at SK channels, is responsible for the relaxant responses to EFS at 2 to 8 Hz in mouse distal colon (Dhaese *et al.*, 2008); this second neurotransmitter, besides NO, is probably ATP or a related purine (Gallego *et al.*, 2012). However, L-NAME plus the selective SK channel blocker apamin failed to influence the relaxant responses by EFS at 2 to 8 Hz in apo-sGC mice and only partially attenuated the relaxations to EFS at 2 to 8 Hz in WT mice, indicating that another neurotransmitter than NO and ATP must be involved in the relaxations at 2 to 8 Hz. The presence of a yet to be defined third neurotransmitter, was also reported in rat distal colon (Van Crombruggen & Lefebvre, 2004). *To summarize*, whereas NO is not the sole inhibitory transmitter at the level of the antrum, pylorus and colon, NO is the principal relaxant neurotransmitter -acting through activation of sGC- in the mouse gastric fundus and jejunum. One thus expects that inducing a heme-free NO-insensitive state of sGC in vivo, will have most influence on motility of the stomach and small intestine.

Motility in the gut. Abolished fundic nitrergic relaxation, essential for gastric accommodation (Desai *et al.*, 1991), in apo-sGC mice should per se lead to enhanced liquid gastric emptying. However, similar to what was observed in nNOS knockout mice (Mashimo *et al.*, 2000) and cGMP-dependent protein kinase (cGKI) knockout mice (Pfeifer *et al.*, 1998), liquid gastric emptying in apo-sGC mice was delayed and this was associated with a marked enlargement of the stomach and hypertrophy of the muscularis externa of the fundus. In nNOS knockout mice, this gastric smooth muscle thickening was suggested to represent work hypertrophy secondary to functional pyloric obstruction (Mashimo *et al.*, 2000).

Indeed, the muscular layer of the pylorus in apo-sGC mice was enlarged and the electrically induced relaxation in pyloric rings of apo-sGC mice was decreased; impairment of pyloric relaxation will counteract the accelerating effect of deficient fundic relaxation on gastric emptying, leading to delayed gastric emptying (Anvari *et al.*, 1998; Mashimo *et al.*, 2000). In addition to and most probably in part resulting from the disturbances in gastric emptying -delaying the gavaged liquid test solution to enter the small intestine-, apo-sGC mice showed delayed *small intestinal transit*. Similarly, impaired intestinal motility was found in several studies with NOS-inhibitors (Karmeli *et al.*, 1997; Chiba *et al.*, 2002; Fraser *et al.*, 2005), in full sGC knockouts (Groneberg *et al.*, 2011) and in cGKI knockout mice (Pfeifer *et al.*, 1998). As the endogenous NANC inhibitory responses in the jejunum showed to be sGC-dependent and fully nitrergic in nature, it seems inevitable that an imbalance between inhibitory (nitrergic) and excitatory (cholinergic) input during peristalsis develops, interrupting the coordinated interplay between ascending contractions and descending relaxations, essential for peristaltic propagation (Waterman *et al.*, 1994). The *colonic transit* was not delayed in apo-sGC mice and decreased colonic transit can thus not contribute to the increase in whole gut transit time seen in apo-sGC mice. This was not a surprising finding as in mouse distal colon, NO -acting via sGC- is only the principal neurotransmitter at a stimulation frequency of 1 Hz and not at higher frequencies.

Conclusions and future perspectives. Gastrointestinal consequences of inducing a heme-free, NO-insensitive state of sGC are most pronounced at the level of the stomach establishing a pivotal role of the activation of sGC by NO in normal gastric functioning. In addition, delayed intestinal transit was observed, indicating that nitrergic activation of sGC also plays a role in the lower gastrointestinal tract. The disturbed gut motility in the apo-sGC mice resembles that in full knockouts of sGC (Friebe *et al.*, 2007), eliminating activation of both sGC isoforms by NO, but also basal sGC activity. An advantage of the apo-sGC mice over the full knockouts is that, despite the reduced life span, these mice are viable with a median survival of 30 weeks (Thoonen, 2010). This is in sharp contrast with the short life span of the full knockout mice, where 60 % of the mice die within the first two days after birth (Friebe *et al.*, 2007). The reduced survival of the full knockouts points to the pivotal role of basal sGC activity; the produced low amounts of cGMP in apo-sGC mice seem vital for survival. The apo-sGC mouse model allows to study heme-free sGC, as seen in oxidative

stress conditions, and to investigate the effect of possible new therapies targeting sGC under oxidative stress conditions.

VII.2   NO-independent sGC stimulators/activators

Impaired nitrergic innervation of gastrointestinal smooth muscle plays a crucial role in several disorders with gastrointestinal dysmotility, such as functional dyspepsia, esophageal achalasia, infantile hypertrophic pyloric stenosis, delayed gastric emptying after vagotomy and Hirschsprung's disease (Goyal & Hirano, 1996; Takahashi, 2003). Although attempts to treat gastrointestinal motor disorders such as esophageal spasms with NO donors have been reported (Tutuian & Castell, 2006), these agents, frequently used in cardiovascular disorders associated with endothelial dysfunction, have not been applied frequently for gastrointestinal dysmotility. This might possibly be related to the well-known attenuation of their effect after long term usage due to the development of tolerance, as was indeed reported in the treatment of achalasia with organic nitrates (Robson & Wilkinson, 1946). Aging and diseases such as colitis and diabetes, that can also lead to enteric nitrergic neuronal dysfunction and motility disturbances (Mizuta *et al.*, 2000; Phillips & Powley, 2007; Zandecki *et al.*, 2008), are conditions associated with oxidative stress (Kashyap & Farrugia, 2011; Cannizzo *et al.*, 2011; Zhu & Li, 2012). ROS interfere with the NO-sGC-cGMP pathway through scavenging of NO and through oxidation of sGC towards an NO-insensitive heme-free status (Fritz *et al.*, 2011). It can be expected that enteric sGC will be driven to the oxidized/heme-free status in these conditions, making it unresponsive towards endogenous NO but also NO donors. Directly targeting sGC in an NO-independent way might thus be useful in some gastrointestinal disorders. During the last 15 years, two novel drug classes have been discovered that seem to address these problems: the heme-dependent sGC stimulators and the heme-independent sGC activators. *sGC stimulators* are capable of directly stimulating the reduced form of sGC, acting in synergy with NO, but they can also stimulate reduced sGC independently of NO, allowing to circumvent conditions with decreased endogenous generation of NO (Stasch & Hobbs, 2009). *sGC activators* preferably activate the oxidized/heme-free enzyme (Schmidt *et al.*, 2009); these drugs should thus target the enzyme more extensively in pathological conditions associated with

oxidative stress. In this thesis, the influence of the sGC stimulator BAY 41-2272 and of the sGC activator cinaciguat on gastrointestinal motility was studied.

