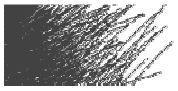

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GEZONDHEIDSWETENSCHAPPEN

Heme Oxygenase in the GI Tract: Physiological Role and Therapeutic Possibilities

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List of Abbreviations

AC	adenylyl cyclase
ADHP	10-acetyl-3,7-dihydroxy-phenoxazine
Amp	amplitude
ANOVA	analysis of variance
5-ASA	5-aminosalicylic acid
ATP	adenosine triphosphate
AUC	area under the curve
BK_{Ca} channel	large-conductance Ca^{2+}-activated K^+ channel
BR	bilirubin
BV	biliverdin
BVR	biliverdin reductase
CCD	charge coupled device
cGK	cGMP-dependent protein kinase
cGMP	guanosine 3', 5'-cyclic monophosphate
CM	colonic manipulation
CO	carbon monoxide
COHb	carboxyhemoglobin
CO_{inf}	carbon monoxide infusion
CO-RMs	carbon monoxide-releasing molecules
COX	cyclooxygenase
CrMP	chromium mesoporphyrin
3D	three dimensional
DMSO	dimethyl sulfoxide
DSS	dextran sulphate sodium
EDTA	ethylenediaminetetraacetic acid
EFS	electrical field stimulation
Egr-1	early growth response-1
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
FAM	6-carboxylfluorescein
FD70	fluorescein-labelled dextran (70 kDa)
GC	geometric center
GI	gastrointestinal
HETAB	hexadecyl-trimethylammonium bromide
HNE	4-hydroxy-2,3-nonenal
5-HT	serotonin
HO	heme oxygenase
HRP	horseradish peroxidase
IAS	internal anal sphincter
ICAM-1	intercellular adhesion molecule-1
IK_{Ca} channel	intermediate-conductance Ca^{2+}-activated K^+ channel
IM	intestinal manipulation
IP	intraperitoneal, intraperitoneally

I/R	ischemia/reperfusion
ICC	interstitial cells of Cajal
IL	interleukin
iNOS	inducible nitric oxide synthase
JNK	Jun N-terminal kinase
K_{Ca} channel	Ca^{2+}-activated K^+ channel
KO	knockout
L-NAME	N^{ω}-nitro-L-arginine methyl ester
LPS	lipopolysaccharide
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MDA	malondialdehyde
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MTD	maximum tolerated dose
MW	molecular weight
NADPH	nicotinamide adenine dinucleotide phosphate
NANC	non-adrenergic non-chloninergic
nNOS	neuronal nitrc oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
OD	optical density
ODQ	1H-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one
PBS	phosphate buffered saline
pGC	particulate guanylyl cyclase
PGF2α	prostaglandin F2α
PKG	protein kinase G
PMA	phorbol 12-myristate 13-acetate
POI	postoperative ileus
PPAR	peroxisome proliferator-activated receptor
RMP	resting membrane potential
ROS	reactive oxygen species
RT-PCR	reverse transcriptase –polymerase chain reaction
RuCl	ruthenium chloride
SEM	standard error of the mean
sGC	soluble guanylyl cyclase
SK_{Ca} channel	small conductance Ca^{2+}-activated K^+ channel
SOD	superoxide dismutase
TAMRA	6-carboxyltetramethylrhodamine
TMB	3,3',5,5'-tetramethylbenzidine
TNBS	trinitrobenzene sulfonic acid
TNF-α	tumor necrosis factor-α
UV	ultraviolet
WB	Western Blotting
WT	wild-type
XO	xanthine oxidase
YC-1	1-benzyl-3-(5'-hydroxymethyl-2'-furyl)-indazol

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Chapter I

LITERATURE SURVEY

Chapter I Literature Survey

I.1 Role of NO in the regulation of gastrointestinal motility

The involvement of the free radical nitric oxide (NO) in relaxant gastrointestinal non-adrenergic non-cholinergic (NANC) neurotransmission became evident in the late 1980s and the beginning of the 1990s. First, it was shown that in rat and mouse anococcygeus, NANC relaxations could be reduced by inhibiting the synthesis of NO (Li & Rand, 1989; Gibson *et al.*, 1990). Evidence further accumulated as immunohistochemistry showed that the NO synthesizing enzyme was present in neural cell bodies and fibres of the rat myenteric plexus (Bredt *et al.*, 1990). Boeckxstaens et al. then showed that the canine ileocolonic junction and rat gastric fundus were able to produce a NO-like compound (Boeckxstaens *et al.*, 1991a; Boeckxstaens *et al.*, 1991b). From that moment on, a vast amount of publications consolidated the importance of NO in the regulation of gastrointestinal motility.

I.1.1 Synthesis of NO

NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). The formation of NO starts with the oxydation of L-arginine leading to the formation of the unstable reaction intermediate N^G-hydroxy-L-arginine. The incorporation of a second oxygen atom then results in the production of NO and L-citrulline; L-citrulline can then be reconverted to L-arginine by the incorporation of an amino group. In order to exert its action, the NOS enzyme requires the presence of two co-substrates i.e., NADPH and O_2 and several co-factors such as flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH_4) and haem protoporphyrin IX (Fig I.1). NO synthase can be regulated by a mechanism of negative feed-back mediated by NO that inhibits NOS by a haem-dependent oxidation of L-arginine.

The NOS enzymes exist in three isoforms of which two can be classified as constitutive enzymes (i.e., NOS-1 or neuronal NOS and NOS-3 or endothelial NOS) and one as an inducible enzyme (NOS-2 or inducible NOS). The constitutive enzymes are activated by a rise in cytosolic Ca^{2+} concentration leading to binding of calmodulin to NOS and subsequent activation. NOS-1 and NOS-3 produce small quantities (nanomolar) of NO in an intermittent way over short periods of time to regulate homeostasis. The inducible enzyme NOS-2 is

activated independently of Ca^{2+} because it has calmodulin tightly bound at all times; it produces large amounts (micromolar) of NO over longer periods of time but after a time lag as it needs a certain time to be expressed in response to immunological stimuli, after which it plays a role in immune defence (Martin *et al.*, 2001). However, NOS-2 is reported to be also constitutively expressed in the gastrointestinal tract where it should be involved in maintaining normal peristalsis (Mancinelli *et al.*, 2001). Besides differences in catalytic properties and biological role, these NOS-isoforms differ in primary structure, inhibitor sensitivity, post-translational modifications and also tissue distribution. As expected NOS-1 is located predominantly in neurons of the central and peripheral nervous system although it may also be present in other cell types such as skeletal and gastrointestinal smooth muscle cells (Nakane *et al.*, 1993; Mule *et al.*, 2001). The other constitutive enzyme NOS-3 appears primarily in endothelial cells, platelets and mesangial renal cells although it is suggested to be also expressed in gastrointestinal smooth muscle cells (Teng *et al.*, 1998; Mule *et al.*, 2001). The expression pattern of NOS-2 is more wide-spread over a variety of mainly immune-related cell types but the most important ones are the macrophages (Xie *et al.*, 1992). In the gastrointestinal tract, NOS-1 localised in the enteric NANC neurons is the most important isoform contributing to inhibitory NANC neurotransmission (Chakder *et al.*, 1997).

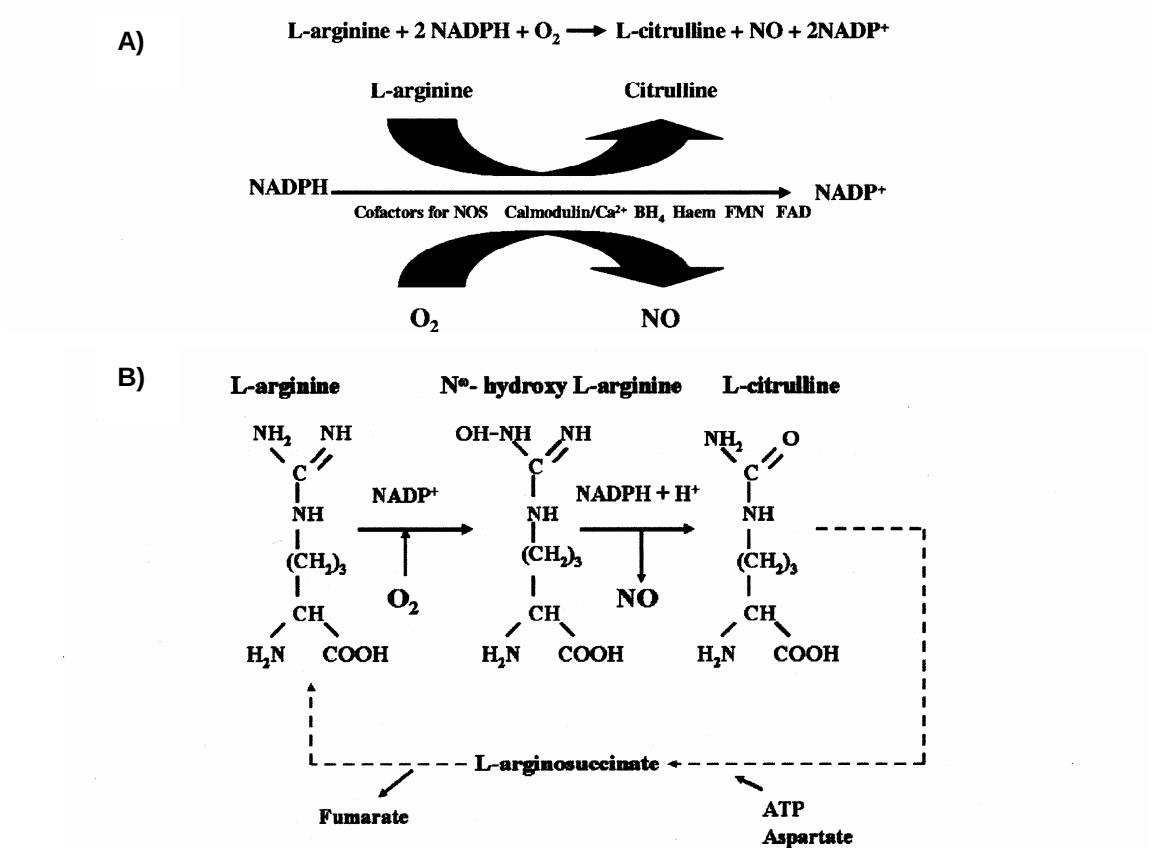

Figure I.1

Biosynthesis of nitric oxide from L-arginine. A) Requirement for a large number of cofactors. B) The formation of L-citrulline and its recycling to L-arginine (Bruckdorfer, 2005).

I.1.2 Role of NO in gastrointestinal motility under physiological conditions

The gastrointestinal canal consists of the oesophagus, the stomach, the small intestine, the colon and the rectum and its main physiological function is to provide nutrients to the body. The major physiological processes needed to obtain this function are secretion, digestion, absorption and motility. The structure of the gastrointestinal wall is similar throughout the gastrointestinal tract, consisting of an inner mucosal layer, the submucosa, the muscle layer and the serosa.

Gastrointestinal motility is achieved by coordinated activity of the smooth muscle cells (SMCs) of the muscle layer regulated by hormonal and neuronal control mechanisms. The SMCs are organised in an outer longitudinal muscle layer and an inner circular muscle layer although regional differences in muscle structure exist f.i. the longitudinal muscle layer is prominent in the distal stomach while the circular muscle layer is prominently present in all gastric regions (Hasler, 2003b). In some species (f.i., humans and pig), also an oblique muscle layer is present in the stomach. This layer is the most inner and least complete muscle layer. It is present on the lesser curvature near the cardia and is continuous with the gastroesophageal junction (Hasler, 2003b). Hormonal regulation occurs via 2 mechanisms i.e., paracrine and endocrine regulation. Paracrine regulation indicates the process by which a chemical released from a sensing cell, diffuses through the interstitial space to influence the function of neighbouring cells. Endocrine regulation is defined as the process by which a sensing cell responds to a stimulus by releasing its contents into the circulation to act on a distant target cell. Neuronal control is regulated by the extrinsic nervous system and the intrinsic nervous system. The extrinsic nervous system consists of a parasympathetic and a sympathetic component while the intrinsic nervous system is embedded in the gastrointestinal wall. The efferent parasympathetic nerves release acetylcholine (Ach) and exert excitatory effects on enteric neurons and SMCs of the intestine. The efferent sympathetic nerves release noradrenaline and reduce gastrointestinal motility in non-sphincter regions by inhibiting the release of Ach from excitatory cholinergic neurons via presynaptic α_2-receptors on cholinergic nerve endings (Raybould *et al.*, 2003). The sympathetic nerves exert excitatory effects in most sphincter regions except f.i. the pylorus where sympathetic stimulation inhibits motility (Allescher *et al.*, 1988).

The regulating neurons of the intrinsic nervous system i.e., the enteric nervous system (ENS) are organised into two plexuses: the myenteric plexus of Auerbach and the submucosal plexus of Meissner. The myenteric plexus of Auerbach is localised between the longitudinal and the circular muscle layers and mainly controls gastrointestinal motility while the submucosal plexus of Meissner is positioned in the submucosal connective tissue and is

involved in secretion and absorption although it also innervates the muscularis mucosae in the small and large intestine (Furness *et al.*, 2003; Fig I.2). The neurons in both plexuses can be classified into the following groups: 1) motor neurons, 2) interneurons, 3) intrinsic primary afferent neurons and 4) secretoneurons. The longitudinal and circular muscle layers are innervated by the motor neurons that can be further classified into excitatory and inhibitory neurons depending on their effect on the SMCs. The primary neurotransmitter released by the excitatory motor neurons is Ach that acts on muscarinic receptors; however also tachykinins eliciting contraction are released. The neurotransmitters involved in the effects of the inhibitory motor neurons are amongst others vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide (PACAP), adenosine triphosphate (ATP), carbon monoxide (CO) and most importantly NO; these neurotransmitters yield relaxation of the gastrointestinal tract. The interneurons supply communication between the afferent neurons and the motor neurons. They are directed orally (ascending) or anally (descending) forming multisynaptic pathways and are involved in the control of secretomotor and local motility reflexes. Important in the activation of reflexes are the intrinsic primary afferent neurons, these neurons project to the mucosa where they become excited by mechanical and chemical stimulation. They enable reflex responses without connection to the central nervous system and are involved in the control of peristalsis, secretion and blood flow. Finally, the secretoneurons regulate secretion by synapsing with endocrine and exocrine cells (Furness, 2000).

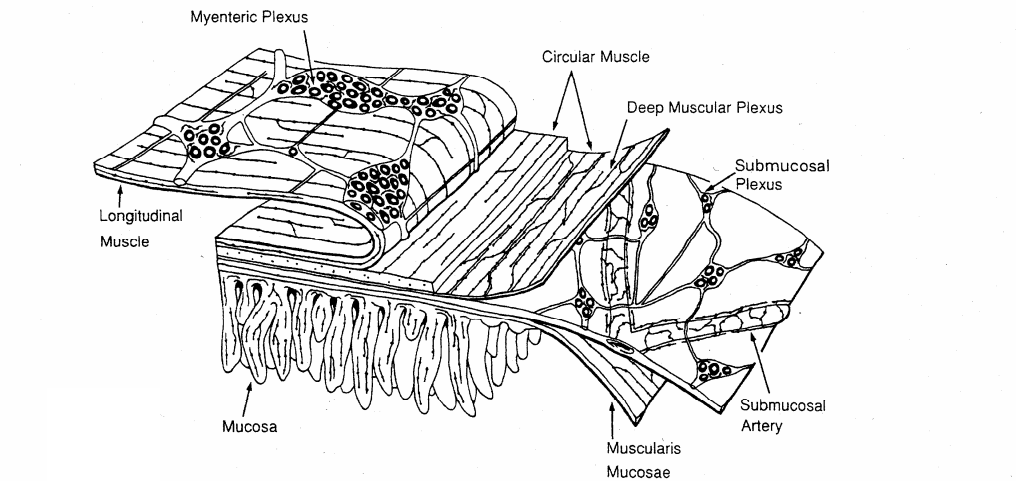

Figure I.2

Schematic representation of the enteric plexuses.

In between the cells of the ENS and the SMCs, a network of interstitial cells of Cajal (ICC) is localised. These cells serve as electrical pacemaker and active propagation pathways for slow waves and are suggested to play a role in reception, conduction and transduction of inputs from the ENS towards the smooth muscle cells. The motor neurotransmission in the

gut does not occur through synaptic structures between neurons and SMC, but rather via synaptic-like contacts that exist between varicose nerve terminals and intramuscular ICCs coupled to SMCs via gap junctions. The ICC plays an important role in cholinergic and nitrergic neurotransmission (Ward *et al.*, 2004).

I.1.2.1 Stomach

Gastric motor activity differs between the fasting and the fed state. In the fed state, food enters the accommodating stomach as large particles containing macromolecules. The large particles are broken down into absorbable material by grinding and mixing the food with various secretions. The stomach content is then emptied into the duodenum at a controlled rate for further digestion. Gastric emptying is regulated by coordinated actions of the different stomach regions i.e., proximal stomach, distal stomach and pylorus and by feed-back control from the small intestine (Hasler, 2003b).

The proximal stomach consists of the cardia, fundus and proximal body and mediates the ability of the stomach to receive and store large amounts of food without major changes in the intraluminal pressure. The proximal stomach is in a state of continuous partial contraction; this tone alters under influence of two neuronally mediated reflexes i.e., receptive relaxation and gastric accommodation. Receptive relaxation is the reduction of proximal gastric tone that occurs with the act of swallowing or mechanical stimulation of the pharynx or oesophagus. Gastric accommodation is the relaxation of the proximal stomach in response to gastric distension mediated by gastric mechanoreceptors. Because of the accommodation reflex, 80 % of food ingested is initially retained in the proximal stomach. Both reflexes are mediated by vagovagal reflex arcs through the nucleus tractus solitarii and have a nitrergic component. Later, tone in the proximal stomach starts to increase, which promotes grinding of solids by propelling them towards the distal stomach (Hasler, 2003b).

The distal stomach consists of the distal body and the antrum and mediates grinding and trituration of solid food and gastric emptying of solids. After eating, the distal stomach displays a fed contractile pattern of variable intensity and duration that produces phasic contractions that begin in the midstomach and move towards the pylorus, a small part of the chyme is emptied into the duodenum while the residual fraction is retropelled back into the stomach (Hasler, 2003b).

The last part the digesting food has to pass before entering the duodenum is the pylorus. This barrier to gastric emptying acts as a mechanical stricture to the passage of large particles. This sphincteric tissue possesses unique smooth muscle and neural properties that distinguish it from the surrounding structures. The resistance to flow is provided by tonic and phasic pyloric motor activity where inhibitory neural input is predominant as it relaxes during electrical stimulation (Hasler, 2003b).

Under fasting conditions, a stereotypical pattern that clears the stomach of undigested debris, mucus and sloughed epithelial cells is seen i.e., the migrating motor complex (MMC) also called the gastrointestinal housekeeper. The MMC consists of four phases: phase I is a period of motor quiescence and is followed by phase II that exhibits increasing but irregular phasic contractions. Phase III consists of a short period of lumenally occlusive, rhythmic contractions induced by the release of the hormone motilin from the duodenal mucosa and is followed by a phase IV transition period displaying irregular contractions before returning to phase I.

NO plays an essential role in the physiological regulation of gastric motility as the most important consequences of knocking out NOS-1 were observed at the level of the stomach. In these NOS-1 knock-out (KO) mice, the most important abnormality is an enlarged stomach with hypertrophy of the pyloric sphincter and the circular muscle layer (Huang *et al.*, 1993). Smooth muscle relaxation induced by electrical field stimulation (EFS) in NANC conditions is reduced in gastric fundus strips of these mice (Dick *et al.*, 2002) and gastric emptying of liquids and solids is delayed (Mashimo *et al.*, 2000). Mashimo et al. (2000) suggested that impairment of the pyloric relaxation counteracting the stimulating effect of decreased gastric accommodation capacity could explain the delayed gastric emptying. Indeed, also pyloric motor relaxation was reported to be mediated mainly by NO (Allescher *et al.*, 1992; Sun *et al.*, 1998). These findings are supported by reports of a delay of solid and liquid gastric emptying induced by NOS inhibitors in rats (Plourde *et al.*, 1994) and dogs (Orihata & Sarna, 1994; Tanaka *et al.*, 2005). In contrast, NOS inhibitors were also reported to increase gastric emptying of liquids in guinea pigs (Desai *et al.*, 1991) and rats (Takahashi & Owyang, 1997). Studies in humans also reported that NOS inhibition enhances the rate of gastric contractions and significantly increases liquid gastric emptying (Konturek *et al.*, 1999) while it had no effect on solid gastric emptying (Hirsch *et al.*, 2000). The enhancing or non-effect of NOS inhibitors on gastric emptying found in the latter studies might be related to the fact that in these studies NOS inhibition had no or less important consequences on pyloric relaxation. The loss of gastric accommodation, pushing ingested food towards the distal stomach, can then indeed lead to an increase in or a non-effect on gastric emptying.

I.1.2.2 Small intestine

The small intestine is divided into three functional regions i.e., duodenum, jejunum and ileum. The duodenum receives the grinded food from the stomach, mixes it with secretions of pancreas and gallbladder to digest the chyme and starts the absorption of small particles. The function of this region is highly regulated by feed-back mechanisms. The major functions of the jejunum are digestion and absorption of nutrients and a significant portion of the water and ions from exogenous and endogenous sources. Also the ileum is a site of

absorption; more specific agents such as cobalamin (vitamin B12) are here absorbed. The small intestine also releases hormones that have important functions in the gut (Hasler, 2003a).

The small intestine exhibits two motor patterns during the fed state i.e., peristalsis and segmentation. Peristalsis is characterized by a wave of relaxation followed by a contraction and is the primary mechanism by which content is moved distally in the digestive period. Segmentation is the process by which rings of contraction develop at uniform intervals, dividing the lumen into segments, which results in a mixing of the intestinal contents with secretions and moving of the small particles towards the mucosa to enhance absorption.

During the fasting state, the small intestine exhibits the same prominent cyclic motor pattern as seen in the stomach. This MMC is an intestinal housekeeper that propels undigested food residue and sloughed enterocytes from the proximal gut into the colon. The small intestinal MMC consists of the same four phases as the gastric MMC and is regulated by extrinsic and intrinsic input. 89% of the MMCs start in the stomach or the proximal duodenum although the first MMC after eating starts distally to the duodenum. This mechanism of starting MMC after the fed phase is poorly understood (Hasler, 2003a).

In NOS-1 KO mice, EFS-induced smooth muscle relaxations are reduced in jejunum strips suggesting an important role for NO in jejunal endogenous inhibitory neurotransmission (Xue *et al.*, 2000). The use of NOS inhibitors to study the role of NO in NANC relaxations of the human small intestine further demonstrated the importance of NO in this tissue (Stark *et al.*, 1993). The role of NO in small intestinal transit was studied by the use of NOS inhibitors in the small intestine of rats (Karmeli *et al.*, 1997), dogs (Chiba *et al.*, 2002) and humans (Fraser *et al.*, 2005). These studies observed a delay in intestinal transit probably because the regular peristalsis was replaced by spastic contractions followed by scarce relaxations. However, besides NO, also other putative NANC neurotransmitters such as ATP, VIP and PACAP are reported to be involved in small intestinal NANC neurotransmission (Matsuyama *et al.*, 2002; Matsuda *et al.*, 2004; Waseda *et al.*, 2005). Each of these individual neurotransmitters exhibits variable effects not only between species and species strains, but also between gut regions and muscle layers (Balsiger *et al.*, 2000; Okishio *et al.*, 2000).

I.1.3 Role of NO in gastrointestinal motility under pathophysiological conditions

Intestinal pathological conditions such as inflammatory bowel diseases (IBD; Crohn's disease and ulcerative colitis) and postoperative ileus (POI) are accompanied by changes in intestinal smooth muscle activity due to intestinal inflammation (Vermillion *et al.*, 1993; Kalff *et al.*, 1998). Various studies based on animal models, indicated that overproduction of NO

evoked by an increased expression of NOS-2 may be involved in gastrointestinal inflammation and that it may have a pathogenic role in IBD (Shah *et al.*, 2004). However, it almost immediately became apparent that NO might have a dichotomous function as both a beneficial and detrimental molecule (McCafferty *et al.*, 1997; Kalff *et al.*, 2000; Thomas *et al.*, 2001). It was hypothesized that both constitutive NOS-isoforms (NOS-1 and NOS-3) were critical for normal physiology while induction of NOS-2 was harmful. Indeed, constitutive NO was found to be protective by maintaining an intact mucosal barrier while NOS-2 induced NO can cause activation of neutrophils and increased production of the highly toxic peroxynitrite radical (Kubes, 1992). However more recent studies have challenged this paradigm as they provide evidence that constitutive NOS-1 plays a role in the development of early dysmotility caused by intestinal manipulation (De Winter *et al.*, 1997a) and NOS-2 might have protective effects by f.i. reducing oxidative stress, leukocyte infiltration and mast cell reactivity (McCafferty *et al.*, 1997).

In this work we will concentrate on POI. POI is defined as a reversible transient impairment of gastrointestinal motility occurring almost universally after surgical interventions especially those involving the opening of the abdominal cavity; however POI can also occur after extra-abdominal surgery (Person & Wexner, 2006). Some authors further classify surgery-induced ileus into postoperative ileus and postoperative paralytic ileus. POI is then defined as the uncomplicated ileus resolving spontaneously within 2 to 3 days while postoperative paralytic ileus is defined as that form of ileus lasting more than 3 days after surgery (Livingston & Passaro, Jr., 1990).

I.1.3.1 Frequency, symptoms and duration of postoperative ileus

During this common and almost obligatory period of gastrointestinal quiescence, motility of the gastrointestinal tract is marked by disorganized electrical activity leading to lack of coordinated propulsion. As a consequence, POI is clinically characterized by abdominal distension, lack of bowel sounds, accumulation of gas and fluids in the bowel and a delayed passage of gas and stool (Person & Wexner, 2006). The clinical picture differs between patients and varies between patients who remain essentially asymptomatic while others display serious symptoms including nausea, vomiting and stomach cramps leading to major postoperative discomfort, a prolonged recovery and hospital stay and an increased morbidity. Determination of the end of POI is somewhat controversial as varying end points are used such as the assessment of electrical activity, focusing on either the return of the MMC or a qualitative change in MMC patterns, occurrence of bowel sounds, the passage of flatus and the passage of stool. Because no objective variable has been found to assess the resolution of POI, the most adequate solution is to assess the patient as a whole (Holte & Kehlet, 2000). POI normally resolves within approximately 3 days, but it may last from 2 days or less

after laparoscopic surgery to 1 week or more after major laparotomies. Because the different anatomical parts of the gastrointestinal tract differ in electrical and mechanical activity, they recover differently from POI. The electrical rhythm and motor activity in the stomach recovers on average within 24 to 48 hours. In the small intestine, early motor activity is disorganized and rarely results in a normal coordinated MMC. This state may last up to 3 to 4 days, after which the small bowel resumes normal motor activity. In the colon, electrical activity is initially characterized by disorganized bursts, which only produce a coordinated, propagated motor response by postoperative day 3 to 4, marking the resolution of ileus (Person & Wexner, 2006).

I.1.3.2 Pathogenesis of postoperative ileus

The pathogenesis of POI has been subscribed to many factors including impairment of normal neural function, inflammation and use of opioids (Holte & Kehlet, 2002).

The findings of experimental animal model studies of POI, which killed animals directly (i.e., 20 to 30 min) or 24 hours after intestinal manipulation, suggest that the activation of inhibitory neural reflexes is causing the acute hypomotility (up to 3 hours) while the inflammatory response initiated by activation of resident macrophages in the intestinal muscularis and sustained by the infiltrating leucocytes is mediating a second prolonged phase of POI (De Winter *et al.*, 1997a; Boeckxstaens *et al.*, 1999; Kalff *et al.*, 1999).

Thus, an important factor believed to cause the acute phase of POI is the impairment of normal function of the autonomic nervous system. The sympathetic nervous system participates in several inhibitory neural reflexes with the afferent limb originating both in the site of skin incision i.e., somatic fibres and in the intestines i.e., visceral fibres. It has efferent influence on the enteric nervous system by inhibiting the release of Ach resulting in decreased motility. An important spinal reflex activated after operation consists of capsaicin-sensitive splanchnic afferents originating in the site of skin incision and in the intestines, and adrenergic efferents. In addition to this adrenergic inhibitory pathway, stimulation of splanchnic afferents triggers an inhibitory non-adrenergic, vagally mediated pathway. In a rat model, the component resistant to adrenergic blockade was reduced by inhibiting nitrergic transmission, suggesting that the neurotransmitter released by the non-adrenergic pathway is NO (De Winter *et al.*, 1997a; Boeckxstaens *et al.*, 1999; Bauer & Boeckxstaens, 2004; Person & Wexner, 2006). The activation of the various reflex pathways depends mainly on the intensity of the nociceptive stimulus. For example, skin incision and laparatomy alone resulted in a brief reduction of intestinal motility, probably due to activation of low-threshold spinal reflexes, while a subsequent evisceration and manipulation of the intestines activated a high-threshold supraspinal pathway involving hypothalamic neurons that resulted in a more prolonged inhibition of gut motility (Holzer *et al.*, 1992; Boeckxstaens *et al.*, 1999). Several

animal studies have shown that blocking these reflexes at various levels of the nervous system resulted in a shorter duration of POI (Holzer *et al.*, 1986; De Winter *et al.*, 1997a). Besides an important nitrergic component in the causative mechanism of POI, other relaxant NANC neurotransmitters such as VIP and calcitonin gene-related peptide (CGRP) are reported to be involved in the pathogenesis of POI. Administration of VIP antagonists as well as the neutralization of CGRP have been shown to improve POI (De Winter *et al.*, 1998; Zittel *et al.*, 1998). However, combining all antagonist treatments of the relaxant neurotransmitters did not lead to further improvement of POI; moreover, the beneficial effect was lost altogether, suggesting that some form of inhibitory neural activity is required for POI to resolve (Zittel *et al.*, 1998).

A second important factor involved in the development of POI is inflammation, which is caused by activation of the resident macrophages present in the intestinal muscle layer and maintained by an important additional recruitment of circulating leukocytes (Kalff *et al.*, 1998; Kalff *et al.*, 1999). Intestinal manipulation occurring during abdominal surgery causes the dormant resident macrophages to secrete numerous active substances including NO produced by NOS-2, several prostaglandins produced by cyclo-oxygenase-2 (COX-2) and a wide spectrum of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) (Kalff *et al.*, 2000; Schwarz *et al.*, 2001; Wehner *et al.*, 2005). The inflammatory cascade evoked by intestinal manipulation has the same pattern as in any other organ system i.e., an external stimulus triggers the activation of macrophages leading to the liberation of pro-inflammatory cytokines causing an up-regulation of various adhesion molecules, which causes chemotaxis of additional circulating leukocytes with a subsequent further release of NO, prostaglandins, cytokines and oxygen free radicals (Person & Wexner, 2006). This sequence of events causes a reduction in postoperative gut motility probably caused by the overproduction of prostaglandins and NO as blocking COX-2 and NOS-2 resulted in an increase of muscle contractility (Josephs *et al.*, 1999; Kalff *et al.*, 2000). Also inhibition of macrophage function prevented inflammation and the inflammation-induced reduction of gastrointestinal motility (Wehner *et al.*, 2006). Besides inhibition of motility, NOS-2-derived NO displays other inflammatory effects. These indirect effects of NO predominate when large amounts of NO are released, and include N_2O_3-formation by a process called autoxidation (i.e., reaction with O_2) and peroxynitrite ($ONOO^-$)-formation by reaction with O_2^-. N_2O_3 is decomposed rapidly to nitrosonium ion (NO^+) and nitrite. Nitrosonium ion causes nitrosylation of an amine, thiol, or hydroxyl aromatic group. Peroxynitrite is responsible for oxidation (when one or two electrons are removed from the substrate) or nitration (when NO_2^+ is added to a molecule) of proteins, lipid and nucleotides (Davis *et al.*, 2001). Oxidation and nitration of f.i. transcription factors,

enzymes and ion channels may be responsible for mitochondrial energy depletion and induction of deoxyribonucleic acid (DNA) strand breaks that may lead to apoptosis (Shah *et al.*, 2004). An interaction between the inflammatory substances and the afferent neural pathways triggering the above mentioned inhibitory reflexes is also suggested as possible explanation for the observation that after abdominal surgery, un-manipulated intestinal segments may also exhibit inhibited motility (Schwarz *et al.*, 2004). A study from De Jonge et al. (2003) supports this hypothesis and additionally proposed that prostaglandins, produced by COX-2 are an important multifactorial neuromodulator in POI. Also the inflammatory mediators released by activated mast cells are involved in the pan-intestinal consequences of intestinal manipulation (de Jonge *et al.*, 2003).

The use of opioids as treatment of moderate to severe postoperative pain can also maintain POI as it is well known that they inhibit gastrointestinal function. These adverse gastrointestinal effects of opioids are mainly mediated by receptors within the bowel, whereas spinal and cerebral opioid receptors play a minor role. The most important classes of opioid receptors as target for the treatment of POI are the μ- and κ-opioid receptors (Lembo, 2006).

In the gastrointestinal tract, μ-opioid receptors are located in the mucosa, submucosal plexus, myenteric plexus and the muscular layers, while κ-opioid receptors can be found in the myenteric plexus and the muscular layers (Sternini *et al.*, 2004). Both classes of opioid receptors are involved in analgesia and gastrointestinal motility. Two novel opioid antagonists, i.e., methylnaltrexone and alvimopan, with selectivity for peripheral μ-opioid receptors were developed for the treatment of POI. Both drugs are able to reverse the opioid-induced slowing of gastrointestinal transit time while not affecting the analgesic effect of opioid analgesics. Methylnaltrexone is well-tolerated and induces mild to moderate abdominal cramping but no serious adverse events. Also alvimopan is well-tolerated and the most common alvimopan-induced adverse events are nausea and vomiting (Kraft, 2007).

Recently, also several peripherally acting κ-opioid agonists have been developed. Peripheral κ-opioid agonists are able to reverse ileus induced by laparotomy and manipulation but also have antinociceptive effects in the gastrointestinal tract. Examples of κ-opioid agonists are fedotozine and asimadoline. These drugs were shown to have antinociceptive effects in colon of patients with IBS but have not yet been used in clinical trials in post-surgical patients (Lembo, 2006). Preclinical data obtained in a rat model of POI showed that fedotozine can enhance the transit after laparotomy plus manipulation indicating that κ-opioid agonists might prove useful in the treatment of POI (De Winter *et al.*, 1997b).

Because of the multifactorial etiology, the management of POI generally consists of a multimodal approach including minimally invasive surgery, followed by aggressive epidural,

opiate-free pain control, early oral nutrition and mobilization. Other treatment options include the use of prokinetic agents, laxatives, non-steroidal anti-inflammatory drugs (NSAIDs) and selective μ_2-opioid receptor antagonists; however no generally applicable pharmacologic therapy of POI currently exists (Bauer & Boeckxstaens, 2004; Person & Wexner, 2006).

I.2 Role of sGC in the regulation of gastrointestinal motility

In 1963, guanosine 3',5'-cyclic monophosphate (cGMP) was isolated in urine and therefore for the first time identified as a molecule of potential biological importance. By 1969, the enzyme responsible for cGMP synthesis i.e., guanine nucleotidyl (guanylyl; guanylate) cyclase (GC) and cGMP breakdown i.e., phosphodiesterase (PDE) had also been identified. The biological role of cGMP was unknown until the 1980s when two important discoveries were made. First, it was discovered that atrial natriuretic peptide (ANP), a peptide synthesized in the heart, could stimulate cGMP synthesis by binding to a transmembrane receptor identified as particulate guanylate cyclase (pGC). Second, the endogenous molecule able to activate soluble guanylate cyclase (sGC) was identified as NO that was at that time still known as the endothelial-derived relaxant factor (EDRF). As NO is an important gastrointestinal neurotransmitter, the NO-sGC-cGMP signalling pathway was also a subject of intensive study in gastrointestinal motility research (Beavo & Brunton, 2002).

I.2.1 Characteristics of sGC

sGC is activated by NO and converts guanosine triphosphate (GTP) into the second messenger cGMP. sGC is a heterodimeric haem-containing protein that is composed of a larger α subunit and a smaller β subunit. Each subunit contains a haem-binding domain, a dimerization domain and a catalytic domain (Fig. I.3A). In the N-terminal haem-binding domain, the histidine 105 residu of the β subunit is the essential amino acid for the binding of the prosthetic haem moiety, which contains a reduced Fe^{2+}. This central ferrous iron is located between the four haem nitrogens and the axial ligand histidine 105, building a penta-coordinated histidyl-haem complex. Binding of NO to this complex results in the formation of a hexa-coordinated histidine-haem-NO intermediate that rapidly decays into a penta-coordinated nitrosyl-haem complex (Fig. I.3B). This change in haem conformation is transduced to the catalytic domain leading to a 200 to 400-fold increase in catalytic activity. However, as not all observed activation characteristics of sGC fit this simple model, a second binding site for NO was proposed (Zhao *et al.*, 1999). This suggestion was confirmed as a virtually inactive form of NO-bound sGC was found in the presence of low concentrations of NO that could be transformed to a fully active enzyme by adding additional NO (Russwurm & Koesling, 2004).

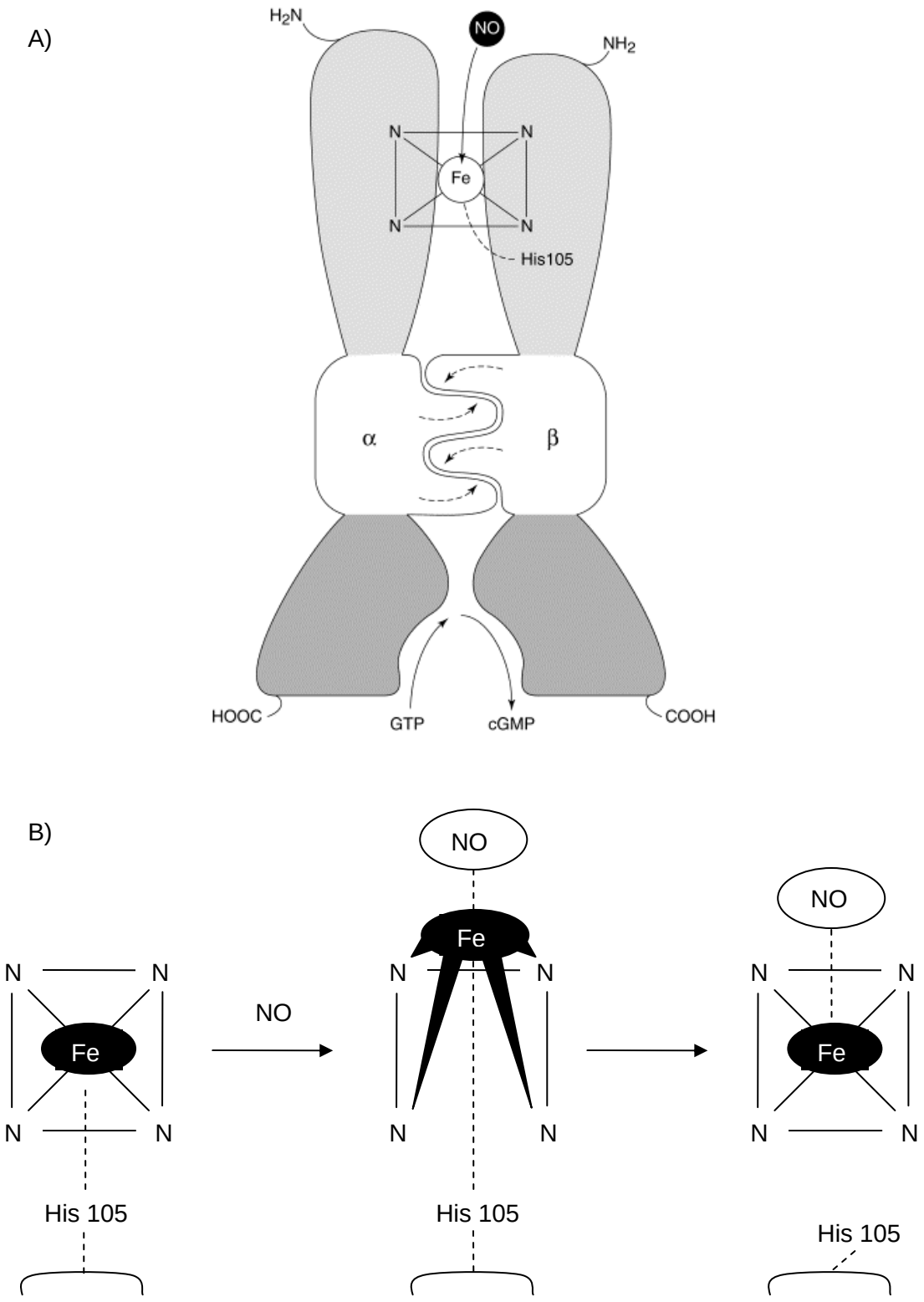

Figure I.3

A) Schematic representation of a sGC heterodimer (Hobbs, 1997).

B) Activation of sGC by NO. NO binds to the prosthetic haem group of the enzyme thereby forming a six-coordinated complex . The NO-dependent conversion into the five-coordinated complex results in rupture of the histidine-iron bond.

The central dimerization domain is involved in the formation of heterodimers, which is a pre-requisite for sGC to exhibit catalytic activity. The C-terminal catalytic domain of the sGC subunits are the most conserved regions. Expression of enzymatic activity requires the presence of the catalytic domains of both the α and β subunit. Deactivation of sGC is considered to be induced by the dissociation of NO (Koesling *et al.*, 2004; Pyriochou & Papapetropoulos, 2005).

Both the α and β subunit exist in 2 isoforms, α_1 and α_2 and β_1 and β_2. The $\alpha_1\beta_1$ and the $\alpha_2\beta_1$ heterodimers seem to be the physiologically active forms with no differences in kinetic properties and sensitivity towards NO between the 2 isoforms. The existence of an active heterodimer containing β_2 remains controversial although an active $\alpha_1\beta_2$ heterodimer has been reported (Gupta *et al.*, 1997). The $\alpha_1\beta_1$ isoform is ubiquitously distributed in mammalian tissues, although some tissues, such as skeletal muscle, bladder, thymus, testis and peripheral leukocytes, display very low levels of expression (Budworth *et al.*, 1999). Expression of $\alpha_2\beta_1$ was high in brain, placenta, pancreas, spleen and uterus (Budworth *et al.*, 1999). In contrast to brain where the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ heterodimers are present in comparable amounts, all other tissues predominantly express the α_1 subunit (Mergia *et al.*, 2003). Also in tissues of the gastrointestinal tract, i.e., stomach, small intestine and colon, $\alpha_1\beta_1$ mRNA expression was reported to predominate, although mRNA coding for the α_2 subunit was also present (Budworth *et al.*, 1999; Mergia *et al.*, 2003). Studies on the cellular distribution of the different sGC subunits in the gut were not found. Besides a different distribution pattern, also a different subcellular localisation of the 2 heterodimers is reported with the $\alpha_1\beta_1$ heterodimer being found in the cytosol and recruited to the membrane upon activation by an elevation of intracellular free calcium concentration while the $\alpha_2\beta_1$ heterodimer is associated to synaptic membranes in a postsynaptic density 95 (PSD-95) dependent manner (Koesling *et al.*, 2004; Pyriochou & Papapetropoulos, 2005).

Besides NO, also CO is able to activate sGC in a haem-dependent manner although to a much lesser extent than NO. New NO-independent activators of sGC have also been described. Based upon their characteristics, these compounds can be classified in a NO-independent but haem-dependent group i.e., the sGC stimulators and a NO-independent and haem-independent group i.e., the sGC activators. The first group of haem-dependent sGC stimulators includes YC-1, BAY 41-2272, BAY 41-8543, CFM-1571 and A-350619. These compounds show a strong synergy with NO and a loss of activation after oxidation or removal of the prosthetic haem moiety of sGC. The second group of haem-independent sGC activators contains BAY 58-2667 and HMR-1766, and have been found to require neither NO nor haem (Evgenov *et al.*, 2006).

I.2.2 Role of sGC in gastrointestinal motility under physiological conditions

The NO-sGC-cGMP signaling pathway exerts its physiological effects by mediation of three major types of intracellular effectors: cGMP-dependent protein kinases I and II (cGKI and II), cGMP-gated ion channels and cGMP-regulated phosphodiesterases (PDEs). The most important mediators of gastrointestinal signaling are cGK type I and PDEs. cGK belongs to the serine/threonine kinase family and exist in two subtypes: the cytosolic cGK type I that has two isoforms i.e., cGKIα and cGKIβ and the membrane-bound cGK type II. cGKI transduces many effects of sGC-derived cGMP while cGKII mediates effects of pGC-derived cGMP. cGKI activation may lead to relaxation by several mechanisms such as the inhibition of inositol 1,4,5-triphosphate (Ins(1,4,5)P_3)-dependent calcium release by phosphorylation of Ins(1,4,5)P_3-receptor-associated cGMP kinase substrate (IRAG), reduction of the synthesis of Ins(1,4,5)P_3 via the regulator of G-protein signaling 2 (RGS2) protein and potentially phospholipase Cβ3 (PLCβ3) and the inhibition of phosphorylation of myosin light chains. In addition, cGKI phosphorylates phospholamban thereby stimulating sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA), which decreases intracellular calcium levels (Fig. I.4; Schlossmann & Hofmann, 2005).

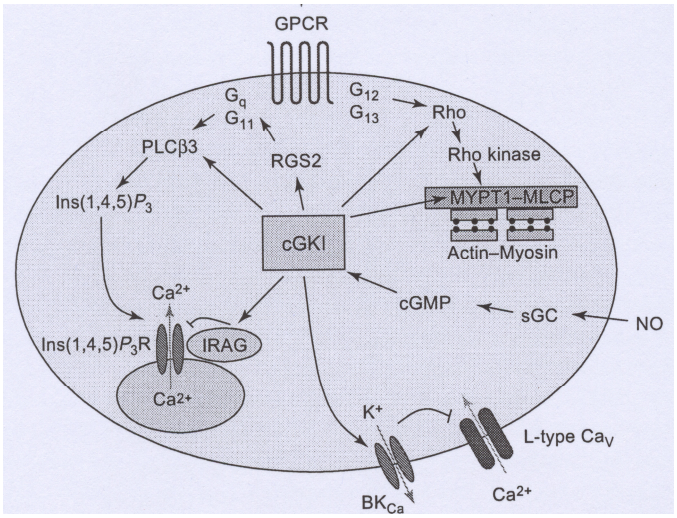

Figure I.4

Mechanisms affected by cGKI. NO-cGMP signaling activates cGKI, which induces smooth muscle relaxation. Smooth muscle contraction is induced via activation of G-protein-coupled receptors (GPCR; Schlossmann & Hofmann, 2005).