VII.2.1 The NO-independent heme-dependent sGC stimulator BAY 41-2272 induces gastrointestinal relaxation also by inhibiting Ca^{2+} entry

Research in cardiovascular, urogenital and respiratory smooth muscle preparations showed that the effect of BAY 41-2272 is related to activation of sGC but additional mechanisms of action were proposed (Bawankule *et al.*, 2005; Teixeira *et al.*, 2006a; Bau *et al.*, 2010; Toque *et al.*, 2010). Limited information is available with regard to the effect of BAY 41-2272 on gastrointestinal smooth muscle activity, so we intended to investigate the effect and mechanism of action of BAY 41-2272 in gastric fundus and colon, with special attention for the role of sGC, the interaction with NO and possible additional cGMP-independent mechanisms.

Mechanism of action. BAY 41-2272 induced concentration-dependent relaxation in both fundus and colon. The discrepant effect of ODQ on the increase in cGMP (abolished) and relaxation (partially reduced) by BAY 41-2272 showed that relaxation by BAY 41-2272 in gastrointestinal tissue is only partially dependent on *sGC activation* (Fig. VII.1). An interaction with small (SK channels) or large (BK channels) conductance Ca^{2+}-activated K^+ channels or with the Na^+/K^+-pump was excluded (Fig. VII.1), as the responses to BAY 41-2272 were not influenced by apamin, charybdotoxin or ouabain. In contrast, a cGMP-independent mechanism that was proposed to contribute to relaxation by BAY 41-2272 in vascular, urinary and tracheal smooth muscle (Teixeira *et al.*, 2006a; Bau *et al.*, 2010; Toque *et al.*, 2010), i.e. *inhibition of extracellular calcium entry* (Fig. VII.1), also contributes to the relaxant effect of BAY 41-2272 in mouse gastric fundus and colon; in these tissues, under conditions of depletion of the intracellular calcium stores and of high K^+ depolarization, BAY 41-2272 concentration-dependently inhibited contractions evoked with extracellular calcium, and this inhibitory effect of BAY 41-2272 was not prevented by the sGC inhibitor ODQ.
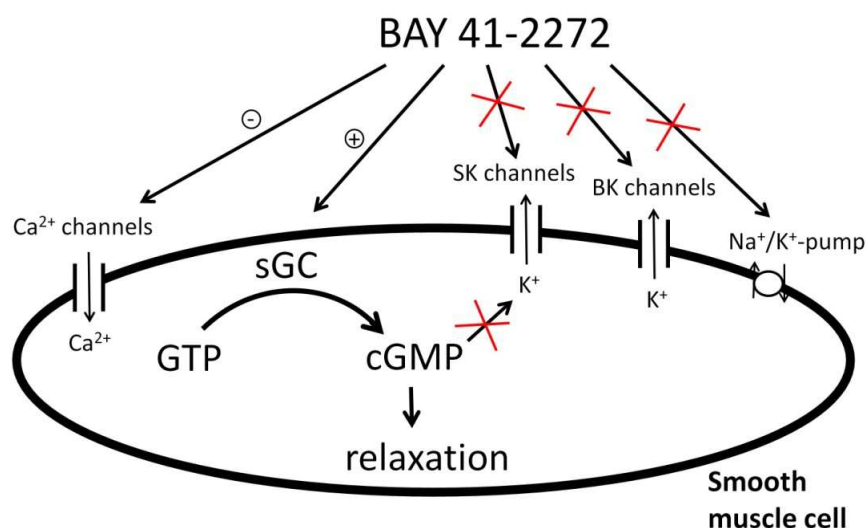
Fig. VII.1 Summary of the pathways that were investigated to elucidate the mechanism of action of the sGC stimulator BAY 41-2272 in mouse gastric fundus and colon. BAY 41-2272-induced relaxation in mouse gastric fundus and colon is only partially sGC-dependent; interaction with the small (SK channels) and large (BK channels) conductance Ca^{2+}-dependent K^+ channels or the Na^+/K^+-pump is excluded, but an sGC-cGMP-independent mechanism involving Ca^{2+} entry blockade is demonstrated.

Contribution of endogenous NO. Endogenous NO seems partially involved in the relaxant effect of BAY 41-2272 in vascular, cavernosal and tracheal tissue (Teixeira *et al.*, 2006a; Teixeira *et al.*, 2006b; Teixeira *et al.*, 2007; Toque *et al.*, 2010). In the gastric fundus however, endogenous NO does not contribute to the relaxation by BAY 41-2272, as L-NAME does not influence it; but in colon, L-NAME had an inhibitory effect on the BAY 41-2272-induced relaxation. Experiments were performed to investigate whether BAY 41-2272 might indeed be able to induce release of NO from nitrergic nerves in colonic strips. The generation of NO in nitrergic nerves is mediated by the Ca^{2+}/calmodulin controlled neuronal NO-synthase, which is normally activated through Ca^{2+} influx in response to an action potential. The N-type Ca^{2+} channel blocker ω-conotoxin and the voltage-gated Na^+ channel blocker tetrodotoxin, that reduce or even abolish EFS-induced nitrergic relaxations in different preparations (Kasakov *et al.*, 1995; Amato *et al.*, 2009), did not influence the relaxations by BAY 41-2272; this corroborates that BAY 41-2272 is not capable of releasing NO from colonic nitrergic neurons. We believe that the reducing effect of L-NAME on the relaxations by BAY 41-2272 in the colon is related to the fact that BAY 41-2272 sensitizes the colon to the effect of tonically released NO. L-NAME will prevent the tonic release of NO so that the part of the relaxation by BAY 41-2272 due to sensitization is gone.

Synergy with endogenous NO. In several tissues, a synergistic interaction of BAY 41-2272 with NO donors or endogenous NO released by EFS was reported (Teixeira *et al.*, 2006a; Teixeira *et al.*, 2006b; Teixeira *et al.*, 2007; Toque *et al.*, 2010). In mouse gastric fundus, EFS-induced relaxations, that were fully nitrergic as abolished by NO synthesis inhibition, were enhanced by BAY 41-2272, illustrating synergy between BAY 41-2272 and endogenous NO. Although the area above the curve of the EFS-induced relaxations in mouse colon was not significantly changed by BAY 41-2272, an effect of BAY 41-2272 was obvious as the rebound contractions -probably tachykininergic in origin (Serio *et al.*, 1998)- occurring at the end of the stimulation train were abolished. We mentioned before that NANC relaxations in mouse distal colon are dependent on NO and another neurotransmitter, acting at SK channels. As the SK channel blocker apamin did not influence the relaxations by BAY 41-2272, the potentiation of the colonic transmitter acting at SK channels, by BAY 41-2272 can be excluded; as also no tachykinin receptor antagonistic effects were reported for BAY 41-2272, the abolishment of the rebound contractions is most probably due to sustained enhancement of the effect of endogenous NO, thereby preventing the breakthrough of the rebound contractions.

Conclusions and future perspectives. The sGC stimulator BAY 41-2272 was now also shown to stimulate sGC in gastrointestinal tissue. In gastric fundus, endogenous NO does not contribute to the relaxation induced by BAY 41-2272, while it does in colon; in both tissues, synergy between BAY 41-2272 and endogenous NO was demonstrated. However, at least one additional cGMP-independent mechanism involving inhibition of Ca^{2+} entry is involved in the relaxing effect of BAY 41-2272. One should thus be careful when thinking of applying BAY 41-2272 as an sGC stimulator for long time usage in gastrointestinal disorders associated with decreased endogenous generation of NO, as Ca^{2+} entry is a process essential for many cell functions, including proliferation, maturation, contraction, and immunity. In addition, the sensitivity of gastrointestinal tissues seems low as relaxation by BAY 41-2272 only started at 0.3 µM in the colon and at 1 µM in the fundus, while relaxation already clearly occurred at 0.01 µM in vascular and tracheal tissue (Bawankule *et al.*, 2005; Toque *et al.*, 2010). This implies that for BAY 41-2272 to have an in vivo gastrointestinal effect, higher doses might be necessary than those used in cardiovascular and respiratory studies (Boerrigter *et al.*, 2003; Evgenov *et al.*, 2004); a possible side effect might then involve

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systemic vasodilatation, causing a sudden decrease in blood pressure. Although doses of sGC stimulators for cardiovascular, respiratory or urogenital disorders might be lower than those required for gastrointestinal disorders, one should still consider possible gastrointestinal side effects. Indeed, another sGC stimulator known as BAY 63-2521 (riociguat or Adempas®) was recently approved by the FDA for treatment of pulmonary hypertension; amongst the most common side effects, nausea, vomiting and diarrhea were reported. BAY 41-2272 however, will never make it this far. BAY 41-2272 displayed low metabolic stability and low oral bioavailability in rats, and showed a strong inhibition as well as induction of metabolizing cytochrome P450 (CYP) enzymes (Mittendorf *et al.*, 2009). Inhibition or induction of CYP enzymes bears the risk of changing the exposure of a second, coadministered drug. Therefore, BAYER decided not to further develop the compound (Follmann *et al.*, 2013).

VII.2.2 The NO- and heme-independent sGC activator cinaciguat is not able to systematically induce relaxation throughout the gastrointestinal tract

In both in vitro and in vivo cardiovascular studies, the sGC activator cinaciguat showed to be more efficient in conditions associated with oxidative stress (Stasch *et al.*, 2006; Erdmann *et al.*, 2012; Salloum *et al.*, 2012; Korkmaz *et al.*, 2012). To assess whether cinaciguat might offer a solution for gastrointestinal conditions associated with oxidative stress, we first investigated the influence of cinaciguat on in vitro muscle tone of gastrointestinal tissues in both WT and apo-sGC mice -which, as mentioned before, can be considered as a model for gut dysmotility under oxidative stress-, and then examined whether cinaciguat could restore the delayed gastric emptying seen in apo-sGC mice.

In vitro effect on gastrointestinal tissues. In *fundus and colon* strips of both WT and apo-sGC mice, cinaciguat induced concentration-dependent relaxations. Although the protein levels of sGCα_1 and sGCβ_1 were lower in apo-sGC fundus and colon, the relaxant responses to cinaciguat in fundus and colon strips were similar or even higher in apo-sGC mice, suggesting that cinaciguat is more efficient when sGC is in the heme-free condition. The data obtained with the sGC inhibitor ODQ, that is thought to inhibit sGC by oxidation of its heme group (Schrammel *et al.*, 1996), corroborate this conclusion: after incubation with

ODQ, the relaxant responses to cinaciguat were increased in WT fundus and colon strips. The efficacy of cinaciguat to activate sGC in the oxidized/heme-free condition was also confirmed by measurement of cGMP: in the absence of ODQ, cGMP levels were only increased by cinaciguat in fundus and colon strips of apo-sGC mice but not of WT mice, but in the presence of ODQ, cinaciguat produced a pronounced increase in cGMP levels in fundus and colon strips of WT mice. When comparing these results in WT fundus and colon to the corresponding functional results and cGMP levels obtained with BAY 41-2272, the difference between sGC activators and sGC stimulators is perfectly illustrated. Whereas BAY 41-2272-induced relaxations were reduced by ODQ, cinaciguat-induced relaxations were increased in the presence of ODQ, and whereas the BAY 41-2272-induced increase in cGMP levels was completely abolished by ODQ, the sGC inhibitor markedly increased cGMP levels of cinaciguat-incubated fundus and colon strips. Correlating with the 'theory' that was based on results in vascular studies (Stasch & Hobbs, 2009; Schmidt *et al.*, 2009), we now also showed in gastrointestinal tissue that the sGC activator cinaciguat preferably activates the oxidized/heme-free sGC enzyme, whereas the sGC stimulator BAY 41-2272 is only capable of activating sGC in its reduced form. In *antrum* strips of WT or apo-sGC mice, cinaciguat was not able to induce relaxations; in *pyloric rings* of WT mice, cinaciguat did not systematically induce relaxations, while in apo-sGC pyloric rings it could not induce relaxation at all. We mentioned before that endogenous NANC relaxation in the antrum and pylorus is partially purinergic; one might thus expect lower amounts of sGC to be present and to be activated, possibly explaining the lack of effect of cinaciguat in these tissues. However, sGC levels in antrum and pylorus were similar to those in other gastrointestinal tissues. Additionally, also for the colon a large part of the inhibitory response is attributed to purinergic signaling (Van Crombruggen & Lefebvre, 2004; Dhaese *et al.*, 2008; Gil *et al.*, 2013), and cinaciguat caused relaxation in this tissue. Still more intriguing, cinaciguat was also not able to induce a relaxation in WT and apo-sGC *jejunum* strips while endogenous NANC inhibitory responses in the jejunum are sGC-dependent and fully nitrergic in nature (Dhaese *et al.*, 2009). Cinaciguat is expected to be more efficient when sGC is in the oxidized condition (Stasch *et al.*, 2006), but oxidative stress levels in fundus and colon were not more pronounced than those in antrum and jejunum. We have thus no explanation for the lack of effect of cinaciguat in WT and apo-sGC antrum and jejunum, nor for its ineffectiveness in the pylorus of apo-sGC mice.

In vivo effect on gastric emptying. Cinaciguat could not restore the delayed gastric emptying seen in apo-sGC mice. This might be explained by the inability of cinaciguat to relax the pylorus of apo-sGC mice; Mashimo *et al.* (2000) similarly suggested that functional pyloric obstruction, due to a loss in nitrergic neurotransmission, contributes to a great extent to the delay in gastric emptying seen in nNOS-KO mice. After intravenous injection of 300 or 100 µg/kg cinaciguat, a clearcut delay in gastric emptying was noted in the WT mice. As cinaciguat is known to cause a decrease in blood pressure at these doses (Vandendriessche *et al.*, 2013), one might expect decreased tissue perfusion and consequently also reduced gastric blood flow, possibly leading to gastric dysmotility and delayed gastric emptying. Cinaciguat was also reported to cause a decrease in blood pressure in the apo-sGC mice (Thoonen, 2010), but as gastric emptying is already severely delayed in these mice, the hypotension will not lead to additional delay.