PDEs function as dimers and cleave the phosphodiester bond of cGMP and adenosine 3',5'-cyclic monophosphate (cAMP), hydrolysing the second messengers to its corresponding nucleotide, 5' monophosphate. The members of the PDE superfamily have significant similarity in their catalytic region but differ in their regulatory domain. As a consequence,

PDE families 1, 2, 3, 10 and 11 hydrolyze both cGMP and cAMP; PDE families 5, 6 and 9 preferentially hydrolyze cGMP while PDE families 4, 7 and 8 specifically cleave cAMP (Lucas *et al.*, 2000). The most important cGMP-regulated PDE in smooth muscle is PDE-5 and inhibition of this PDE induces relaxation by cGMP accumulation.

NO signaling can also occur via non-cGMP mediated mechanisms in physiological conditions. The reaction of NO with haem-containing proteins is the most physiologically relevant and includes an interaction with cytochrome c oxidase. However, NO can also interact with non-haem iron-containing proteins such as ferritin (protein crucial in the regulation of the cellular iron pool availability) and aconitase (bifunctional protein that regulates enzyme function and acts as a posttranscriptional regulator) and zinc-containing proteins such as Zn-finger proteins and tumor suppressor p53. NO may also be involved in termination of lipid oxidation (Davis *et al.*, 2001). The indirect effects of NO, produced through the interaction of NO with either O_2 or O_2^-, include nitration, oxidation and nitrosylation and were initially proposed to be mainly involved in pathological conditions. However, there is increasing evidence that nitration may also occur in normal cells although at a much lower level. It is possible that these nitrations may play a role in signal transduction processes but this is still a matter of speculation (Bruckdorfer, 2005). Also S-nitrosylation is reported to be involved in physiological signaling; nitrosylation targets of NO include metabolic, structural and signaling proteins that may be effectors for NOS-1-derived NO (Jaffrey *et al.*, 2001).

I.2.2.1 Stomach

The role of sGC in gastric emptying remains still to be explored. However, the effect of the PDE-5 inhibitor sildenafil has been assessed repeatedly with equivocal results. Investigators reported no change or a delay of liquid gastric emptying and no change of solid gastric emptying in humans (Madsen *et al.*, 2004; Sarnelli *et al.*, 2004). As a decreased proximal volume and a significantly increased distal volume of the stomach was found, it was suggested that sildenafil alters the intragastric distribution of food rather than causing gastric stasis (Cho *et al.*, 2006). Reports studying the effect of sildenafil on the interdigestive motor activity of the distal stomach in humans revealed an inhibition of antral motility (Bortolotti *et al.*, 2001). In-vitro experiments suggested a role for sGC in NANC relaxations of the stomach as the sGC inhibitor ODQ significantly inhibited EFS-induced relaxations in longitudinal muscle of the rat gastric fundus (Lefebvre, 1998) and circular muscle of the guinea-pig antrum (Kim *et al.*, 2003). In pyloric tissue, it was reported that the hyperpolarization induced by NO was also mediated by sGC activation as NO-administration evoked an enhanced production of cGMP in canine pyloric circular muscle (Bayguinov & Sanders, 1993). Knocking out cGMP's effector, cGKI, induced dilation of the stomach and

signs of pyloric stenosis; electrically induced relaxation of gastric fundus strips was impaired, as well as gastric emptying of a barium suspension (Pfeifer *et al.*, 1998). When summarized, these findings suggest an important role for the NO-sGC-cGMP-cGKI signaling pathway in the stomach.

I.2.2.2 Small intestine

The role of sGC in intestinal transit has not been explored yet; however, the use of a cGKI KO model revealed that interruption of the NO-sGC-cGMP-cGKI signaling pathway delayed small intestinal transit of a barium suspension. Instead of regular peristalsis, spastic contractions of long intestinal segments followed by scarce and slow relaxations were observed (Pfeifer *et al.*, 1998).

In-vitro studies also showed that NANC relaxations were mediated by sGC as the sGC inhibitor ODQ reduced the EFS-induced relaxations in longitudinal muscle of mouse duodenum (Serio *et al.*, 2003), jejunum and ileum (Ueno *et al.*, 2004). Similar findings were reported for human jejunal longitudinal muscle (Zyromski *et al.*, 2001).

I.2.3 Role of sGC in gastrointestinal motility under pathophysiological conditions

As mentioned above (see I.1.3.), the role of NO in pathologies is considered to be ambiguous as it is reported to have both beneficial and detrimental effects. Generally, activation of the constitutive NOS-isoforms (NOS-1 and NOS-3) was assumed to be salutary while induction of NOS-2 was proposed to be harmful. Still, in experimental IBD models, inhibition of NOS by non-selective NOS inhibitors caused equivocal results as it ameliorated tissue injury (Rachmilewitz *et al.*, 1995), corresponding with a detrimental role for NO, it evoked no effect (Armstrong *et al.*, 2000) as well as it exaggerated inflammatory parameters with an increase of mucosal damage (Pfeiffer & Qiu, 1995; Yoshida *et al.*, 2000), corresponding with a beneficial effect of NO. It was thought that the amelioration might be caused by the inhibition of the “harmful" NOS-2 by the non-selective NOS inhibitor, while the exaggeration of tissue injury might be related to the inhibition of beneficial constitutive NOS. However, the use of selective NOS-2 inhibitors also showed this ambiguous pattern as they were shown to ameliorate intestinal inflammation (Kankuri *et al.*, 2001), to evoke no effect (Ribbons *et al.*, 1997) or to increase mucosal injury (Blanchard *et al.*, 2001). Similar results were obtained by the use of NOS-2 KO mice, as either a beneficial role (Vallance *et al.*, 2004), no effect (McCafferty *et al.*, 2000) or a detrimental role (Krieglstein *et al.*, 2001) for NOS-2-derived NO was suggested. This clearly illustrated that NOS-2-derived NO can have both beneficial and detrimental effects. But the use of KO animals of both constitutive NOS

isoforms also revealed both salutary and harmful effects of NO derived from NOS-1 and NOS-3 (Beck *et al.*, 2004; Vallance *et al.*, 2004). Correspondingly, activation of constitutive NOS was found to cause negative effects in an animal model of POI as evidence was provided that constitutive NOS-1 plays a role in the development of early dysmotility caused by intestinal manipulation (De Winter *et al.*, 1997a). As NO derived from constitutive and inducible NOS exerts both positive and negative effects, the above mentioned black-white paradigm is thus seriously challenged. sGC might play an important role in the mystery of the positive/negative effects of NO. Indeed, activation of sGC by endotoxin-induced NO production and probably an additional sGC-stimulating mediator, was suggested to be responsible for negative vascular effects such as vascular hyporeactivity in an endotoxin-induced shock model (Wu *et al.*, 1998). Also, a study of Cauwels et al. (2000) suggested that the detrimental vascular effects in a TNF-induced lethal shock model were caused by sGC activation; results of this study also suggested that NOS-2-derived NO was protective via sGC-independent mechanisms. Although the sGC-independent effects of NO, produced through the interaction of NO with either O_2 or O_2^- leading to either N_2O_3 or peroxynitrite formation, were initially proposed to be mainly involved in pathological effects, there is increasing evidence that they may also occur in physiological processes subsequently leading to beneficial effects. The nature of these beneficial sGC-independent mechanisms still remain a matter of investigation but might include nitration and S-nitrosylation of proteins.
In a murine model of endotoxin-induced gastrointestinal motility disturbances, it was found that application of the sGC inhibitor ODQ improved the endotoxin-induced delay in gastric emptying. Similar improvement was found when the specific NOS-2 antagonist 1400 W was administered, suggesting that the endotoxin-induced hypomotility was caused by NOS-2-derived NO acting via sGC (De Winter *et al.*, 2002). However, note that the improving effects of ODQ might be due to the antioxidant effects of ODQ's solvent, i.e., DMSO (De Winter *et al.*, 2005). Nevertheless, as similar improving effects could be obtained with another guanylate cyclase inhibitor, i.e., NS2028 that was not dissolved in DMSO, sGC activation is likely to play a detrimental role in endotoxin-induced gastrointestinal motility disturbances (De Winter et al., Gastroenterology, 2002, 122 (4), A-555, nr W1047). Also in endotoxin-treated rats, endotoxin-evoked gastric hypocontractility was partly mediated by sGC activation, but now due to an increased NOS-1-derived NO synthesis in postganglionic myenteric neurons (Quintana *et al.*, 2004).
Although the above mentioned studies strongly suggest that sGC activation causes the negative effects of NO in sepsis and shock models, also positive effects of NO are reported to be mediated by sGC activation such as the inhibitory effect of NO on neutrophil rolling, adhesion and migration (Dal Secco *et al.*, 2006). This key anti-inflammatory role of sGC is

achieved by inhibiting the expression of several adhesion molecules thereby preventing leukocyte recruitment (Ahluwalia *et al.*, 2004). In a model of TNF-α induced cellular injury, NO-induced protection against TNF-α evoked cellular injury was reported to be mediated by a cGMP-dependent pathway (Polte *et al.*, 1997). A more recent report supported this finding as they also suggested a protective role for sGC activation in a murine model of lipopolysaccharide (LPS)-induced acute lung injury (Glynos *et al.*, 2007).

In summary, it appears that also sGC activation might have a dual role in pathological conditions.

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Chapter II

AIMS

Chapter II Aims

Gastrointestinal motility is regulated by hormonal and neuronal control mechanisms. Within the enteric nervous system, nitrergic neurons containing NOS-1 play a very important role in the regulation of relaxation of gastrointestinal SMCs. Although, depending upon species and region of the gastrointestinal tract, also other inhibitory neurotransmitters such as VIP, ATP and CO are involved in gastrointestinal inhibitory neurotransmission. The principal target of NOS-1-derived NO to induce relaxation in gastrointestinal smooth muscle is sGC. But NO has also sGC-independent effects such as nitration and S-nitrosylation of proteins. Upon inflammation of the gastrointestinal tract, NOS-2 is induced releasing larger amounts of NO, similar to what is observed in non-gastrointestinal models of inflammation such as shock. NOS-2-derived NO was originally thought to only induce detrimental effects i.e., aggravation of the inflammation and inhibition of gastrointestinal motility, but several reports suggested that it might also be beneficial. Cauwels et al. (2000) suggested that the NOS-2 induced detrimental vascular effects in a TNF model of lethal shock were caused by sGC activation, while NOS-2-derived NO also had a protective role in a sGC-independent way. Still, it was reported that NOS-2-derived NO has anti-inflammatory properties via a sGC-dependent pathway in a model of endotoxin-induced inflammation (Glynos *et al.*, 2007). In view of the positive/negative effects of NO and sGC, the aim of our study was to investigate the role of sGC in the effect of NO on gastrointestinal motility in physiological conditions and during POI.

The small intestine belongs to the affected area in human POI. Rodent models are commonly used to investigate the pathogenesis of the gastrointestinal disturbed motility in POI, and a Wistar rat model was selected to investigate the role of sGC in POI, concentrating on jejunal tissue. As it has been suggested that stimulation of inhibitory neurotransmission can be involved in POI, at least during the first hours after manipulation (De Winter *et al.*, 1997; De Winter *et al.*, 1998), it is important to delineate the regulation of inhibitory neurotransmission under normal conditions. Conflicting results have indeed been reported on the role of NO in inhibitory neurotransmission of rat jejunum, depending upon rat strain used and the study of circular or longitudinal muscle (Niioka *et al.*, 1997; Balsiger *et al.*, 2000; Okishio *et al.*, 2000). Our first aim was therefore to investigate the effect and the contribution of NO, ATP, VIP and PACAP to inhibitory neurotransmission in the circular

muscle of Wistar rat jejunum. Also the mechanism of action of the inhibitory neurotransmitters, concentrating on the role of sGC, was investigated. These results are summarised in **Chapter III.**

The involvement of NO in both the early and prolonged phase of POI has already been assessed in several studies using NOS inhibitors or NOS-2 KO mice (De Winter *et al.*, 1997; Kalff *et al.*, 2000; Korolkiewicz *et al.*, 2004; Turler *et al.*, 2006). As these reports did not study the role of nitrergic inhibitory neurotransmission and of NO's main effector, sGC, in the effects of NO in the second more prolonged inflammatory phase POI and as the role of NOS and sGC in pathological conditions remains ambiguous, the aim of our second study was to investigate the role of NO and the effect of sGC inhibition on inflammation and NANC neurotransmission in jejunal tissue of a Wistar rat model of POI. The results of this study are summarised in **Chapter IV**.

The principal target of the relaxant neurotransmitter NO in the gastrointestinal tract is sGC. Still, NO has sGC-independent effects such as nitration and S-nitrosylation of proteins and sGC can be stimulated by other stimuli than NO such as CO. sGC exists in 2 isoforms i.e., sGC$\alpha_1\beta_1$ and sGC$\alpha_2\beta_1$ of which the $\alpha_1\beta_1$-isoform predominates in the gastrointestinal tract. Therefore the consequences of knocking out the α_1-subunit of sGC were studied at the level of the stomach, where NO is involved in the control of gastric emptying, and at the level of the jejunum, a tissue displaying phasic activity where NO is involved in peristalsis. These experiments are described in **Chapter V** (stomach) and **Chapter VI** (jejunum).

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Chapter V

INVESTIGATION OF A POSSIBLE INTERACTION BETWEEN THE HEME OXYGENASE/BILIVERDIN REDUCTASE AND NITRIC OXIDE SYNTHASE PATHWAY IN MURINE GASTRIC FUNDUS AND JEJUNUM

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Chapter III Investigation of a Possible Interaction between the HO/BVR and NOS pathway in Murine Gastric Fundus and Jejunum

III.1 Abstract

This study investigated the possible interaction between the heme oxygenase (HO)/biliverdin reductase (BVR) and nitric oxide synthase (NOS) pathway in murine gastric fundus and jejunum, since previous studies have shown that both HO-2 and BVR are expressed in interstitial cells of Cajal (ICCs) and co-localized with neuronal NOS in a large proportion of myenteric neurons along the gastrointestinal tract. Neither HO inhibition by chromium mesoporphyrin (CrMP) nor co-incubation with CO or biliverdin/bilirubin affected nitrergic neurotransmission – i.e. relaxations induced by non-adrenergic non-cholinergic (NANC) nerve stimulation or exogenous NO – under normal physiological conditions. However, biliverdin/bilirubin reversed the inhibitory effect of the superoxide generator LY83583 on exogenous NO-induced relaxations in both tissues. When gastric fundus muscle strips were depleted of the endogenous antioxidant Cu/Zn superoxide dismutase (SOD) by the Cu-chelator DETCA, electrically induced NANC relaxations were also affected by LY82583; however, biliverdin/bilirubin could not substitute for the loss of Cu/Zn SOD when this specific antioxidant enzyme was depleted. In jejunal muscle strips, the combination DETCA plus LY83583 nearly abolished contractile phasic activity and, hence, did not allow studying nitrergic relaxation in these experimental conditions. In conclusion, this study does not establish a role for HO/CO in inhibitory NANC neurotransmission in murine gastric fundus and jejunum under normal physiological conditions. However, the antioxidants biliverdin/bilirubin might play an important role in the protection of the nitrergic neurotransmitter against oxidative stress.

III.2 Introduction

Heme oxygenase (HO – EC 1.14.99.3) is the rate-limiting enzyme in the degradation of heme, catalyzing the oxidative cleavage of heme to carbon monoxide (CO), biliverdin and ferrous iron. Biliverdin is subsequently reduced to bilirubin by biliverdin reductase (BVR – EC 1.3.1.24). The HO family consists of two distinct isozymes: the stress-inducible HO-1, which is upregulated by a variety of different stimuli; and the constitutive HO-2, which is expressed under basal conditions (Maines, 1997; Ryter et al., 2006; Wu and Wang, 2005).

HO-2 is widely expressed in the gastrointestinal tract and more specifically in myenteric and submucosal enteric neurons, interstitial cells of Cajal (ICCs), and mucosal epithelial cells (Gibbons and Farrugia, 2004). To a varying extent, this HO-2 isoform has been found to be co-localized with neuronal nitric oxide synthase (nNOS) in the enteric plexuses of pig gastric fundus and murine small intestine (100% co-localisation; Colpaert et al., 2002a; Zakhary et al., 1997), canine and feline lower oesophageal sphincter and ileum (50% co-localisation; Ny et al., 1996, 1997), and human gastric antrum and jejunum (10-40% co-localisation; Miller et al., 2001). Based on 1/ the immunohistochemical co-localisation of HO-2 with nNOS in myenteric neurons; 2/ the effects of some non-selective HO inhibitors on neurally-evoked non-adrenergic non-cholinergic (NANC) relaxations (Alcon et al., 2001; Grundemar et al., 1997; Rattan and Chakder, 1993; Zakhary et al., 1997); and 3/ a study reporting reduced neurally-evoked ileal smooth muscle relaxations in HO-$2^{-/-}$ mice (Zakhary et al., 1997), the suggestion was made that NO and CO might function as co-neurotransmitters in the enteric nervous system. More recent studies, however, do not confirm a neural role for CO in enteric neurotransmission but indicate that CO – generated by HO-2 in the ICCs – more likely acts as an endogenous hyperpolarizing factor in the gastrointestinal tract (Farrugia et al., 2003; Sha et al., 2007).

As both HO-2 and BVR are expressed in ICCs and co-localized with nNOS in a large proportion of myenteric neurons (Colpaert et al., 2002a, 2002b; Miller et al., 1998, 2001; Ny et al., 1996, 1997; Zakhary et al., 1997), (a) possible interaction(s) between the HO/BVR and NOS pathway should be considered. Thus, the aim of our study was to investigate the role of the HO/BVR pathway in NANC neurotransmission and to elucidate the influence of CO and/or biliverdin-bilirubin on nitrergic neurotransmission in murine gastric fundus and jejunum.

III.3 Materials & Methods

III.3.1 Animals

Male Swiss (SPF Orl) mice (6-8 weeks, 30-35 g) were kept and cared for in standard cages in a 12h:12h light-darkness cycle with free access to water and pellets. All experimental procedures were approved by the Ethical Committee for Animal Experiments, Faculty of Medicine and Health Sciences, Ghent University, Belgium.

III.3.2 Tissue preparation & mounting

Mice were sacrificed by cervical dislocation and the stomach and a ± 8 cm long jejunal segment starting 5 cm distally from the ligament of Treitz were removed. Two full-thickness muscle strips (2 x 15 mm) were prepared from the gastric fundus by cutting in the direction of the circular muscle layer. The jejunum was opened along the mesenteric border and pinned mucosa side up. The mucosa was removed by sharp dissection under a microscope and full-thickness muscle strips (2 x 7 mm) were cut along the circular axis. The muscle strips were mounted vertically between two platinum plate electrodes in 10 ml organ baths that were filled with Krebs solution (composition in mM: NaCl 188.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1), maintained at 37°C and aer ated by 95% O_2/5% CO_2. The experiments were carried out in the continuous presence of atropine (1 µM) and guanethidine (4 µM) to block respectively cholinergic and noradrenergic responses. Changes in tension were measured using Radnoti isometric transducers (Radnoti, Monrovia, USA) and recorded with Chart software and a PowerLab/8sp data recording system (AD Instruments, U.K.). Electrical field stimulation (EFS; 40 V, pulse width: 0.1 ms, pulse train: 10 s) was applied by means of a Grass S88 stimulator. The strips were brought to their optimal point of length-tension relationship (gastric fundus at 1.5 g; jejunum at 0.2 g – as determined in preliminary experiments) and then allowed to equilibrate for 60 min with rinsing every 15 min before starting the experiment.

III.3.3 Functional experiments

A first series of experiments was performed to study the influence of the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) and/or the HO inhibitor chromium mesoporphyrin (CrMP) on relaxations induced by electrical field stimulation (EFS) of NANC nerves *or* exogenous NO. Therefore, all strips were precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 300 nM) and relaxations were induced by EFS (0.5-8 Hz, 40 V, pulse width: 0.1 ms, pulse train: 10 s) *or* the addition of NO (0.1 µM-10 µM) at 5-min intervals. Tissues were then

repetitively rinsed for 60 min and L-NAME (300 µM) and/or CrMP (10 µM) were incubated for 20 min. Contraction was induced again with $PGF_{2\alpha}$ and the relaxant stimuli were repeated. In addition, the influence of a submaximal concentration of L-NAME (1 µM) as well as the combination of L-NAME (1 µM) plus CrMP (10 µM) on EFS-induced NANC relaxations was studied.

In a second series of experiments, the influence of CO, biliverdin, bilirubin and superoxide dismutase (SOD) *per se* on relaxations induced by nitrergic nerve stimulation and exogenous NO were studied as follows. All strips were precontracted with $PGF_{2\alpha}$ and two relaxant stimuli were consecutively studied with a 5-min interval in between: EFS (2 Hz, 40 V, pulse width: 0.1 ms, pulse train: 10 s) and NO (1 µM). Biliverdin (200 µM), bilirubin (200 µM) or SOD (1000 U/ml) was injected into the organ bath 20 min before the second $PGF_{2\alpha}$-induced contraction, at the top of which the two relaxant stimuli were repeated. In contrast, CO-releasing molecule (CORM)-2 (30 µM) or its control compound ruthenium chloride hydrate (RuCl; 60 µM) were added on top of the second contraction and – after a 3 min incubation period – the two relaxant stimuli (EFS, NO) were studied for the second time. In a similar way, the influence of a continuous infusion of CO (CO_{inf}) on nitrergic responses was studied by infusing a CO-saturated Krebs solution (1 mM) into the organ bath *via* a Braun infusion pump (1 ml/min); CO_{inf} was started on top of the second contraction and – after 3 min of infusion – the two relaxant stimuli (EFS, NO) were repeated; as the half-life of CO is not known in these experimental settings, we cannot calculate the obtained concentration in the organ bath.

In a third series of experiments, the influence of CO, biliverdin, bilirubin and SOD on EFS- and exogenous NO-induced relaxations was studied in the presence of the superoxide generator LY83583 and/or the Cu/Zn superoxide dismutase (Cu/Zn SOD) depletor diethyldithiocarbamate acid (DETCA), a Cu-chelator irreversibly inhibiting endogenous Cu/Zn SOD. All strips were precontracted with $PGF_{2\alpha}$ and two relaxant stimuli were consecutively studied with a 5-min interval in between: EFS (2 Hz, 40 V, pulse width: 0.1 ms, pulse train: 10 s) and NO (1 µM). Tissues were then repetitively rinsed and DETCA (1 mM) was administered and left in contact with some of the tissues for 60 min; this compound was then washed from the organ bath. Subsequently, the superoxide generator LY83583 (10 µM) was added and 15 min later a second contraction was induced by $PGF_{2\alpha}$ and the two relaxant stimuli were repeated. Biliverdin (200 µM), bilirubin (200 µM) or SOD (1000 U/ml) was added 5 min before LY83583, whereas CO_{inf} was started on top of the second contraction, 3 min before the two relaxant stimuli (EFS, NO) were repeated.

In all protocols studying the influence of CrMP, biliverdin or bilirubin, the organ baths were covered with aluminium foil to avoid light exposure.

III.3.4 Measurement of HO activity

Gastric fundus and jejunal muscle strips were prepared and mounted as described above for the functional experiments and incubated with CrMP (10 µM; for jejunal muscle strips also 100 µM) for 20 min. Parallel preparations from the same animal, without CrMP treatment, served as controls. HO activity was measured by bilirubin generation as previously described (Farrugia *et al*., 2003). In brief, gut tissues from 6 mice were pooled and homogenized in 5 vol of 0.1 M potassium phosphate buffer (pH 7.4), followed by sonication on ice for 30s. The homogenates were centrifuged at 3,000 *g* at 4℃ for 10 min, and the supernatant was subsequently centrifuged at 12,000 *g* at 4℃ for 20 min. Microsomes were pelleted from the resulting supernatant by centrifugation at 105,000 *g* at 4℃ for 60 min. The microsomal pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 2 mM $MgCl_2$. An aliquot of the microsomal suspension (200 µg of protein) was added to a reaction mixture containing hemin (25 µM), glucose 6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (1 U), and mouse liver cytosol (as a source of biliverdin reductase, prepared from 105,000 *g* supernatant). The reaction was initiated by addition of NADPH (1 mM) and conducted in the dark at 37℃ for 60 min; placing the samples on ice terminated the reaction. Bilirubin concentration was calculated by the difference in absorbance at 470 and 530 nm, using an extinction coefficient of 40 mM^{-1} cm^{-1}. HO activity was expressed as picomoles of bilirubin formed per hour per milligram of protein, as determined by Bradford assay.

III.3.5 Measurement of Cu/Zn SOD activity

Gastric fundus and jejunal muscle strips were prepared and mounted as described above for the functional experiments and incubated with DETCA (1 mM) for 1 h. Parallel preparations from the same animal, without DETCA treatment, served as controls. Total Cu/Zn SOD activity in each muscle strip was determined with a commercially available Cu/Zn SOD activity assay kit (Cayman Chemical) according to the manufacturer's instructions, and expressed as U/ml.

III.3.6 Data analysis

In the first series of experiments, relaxations induced by EFS and NO were expressed as percentage of $PGF_{2\alpha}$-induced contraction (see V.4.1.). In all other functional experiments, responses in the presence of interfering drugs are related to those obtained before administration of these drugs. Experimental data are expressed as means ± SEM. Results within tissues are compared by a paired *t*-test. Results between tissues are

compared by an unpaired *t*-test. When more than 2 groups have to be compared, one-way analysis of variance (ANOVA) is performed; if statistical significance is reached ($P < 0.05$), comparison per 2 groups is performed by a *t*-test, corrected for multiple comparisons (Bonferroni procedure). A difference is considered statistically significant at $P < 0.05$.

III.3.7 Drugs & Materials

The following drugs were used (supplied by Sigma unless stated otherwise): 6-anilino-5,8-quinolinedione (LY83583; Calbiochem), atropine sulphate, bilirubin ditaurate (Calbiochem), biliverdin hydrochloride (ICN biomedicals), chromium mesoporphyrin (CrMP; ICN biomedicals), diethyldithiocarbamic acid (DETCA), guanethidine sulphate, N^{ω}-nitro-L-arginine methyl ester (L-NAME), prostaglandin F2α ($PGF_{2\alpha}$), ruthenium chloride hydrate (RuCl), superoxide dismutase (SOD) from bovine erythrocytes, tricarbonyldichlororuthenium dimer (CORM-2). Drugs were dissolved in deionized water except LY83583 which was dissolved in ethanol, CORM-2 which was dissolved in dimethylsulfoxide, and biliverdin/bilirubin which were dissolved in 0.2% NaOH. Solvents themselves were without significant effect at the concentrations used in the experiments. Stock solutions were made of LY83583 (10 mM); other solutions were prepared on the day of experiment. CORM-2 was always freshly prepared just before administration into the organ bath. A saturated NO (2 mM) or CO (1 mM) solution was prepared from 99.9% NO or CO gas (Air Liquide, Belgium) as described by Kelm and Schrader (1990) and Schröder et al. (2002).

III.4 Results

III.4.1 Influence of L-NAME and CrMP on EFS- and NO-induced relaxations

In gastric fundus muscle strips, EFS (0.5-8 Hz) induced transient and frequency-dependent NANC relaxations which were abolished by the NOS inhibitor L-NAME (300 µM; Fig V.1A). In jejunal muscle strips, EFS evoked similar, transient relaxations which were abolished by L-NAME (300 µM) at low frequencies (≤ 2 Hz); however, residual acute twitch relaxations were still observed at higher frequencies (4-8 Hz; Fig V.1B). The combination of L-NAME (300 µM) with CrMP (10 µM) did not reduce jejunal NANC relaxations (4-8 Hz) in a more pronounced way than L-NAME (300 µM) alone (*n* = 4; data not shown). In both tissues, neurally evoked NANC relaxations (0.5-8 Hz) were unaffected by the HO inhibitor CrMP (10 µM; Fig V.1A, V.1B). Also the combination of a submaximal concentration of L-NAME (1 µM) – inhibiting EFS-induced NANC relaxations by ± 60% – with CrMP (10 µM) did not reduce NANC relaxations in a more pronounced way than L-NAME (1 µM) alone (*n* = 4; data not shown). The administration of NO (0.1-10 µM) induced transient and concentration-dependent relaxations which were unaffected by L-NAME (300 µM) and CrMP (10 µM; Fig V.1C, V.1D).

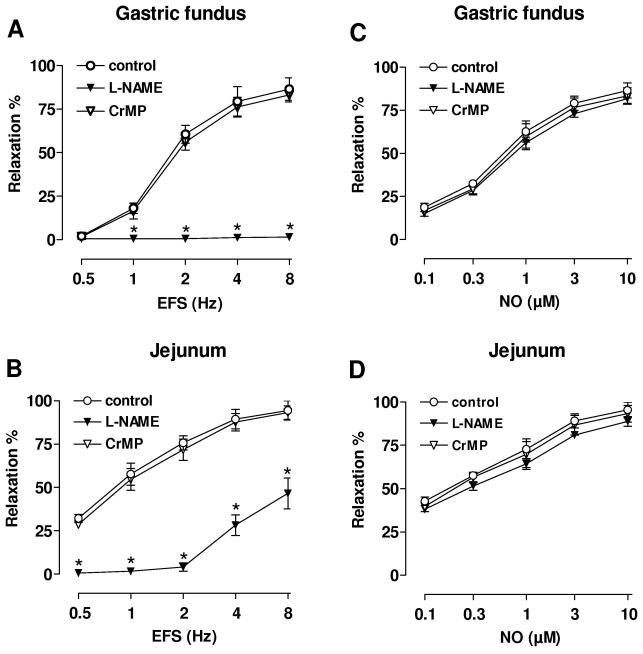

Figure V.1

Relaxant responses to EFS (0.5-8 Hz; left panels) and exogenous NO (0.1-10 µM; right panels) in murine gastric fundus (upper panels) and jejunal (lower panels) circular smooth muscle strips in control conditions (**o**) and in the presence of L-NAME (300 µM, ▼) or CrMP (10 µM, ∇). Relaxations are expressed as percentage of $PGF_{2\alpha}$-induced contraction and shown as mean ± s.e.mean of *n* = 6 experiments. * *P* < 0.05: significantly different from control (Student's *t*-test).

The HO activity in control preparations was 48.8 pmol/hr/mg protein in gastric muscle strips and 45.2 pmol/hr/mg protein in jejunal muscle strips. After incubation with CrMP (10 µM), HO activity was markedly reduced to, respectively, 13.9 pmol/hr/mg protein in gastric muscle strips and 14.2 pmol/hr/mg protein in jejunal muscle strips (as measured in 6 pooled tissue samples). These results confirm the effectiveness of CrMP (10 µM) as a potent HO inhibitor in our experiments. When jejunal tissues were incubated with a ten-fold higher concentration of CrMP (100 µM), HO activity was reduced to 12.8 pmol/hr/mg protein.

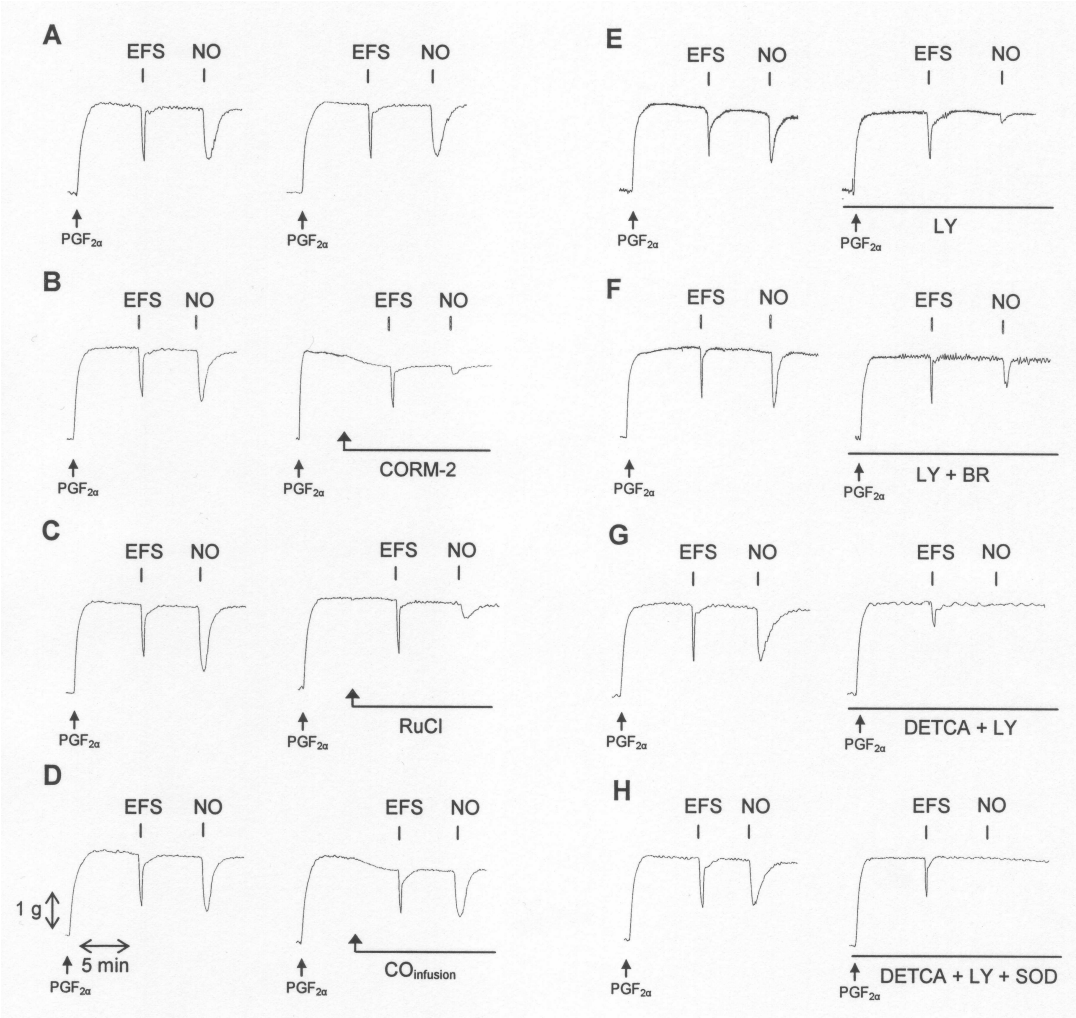

Figure V.2

Representative traces showing $PGF_{2\alpha}$ (300 nM)-induced contraction and the response to EFS (2Hz) and exogenous NO (1 µM) in murine gastric fundus circular smooth muscle strips: (A) control tissue showing the reproducibility of EFS- and NO-induced relaxations; (B-D): the influence of CORM-2 (30 µM), RuCl (60 µM) or $CO_{infusion}$ (1 ml/min) on EFS- and NO-induced relaxations; (E-F): the influence of LY83583 (10 µM) on EFS- and NO-induced relaxations and the partial protective effect of bilirubin (200 µM) on NO-induced relaxations in these conditions; (G-H): the influence of DETCA (1 mM) plus LY83583 on EFS- and NO-induced relaxations and the partial protective effect of SOD (1000 U/ml) on EFS-induced NANC relaxations in these conditions.

III.4.2 Influence of CO and biliverdin/bilirubin on nitrergic relaxations per se

The relaxant responses to EFS (2 Hz) and NO (1 µM) in gastric fundus muscle strips were well maintained in control tissues (Fig V.2A, V.3A). Administration of CORM-2 (30 µM) moderately decreased $PGF_{2\alpha}$-induced tone (decrease of 16.1 ± 2.8%; *n* = 7) and reduced exogenous NO-induced relaxation to 33.9 ± 8.8 % (*n* = 7) of the response before its administration. However, CORM-2 (30 µM) did not affect EFS-induced relaxations (Fig V.2B). A similar discriminatory effect on exogenous NO and endogenous nitrergic neurotransmission was obtained with the control compound RuCl (60 µM); however, RuCl did not influence $PGF_{2\alpha}$-induced tone (Fig V.2C). In contrast, continuous infusion of a CO-saturated Krebs solution (CO_{inf},1 ml/min) also moderately decreased $PGF_{2\alpha}$-induced tone (decrease of 19.6 ± 5.1%; *n* = 6) but did not affect EFS- or exogenous NO-induced relaxant responses (Fig V.2D, V.3A). Also pre-incubation with biliverdin (200 µM) or bilirubin (200 µM) did not significantly affect relaxations induced by EFS or exogenous NO in murine gastric muscle strips (Fig V.3A).

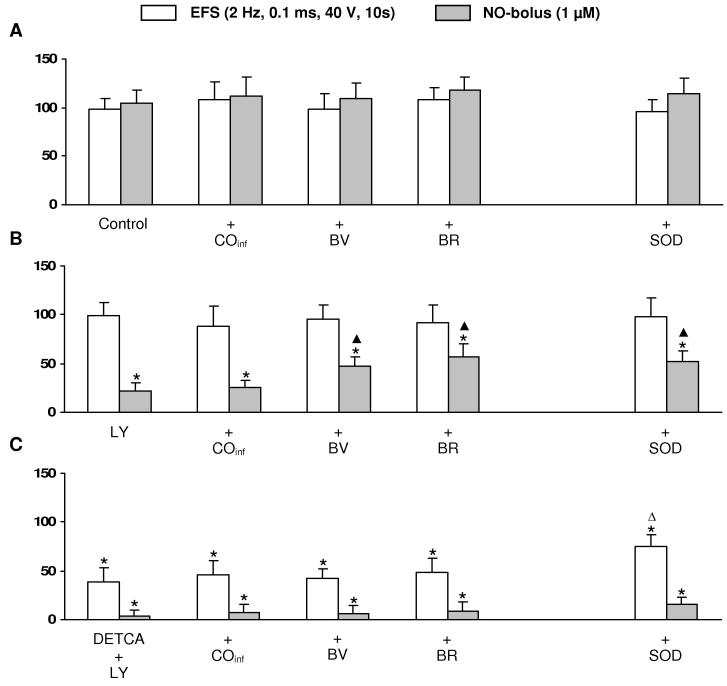

Figure V.3

Relaxant responses to EFS (2 Hz) and exogenous NO (1 µM) in murine gastric fundus circular smooth muscle strips: (A) the influence of $CO_{infusion}$ (1 ml/min), biliverdin (BV; 200 µM), bilirubin (BR; 200 µM), or SOD (1000 U/ml) on EFS- and NO-induced relaxations *per se*; (B) the influence of $CO_{infusion}$, BV, BR or SOD on the same relaxant stimuli in the presence of LY83583 (10 µM); (C) the influence of $CO_{infusion}$, BV, BR or SOD on the same relaxant stimuli in the presence of DETCA (1 mM) plus LY83583. Relaxant responses are expressed as a percentage of the response to the same stimulus before administration of interfering drugs. Means ± s.e.mean of n = 6-8 are shown. * $P < 0.05$: significantly different from the response in the same tissue before administration of drugs (Student's *t*-test); ▲ $P < 0.05$: significantly different from pretreatment with LY83583 alone; Δ $P < 0.05$: significantly different from pretreatment with DETCA plus LY83583 (ANOVA with Bonferroni post-hoc test).

Similar results were obtained for the influence of CO – administered as CORM-2 or CO_{inf} – and biliverdin/bilirubin on nitrergic relaxations *per se* in jejunal muscle strips (Fig V.4A).

III.4.3 Influence of CO and biliverdin/bilirubin on nitrergic relaxations in the presence of the superoxide generator LY83583

In gastric fundus muscle strips, incubation with LY83583 (10 µM) markedly reduced the relaxant response elicited by exogenous NO (1 µM) to 21.2 ± 8.7 % (*n* = 7) of the response before its administration; however, no inhibitory effect on the response to EFS (2 Hz) was observed (Fig V.2E, V.3B). This inhibitory effect of LY83583 on relaxations induced by exogenous NO was partially antagonized by co-incubation with biliverdin or bilirubin (Fig V.2F, V.3B). The response to exogenous NO increased from 21.2 ± 8.7 % (*n* = 7) in the presence of LY83583 alone to 47.3 ± 10.1 % (n = 7; P < 0.05) and 57.2 ± 12.3% (*n* = 7; P < 0.05) in the combined presence of LY83583 and, respectively, biliverdin (200 µM) and bilirubin (200 µM). Also exogenously administered SOD (1000 U/ml) partially protected exogenous NO-induced relaxations versus LY83583 (Fig V.3B). Co-infusion of a CO-saturated Krebs solution (CO_{inf},1 ml/min) did not affect EFS- or exogenous NO-induced relaxations in the presence of LY83583 (Fig V.3B).

Similar findings were observed with respect to the influence of CO and biliverdin/bilirubin on nitrergic relaxations in the presence of LY83583 in jejunal muscle strips (Fig V.4B, V.5C, V.5D).

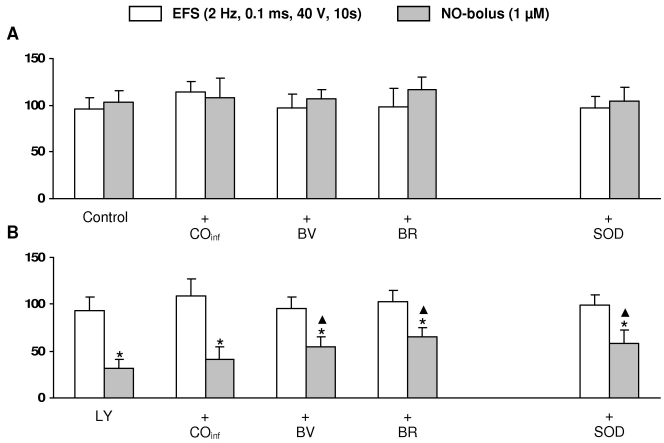

Figure V.4

Relaxant responses to EFS (2 Hz) and exogenous NO (1 µM) in murine jejunal circular smooth muscle strips: (A) the influence of $CO_{infusion}$ (1 ml/min), biliverdin (BV; 200 µM), bilirubin (BR; 200 µM), or SOD (1000 U/ml) on EFS- and NO-induced relaxations *per se*; (B) the influence of $CO_{infusion}$, BV, BR or SOD on the same relaxant stimuli in the presence of LY83583 (10 µM). Relaxant responses are expressed as a percentage of the response to the same stimulus before administration of interfering drugs. Means ± s.e.mean of n = 6-8 are shown. * *P* < 0.05: significantly different from the response in the same tissue before administration of drugs (Student's *t*-test); ▲ *P* < 0.05: significantly different from pretreatment with LY83583 alone (ANOVA with Bonferroni post-hoc test).

III.4.4 Influence of CO and biliverdin/bilirubin on nitrergic relaxations in the presence of the Cu/Zn depletor DETCA plus LY83583

The total Cu/Zn SOD activity in control preparations was 61.3 ± 8.1 U/ml in gastric muscle strips and 48.9 ± 7.8 U/ml in jejunal muscle strips (*n* = 6). After incubation with DETCA (1 mM), the Cu/Zn SOD activity was markedly reduced to, respectively, 14.4 ± 3.5 U/ml in gastric muscle strips and 8.9 ± 1.4 U/ml in jejunal muscle strips (*n* = 6; $P < 0.001$) confirming the effectiveness of the DETCA depletion method.

In gastric fundus muscle strips, incubation with DETCA (1 mM) had no influence *per se* on nitrergic relaxations elicited by EFS or exogenous NO (data not shown). After DETCA-pretreatment, the superoxide generator LY83583 (10 µM) now also significantly reduced EFS-induced NANC relaxations, whereas the inhibitory effect of LY83583 on relaxations induced by exogenous NO was even further increased (Fig V.2G, V.3C). The inhibitory effect of DETCA plus LY83583 on EFS- and exogenous NO-induced relaxations was not influenced by co-incubation with bile pigments nor co-infusion with a CO-saturated Krebs solution (Fig V.3C). In contrast, addition of exogenous SOD (1000 U/ml) partially reversed the inhibitory effect of DETCA plus LY83583 on EFS-induced NANC relaxations (Fig V.2H, V.3C). The combination DETCA plus LY83583 did not affect $PGF_{2\alpha}$-induced tone in murine gastric fundus muscle strips.

In jejunal muscle strips, incubation with DETCA (1 mM) had no influence *per se* on $PGF_{2\alpha}$-induced contractile activity nor on nitrergic relaxations induced by EFS or exogenous NO (Fig V.5B). However, the combination DETCA plus LY83583 nearly abolished basal and $PGF_{2\alpha}$-induced contractile phasic activity (from an area under the curve $AUC(PGF_{2\alpha})_{5min}$ = 123.3 ± 23.6 g.s in control conditions to $AUC(PGF_{2\alpha})_{5min}$ = 24.5 ± 8.0 g.s in the presence of DETCA plus LY83583). Therefore, nitrergic relaxations could not be studied in the presence of DETCA plus LY83583 in jejunal muscle strips (Fig V.5E). Remarkably, we observed that exogenously administered SOD (1000 U/ml) partially restored basal and $PGF_{2\alpha}$-induced contractile activity in the presence of DETCA plus LY83583 (from $AUC(PGF_{2\alpha})_{5min}$ = 24.5 ± 8.0 g.s in the presence of DETCA plus LY83583 to $AUC(PGF_{2\alpha})_{5min}$ = 71.6 ± 16.8 g.s when co-incubated with 1000 U/ml SOD; Fig V.5F).

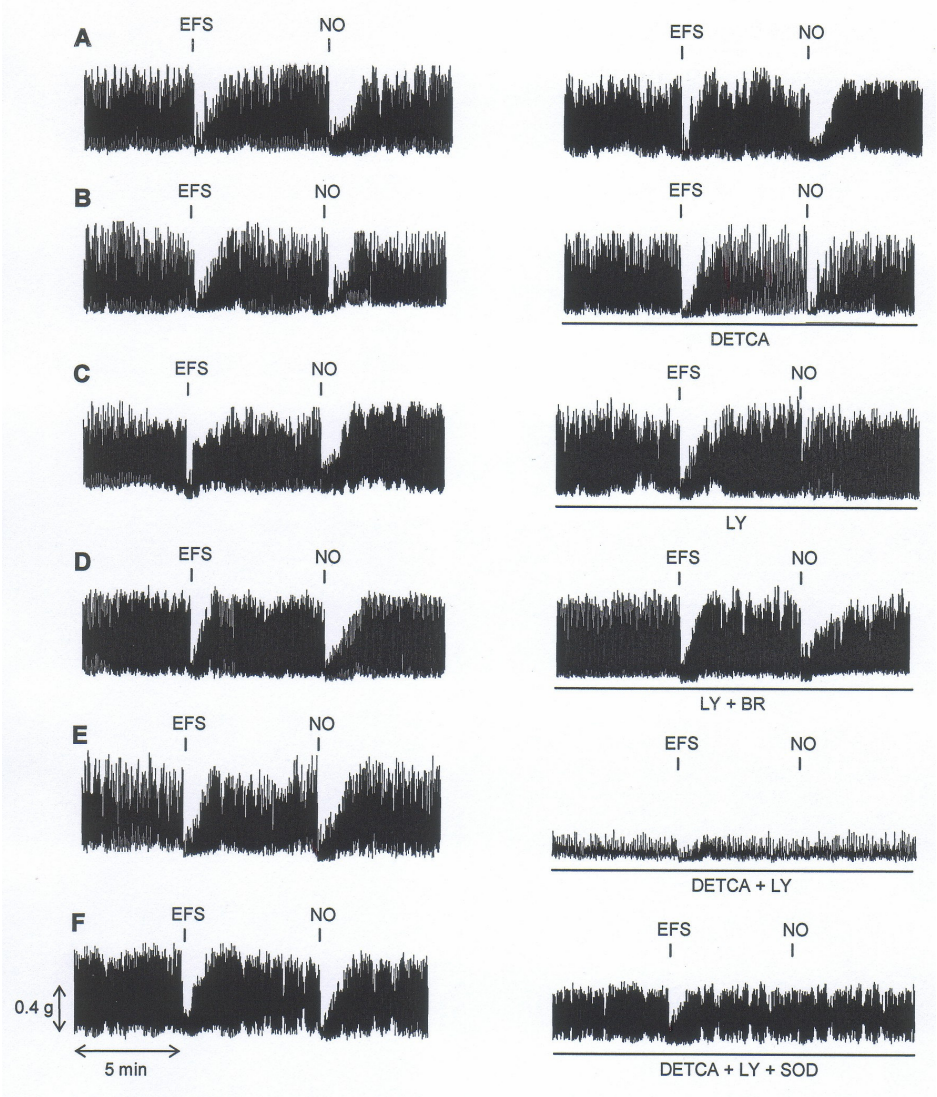

Figure V.5

Representative traces showing $PGF_{2\alpha}$ (300 nM)-induced contractile activity and the response to EFS (2Hz) and exogenous NO (1 µM) in murine jejunal circular smooth muscle strips: (A) control tissue showing the reproducibility of EFS- and NO-induced relaxations; (B): the influence of DETCA (1 mM) on EFS- and NO-induced relaxations; (C-D): the influence of LY83583 (10 µM) on EFS- and NO-induced relaxations and the partial protective effect of bilirubin (200 µM) on NO-induced relaxations in these conditions; (E-F): the detrimental effect of DETCA plus LY83583 on jejunal contractile activity and the partial protective effect of SOD (1000 U/ml) in these experimental conditions.