Conclusions and future perspectives. The sGC activator cinaciguat relaxes the fundus and colon efficiently when sGC is in the heme-free condition, corresponding to its preferential activation of heme-free sGC in vascular tissue. But it is not able to systematically induce relaxation throughout the gastrointestinal tract as it is unable to relax the antrum, pylorus and jejunum of NO-insensitive, heme-free apo-sGC mice (Fig. VII.2). More importantly, it did not improve gastric emptying in apo-sGC mice and thus not seems a viable solution for the treatment of gastrointestinal conditions associated with oxidative stress. In addition, the action of cinaciguat upon clinical application already seems associated with a serious side effect; in agreement with the hypotension reported in mice, when going through phase II clinical trials testing the effect of cinaciguat in patients with acute decompensated heart failure, the clinical development had to be stopped as even at low doses, cinaciguat caused a serious decrease in blood pressure (Gheorghiade *et al.*, 2012; Erdmann *et al.*, 2012).

MODEL FOR GUT DYSMOTILITY UNDER OXIDATIVE STRESS

NO

Oxidized / heme-free sGC

Apo-sGC mice
(sGCβ$_1^{His105Phe}$ knockin)

↓

delayed gastric emptying
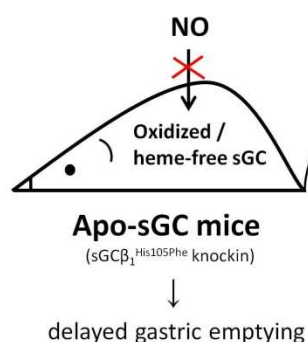
CINACIGUAT?
(NO- and heme-independent sGC activator)
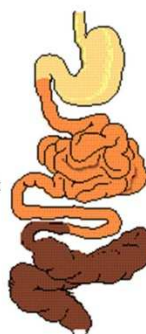
In vitro studies

Relaxation to cinaciguat in

	WT	Apo-sGC mice
FUNDUS:	+	++
ANTRUM:	-	-
PYLORUS:	+/-	-
JEJUNUM:	-	-
COLON:	+	++

In vivo studies

Cinaciguat did not improve the delayed gastric emptying seen in apo-GC mice

Fig. VII.2 Schematic overview of the results obtained in our study with the sGC activator cinaciguat. Cinaciguat relaxes the fundus and colon efficiently when sGC is in the heme-free condition, but it is unable to relax the antrum, pylorus and jejunum of NO-insensitive, heme-free apo-sGC mice. The non-effect of cinaciguat in pylorus explains its inability to improve the delayed gastric emptying in apo-sGC mice.

VII.3 Nitrite reduces postoperative ileus via sGC activation

Next to the sGC stimulators/activators as alternatives to classic NO pharmacotherapy, we also looked at the inorganic anion nitrite (NO_2^-), which has been reported to be a source of NO under hypoxic conditions (Lundberg *et al.*, 2008). Postoperative ileus (POI), a transient impairment of gastrointestinal motility, is a common complication seen after abdominal surgery for which no single preventive means exist. As the pathogenesis of POI involves inflammation and oxidative stress (Bauer & Boeckxstaens, 2004; De Backer *et al.*, 2009), similar to ischemia/reperfusion (I/R) injury (Kalogeris *et al.*, 2012) that can be counteracted with nitrite in an NO-dependent way (Duranski *et al.*, 2005; Shiva *et al.*, 2007; Dezfulian *et al.*, 2007; Raat *et al.*, 2009), we investigated whether nitrite can protect against POI.

Effect of nitrite in POI. As reported before (Schmidt *et al.*, 2012), manipulation of the intestine increased inflammatory cytokines and chemokines, caused an influx of neutrophils and an increase in iNOS activity in the intestinal muscularis. In addition, we found that surgical handling of the intestine increased reactive oxygen species (ROS), corroborating previous reports (Anup *et al.*, 1999; De Backer *et al.*, 2009). Pre-treatment with exogenous nitrite attenuated POI in mice, as it almost completely prevented the delay in intestinal

transit following intestinal manipulation and as it reduced the associated inflammatory response and oxidative stress increase in the intestinal smooth muscle.

Mechanism of action. The protective effects of nitrite were abolished in the presence of the NO scavenger carboxy-PTIO, supporting the idea of a mechanism that requires the reduction of nitrite to NO to protect against POI. Two possible mechanisms of action have been proposed for the protective effect of nitrite-derived NO against I/R injury: reversible inhibition of mitochondrial complex I by S-nitrosation and activation of sGC (Duranski *et al.*, 2005; Jung *et al.*, 2006; Shiva *et al.*, 2007). Nitrite-induced protection in the POI model was shown not to be mediated via reversible inhibition of mitochondrial complex I; therefore, a possible mechanism via the NO-sGC-cGMP pathway was studied. After surgical handling of the intestine, cGMP levels were significantly decreased in the intestinal muscularis, but pre-treatment with nitrite increased these cGMP levels, supporting the idea that the protective effect of nitrite in POI might be dependent on sGC, generating cGMP upon activation. Both the NO-scavenger carboxy-PTIO and the sGC inhibitor ODQ brought intestinal cGMP levels in nitrite-treated manipulated mice back to those of non-treated manipulated mice; they prevented nitrite-induced protection on manipulation-induced intestinal dysmotility and nitrite-induced reduction of manipulation-induced inflammation and oxidative stress, corroborating that the nitrite-induced protection in the POI model must be mediated via sGC.

Conclusions and future perspectives. An sGC-dependent protective effect of nitrite-derived NO was demonstrated in a mouse model of POI. Whether this can be translated to humans will need clinical investigation. Long-term intravenous infusion of sodium nitrite proved to be safe in healthy volunteers (48 h; Pluta *et al.*, 2011) and in patients with subarachnoid hemorrhage (14 days; Oldfield *et al.*, 2013). A principal concern about the use of sodium nitrite in humans might be the formation of carcinogenic nitrosamines (formed from the reaction of nitrite with secondary amines); in studies performed in the 1950s-1980s, nitrosamines were reported to be associated with malignancies, most notably gastric cancer. However, the European Food Safety Authority stated in their 2008 survey that the evidence that nitrite might be associated with increased cancer risk is ambiguous and that it mainly involves high doses of nitrite or chronic exposure (Alexander et al., 2008). In a study