III.5 Discussion

In the present study, we investigated the role of the HO/BVR pathway in NANC neurotransmission and its possible interaction with nitrergic neurotransmission in the murine gastric fundus and jejunum.

Over the past decade, confusing data have been published regarding a possible neural role for CO in the enteric nervous system. Based on the effects of some non-selective HO inhibitors – also affecting NOS activity (Grundemar et al., 1997) – on EFS-induced NANC relaxations (Alcon et al., 2001; Rattan and Chakder, 1993; Zakhary et al., 1997) and the observation that neurally-evoked NANC relaxations of ileal and internal anal sphincter (IAS) muscle strips were reduced in HO-2$^{-/-}$ mice (Watkins et al., 2004; Zakhary et al., 1997), it was suggested that CO might function as an inhibitory neurotransmitters in the enteric nervous system. In contrast, Rattan et al. (2005) reported that EFS-induced NANC relaxations of IAS did not differ between WT and HO-2$^{-/-}$ mice and, thus, could not confirm a neural role for CO in NANC neurotransmission. More recent studies indicate that CO – generated by HO-2 in the ICCs – acts as an endogenous hyperpolarizing factor in the gastrointestinal tract (Farrugia et al., 2003; Sha et al., 2007). In this context, it has recently been shown that the selective HO inhibitor CrMP abolishes the resting membrane potential (RMP) gradient across the intestinal wall in murine circular smooth muscle strips (Appleton et al., 1999; Sha et al., 2007). Nevertheless, CrMP (10 µM) did not affect EFS-induced NANC relaxations of gastric and jejunal smooth muscle strips in our study, further supporting the view that HO/CO is not involved in NANC neurotransmission under normal physiological conditions.

As neurally-evoked NANC relaxations (0.5-8 Hz) were completely abolished by L-NAME (300 µM) in murine gastric fundus, we can assume that these responses are 100% nitrergic in origin. In jejunal muscle strips, EFS-induced NANC relaxations were fully suppressed by NOS inhibition at low frequencies (≤ 2 Hz) but residual twitch relaxations were still observed in the presence of L-NAME at higher frequencies (4-8 Hz). Very recently, it has been demonstrated that co-incubation of L-NAME with the purinoceptor blocker suramin abolishes EFS-induced NANC relaxations of jejunal muscle strips at all frequencies (1-8 Hz), indicating that these residual twitch relaxations in the presence of L-NAME are purinergic in origin (De Man et al., 2007).

To examine a possible modulatory effect of CO on nitrergic neurotransmission in gastric fundus and jejunum, we studied the relaxant responses to both endogenous and exogenous NO in the presence of the CO-releasing molecule (CORM)-2. We have previously shown that CORM-2 concentration-dependently relaxes gastric and jejunal muscle strips, at least in part, by the same mechanism as NO, i.e. activation of soluble guanylyl cyclase (sGC; De Backer and Lefebvre, 2007). In this study, CORM-2 was administered at a concentration of 30 µM,

inducing only moderate relaxation of $PGF_{2\alpha}$-induced tone. Surprisingly, we observed that relaxant responses to exogenous NO were markedly inhibited by the concomitant presence of CORM-2, while EFS-induced NANC relaxations were not affected. In order to further explore these findings, we also studied EFS- and exogenous NO-induced relaxant responses during continuous infusion of a CO-saturated Krebs solution. Remarkably, neither EFS- nor exogenous NO-induced relaxations were affected in these experimental conditions. This led us to the hypothesis that the transition metal compound, i.e. ruthenium, of the CO-releasing molecule was responsible for the inhibiting effect of CORM-2 on the exogenous NO-induced responses. Indeed, relaxations elicited by exogenous NO were also markedly inhibited in the presence of RuCl; this finding confirms other studies reporting the NO-scavenging properties of ruthenium (Hutchings et al., 2005; Marmion et al., 2004; Mosi et al., 2002). In parallel, other NO-scavengers (e.g. oxyhemoglobin, hydroxocobalamin) have been shown to affect exogenous NO-mediated relaxations but not EFS-evoked NANC relaxations (Selemidis et al., 1997; Colpaert et al., 2002c). Taken together, we can conclude that CO does not influence nitrergic responses in murine gastric fundus and jejunum. In contrast, CO has been reported to modulate the NOS/NO system in several other studies (Wu and Wang, 2005). While some studies indicated that CO could cause a release of NO from its 'intracellular pool' (Thorup et al., 1999; Thom et al., 1997), there is no report to date that CO can directly activate NOS. In contrast, it has been shown that CO can directly bind to and inactivate neuronal NOS (nNOS), endothelial NOS (eNOS) as well as inducible NOS (iNOS; Ding et al., 1999; Thorup et al., 1999; Turcanu et al., 1998; Willis et al. 1995). Also a possible reduction of the NOS/NO system due to competition between CO and NO for the same binding site on the sGC complex has been suggested (Ingi et al., 1996). However, the results in this study do not support any of these proposed interactions between the HO/CO and NOS/NO system.

Next, we investigated the effect of the bile pigments on nitrergic relaxations *per se* in murine gastric fundus and jejunum. Neither biliverdin nor bilirubin significantly affected EFS- or exogenous NO-induced relaxations in this study. In contrast, Colpaert and Lefebvre (2000) showed that bilirubin markedly enhanced the amplitude of relaxations induced by exogenous NO in pig gastric fundus smooth muscle strips. Although the explanation for this difference is unclear, we speculate that species differences may be involved. Moreover, Koglin et al. (2002) reported that biliverdin – but *not* bilirubin – potently inhibited basal and NO-stimulated sGC activity using a purified α1/β1 isoform of sGC. However, since biliverdin – unlike bilirubin – is not lipophilic and does not cross the cell membrane lipid bilayer (Maines, 2005), it may be possible that biliverdin was not able to reach the intracellular sGC apparatus in our experiments using circular smooth muscle strips.

As biliverdin/bilirubin have been shown to be potent antioxidants – due to their ability to scavenge reactive oxygen species (ROS; Stocker et al., 1987a, 1987b; Baranano et al., 2002) – we also investigated whether these bile pigments might protect free radical NO against superoxide anions generated by LY82583 (10 µM) in gastric fundus and jejunum (Abi-Gerges et al., 2001; Kubo et al., 2008; Støen et al., 1997). It has been shown before that superoxide generators inhibit relaxations induced by exogenous NO; however, are not capable of reducing EFS-evoked NANC relaxations (Barbier and Lefebvre, 1992; Colpaert et al., 2002c). In this study, we demonstrated that both exogenously administered biliverdin as well as bilirubin were capable of partially reversing the inhibitory effect of LY83583 on exogenous NO-induced relaxations in both tissues (bilirubin > biliverdin). These results suggest that both bile pigments might play an important role in the protection of the nitrergic neurotransmitter against oxidative stress in the gastrointestinal tract.

As the endogenous nitrergic neurotransmitter is protected by tissue antioxidants – fencing off superoxide attack by LY83583 – the same experimental protocols were also repeated in the presence of Cu-chelator DETCA (1 mM), irreversibly inhibiting endogenous Cu/Zn SOD. This antioxidant enzyme has been shown before to play an important role in the protection of the nitrergic neurotransmitter in several other tissues (Martin et al., 1994; Lefebvre, 1996; De Backer et al., 2004). As expected, treatment with DETCA rendered EFS-evoked NANC relaxations sensitive to the superoxide generator LY83583 in murine gastric fundus; however, neither biliverdin nor bilirubin were able to reverse the inhibitory action of DETCA plus LY83583 on EFS- and NO-induced relaxations. This lack of protection of nitrergic neurotransmission by biliverdin/bilirubin in these experimental conditions is unclear; however, we assume that neither biliverdin nor bilirubin can substitute for the loss of Cu/Zn SOD when this specific antioxidant enzyme is depleted by the Cu-chelator DETCA. In contrast, we observed a partial reversion of the inhibitory effect of DETCA plus LY83583 on EFS-induced NANC-relaxations, when gastric fundus muscle strips were co-incubated with exogenous SOD (1000 U/ml). In murine jejunal muscle strips, pretreatment with DETCA plus LY83583 nearly abolished basal and $PGF_{2\alpha}$-induced contractile phasic activity and, hence, did not allow studying nitrergic relaxations in their presence. But remarkably, we observed that co-incubation with exogenous SOD partially restored basal and $PGF_{2\alpha}$-induced contractile phasic activity in these experimental conditions. Interestingly, it has been shown before that pharmacological inhibition of Cu/Zn SOD exacerbated intestinal motility disturbances in an *in vitro* model of ischemia-reperfusion injury ('oxidative stress'), while the overexpression of this radical-scavenging enzyme in SOD transgenic mice increased the likelihood of functional recovery from this ischemia/reperfusion injury (Bielefeldt and Conklin, 1997).

In conclusion, our data do not establish a role for HO/CO in NANC neurotransmission in murine gastric fundus and jejunum under normal physiological conditions. However, the antioxidants biliverdin/bilirubin might play an important role in the protection of the nitrergic neurotransmitter against oxidative stress. Moreover, ruthenium-based CO-RMs should be used with caution when investigating nitrergic responses, due to NO-scavenging properties of their transition metal compound.

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Chapter III

MECHANISMS OF RELAXATION BY CARBON MONOXIDE-RELEASING MOLECULE-2 IN MURINE GASTRIC FUNDUS AND JEJUNUM

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Chapter IV Mechanisms of Relaxation by CORM-2 in Murine Gastric Fundus and Jejunum

IV.1 Abstract

This study investigated the effects and mechanisms of action of carbon monoxide-releasing molecule-2 (CORM-2), compared to those of carbon monoxide (CO), in murine gastric fundus and jejunal circular smooth muscle. Functional in vitro experiments and cGMP measurements were conducted.

In both tissues, CO and CORM-2 induced concentration-dependent relaxations. CO-induced relaxations were abolished by the soluble guanylyl cyclase (sGC) inhibitor ODQ, while CORM-2-evoked inhibitory responses were only partly prevented by ODQ. Relaxations elicited by CO (300 µM) were associated with a significant increase in cGMP levels, whereas for CORM-2 (300 µM) no significant increase in cGMP levels could be measured. The sGC sensitizer YC-1 was able to accelerate and potentiate both CO- and CORM-2-induced relaxations. Furthermore, the intermediate- and large-conductance Ca^{2+}-activated K^+ (IK_{Ca}-BK_{Ca}) channel blocker charybdotoxin significantly reduced CO- and CORM-2-induced relaxations in jejunal tissue; this same effect was observed with the BK_{Ca} channel blocker iberiotoxin. The combination of apamin plus charybdotoxin significantly reduced relaxations in gastric fundus and had synergistic inhibitory effects in jejunum. The NOS inhibitor L-NAME had no effect on the induced relaxations in gastric fundus, but significantly reduced CO- and CORM-2-evoked relaxations in jejunum.

In conclusion, these results demonstrate that CO and CORM-2 produce relaxation in gastric fundus and jejunum *via* sGC and activation of K_{Ca} channels, and a nitric oxide (NO)-mediated amplification of CO signaling in jejunum is suggested.

IV.2 Introduction

Carbon monoxide (CO) is produced endogenously by heme oxygenase (HO) enzymes as a product in the catabolism of heme to CO, biliverdin, and iron. It has been shown that CO exerts potent anti-inflammatory effects, prevents apoptosis, and promotes protection against hyperoxic as well as ischemic injury (Gibbons and Farrugia, 2004; Ryter et al., 2006). The recent discovery that certain transition metal carbonyls function as CO-releasing molecules has made it possible to deliver CO in a more practical and accurate way to the target site, compared to delivery of CO as gas. These characteristics have accelerated the use of these molecules in pharmacological research (Clark et al., 2003; Foresti et al., 2004; Motterlini et al., 2002, 2005). When one considers the use of CO-releasing molecules as therapeutic drugs, the effects on gastrointestinal smooth muscle will have to be taken into account.

Previous studies have shown that CO relaxes gastrointestinal smooth muscle and a possible role in inhibitory neurotransmission has been suggested (Battish et al., 2000; Miller et al., 2001; Gibbons and Farrugia, 2004; Ny et al., 1996; Rattan et al., 1993; Xue et al., 2000; Zakhary et al., 1997). However, more recent findings indicate that CO does not play a significant role in the non-adrenergic non-cholinergic (NANC) relaxation (Rattan et al., 2005). On the other hand, it has been demonstrated that HO-2 knockout mice have depolarized intestinal smooth muscle cells and that the membrane potential gradient in the gut (along the long axis of the gastrointestinal tract as well as across the gut wall) is abolished. As HO-2 is abundantly present in the interstitial cell networks associated with the enteric plexuses, Farrugia et al. (2003) hypothesized that interstitial cells of Cajal (ICCs) might be the source of the hyperpolarizing CO. Thus, CO may function as a messenger molecule between ICCs and smooth muscle cells (Farrugia et al., 2003; Miller et al., 1998).

As the effects of CO-releasing molecules on the gastrointestinal tract are still largely unknown and CO-releasing molecules do not always behave as authentic CO in vascular tissue (Motterlini et al., 2002; Musameh et al., 2006), the aim of this study was to investigate the effects and mechanisms of action of the fast CO-releasing molecule-2 (CORM-2) ($[Ru(CO)_3Cl_2]_2$) - in comparison with those of CO - in two different gastrointestinal regions, namely murine gastric fundus (a tissue with *tonic* activity) and jejunum (a tissue with *phasic* activity).

IV.3 Materials & Methods

IV.3.1 Animals

Male Swiss (SPF Orl) mice (6-8 weeks, 28-40 g) were purchased from Janvier, Le Genest St-Isle, France. All experimental procedures were approved by the Ethical Committee for Animal Experiments, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium.

IV.3.2 Tissue preparation & mounting

Experiments were carried out on isolated circular smooth muscle strips of the murine gastric fundus and jejunum. Mice were killed by cervical dislocation and the stomach and a ± 8 cm long segment of jejunum starting 5 cm distally from the ligament of Treitz were removed. Two full-thickness muscle strips (2 x 15 mm) were prepared from the gastric fundus by cutting in the direction of the circular muscle layer. The jejunum was opened along the mesenteric border and pinned mucosa side up. The mucosa was removed by sharp dissection under a microscope and full-thickness muscle strips (4 x 7 mm) were cut along the circular axis. The muscle strips were mounted in 10 ml organ baths that were filled with Krebs solution (composition in mM: NaCl 188.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1), maintained at 37℃ and aer ated by a mixture of 95 % O_2 and 5% CO_2. The experiments were carried out in the continuous presence of atropine (1 µM) and guanethidine (4 µM) to block respectively cholinergic and noradrenergic responses. Changes in tension were measured using Radnoti isometric transducers (Radnoti, Monrovia, USA) and recorded with Chart software and a PowerLab/8sp data recording system (AD Instruments, U.K.). The strips were brought to the optimal point of length-tension relationship (gastric fundus at 1.4 g; jejunum at 0.2 g – as determined in preliminary experiments) and then allowed to equilibrate for 60 min with rinsing every 15 min before starting the experiment.

IV.3.3 Functional experiments

After the equilibration period, all strips were pre-contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 300 nM). In gastric fundus, $PGF_{2\alpha}$ induced an increase in tone and when a stable plateau contraction was obtained after ± 5 min, a single concentration of the relaxant stimulus CO (10-300 µM) or CORM-2 (30-600µM) was applied. Cumulative application of these relaxant stimuli was avoided, since the $PGF_{2\alpha}$-induced contraction progressively declined after ± 5 min. Responses to CO and CORM-2 were reproducible, and neither the

solvent of CORM-2 (dimethyl sulfoxide, DMSO) nor the CORM-2 control compound ruthenium (III) chloride hydrate ($RuCl_3$) had any influence per se on $PGF_{2\alpha}$-induced tone. To study the influence of the soluble guanylyl cyclase inhibitor 1*H*-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) on CO- or CORM-2-induced relaxation, gastric fundus strips were repetitively rinsed for 60 min and ODQ (10 µM) or its solvent (ethanol) was incubated for 20 min. Tone was raised again with $PGF_{2\alpha}$ and when a stable plateau contraction was obtained, the relaxant stimulus was repeated. In a similar way, the influence of the soluble guanylyl cyclase sensitizer 1-benzyl-3-(5'-hydroxymethyl-2'-furyl-)-indazol (YC-1; 10 µM), the nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 300 µM), the ATP-sensitive K^+ channel blocker glibenclamide (10 µM), the small conductance Ca^{2+}-activated K^+ (SK_{Ca}) channel blocker apamin (500 nM), the intermediate- and large-conductance Ca^{2+}-activated K^+ (IK_{Ca}-BK_{Ca}) channel blocker charybdotoxin (100 nM) or some combinations were tested *versus* a single concentration of CO (300 µM) or CORM-2 (300 µM).

In jejunal strips, $PGF_{2\alpha}$ induced an increase of contractile phasic activity and when a stable level of phasic activity was obtained after ± 10 min, CO (10-300 µM) or CORM-2 (30-600µM) was applied cumulatively with 5 min intervals. The concentration-response curve to CO was reproducible. Thus, to study the influence of the inhibitors mentioned above and the large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel blocker iberiotoxin (100 nM) on CO-induced relaxation, jejunal muscle strips were repetitively rinsed for 60 min and the inhibitor(s) or its solvent(s) was/were incubated for 20 min. $PGF_{2\alpha}$ was applied again and when a stable level of phasic activity was obtained, a second concentration response-curve to CO was obtained. As preliminary experiments showed that responses to CORM-2 (30-600µM) were not reproducible, the influence of all inhibitors was tested by constructing one concentration-response curve to CORM-2 in parallel muscle strips, in the presence of the inhibitor(s) or their solvent(s). Parallel control strips, studied in the absence of inhibitor(s) or their solvent(s), received CORM-2 or its solvent DMSO (0.3-60µl), as cumulative administration of DMSO yielded per se a moderate inhibitory effect on jejunal $PGF_{2\alpha}$-induced phasic activity (16.72 ± 3.28% with 60µl DMSO). The inhibitory effects of CORM-2 in jejunal muscle strips were always corrected for this DMSO-effect. CORM-2 control compound ruthenium (III) chloride hydrate had no influence per se on $PGF_{2\alpha}$-induced phasic activity.

IV.3.4 cGMP analysis

Circular muscle strips of gastric fundus and jejunum were prepared as described above, except that Krebs solution also contained the phosphodiesterase-5 inhibitor zaprinast (10 µM). Strips were pre-contracted with $PGF_{2\alpha}$ (300nM) and 5 min (gastric fundus) or 10 min later (jejunum), strips were administered CO (300 µM), CORM-2 (300 µM) or nothing (basal

conditions). Upon maximal relaxation (i.e. 90 sec after administration for CO and 3 min for CORM-2) or the corresponding moment in the basal strip, strips were snap-frozen in liquid nitrogen and stored at – 80 ℃ until further proces sing. The cGMP content in each muscle strip was determined using an enzyme immunoassay kit (cGMP Biotrak EIA System, Amersham Biosciences, UK). The tissue cGMP concentration was expressed as pmol/gram tissue wet weight (as measured before mounting the tissues).

IV.3.5 Data analysis

In gastric fundus, CO and CORM-2 induced tonic relaxant responses; all peak relaxations were expressed as percentage of $PGF_{2\alpha}$-induced tone. In jejunum, CO and CORM-2 induced a decrease in phasic activity; this was expressed as percentage reduction of $PGF_{2\alpha}$-induced phasic activity (by measuring the area under the curve (AUC) for 3 min before ($AUC_{PGF2\alpha}$) and 3 min after application of CO (AUC_{CO}) or CORM-2 (AUC_{CORM}) and calculating the % relaxation according to the formula [($AUC_{PGF2\alpha}$–$AUC_{CO(RM)}$)/$AUC_{PGF2\alpha}$]*100). Responses to CO or CORM-2 in the presence of interfering drugs (or its solvents) are related to those obtained before administration of these drugs; except for jejunal muscle strips, whereby relaxant responses to CORM-2 in the presence of inhibitors (or its solvents) are related to those obtained in a parallel control strip. Experimental data are expressed as means ± SEM. Results within tissues of the same animal are compared by a paired *t*-test. When more than 2 groups have to be compared, one-way analysis of variance (ANOVA) is performed; if statistical significance is reached ($P < 0.05$), comparison per 2 groups is performed by a *t*-test, corrected for multiple comparisons (Bonferroni procedure). A difference is considered statistically significant at $P < 0.05$.

IV.3.6 Drugs & Materials

The following drugs were used: atropine, glibenclamide, guanethidine sulphate, L-NAME, PGF2α, ruthenium (III) chloride hydrate, tricarbonyldichlororuthenium(II)dimer (CORM-2) (all obtained from Sigma), ODQ (from Tocris Cookson), YC-1 (from Alexis biochemicals), apamin, charybdotoxin, iberiotoxin (from Alomone Labs), zaprinast (from UCB). All drugs except CORM-2, glibenclamide, ODQ, YC-1 and zaprinast were dissolved in deionised water. Glibenclamide (3 mM stock) and ODQ (10 mM stock) were dissolved in 100% ethanol. CORM-2 (100 mM stock) and YC-1 (10 mM stock) were dissolved in DMSO. Zaprinast (10 mM stock) was dissolved in 2% triethanolamine. Further dilutions were made with distilled water. A saturated CO solution (1 mM) was prepared from CO gas as described by Schröder et al. (2002). CORM-2 was always freshly prepared before administration.

IV.4 Results

IV.4.1 Effects of CO and CORM-2 in gastric fundus

IV.4.1.1 Influence of ODQ, YC-1 and L-NAME

In gastric fundus, both CO and CORM-2 induced concentration-dependent relaxations amounting to respectively 43.6 ± 5.6% (300 µM CO) and 38.8 ± 3.4% (300 µM CORM-2) of $PGF_{2\alpha}$-induced tone (Fig III.1A, III.1C). CORM-2 control compound ruthenium (III) chloride hydrate had no influence on $PGF_{2\alpha}$-induced tone (n = 4; data not shown). CO-induced relaxations were abolished by 10 µM ODQ (Fig III.1B, III.2A). In contrast, ODQ (10 µM) significantly reduced – but did *not* abolish - the relaxant responses to CORM-2 (Fig III.1D, III.2D); the reduction of the response to 300 µM CORM-2 was not more pronounced with 100 µM ODQ (reduction of CORM-2 (300 µM)-induced responses to respectively 53.7 ± 4.5% by 10 µM ODQ and 56.2 ± 5.3% by 100 µM ODQ; n = 4).

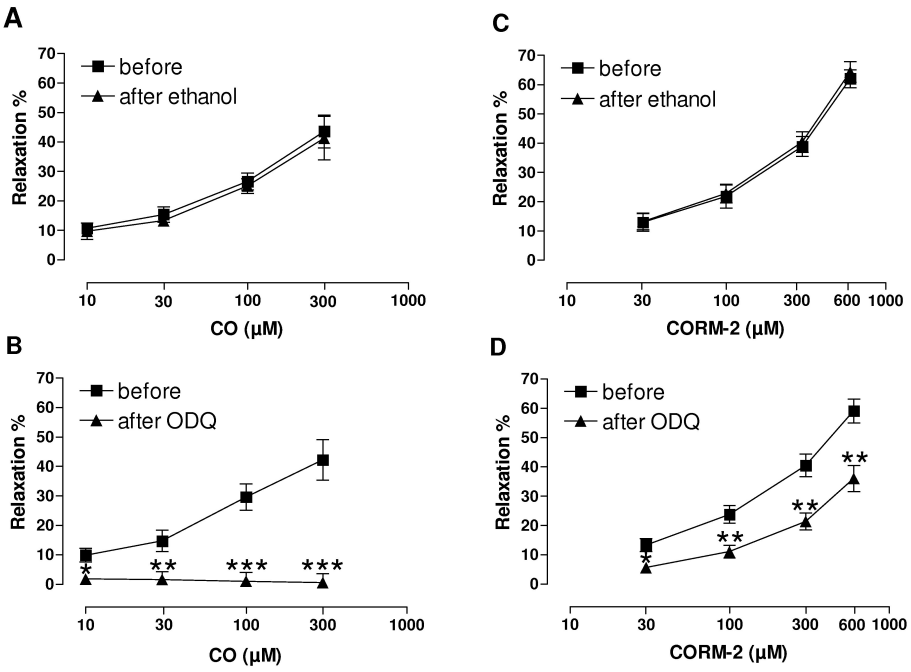

Figure III.1

Effects of exogenously applied CO (10-300 µM) and CORM-2 (30-600 µM) in gastric fundus. Relaxations induced by exogenous CO and CORM-2 before (■) and after the incubation (▲) with 10 µM ODQ (B, D) or its solvent ethanol (A, C). The response measured was expressed as a percentage of the $PGF_{2\alpha}$-induced contraction plateau before administration of CO or CORM-2. Means ± s.e.mean of n = 6 are shown. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$: significantly different *vs*. before.

YC-1 (10 µM) accelerated and potentiated both CO- and CORM-2-induced relaxations to respectively 216.0 ± 19.2% (300 µM CO) and 147.3 ± 8.0% (300 µM CORM-2; Fig III.2A, III.2D, III.4B, III.4D). In the presence of ODQ, YC-1 was not able to enhance the effect of neither CO nor CORM-2; thereby ODQ (10µM) inhibited CO- and CORM-2-evoked relaxations to the same extent as in the absence of YC-1 (Fig III.2A, III.2D). L-NAME (300 µM) had no inhibiting effect on CO- and CORM-2-induced relaxations in gastric fundus, and the presence of L-NAME did not prevent the potentiating effect of YC-1 (Fig III.2B, III.2E). ODQ, YC-1 and L-NAME did not affect basal or PGF2α-induced tone of the preparations.

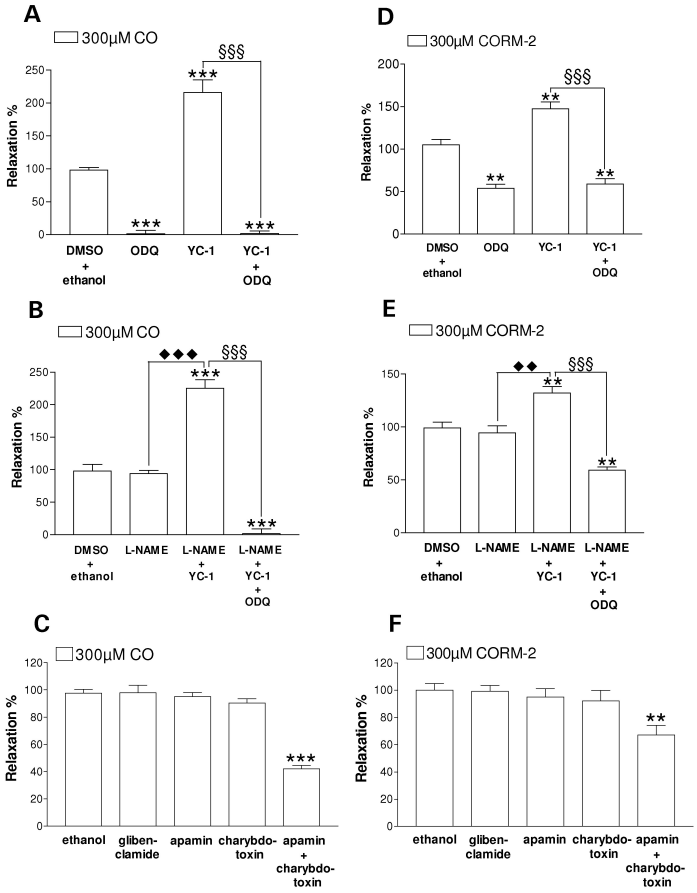

Figure III.2

Effects of exogenously applied CO (300 µM) and CORM-2 (300 µM) in gastric fundus. Panel A, D: influence of DMSO + ethanol (parallel time-control), ODQ (10 µM), YC-1 (10 µM), and YC-1 + ODQ on relaxant responses induced by CO (A) and CORM-2 (D). Panel B, E: influence of DMSO + ethanol (parallel time-control), L-NAME (300 µM), L-NAME + YC-1 (10 µM), and L-NAME + YC-1 + ODQ (10 µM) on CO (B)- and CORM-2 (E)-evoked relaxations. Panel C, F: influence of ethanol (parallel time-control), glibenclamide (10 µM), apamin (500 nM), charybdotoxin (100 nM), and apamin + charybdotoxin on relaxant responses induced by CO (C) and CORM-2 (F). Relaxant responses are expressed as a percentage of the response to the same stimulus before administration of interfering drugs. Means ± s.e.mean of n = 4-8 are shown. ** $P < 0.01$, *** $P < 0.001$: significantly different from the response in parallel time-control (ethanol ± DMSO); ♦♦ $P < 0.01$, ♦♦♦ $P < 0.001$: significantly different from the response in the presence of L-NAME; §§§ $P < 0.001$: significantly different from the response in the presence of L-NAME + YC-1 or YC-1 alone (ANOVA followed by Bonferroni multiple comparison *t*-test).

The relaxations elicited by 300 µM CO were associated with a significant increase in cGMP levels, but - remarkably - CORM-2 (300 µM)-evoked relaxations were *not* accompanied with a measurable increase in cGMP levels (Fig III.3A).

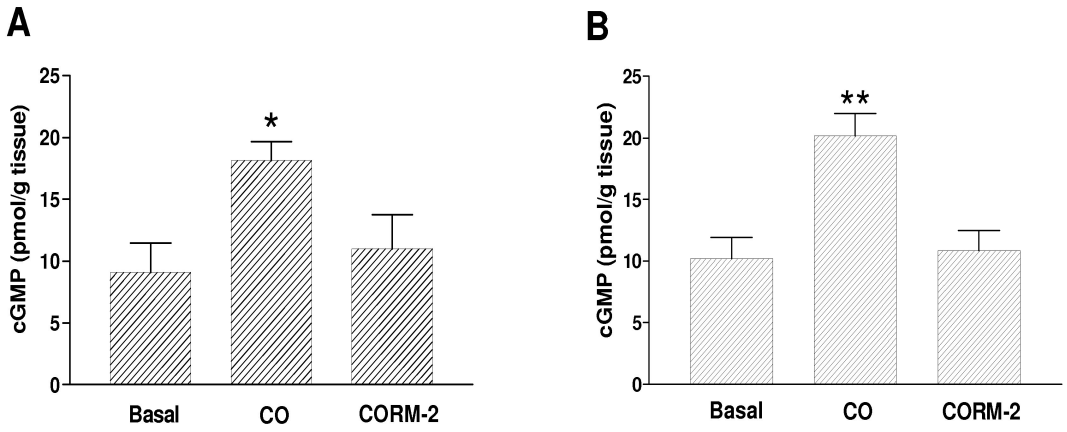

Figure III.3

cGMP levels in gastric fundus (A) and jejunum (B) in basal conditions (parallel control) and stimulated with CO (300 µM) and CORM-2 (300 µM). Means ± s.e.mean of n = 4-6 are shown. * $P < 0.05$, ** $P < 0.01$: significantly different from basal (parallel control; ANOVA followed by Bonferroni multiple comparison t-test). Influence of K^+-channel blockers.

IV.4.1.2 Influence of K^+-channel blockers

Glibenclamide (10 µM), apamin (500 nM) and charybdotoxin (100 µM) had no effects on CO- and CORM-2-induced relaxations in gastric fundus. However, the combination of apamin plus charybdotoxin caused significant inhibition of the relaxations evoked by CO and CORM-2 (to respectively 42.0 ± 4.6 % for 300 µM CO and 67.2 ± 7.1 % for 300 µM CORM-2; Fig III.2C, III.2F). The combination of ODQ (10 µM) with apamin plus charybdotoxin did not cause a more pronounced inhibition of CORM-2-induced relaxations than did ODQ alone (to respectively 53.7 ± 4.5% by ODQ and 51.9 ± 6.2% by ODQ + apamin + charybdotoxin, n = 6, data not shown). None of the K+-channel blockers influenced basal or PGF2α-induced tone.

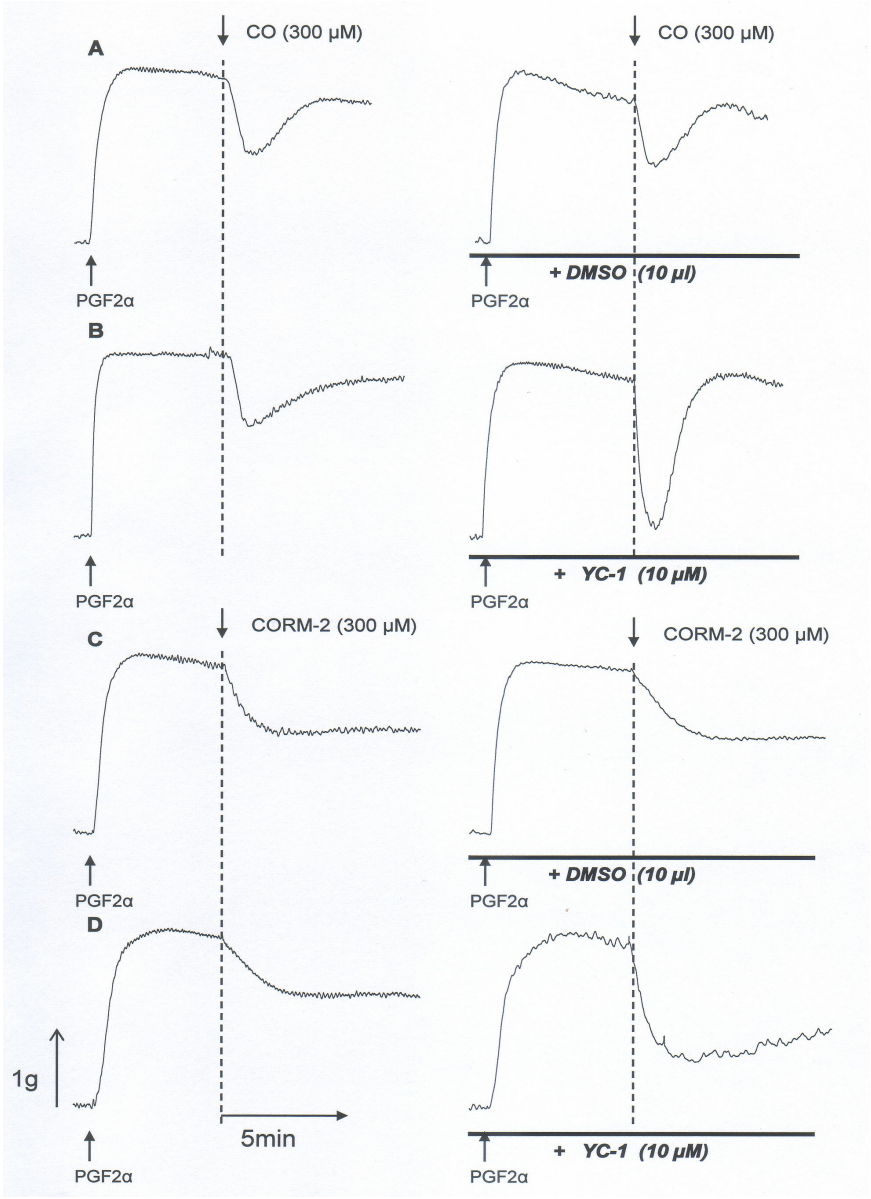

Figure III.4

Original recordings showing the responses to CO (300 µM) and CORM-2 (300 µM) in gastric fundus before and in the presence of DMSO (10 µl, parallel time-control, A, C), and before and in the presence of YC-1 (10 µM, B, D). Maximal relaxations are reached ± 3 min after administration of CORM-2, whereas CO-induced relaxations already reach maximal relaxation after ± 90 sec. After reaching maximum, the effect of CORM-2 was sustained, whereas CO-induced relaxations are almost completely reversed after ± 5 min. Five min prior to the addition of CO or CORM-2, gastric fundus muscle strips were pre-contracted with 300 nM $PGF_{2\alpha}$.

IV.4.2 Effects of CO and CORM-2 in jejunum

IV.4.2.1 Influence of ODQ, YC-1 and L-NAME

Both CO and CORM-2 evoked concentration-dependent inhibitory responses in the murine jejunal circular muscle, whereas CORM-2 control ruthenium (III) chloride hydrate had no influence on $PGF_{2\alpha}$-induced contractile phasic activity (n = 4; data not shown). Also in jejunum, ODQ (10 µM) abolished CO-induced relaxations, but only partly prevented CORM-2-evoked inhibitory responses (Fig III.5A, III.5D). Relaxations elicited by 300 µM CO were associated with a significant increase in cGMP levels; whereas for CORM-2 (300 µM) no significant increase in cGMP levels could be measured (Fig III.3B). In contrast with gastric fundus, CO- and CORM-2-induced responses were partly inhibited by L-NAME (300µM; Fig III.5B, III.5E, III.6D). As incubation with L-NAME increased basal (spontaneous) phasic activity in murine jejunal preparations, a lower concentration of $PGF_{2\alpha}$ (starting with 10nM) was added and then progressively increased ($PGF_{2\alpha}$ 10-300nM) as required, until a stable degree of phasic activity (AUC_{PGF2a}) equal to that *before* incubation of L-NAME was obtained. In this way, the level of phasic activity induced by $PGF_{2\alpha}$ did not alter within the same jejunal strip *before* and *after* the incubation of L-NAME ($AUC_{PGF2\alpha}$-*after*/$AUC_{PGF2\alpha}$-*before*)*100 = 96.3 ± 7.6 %). Moreover, we also tested the influence of L-NAME on NO-induced relaxations in the same experimental conditions and, thereby, we could not show any influence of L-NAME on NO-induced relaxations (relaxant response of NO (10 µM) was 95.6 ± 7.3 % of the response to the same stimulus before administration of L-NAME, n = 6, data not shown). Thus, the possibility that the reduction of CO- and CORM-2-induced inhibitory responses by L-NAME occurs as a consequence of an increase in mechanical activity can be excluded. The evaluation of jejunal strips incubated with YC-1 (10µM) alone was not possible, as YC-1 reduced heavily the $PGF_{2\alpha}$-induced phasic activity ($AUC_{PGF2\alpha}$), probably due to potentialisation of the basal inhibitory effect by endogenously produced NO. Only by combining YC-1 with L-NAME, $PGF_{2\alpha}$ was able to induce the same level of phasic activity as usual. In the presence of L-NAME, YC-1 resulted in a significant acceleration and potentialisation of both CO- and CORM-2-induced relaxations, compared to the response in the presence of L-NAME alone (Fig III.5B, III.5E, III.6E). The addition of ODQ abolished the CO-evoked relaxations despite the presence of YC-1, and reduced CORM-2-evoked relaxations to the same extent as in the absence of YC-1 (Fig III.5B, III.6F).

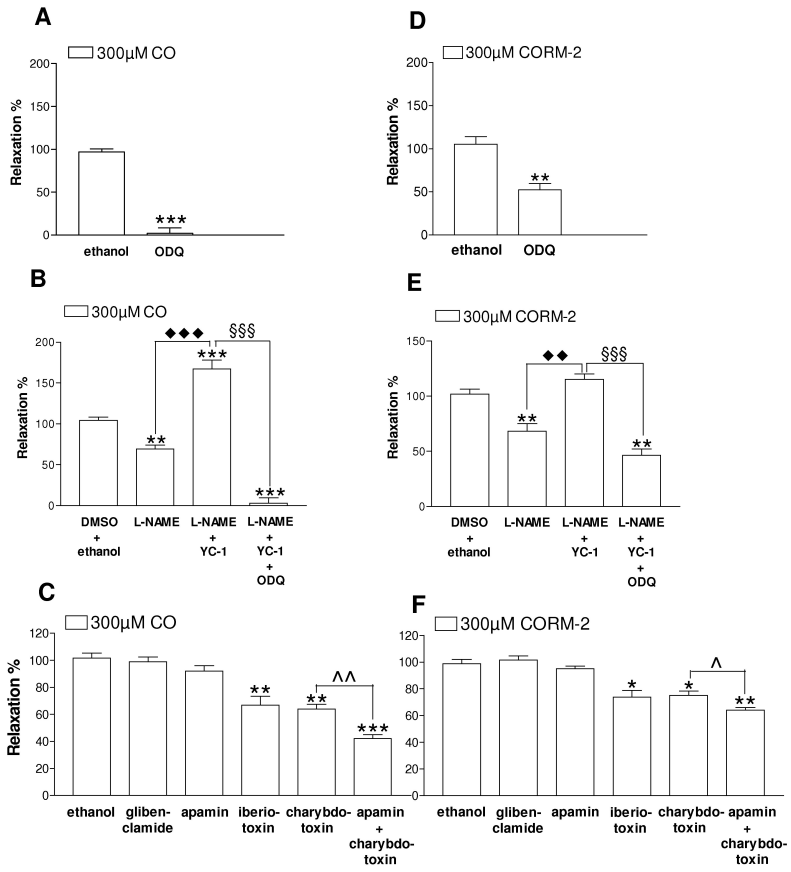

Figure III.5

Effects of exogenously applied CO (300 µM) and CORM-2 (300 µM) in jejunum. Panel A, D: influence of ethanol (parallel control) and ODQ (10 µM) on relaxant responses induced by CO (A) and CORM-2 (D). Panel B, E: influence of DMSO + ethanol (parallel control), L-NAME (300 µM), L-NAME + YC-1 (10 µM), and L-NAME + YC-1 + ODQ (10 µM) on CO (B) and CORM-2 (E)-evoked relaxations. Panel C, F: influence of ethanol (parallel control), glibenclamide (10 µM), apamin (500 nM), iberiotoxin (100 nM), charybdotoxin (100 nM), and apamin + charybdotoxin on relaxant responses induced by CO (C) and CORM-2 (F). *Panel A-C*: Relaxant responses are expressed as a percentage of the response to the same stimulus before administration of interfering drugs. *Panel D-F*: Relaxant responses are expressed as a percentage of the response to the same stimulus in a parallel strip without administration of interfering drugs. Means ± s.e.mean of n = 4-8 are shown. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$: significantly different from the response in parallel control (ethanol ± DMSO) ; ♦♦ $P < 0.01$, ♦♦♦ $P < 0.001$: significantly different from the response in the presence of L-NAME; §§§ $P < 0.001$: significantly different from the response in the presence of L-NAME + YC-1; ^ $P < 0.05$, ^^ $P < 0.01$: significantly different from the response in the presence of charybdotoxin alone (ANOVA followed by Bonferroni multiple comparison *t*-test).

IV.4.2.2 Influence of K^+-channel blockers

Treatment with glibenclamide (10 µM) or apamin (500 nM) had no effects on CO- and CORM-2-evoked relaxations in jejunum. But - in contrast with gastric fundus - charybdotoxin (100 nM) per se significantly reduced CO- and CORM-2-induced relaxations; this same effect was observed with iberiotoxin (100nM). Combination of apamin plus charybdotoxin caused a more pronounced inhibition than charybdotoxin alone (Fig III.5C,

III.5F). The addition of apamin plus charybdotoxin to ODQ (10 µM) had no additional inhibitory effect on CORM-2-evoked responses, in comparison with ODQ alone (reduction of CORM-2 (300 µM)-induced responses to respectively 52.0 ± 7.6% by ODQ and 48.7 ± 5.3% by ODQ + apamin + charybdotoxin, n = 6, data not shown). Similarly to L-NAME in jejunum, all K_{Ca} channel blockers markedly raised basal phasic activity in jejunal strips, and therefore, in the presence of K_{Ca} channel blockers, a lower concentration of $PGF_{2\alpha}$ was added to ensure a level of phasic activity equal to that before incubation of the K_{Ca} channel blockers.

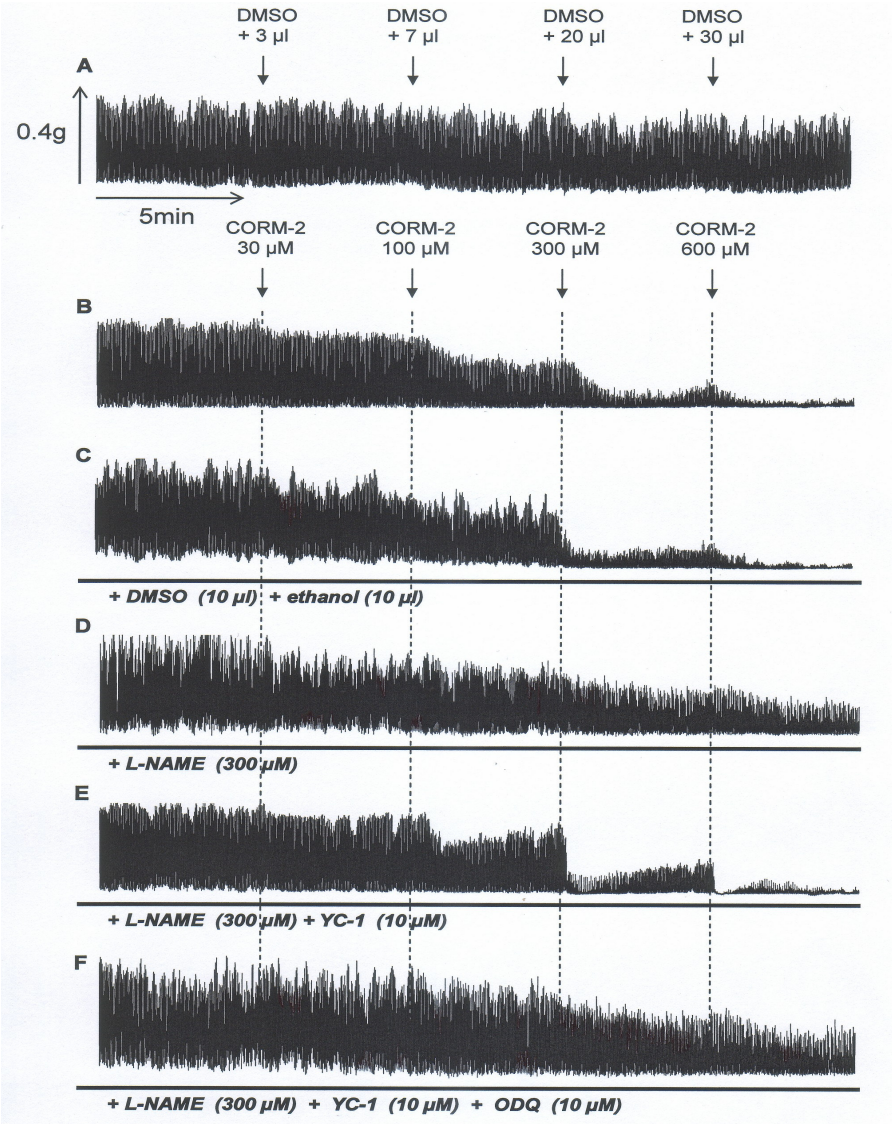

Figure III.6

Representative traces showing the responses to cumulative addition of DMSO (3-60µl, A) in jejunum; and the responses to cumulative addition of CORM-2 (30-600 µM) in the absence of any interfering drug (B), in the presence of DMSO (10µl) + ethanol (10µl; C), and in the presence of L-NAME (300µM; D), L-NAME + YC-1 (10µM; E), and L-NAME + YC-1 + ODQ (10µM; F). About 10 min prior to the addition of CORM-2 (30 µM) or DMSO (3 µl), all jejunal muscle strips were activated with 300 nM $PGF_{2\alpha}$.

IV.5 Discussion

This study reports for the first time the relaxant effects and mechanisms of action of CORM-2 - in comparison with those of CO - in murine gastric fundus and jejunal smooth muscle. As CORM-2 control ruthenium (III) chloride hydrate had no influence on $PGF_{2\alpha}$-induced tone or contractile activity, we could confirm that CO is indeed the mediator of the observed pharmacological effects by CORM-2 in this study. In the gastrointestinal tract, exogenous CO has been shown to relax smooth muscle of lower oesophagogastric junction, gastric fundus, small intestine and internal anal sphincter of different species. Similar to nitric oxide (NO), CO has been demonstrated to act *via* activation of sGC, leading to an increase in intracellular cGMP (Colpaert et al., 2002; Farrugia et al., 1998; Kwon et al., 2001; Rattan et al., 2004; Werkström et al., 1997). Our study confirms the involvement of sGC in CO-evoked responses: (1) the sGC inhibitor ODQ abolished CO-induced relaxations; (2) the sGC sensitizer YC-1 was able to accelerate and potentiate CO-evoked inhibitory responses; and (3) the relaxant response evoked by CO (300µM) was associated with a significant increase in cGMP. With regard to the mechanisms of relaxation by CORM-2, we observed some remarkable differences as CORM-2-induced relaxations were only *partly* prevented by ODQ, and *no* significant increase in cGMP could be measured for CORM-2.

The observation that ODQ only *partly* reduced CORM-2-induced relaxations (by 40-50%) in murine gastric fundus and jejunum is similar to results obtained by Motterlini et al. (2002) and Rattan et al. (2004) in rat aorta and internal anal sphincter. These authors suggested that ODQ causes competitive antagonism of CORM-2-evoked relaxations. On the other hand, these results could suggest that the relaxant effect by CORM-2 is not solely mediated by activation of sGC, but also by other (independent) mechanisms working in parallel. This latter hypothesis seems supported by our observation that a higher concentration of ODQ (100 µM) did not inhibit CORM-2-induced responses in a more pronounced way than 10 µM ODQ. In addition, the observation that CORM-2 did *not* cause a significant increase in cGMP might further strengthen this possibility. The reason why CORM-2 did not induce a significant cGMP increase might alternatively be related to the more progressive delivery of CO by CORM-2, in comparison to CO given in bolus; or to a different subcellular localization ('compartmentalized increase') of cGMP raised by CO given in bolus or progressively released by CORM-2 (Mergia et al., 2006). There are indeed several examples where equally sized nitrergic relaxant responses do not induce a similar degree of cGMP increase (e.g. Garcia-Pascual & Triguero, 1994). Interestingly, vasodilatation in rat aorta elicited by CORM-3 was found to be accompanied by a small cGMP increase following a first and second addition of CORM-3 (100µM), but addition of a third bolus of CORM-3 did not change

cGMP levels compared to a control strip, even though a higher level of vasorelaxation was measured (Foresti et al., 2004).

Although it is well established that the relaxant effect of CO on smooth muscle involves sGC activation, the more 'downstream' mechanisms are less well known. Some possible mechanisms of CO-induced relaxation in the GI tract, that are not mutually exclusive, have been proposed: (1) a sGC/cGMP-dependent decrease in Ca^{2+} sensitivity of contractile elements (Kwon et al., 2001); and/or (2) the modulation of whole cell K^+ currents and hyperpolarization of the smooth muscle cell membrane (Farrugia et al., 1993, 1998, 2003). In our study, we observed that neither the ATP-sensitive K^+ channel blocker glibenclamide, nor the SK_{Ca} channel blocker apamin influenced CO-induced relaxations. In jejunum, the IK_{Ca}-BK_{Ca} channel blocker charybdotoxin per se partly reduced CO-induced relaxations; this same effect was observed with the more specific BK_{Ca} channel blocker iberiotoxin. Only a combination of apamin plus charybdotoxin significantly reduced CO-evoked relaxations in gastric fundus, and had a more pronounced inhibitory effect than charybdotoxin alone in jejunum. Thus, as ODQ abolished CO-induced relaxations - whereas a combination of K_{Ca} channel blockers only partly reduced CO-evoked relaxations - we can conclude that: (1) K_{Ca} channel activation is sequential to cGMP generation; and (2) the mechanism of relaxation 'downstream' of sGC/cGMP involves not only activation of K_{Ca} channels, but also other mechanisms such as decreasing Ca^{2+} sensitivity of the contractile apparatus or a still unknown mechanism. The K_{Ca} channel blockers apamin and charybdotoxin yielded similar inhibitory effects versus CORM-2 in gastric fundus and jejunum. As ODQ only partially reduced the effects of CORM-2, CORM-2 might possibly activate K_{Ca} channels in a cGMP-independent way, as reported for NO in rat and murine duodenum (Martins et al., 1995; Serio et al., 2003). However, as the combination of ODQ with apamin plus charybdotoxin did not cause a more pronounced inhibition of CORM-2-induced relaxations than did ODQ alone, we can exclude the direct activation of K_{Ca} channels by CORM-2 as a mechanism working in parallel to sGC activation (see above). The requirement for the combination of apamin plus charybdotoxin to reduce relaxations in gastric fundus and their synergistic inhibitory effects seen in jejunum can be explained as follows: at least two channels, an IK_{Ca} and/or BK_{Ca} channel blocked by charybdotoxin and a SK_{Ca} channel blocked by apamin, are involved in the relaxant effect of CO and CORM-2 - whereby an interplay between these different K_{Ca} channels might occur (Doughty et al., 1999); alternatively, Zygmunt et al. (1997) reported that apamin can significantly enhance charybdotoxin binding. Thus it is possible that, by combining the two K^+ channel blockers, apamin increases charybdotoxin binding *via* an allosteric effect, rather than by acting independently to block an apamin-sensitive channel.

One of the most surprising findings of this study is that the relaxant properties of CO and CORM-2 are significantly reduced in jejunum, when nitric oxide synthase (NOS) activity is blocked by L-NAME. Importantly, this inhibitory effect by L-NAME does not occur in gastric fundus, raising the possibility that this difference should be ascribed to a cell type that is absent or altered in the gastric fundus. Interestingly, ICCs in the fundus are morphologically different from ICCs in the rest of the gastrointestinal tract and have a different function (Burns et al., 1996; Farrugia et al., 2003). ICCs generate rhythmic changes in membrane potential and are essential for GI motility (Huizinga et al., 1995; Ward et al., 1994). The murine small intestine shows strong HO-2-immunoreactivity in ICCs, whereas ICCs in the fundus do not express significant amounts of HO-2. It has been suggested by Farrugia et al. (2003) that HO-2 expressed by ICCs is probably the major source for endogenous CO in the small intestine and that CO released from these cells acts as a smooth muscle hyperpolarizing factor. Taken all together, as both HO-2 and NOS are present in small intestine ICCs (Berezin et al., 1994; Donat et al., 1999; Matini et al., 1997; Miller et al., 1998) and an amplification pathway (involving NOS/NO) in the ICCs has been described (Publicover et al., 1993), we hypothesize that CO signaling in jejunum might be amplified by NOS/NO in the ICCs, similar to the observation by Lim et al. (2005) that CO can activate the NOS/NO/cGMP pathway in intestinal smooth muscle cells.

In conclusion, our study demonstrates that CO and CORM-2 produce relaxation in murine gastric fundus and jejunal smooth muscle *via* sGC and subsequent activation of K_{Ca} channels, whereby an interplay between K_{Ca} channels might occur. In contrast with gastric fundus, both CO- and CORM-2-induced relaxations were significantly inhibited by L-NAME in jejunum, suggesting a NO-mediated amplification of CO signaling in the jejunum. These results are of importance when we consider the use of CO-releasing molecules as therapeutic drugs in inflammatory disorders.

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Chapter IV

ROLE OF SOLUBLE GUANYLYL CYCLASE α1/α2 SUBUNITS IN THE RELAXANT EFFECT OF CO AND CORM-2 IN MURINE GASTRIC FUNDUS

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Chapter V Role of Soluble Guanylyl Cyclase α1/α2 Subunits in the Relaxant Effect of CO and CORM-2 in Murine Gastric Fundus

V.1 Abstract

Carbon monoxide (CO) has been shown to cause enteric smooth muscle relaxation by activating soluble guanylyl cyclase (sGC). In gastric fundus, the $sGC\alpha_1\beta_1$ heterodimer is believed to be the most important isoform. The aim of our study was to investigate the role of the $sGC\alpha_1/\alpha_2$ subunits in the relaxant effect of CO and CORM-2 in murine gastric fundus using wild-type (WT) and $sGC\alpha_1$ knockout (KO) mice.

In WT mice, CO (bolus)-induced relaxations were abolished by the sGC inhibitor ODQ, while CORM-2- and CO (infusion)-induced relaxations were only partially inhibited by ODQ. In $sGC\alpha_1$ KO mice, relaxant responses to CO and CORM-2 were significantly reduced when compared with WT mice, but ODQ still had an inhibitory effect. The sGC sensitizer YC-1 was able to potentiate CO- and CORM-2-induced relaxations in WT mice, but lost this potentiating effect in $sGC\alpha_1$ KO mice. Both in WT and $sGC\alpha_1$ KO mice, CO-evoked relaxations were associated with a significant cGMP increase; however, basal and CO-elicited cGMP levels were markedly lower in $sGC\alpha_1$ KO mice.

These data indicate that besides the predominant $sGC\alpha_1\beta_1$ isoform also the less abundantly expressed $sGC\alpha_2\beta_1$ isoform plays an important role in the relaxant effect of CO in murine gastric fundus; however, the sGC stimulator YC-1 loses its potentiating effect towards CO in $sGC\alpha_1$ KO mice. Prolonged administration of CO – either by the addition of CORM-2 *or* by continuous infusion of CO – mediates gastric fundus relaxation in both a sGC-dependent and sGC-independent manner.

V.2 Introduction

Carbon monoxide (CO), which is classically thought of as a toxic molecule, has been increasingly recognized as an important signaling molecule with diverse physiological functions (Wu and Wang 2005; Ryter et al. 2006). Mammalian cells generate CO *via* the endogenous degradation of heme by a family of constitutive (HO-2) and inducible (HO-1) heme oxygenase enzymes. Endogenous CO has been shown to be a regulator of vascular tone in (patho)physiological conditions and appears to be a major hyperpolarizing factor in the gastrointestinal (GI) tract (Gibbons and Farrugia 2004; Wu and Wang 2005). In addition, exogenously applied CO exerts potent anti-inflammatory, anti-oxidative and anti-apoptotic effects (Ryter et al. 2006). The recent discovery of CO-releasing molecules (CO-RMs) has accelerated the use of these molecules in pharmacological research and resulted into a new class of potential therapeutics (Motterlini et al. 2007). However, when one considers the use of CO-RMs as therapeutic drug, their effects on enteric smooth muscle will have to be taken into account as CO is well known to cause direct smooth muscle relaxation in a number of GI preparations – including oesophagus, gastric fundus, small intestine and internal anal sphincter – when administered exogenously (Ny et al. 1996; Farrugia et al. 1998; Colpaert et al. 2002; Rattan et al. 2004).

Similar to nitric oxide (NO), CO can activate soluble guanylyl cyclase (sGC), although CO stimulates sGC activity only 4-fold *vs*. 200-fold by NO stimulation (Kharitonov et al. 1995). In a previous study, we demonstrated that the CO-sGC interaction is critical for the relaxant effect of CO and plays a major role in the relaxant effect of CORM-2 in murine gastric fundus (De Backer and Lefebvre 1997). sGC is a heterodimeric hemoprotein composed of a larger α (sGCα) and a smaller β (sGCβ) subunit, both necessary for catalytic activity (Harteneck et al. 1990). Two isoforms for each subunit (α_1/α_2 and β_1/β_2) have been identified in various species (Harteneck et al. 1991). Theoretically, the association of the α and β subunits could result in four different heterodimers; however, only the sGC$\alpha_1\beta_1$ (cytosolic) and sGC$\alpha_2\beta_1$ (membrane-associated) isoforms are reported to be physiologically active (Russwurm et al. 1998, 2001). In the brain, the levels of both isoforms are comparable, but in all other tissues the sGC$\alpha_1\beta_1$ isoform is predominant (Mergia et al. 2003). Due to the lack of sGC isoform-specific inhibitors, little is known about the specific role and relative importance of the sGC isoforms in CO-induced responses. Recently developed sGCα_1 knockout (KO) mice allow to explore this (Buys et al. 2008). In the present study, we investigated the functional importance of both sGC isoforms in the relaxant effect of CO and CORM-2 using gastric fundus muscle strips from wild type (WT) and sGCα_1 KO mice.

V.3 Materials & Methods

V.3.1 Animals

All experiments were performed on male wild type (WT) and homozygous soluble guanylyl cyclase α_1 knockout ($sGC\alpha_1$ KO) mice, bred in the Department of Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology, Ghent, Belgium (genetic background: mixed Swiss-129; 25-40 g). The $sGC\alpha_1$ KO mice lack exon 6 of the $sGC\alpha_1$ gene, which codes for an essential part in the catalytic domain (Buys et al. 2008). Mice were kept and cared for in standard cages in a 12-12h light-darkness cycle with free access to water and pellets. All experimental procedures were approved by the Ethical Committee for Animal Experiments, Faculty of Medicine and Health Sciences, Ghent University, Belgium.

V.3.2 Tissue preparation & mounting

Mice were killed by cervical dislocation, the stomach was removed, and two circular smooth muscle strips (2 mm x 15 mm) were prepared from the gastric fundus. The muscle strips were mounted in 10 ml organ baths that were filled with Krebs solution (composition in mM: NaCl 188.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1), maintained at 37°C and aerated by 95% O_2/5% CO_2. The experiments were carried out in the continuous presence of atropine (1 µM) and guanethidine (4 µM) to block respectively cholinergic and noradrenergic responses. Changes in tension were measured using Radnoti isometric transducers (Radnoti, Monrovia, USA) and recorded with Chart software and a PowerLab/8sp data recording system (AD Instruments, U.K.). The strips were brought to their optimal point of length-tension relationship (1.5 g) and then allowed to equilibrate for 60 min with rinsing every 15 min before starting the experiment.

V.3.3 Functional experiments

After the equilibration period, all strips were pre-contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 300 nM). When a stable plateau contraction was obtained after ± 5 min, a single bolus of CO (100-4300 µL of a CO-saturated (1 mM) Krebs solution) *or* CORM-2 (3-60 µL of a 100 mM stock) was applied, yielding organ bath concentrations of 10-300 µM (CO) *or* 30-600 µM (CORM-2). Next, strips were repetitively rinsed for 40 min and interfering drugs were then incubated for 20 min. Tone was raised again with $PGF_{2\alpha}$ and the responses to CO *or* CORM-2 were studied again in the presence of the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ, 10 µM), the soluble guanylyl cyclase stimulator 1-benzyl-3-(5'-hydroxymethyl-2'-furyl-)-indazol (YC-1, 10 µM), *or* the protein kinase

G inhibitor KT5823 (1 µM); KT5823 was also tested versus the relaxant effects of NO (1-30 µM). In a similar way, we also studied the influence of the particulate guanylyl cyclase inhibitors cystamine (1 mM) and phorbol 12-myristate 13-acetate (PMA; 10 µM), the adenylyl cyclase inhibitor SQ22536 (10 µM), as well as their combination with ODQ (10 µM) on the relaxant responses evoked by CORM-2. The reproducibility of the responses to CO *or* CORM-2 was evaluated by running time-control strips in parallel that received the solvent of these interfering drugs.

In a separate series of experiments, CO was continuously administered to the gastric tissues for 10 min by infusing a CO-saturated Krebs solution (1 mM) into the organ bath via a Braun infusion pump (CO_{inf}, 5 ml/min). As the half-life of CO is not known in these experimental settings, we can not calculate the obtained concentration in the organ bath. The influence of ODQ (10 µM) on the relaxant response evoked by CO_{inf} was studied in a similar way as described above.

V.3.4 cGMP analysis

Circular muscle strips of gastric fundus were prepared and mounted as described above, except that each muscle strip was still divided into two (resulting in 4 muscle strips of 2 mm x 7.5 mm per stomach) and Krebs solution also contained the phosphodiesterase 5 inhibitor zaprinast (10 µM). Strips were pre-contracted with $PGF_{2\alpha}$ (300nM; basal condition) and subsequently relaxed by administration of CO (300 µM), CORM-2 (300 µM) *or* CO_{inf}. Strips were snap-frozen in liquid nitrogen in basal condition (basal), at maximal relaxation by CO, CORM-2 *or* CO_{inf} (i.e. respectively 90 sec after administration for CO *vs*. 3 min for CORM-2 and CO_{inf}), or 10 min after application of CORM-2 *or* CO_{inf} as these stimuli induced sustained relaxations. These experiments were also conducted after pre-incubation with ODQ (10 µM). The cGMP content in each muscle strip was determined using an enzyme immunoassay kit (cGMP Biotrak EIA System, Amersham Biosciences, UK). Tissue cGMP concentrations were expressed as pmol/gram tissue wet weight (as measured before mounting the tissues).

V.3.5 Western Blotting analysis

Gastric fundus muscle tissues were homogenized in denaturing cell extraction buffer (Invitrogen), subjected to NuPAGE electrophoresis on Novex 4-12% Bis-Tris gels (Invitrogen) and subsequently transferred to nitrocellulose membranes (Amersham Bioscience). Blots were incubated with the following antibodies: rabbit polyclonal antibody specific for $sGC\alpha_1$ (dilution 1:2500), $sGC\alpha_2$ (dilution 1:200), $sGC\beta_1$ (dilution 1:500) and β-tubulin (dilution 1:500) (Abcam). After incubation with anti-rabbit IgG HRP-linked antibody

(Cell signaling Technology), blots were developed by SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and quantification of the bands was done by densitometry (ImageJ). To determine $sGC\alpha_2$ in gastric fundus, protein homogenates were further ultracentrifuged at 100,000*g* (60 min, 4℃) to obtain the isolated membrane frac tion.

V.3.6 Data analysis

Maximal relaxation by CO (90 sec after administration) and CORM-2 (3-5 min after administration) was measured. For CO_{inf}, relaxation was measured at 1 min intervals during the 10 min of infusion. The relaxant responses were expressed as percentage of $PGF_{2\alpha}$-induced tone. Experimental data are expressed as means ± SEM. Comparison between WT and $sGC\alpha_1$ KO mice *or* between parallel tissues of either WT or $sGC\alpha_1$ KO mice was done with an unpaired Student's *t*-test. Comparison within tissues of either WT or $sGC\alpha_1$ KO mice was done by a paired Student's *t*-test. A difference is considered statistically significant at $P < 0.05$.

V.3.7 Drugs & Materials

The following drugs were used: atropine sulphate, cystamine, guanethidine sulphate, PMA, $PGF_{2\alpha}$, $RuCl_3$, SQ22536, CORM-2 (tricarbonyldichlororuthenium(II)dimer; all obtained from Sigma), KT5823 (from Calbiochem), ODQ (from Tocris Cookson), YC-1 (from Alexis biochemicals), zaprinast (from UCB). All drugs except CORM-2, ODQ, YC-1 and zaprinast were dissolved in deionised water. CORM-2 (100 mM stock), ODQ (10 mM stock), YC-1 (10 mM stock) and KT5823 (1 mM stock) were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the organ baths did not exceed 0.6 % and had no effects on $PGF_{2\alpha}$-induced tone. CORM-2 was always freshly prepared before administration into the organ bath. Zaprinast (10 mM stock) was dissolved in 2% triethanolamine. Further dilutions were made in deionised water. Saturated CO (1mM) or NO (2mM) solutions were prepared by bubbling deoxygenated Krebs solution with, respectively, 99.9% CO or NO gas (De Backer and Lefebvre 2007). The CO-saturated Krebs solution also contained $PGF_{2\alpha}$ (300 nM) and was maintained at 37℃.

V.4 Results

V.4.1 Protein expression of the sGC subunits in gstric fundus

In order to validate the phenotype of $sGC\alpha_1$ KO mice, protein expression levels of the three sGC subunits were determined in gastric fundus. Remarkably, $sGC\alpha_2$ protein was not measurable in whole tissue homogenates (data not shown) and could only be detected in the isolated membrane fraction of gastric tissue samples. As shown in Fig IV.1A, $sGC\alpha_1$ protein was completely absent in $sGC\alpha_1$ KO mice. The targeted deletion of $sGC\alpha_1$ also led to a moderate reduction in $sGC\beta_1$ protein expression, but did not affect $sGC\alpha_2$ protein expression when compared with gastric fundus of WT mice. The quantitative analysis of the signals obtained in several immunoblots is shown in Fig IV.1B. However, as $sGC\alpha_2$ protein was only detected in the membrane fraction and the antibodies differ in sensitivity, the signals detected for the $sGC\alpha_1$ and $sGC\alpha_2$ subunit can not be compared directly.

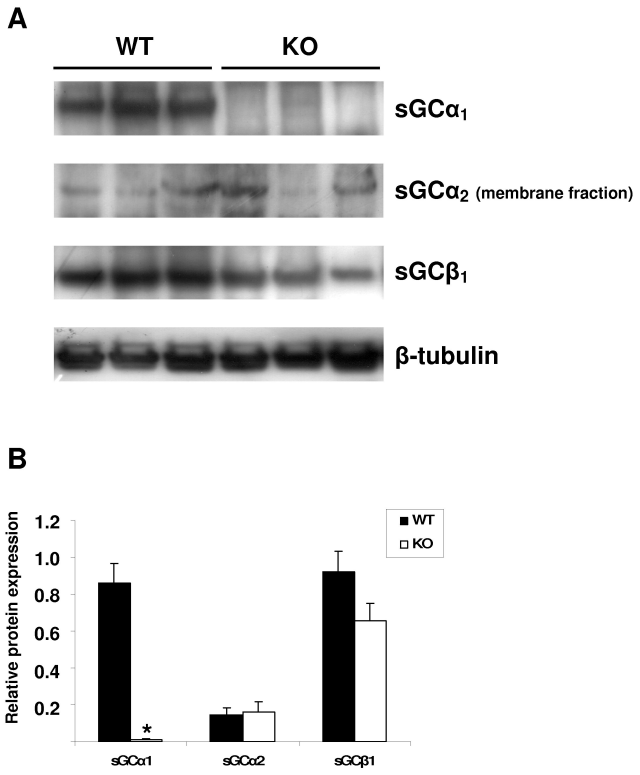

Figure IV.1

Protein expression levels of the soluble guanylyl cyclase (sGC) subunits in murine gastric fundus. (**a**) Representative immunoblots showing the protein expression of $sGC\alpha_1$, $sGC\beta_1$, β-tubulin (*whole homogenates*) and $sGC\alpha_2$ (*membrane fraction*) in murine gastric fundus. (**b**) Quantification of protein levels after normalisation to β-tubulin. Means ± SEM of n = 5-6 are shown. * $P < 0.05$: KO *vs*. WT (unpaired Student's *t*-test).

V.4.2 Inhibitory responses to CO

The administration of CO induced concentration-dependent, short-lasting relaxations amounting to respectively 44.4 ± 6.1 % in WT and 18.8 ± 3.3 % in $sGC\alpha_1$ KO strips for 300 µM CO (n = 6; P < 0.05: KO *vs*. WT; Fig. 2A). These responses were abolished by the sGC inhibitor ODQ (10 µM) in both WT and $sGC\alpha_1$ KO strips (Fig IV.2B). The sGC stimulator YC-1 (10 µM) significantly accelerated and potentiated CO-induced relaxations in WT strips (to 91.2 ± 7.2 % for 300 µM CO; n = 6), but had no potentiating effect in $sGC\alpha_1$ KO strips (Fig IV.2C, IV.3A). Responses to CO were reproducible in control strips, and none of the interfering drugs tested affected basal *or* $PGF_{2\alpha}$-induced tone of the preparations.

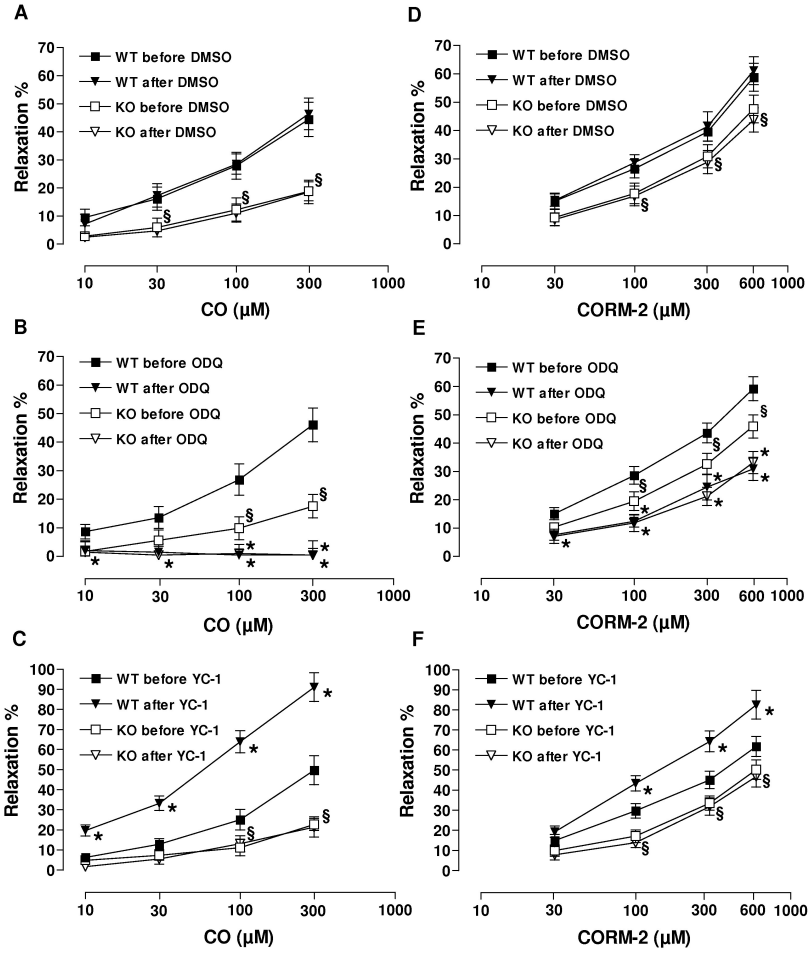

Figure IV.2

Relaxant effects of exogenously applied CO (10-300 µM) and CORM-2 (30-600 µM) before (■,□) and after the incubation (▼,▽) with ODQ (10 µM; **b, e**), YC-1 (10 µM; **c, f**), *or* their solvent DMSO (**a, d**) in gastric fundus of wild-type (WT, black symbols) and knock-out (KO, open symbols) mice. Data are expressed as % relaxation of $PGF_{2\alpha}$-induced tone. Means ± SEM of n = 6 are shown. * *P* < 0.05: after ODQ/YC-1 *vs*. before ODQ/YC-1 (paired Student's *t*-test); § *P* < 0.05: KO before *vs*. WT before (unpaired Student's *t*-test).

V.4.3 Inhibitory responses to CORM-2

The administration of CORM-2 induced concentration-dependent, sustained relaxations to respectively 39.6 ± 3.4 % in WT and 30.9 ± 4.1 % in sGCα_1 KO strips for 300 µM CORM-2 (n = 6; P < 0.05: KO *vs*. WT; Fig IV.2D). Neither the solvent of CORM-2 (DMSO) nor the CORM-2 control compound ruthenium chloride hydrate ($RuCl_3$) had any influence per se on $PGF_{2\alpha}$-induced tone, and thus confirmed CO as the mediator of the observed pharmacological effects. In contrast with CO-evoked responses, CORM-2-induced relaxations were significantly reduced, but *not* abolished, by 10 µM ODQ (from 43.6 ± 3.5 % to 24.5 ± 4.6 % in WT strips and from 32.6 ± 3.8 % to 21.2 ± 3.2 % in sGCα_1 KO strips for 300 µM CORM-2; n = 6; Fig IV.2E, IV.3B); the reduction of CORM-2-induced responses was not more pronounced with 100 µM ODQ (n = 4; data not shown).

In an attempt to block the more downstream protein kinase G (PKG) of the sGC/cGMP/PKG pathway, strips were pre-incubated with the PKG inhibitor KT5823. However, KT5823 (1 µM) did not affect relaxations evoked by CORM-2, nor did it influence CO- or NO-evoked relaxations (n = 4; data not shown). As the role of PKG in NO-induced gastric relaxation has unequivocally been proven in cGKI (PKG) knockout mice (Ny et al. 2000), we can suggest that KT5823 may not be useful as a reliable PKG inhibitor in these experimental conditions.

In addition, the particulate guanylyl cyclase (pGC) inhibitors cystamine (10 mM) and PMA (10 µM), as well as the adenylyl cyclase (AC) inhibitor SQ22536 (10 µM) did not affect CORM-2-induced relaxations in murine gastric fundus. Also the combination of these pGC and AC inhibitors with ODQ could not inhibit CORM-2-evoked relaxations in a more pronounced way than ODQ alone (n = 4; data not shown).

Pre-incubation with YC-1 (10 µM) significantly accelerated and potentiated CORM-2-mediated responses in WT strips, but did not influence CORM-2-induced relaxations in sGCα_1 KO strips (Fig IV.2F). Responses to CORM-2 were reproducible in control strips, and none of the interfering drugs tested affected basal *or* $PGF_{2\alpha}$-induced tone of the gastric fundus muscle preparations.

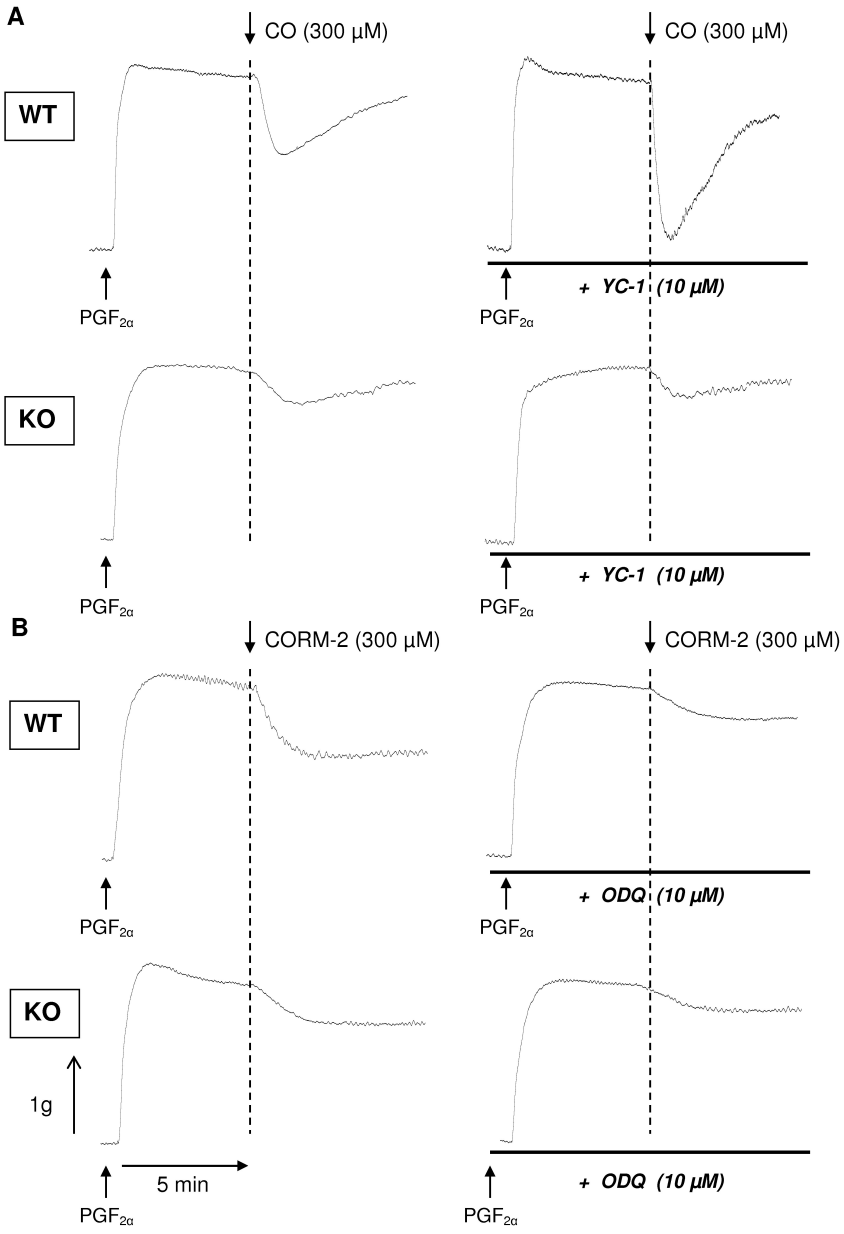

Figure IV.3

Original recordings showing the response to CO (300 µM) before and in the presence of YC-1 (10 µM, **a**) and the response to CORM-2 (300 µM) before and in the presence of ODQ (10 µM, **b**) in gastric fundus of wild-type (WT) and knock-out (KO) mice. Maximal relaxation was reached ± 90 sec after administration of CO, whereas CORM-2-induced relaxations reached maximal relaxation after ± 3-5 min. The relaxant effect of CO was almost completely reversed after ± 5 min, whereas CORM-2 induced sustained relaxations. Five min prior to the addition of CO or CORM-2, gastric fundus muscle strips were pre-contracted with $PGF_{2\alpha}$ (300 nM).

V.4.4 Inhibitory responses to continuous infusion of CO

In WT strips, continuous infusion of CO (CO_{inf}) induced a progressive relaxation during the first 2-3 min of infusion (relaxation of 72.3 ± 6.2 % at 3 min of infusion) and this relaxation was then sustained during the entire infusion-period (relaxation of 66.8 ± 5.1 % at 10 min of infusion). In $sGC\alpha_1$ KO strips, the relaxant response to CO_{inf} occurred at a markedly slower rate (relaxation of 35.6 ± 6.4 % at 3 min of infusion) and the amplitude of the sustained relaxation was slightly but significantly reduced compared to WT mice (relaxation of 52.1 ± 4.8 % at 10 min of infusion; Fig IV.4A). The sGC inhibitor ODQ (10 µM) abolished the response to CO_{inf} during the first 2 min of infusion, but relaxation then slowly occurred reaching 38.7 ± 4.6 % and 34.9 ± 4.0 % in, respectively, WT and $sGC\alpha_1$ KO strips at 10 min of infusion (Fig IV.4B). Responses to CO_{inf} were reproducible in control strips, and $PGF_{2\alpha}$-induced tone returned back to its original level within 2-3 min after CO_{inf} was ceased.

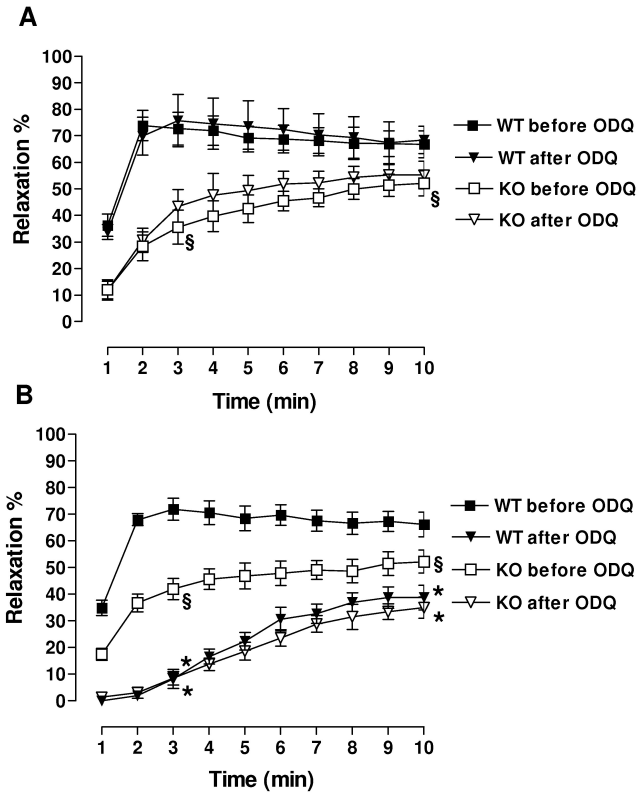

Figure IV.4

Responses – measured at 1 min interval – to continuous infusion (10 min) of a saturated CO-solution (CO_{inf}) before (■,□) and after the incubation (▼,▽) with ODQ (10 µM; **b**) *or* its solvent DMSO (**a**) in gastric fundus of wild-type (WT, black symbols) and knock-out (KO, open symbols) mice. Data are expressed as % relaxation of $PGF_{2\alpha}$-induced tone. Means ± SEM of n = 6 are shown. Statistical analysis was only performed for the results obtained after 3 min and 10 min of CO_{inf}. * $P < 0.05$: after ODQ *vs*. before ODQ (paired Student's *t*-test); § $P < 0.05$: KO before *vs*. WT before (unpaired Student's *t*-test).

V.4.5 cGMP analysis

Both in WT and sGCα_1 KO strips, cGMP levels were significantly increased by CO given in bolus (300 µM); however, basal and CO-elicited cGMP levels were markedly lower in sGCα_1 KO strips (Fig IV.5A). In contrast, cGMP levels were only slightly but not significantly increased by CORM-2 (300 µM) in both WT and sGCα_1 KO strips (Fig IV.5D). In the presence of ODQ (10 µM), basal cGMP levels were markedly lower in WT strips, and no cGMP increase by CO nor CORM-2 could be measured (Fig IV.5B, IV.5E). When WT strips were pre-incubated with YC-1 (10 µM), basal cGMP levels were slightly higher and the cGMP increase elicited by CO was markedly potentiated (Fig IV.5C). Moreover, YC-1 resulted in a significant increase in cGMP levels generated by CORM-2, when WT strips were snap-frozen 10 min after the administration of CORM-2 (Fig IV.5F). Remarkably, YC-1 did not potentiate the cGMP increase by CO, nor did it influence cGMP levels in response to CORM-2 in sGCα_1 KO strips (Fig V.5C, IV.5F).

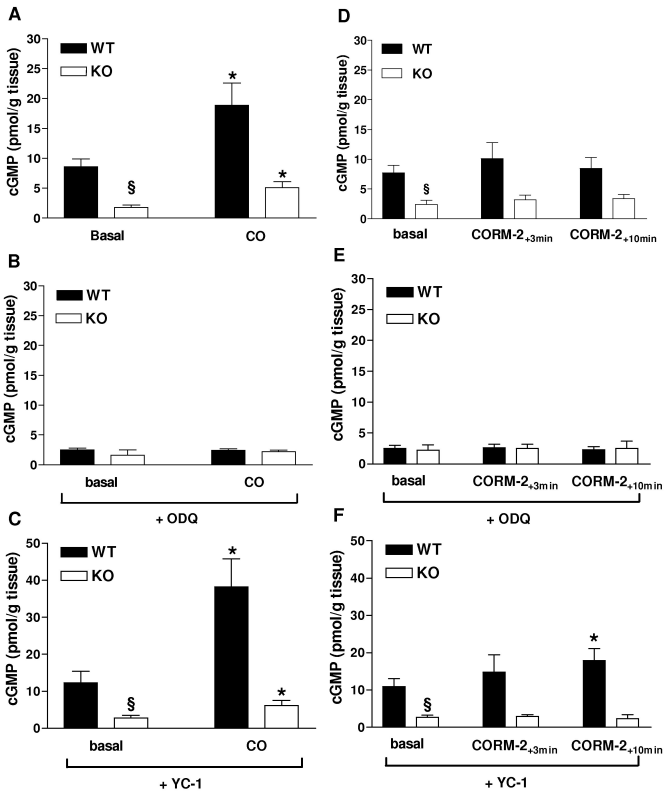

Figure IV.5

cGMP levels in gastric fundus of wild-type (WT, black bars) and knock-out (KO, open bars) mice in basal conditions (basal, parallel control) and stimulated with CO (300 µM, **a-c**) or CORM-2 (300 µM, **d-f**). Panel **a, d**: in the absence of any interfering drug. Panel **b, e**: in the presence of ODQ (10 µM). Panel **c, f**: in the presence of YC-1 (10 µM). Strips were snap-frozen at maximal relaxation by CO or CORM-2 (i.e. respectively 90 sec after administration for CO *vs.* 3 min for CORM-2$_{+3min}$) *or* 10 min after application of CORM-2$_{+10min}$ – as CORM-2 induced sustained relaxations. Means ± SEM of n = 5-6 are shown. * $P < 0.05$: significantly different from basal (unpaired Student's *t*-test); § $P < 0.05$: KO basal *vs*. WT basal (unpaired Student's *t*-test).

Continuous infusion of CO (CO_{inf}) increased cGMP levels, with a similar increase after 3 min (25.1 ± 3.8 pmol/g tissue) and 10 min (26.2 ± 2.9 pmol/g tissue) of infusion (Fig IV6A). In the presence of ODQ (10 µM), basal cGMP levels were again lower in WT strips, and no increase in cGMP levels could be measured, neither at 3 min nor at 10 min of CO_{inf} (Fig IV.6B).

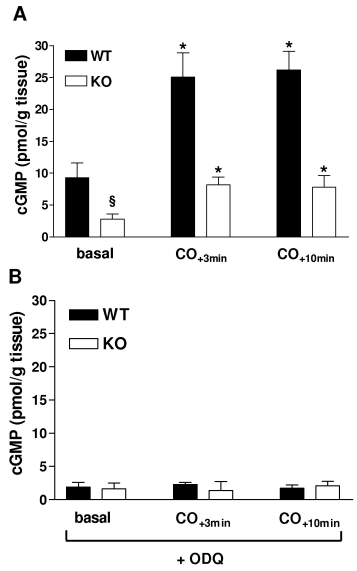

Figure IV.6

cGMP levels in gastric fundus of wild-type (WT, black bars) and knock-out (KO, open bars) mice in basal conditions (basal, parallel control) and stimulated with continuous infusion of CO (CO_{inf}). Panel **a**: in the absence of any interfering drug. Panel **b**: in the presence of ODQ (10 µM). Strips were snap-frozen at 3 min (CO_{+3min}) *or* 10 min (CO_{+10min}) after the start of the continuous CO infusion – as CO_{inf} induced sustained relaxations. Means ± SEM of n = 5-6 are shown. * $P < 0.05$: significantly different from basal (unpaired Student's *t*-test); § $P < 0.05$: KO basal *vs*. WT basal (unpaired Student's *t*-test).

V.5 Discussion

To date, the specific role and relative importance of the different sGC isoforms in CO-mediated responses has remained unclear. In this study, we investigated the functional importance of the sGC isoforms in the relaxant effect of CO and CORM-2 in murine gastric fundus using WT and sGCα_1 KO mice.

The importance of the sGCα_1 subunit in CO-induced smooth muscle relaxation is illustrated by the significantly reduced responses to CO in gastric fundus muscle strips from sGCα_1 KO mice. However, CO still elicited a substantial relaxation and cGMP increase in sGCα_1 KO strips, which were abolished by the sGC inhibitor ODQ. These results indicate that, besides the predominant sGCα_1 subunit, also the less abundantly expressed sGCα_2 subunit plays an important role in CO-induced responses in murine gastric fundus. A similar result has been reported by our group for exogenous NO in gastric fundus of sGCα_1 KO mice (Vanneste et al. 2007).

In a recent study, Mergia et al. (2006) showed that NO-mediated vasorelaxation was preserved in isolated aortic rings of sGCα_1 KO mice, although NO-stimulated sGC activity was reduced by 94%. These authors concluded that the majority of NO-sensitive sGC activity is not required for cGMP synthesis and that sGC$\alpha_2\beta_1$ was responsible for the residual NO-mediated relaxation in isolated aortic rings. The present protein expression data do not suggest a compensatory increase in sGCα_2 gene function in gastric fundus of sGCα_1 KO mice. Thus, we conclude that the normal sGCα_2 amount is sufficient to mediate the substantial residual response to CO in sGCα_1 KO muscle strips.

Remarkably, sGCα_2 protein was not measurable in whole homogenates of gastric tissue, but could only be detected in the isolated membrane fraction. In contrast, sGCα_2 protein has been demonstrated in whole homogenates of lung tissue (Vermeersch et al. 2007). This discrepancy can be explained by the higher sGC content in lung tissue when compared with GI tissue (Mergia et al. 2003).

Confirming previous studies (McLaughlin et al. 2000; Colpaert et al. 2002), the sGC stimulator YC-1 markedly potentiated CO-induced relaxations and cGMP increases in WT mice, but surprisingly lost this potentiating effect in sGCα_1 KO mice. The reason for this finding is unclear. It is well known that YC-1 stimulates sGC in a NO-independent manner. However, the exact mechanism of sGC stimulation explaining the synergistic effect of YC-1 and CO remains uncertain (Cary et al. 2006; Friebe et al. 1998). Kinetic studies utilizing flash photolysis of GC complexed with CO showed that YC-1 has a profound effect on bimolecular association kinetics and a smaller but significant effect on ligand affinity (Kharitonov et al. 1999; Sharma et al. 1999). Given the fact that YC-1 did not potentiate CO-induced responses in sGCα_1 KO strips, we suggest that YC-1 mainly increases the binding of CO to

$sGC\alpha_1\beta_1$, but *not* $sGC\alpha_2\beta_1$, in gastric fundus muscle strips. This might be related to the different subcellular localisation of the 2 heterodimers, with the $sGC\alpha_1\beta_1$ isoform being found in the cytosol and recruited to the membrane upon activation, while the $sGC\alpha_2\beta_1$ isoform has been shown to be membrane-associated (Russwurm et al. 2001; Zabel et al. 2002).