on hepatic ischemia/reperfusion, the dose of nitrite used in the mouse model of POI was shown to induce hepatic nitrite levels lower than those present in the liver before induction of ischemia (Duranski et al., 2005); there is thus no induction of supraphysiological nitrite concentrations. Additionally, a single injection of this dose is sufficient to obtain the protective effect in POI. We therefore believe that testing of sodium nitrite for POI in humans can be considered without major safety issues. There is some evidence of intestinal microvascular dysfunction during abdominal surgery with hypoperfusion of the microvasculature by a combination of factors such as surgical intervention, anesthesia and therapeutic interventions (Vellinga et al., 2010; Urbanavicius et al., 2011), thus promoting the conditions necessary to reduce nitrite towards NO (low O_2). In the operative setting, nitrite will thus be reduced to NO at the time and location needed, which probably provides an advantage over the classical NO donors. The fact that NO donors lack this property might explain the conflicting results obtained with classical NO donors in I/R studies, showing beneficial, no or even detrimental effects (Hoshida et al., 1996; Zhu et al., 1996; Mori et al., 1998; Lozano et al., 2005; Li et al., 2009). The exact mechanism by which the nitrite-NO-sGC-cGMP pathway exerts its protective effects in POI downstairs of cGMP is still to be elucidated. In an I/R model of the brain it was demonstrated that the protective effect of nitrite-derived NO via sGC activation was dependent upon its vasodilatory effects (Jung et al., 2006). It might therefore be interesting to look at the microcirculation in the small intestine e.g. via intravital microscopy, to assess whether nitrite similarly causes vasodilatation of the intestinal microcirculation. Another possibility would be to investigate the contribution of mitochondrial K_{ATP} channels in the protective effect of nitrite, as in a model of I/R injury in isolated mouse heart (Bell et al., 2003) activation of sGC by an NO donor led to opening of the mitochondrial K_{ATP} channels thereby preserving mitochondrial function (Korge et al., 2002); also enterocyte mitochondrial dysfunction was shown to be associated with surgical manipulation of the intestine and this dysfunction was shown to be prevented in the presence of the NOS substrate L-arginine (Thomas et al., 2001; Anup et al., 2001). A mitochondrial selective K_{ATP} channel antagonist such as 5-hydroxydecanoate (5-HD) could be used to assess if the nitrite-induced protective effects in the POI model are related to mitochondrial K_{ATP} channel opening. As the protective effect of nitrite-derived NO correlates with attenuation of the inflammatory response, it might also be interesting to try to elucidate the underlying molecular mechanisms. NO donors showed to inhibit

transcription factor NF-κB (Matthews *et al.*, 1996; Shin *et al.*, 1996; Bogdan, 2001) and this was associated with a variety of anti-inflammatory effects (Phillips *et al.*, 2009). A possibility in the POI model thus seems that nitrite-derived NO inhibits transcription factor NF-κB.

VII.4   General conclusion

We started this thesis by establishing the importance of nitrergic activation of sGC in the regulation of gastrointestinal motility by using genetically manipulated mice, the so called apo-sGC mice, which express heme-free sGC that has basal activity, but cannot be stimulated by NO anymore. Gastrointestinal consequences of inducing a heme-free state of sGC were most pronounced at the level of the stomach, showing delayed gastric emptying. Subsequently, the gastrointestinal effects of the NO-independent heme-dependent sGC stimulator BAY 41-2272 and the NO- and heme-independent stimulator cinaciguat were investigated. BAY 41-2272 induced relaxation in both fundus and colon, but it was shown to only partially depend upon activation of sGC; a cGMP-independent mechanism involving inhibition of Ca^{2+} entry was demonstrated. Cinaciguat was not able to systematically induce relaxation throughout the gastrointestinal tract; it relaxed the fundus and colon efficiently, preferably when sGC is in the heme-free condition, but it was unable to relax the antrum, pylorus and jejunum of NO-insensitive, heme-free apo-sGC mice. Its inability to relax the pylorus in apo-sGC explains its inability to restore the delayed gastric emptying seen in apo-sGC mice. In a final study, nitrite-derived NO was shown to protect against postoperative ileus through a mechanism dependent on activation of sGC.

VII.5   References

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Chapter VIII

SUMMARY

Chapter VIII   Summary

The gastrointestinal tract exerts a variety of physiological processes, such as motility, digestion, secretion, absorption and elimination. The different gastrointestinal functions are regulated by both hormonal and neuronal control mechanisms. Neuronal control mechanisms, mediated via the extrinsic nervous system and the enteric nervous system (ENS), play an important role in the regulation of gastrointestinal motility. The myenteric plexus, a part of the ENS that lies between the longitudinal and circular smooth muscle layers, controls the contraction and relaxation of gastrointestinal smooth muscle through the release of respectively excitatory and inhibitory neurotransmitters. Besides excitatory cholinergic and inhibitory adrenergic pathways, also non-adrenergic non-cholinergic (NANC) pathways are involved in the neuronal control of gastrointestinal motility. Substance P is accepted as the primary excitatory NANC neurotransmitter and nitric oxide (NO), adenosine triphosphate (ATP) and vasoactive intestinal peptide (VIP) mediate the inhibitory NANC responses; NO being the primary inhibitory NANC neurotransmitter. The principal intracellular receptor for NO as smooth muscle cell relaxant, is soluble guanylate cyclase (sGC). In both physiologically functional isoforms of sGC (the predominantly expressed $sGC\alpha_1\beta_1$ and the less abundantly expressed $sGC\alpha_2\beta_1$), NO binds to heme that is linked to histidine 105 in the β_1 subunit. This will generate cGMP, that mediates smooth muscle cell relaxation.

Given the important role of NO in gastrointestinal motility, it is not surprising to find that in several disorders with gastrointestinal dysmotility, such as functional dyspepsia, esophageal achalasia, infantile hypertrophic pyloric stenosis, delayed gastric emptying after vagotomy and Hirschsprung's disease, impaired nitrergic innervation of gastrointestinal smooth muscle plays a crucial role. NO donors, frequently used in cardiovascular disorders associated with endothelial dysfynction, have not been applied frequently in gastrointestinal disorders, possibly because of the well-known attenuation of their effect after long term usage due to the development of tolerance. Aging and diseases such as colitis and diabetes, can also lead to enteric nitrergic neuronal dysfunction and motility disturbances. These conditions are associated with oxidative stress; ROS interfere with the

NO-sGC-cGMP pathway through scavenging of NO and oxidation of sGC towards an NO-insensitive heme-free status. It can be expected that enteric sGC will be driven to the oxidized/heme-free status in these conditions, making it unresponsive towards endogenous NO but also NO donors.

We investigated **(Chapter III)** the consequences on gastrointestinal nitrergic signalling and motility of inducing a heme-free status of sGC, as induced by oxidative stress. This was done by using sGCβ$_1$^{His105Phe} knockin (apo-sGC) mice. The histidine 105 residue of the β$_1$ subunit is a crucial amino acid for the binding of the heme group to sGC; the resulting heme-deficient sGC isoforms retain their basal activity but can no longer be activated by NO. Correspondingly, nitrergic signalling was completely abolished in the apo-sGC mice and it was confirmed that whereas NO is not the sole inhibitory transmitter at the level of the antrum, pylorus and colon, NO is the principal relaxant neurotransmitter -acting through activation of sGC- in the mouse gastric fundus and jejunum. Inducing a heme-free, NO-insensitive state of sGC should thus have most influence on in vivo motility of the stomach and small intestine. Indeed, the apo-sGC mice showed delayed gastric emptying and intestinal transit, while the colonic transit was not influenced. The apo-sGC mice also showed a marked enlargement of the stomach and hypertrophy of the muscularis externa of the fundus, suggested to represent work hypertrophy secondary to deficient pyloric relaxation.