When investigating the functional importance of the different sGC subunits in CORM-2-mediated responses, we were confronted with a more complex picture. CORM-2, an exogenous donor of CO, caused more sustained relaxations, which were only slightly but significantly reduced in $sGC\alpha_1$ KO mice. Administration of the sGC inhibitor ODQ further reduced CORM-2-induced responses in $sGC\alpha_1$ KO mice, indicating that both sGC isoforms are involved. However, as CORM-2-evoked relaxations were not completely suppressed by the sGC inhibitor ODQ (10-100 µM), we conclude that CORM-2-induced responses are not solely mediated by sGC activation.

A similar discrepancy between gaseous CO and CORM-3 has recently been reported by Chlopicki et al. (2006) demonstrating that inhibition of platelet aggregation by CORM-3 was sGC-independent, whereas bolus administration of gaseous CO has been shown to inhibit platelet aggregation *via* sGC activation (Brüne and Ullrich 1987; Friebe et al 1998). In the present study, however, we can not ignore the partial involvement of sGC in CORM-2-mediated responses, as CORM-2-induced relaxations were significantly attenuated in $sGC\alpha_1$ KO strips and even further reduced by ODQ, whereas the sGC stimulator YC-1 significantly potentiated CORM-2-evoked responses in gastric muscle strips of WT mice.

In order to investigate whether these observed differences between CO and CORM-2 might be related to the more prolonged CO release by CORM-2 towards the tissues, we studied the relaxant effects evoked by continuous infusion of CO (CO_{inf}) in both WT and $sGC\alpha_1$ KO mice. The progressive relaxation during the first 2 min of CO_{inf} was significantly suppressed in $sGC\alpha_1$ KO strips and abolished by ODQ in both WT and $sGC\alpha_1$ KO strips, pointing to sGC activation as the sole mechanism. However, the sustained relaxation induced by CO_{inf}, which was slightly but significantly reduced in $sGC\alpha_1$ KO strips, was only partially sensitive to ODQ; the same degree of relaxation was maintained in WT and $sGC\alpha_1$ KO strips at 10 min of infusion. Importantly, these CO_{inf}-induced relaxations in the presence of ODQ were not accompanied by a cGMP increase, indicating that prolonged administration of CO indeed induces gastric relaxation in both a sGC-dependent and sGC-independent manner.

In literature, CO has been reported to induce vasorelaxation by both a cGMP signaling pathway as well as direct activation of Ca^{2+}-activated K^+ (K_{Ca}) channels (Wang et al. 1997; Xi et al. 2004). However, as we previously demonstrated that the combination of ODQ with K_{Ca} channel inhibitors did not cause a more pronounced inhibition of CORM-2-mediated gastric relaxation than did ODQ alone, we can exclude the direct activation of K_{Ca} channels by

CORM-2 as a possible mechanism working in parallel (De Backer and Lefebvre 2007). In addition, we excluded the possible interaction of CO with the nitric oxide synthase (NOS) pathway, as NOS inhibitor L-NAME did not affect CORM-2-induced relaxations in murine gastric fundus (De Backer and Lefebvre 2007).

In this study, we also explored the possible involvement of the particulate GC (pGC) and adenylyl cyclase (AC) signaling pathways in CORM-2-mediated responses, as cross-talk between these pathways and the sGC/cGMP signaling pathway has been described (Hussain et al. 2001; Lim et al. 2005). Neither the (non-selective) pGC inhibitors cystamine *or* PMA nor the AC inhibitor SQ22536 were able to suppress CORM-2-induced relaxations. Taken together, we have to conclude that the origin of this sGC-independent part of CORM-2-induced responses remains unravelled. Other possible mechanisms by which prolonged CO release might cause gastric relaxation are the following: 1/ membrane hyperpolarization, which may be related to the Na^+/K^+-ATPase electrogenic pump *or* an inward rectifying K^+-current (Sha et al. 2007); 2/ modulation of Ca^{2+} currents by inhibition and/or activation of (L-type) Ca^{2+} channels (Lim et al. 2005; Wu and Wang 2005); and 3/ interaction with other ion channels (e.g. Na+ channels; Muraki et al. 1991; Wu and Wang 2005). Future studies will be needed to elucidate the additional mechanism(s) by which prolonged administration of CO induces gastric relaxation.

In conclusion, our findings indicate that besides the predominant $sGC\alpha_1\beta_1$ isoform also the less abundantly expressed $sGC\alpha_2\beta_1$ isoform plays an important role in the relaxant effect of CO in murine gastric fundus; however, the sGC stimulator YC-1 loses its potentiating effect towards CO in $sGC\alpha_1$ KO mice. Prolonged administration of CO – either by the addition of CORM-2 *or* by continuous infusion of CO – mediates gastric fundus relaxation in both a sGC-dependent and sGC-independent manner.

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Chapter VI

A NOVEL METHOD FOR THE EVALUATION OF INTESTINAL TRANSIT AND CONTRACTILITY IN MICE USING FLUORESCENCE IMAGING AND SPATIOTEMPORAL MOTILITY MAPPING

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Chapter VI A Novel Method for the Evaluation of Intestinal Transit and Contractility in Mice using Fluorescence Imaging and Spatiotemporal Motility Mapping

VI.1 Abstract

This study introduces a novel, simplified method for the evaluation of murine intestinal transit and contractility using fluorescence and video imaging. Intestinal transit was measured by evaluating the intestinal distribution of non-absorbable fluorescein-labeled dextran (70kDa, FD70) along the GI tract. After excision of the gastrointestinal (GI) tract, two full-field images – one in normal illumination mode and another in fluorescent mode – were taken with a CCD camera and subsequently matched for calculation of fluorescence distribution along the GI tract. Immediately after, intestinal contractility was evaluated in different regions of the intact intestine by spatiotemporal motility mapping (i.e. video imaging). In control mice, the small intestine showed vigorous oscillatory contractions and FD70 was primarily distributed within the terminal ileum/caecum at 90 min post-gavage. As validation step, the effect of intestinal manipulation (IM, surgical procedure) and two pharmacological agents – known to alter GI motility – was tested. At 24h postoperatively, spontaneous contractile activity of the small intestine was nearly abolished in IM mice, leaving the small intestine distended and resulting in a significantly delayed intestinal transit. In accordance, spontaneous mechanical activity of circular muscle strips in standard organ baths was significantly reduced in IM mice compared to control mice. Administration of atropine (1-3 mg/kg, IP) suppressed spontaneous contractile activity along the entire intestinal tract and induced a dose-related delay in intestinal transit. In contrast, metoclopramide (3-10 mg/kg, IP) markedly increased contractile activity – however only in the upper GI tract – and accelerated intestinal transit in a dose-dependent manner.

VI.2 Introduction

Intestinal transit and contractility are important parameters in gastrointestinal (GI) motility studies. At present, intestinal transit in rodents is usually measured by gavage of a colorimetric, fluorescent *or* radioactive marker, and the subsequent determination of this marker in the divided intestinal segments of the GI tract (Gan et al., 2007; Seerden et al., 2005; Moore et al., 2003; Capasso et al., 2007; De Jonge et al., 2003; Fukuda et al., 2006). Therefore, these methods do not allow to evaluate intestinal contractility in the intact intestine of the same animal. This study describes a novel method to measure both intestinal transit and contractility in the intact intestine quasi-simultaneously using fluorescence and video imaging. In order to validate this newly developed method, the effect of abdominal surgery and two different pharmacological agents – known to alter GI motility – was tested (Fukuda et al., 2005; Kalff et al., 1998; Kehlet & Holte, 2001; Galligan & Burks, 1986; Suchitra et al., 2003).

VI.3 Materials & Methods

VI.3.1 Animals

Male C57/Bl6 mice (20-25g) were purchased from Janvier (Le Genest St-Isle, France) and maintained in standard cages with free access to water and pellets. All experimental procedures were approved by the Ethical Committee for Animal Experiments, Ghent University, Belgium.

VI.3.2 Surgical procedure

Mice were anesthetized with inhaled isoflurane (induction 5%, maintenance 2%) and the abdomen was opened by midline laparotomy. The small intestine was eventrated and then compressed for 5 minutes along its entire length by using sterile moist cotton applicators (intestinal manipulation, IM). The bowel was repositioned in the abdominal cavity and the incision was closed by 2 layers of continuous sutures. Mice were gavaged at 22h30 postoperatively and killed by cervical dislocation 90 minutes post-gavage.

VI.3.3 Drug administration

In a separate set of experiments, atropine (1-3 mg/kg; cholinergic antagonist) *or* metoclopramide (3-10 mg/kg; 5-HT_4 receptor agonist) was given intraperitoneally (IP) 30 minutes before gavage and animals were killed by cervical dislocation 30 minutes post-gavage (Galligan & Burks, 1986; Suchitra et al., 2003).

VI.3.4 Intestinal transit

Intestinal transit was measured by evaluating the intestinal distribution of non-absorbable fluorescein-labeled dextran (70 kDa, FD70, Invitrogen, Merelbeke, Belgium) along the GI tract. Mice were killed by cervical dislocation, respectively, 90 minutes (IM) *or* 30 minutes (pharmacological agents) after the oral ingestion of FD70 (200 µl, 25 mg/ml). The abdomen was cut open, a ligature was placed around the lower oesophagus (just above the cardia) and rectum, and the entire GI tract was excised. Next, the mesenterium was removed and the GI tract (length: ± 25-30 cm) was pinned down with pins at 7.5 cm intervals in a custom-made Petri dish (5 x 30 cm) filled with Krebs solution (composition in mM: NaCl 188.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0, glucose 10.1), containing 1 mM PMSF and aerated with 95% O_2/5% CO_2. Immediately after, FD70 was visualized using the Syngene GeneFlash system (Syngene, Cambridge, UK). The system consisted of a UV-light source, an excitation filter (410-510 nm conversion screen), a 8-bit monochrome CCD camera equipped with a f/1.2 8-48mm zoom lens, and an emission bandpass filter to detect fluorescence (550-600 nm emission; Fig VI.1). Two full-field images – one in normal illumination mode and another in fluorescent mode – were taken and subsequently matched for analysis (Fig VI.2A); the fluorescent intensity throughout the entire GI tract was analyzed and calculated using custom-made software (Intestinal Transit software, written as ImageJ plugins which can be downloaded from http://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 formula: Σ (% FD70 per segment*segment number)/100 (Miller et al., 1981). In order to validate this new imaging technique, intestinal transit was also evaluated as described previously. Therefore, the GI tract had to be cut into 14 segments of equal length and the fluorescent signal in each sample was determined by using a spectrofluorometer (Victor, Perkin-Elmer, MA, USA).

VI.3.5 Contractility imaging and spatiotemporal motility mapping

Immediately after the evaluation of intestinal transit by fluorescence imaging – which took only 10 seconds (two full-field images) – the spontaneous contractile activity (i.e. oscillatory contractions) in different regions of the GI tract (duodenum, jejunum, ileum, and colon) was recorded using the same 8-bit monochrome CCD camera, a Pinnacle Dazzle Platinum video acquisition device, a HP Pavillion zd8000 notebook, and a commercially available software package (Pinnacle Studio 10). Recordings were analyzed according to a modified method described by Seerden et al. (2005). Briefly, a 6-cm-long segment was recorded for 30 seconds (s) and the video files were imported in ImageJ at 25 frames/s (40-

ms interframe interval; 8-bit greyscale images). After the contrast threshold value was set, the images (i.e. 30 x 25 images) were converted to black-and-white and the mean diameter ($Ø_{mean}$) of the intestinal segment under study was measured in the first frame #1. Next, all 750 frames were sequentially analyzed using Amplitude Profiler software – written as ImageJ plugin. The change in intestinal diameter within this 30s-period for every pixel (768 pixels) along this 6-cm-long intestinal segment was calculated by the following equation: [(maximal diameter - minimal diameter)/maximal diameter)]*100 and expressed as % contraction amplitude. The 5^{th}, 25^{th}, 50^{th}, 75^{th} and 95^{th} percentile was determined among these 768 amplitude values and the mean value of these 768 amplitude values was calculated (n = 1; Fig VI.3A); as the colon was sometimes less than 6 cm long, the same calculations were done for 600-768 amplitude values. Finally, the mean of these respective percentiles and mean values was calculated for each experimental group (n = 6-8) and represented in a box-and-whisker plot (Fig VI.3B, VI.4B). The oscillatory changes in intestinal diameter were also represented in a three-dimensional (3-D) plot using Spatiotemporal Motility Mapping software – written as ImageJ plugin with a GnuPlot backend – allowing to see contractility in function of time (Fig VI.3C). Sample recordings of these oscillatory contractions as well as the ImageJ plugins can be downloaded from http://www.heymans.ugent.be/En/DownloadsEn.htm.

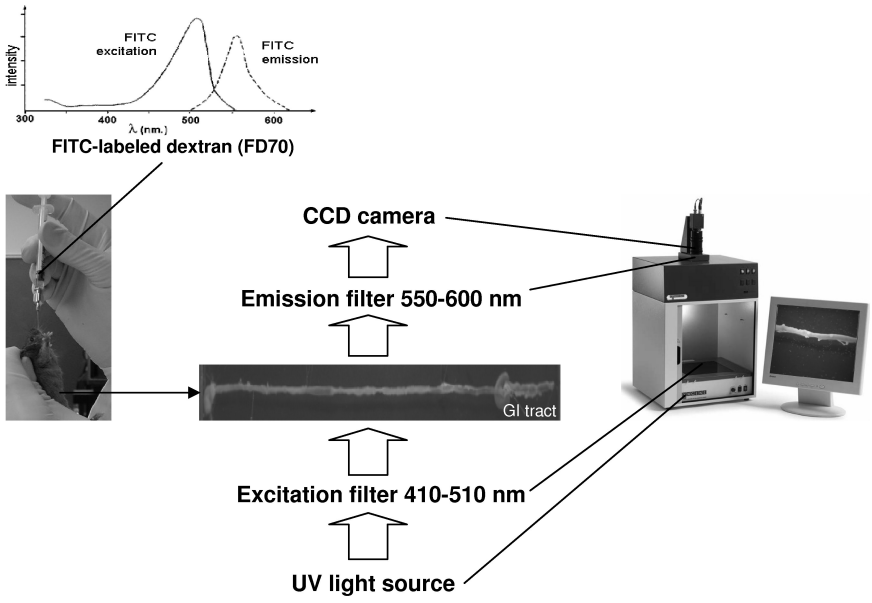

Figure VI.1

Experimental set up and schematic protocol. Ninety minutes (surgical procedure) *or* 30 minutes (pharmacological agents) after oral administration of fluorescein-labeled dextran (FD70), mice were killed by cervical dislocation and the GI tract was excised. Intestinal transit was evaluated by fluorescence imaging of FD70 along the GI tract, and high-resolution spatiotemporal motility mapping – based on real-time video recordings – was used to study intestinal contractility. The imaging system consisted of a UV-light source, an excitation filter (410-510 nm conversion screen), an 8-bit monochrome CCD camera equipped with a f/1.2 8-48mm zoom lens, and an emission bandpass filter to detect fluorescence (550-600 nm emission).

VI.3.6 Organ bath muscle contractility

A mid-jejunal segment was isolated to study spontaneous mechanical activity of circular (mucosa-free) muscle strips in standard organ baths. Contractile activity was calculated as g*s/mm^{-2}. Cross-section area (mm^2) was determined as tissue wet weight (mg)/[tissue length at optimal load (mm)*density (1.05 mg mm^{-3})] (Moore et al., 2003).

VI.3.7 Measurement of inflammatory parameters

Tissue myeloperoxidase (MPO) activity – marker of leukocytic infiltration – was measured as described previously (De Jonge et al., 2003). MDA/HNE levels – markers of oxidative stress – were determined using the Lipid Peroxidation Assay kit (Oxford Biomedical, USA). IL-1β/IL-6 levels were determined by ELISA (Invitrogen, Belgium).

VI.3.8 Data analysis

Data are expressed as means ± SEM (n = 6-8). Statistical analysis was performed using an unpaired Student's *t* test. $P < 0.05$ was considered statistically significant.

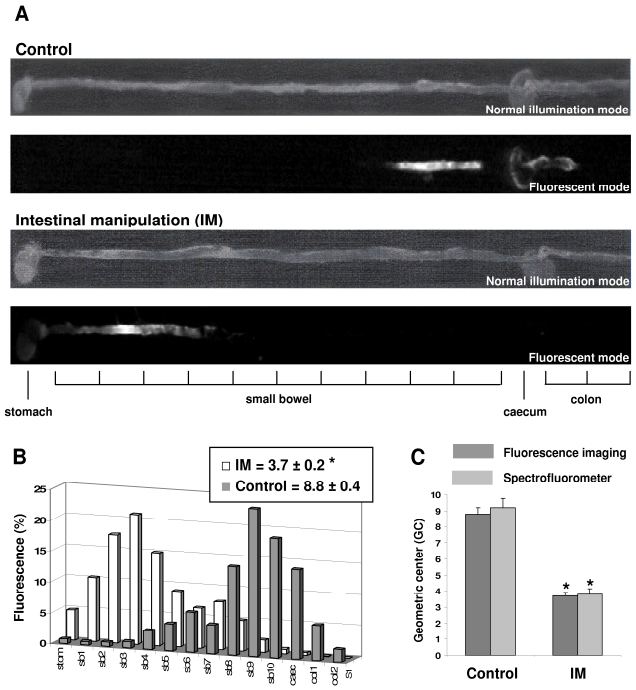

Figure VI.2

Intestinal transit study by fluorescence imaging. **(A)** Representative images showing the macroscopic appearance of the excised GI tract (normal illumination mode) and the distribution of FD70 along the GI tract (fluorescent mode) 90 min after oral administration of FD70. **(B)** Transit histograms for the distribution of FD70 along the GI tract (stom, stomach; sb, small bowel; caec, caecum; col, colon) 90 min post-gavage. IM causes a significant delay in intestinal transit (geometric center (GC) = 3.7 ± 0.2 *vs.* GC = 8.8 ± 0.4 for control). **(C)** Geometric center (GC) as measured by fluorescence imaging (dark grey bars) *or* spectrofluorometer (light grey bars). GCs are given as means ± SEM (n = 6-8). * indicates $P < 0.05$ *vs.* control.

VI.4 Results

VI.4.1 Intestinal transit

In control mice, FD70 was primarily distributed within the terminal ileum and caecum when mice were killed 90 minutes after ingestion of FD70 (geometric center (GC) = 8.8 ± 0.4, as measured by fluorescence imaging). After intestinal manipulation (IM), the fluorescent signal was confined to the more proximal segments of the small intestine (GC = 3.7 ± 0.2), a finding that is consistent with the presence of postoperative ileus (Fig VI.2A, VI.2B).
When evaluating the effect of pharmacological agents on intestinal transit, mice were killed 30 minutes post-gavage, which resulted in a GC = 4.5 ± 0.3 for control mice. Administration of atropine (1-3 mg/kg, IP) induced a dose-related delay in intestinal transit, while metoclopramide (3-10 mg/kg, IP) significantly accelerated intestinal transit in a dose-dependent manner (Fig VI.4A).

Fluorescence imaging and spectrofluorometric determination of intestinal transit yielded comparable results (Fig VI.2C, VI.4A).

VI.4.2 Contractility imaging and spatiotemporal motility mapping

In control mice, the jejunum showed vigorous contractile activity, resulting in a mean contraction amplitude of 21.7 % and a mean diameter ($Ø_{mean}$) of 2.4 ± 0.1 mm. Surgical manipulation of the intestine nearly abolished these oscillatory contractions (mean contraction amplitude = 5.1 %), leaving the small intestine distended ($Ø_{mean}$ = 3.4 ± 0.2 mm; Fig VI.3B, VI.3C). In addition, contractile activity of jejunal smooth muscle strips was investigated in standard organ baths. Circular muscle strips from control mice generated spontaneous phasic activity with a mean contractile force of 57.4 ± 10.2 g.s/mm^2, while IM significantly suppressed contractile activity to a mean contractile force of 16.7 ± 2.6 g.s/mm^2 ($P < 0.05$).

In order to evaluate the effect of pharmacological agents on intestinal contractility, the contractile activity in different regions along the GI tract (duodenum, jejunum, ileum, and colon) was recorded using video-imaging. Figure VI.4B shows that atropine (1-3 mg/kg, IP) suppressed spontaneous contractile activity along the entire GI tract, while metoclopramide (3-10 mg/kg, IP) increased contractile activity only in the upper GI tract.

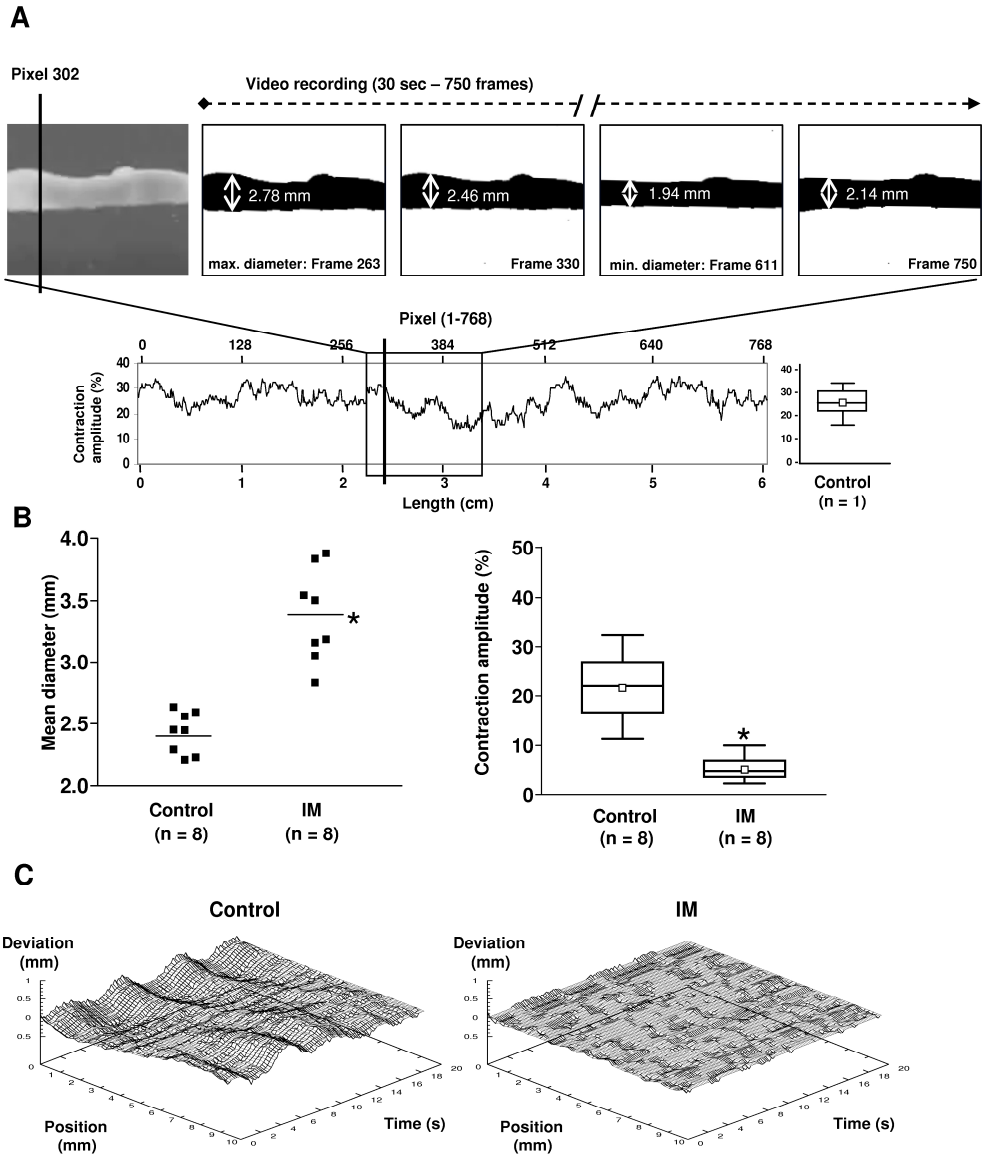

Figure VI.3

Intestinal contractility study by spatiotemporal motility mapping. **(A)** Schematic diagram showing an example of the oscillatory contractions of the small intestine as recorded in a control animal. The change in intestinal diameter (% contraction amplitude) within a 30s-period was calculated for every pixel along a 6 cm long intestinal segment (768 pixels). Among these values, the 5th, 25th, 50th, 75th, and 95th percentile was determined for the intestinal segment under study (n = 1). **(B)** The left panel shows a scatter plot representing the mean diameter (mm) of the mid-jejunal segment; the solid line represents the mean of all measured values within each experimental group (n = 8). The right panel shows a box-and-whisker plot representing the contraction amplitude (%) of the spontaneous oscillatory contractions in the same mid-jejunal segments; upper and lower ends of boxes represent 25/75th percentiles; whiskers represent 5/95th percentiles; the median is a solid line within the box; the mean value is represented by the □ symbol (n = 8). * indicates $P < 0.05$ *vs.* control. **(C)** Representative contractility traces showing the spontaneous oscillatory contractions in a 10 mm mid-jejunal segment (X-axis) as deviations in mm (Y-axis) for a period of 20 seconds (Z-axis); the intestinal diameter measured at t = 20 s was used as reference value. Oscillatory contractions were vigorous in control mice, but almost completely abolished after intestinal manipulation (IM).

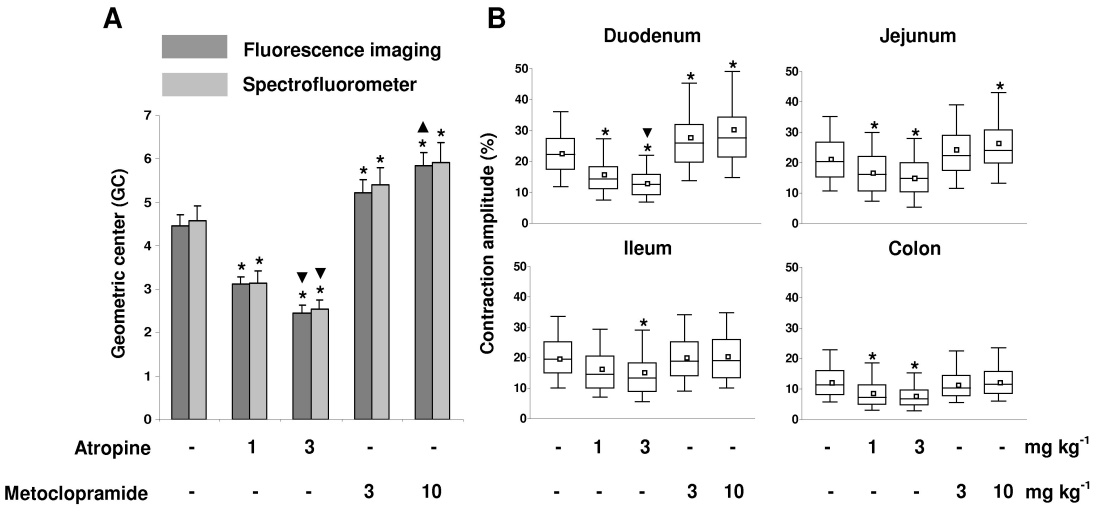

Figure VI.4

Effect of atropine and metoclopramide on intestinal transit and contractility. **(A)** Geometric center for the distribution of FD70 along the GI tract 30 min after oral administration of FD70. GCs are given as means ± SEM (n = 6-8). * indicates $P < 0.05$ *vs.* control; ▼ indicates $P < 0.05$: atropine 3 mg/kg *vs.* atropine 1 mg/kg; ▲ indicates $P < 0.05$: metoclopramide 10 mg/kg *vs.* metoclopramide 3 mg/kg. **(B)** Box-and-whisker plots representing the contraction amplitude (%). Upper and lower ends of boxes represent 25/75th percentiles; whiskers represent 5/95th percentiles; the median is a solid line within the box; the mean value is represented by the □ symbol (n = 6-8). * indicates $P < 0.05$ *vs.* control; ▼ indicates $P < 0.05$: atropine 3 mg/kg *vs.* atropine 1 mg/kg (mean values).

VI.4.3 Inflammatory parameters

All measured inflammatory parameters were significantly increased after IM: MPO activity, MDA/HNE, IL-1β and IL-6 levels in intestinal tissue of IM mice were, respectively, 3.6-, 3.1-, 2.8- and 13.3-fold higher compared to control mice (Table VI.1).

Table VI.1 Inflammatory parameters in intestinal tissue homogenates.

	MPO[†]	MDA/HNE[‡]	IL-1β[§]	IL-6[§]
Control	39.2 ± 6.5	13.5 ± 2.	231.9 ± 28.0	8.3 ± 0.9
IM	141.8 ± 10.8*	41.4 ± 3.9*	641.7 ± 53.9*	110.6 ± 20.3*

[†] MPO activity ~ index of leukocytic infiltration, expressed as U/g tissue; [‡] MDA/HNE levels ~ markers of oxidative stress, expressed as nmol/mg protein; [§] Protein expression levels of IL-1β and IL-6 are expressed as pg/100 mg tissue; * indicates $P < 0.05$ for intestinal manipulated (IM) *vs.* control mice.

VI.5 Discussion

In the present study, we introduce a novel method to measure both intestinal transit and contractility quasi-simultaneously in the murine intestinal tract. Evaluation of intestinal transit by fluorescence imaging avoids the division of the GI tract in separate segments, reducing manipulation and processing time. Intestinal contractility can be measured in different regions of the intact intestine without the necessity of preparing isolated smooth muscle strips.

Intestinal transit was evaluated by fluorescence imaging of FD70 in the intact intestine (i.e. within the intestinal lumen). As this method only required two full-field images – one taken in normal illumination mode and one in fluorescent mode – the processing time for intestinal transit studies was dramatically reduced compared to the traditional methods of measuring intestinal transit in rodents. Thereby, the intestine is typically divided into a specific number of equal segments and the colorimetric (e.g. phenol red, Evans blue), fluorescent (e.g. FD70, rhodamine-dextran) *or* radioactive (e.g. ^{99m}Tc, ^{51}Cr) signal in each sample is subsequently determined by using a spectrophoto(fluoro)meter *or* gamma counter (Gan et al., 2007; Seerden et al., 2005; Moore et al., 2003; Capasso et al., 2007; De Jonge et al., 2003; Fukuda et al., 2006). Recently, a novel method monitoring the progression of a small magnetic pill through the entire GI tract (Magnet Tracking) has been proposed for *in vivo* studies of GI motility in the rat (Guignet et al., 2006). An alternative method to evaluate *in vivo* intestinal transit is the determination of the whole gut transit time using radiopaque markers or carmine red (Zakhary et al., 1997; Carai et al., 2006).

In the present study, surgical manipulation of the intestine significantly delayed intestinal transit – as measured 24h postoperatively – and, thus, resulted in a significantly reduced geometric center value. Administration of atropine (1-3 mg/kg, IP) induced a dose-related delay in intestinal transit, while metoclopramide (3-10 mg/kg, IP) markedly accelerated intestinal transit in a dose-dependent manner. The results obtained by fluorescence imaging correlated with those obtained by spectrofluorometry (i.e. traditional method – GI tract divided into 14 equal segments) and are consistent with data in literature.(Moore et al., 2003; De Jonge et al., 2003; Galligan & Burks, 1986; Suchitra et al., 2003)

The most common way to evaluate intestinal contractility in laboratory animals is the analysis of *in vitro* smooth muscle mechanical activity in standard organ baths.(Moore et al., 2003; De Jonge et al., 2003; Kalff et al., 1998). In recent years, high-resolution spatiotemporal motility mapping has been introduced in GI research (Seerden et al., 2005; Lammers et al., 1996; Hennig et al., 1999). In this study, we used high-resolution spatiotemporal mapping – based on real-time video recordings – to study spontaneous contractile activity in the murine intestinal tract. In control mice, the jejunum showed

pronounced contractile activity; however, surgical manipulation of the intestine nearly abolished these spontaneous oscillatory contractions; a finding that was confirmed by our contractility studies in standard organ baths. We also demonstrated that the intestine of operated mice was significantly dilated, an observation that has been previously reported in rat small intestine (Kreiss et al., 2003) and is commonly used as a diagnostic feature of postoperative ileus in larger animals and humans (Moore et al., 2005; Althausen et al., 2001) Moreover, we showed that atropine (1-3 mg/kg, IP) suppressed spontaneous contractile activity along the entire GI tract, while metoclopramide (3-10 mg/kg, IP) increased contractile activity only in the upper GI tract. These findings are supported by previous studies, reporting the inhibitory effect of atropine on intestinal contractility along the entire GI tract (Galligan & Burks, 1986; Kitazawa et al., 2005; Sanmiguel et al., 2006), whereas metoclopramide has been shown to enhance only upper GI tract motor activity in rats and dogs (Kishibayashi & Karasawa,1995; Yoshida et al., 1991)

As our method to evaluate intestinal transit and contractility could record both parameters within a very short time – and, hence, required minimal tissue manipulation – intestinal tissue samples were processed for the determination of leukocytic infiltration (MPO), oxidative stress (MDA/HNE), and protein expression levels of IL-1β and IL-6. In accordance with literature, all of these parameters were markedly increased in tissue samples of the IM group.(Moore et al., 2003; De Jonge et al., 2003; Kalff et al., 1999; Anup et al., 1999; Wehner et al., 2005; Wehner et al., 2007).

In conclusion, we introduce a novel method to evaluate murine intestinal transit and contractility in a fast, accurate, and easy-to-implement manner. As this method – based on fluorescence imaging and spatiotemporal motility mapping – allows measuring both parameters very rapidly and without major manipulation of the gut, the intestine can still be used to determine other *in vitro* parameters. Therefore, this method may facilitate the study of GI motility under both normal and disease conditions.

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Chapter VII

WATER-SOLUBLE CARBON MONOXIDE-RELEASING MOLECULES REDUCE THE DEVELOPMENT OF POSTOPERATIVE ILEUS VIA MODULATON OF MAPK/HO-1 SIGNALLING AND REDUCTION OF OXIDATIVE STRESS

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Based on

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Chapter VII Water-Soluble CO-RMs Reduce the Development of Postoperative Ileus via Modulation of MAPK/HO-1 signalling and Reduction of Oxidative Stress

VII.1 Abstract

Background and aims: Treatment with CO-inhalation has been shown to ameliorate postoperative ileus (POI) in rodents and swine. The aim of this study was to investigate whether CO liberated from water-soluble CO-releasing molecules (CO-RMs) can protect against POI in mice and to elucidate the mechanisms involved.

Methods: Ileus was induced by surgical manipulation of the small intestine (IM). Intestinal contractility-transit was evaluated by video-fluorescence imaging. Leukocyte infiltration (myeloperoxidase), inflammatory parameters (ELISA), oxidative stress (lipid peroxidation), and heme oxygenase (HO)/inducible NO synthase (iNOS) enzyme activity were measured in the intestinal mucosa and muscularis propria.

Results: Intestinal contractility and transit were markedly restored when manipulated mice were pre-treated with CO-RMs. Intestinal leukocyte infiltration, expression levels of IL-6, MCP-1 and ICAM-1, as well as iNOS activity were reduced by CORM-3-treatment; whereas expression of IL-10/HO-1 was further increased when compared to non-treated manipulated mice. Moreover, CORM-3-treatment markedly reduced oxidative stress and ERK1/2 activation in both mucosa (early response) and muscularis (biphasic response). The p38 MAPK-inhibitor SB203580 abolished CORM-3-mediated HO-1-induction. The HO-inhibitor CrMP only partially reversed the protective effects of CORM-3 on inflammation/oxidative stress in the muscularis, but completely abrogated CORM-3-mediated inhibition of the early ‘oxidative burst’ in the mucosa.

Conclusions: Pre-treatment with CO-RMs markedly reduced IM-induced intestinal muscularis inflammation. These protective effects are, at least in part, mediated through induction of HO-1 – in a p38-dependent manner – as well as reduction of ERK1/2 activation. In addition, CORM-induced HO-1 induction reduces the early ‘oxidative burst’ in the mucosa following IM.

VII.2 Introduction

Ileus, a transient impairment of bowel motility, is a common complication of major abdominal surgery which causes abdominal discomfort, nausea, and vomiting. If prolonged, postoperative ileus (POI) can lead to increased morbidity, length of hospital stay, and healthcare costs (Kehlet & Holte, 2001). The pathogenesis of POI is complex, involving neurogenic and inflammatory mechanisms. In the acute postoperative phase, mainly neural reflex pathways are activated. More recent studies, however, show that the prolonged phase of POI is caused by an enteric molecular inflammatory response and the recruitment of leukocytes into the intestinal muscularis (Bauer & Boeckxstaens, 2004). Despite these recent advances in the understanding of the pathogenesis of POI, treatment has been rather disappointing and limited to predominantly supportive measures (Kehlet & Holte, 2001).

Heme oxygenase (HO) is the rate-limiting enzyme in the degradation of heme, converting heme to free iron, biliverdin and carbon monoxide (CO). Biliverdin is subsequently reduced to bilirubin by biliverdin reductase. Three HO isoforms have been identified: the stress-inducible HO-1 isoform and the constitutive HO-2/HO-3 isoforms which are expressed under basal conditions. The adaptive response of HO-1 to various stimuli suggests that HO-1 may play an important role in conferring protection against different types of stress (Abraham & Kappas, 2008). Accordingly, HO-$1^{-/-}$ mice show an increased systemic inflammatory response and decreased survival after lipopolysaccharide (LPS) challenge and renal ischemia (Poss & Tonegawa, 1997; Tracz et al., 2007), whereas HO-1 induction has been shown to protect tissues against inflammatory and oxidative stress injury (Abraham & Kappas, 2008).

Several lines of evidence indicate that CO mediates many of the biological actions of HO-1 (Ryter et al., 2006). In the gastrointestinal (GI) tract, CO-inhalation has been shown to reduce ischemia/reperfusion injury of intestinal grafts and chronic colitis in IL-$10^{-/-}$ mice (Nakao et al., 2003; Nakao et al., 2003; Hegazi et al., 2005). Moreover, it has been reported that CO-inhalation protects against the development of POI and necrotizing enterocolitis (Moore et al., 2003; Moore et al., 2005; Zuckerbraun et al., 2005).

Recently, water-soluble CO-releasing molecules (CO-RMs) have been used to deliver CO in a more practical and controllable manner to biological systems, thereby reducing the risk of systemic toxicity (Alberto & Motterlini, 2007; Motterlini, 2007). As a consequence, these molecules have received increasing attention for potential pharmaceutical application. In cell cultures, the ‘fast’ CO-releaser CORM-3 – a transition metal carbonyl that releases CO very rapidly – has been shown to suppress cytokine and nitrite production in RAW264.7 macrophages and microglia cells (Bani-Hani et al., 2006; Sawle et al., 2005). In addition, CORM-3 exerts anti-inflammatory effects *in vivo* by diminishing leukocyte-endothelial

interactions and, hence, leukocyte infiltration into tissues (Urquhart et al., 2007). More recently, the 'slow' CO-releaser CORM-A1 – a newly identified boron-containing carbonate that liberates CO at a much slower rate compared to CORM-3 – has also shown promising results *in vitro* (Motterlini et al., 2005; Sandouka et al., 2006; Basuroy et al., 2006; Parfenova et al., 2006; Barbagallo et al., 2008; Kelsen et al., 2008); however, thus far its pharmacological effects in animal disease models have been barely explored. Thus, the aim of our study was to evaluate whether CO liberated from CO-RMs can protect against POI in mice and to elucidate the mechanisms involved.

VII.3 Materials & Methods

VII.3.1 Animals

Male C57/Bl6 mice weighing 20-25g (Janvier, Le Genest St-Isle, France) were used in this study. All experiments were performed in accordance with EU regulations for the handling and use of laboratory animals. The protocols were approved by the Ethical Committee for Animal Experiments, Faculty of Medicine, Ghent University, Belgium.

VII.3.2 Surgical procedure

Mice were anesthetized with inhaled isoflurane (Abbott, Belgium) and the abdomen was opened by midline laparotomy. The small intestine was eventrated and then compressed for 5 min along its entire length by using sterile moist cotton applicators. The bowel was repositioned in the abdominal cavity and the incision was closed by 2 layers of continuous sutures. The total duration of the procedure was approximately 20-25 minutes. Mice were sacrificed at different time intervals after surgery and the GI tract was removed. The small intestine was flushed with ice-cold Krebs, divided into 10 segments, and the mucosa was gently scraped off the underlying muscularis by using a glass slide. Both isolated layers were stored at -70°C, until further processing.

VII.3.3 Preparation of CO-RMs

Two different CO-RMs were used in this study, i.e. the 'fast' CO-releaser CORM-3 (rate of CO-release: $t_{½}$ ~ 1 min) and the 'slow' CO-releaser CORM-A1 (rate of CO-release: $t_{½}$ ~ 21 min). CORM-3 (MW 295 g/mol) and CORM-A1 (MW 104 g/mol) were freshly prepared in sterile water and saline, respectively, and were immediately administered by intraperitoneal (IP) injection. The doses of CORM-3 (1.3 - 40 mg/kg) and CORM-A1 (0.5 - 15 mg/kg) utilized in this study were such that an equivalent amount of CO was delivered in both cases. To prepare the inactive compounds (iCO-RMs, negative controls), CORM-3 was

dissolved in PBS and left at room temperature for 48 h; nitrogen was then bubbled to remove any residual CO gas. CORM-A1 was inactivated by dissolving it in water (using half of the volume necessary to prepare the stock solution). Then, 10 µL HCl (1M) was added, nitrogen was bubbled into the solution for 10 min, and the solution was brought to volume with PBS.

VII.3.4 Blood carboxyhemoglobin (COHb) levels

In a preliminary series of experiments, the effect of IP injection of CORM-3 and CORM-A1 on blood COHb levels was evaluated. Blood samples were taken at indicated time points and blood COHb levels were measured by hemoximeter (Radiometer, Denmark).

VII.3.5 Experimental protocols

Mice were randomly assigned to six experimental groups. *Group I* served as control (non-treated, non-operated). *Group II* underwent surgical manipulation of the small intestine (IM). *Group III* received CORM-3 (40 mg/kg) at 3h and 1h before the surgical procedure. *Group IV* received iCORM-3 at the same dose/schedule as in *group III*. *Group V* received CORM-A1 (15 mg/kg) at 4h30 and 1h30 before the surgical procedure. *Group VI* received iCORM-A1 at the same dose/schedule as in *group V*. The dose/schedule selection was based on preliminary experiments, in which both toxicity and efficacy endpoints were considered (see Fig VII.S1, VII.S2). Mice of *Group II-III* were sacrificed at 1, 3, 6, and 24h after surgery; *Group IV-VI* at 24h after surgery.

Table VII.S1 Effect of chromium mesoporphyrin (CrMP, 30 mg/kg i.p.)* on intestinal heme oxygenase (HO) activity.

	Time after administration (h)				
	0	1	3	6	24
Intestinal mucosa	115 ± 12	51 ± 9	34 ± 7	39 ± 10	56 ± 13
Intestinal muscularis	67 ± 11	43 ± 7	22 ± 8	29 ± 9	41 ± 12

* Dose selection based on Johnson FK, Teran FJ, Prieto-Carrasquero M, *et al.* Vascular effects of a heme oxygenase inhibitor are enhanced in the absence of nitric oxide. *Am J Hypertens* 2002;15:1074-80.

In an additional set of experiments, we studied the influence of p38 MAPK inhibitor SB203580 (10 mg/kg, IP) and HO inhibitor chromium mesoporphyrin (CrMP, 30 mg/kg, IP) on CORM-3-mediated effects in our POI model. *Group VII* consisted of manipulated mice pre-treated with SB203580 alone. *Group VIII* consisted of manipulated mice pre-treated with CORM-3 (at 3h and 1h before IM) + SB203580 (at 4h before IM). *Group IX* consisted of manipulated mice pre-treated with CrMP alone. *Group X* consisted of manipulated mice pre-treated with CORM-3 (at 3h and 1h before IM) + CrMP (at 6h before IM; see Table VII.S1). Mice of *Group VII-X* were sacrificed at 1 and 6h after surgery.

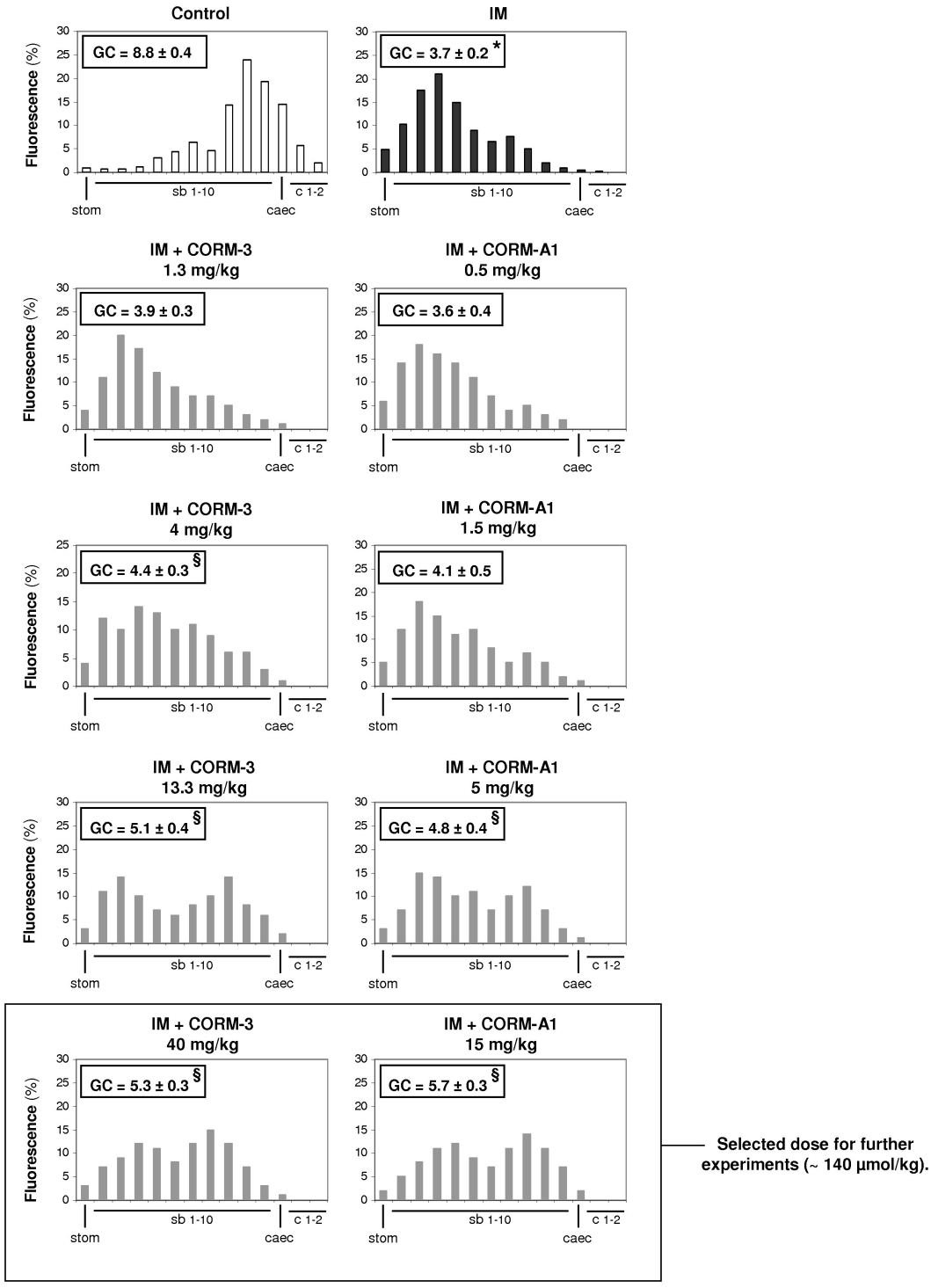
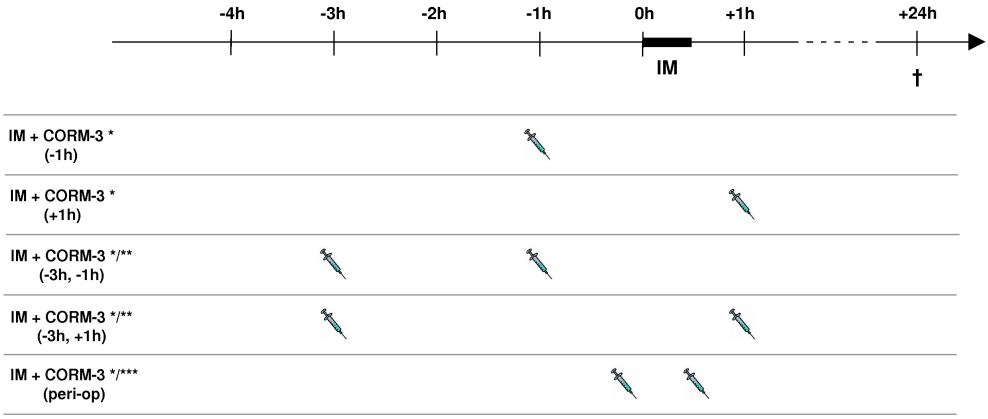


Figure VII.S1

Preliminary experiments – selection of optimal dose. Transit histograms showing the distribution of FD70 along the GI tract (stom, stomach; sb, small bowel; caec, caecum; col, colon) 90 min after oral gavage of FD70 (as measured at 24h after IM). Mean calculated geometric centers (GC) are given as means ± SEM (n = 4-8) in the boxes. * indicates $P < 0.05$ for intestinal manipulated (IM, ■) *vs.* control (□) mice; § indicates $P < 0.05$ for CORM-treated (■) *vs.* non-treated (■) IM mice.