During the last 15 years, two novel drug classes have been discovered that seem to address the problem of reduced availability of NO and/or oxidation of sGC towards the NO-insensitive, heme-free status: the heme-dependent sGC stimulators and the heme-independent sGC activators. sGC stimulators are capable of directly stimulating the reduced form of sGC, acting in synergy with NO, but they can also stimulate reduced sGC independently of NO, allowing to circumvent conditions with decreased endogenous generation of NO. sGC activators preferably activate the oxidized/heme-free enzyme; these drugs should thus target the enzyme more extensively in pathological conditions associated with oxidative stress. Reports on the gastrointestinal effects of sGC stimulators/activators are limited.

We first investigated the gastrointestinal effects of the sGC stimulator BAY 41-2272 **(Chapter IV)**. Its effect and mechanism of action was studied in mouse gastric fundus and colon. BAY 41-2272 induced concentration-dependent relaxation in both tissues and

increased cGMP levels. The sGC inhibitor ODQ totally inhibited this BAY 41-2272-induced increase of cGMP, but only partially reduced the corresponding relaxation, suggesting that the mechanism of action of BAY 41-2272 is indeed dependent on sGC activation, but also that additional cGMP-independent mechanisms must be involved. Responses to BAY 41-2272 were not significantly influenced by apamin, charybdotoxin or ouabain, excluding interaction with small and large conductance Ca^{2+}-activated K^+ channels and with the Na^+/K^+-pump. Under depletion of intracellular calcium, $CaCl_2$-induced contractions were significantly reduced by BAY 41-2272 in an ODQ-insensitive way. This suggested a cGMP-independent mechanism for BAY 41-2272, involving inhibition of Ca^{2+} entry. In colon, but not in fundus, the NO synthase inhibitor L-NAME caused a significant decrease in BAY 41-2272-induced responses; we believe that BAY 41-2272 sensitizes the colon to the effect of tonically released NO, an effect that is abolished in the presence of L-NAME. In both fundus and colon, BAY 41-2272 can enhance the effect of endogenous NO; NANC relaxation by electrical field stimulation in the fundus was increased in the presence of BAY 41-2272, while in colon, rebound contraction at the end of the stimulation train was no longer visible. This suggests synergy of BAY 41-2272 with endogenously released NO.

As sGC activators were reported to preferably activate the oxidized/heme-free enzyme, the influence of the sGC activator cinaciguat **(Chapter V)** was assessed on muscle tone in the different parts of the gastrointestinal tract i.e. fundus, antrum, pylorus, jejunum and colon, and on gastric emptying in both wild type (WT) and apo-sGC mice. Although the protein levels of the sGC subunits were lower in gastrointestinal tissues of apo-sGC mice, cinaciguat induced concentration-dependent relaxations in apo-sGC fundus and colon to a similar or greater extent than in WT mice, suggesting that cinaciguat is more efficient when sGC is in the heme-free condition. The data obtained with ODQ -inhibiting sGC by oxidizing the heme group- corroborate this conclusion: after incubation with ODQ, cinaciguat-induced relaxations were greatly increased in WT fundus and colon. The efficacy of cinaciguat to activate sGC in the oxidized/heme-free condition was also confirmed by measurement of cGMP: in the absence of ODQ, cGMP levels were only increased by cinaciguat in fundus and colon of apo-sGC mice but not of WT mice, but in the presence of ODQ, cinaciguat produced a pronounced increase in cGMP levels in fundus and colon of WT mice. The preferential activation of heme-free sGC by cinaciguat was now thus also confirmed in gastrointestinal tissue. However, in apo-sGC antrum, pylorus and jejunum, cinaciguat was not able to induce

relaxations; we do not have an explanation for this inconsistency of cinaciguat in the gastrointestinal tract. The non-effect of cinaciguat in the pylorus explains the inability of cinaciguat to improve the delayed gastric emptying in apo-sGC mice.

Next to the sGC stimulators/activators as alternatives to classic NO pharmacotherapy, the inorganic anion nitrite (NO_2^-), which has been reported to be a source of NO under hypoxic conditions, can be considered. We studied nitrite in a model of postoperative ileus **(Chapter VI)**. Exogenous administration of nitrite showed to protect the heart, liver, kidney and brain from ischemia/reperfusion injury; a possible mechanism of action is activation of sGC by NO, produced from nitrite under hypoxic conditions. Postoperative ileus is a transient impairment of gastrointestinal motility commonly seen after abdominal surgery. The surgical handling of the bowel during abdominal surgery leads to muscular inflammation and oxidative stress, two factors known to also play a major role in ischemia/reperfusion injury. The aim of our last study was therefore to investigate whether nitrite also has a protective, possibly sGC dependent, effect in postoperative ileus. Corresponding to previous studies on ileus, we found that manipulation of the intestine increased inflammatory cytokines and chemokines, caused an influx of neutrophils, an increase in iNOS activity and increased ROS in the intestinal muscularis. Pre-treatment with nitrite markedly improved the delay in intestinal transit and reduced the associated inflammatory response and oxidative stress in the intestinal smooth muscle. This protective effect of nitrite was shown to require the reduction of nitrite to NO, as in the presence of the NO scavenger carboxy-PTIO, the protective effects of nitrite were completely abolished. Moreover, the involvement of the NO-sGC-cGMP pathway was demonstrated (1) as the manipulation-induced decrease in cGMP levels in the intestinal muscularis was again increased after pre-treatment with nitrite, (2) as both the NO-scavenger carboxy-PTIO and the sGC inhibitor ODQ brought intestinal cGMP levels in nitrite-treated manipulated mice back to the cGMP levels of non-treated manipulated mice, and (3) as both carboxy-PTIO and ODQ prevented nitrite-induced protection on manipulation-induced intestinal dysmotility and nitrite-induced reduction of manipulation-induced inflammation and oxidative stress.

Conclusions. The pivotal role of the activation of sGC by NO in normal gastric motility and small intestinal transit was established, and apo-sGC mice, which express heme-free sGC, were shown to be a model of gut dysmotility under oxidative stress. The NO-

independent heme-dependent sGC stimulator BAY 41-2272 was shown to exert its relaxing effect in mouse gastric fundus and colon partially through a cGMP-dependent mechanism and at least one additional cGMP-independent mechanism involving Ca^{2+} entry blockade. The NO- and heme-independent activator cinaciguat relaxed the mouse fundus and colon efficiently when sGC is in the heme-free condition, corresponding to its preferential activation of heme-free sGC in vascular tissue, but it was unable to relax the antrum, pylorus and jejunum of NO-insensitive, heme-free apo-sGC mice. The non-effect of cinaciguat in pylorus explains its inability to improve the delayed gastric emptying in apo-sGC mice. Finally, an sGC-dependent protective effect of nitrite-derived NO was demonstrated in a model of postoperative ileus.

Chapter IX

SAMENVATTING

Chapter IX   Samenvatting

Het gastro-intestinale stelsel is betrokken in verschillende fysiologische processen zoals motiliteit, vertering, secretie, absorptie en eliminatie. De verschillende gastro-intestinale functies worden geregeld door zowel hormonale als neuronale controlemechanismen. Neuronale controlemechanismen, gemedieerd via zowel het extrinsieke als het intrinsieke of enterische zenuwstelsel, spelen een belangrijke rol in de regeling van de gastro-intestinale motiliteit. De myenterische plexus, een onderdeel van het enterische zenuwstelsel die gelegen is tussen de longitudinale en circulaire gladde spierlagen, regelt de contractie en relaxatie van de gastro-intestinale gladde spieren door het vrijstellen van respectievelijk contractiele en relaxerende neurotransmitters. Naast contractiele cholinerge en relaxerende adrenerge neurotransmitters, zijn ook zogenaamde niet-adrenerge niet-cholinerge (NANC) neurotransmitters betrokken in de controle van de gastro-intestinale motiliteit. Substantie P is aanvaard als de primaire contractiele NANC neurotransmitter en stikstofmonoxide (NO), adenosine trifosfaat (ATP) en vaso-actief intestinaal peptide (VIP) vertegenwoordigen de relaxerende NANC neurotransmitters, waarbij NO de belangrijkste is. NO veroorzaakt relaxatie van gladde spiercellen door activering van oplosbaar guanylaatcyclase (sGC), zijn belangrijkste intracellulaire receptor. In beide fysiologisch functionele isovormen van sGC (sGC$\alpha_1\beta_1$ en sGC$\alpha_2\beta_1$, waarvan sGC$\alpha_1\beta_1$ de meest voorkomende is in het gastro-intestinale stelsel), zal NO binden aan de heemgroep van sGC die is gekoppeld aan histidine 105 van de β_1 subeenheid. Dit zal leiden tot de generatie van cGMP, welke zal zorgen voor de relaxatie van de gladde spiercellen.

Gezien het belang van NO in de gastro-intestinale motiliteit, is het niet onverwacht dat in verschillende aandoeningen met verminderde gastro-intestinale motiliteit, zoals functionele dyspepsie, achalasie van de slokdarm, infantiele hypertrofische pylorusstenose, vertraagde maaglediging na vagotomie en de ziekte van Hirschsprung, verminderde nitrerge innervatie van de gastro-intestinale gladde spier een cruciale rol speelt. NO-donoren, veelvuldig gebruikt bij cardiovasculaire aandoeningen geassocieerd met endotheliale dysfunctie, worden niet vaak aangewend voor gastro-intestinale stoornissen, mogelijk vanwege de bekende vermindering van het effect bij langdurig gebruik door de ontwikkeling

van tolerantie. Veroudering en ziektes zoals colitis en diabetes kunnen ook leiden tot enterische nitrerge neuronale dysfunctie en stoornissen in de motiliteit. Deze aandoeningen zijn geassocieerd met oxidatieve stress; reactieve zuurstofspecies (RZS) interfereren met de signaalweg van NO-sGC-cGMP door het wegvangen van NO en door oxidatie van sGC naar een NO-ongevoelige, heem-vrije status. Men kan dus verwachten dat sGC naar de geoxideerde/heem-vrije status zal worden gedreven bij deze aandoeningen, waardoor het ongevoelig wordt voor endogeen NO maar ook voor NO-donoren.

We onderzochten **(Hoofdstuk III)** de gevolgen van het induceren van een heem-vrije status van sGC, zoals veroorzaakt door oxidatieve stress, op de gastro-intestinale nitrerge signalisatie en de gastro-intestinale motiliteit. Dit werd gedaan met behulp van $sGC\beta_1^{His105Phe}$ knock in (apo-sGC) muizen. Histidine 105 van de β_1 subeenheid is een cruciaal aminozuur voor de binding van de heemgroep van sGC; de resulterende heem-vrije isovormen van sGC behouden hun basale activiteit, maar kunnen niet meer worden geactiveerd door NO. Bijgevolg was de nitrerge relaxatie dan ook volledig opgeheven in de apo-sGC muizen en werd bevestigd dat NO niet de enige relaxerende neurotransmitter is in antrum, pylorus en colon, maar wel in fundus en jejunum, en dat de desbetreffende nitrerge relaxatie volledig berust op activatie van sGC. Het induceren van een heem-vrije, NO-ongevoelige toestand van sGC zal dus het meest invloed hebben op de in vivo motiliteit van de maag en de dunne darm. Inderdaad, de apo-sGC muizen vertoonden een vertraagde maaglediging en vertraagde transit in de dunne darm, terwijl de transit in de dikke darm niet was beïnvloed. De apo-sGC muizen vertoonden ook een duidelijke vergroting van de maag en hypertrofie van de buitenste spierlaag van de fundus, voor dewelke gesuggereerd werd dat dit "werk" hypertrofie voorstelt, secundair aan gebrekkige relaxatie van de pylorus.

Gedurende de laatste 15 jaar werden twee nieuwe klasses geneesmiddelen ontdekt die het probleem van de verminderde beschikbaarheid van NO en/of oxidatie van sGC naar de NO-ongevoelige, heem-vrije status lijken aan te pakken: de heem-afhankelijke sGC stimulatoren en de heem-onafhankelijk sGC activatoren. sGC stimulatoren kunnen de gereduceerde vorm van sGC stimuleren, in synergie met NO, maar ook onafhankelijk van NO, waardoor ze een aanwinst zouden kunnen zijn bij aandoeningen geassocieerd met verminderde endogene productie van NO. sGC activatoren activeren bij voorkeur de geoxideerde/heem-vrije vorm van het sGC enzym; deze zouden dus sGC efficiënter moeten

activeren bij pathologische aandoeningen geassocieerd met oxidatieve stress. Informatie over de gastro-intestinale effecten van sGC stimulatoren en activatoren is echter beperkt.

We onderzochten eerst de gastro-intestinale effecten van de sGC stimulator BAY 41-2272 **(Hoofdstuk IV)**. Het effect en het werkingsmechanisme van BAY 41-2272 werd bestudeerd in de maagfundus en het colon van de muis. BAY 41-2272 induceerde concentratie-afhankelijke relaxatie in beide weefsels en verhoogde het gehalte van cGMP. De sGC inhibitor ODQ verhinderde deze BAY 41-2272-geïnduceerde toename van cGMP volledig, maar reduceerde slechts gedeeltelijk de overeenkomstige relaxatie, wat suggereert dat het werkingsmechanisme van BAY 41-2272 inderdaad afhankelijk is van sGC activering, maar ook dat aanvullende cGMP-onafhankelijke mechanismen betrokken moeten zijn. De BAY 41-2272-geïnduceerde relaxaties werden niet significant beïnvloed door apamine, charybdotoxine of ouabaïne, wat interactie uitsluit met de Ca^{2+}-geactiveerde K^+-kanalen met lage (SK kanalen) en hoge geleiding (BK kanalen) en met de Na^+/K^+-pomp. Onder depletie van intracellulair calcium, werden $CaCl_2$-geïnduceerde contracties aanzienlijk verminderd door BAY 41-2272 op een ODQ-ongevoelige manier. Dit suggereert een cGMP-onafhankelijk mechanisme voor BAY 41-2272, waarbij inhibitie van de Ca^{2+} influx betrokken is. In het colon, maar niet in de fundus, veroorzaakte de NO-synthase inhibitor L-NAME een significante reductie van de BAY 41-2272-geïnduceerde responsen; wij menen dat BAY 41-2272 het colon sensibiliseert voor het effect van tonisch vrijgegeven NO, een effect dat wordt tenietgedaan in aanwezigheid van L-NAME. In zowel fundus als colon kan BAY 41-2272 het effect van endogeen NO versterken; NANC relaxatie door elektrische veldstimulatie in de fundus was verhoogd in aanwezigheid van BAY 41-2272, terwijl in het colon de contractie aan het einde van de stimulaties niet meer zichtbaar was. Dit suggereert synergie van BAY 41-2272 met endogeen vrijgesteld NO.