* A single dose of CORM-3 was administered 1h before IM for these dose-selection experiments; the 'slow' CO-releaser CORM-A1 was administered 1h30 before IM in order to assure a CO-release of > 90% (i.e. CO-release rate: $t_{1/2}$ ~ 21 min).



* The dose used in these experiments was 40 mg/kg for every single i.p. injection of CORM-3.

** Treatment scheme based on Moore BA, Overhaus M, Whitcomb J, *et al.* Brief inhalation of low-dose carbon monoxide protects rodents and swine from postoperative ileus. *Crit Care Med* 2005;33:1317-26.

*** Treatment scheme based on Nakao A, Schmidt J, Harada T, *et al.* A single intraperitoneal dose of carbon monoxide-saturated ringer's lactate solution ameliorates postoperative ileus in mice. *J Pharmacol Exp Ther* 2006;319:1265-75.

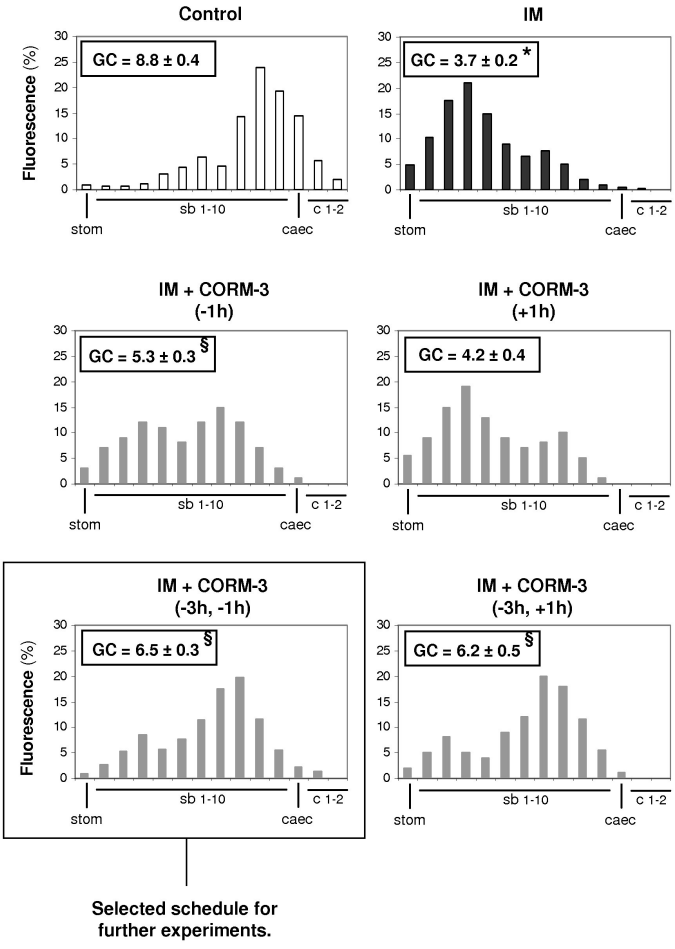


Figure VII.S2

Preliminary experiments – selection of optimal schedule. Transit histograms showing the distribution of FD70 along the GI tract (stom, stomach; sb, small bowel; caec, caecum; col, colon) 90 min after oral gavage of FD70 (as measured at 24h after IM). Mean calculated geometric centers (GC) are given as means ± SEM (n = 4-8) in the boxes. * indicates $P < 0.05$ for intestinal manipulated (IM, ■) *vs.* control (□) mice; § indicates $P < 0.05$ for CORM-treated (■) *vs.* non-treated (■) IM mice.

VII.3.6 Evaluation of intestinal motility

Intestinal contractility (contraction amplitude, Amp_{mean}%) and intestinal transit (geometric center, GC) were evaluated 24h postoperatively using spatiotemporal motility mapping and fluorescence imaging, as described previously (De Backer et al., 2008).

VII.3.7 Leukocyte infiltration

Myeloperoxidase (MPO) activity was measured as an index of leukocyte infiltration, according to a previously descrived protocol (De Backer et al., 2008). Frozen tissue samples were pulverized with a Mikro-Dismembrator (B-Braun, Melsungen, Germany) and dissolved in 10X 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 0.7s at full power) and subsequently subjected to freeze/thaw. The suspension was centrifuged (14000g, 20 min, 4 ℃) and the supernat ant was discarded. A 10 µl aliquot 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 (OD) was immediately read at 620 nm. The reaction was then allowed to proceed for 3 min at 37 ℃. The reaction was stopped by placing the 96-well plate on ice, and the OD values were measured again. One unit of MPO activity was defined as the amount of enzyme that produces a change in optical density (ΔOD) of 1.0 per minute at 37 ℃. Results were normal ized to total protein content (Bradford) and expressed as U/mg protein.

VII.3.8 Protein expression levels

Protein expression levels of interleukin (IL)-6, IL-10, MCP-1, ICAM-1 (R&D systems, Abingdon, UK), HO-1 (Takara Bio Inc., Shiga, Japan), phospho-p38, phospho-ERK1/2, and phospho-JNK (Invitrogen, Merelbeke, Belgium) were determined by ELISA, according to the manufacturer's protocols.

VII.3.9 Measurement of iNOS/HO enzyme activity

Inducible NO synthase (iNOS) enzyme activity was assayed by measuring the conversion of [^{3}H]-arginine to [^{3}H]-citrulline using a NOS activity assay kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's protocol.

Total HO enzyme activity was measured by spectrophotometric determination of bilirubin generation, according to a protocol by Farrugia et al. (2003). Frozen tissue samples were pulverized with a Mikro-Dismembrator (B-Braun, Melsungen, Germany) and dissolved in 5X volumes of 0.1 M potassium phosphate buffer (pH 7.4). The homogenate was sonicated on

ice and centrifuged at 3000*g* for 10 min at 4℃. The supernatant was subsequently centrifuged at 12000*g* for 20 min at 4℃. Microsomes were pelleted from the resulting supernatant by centrifugation at 105000*g* for 60 min at 4℃. The microsomal pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 2 mM $MgCl_2$. An aliquot of the microsomal suspension (200 µg of protein) was added to a reaction mixture containing hemin (25 µM), glucose 6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (1 U), and mouse liver cytosol (as a source of biliverdin reductase, prepared from 105000g supernatant). The reaction was initiated by addition of NADPH (1 mM) and conducted in the dark for 1 h at 37℃; placing the samples on ice terminated the reaction. Bilirubin concentration was calculated by the difference in absorbance at 470 and 530 nm, using an extinction coefficient of 40 mM^{-1} cm^{-1}. HO activity was expressed as picomoles of bilirubin/hour/mg protein.

VII.3.10 Oxidative stress levels

Levels of malondialdehyde/4-hydroxy-2-non-enal (MDA/HNE - indicator of oxidative stress) and trolox-equivalent antioxidant capacity were measured by, respectively, Lipid Peroxidation assay (Oxford Biomedical Research, Oxford, MI, USA) and Total Antioxidant assay (Sigma-Aldrich, Schnellendorf, Germany), according to the manufacturer's protocols.

VII.3.11 Data analysis

Data are expressed as means ± SEM, unless stated otherwise. Statistical analysis was performed using one-way ANOVA, *or* two-way ANOVA with time and treatment as the factors (followed by Bonferroni's multiple comparison test). A probability level of $P < 0.05$ was considered statistically significant.

VII.4 Results

VII.4.1 Effect of CO-RMs on blood COHb levels

In a preliminary series of experiments, we evaluated the effect of IP administration of CO-RMs on systemic COHb levels in order to limit their potential toxicity (COHb < 10%; Von Burg, 1999). The 'slow' CO-releaser CORM-A1 exceeded the toxicity limit at doses > 15 mg/kg (maximum tolerated dose (MTD)). Administration of 15 mg/kg CORM-A1 increased COHb levels to a peak value of 8.4% (measured 20 min after IP injection); the COHb levels then gradually decreased to the baseline level by 180 min after administration. In contrast, an equimolar dose of the 'fast' CO-releaser CORM-3 (40 mg/kg) only induced minimal changes in COHb levels (Table VII.1).

Table VII.1 Blood carboxyhemoglobin (COHb%) levels after intraperitoneal (IP) administration of CO-RMs

	Dose		Time after administration (min)					
	mg/kg	~ µmol/kg	0	10	20	60	120	180
CORM-3								
	1.3	5	0.7	0.5				
	4.0	15	0.7	0.6				
	13.3	45	0.7	1.0	0.4			
	40.0	140	0.7	2.3	1.5	0.9	0.5	0.7
CORM-A1								
	0.5	5	0.3	0.4	0.3			
	1.5	15	0.3	0.6	0.5			
	5.0	45	0.3	1.7	2.5	1.2	0.7	
	15.0	140	0.3	5.3	8.4	4.7	2.8	1.4
	20.0*	185			15.0			

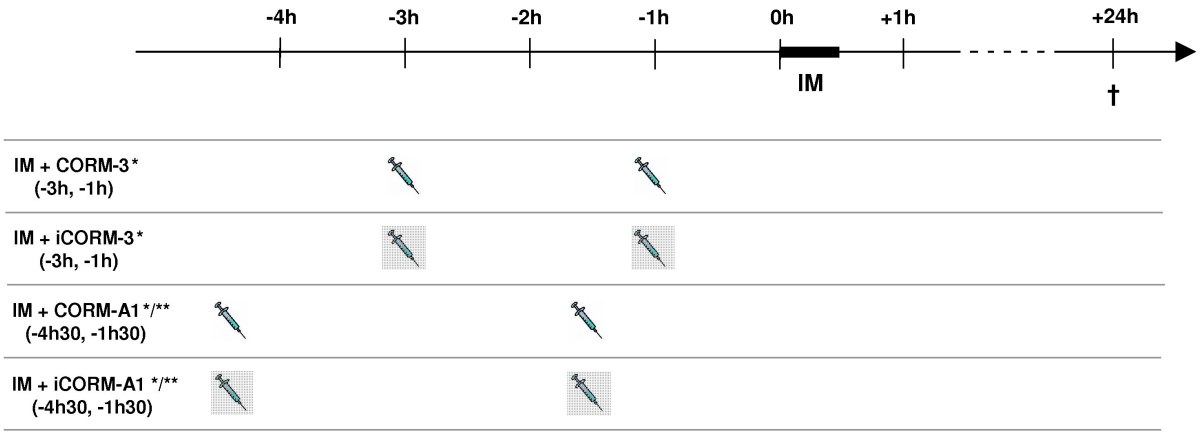
* Administration of 45 mg/kg, i.p. (CORM-A1) induced loss of consciousness, convulsions, respiratory insufficiency and, ultimately, led to death.

VII.4.2 Effect of CO-RMs on surgically-induced intestinal dysmotility

In control mice, the small intestine showed vigorous contractile activity, resulting in a mean contraction amplitude (Amp-$P50_{mean}$) of 21.7% and a mean diameter ($Ø_{mean}$) of 2.4 mm. Surgical manipulation of the small intestine nearly abolished these oscillatory contractions (Amp-$P50_{mean}$ of 5.1%), leaving the small intestine distended ($Ø_{mean}$ > 3 mm). The severity of POI was significantly reduced when mice were pre-treated with CO-RMs, as evidenced by a partial restoration of intestinal contractility (Amp-$P50_{mean}$ of, respectively, 14.1% for CORM-3 and 12.7% for CORM-A1) and, hence, a less distended bowel ($Ø_{mean}$ < 3 mm) (Fig VII.1A, VII.1B).

These results obtained by spatiotemporal motility mapping were confirmed by intestinal transit studies. In control mice, FD70 was rapidly moved aborally – through the jejunum to the ileum – with the peak fluorescence signal in the distal ileum (GC = 8.8 ± 0.4). In non-treated IM mice, we visualised the highest fluorescence intensity in the more proximal segments of the GI tract (GC = 3.7 ± 0.2), a finding that is consistent with the presence of POI. This surgically induced delay of intestinal transit was significantly reduced in mice pre-treated with CO-RMs, as indicated by the higher GC values (Fig VII.1C). The inactive compounds (iCO-RMs), which are unable to release CO, did not protect against the development of POI.

Based on the above results, we selected the CORM-3-treated group for further investigation of the molecular mechanisms implicated in their protective effect against surgicallyinduced ileus.



* Equimolar doses of CORM-3 (40 mg/kg, i.p.) and CORM-A1 (15 mg/kg, i.p.) were used in these experiments.

** CORM-A1 was administered 1h30 before surgery in order to assure a CO-release of > 90%; a 3-hour dosing interval was chosen in order to keep blood COHb levels < 10%.

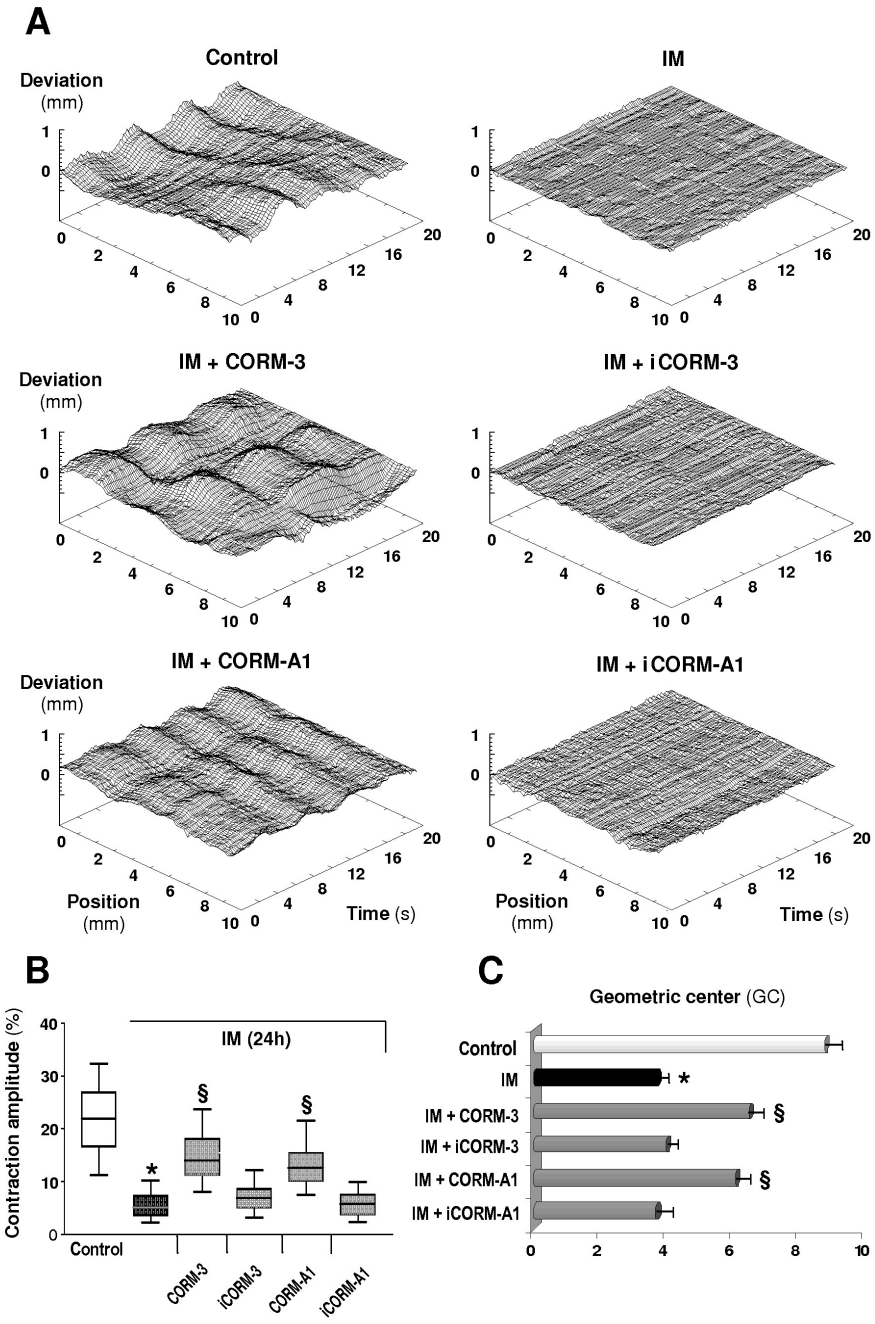


Figure VII.1

Intestinal contractility and transit as measured by spatiotemporal motility mapping and fluorescence imaging (24h after surgery). (A) Representative motility maps showing the spontaneous oscillatory contractions in a 10 mm mid-jejunal segment (X-axis) as deviations in mm (Y-axis) for a period of 20 seconds (Z-axis); the intestinal diameter measured at t = 20s was used as reference value. (B) Box-and-whisker plots representing the contraction amplitude of the spontaneous oscillatory contractions. Upper and lower ends of boxes represent 75th and 25th percentiles. Whiskers represent 95th and 5th percentiles. The median is a solid line within the box (n = 6-8). (C) Mean calculated geometric center for the distribution of FD70 along the GI tract 90 min after oral gavage of FD70 (n = 6-8). * $P < 0.05$ for intestinal manipulated (IM, ■) *vs.* control (□) mice; § $P < 0.05$ for treated (■) *vs.* non-treated (■) IM mice.

VII.4.3 Effect of CORM-3 on inflammatory responses

In accordance with previous studies (Kalff et al., 1999; Schwarz et al., 2002), we found that leukocyte infiltration into the intestinal muscularis begins between 3-6h after IM. This IM-induced leukocyte accumulation into the muscular layer was markedly reduced when mice were pre-treated with CORM-3. Remarkably, IM did not evoke leukocyte infiltration into the mucosal layer (Fig VII.2).

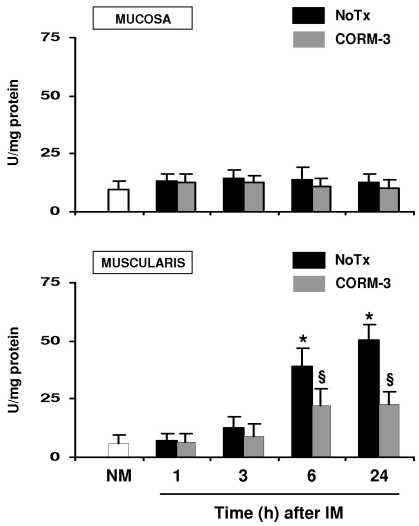


Figure VII.2

Leukocyte infiltration – as measured by myeloperoxidase (MPO) activity – into the mucosa (upper panel) and muscularis (lower panel) of the small intestine following surgical manipulation. Intestinal tissue samples were obtained at 1, 3, 6, and 24h after surgery. Non-manipulated (NM) mice were used as controls. Data represent means ± SEM (n = 5-7). * $P < 0.05$ for intestinal manipulated (IM, black bars) *vs.* non-manipulated (NM, white bars) mice; § $P < 0.05$ for CORM-3-treated (CORM-3, grey bars) *vs.* non-treated (NoTx, black bars) IM mice.

As shown in fig 3, surgical manipulation of the small intestine markedly increased IL-6, IL-10, MCP-1 and ICAM-1 protein expression in the intestinal muscularis from 3h postoperatively. Pre-treatment with CORM-3 significantly reduced the IM-induced increase in cytokine/chemokine release; with the exception of the anti-inflammatory IL-10 which was further up-regulated by CORM-3-treatment when compared to non-treated IM mice. Note that mucosal changes in cytokine/chemokine expression following IM were almost negligible when compared to the observed increases in the muscularis externa and only reached a small but significant increase in IL-6 and IL-10 levels at 3-24h after surgery (Fig VII.3).

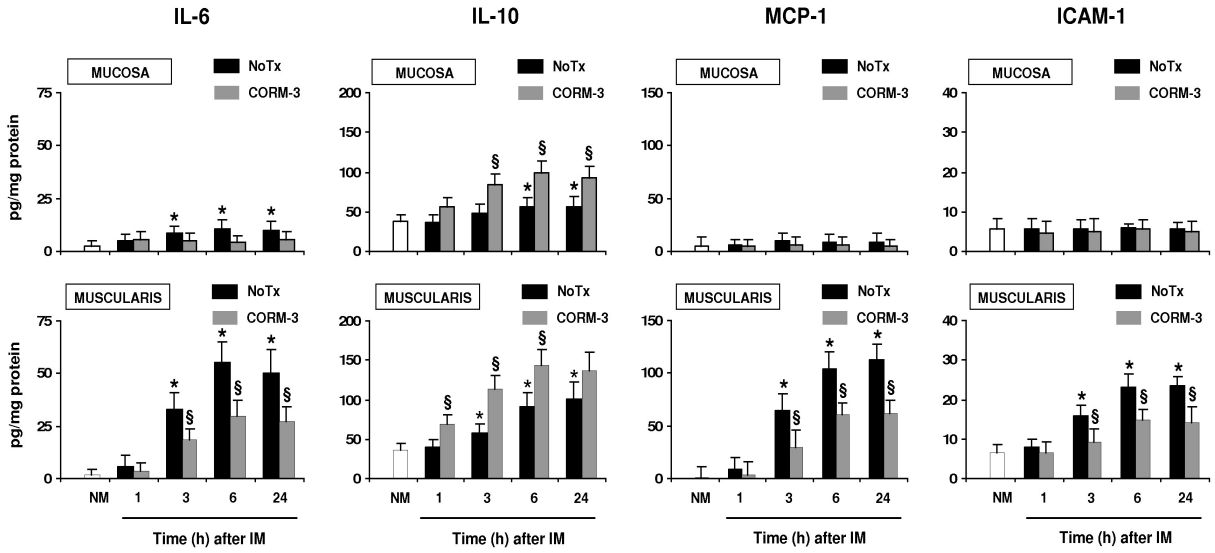


Figure VII.3

Protein expression of IL-6, IL-10, MCP-1 and ICAM-1 in the mucosa (upper panels) and muscularis (lower panels) of the small intestine following surgical manipulation. Intestinal tissue samples were obtained at 1, 3, 6, and 24h after surgery. Non-manipulated (NM) mice were used as controls. Data represent means ± SEM (n = 5-7). * $P < 0.05$ for intestinal manipulated (IM, black bars) *vs.* non-manipulated (NM, white bars) mice; § $P < 0.05$ for CORM-3-treated (CORM-3, grey bars) *vs.* non-treated (NoTx, black bars) IM mice.

In parallel with these findings, surgical manipulation also significantly increased iNOS enzyme activity in the intestinal muscularis at 3-24h after surgery, which was significantly reduced by treatment with CORM-3 (Fig VII.4).

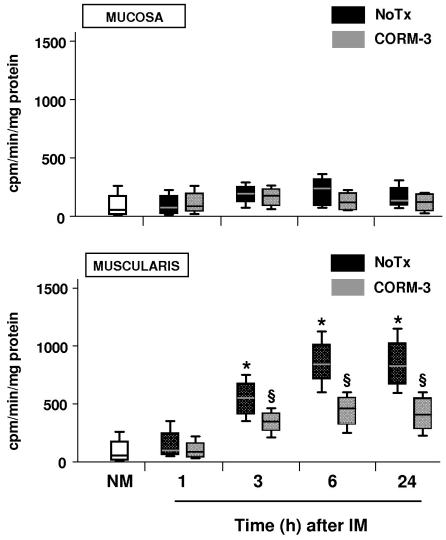


Figure VII.4

Inducible NO synthase (iNOS) enzyme activity in the mucosa (upper panel) and muscularis (lower panel) of the small intestine following surgical manipulation. Intestinal tissue samples were obtained at 1, 3, 6, and 24h after surgery. Non-manipulated (NM) mice were used as controls. Data represent percentiles (n = 5-7). * $P < 0.05$ for intestinal manipulated (IM, black boxes) *vs.* non-manipulated (NM, white boxes) mice; § $P < 0.05$ for CORM-3-treated (CORM-3, grey boxes) *vs.* non-treated (NoTx, black boxes) IM mice.

VII.4.4 Effect of CORM-3 on oxidative stress

Oxidative stress levels markedly increased following IM; however, a different time-course was observed in the different layers of the intestine. An early and transient increase of oxidative stress ('oxidative burst' – $peak_{IM+1h}$) was measured in the mucosa, whereas oxidative stress levels in the muscularis were also markedly elevated in the immediate postoperative phase but further increased until the end of the experiment. Pre-treatment with CORM-3 significantly reduced oxidative stress levels in both intestinal layers when compared to non-treated IM mice. In addition, changes in oxidative stress were associated with reciprocal changes in antioxidant capacity (Fig VII.5).

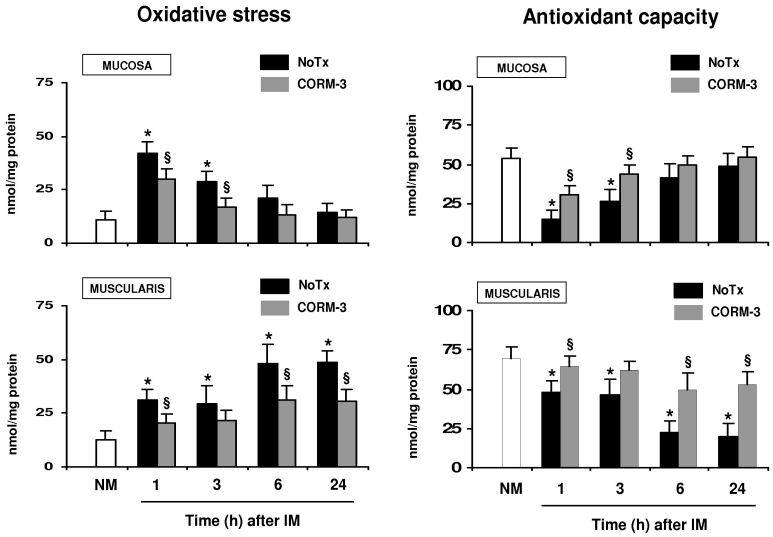


Figure VII.5

Oxidative stress levels (MDA/HNE) and antioxidant capacity levels in the mucosa (upper panel) and muscularis (lower panel) of the small intestine following surgical manipulation. Intestinal tissue samples were obtained at 1, 3, 6, and 24h after surgery. Non-manipulated (NM) mice were used as controls. Data represent means ± SEM (n = 5-7). * $P < 0.05$ for intestinal manipulated (IM, black bars) *vs.* non-manipulated (NM, white bars) mice; § $P < 0.05$ for CORM-3-treated (CORM-3, grey bars) *vs.* non-treated (NoTx, black bars) IM mice.

VII.4.5 Effect of CORM-3 on MAPK signalling

Surgical manipulation of the intestine resulted in a transient activation of all three subtypes of MAPKs in both the mucosa ($peak_{IM+1h}$) and muscularis (first $peak_{IM+1h}$ followed by a second $peak_{IM+6h}$). Pre-treatment with CORM-3 significantly reduced IM-induced ERK1/2 activation; only the early reduction of mucosal ERK1/2 activation did not reach significance. Remarkably, administration of CORM-3 also induced a rapid but transient (additional) activation of p38 MAPK in both intestinal layers. JNK was activated in the manipulated intestine but was not significantly regulated by CORM-3 (Fig VII.6).

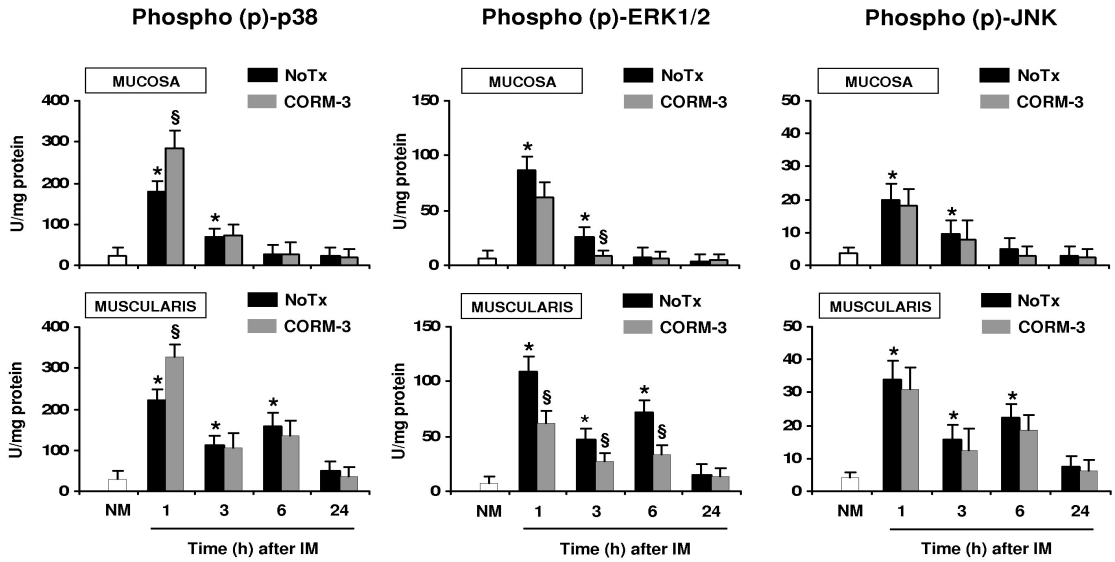


Figure VII.6

Activation of p38, ERK1/2, and JNK MAPKs in the mucosa (upper panels) and muscularis (lower panels) of the small intestine following surgical manipulation. Intestinal tissue samples were obtained at 1, 3, 6, and 24h after surgery. Non-manipulated (NM) mice were used as controls. Data represent means ± SEM (n = 5-7). * $P < 0.05$ for intestinal manipulated (IM, black bars) *vs.* non-manipulated (NM, white bars) mice; § $P < 0.05$ for CORM-3-treated (CORM-3, grey bars) *vs.* non-treated (NoTx, black bars) IM mice.

VII.4.6 Effect of CORM-3 on HO-1 expression/signalling

Intestinal HO-1 protein expression was significantly up-regulated at 3-24h after IM when compared to control mice (muscularis externa >> mucosa). This up-regulation of HO-1 expression was associated with a similar increase in total HO activity; except for a remarkable depression (~48%) in the enzyme activity level at 1h after IM in the intestinal mucosa. Administration of CORM-3 significantly restored this IM-induced decrease in mucosal HO activity and induced a further increase in HO-1 expression/activity at 1-6h after IM in both intestinal layers when compared to non-treated IM mice (Fig VII.7).

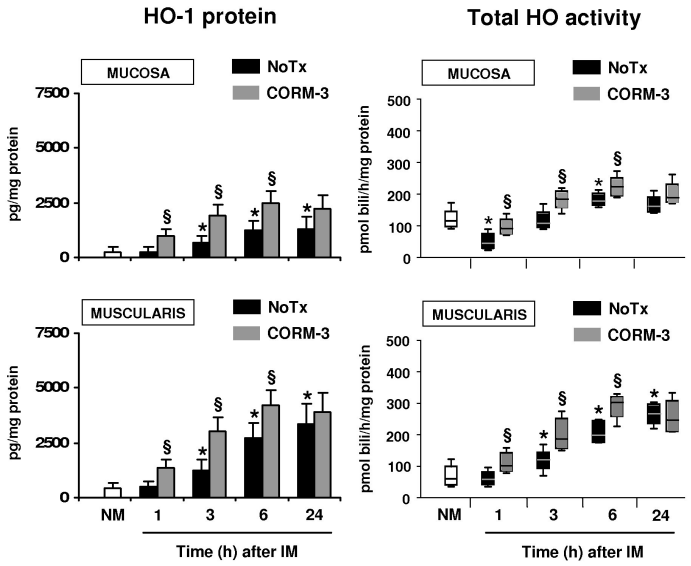


Figure VII.7

Protein expression of HO-1 and total HO activity in the mucosa (upper panels) and muscularis (lower panels) of the small intestine following surgical manipulation. Intestinal tissue samples were obtained at 1, 3, 6, and 24h after surgery. Non-manipulated (NM) mice were used as controls. Data represent means ± SEM (bars) *or* percentiles (boxes; n = 5-7). * $P < 0.05$ for intestinal manipulated (IM, black bars/boxes) *vs.* non-manipulated (NM, white bars/boxes) mice; § $P < 0.05$ for CORM-3-treated (CORM-3, grey bars/boxes) *vs.* non-treated (NoTx, black bars/boxes) IM mice.

The increase in HO-1 expression induced by CORM-3 was, however, abolished by co-administration of the p38 MAPK inhibitor SB203580. Administration of SB203580 alone did not change IM-induced HO-1 expression (Fig VII.8).

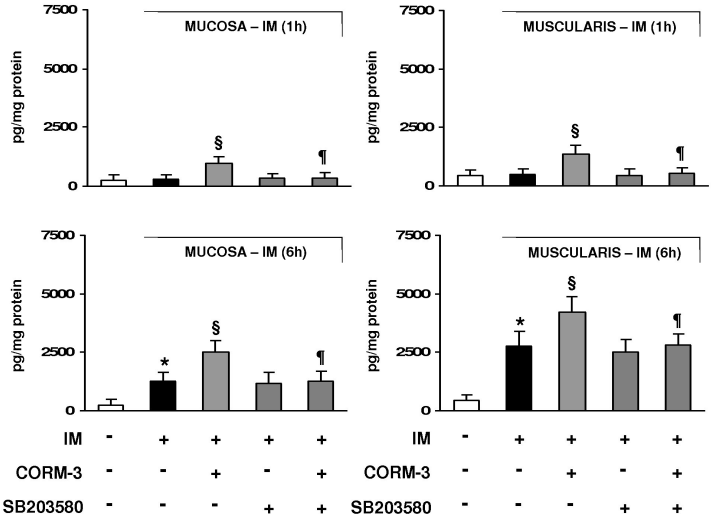


Figure VII.8

Influence of p38 MAPK inhibitor SB203580 on intestinal manipulation (IM)- and CORM-3-mediated HO-1 expression in the mucosa (left panels) and muscularis (right panels) of the small intestine. Intestinal tissue samples were obtained at 1 and 6h after surgery. Data represent means ± SEM (n = 4-7). * $P < 0.05$ for IM *vs.* control mice; § $P < 0.05$ for IM/CORM-3 *vs.* IM mice; ¶ $P < 0.05$ for IM/CORM-3/SB203580 *vs.* IM/CORM-3 mice.

VII.4.7 The role of HO-1 in CORM-3-mediated effects

Administration of CrMP alone to manipulated mice aggravated intestinal tissue injury, indicating that endogenous HO-1 plays an important role in intestinal protection following POI. Despite HO inhibition, administration of CORM-3 still partially reduced inflammation/oxidative stress in the muscularis externa when compared to CrMP-treated IM mice. In contrast, CORM-3 did not affect the early 'oxidative burst' in the mucosa when the HO-inhibitor CrMP was co-administered (Fig VII.9).

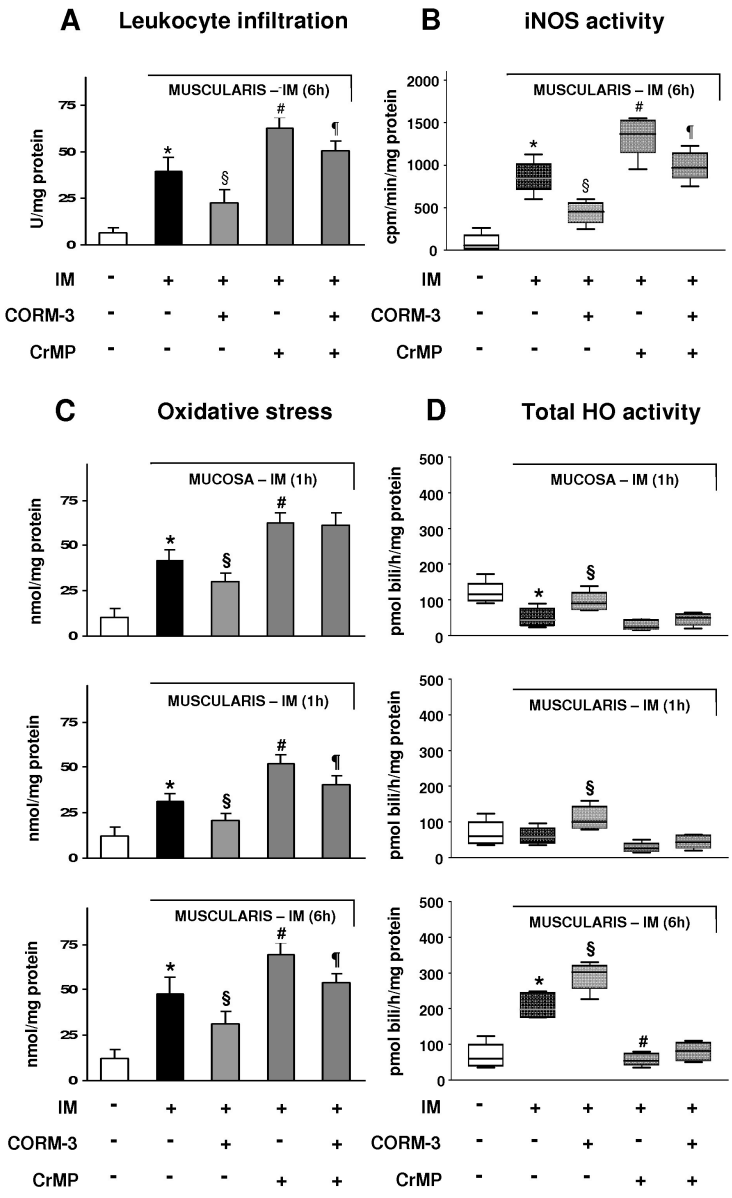


Figure VII.9

Influence of HO inhibitor CrMP on CORM-3-mediated protective effects against intestinal manipulation (IM)-induced changes in leukocyte infiltration (MPO), iNOS enzyme activity, oxidative stress (MDA/HNE) and total HO enzyme activity. Intestinal tissue samples were obtained at 1 and 6h after surgery. Data represent means ± SEM (bars) *or* percentiles (boxes; n = 4-7). * $P < 0.05$ for IM *vs.* control mice; § $P < 0.05$ for IM/CORM-3 *vs.* IM mice; # $P < 0.05$ for IM/CrMP *vs.* IM mice; ¶ $P < 0.05$ for IM/CORM-3/CrMP *vs.* IM/CrMP mice.

VII.5 Discussion

In the present study, we demonstrated that pre-treatment with CO-releasing molecules (CO-RMs) reduces the development of POI in mice. These beneficial effects were associated with: *(1)* the modulation of MAPK signaling pathways; *(2)* the induction of HO-1 expression/activity; *(3)* the reduction of oxidative stress in both mucosa/muscularis; and *(4)* the suppression of IM-induced inflammatory responses in the muscularis.

Two molecules with different biochemical characteristics were used in this study, i.e. the 'fast' CO releaser CORM-3 and the 'slow' CO releaser CORM-A1 (Alberto & Motterlini, 2007; Motterlini, 2007). To date, little is known about the pharmacokinetics and toxicity of these molecules *in vivo*. Therefore, we first evaluated the effect of IP administration of CO-RMs on systemic COHb levels, in order to guarantee their non-toxicity (COHb < 10%; Von Burg, 1999). Remarkably, the maximum tolerated dose (MTD) of CORM-A1 was determined to be 15 mg/kg/dose, whereas an equimolar dose of CORM-3 hardly changed COHb levels. This observation indicates that the difference in CO-release rate has an impact on blood COHb levels. In accordance, the Coburn-Forster-Kane equation states that COHb levels are a function of both the 'concentration of CO' as well as the 'time of exposure' to this concentration of CO (Coburn et al, 1965; Bruce & Bruce, 2003). Thus, we hypothesize that the longer half-life for CO-release from CORM-A1 gives rise to higher blood COHb levels; however, additional pharmacokinetic studies are needed to fully uncover the underlying mechanisms.

Previous studies have shown that surgical manipulation of the small intestine activates the dense network of residential macrophages in the muscularis externa, resulting in the production and release of several cytokines, chemokines and adhesion molecules (Wehner et al., 2007). This local molecular inflammatory response is followed by a cellular inflammatory phase with the additional recruitment of circulating leukocytes and the subsequent further release of inflammatory mediators (biphasic response; Kalff et al., 1999). In rodents, inactivation/depletion of the muscularis macrophage network (Wehner et al., 2007) as well as inhibition of leukocyte infiltration by ICAM-1 blockade (The et al., 2005) has been reported to reduce POI, thereby underscoring the importance of IM-induced inflammation in the pathogenesis of POI. In the present study, treatment with CORM-3 was found to reduce the IM-induced inflammatory response, as evidenced by a marked reduction of inflammatory mediators and leukocyte infiltration. The only exception to this was the observation that protein expression of the anti-inflammatory IL-10 was further increased in CORM-3-treated IM mice. Moreover, CORM-3-treatment was shown to inhibit the IM-induced increase in iNOS enzyme activity in the muscularis externa. These findings are consistent with previous studies demonstrating the potent anti-inflammatory properties of these

compounds both *in vitro* (Bani-Hani et al., 2006; Sawle et al., 2005; Guillén et al., 2008; Megias et al., 2007) and *in vivo* (Urquhart et al., 2007; Cepinskas et al., 2008; Ferrandiz et al., 2008).

Most studies investigating POI have focused on this inflammatory process in the intestinal muscularis following IM. However, in the present study, we also demonstrated that molecular changes in the intestinal mucosa play an important role in the pathogenesis of POI. In particular, an early and transient increase in oxidative stress – with peak value at 1h after IM – was detected in the mucosa. Interestingly, Anup et al. (1999) reported before that surgical manipulation of the intestine resulted in a transient increase in xanthine oxidase activity in the enterocytes and, hence, led to increased mucosal permeability (Anup et al., 1999; Thomas & Balasubramanian, 2004). This loss of barrier function may cause translocation of intraluminal endotoxins into the muscularis externa (Schwarz et al., 2004; Türler et al., 2007) – presumably by way of the lymphatic system (Schwarz et al., 2002) – and, subsequently, exacerbate and/or prolong the inflammatory process in the muscular layer. In parallel with the activation/ infiltration of macrophages, we also measured an increase in oxidative stress in the intestinal muscularis. Importantly, CORM-3 significantly reduced oxidative stress levels in the manipulated intestine, whereas antioxidant capacity levels were partially restored. In accordance, CO-RMs have been shown to reduce reactive oxygen species (ROS) production in a wide variety of cell types in recent *in vitro* experiments (Basuroy et al., 2006; Parfenova et al., 2006; Barbagallo et al., 2008; elsen et al., 2008; Guillén et al., 2008; Srisook et al., 2006; Matsumoto et al., 2006; Masini et al., 2008).

To further explore the molecular mechanisms underlying the beneficial effects of CO-RMs in POI, we examined the possible involvement of p38, ERK1/2 and JNK MAPK signaling pathways (Ryter et al., 2006). These MAPKs are activated by a wide range of extracellular stimuli and have been shown to be key regulators of inflammatory cytokine/chemokine expression. In parallel with changes in oxidative stress, IM led to an increased activation of all three MAPKs in both the mucosa (early response) and muscularis externa (biphasic response). In a previous study, Nakao et al. (2006) demonstrated that intraoperative administration of a CO-saturated Ringer's lactate solution down-regulated ERK1/2 and JNK signaling pathways but did not affect phosphorylation of p38 MAPK (as measured at 6h after surgery; Nakao et al., 2006). In the present study, however, treatment with CORM-3 was only found to reduce IM-induced ERK1/2 activation, suggesting that ERK1/2 plays a pivotal role in the CORM-3-mediated protection against POI. Remarkably, administration of CORM-3 also caused a rapid but transient (additional) activation of p38 MAPK, indicating that CO modulates the inflammatory response by differential regulation of the MAPK signaling pathway.

Another important finding of this study is that CORM-3 potently induced HO-1 expression/activity in the manipulated intestine. In contrast, CORM-2 has been reported not to affect HO-1 expression in Caco-2 cells under basal conditions; however, several other studies have shown that CO-RMs potently induce HO-1 expression both *in vitro* (Sawle et al., 2005; Lee et al., 2006; Kim et al., 2007) and *in vivo* (Foresti et al., 2005; Rodella et al., 2006; Vera et al., 2005). Remarkably, total HO activity exhibited a biphasic response over time in the intestinal mucosa; the increase in HO activity at 6h after IM followed a significant depression in the enzyme activity level at 1h after IM. As HO-1 protein expression was not altered at this early time point following IM, we assume that this IM-induced decrease in HO activity is caused by posttranslational modifications of HO-1 (*or* HO-2, the constitutive isoform which is highly expressed in the intestinal mucosa – as total HO activity measures enzyme activity of both enzymes). Importantly, CORM-3 markedly restored this early decrease in HO activity in the mucosa and induced a further increase in HO-1 expression/activity in both intestinal layers at 1-6h after IM when compared to non-treated IM mice. As several studies have implicated a major role for MAPKs in HO-1 induction (Ryter et al., 2006), we hypothesized that p38 MAPK activation might be involved in HO-1 induction by CORM-3. Indeed, co-administration of p38 MAPK inhibitor SB203580 abolished CORM-3-mediated HO-1 induction, confirming a critical role for p38 MAPK in this CO/HO-1 positive feedback loop.