Aangezien voor sGC activatoren werd gemeld dat deze bij voorkeur het geoxideerde/heem-vrije sGC enzym activeren, bestudeerden we de invloed van de sGC activator cinaciguat **(Hoofdstuk V)** op de spiertonus in verschillende delen van het gastro-intestinale stelsel, meer bepaald in fundus, antrum, pylorus, jejunum en colon, en op de maaglediging van zowel wild type (WT) als apo-sGC muizen. Hoewel de eiwitgehaltes van de sGC subeenheden lager waren in de gastro-intestinale weefsels van apo-sGC muizen, induceerde cinaciguat in apo-sGC muizen concentratie-afhankelijke relaxaties in fundus en colon van een vergelijkbare of zelfs grotere orde dan deze in WT muizen, wat suggereert dat

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cinaciguat efficiënter is wanneer sGC zich in heem-vrije toestand bevindt. De data verkregen met ODQ -dat sGC inhibeert door oxidatie van zijn heemgroep- bevestigen deze conclusie: na incubatie met ODQ waren de cinaciguat-geïnduceerde relaxaties sterk toegenomen in fundus en colon van WT muizen. Het vermogen van cinaciguat om sGC in de geoxideerde/heem-vrije toestand te activeren werd ook bevestigd via de metingen van cGMP: in afwezigheid van ODQ werden de gehaltes van cGMP enkel verhoogd door cinaciguat in fundus en colon van apo-sGC muizen en niet in die van WT muizen, terwijl in de aanwezigheid van ODQ er ook een uitgesproken toename van cGMP-gehaltes veroorzaakt werd door cinaciguat in fundus en colon van WT muizen. De preferentiële activering van heem-vrij sGC door cinaciguat werd nu dus ook bevestigd in gastro-intestinale weefsels. In antrum, pylorus en jejunum van apo-sGC muizen, kon cinaciguat evenwel geen relaxaties induceren; we hebben geen verklaring voor deze inconsistentie van cinaciguat in het gastro-intestinale stelsel. Het niet relaxeren van de pylorus door cinaciguat verklaart wel het onvermogen van cinaciguat om de vertraagde maaglediging in apo-sGC muizen te herstellen.

Naast de sGC stimulatoren/activatoren als alternatief voor de klassieke farmacotherapie met NO, kan ook het anorganische anion nitriet (NO_2^-), waarvan gemeld is dat het een bron van NO is onder hypoxische omstandigheden, in aanmerking worden genomen. We bestudeerden nitriet in een model van postoperatieve ileus **(Hoofdstuk VI)**. Exogene toediening van nitriet toonde reeds aan hart, lever, nieren en hersenen te beschermen tegen schade door ischemie/reperfusie; een mogelijk mechanisme is de activering van sGC door NO, gegenereerd uit nitriet onder hypoxische omstandigheden. Postoperatieve ileus is een tijdelijke vermindering van de gastro-intestinale motiliteit, vaak voorkomend na abdominale operaties. De chirurgische manipulatie van de darm tijdens abdominale operaties leidt tot inflammatie en oxidatieve stress, twee factoren die ook een belangrijke rol spelen bij schade door ischemie/reperfusie. Het doel van onze laatste studie was dan ook om te onderzoeken of nitriet ook een beschermend, eventueel sGC-afhankelijk, effect heeft bij postoperatieve ileus. Overeenkomstig met vorige studies over ileus, vonden we dat manipulatie van de darm leidde tot een verhoging van de inflammatoire cytokines en chemokines, een instroom van neutrofielen, een toename van de iNOS activiteit en verhoogde RZS in de intestinale muscularis. Voorbehandeling met nitriet verbeterde de vertraging in de darmtransit aanzienlijk en verminderde de bijhorende inflammatoire

respons en oxidatieve stress in de intestinale gladde spieren. Voor dit beschermende effect van nitriet bleek de reductie van nitriet naar NO nodig, aangezien in de aanwezigheid van carboxy-PTIO, dat NO "wegvangt", de beschermende effecten van nitriet volledig werden tenietgedaan. Bovendien werd de betrokkenheid van NO en activatie van sGC aangetoond (1) aangezien het gehalte van cGMP in de intestinale muscularis dat daalde door manipulatie, terug was toegenomen na voorbehandeling met nitriet, (2) aangezien zowel carboxy-PTIO als de sGC inhibitor ODQ de intestinale gehaltes van cGMP in nitriet-behandelde gemanipuleerde muizen terug naar de gehaltes van cGMP van niet-behandelde gemanipuleerde muizen bracht, en (3) aangezien zowel carboxy-PTIO als ODQ de nitriet-geïnduceerde bescherming tegen manipulatie-geïnduceerde intestinale dysmotiliteit en de nitriet-geïnduceerde daling van manipulatie-geïnduceerde inflammatie en oxidatieve stress verhinderden.

Conclusies. De centrale rol voor de activering van sGC door NO in de motiliteit van de maag en in de transit van de dunne darm werd bevestigd, en apo-sGC muizen, die over heem-vrij sGC beschikken, kunnen als een model voor verstoorde gastro-intestinale motiliteit onder oxidatieve stress voorgesteld worden. Voor de NO-onafhankelijke heem-afhankelijke sGC stimulator BAY 41-2272 werd aangetoond dat het relaxerend effect in maagfundus en colon gedeeltelijk via een cGMP-afhankelijk mechanisme verloopt en ten minste via één extra cGMP-onafhankelijk mechanisme, waarbij Ca^{2+} influx geblokkeerd wordt. De NO- en heem-onafhankelijke activator cinaciguat relaxeerde de fundus en het colon efficiënt wanneer sGC in de heem-vrije toestand was, wat overeenkomt met zijn preferentiële activering van heem-vrij sGC in vaatweefsel, maar het was niet in staat om antrum, pylorus en jejunum van NO-ongevoelige, heem-vrije apo-sGC muizen te relaxeren. Het ontbreken van een relaxerend effect in de pylorus verklaart het onvermogen van cinaciguat om de vertraagde maaglediging in apo-sGC muizen te herstellen. Ten slotte werd een sGC-afhankelijk beschermend effect voor nitriet aangetoond in postoperatieve ileus, waarbij nitriet eerst gereduceerd moet worden tot NO teneinde een beschermend effect te vertonen.

Chapter X

DANKWOORD

Dankwoord

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