To determine whether induction of HO-1 – and, hence, the endogenous production of CO and/or biliverdin – is essential for CORM-3-mediated cytoprotection, we investigated the influence of HO inhibitor CrMP in our study. Interestingly, administration of CrMP alone to IM mice aggravated intestinal tissue injury, indicating that endogenous HO plays an important role in intestinal protection following POI. Despite HO inhibition, treatment with CORM-3 still partially reduced inflammation/oxidative stress in the muscularis externa, confirming a direct protective effect of CO in addition to the ability to induce HO-1 (‘dual effect’). In line with these findings, Rodella et al. (2006) reported before that CORM-3 decreased endothelial sloughing in diabetic rats by two additional mechanisms, i.e. the release of CO and induction of HO-1. In contrast, inhibition of the early ‘oxidative burst’ in the mucosa by CORM-3 seems to be fully dependent on the induction of HO-1, as this CORM-3-mediated effect was completely abrogated by HO inhibition. Although there is no clear explanation for this discrepancy, we suggest this might be related to the different source of ROS generation in the different layers of the intestine, i.e. epithelial cells (xanthine oxidase) in the mucosa *vs.* residential/infiltrated macrophages (NADPH oxidase) in the muscular layer. In this regard, the biliverdin/bilirubin redox cycle has been reported to possess ‘broad’ ROS-scavenging properties (Stocker et al., 1987; Baranano et al., 2002; Deguchi et al., 2008), whereas the antioxidant actions of CO are most likely limited to the inhibition of NADPH oxidase (Kelsen

et al., 2008; Taillé et al., 2005; Nakahira et al., 2006; Wang et al., 2007) (but not xanthine oxidase; Benowitz et al., 2003). In addition, biliverdin has been shown to protect intestinal epithelial cell monolayers against ROS-induced barrier dysfunction (Nagira et al., 2006).

In summary, we demonstrated that CO-RMs prevent POI in mice by reduction of IM-induced local intestinal muscle inflammation. These protective effects appear to be, at least in part, mediated through induction of HO-1 – in a p38-dependent manner – and the (subsequent) reduction of ERK1/2 activation. In addition, CORM-induced HO-1 induction was shown to reduce the early 'oxidative burst' in the mucosa following IM, thereby protecting the integrity of the mucosal barrier and, hence, preventing endotoxin translocation into the muscularis propria. Taken together, these data indicate that CO-RMs may be useful agents for the management of POI in the clinical setting.

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Chapter VIII

PPARγ ACTIVATION ALLEVIATES POSTOPERATIVE ILEUS BY INHIBITION OF EGR-1 EXPRESSION AND ITS DOWNSTREAM TARGET GENES

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Based on

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Chapter VIII PPARγ Activation Alleviates Postoperative Ileus by Inhibition of Egr-1 Expression and its Downstream Target Genes

VIII.1 Abstract

Background & aims: Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor whose activation has been linked to several physiological pathways including those related to the regulation of intestinal inflammation. This study aimed to evaluate pharmacological activation of PPARγ in a murine model of postoperative ileus (POI) using the PPARγ agonist rosiglitazone.

Methods: Mice were pretreated with rosiglitazone 1 hour before surgical manipulation of the colon. Intestinal tissue was analyzed for gene expression, transcriptional activity, inflammatory parameters/enzyme activity, leukocyte infiltration and oxidative stress levels. Intestinal contractility and transit were evaluated by video-fluorescence imaging.

Results: Surgical manipulation induced a rapid phosphorylation and subsequent degradation of PPARγ within both intestinal layers of the colon. Accompanying these modifications, there was a decrease in PPARγ DNA-binding activity which was significantly restored by rosiglitazone treatment. The functional severity of POI was significantly ameliorated in mice pretreated with rosiglitazone; this was associated with a down-regulation of inflammatory parameters, iNOS/COX-2 enzyme activity as well as a decrease in leukocyte recruitment into the intestinal muscularis of both colon and jejunum. These anti-inflammatory effects were preceded by a PPARγ-dependent inhibition of surgically-induced early growth response-1 (Egr-1) induction. In addition, treatment with rosiglitazone markedly reduced the early oxidative burst in the mucosa following colonic manipulation; an effect that appeared to be independent of PPARγ.

Conclusions: These data demonstrate that PPARγ occupies a key role in the pathogenesis of POI and that rosiglitazone prevents POI by suppression of the muscularis inflammatory cascade through a PPARγ-dependent down-regulation of Egr-1.

VIII.2 Introduction

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated nuclear transcription factors, members of the nuclear hormone receptor superfamily (Evans et al., 1988). The PPARγ subtype is predominantly expressed in adipose tissue and colon, and to a lesser extent in macrophages, kidney, liver, small intestine and pancreas (Dubuquoy et al., 2006; Mansén et al., 1996; Clark, 2002). Besides its well-known role in adipocyte differentiation, lipid storage and glucose metabolism, PPARγ has also been demonstrated to play a pivotal role in the regulation of inflammatory/immune responses (Clark, 2002; Straus & Glass, 2007). In this context, it has been shown that synthetic PPARγ agonists attenuate colonic damage in several models of colitis (reviewed in Dubuquoy et al., 2006), whereas targeted deletion of PPARγ in either intestinal epithelial cells (Adachi et al., 2006) or inflammatory cells (Shah et al., 2007) leads to further aggravation of dextran sodium sulphate (DSS-induced colitis. Moreover, a recent study identified PPARγ as the main target of 5-aminosalicylic acid (5-ASA), mediating the anti-inflammatory effects of 5-ASA in inflammatory bowel disease (Rousseaux et al., 2005). In an experimental model of intestinal ischemia-reperfusion (I/R) injury, a more severe injury was observed in PPARγ$^{-/-}$ mice, whereas local and remote tissue injury was markedly attenuated in mice treated with a PPARγ agonist. Thereby, it was demonstrated that PPARγ ligands can inhibit the inflammatory response by decreasing interleukin (IL)-8, tumor necrosis factor-α (TNF-α) and inducible NO synthase (iNOS) expression (Nakajima et al., 2001).

Recent studies have demonstrated that a complex cascade of inflammatory responses occupies a key position in the pathogenesis of postoperative ileus (POI; Bauer & Boeckxstaens, 2004). Activation of macrophages that reside in the intestinal muscle layer has been implicated to play a critical role in the initiation of the surgically-induced muscle inflammation, resulting in the release of inflammatory cytokines, chemokines and cellular adhesion molecules, and the secretion of kinetically active inhibitory substances such as nitric oxide (iNOS) and prostaglandins (cyclooxygenase [COX]-2; Kalff et al., 1999, 2000; Schwarz et al., 2001; Wehner et al., 2005, 2007; Türler et al., 2006). This local molecular inflammatory response is followed by a cellular inflammatory phase with the additional recruitment of circulating leukocytes and the subsequent further release of inflammatory mediators (Kalff et al., 1999) In rodents, inactivation/depletion of the muscularis macrophage network (Wehner et al., 2007) as well as inhibition of leukocyte infiltration by ICAM-1 blockade (The et al., 2005) has been reported to reduce surgically-induced ileus, thereby underscoring the importance of intestinal inflammation in the pathogenesis of POI.

Previous studies have demonstrated that heme oxygenase-1 (HO-1) plays an important protective role in the pathogenesis of POI. Thereby, it was shown that pretreatment of mice

with the HO-1 end product carbon monoxide (CO) – either by inhalation of CO gas *or* by intraperitoneal administration of CO-releasing molecules (CO-RMs) – markedly reduces the development of surgically-induced ileus (Moore et al., 2003; De Backer et al., 2009). Recently, Bilban et al. (2006) reported that CO exerts its anti-inflammatory effects in macrophages through the induction of PPARγ. In accordance, Hoetzel et al. (2008) demonstrated that inhaled CO protects against ventilator-induced lung injury via activation of PPARγ and the subsequent inhibition of early growth response-1 (Egr-1). These observations led us to specifically investigate the role of PPARγ in the pathogenesis of POI. Our data demonstrate that PPARγ function is markedly impaired following colonic manipulation; however, treatment with rosiglitazone prevents POI by suppression of the muscularis inflammatory cascade through a PPARγ-dependent down-regulation of Egr-1. These data suggest that PPARγ activation may be a new therapeutic strategy for the treatment of surgically-induced ileus.

VIII.3 Materials & Methods

VIII.3.1 Animals

Male C57/Bl6 mice (Janvier, France), 8-12 weeks, were kept under environmentally controlled conditions (12:12h light/darkness cycle; temperature 20-23°C; standard mouse chow and water ad libitum). All experiments were performed in accordance with EU regulations for the handling and use of laboratory animals and approved by the Ethical Committee for Animal Experiments, Faculty of Medicine & Health Sciences, Ghent University, Belgium.

VIII.3.2 Surgical procedure

Mice were anesthetized by inhalation of isoflurane (Forene, Abbott Labs, Belgium) at 5% induction and 2% maintenance dose. The surgical procedure was performed under sterile conditions. Mice underwent laparotomy and colonic manipulation (CM) as described previously (Türler et al., 2002). In brief, the colon was exteriorized carefully and then compressed for 5 minutes using sterile moist cotton applicators. After repositioning of the bowel, the abdomen was closed using a 2-layer continuous suture (Flexocrin 4/0, Aesculap, Germany). Mice recovered from surgery in a temperature-controlled cage with free access to water, but not food.

VIII.3.3 Study protocol

Mice were randomly assigned to five experimental groups. *Group I* served as control (non-treated, non-operated). *Group II* underwent surgical manipulation of the colon (CM). *Group III* consisted of manipulated mice pretreated with the PPARγ agonist rosiglitazone (3 mg/kg, IP) at 1h before surgery. *Group IV* consisted of manipulated mice pretreated with the PPARγ antagonist GW9662 (1 mg/kg, IP) at 1h30 before surgery. *Group V* consisted of manipulated mice pretreated with GW9662 + rosiglitazone at the same dose/time point as mentioned above. Dose selection for rosiglitazone (0.3-10 mg/kg) resulted from preliminary experiments by using intestinal transit as experimental endpoint (*see* Figure VIII.S1).

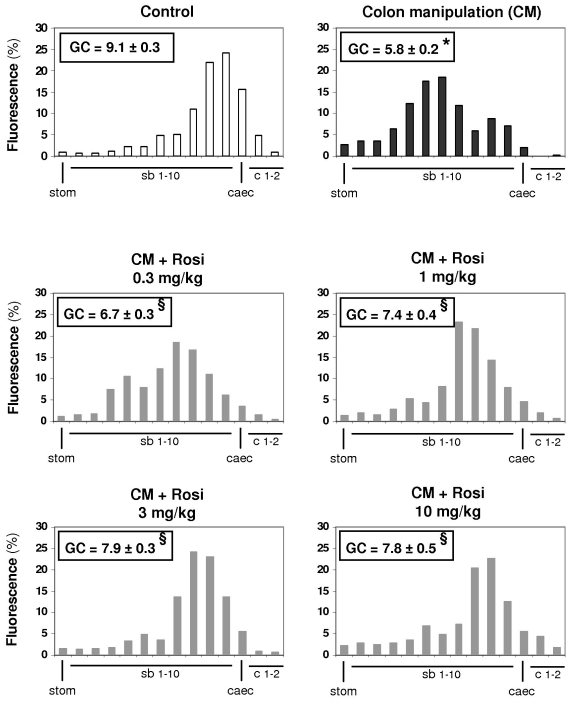

Figure VIII.S1

Transit histograms showing the distribution of fluorescein-labelled dextran (70 kDa, FD70) along the GI tract (stom, stomach; sb, small bowel; caec, caecum; col, colon) at 90 min after gavage (as measured at 24 hours after surgery). In rosiglitazone-treated manipulated mice, a single dose of rosiglitazone (0.3-10 mg/kg, IP) was administered 1 hour before surgery. Mean calculated geometric centres (GC) are given as mean ± SEM (n = 6) in the boxes. * $P < .05$ for colon manipulated (CM, ■) *vs.* control (□) mice; § $P < .05$ for rosiglitazone (Rosi)-treated (■) *vs.* non-treated (■) CM mice.

Animals were killed at 1, 6 *or* 24h after the surgical procedure. The gastrointestinal (GI) tract was removed, opened along the mesenteric border and washed with ice-cold Krebs-Henseleit buffer. The mucosa was separated from the muscularis by scraping off the mucosa with a glass slide and both isolated layers were snap-frozen in liquid nitrogen and stored at -80℃ *or* fixed in ethanol 100% for 10 minutes, until further analysis.

VIII.3.4 Evaluation of intestinal motility

Intestinal contractility and transit were evaluated 24h postoperatively using spatiotemporal motility mapping and fluorescence imaging (De Backer et al., 2008).

Intestinal contractile activity was evaluated by measuring the contraction amplitude of the spontaneous oscillatory contractions in the small intestine. Therefore, the change in intestinal diameter (Ø) within a 30s-period for every pixel along a 6-cm long mid-jejunal segment was calculated by the following equation: $[(Ø_{max} - Ø_{min})/Ø_{max})]*100$ and expressed as % contraction amplitude. The oscillatory changes in intestinal diameter can also be represented in a 3D plot using Spatiotemporal Motility Mapping software allowing to see contractility in function of time (can be downloaded from *www.heymans.ugent.be/En/DownloadsEn.htm*).

Intestinal transit was evaluated by measuring the intestinal distribution of fluorescein-labeled dextran (70kDa, FD70). Mice were gavaged with 200 µl of FD70 dissolved in distilled water (25 mg/ml) at 22h30 after surgery. Ninety minutes later, animals were killed by cervical dislocation, the GI tract was excised, and the fluorescent signal (λ excitation 410-510 nm; λ emission 550-600 nm) along the GI tract was measured by fluorescence imaging (CCD camera). Data were expressed as % fluorescence intensity per segment (stom, stomach; sb, small bowel segments 1-10; caec, caecum; col, colon segments 1-2) and plotted in a histogram (see Figure VIII.S1). The geometric center (GC) was calculated by the formula: Σ (%FD70 per segment*segment number)/100.

VIII.3.5 Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from intestinal tissue samples using the RNeasy Plus Mini kit (Qiagen, The Netherlands) and reverse-transcribed to cDNA using Superscript III First-Strand Synthesis SuperMix (Invitrogen, Belgium). Quantitative PCR amplification (40 cycles) was performed on the ABI Prism 7700 sequence detector (Applied Biosystems, CA, USA) using Platinum qPCR SuperMix-UDG with ROX (Invitrogen, Belgium). The primers and Taqman probes (Eurogentec, Belgium) used were as follows: β-actin (*NM_007393*) forward 5' TCCTTCCTGGGTATGGAATC 3', reverse 5' GCACTGTGT TGGCATAGA GG 3', probe 5' 6FAM-ACGGATGTCAACGTCACACTTCATGA-TAMRA 3'; PPARγ1 (*U_01664*) forward 5' TTTAAAAACAAGACTACCCTTTACTGAAATT 3', reverse 5' AGAGGTCCACAGAGCTGATT C 3', probe 5' 6FAM-AGAGATGCCATTCTGGCCCACCAACTT-TAMRA 3'; PPARγ2 (*NM_011146)* forward 5' CTGATGCACTGCCTATGAGCACTT 3', reverse 5' AGAGGTCCAC AGAGCTGATTC 3', probe 5' 6FAM-AGAGATGCCATTCTGGCCCACCAACTT-TAMRA 3'.

PPARγ1 (U_01664)

Primer sequences used in Taqman PCR

Forward	**5′-TTTAAAAACAAGACTACCCTTTACTGAAATT-3′**
Reverse	**5′-AGAGGTCCACAGAGCTGATTC-3′**
Probe	**5′-6FAM-AGAGATGCCATTCTGGCCCACCAACTT-TAMRA3′**

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   1 ggaattccgc cggaggacgc ggaagaagag acctggggcg ctgcgctggg gtattgggtc
  61 gcgcgcagcc tgaggggacc gagtgtgacg acaagatttg aaagaagcgg tgaaccactg
 121 atatcaggac atttttaaaa acaagactac cctttactga aattaccatg gttgacacag
 181 agatgccatt ctggcccacc aacttcggaa tcagctctgt ggacctccc gtgatggaag
 241 accactcgca ttcctttgac atcaagccct ttaccacagt tgatttctcc agcatttctg
 301 ctccacacta tgaagacatt ccattcacaa gagctgaccc aatggttgct gattacaaat
 361 atgacctgaa gctccaagaa taccaaagtg cgatcaaagt agaacctgca tctccacctt
 421 attattctga aaagacccag ctctacaaca ggcctcatga agaaccttct aactccctca
 481 tggccattga gtgccgagtc tgtggggata aagcatcagg cttccactat ggagttcatg
 541 cttgtgaagg atgcaagggt tttttccgaa gaaccatccg attgaagctt atttatgata
 601 ggtgtgatct taactgccgg atccacaaaa aaagtagaaa taaatgtcag tactgtcggt
 661 ttcagaagtg ccttgctgtg gggatgtctc acaatgccat caggtttggg cgggatcgac
 721 aggccgagaa ggagaagctg ttggcggaga tctccagtga tatcgaccag ctgaacccag
 781 agtctgctga tctgcgagcc ctggcaaagc atttgtatga ctcatacata aagtccttcc
 841 cgctgaccaa agccaaggcg agggcgatct tgacaggaaa gacaacggac aaatcaccat
 901 ttgtcatcta cgacatgagt tcctttatga tgggagaaga taaaatcaag ttcaaacata
 961 tcacccccct gcaggagcag agcaaagagg tggccatccg aatttttcaa gggtgccagt
1021 ttcgatccgt agaagccgtg caagagatca cagagtatgc caaaaatatc cctggtttca
1081 ttaaccttga tttgaatgac caagtgactc tgctcaagta tggtgtccat gagatcatct
1141 acacgatgct ggcctccctg atgaataaag atggagtcct catctcagag ggccaaggat
1201 tcatgaccag ggagttcctc aaaagcctgc ggaagccctt tggtgacttt atggagccta
1261 agtttgagtt tgctgtgaag ttcaatgcac tggaattaga tgacagtgac ttggctatat
1321 ttatagctgt cattattctc agtggagacc gcccaggctt gctgaacgtg aagcccatcg
1381 aggacatcca agacaacctg ctgcaggccc tggaactgca gctcaagctg aatcacccag
1441 agtcctctca gctgttcgcc aaggtgctcc agaagatgac agacctcagg cagatcgtca
1501 cagagcacgt gcagctactg catgtgatca agaagacaga gacagacatg agccttcacc
1561 ccctgttcca ggagatctac aaggacttgt attagcagga aagtcccacc cgctgacaac
1621 gtgttccttc tattgattgc actattattt tgagggaaaa aaaatctgac acctaagaaa
1681 atttactgtg aaaaaagcat ttaaaaaaca aaaaagtttt agaacatgat ctattttatg
1741 catattgttt attaaaagat acatttacaa ttacttttaa tattaaaaat taccacatta
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PPARγ2 (NM_011146)

Primer sequences used in Taqman PCR

Forward	**5′–CTGATGCACTGCCTATGAGCACTT–3′**
Reverse	**5′–AGAGGTCCACAGAGCTGATTC–3′**
Probe	**5′–6FAM–AGAGATGCCATTCTGGCCCACCAACTT–TAMRA3′**

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   1 ccagtgtgaa ttacagcaaa tctctgtttt atgctgttAT Gggtgaaact ctgggagatt
  61 ctcctgttga cccagagcat ggtgccttcg ctgatgcact gcctatgagc acttcacaag
 121 aaattaccat ggttgacaca gagatgccat tctggcccac caacttcgga atcagctctg
 181 tggacctctc cgtgatggaa gaccactcgc attcctttga catcaagccc tttaccacag
 241 ttgatttctc cagcatttct gctccacact atgaagacat tccattcaca agagctgacc
 301 caatggttgc tgattacaaa tatgacctga agctccaaga ataccaaagt gcgatcaaag
 361 tagaacctgc atctccacct tattattctg aaaagaccca gctctacaac aggcctcatg
 421 aagaaccttc taactccctc atggccattg agtgccgagt ctgtggggat aaagcatcag
 481 gcttccacta tggagttcat gcttgtgaag gatgcaaggg ttttttccga agaaccatcc
 541 gattgaagct tatttatgat aggtgtgatc ttaactgccg gatccacaaa aaaagtagaa
 601 ataaatgtca gtactgtcgg tttcagaagt gccttgctgt ggggatgtct cacaatgcca
 661 tcaggtttgg gcggatgcca caggccgaga aggagaagct gttggcggag atctccagtg
 721 atatcgacca gctgaaccca gagtctgctg atctgcgagc cctggcaaag catttgtatg
 781 actcatacat aaagtccttc ccgctgacca aagccaaggc gagggcgatc ttgacaggaa
 841 agacaacgga caaatcacca tttgtcatct acgacatgaa ttccttaatg atgggagaag
 901 ataaaatcaa gttcaaacat atcacccccc tgcaggagca gagcaaagag gtggccatcc
 961 gaatttttca agggtgccag tttcgatccg tagaagccgt gcaagagatc acagagtatg
1021 ccaaaaatat ccctggtttc attaaccttg atttgaatga ccaagtgact ctgctcaagt
1081 atggtgtcca tgagatcatc tacacgatgc tggcctccct gatgaataaa gatggagtcc
1141 tcatctcaga gggccaagga ttcatgacca gggagttcct caaaaacctg cggaagccct
1201 ttggtgactt tatggagcct aagtttgagt ttgctgtgaa gttcaatgca ctggaattag
1261 atgacagtga cttggctata tttatagctg tcattattct cagtggagac cgcccaggct
1321 tgctgaacgt gaagcccatc gaggacatcc aagacaacct gctgcaggcc ctggaactgc
1381 agctcaagct gaatcaccca gagtcctctc agctgttcgc caaggtgctc cagaagatga
1441 cagacctcag gcagatcgtc acagagcacg tgcagctact gcatgtgatc aagaagacag
1501 agacagacat gagccttcac cccctgctcc aggagatcta caaggacttg tatTAGcagg
1561 aaagtcccac ccgctgacaa cgtgttcctt ctattgattg cactattatt ttgagggaaa
1621 aaaatctgac acctaagaaa tttactgtga aaaagcattt aaaaacaaaa gttttagaag
1681 atgatgatct attttatgca tattgtttat aaagatacat ttacaattta cttttaatat
1741 taaaaattac cacattataa aattaaaaaa aaaaaaaaa
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VIII.3.6 Western Blotting analysis

Nuclear and cytoplasmic extracts were prepared using the NE-PER Nuclear and Cytoplasmic Reagent's kit (Pierce Biotechnology, Belgium) containing protease- (Roche, Switzerland) and phosphatase-inhibitor cocktail (Sigma-Aldrich, Germany). Equal amounts of each fraction (10 µg) were loaded onto NuPAGE Novex 4-12% Bis-Tris electrophoresis gels (Invitrogen, Belgium) and blotted onto nitrocellulose membranes (Amersham Bioscience, Germany). Membranes were blocked in Tris-buffered saline/0.1% Tween-20 containing 5% non-fat dry milk and incubated overnight with appropriate antibodies for the detection of PPAR*γ* (1:200), Egr-1 (1:200), β-actin (1:500; all from Santa Cruz Biotechnology Inc., CA, USA), and phospho (Ser82)-PPAR*γ* (1:500; Millipore, Belgium). Horseradish peroxidase (HRP)-linked secondary antibodies (Cell Signaling Technology, MA, USA) were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Belgium). Quantification of the bands was done by densitometry analysis (ImageJ).

VIII.3.7 Enzyme-linked immunosorbent assay

Intestinal tissue samples were homogenized in phosphate-buffered saline (PBS, pH 7.4) containing protease-inhibitor cocktail (Roche, Switzerland) and centrifuged at 10,000 *g* for 15 min at 4℃. Protein expression levels of interleukin (IL)-1β, IL-6, monocyte chemoattractant protein (MCP)-1 (all from Invitrogen, Belgium), intercellular adhesion molecule (ICAM)-1 (R&D systems, UK) and HO-1 (Takara Bio Inc., Japan) were determined in the resulting supernatants by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocols. The 96-well microplates were read at 450 nm in a spectrophotometer (Biotrak II, Amersham Biosciences, USA).

VIII.3.8 Chemiluminescence assay

Tissue reactive oxygen species (ROS) levels were quantified using L-012, as described previously (Castier et al., 2005). Intestinal tissue samples were homogenized in 50 mmol/l Tris-HCl buffer (pH 7.5) and centrifuged at 10,000 *g* for 15 min at 4℃. Supernatants were then incubated with L-012 (100 µmol/l; Wako, Japan) and luminescence was counted (Victor Wallac, PerkinElmer, USA) after a 10 min interval, allowing the plate to dark-adapt.

VIII.3.9 Measurement of PPARγ/Egr-1 DNA-binding activity

Binding of PPARγ to the PPRE consensus binding sequence 5'-AACTAGGTCAAAG GTCA-3' (Active Motif, Belgium) and binding of Egr-1 to the Egr-1 consensus binding sequence 5'-GGATCCAGCGGGGGCGAGCGGGGGCGA-3' (Panomics

Srl, Italy) was measured by ELISA-based assay using tissue nuclear extracts. These assays are performed in 96-well plates coated with an oligonucleotide containing the binding consensus sequence. The active form in nuclear extracts is detected using antibodies specific for an epitope that is accessible only when the appropriate subunit is activated and bound to its target DNA. Addition of HRP-linked secondary antibodies provided a colorimetric readout that was quantified in a spectrophotometer (Biotrak, Amersham Biosciences, USA).

VIII.3.10 Measurement of iNOS/COX-2 enzyme activity

Inducible NO synthase (iNOS) enzyme activity in intestinal tissue samples was assayed by measuring the conversion of [^{3}H]-arginine to [^{3}H]-citrulline using a NOS activity assay (Cayman Chem., USA), according to the manufacturer's protocol. [^{3}H]-citrulline formation was measured using a liquid scintillation counter (Packard Tri-Carb 2100 TR, Packard Canberra, USA).

Cyclooxygenase (COX)-2 enzyme activity was measured by COX activity assay (Cayman Chem., USA) using arachidonic acid as a substrate and 10-acetyl-3,7-dihydroxy-phenoxazine (ADHP) as a fluorometric co-substrate. The reaction between COX-derived prostaglandin G_2 and ADHP produces the highly fluorescent compound resorufin, which was measured in a spectrofluorometer (Victor Wallac, PerkinElmer, USA).

VIII.3.11 Leukocyte infiltration

Polymorphonuclear leukocyte infiltration was assessed by measuring myeloperoxidase (MPO) activity, according to a previously described protocol (De Backer et al., 2009). Intestinal tissue samples were homogenized in 50 mM KH_2PO_4 buffer (pH 6.0), sonicated on ice, subjected to freeze/thaw, centrifuged at 14,000 *g* for 20 min at 4°C, and a 10 µl aliquot of the supernatant was added to an assay mixture (200 µl), containing ready-to-use tetramethylbenzidine substrate and 0.5% hexadecyltrimethylammonium bromide. Absorbance at 620 nm was read at t = 0 and t = 3 min.

In addition, histochemical analysis was performed on whole mounts of the intestinal muscularis as described previously. MPO-positive cells were detected using a mixture of Hanker-Yates reagent (Polysciences, USA), Krebs-Ringer buffer and 3% hydrogen peroxide (Sigma-Aldrich, Germany) for 10 minutes (Moore et al., 2003).

VIII.3.12 Statistics

Data are expressed as mean ± standard error of mean (SEM), unless stated otherwise. Statistical analysis was performed using one-way or two-way ANOVA followed by Bonferroni's multiple comparison test. *P* values < .05 were considered statistically significant.

VIII.4 Results

VIII.4.1 Rosiglitazone restores CM-induced PPARγ dysfunction

Initially, we examined the time-course of PPARγ mRNA expression in colonic tissue samples following surgical manipulation. PPARγ exists as two isoforms, γ1 and γ2, generated by alternative promoter usage and differential splicing of at least three different transcripts from the PPARγ gene (Zhang et al., 1995). Colonic manipulation did not affect PPARγ1 mRNA expression in the colon - neither in the mucosa nor in the muscularis; PPARγ2 mRNA was not detectable after 40 amplification cycles (data not shown).

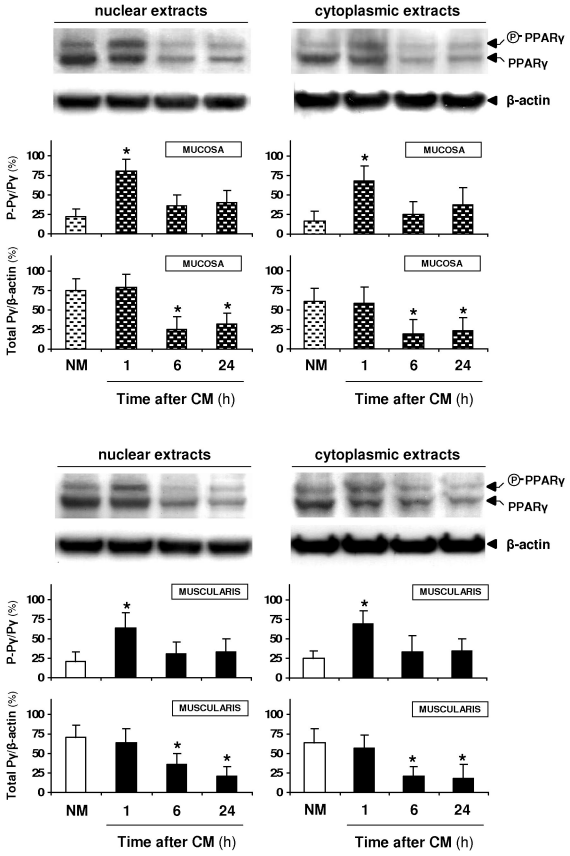

Figure VIII.1

Expression of PPARγ protein in colonic tissue samples following surgical manipulation. Surgical manipulation induces a rapid, transient phosphorylation of PPARγ in both the mucosa and muscularis of the manipulated colon, as indicated by an increased intensity of the phosphorylated PPARγ (*slower migrating form*) in nuclear and cytoplasmic extracts at 1 hour after surgery. The histograms (*upper panels*) show a significantly increased phosho-(P)-PPARγ/PPARγ ratio at this particular time point. In the late postoperative phase, expression levels of PPARγ protein markedly decrease in mucosal and muscularis tissue samples of the manipulated colon. The histograms (*lower panels*) show a significant decrease of total PPARγ protein in nuclear and cytoplasmic extracts at 6-24 hours after surgery. β-actin served as a loading control. Chemiluminescence films were quantified by Image J analysis. Data are expressed as mean ± SEM (n = 4). * $P < .05$ for colon manipulated (CM) *vs*. non-manipulated (NM) mice; one-way ANOVA with Bonferoni post-hoc test.

Next, we performed Western Blotting (WB) analysis to determine whether surgical manipulation of the colon was associated with changes in PPARγ protein expression. As shown in Figure VIII.1, nuclear and cytoplasmic PPARγ protein was markedly decreased in both the mucosa and muscularis of the manipulated colon, as measured at 6 to 24 hours after surgery. Remarkably, we detected a dual band for PPARγ in our WB analysis, known to correspond to non-phosphorylated and phosphorylated forms of PPARγ (Han et al., 2002). Interestingly, surgical manipulation induced a rapid phosphorylation of PPARγ in both intestinal layers of the manipulated colon, as indicated by an increased intensity of the slower migrating phospho-PPARγ at 1 hour after surgery. This observation was confirmed by WB analysis using the phospho-specific anti-PPARγ (Ser82) antibody (Figure SVIII.2).

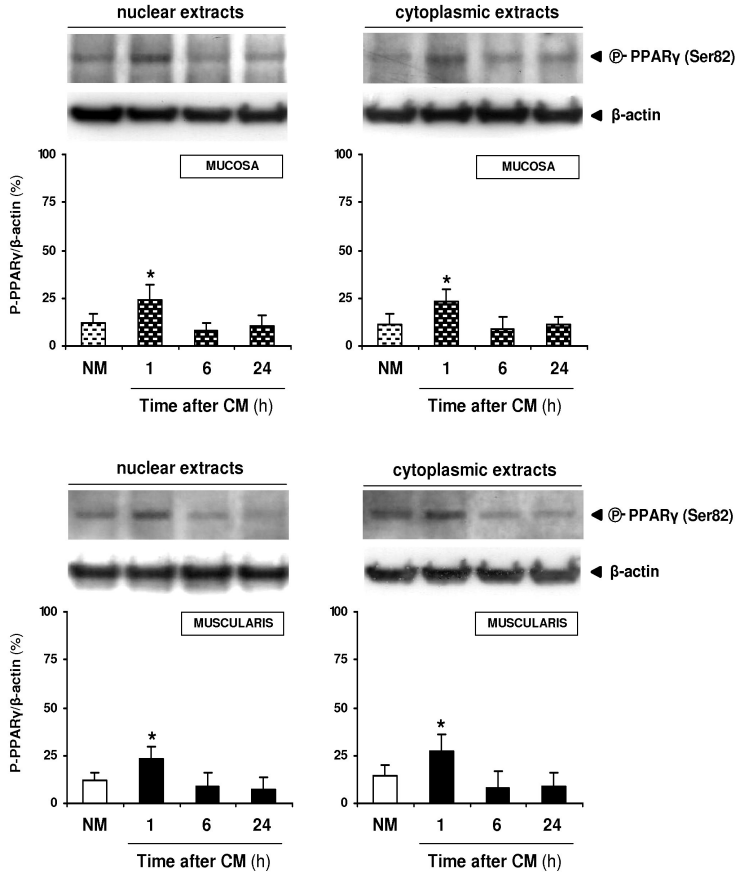

Figure SVIII.2

Expression of phospho-(P)-PPARγ protein in colonic tissue samples following surgical manipulation, as determined by WB analysis using the phospho-specific anti-PPARγ (Ser82) antibody. Surgical manipulation induces a rapid, transient phosphorylation of PPARγ in both the mucosa and muscularis of the manipulated colon, as indicated by an increased intensity of P-PPARγ in nuclear and cytoplasmic extracts at 1 hour after surgery. β-actin served as a loading control. Chemiluminescence films were quantified by Image J analysis. Data are expressed as mean ± SEM (n = 4). * $P < .05$ for colon manipulated (CM) *vs*. non-manipulated (NM) mice; one-way ANOVA with Bonferoni post-hoc test.

In order to measure transcriptional activity of PPARγ, we also performed DNA-binding experiments using nuclear extracts of colonic tissue samples. As shown in Figure VIII.2, surgical manipulation led to a rapid, significant reduction in PPARγ DNA-binding activity in both intestinal layers of the manipulated colon, which remained suppressed for up to 24 hours after surgery. Pretreatment with the synthetic PPARγ agonist rosiglitazone significantly restored this surgically-induced PPARγ dysfunction to even higher levels than in control mice ($P < .05$; n = 4; Figure 2).

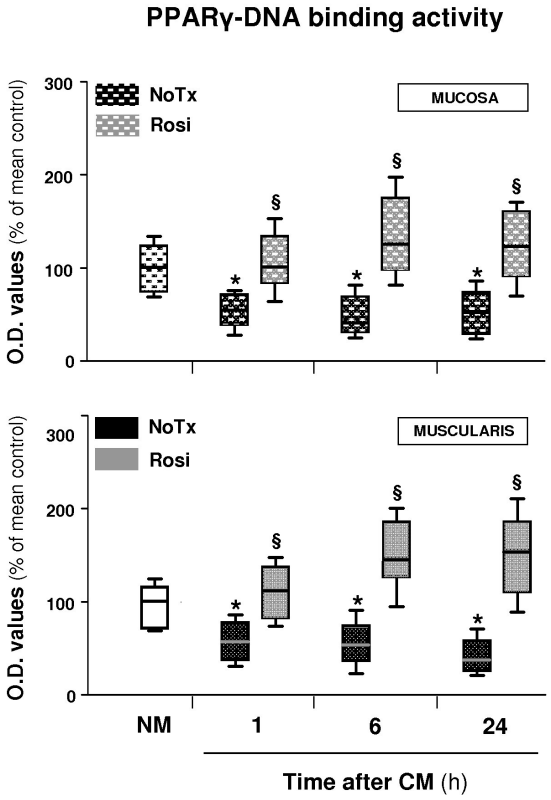

Figure VIII.2

Effect of rosiglitazone on PPARγ transcriptional activity following colonic manipulation; the DNA-binding activity of PPARγ is measured in nuclear extracts of colonic tissue samples by ELISA-based TransAM PPARγ TF assay (*see* Methods & Materials). Surgical manipulation leads to a rapid, significant reduction in PPARγ DNA-binding activity in both the mucosa and muscularis of the manipulated colon, which remains suppressed for up to 24 hours after surgery. This surgically-induced PPARγ dysfunction is, however, significantly restored in mice pretreated with rosiglitazone. Data are expressed as % of mean control value (n = 6). * $P < .05$ for colon manipulated (CM) *vs*. non-manipulated (NM) mice, § $P < .05$ for rosiglitazone (Rosi)-treated *vs*. non-treated (NoTx) CM mice; two-way ANOVA with Bonferoni post-hoc test.

VIII.4.2 Rosiglitazone reduces CM-induced oxidative stress levels

Previous studies have shown that oxidative stress plays an important role in the pathogenesis of POI, based on postsurgical increases in lipid peroxidation (Prabhu et al., 2002; De Backer et al., 2009). In this study, we introduced a more direct and physiologically relevant measure of oxidative stress using the superoxide-sensitive chemiluminescence dye L-012 (Castier et al., 2005). Oxidative stress markedly increased following colonic manipulation; however, a different time-course was observed in the different layers of the colon. An early, but transient, increase in oxidative stress ('oxidative burst' - $peak_{CM+1h}$) was measured in the mucosa, whereas ROS levels in the muscularis were also markedly elevated in the immediate postoperative phase but further increased up to 24 hours after surgery (Figure VIII.3). Pretreatment of mice with rosiglitazone significantly reduced ROS levels in both colonic layers ($P < .05$; n = 6); only the early increase in oxidative stress in the muscularis was not reduced by rosiglitazone treatment.

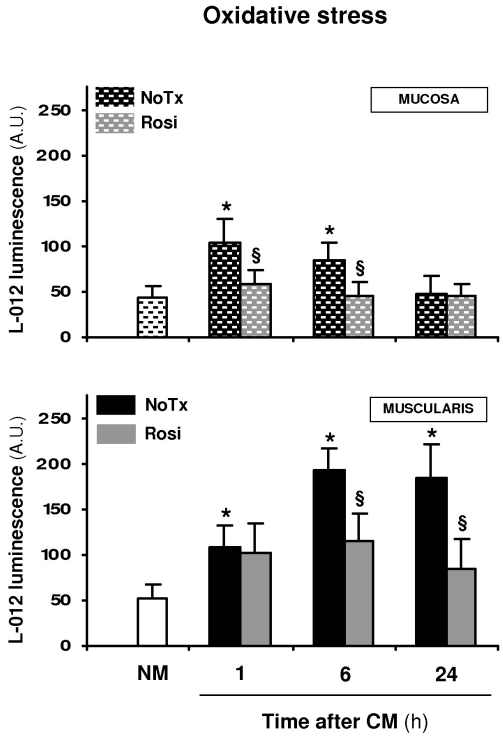

Figure VIII.3

Effect of rosiglitazone on oxidative stress levels following colonic manipulation; levels of oxidative stress (ROS) are determined in colonic tissue samples by L-012-based chemiluminescence assay (*see* Methods & Materials). Surgical manipulation causes an early, but transient, increase in ROS levels in the mucosa, as measured at 1-6 hours after surgery; muscular ROS levels are also increased in the immediate postoperative phase but further increase up to 24 hours after surgery. Levels of oxidative stress are significantly reduced in mice pretreated with rosiglitazone; only the early increase in oxidative stress in the colonic muscularis is not reduced by rosiglitazone treatment. Data are expressed as mean ± SEM (n = 6). * $P < .05$ for colon manipulated (CM) *vs*. non-manipulated (NM) mice, § $P < .05$ for rosiglitazone (Rosi)-treated *vs*. non-treated (NoTx) CM mice; two-way ANOVA wit Bonferoni post-hoc test.

VIII.4.3 Rosiglitazone reduces CM-induced inflammatory responses

Surgical manipulation of the colon initiates a complex inflammatory cascade within the colonic muscularis, which is characterized by the release of inflammatory mediators and the recruitment of circulating leukocytes (Türler et al., 2002). Recently, it was shown that selective colonic manipulation also initiates a distant inflammatory response in the small intestinal muscularis that significantly contributes to the panenteric development of POI (Türler et al., 2007). Therefore, we sought to determine whether the molecular and cellular inflammatory cascade within both the colonic and small intestinal muscularis would be altered by rosiglitazone treatment.

The role of transcription factor Egr-1 in the early phase of this postoperative inflammatory cascade was investigated by WB analysis. Egr-1 is a member of the immediate-early response transcription factor family and functions as a 'master switch' that triggers the expression of numerous key inflammatory mediators (Yan et al., 2000). Surgical manipulation of the colon caused a rapid, significant up-regulation of Egr-1 protein expression in nuclear extracts of colonic tissue samples, which remained elevated up to 6 hours after surgery. In contrast, Egr-1 expression was only increased at the 6-hour time point in nuclear extracts of jejunal tissue samples (Figure VIII.4A). In rosiglitazone-treated manipulated mice, nuclear Egr-1 expression was significantly reduced compared to non-treated manipulated mice ($P < .05$; n = 4). A similar result was observed in the cytoplasmic extracts from the same tissue samples (data not shown), demonstrating that PPARγ activation leads to a down-regulation of Egr-1 expression and not merely a reduced translocation of Egr-1 from the cytoplasm to the nucleus.

Previous studies have demonstrated the functional importance of the kinetically active substances, NO and prostaglandins, in the pathogenesis of POI (Kalff et al., 2000; Schwarz et al., 2001; Türler et al., 2006). Therefore, we wanted to determine whether iNOS and COX-2 enzyme activity levels would be altered by rosiglitazone treatment. As expected, surgical manipulation of the colon caused a significant increase in iNOS and COX-2 enzyme activity in the intestinal muscularis of both colon and jejunum; however, enzyme activity levels were markedly higher in colonic tissue samples when compared to jejunal tissue samples (Figure VIII.4B, VIII.4C). When manipulated mice were pretreated with rosiglitazone, we observed an overall reduction in iNOS and COX-2 enzyme activity levels compared to non-treated manipulated mice ($P < .05$; n = 6).

The regulation by Egr-1 of other downstream inflammatory mediators involved in the pathogenesis of POI was studied using enzyme-linked immunosorbent assay. As shown in figure VIII.4D, colonic manipulation resulted in a significant increase of IL-1β, IL-6, MCP-1, and ICAM-1 expression in the isolated muscularis of both colon and jejunum. In accordance

with the potent down-regulation of Egr-1 expression by rosiglitazone shown above, this surgical manipulation-induced increase of inflammatory parameters was significantly reduced in mice pretreated with rosiglitazone ($P < .05$; $n = 6$); only the reduction of IL-1β in jejunal tissue samples did not reach significance.

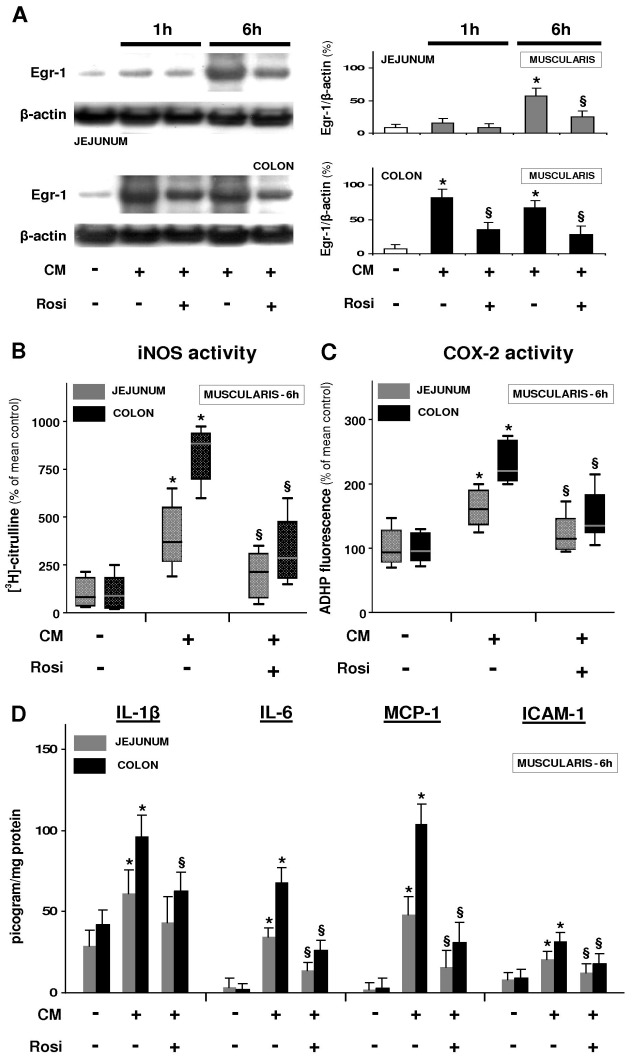

Figure VIII.4

Effect of rosiglitazone treatment on intestinal muscularis inflammatory responses following colonic manipulation. (A) Histograms of the densitometry analysis show the rapid up-regulation of Egr-1 protein expression in nuclear extracts of colonic tissue samples. In contrast, Egr-1 expression is only increased at the 6-hour time point in nuclear extracts of jejunal tissue samples. In rosiglitazone-treated manipulated mice, nuclear Egr-1 expression is significantly reduced compared to non-treated manipulated mice. (B-C) Surgical manipulation of the colon causes a significant increase in iNOS and COX-2 enzyme activity in the intestinal muscularis; however, enzyme activity levels are markedly higher in colonic tissue samples compared to jejunal tissue samples. In rosiglitazone-treated manipulated mice, iNOS and COX-2 enzyme activity levels are significantly reduced compared to non-treated manipulated mice. (D) Colonic manipulation results in a significant increase of IL-1β, IL-6, MCP-1, and ICAM-1 expression in the muscularis of both colon and jejunum. In rosiglitazone-treated manipulated mice, we observed a marked reduction in inflammatpry parameters compared to non-treated manipulated mice. Data are expressed as mean ± SEM *or* as % of mean control value (n = 4-6). * *P* < .05 for colon manipulated (CM) *vs*. control mice, § *P* < .05 for rosiglitazone (Rosi)-treated *vs*. non-treated CM mice; one-way ANOVA with Bonferoni post-hoc test.

As a result of the increased chemokine/adhesion molecule expression, this early molecular inflammatory response is followed by the subsequent infiltration of circulating leukocytes into the intestinal muscularis (Kalff et al., 1999). In this study, we demonstrate that colonic manipulation results in a massive recruitment of leukocytes into the intestinal muscularis of both colon and jejunum; with the highest density of leukocytes in muscularis whole mounts of the manipulated colon. This cellular infiltration was markedly reduced in mice pretreated with rosiglitazone (Figure VIII.5A). Figure VIII.5B shows the quantification of MPO-positive leukocytes in muscularis tissue samples, as determined by MPO assay. Statistical analysis shows a significant increase in colonic and jejunal MPO activity following colonic manipulation, and a significant reduction of leukocyte infiltration in rosiglitazone-treated manipulated mice compared to non-treated manipulated mice ($P < .05$; n = 6).

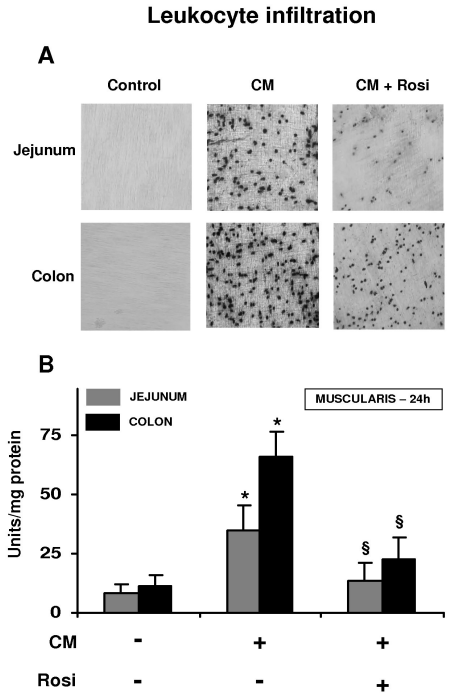

Figure VIII.5

Effect of rosiglitazone treatment on leukocyte infiltration following colonic manipulation. (A) MPO-positive leukocytes within Hanker-Yates stained muscularis whole mounts (x 200 magnification) prepared from the jejunum and colon at 24 hours after surgery. Surgical manipulation of the colon results in the massive recruitment of leukocytes in the intestinal muscularis; with the highest density of leukocytes in muscularis whole mounts of the manipulated colon. Treatment with rosiglitazone markedly reduces the number of infiltrated leukocytes into the muscularis. (B) Histogram shows the quantification of leukocytes in intestinal muscularis tissue samples, as determined by MPO activity assay (*see* Methods & Materials). Statistical analysis shows a significant increase in colonic and jejunal MPO activity following colonic manipulation, and a significant reduction of leukocyte infiltration in rosiglitazone-treated manipulated mice compared to non-treated manipulated mice. Data are expressed as mean ± SEM (n = 6). * $P < .05$ for colon manipulated (CM) *vs*. control mice, § $P < .05$ for rosiglitazone-treated *vs*. non-treated CM mice; one-way ANOVA with Bonferoni post-hoc test.

VIII.4.4 Influence of GW9662 on rosiglitazone-mediated protective effects

Additional confirmation of a role for PPARγ in mediating protection against POI was determined in mice treated with GW9662. Administration of GW9662 to manipulated mice did not alter oxidative stress levels, neither in the mucosa nor in the muscularis, compared to non-treated manipulated mice. When GW9662 was co-administered with rosiglitazone, the PPARγ agonist partially lost its ability to diminish manipulation-induced ROS levels in the muscularis externa ($P < .05$; n = 6). In contrast, GW9662 did not prevent rosiglitazone-mediated antioxidant effects in the mucosa following colonic manipulation (Figure VIII.6).

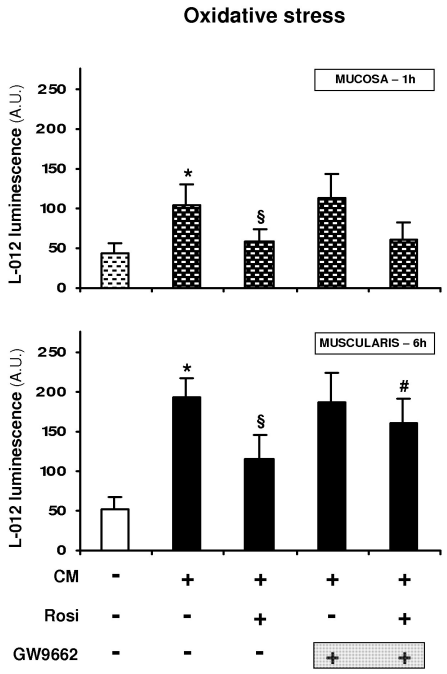

Figure VIII.6

Effect of GW9662 on rosiglitazone-mediated reduction of oxidative stress. Administration of GW9662 alone had no significant effect on surgically-induced oxidative stress levels, neither in the mucosa nor in the muscularis of the manipulated colon. When co-administered with GW9662, rosiglitazone partially lost its ability to reduce oxidative stress in the muscularis following colonic manipulation. In contrast, GW9662 did not affect the rosiglitazone-mediated reduction of oxidative stress in the mucosa following colonic manipulation. Data are expressed as mean ± SEM (n = 6). * $P < .05$ for colon manipulated (CM) *vs*. control mice, § $P < .05$ for rosiglitazone (Rosi)-treated *vs*. non-treated CM mice; # $P < .05$ for CM/Rosi/GW9662 *vs*. CM/Rosi mice; one-way ANOVA with Bonferoni post-hoc test.

Next, we also investigated the influence of GW9662 on the molecular inflammatory response in the intestinal muscularis. As shown in Figure VIII.7A, GW9662 alone did not affect the surgically-induced increase in Egr-1 DNA-binding activity; whereas co-administration of GW9662 and rosiglitazone abrogated the suppression of Egr-1 DNA-

binding activity by rosiglitazone treatment ($P < .05$; n = 6). A similar result was observed when studying the influence of GW9662 on iNOS and COX-2 enzyme activity following colonic manipulation (Figure VIII.7B, VIII.7C); GW9662 did not exacerbate intestinal inflammation when administered alone; however, significantly reversed the anti-inflammatory effects of rosiglitazone in our POI model ($P < .05$; n = 6).

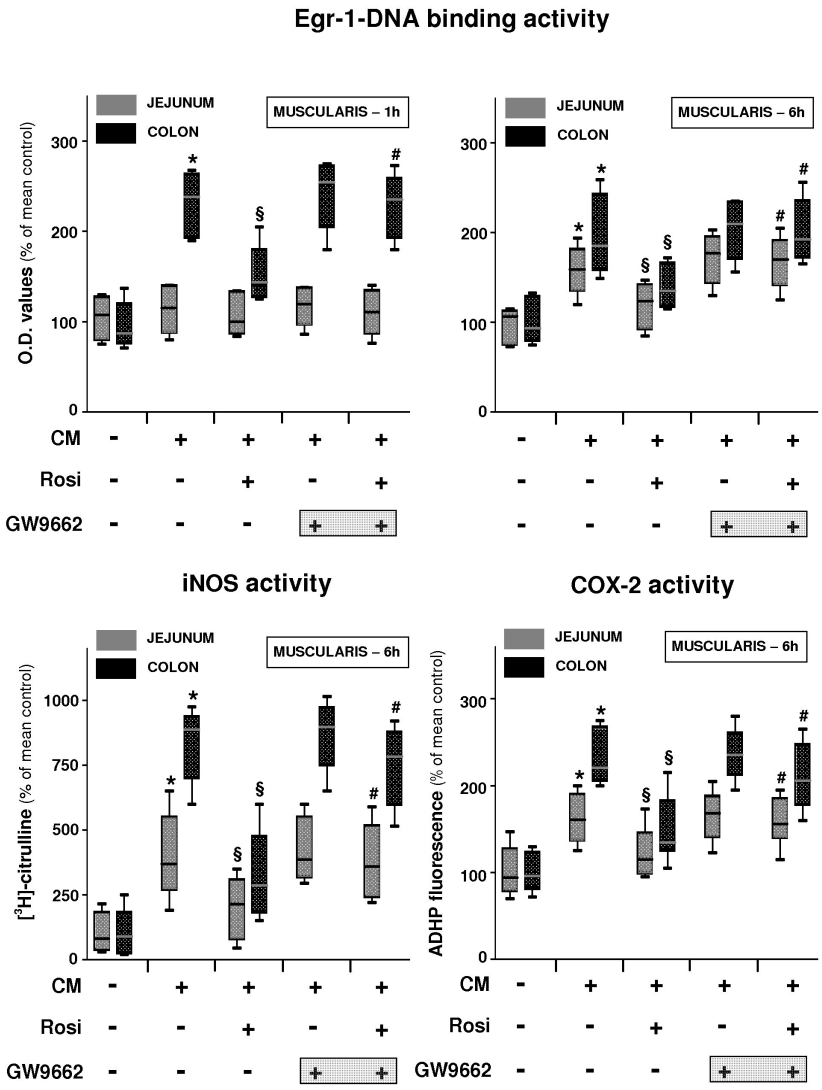

Figure VIII.7

Effect of GW9662 on rosiglitazone-mediated reduction of intestinal muscularis inflammatory responses. Administration of GW9662 alone did not affect the surgically-induced increase in Egr-1 DNA-binding activity; whereas co-administration of GW9662 with rosiglitazone abrogated the suppression of Egr-1 DNA-binding activity by rosiglitazone treatment. Accordingly, GW9662 alone did not affect iNOS and COX-2 enzyme activity following colonic manipulation; however, resulted in a significant reversion of rosiglitazone-mediated inhibition of iNOS and COX-2 activity levels. Data are expressed as % of mean control value (n = 6). * $P < .05$ for colon manipulated (CM) *vs*. control mice, § $P < .05$ for rosiglitazone (Rosi)-treated *vs*. non-treated CM mice; # $P < .05$ for CM/Rosi/GW9662 *vs*. CM/Rosi mice; one-way ANOVA with Bonferoni post-hoc test.

VIII.4.5 Activation of PPARγ prevents surgically-induced ileus

The primary efficacy endpoint of this study was restoration of intestinal transit following colonic manipulation; improvement of surgically-induced intestinal contractile dysfunction was the secondary endpoint – as measured at 24 hours postoperatively.

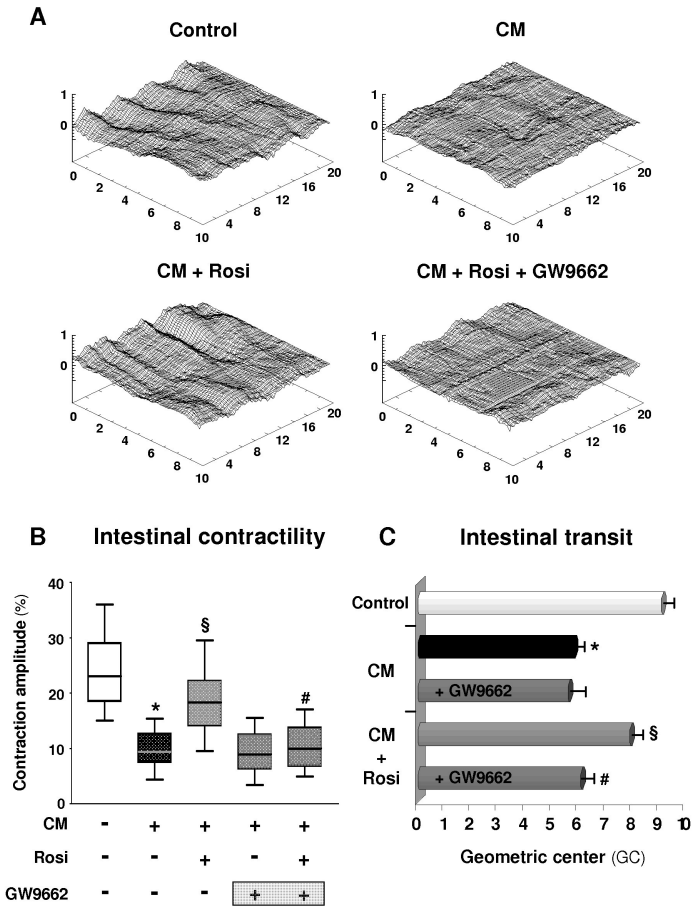

Figure VIII.8

Activation of PPARγ prevents surgically-induced intestinal dysmotility. (A) Intestinal contractile activity in the mid-jejunum was evaluated by spatiotemporal motility mapping (*see* Methods & Materials). Surgical manipulation of the colon results in a marked suppression of the spontaneous oscillatory contractions in the small intestine when compared to control mice. The severity of this surgically-induced intestinal dysmotility is significantly reduced when mice are pretreated with rosiglitazone; however, the protective effect of rosiglitazone is nearly abrogated by co-administration of GW9662. Box-and-whisker plots represent median [line-in-the-box], 25/75th percentiles [box], and 5/95th percentiles [whiskers] (n = 6). (B) Intestinal transit was evaluated by measuring the gastrointestinal distribution of orally fed fluorescein-labelled dextran using an optical fluorescence imaging system (*see* Methods & Materials). Surgical manipulation of the colon causes a significant delay in intestinal transit, as measured 24 hours postoperatively. This surgically-induced delay in intestinal transit is significantly prevented by rosiglitazone; however, co-administration of GW9662 abolishes this salutary effect of rosiglitazone treatment. The calculated geometric centres (GC) are given as mean ± SEM (n=6). * $P < .05$ for colon manipulated (CM) *vs*. control mice, § $P < .05$ for rosiglitazone (Rosi)-treated *vs*. non-treated CM mice; # $P < .05$ for CM/Rosi/GW9662 *vs*. CM/Rosi mice; one-way ANOVA with Bonferoni post-hoc test.

Intestinal transit was evaluated by measuring the gastrointestinal distribution of orally fed fluorescein-labelled dextran using an optical fluorescence imaging system (De Backer et al., 2008). In control mice, the non-absorbable fluorescent dextran was primarily localized in the distal segments of the small intestine at 90 minutes after gavage (GC = 9.0 ± 0.3). In non-treated manipulated mice, we visualised the highest fluorescence intensity in the more proximal segments of the intestinal tract (GC = 5.8 ± 0.2), a finding that is consistent with the presence of ileus. This surgically-induced delay in intestinal transit was significantly reduced in mice pretreated with rosiglitazone (GC = 7.8 ± 0.3); co-administration of GW9662, however, abolished this salutary effect of rosiglitazone treatment (Figure VIII.8C).

In parallel to these intestinal transit studies, we also evaluated spontaneous contractile activity in the small intestine using spatiotemporal motility mapping (De Backer et al., 2008). As shown in Figure VIII.8B, the small intestine showed vigorous contractile activity in control mice, resulting in a mean contraction amplitude (Amp-$P50_{mean}$) of 21.7%. As reported previously (Türler et al., 2007), selective colonic manipulation caused a marked suppression of small intestinal contractile activity (Amp-$P50_{mean}$ of 5.1 %). This surgically-induced impairment of intestinal contractility was significantly restored when mice were pretreated with rosiglitazone (Amp-$P50_{mean}$ of 15.1 %); however, this salutary effect of rosiglitazone was nearly abrogated by co-administration of GW9662.

VIII.5 Discussion

Ileus, a transient impairment of bowel motility, occurs in virtually every patient who undergoes major abdominal surgery and contributes to postoperative morbidity, increased patient discomfort, and prolonged hospitalization (Kehlet & Holte, 2001). Recent evidence indicates that a complex cascade of inflammatory responses within the intestinal muscularis can be considered the major cause of POI after abdominal surgery (Bauer & Boeckxstaens, 2004). In this study, we focused on transcriptional regulatory mechanisms that are involved in this postoperative inflammatory cascade. The above data show that 1/ PPARγ function is markedly impaired following colonic manipulation; 2/ treatment with rosiglitazone attenuates POI by suppression of the inflammatory cascade through a PPARγ-dependent down-regulation of Egr-1; and 3/ treatment with rosiglitazone markedly reduces the early oxidative burst in the mucosa following surgical manipulation, an effect that is independent of PPARγ. These findings suggest that rosiglitazone treatment may represent a potential new pharmacological approach to prevent POI.

Although PPARγ was initially thought to mainly regulate adipogenesis and glucose homeostasis, more recently, its role as an endogenous regulator of intestinal inflammation has been revealed (Clark, 2002; Straus & Glass, 2007). Here, we demonstrate that PPARγ was rapidly phosphorylated and subsequently degraded within both the mucosa and muscularis of the mouse colon following surgical manipulation. In accordance with WB results, we measured a rapid and sustained reduction in PPARγ DNA-binding activity, which was significantly restored by rosiglitazone treatment. This finding is consistent with prior studies reporting a marked decrease in PPARγ expression and/or activity in irradiation- and TNBS-induced colitis (Linard et al., 2008; Zhang et al., 2006), as well as in patients with ulcerative colitis (Dubuquoy et al., 2003). In contrast, Sato et al. (2005) reported a markedly increased PPARγ activity in a model of mesenteric ischemia/reperfusion (I/R) injury.

Similar to other nuclear receptors, PPARγ has been reported to be phosphorylated by mitogen-activated protein (MAP) kinases (reviewed in Diradourian et al., 2005; Burns & Vanden Heuvel, 2007); the MAP kinase phosphorylation site - which can be used by both ERK and JNK (Camp et al., 1999) - is mapped at Ser82 of mouse PPARγ1 (Shao et al., 1998). This modification decreases transcriptional activity of PPARγ (Adams et al., 1997). In addition, extracellular signals which activate intracellular phosphorylation pathways also influence the degradation process of PPARγ. For example, treatment with an inhibitor of MEK kinases inhibits proteasomal degradation of PPARγ (Floyd & Stephens, 2002). Since we previously demonstrated the involvement of MAP kinases in the early postoperative phase following intestinal manipulation (De Backer et al., 2009), we assume that surgical

manipulation-induced MAP kinase activation is responsible for the observed phosphorylation and subsequent degradation of PPARγ in our POI model (Figure 9).

Paralleling this early activation of MAP kinases, there is a marked increase in oxidative stress following bowel manipulation. Confirming our previous results (De Backer et al., 2009), an early but transient increase in oxidative stress - with peak value at 1 hour after surgery - was detected in the mucosal layer. Interestingly, Anup et al. (1999) reported before that mild intestinal handling induces a transient increase in xanthine oxidase (XO) activity in the enterocytes, which leads to increased mucosal permeability due to widening of the intercellular spaces (Anup et al., 1999, 2000). This loss of intestinal barrier function promotes bacterial translocation (Thomas & Balasubramanian) and contributes to the panenteric development of POI following colonic manipulation (Türler et al., 2007). As shown above, treatment with rosiglitazone significantly reduces this early oxidative burst in the mucosa following colonic manipulation. Remarkably, this rosiglitazone-mediated antioxidant effect was not affected by the PPARγ antagonist GW9662, indicating that this effect is independent of PPARγ. In accordance, rosiglitazone has been shown to reduce XO activity in the gastric mucosa following I/R injury; however, it was not reported whether this was a PPARγ-dependent *or* -independent effect (Villegas et al., 2004). In the muscularis layer, rosiglitazone treatment did not reduce the early increase in oxidative stress; however, significantly reduced surgically-induced ROS levels in the late post-operative phase. This latter effect was significantly reduced by GW9662 and paralleled the reduced cellular infiltration into the muscularis (*see* above).

Recent studies have demonstrated that surgical manipulation of the colon initiates an inflammatory cascade within both the colonic and small intestinal muscularis that consists of the induction of pro-inflammatory cytokines, chemokines and cellular adhesion molecules, the recruitment of circulating leukocytes, and the release of kinetically active substances that directly inhibit smooth muscle contractility (Türler et al., 2002, 2007). Here, we show that pretreatment of mice with rosiglitazone significantly attenuates these intestinal muscularis inflammatory responses, an effect that was abrogated by GW9662. In contrast to earlier studies reporting aggravation of intestinal injury in PPARγ$^{-/-}$ mice following intestinal I/R injury (Nakajima et al., 2001) *or* DSS-induced colitis (Adachi et al., 2006; Shah et al., 2007), we did not observe worsening of intestinal injury in mice pretreated with GW9662 alone compared to non-treated manipulated mice. A search of the literature, however, revealed that GW9662 modifies a conserved cysteine residue in the PPARγ molecule, interfering with ligand-binding but not with DNA-binding (Leesnitzer et al., 2002; Todorov et al., 2007). Therefore, GW9662 does not affect basal PPARγ activity but only its ligand-induced activation.

Very recently, it has been demonstrated that Egr-1 occupies a key position in the intestinal muscularis inflammatory cascade following surgical manipulation (Schmidt et al.,

2008). Egr-1, a zinc-finger transcription factor, represents an important pro-inflammatory transcriptional regulator that coordinates inflammatory responses in various cell types, including macrophages (Yan et al., 2000). The functional severity of POI was significantly ameliorated in Egr-1$^{-/-}$ mice and chimera wild-type mice transplanted with Egr-1$^{-/-}$ bone marrow. Based on these results, the authors concluded that 'the Egr-1 transcription factor could be a promising checkpoint for the therapeutic targeting of POI' (Schmidt et al., 2008). Our current results demonstrate that Egr-1 expression/activity is highly up-regulated in the manipulated colon as early as after 1 hour after surgery. In contrast, Egr-1 expression/activity was only increased at the 6-hour time point in the jejunum, indicating that this distant inflammatory response only occurs after a certain time interval. Pretreatment of mice with rosiglitazone significantly prevented this surgically-induced Egr-1 induction, an effect that was abrogated by GW9662. This finding is consistent with prior studies reporting that PPARγ counteracts Egr-1 in order to provide an anti-inflammatory transcriptional control mechanism. In this context, it has been demonstrated that activation of PPARγ suppresses ischemic induction of Egr-1 and its inflammatory gene targets, thereby providing protection against I/R-induced lung injury (Okada et al., 2002). Very recently, Wu et al. (2009) demonstrated that rosiglitazone attenuates bleomycin-induced skin inflammation as well as profibrotic responses by inhibiton of Egr-1 induction. Our findings are further supported by recent studies reporting that Egr-1 is suppressed by CO through a PPARγ-dependent signalling pathway. In murine macrophage cultures, the inhibitory effect of CO on Egr-1 expression and function was abrogated by PPARγ inhibition, either pharmacologically *or* genetically. In the same study, CO also protected mice against endotoxin-induced lung injury by inhibition of Egr-1, an effect that was blocked by GW9662 (Bilban et al., 2006). Very recently, Hoetzel et al. (2008) also demonstrated that inhaled CO protects against ventilator-induced lung injury via PPARγ activation and the subsequent inhibition of Egr-1.

The above studies suggest a role for PPARγ as a downstream mediator of CO-mediated anti-inflammatory effects (Bilban et al., 2006; Hoetzel et al., 2008). In contrast, other studies recently identified HO-1 as a target gene of PPARγ (Lin et al., 2006; Ptasinska et al., 2007; Krönke et al., 2007); this opens up the possibility that the protective effects of rosiglitazone as seen in this study should be ascribed to the HO-1/CO signalling pathway. In our current model, HO-1 expression was significantly up-regulated following colonic manipulation; however, rosiglitazone treatment partially prevented this surgically-induced HO-1 induction (Figure VIII.S3). This finding is consistent with the role of HO-1 as an inducible stress protein that is up-regulated as a consequence of systemic stress and inflammatory tissue injury (Ryter et al., 2006); however, it does not support the existence of a positive feedback loop between PPARγ and HO-1/CO. Nevertheless, this result does not exclude the possibility that CO-mediated protection against POI is mediated by PPARγ.

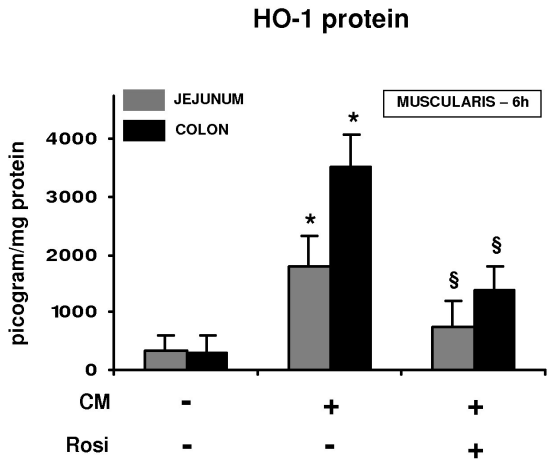

Figure SVIII.3

Effect of rosiglitazone treatment on HO-1 protein expression following colonic manipulation. Colonic manipulation results in a significant increase of HO-1 protein expression in the muscularis of both colon and jejunum. In rosiglitazone-treated manipulated mice, we observed a significant reduction of HO-1 protein expression compared to non-treated manipulated mice. Data are expressed as mean ± SEM (n = 4-6). * $P < .05$ for colon manipulated (CM) *vs*. control mice, § $P < .05$ for rosiglitazone (Rosi)-treated *vs*. non-treated CM mice; one-way ANOVA with Bonferoni post-hoc test.

Although we demonstrate that the anti-inflammatory effects of rosiglitazone within the intestinal muscularis are related to the stimulation of PPARγ, our experiments do not define the cellular target by which rosiglitazone limits the postoperative inflammatory responses. In the above mentioned study, reporting the critical role of Egr-1 in the development of POI, surgical manipulation-induced intestinal injury was nearly abolished in chimera wild-type mice transplanted with Egr-$1^{-/-}$ bone marrow. This led the authors to conclude that leukocyte-derived Egr-1 plays a critical role in the initiation of the postoperative inflammatory cascade. In the early postoperative phase, however, intense immunoreactivity for Egr-1 was observed in the nuclei of smooth muscle cells and a population of enteric ganglionic cells. This early expression of Egr-1 in the smooth muscle cells and enteric neurons waned substantially 24 hours after surgery and became more evident in the infiltrated leukocytes in the late postoperative phase (Schmidt et al., 2008). The data reported here do not distinguish between changes in PPARγ expression/activity in macrophages and/or smooth muscle cells. Since previous studies have shown that monocytes/macrophages are the major source of the inflammatory mediators contributing to POI, we suggest that the rosiglitazone-mediated anti-inflammatory effects should primarily be ascribed to their effect on PPARγ in cells of the monocyte/macrophage lineage. However, we can not exclude the possibility that activation of PPARγ in intestinal smooth muscle cells might contribute to the early protective effects of rosiglitazone (Lefebvre et al., 1999), as surgically-induced up-regulation of Egr-1 expression/activity at 1 hour after surgery was also reduced by rosiglitazone treatment.

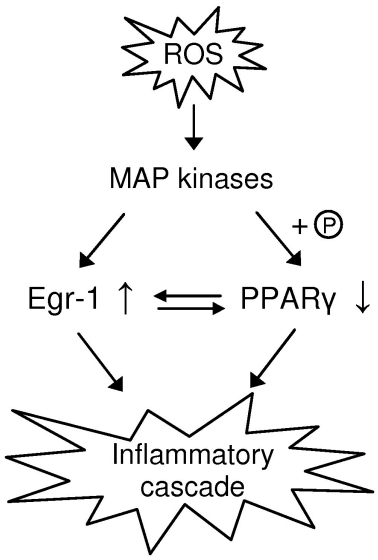

Figure VIII.9

Scheme of the proposed mechanism of PPARγ involvement in inflammatory responses generated by surgical manipulation of the bowel.

In conclusion, our study demonstrates for the first time that PPARγ plays a key role in regulating inflammatory responses within the intestinal muscularis following surgical manipulation. Pretreatment with rosiglitazone significantly attenuates POI by inhibiting the initiation of the muscularis inflammatory cascade through a PPARγ-dependent down-regulation of Egr-1. In addition, rosiglitazone markedly reduces the early oxidative burst in the mucosa following colonic manipulation; an effect that appears to be independent of PPARγ. These data encourage further clinical evaluation of rosiglitazone and other PPARγ agonists as pharmacological tool to prevent POI.

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Chapter IX

GENERAL DISCUSSION AND CONCLUSION

Chapter IX General Discussion and Conclusion

IX.1 Role of NO in the regulation of jejunal motility under physiological conditions and in postoperative inflammation

The neuronal component regulating gastrointestinal motility consists of contractile and inhibitory pathways. The main pathway evoking relaxations is the nitrergic pathway, which uses nitric oxide (NO) as inhibitory neurotransmitter. NO is produced by neuronal NO synthase-1 (NOS-1) and induces relaxations by activating soluble guanylate cyclase (sGC) (Rand & Li, 1995). However, depending upon species and region of the gastrointestinal tract, also other inhibitory neurotransmitters such as vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating polypeptide (PACAP), adenosine triphosphate (ATP) and carbon monoxide (CO) are involved in gastrointestinal inhibitory neurotransmission.

Patients undergoing surgical interventions, especially those involving the opening of the abdominal cavity, frequently experience postoperative ileus (POI). Postoperative ileus is defined as a reversible transient impairment of gastrointestinal motility (Person & Wexner, 2006). During this common period of gastrointestinal quiescence, motility of the gastrointestinal tract is marked by a lack of coordinated propulsion caused by disorganized electrical activity. As a consequence, POI is clinically characterized by abdominal distension, lack of bowel sounds, accumulation of gas and fluids in the bowel and a delayed passage of gas and stool. The clinical picture differs between patients and varies between patients who remain almost asymptomatic while others display serious symptoms including nausea, vomiting and stomach cramps leading to major postoperative discomfort and a prolonged recovery and hospital stay (Person & Wexner, 2006). This manipulation-induced impairment of gastrointestinal motility is caused by stimulation of inhibitory neurotransmission in the acute phase of POI while inflammation is the cause of hypomotility in the second more prolonged phase of POI. The most commonly studied region in the pathogenesis of POI is the small intestine. Circular muscle motility of the jejunum was inhibited in a pathological model of POI in rats (Kalff *et al.*, 1998). However, also other parts of the gastrointestinal tract are involved.

As our aim was to study the role of NO in POI and more specific in manipulation-induced inflamed jejunum, it is important to delineate the contribution of NO in the regulation of inhibitory neurotransmission in jejunal tissue under normal conditions. Therefore, our first aim

was to investigate the effect and the contribution of NO, ATP, VIP and PACAP to inhibitory neurotransmission under normal physiological conditions in the jejunum circular muscle of Wistar rat, the species intended to use in the POI model.

Upon inflammation of the gastrointestinal tract, NOS-1-derived NO was reported to induce beneficial effects, i.e., maintaining an intact mucosal barrier, while inducible NOS-2-derived NO was originally thought to only induce detrimental effects i.e., aggravation of the inflammation and inhibition of gastrointestinal motility. However, several reports suggested that NOS-2-derived NO might also be beneficial and that NOS-1-derived NO has detrimental effects. As the involvement of nitrergic inhibitory neurotransmission in the second prolonged phase of POI is unknown, the second aim was to investigate the effect of inflammation on nitrergic inhibitory neurotransmission in jejunal tissue of a rat model of POI.

IX.1.1 Role of NO in jejunal motility under physiological conditions

Conflicting results have been reported on the role of NO in inhibitory neurotransmission of rat jejunum. NO was reported to mediate non-adrenergic non-cholinergic (NANC) inhibitory responses in longitudinal muscle of rat jejunum in Sprague Dawley and Wistar ST rats (Niioka *et al.*, 1997; Okishio *et al.*, 2000). However, in circular muscle of Lewis rat jejunum, it was reported that NO is not involved in NANC inhibitory responses (Balsiger *et al.*, 2000). In general, the primary transduction pathway reported for NO involves the activation of sGC with subsequent production of guanosine 3',5'-cyclic monophosphate (cGMP) (Tanovic *et al.*, 2001). The increased cGMP levels induce relaxation via activation of cGMP-dependent protein kinases that result in the activation of apamin-sensitive small conductance Ca^{2+}-activated K^{+}-channels (SK_{Ca}-channels), leading to hyperpolarisation, inhibition of voltage-operated calcium channels and subsequent relaxation by a decrease in Ca^{2+} influx (Keef *et al.*, 1993; Koh *et al.*, 1995; Nelson & Quayle, 1995; Cayabyab & Daniel, 1996; Shuttleworth *et al.*, 1999). In rat small intestine, sGC activation was indeed reported to be involved in nitrergic relaxations; however, NO was also reported to induce relaxations by activation of sGC-independent pathways (Kanada *et al.*, 1992; Martins *et al.*, 1995).

ATP was reported to be involved in NANC inhibitory responses in longitudinal muscle of Wistar rat jejunum (Waseda *et al.*, 2005). Data on the involvement of ATP in inhibitory neurotransmission of circular muscle of rat jejunum were not found. ATP is a ligand for P2 purinoceptors, which exist in two main subtypes; i.e., the P2X receptors that are ligand-gated ion channels and the P2Y receptors that are coupled to G-proteins (Ralevic & Burnstock, 1998). Inhibitory responses are mainly mediated via P2Y receptors (Dalziel & Westfall, 1994; Kennedy, 2000).

In longitudinal muscle of Sprague Dawley and Wistar ST rat jejunum, PACAP and VIP do not contribute to NANC inhibitory responses (Niioka *et al.*, 1997; Okishio *et al.*, 2000). In circular

muscle of Lewis rats, electrical field stimulation (EFS)-induced contractions were increased by a VIP antagonist suggesting a role for VIP in inhibitory neurotransmission (Kasparek *et al.*, 2007). The neuropeptides VIP and PACAP interact with $VPAC_1$ and $VPAC_2$ receptors while only PACAP displays high affinity for the PAC_1 receptor (Harmar *et al.*, 1998).

We investigated the effect and the contribution of NO, ATP, VIP and PACAP to inhibitory neurotransmission in the circular muscle of Wistar rat jejunum. We showed that in Wistar rat jejunum, EFS induced frequency-dependent relaxations that were reduced by the nerve conduction blocker tetrodotoxin indicating the involvement of nerve activation. Activation of mechanisms at the varicosities of the NANC inhibitory neurons that require N-type Ca^{2+} channel activation but not action potential propagation via tetrodotoxin-sensitive Na^+ channels is not involved in these EFS-evoked responses as the N-type Ca^{2+} channel blocker ω-conotoxin GVIA reduced these responses similarly to tetrodotoxin. These results are in agreement with de Luca et al. (1990) who reported that ω-conotoxin GVIA did not decrease the EFS-induced relaxations to a more pronounced extent than tetrodotoxin in longitudinal muscle of Sprague Dawley rat jejunum. Capsaicin-sensitive sensory nerves with tetrodotoxin-resistant Na^+ channels were not involved in the EFS-induced relaxations as capsaicin only had a minor influence on the EFS-induced responses.

Exogenously applied ATP induced relaxation via P2Y receptors, exogenously applied VIP by interaction with both $VPAC_2$ and PAC_1 receptors and exogenously applied PACAP only interacted with PAC_1 receptors. But the P2Y receptor antagonist reactive blue 2, the $VPAC_2$-receptor antagonist PG99-465 and the PAC-receptor antagonist $PACAP_{6-38}$ had no influence on the EFS-induced relaxations. This excludes the involvement of ATP, VIP, and PACAP in jejunal inhibitory neurotransmission of Wistar rat jejunum.

Relaxations evoked by EFS were nearly abolished by the NOS inhibitor L-NAME, the sGC inhibitor ODQ and the SK_{Ca}-channel blocker apamin indicating the involvement of NO, and activation of sGC and SK_{Ca}-channels. Also the relaxation evoked by exogenously applied NO was reduced by ODQ and apamin indicating the involvement of sGC activation and of apamin-sensitive SK_{Ca}-channels in NO-evoked inhibitory responses in circular muscle of Wistar rat jejunum. The reduction of the EFS-evoked responses and of the relaxation induced by exogenous NO by ODQ plus apamin was not more pronounced than by ODQ alone except at a stimulation frequency of 4 Hz and at a concentration of 100 µmol/L NO. For the lower concentrations of NO released during EFS at lower frequencies or added exogenously, there is a serial link between the rise of cGMP and the activation of SK_{Ca}-channels. However, direct activation of SK_{Ca}-channels by higher concentrations of NO released at higher stimulation frequencies cannot be excluded. SK_{Ca}-channels can indeed be directly activated by NO through covalent modifications without interference of cGMP (Bolotina *et al.*, 1994; Koh *et al.*, 1995). Our results thus showed that we could fully

concentrate on nitrergic neurotransmission, when examining the inhibitory innervation of the jejunum of the Wistar rat in the postoperative period.

Also in murine small intestine, NO is an important neurotransmitter as L-NAME abolished the responses to EFS in jejunum of WT mice. The responses induced by endogenous and exogenous NO were clearly reduced by ODQ indicating that also in murine jejunum NO induces relaxation by activation of sGC.

IX.1.2 Role of NO in postoperative jejunal motility

The use of animal models of POI revealed that acute hypomotility is caused by an increase of adrenergic and nitrergic neuronal activity as these pathways are activated by an important manipulation-induced spinal reflex, while hypomotility in the second more prolonged inflammatory phase is caused by the secretion of NO and prostaglandins by activated resident macrophages and recruited leukocytes as blocking NOS-2 and cyclo-oxygenase-2 (COX-2) resulted in an increase of muscle contractility (Josephs *et al.*, 1999; Kalff *et al.*, 2000; Bauer & Boeckxstaens, 2004; Person & Wexner, 2006).

As the effect of manipulation-induced inflammation on the intestinal nitrergic innervation during the second phase of POI has not yet been examined, we investigated the influence of laparotomy and intestinal manipulation in muscle strips of Wistar rats 24 hours postoperatively. Nitrergic nerve activity was assessed by NOS-1 immunohistochemistry and by evaluation of the muscle responses to EFS and NO.

In strips of operated-saline animals, relaxations induced by EFS at 4 and 8 Hz were reduced compared to those evoked by EFS in strips of non-operated control animals indicating a decreased release of NO and/or an impaired functioning of the nitrergic pathway. As relaxations evoked by exogenous NO did not differ between strips of non-operated versus operated-saline animals, impaired functioning of the downstream pathway of NO can be excluded. However, as EFS-induced responses were only moderately reduced by L-NAME in strips of operated animals treated with saline in contrast to the pronounced reduction in strips of non-operated control rats, reduction of NO release does seem to be involved.

The decreased release of NO is probably caused by a reduced efficiency of the NOS-1 enzyme within the nitrergic neurons as neither the number of submucosal and myenteric neurons, nor their percentage with NOS-1 immunoreactivity differed between non-operated control and operated animals. Kurjak et al. (1999) reported that exogenous NO inhibited NOS activity in enteric synaptosomes. Large amounts of NO produced by activation of inducible NOS-2 were indeed reported to play an important role in the pathogenesis of gastrointestinal motility disturbances 24 hours after intestinal manipulation (Kalff *et al.*, 2000). The high amounts of NOS-2-derived NO can diffuse to the myenteric plexus where it may inhibit NOS-1 activity. An additional argument for reduced efficiency of the NOS-1 enzyme

during the inflammatory phase of POI was obtained by the result that L-NAME and ODQ + apamin increased basal tone in strips of non-operated animals while they did not influence basal tone in strips of operated animals, suggesting that the tonic release of NO acting through sGC and/or SK_{Ca}-channels in control strips is diminished or even abolished in strips of operated animals. The effect of L-NAME indeed corresponds to that seen with NOS-inhibitors in other rat gastrointestinal smooth muscle preparations, which was ascribed to inhibition of the tonic release of NO from nitrergic nerves (Li & Rand, 1990; Boeckxstaens *et al.*, 1991).

Our findings thus suggest that the increase of nitrergic activity caused by the activation of an important manipulation-induced spinal reflex reported in the early postoperative phase (De Winter *et al.*, 1997; Meile *et al.*, 2006) might switch during the second prolonged phase of POI to decreased nitrergic neuronal activity caused by a reduced efficiency of the NOS-1 enzyme, possibly in reaction to increased quantities of NOS-2-derived NO. This might contribute to the hypomotility during POI as inhibition of the nitrergic inhibitory influences has been shown to delay small intestinal transit in rats (Karmeli *et al.*, 1997) and healthy humans (Fraser *et al.*, 2005).

IX.2 Role of sGC in the regulation of gastrointestinal motility under physiological conditions and in postoperative inflammation

The principal target of the relaxant neurotransmitter NO is sGC, which is a heterodimeric protein that contains an α and a β subunit, each containing a haem-binding, a catalytic and a dimerization domain (Hobbs, 1997). NO stimulates sGC through the formation of a nitrosyl-haem complex, leading to an increase in catalytic activity (Stone & Marletta, 1996). Both the α and β subunit exist in 2 isoforms, α_1 and α_2 and β_1 and β_2. The $\alpha_1\beta_1$ and the $\alpha_2\beta_1$ heterodimers seem to be the physiologically active forms with no differences in kinetic properties and sensitivity towards NO (Russwurm *et al.*, 1998; Mergia *et al.*, 2003). Of the 2 isoforms, sGC$\alpha_1\beta_1$ is thought to predominate in the gastrointestinal tract but sGC$\alpha_2\beta_1$ is also present (Mergia *et al.*, 2003). Therefore the consequences of knocking out the α_1-subunit of sGC were studied at the level of the stomach, where NO is involved in the control of gastric emptying, and at the level of the jejunum, where NO is involved in peristalsis.

The detrimental effects of NOS-2-derived NO are usually ascribed to sGC-independent mechanisms (Davis *et al.*, 2001). Moreover, NOS-2-derived NO was reported to possess anti-inflammatory properties via a sGC-dependent pathway in a murine model of endotoxin-induced lung injury (Glynos *et al.*, 2007). In contrast, Cauwels et al. (2000) suggested that in a TNF model of lethal shock, NOS-2-derived NO induced detrimental vascular effects caused by sGC activation, while it also had a protective role in a sGC-independent way. As the

induction of NOS-2 plays an important role in the second prolonged phase of POI (Kalff *et al.*, 2000) and as the role of sGC in this phase of POI has not yet been assessed, our last aim was to investigate the effect of postoperative inflammation on sGC functioning in jejunal tissue of the Wistar rat model of POI.

IX.2.1 Role of sGC in gastric and jejunal motility under physiological conditions

We investigated the consequences of knocking out the α_1-subunit of sGC at the level of the stomach and the jejunum using cGMP level determination and muscle tension experiments. The sGCα_1 KO mice were generated by targeting exon 6 of the sGCα_1 gene (or exon 5 when the first 5' non-coding exon is not taken into account), which codes for an essential part of the catalytic domain. When studying the cardiovascular consequences in these sGCα_1 KO mice, Buys et al. (2008) observed a gender-specific phenotype, with male KO mice showing testosteron-dependent hypertension. The gastric fundus did not display differences in nitrergic responses between male and female KO mice. However, the pylorus and small intestine of KO mice did show gender-related alterations in gastrointestinal motility. The results obtained in male sGCα_1 KO mice will be discussed first, followed by the differences observed in female mice.

sGC functioning in male mice

Stomach

In gastric circular muscle strips of male wild-type (WT) mice, NO induced concentration-dependent relaxations that were abolished by ODQ at the lowest concentration of NO (i.e., 1 µmol/L) and reduced by ODQ at the higher concentrations of NO (i.e., 10 and 100 µmol/L). Also the increase in cGMP levels induced by 10 µmol/L NO was almost abolished by ODQ suggesting that NO indeed activates sGC in gastric fundus of male WT mice. In KO mice, the responses to NO were moderately decreased compared to WT mice and were still sensitive to ODQ indicating that NO can relax gastric fundus strips of male KO mice through activation of sGC$\alpha_2\beta_1$. This is corroborated by the observation that NO still induced an increase in cGMP levels that was reduced by ODQ in male KO strips. Although the NO-induced increase in cGMP levels was much smaller in KO than in WT tissues, these lower cGMP levels were able to induce almost the same degree of relaxation suggesting that the subcellular localization of cGMP produced by sGC$\alpha_2\beta_1$ in KO strips differs from that by activation of sGC$\alpha_1\beta_1$ in WT strips. A different subcellular localisation of the 2 heterodimers has indeed been reported with the $\alpha_1\beta_1$ heterodimer being found in the cytosol while the $\alpha_2\beta_1$ heterodimer is reported to be associated to synaptic membranes in brain and the apical segment of epithelial cells in human colon (Russwurm *et al.*, 2001; Zabel *et al.*, 2002;

Bellingham & Evans, 2007). Subsequently, $sGC\alpha_2\beta_1$ activation may increase the efficiency of NO signalling at membrane targets, such as cGMP-gated ion channels, which might explain the same degree of relaxation via a smaller amount of measurable cGMP.

The results with EFS indicate that also endogenous NO can relax gastric fundus strips through $sGC\alpha_2\beta_1$ in $sGC\alpha_1$ KO mice, at least when released for a sufficiently long time. Relaxations induced by EFS with 10 s trains were abolished by L-NAME and ODQ in WT mice indicating NOS-1-derived NO release and activation of sGC. Also the relaxations by EFS with 60 s trains at 1 to 8 Hz were abolished by L-NAME and ODQ, indicating that they are fully nitrergic and that NO release is thus maintained for 60 s. This is also supported by the L-NAME- and ODQ-sensitive rise in cGMP measured for EFS (60 s train) at 16 Hz. In $sGC\alpha_1$ KO mice, relaxant responses to 10 s trains of EFS were very small or absent, suggesting that $sGC\alpha_1\beta_1$ is essential for a fast nitrergic response. Relaxant responses to EFS with 60 s trains (2-16 Hz) were well maintained in KO mice. As in WT tissues, relaxations induced by EFS (60 s train) at 2 to 8 Hz in KO strips were abolished by L-NAME and ODQ. This suggests that more prolonged release of endogenous NO can also lead to activation of $sGC\alpha_2\beta_1$ in gastric fundus strips of $sGC\alpha_1$ KO mice; this is supported by the modest but significant increase in cGMP upon EFS (60 s train) at 16 Hz.

No adaptive changes occurred in the relaxant pathway downstream of sGC as the relaxant response to the cell-permeable cGMP analog 8-Br-cGMP was not different between WT and KO mice. These observations are in agreement with observations in vascular tissue of the $sGC\alpha_1$ KO mice with an exon 6 (5) deletion, where relaxations induced by a cGMP-analog were not altered between WT and KO mice (Nimmegeers *et al.*, 2007). In contrast, increased sensitivity to a cGMP-analog was observed in vascular tissue of $sGC\alpha_1$ KO mice with an exon 4 deletion (Mergia *et al.*, 2006).

In male $sGC\alpha_1$ KO mice, liquid gastric emptying was accelerated 15 min after gavage. This is probably caused by a reduction of the nitrergic storage capacity in the fundus as it was reported that nitrergic relaxation in the fundus is important for gastric accommodation (Desai *et al.*, 1991). However, NOS-1 and cGMP-dependent protein kinase I (cGKI) KO mice show delayed gastric liquid emptying (Pfeifer *et al.*, 1998; Mashimo *et al.*, 2000), which was ascribed to increased pyloric tone by inhibition of nitrergic relaxation in the pylorus. This will counteract the stimulating effect of decreased gastric accommodation capacity on liquid emptying (Anvari *et al.*, 1998), leading to gastric dilation (Huang *et al.*, 1993; Ny *et al.*, 2000). As the $sGC\alpha_1$ KO mice did not show gastric enlargement, less important consequences at the pyloric level of knocking out $sGC\alpha_1$ were expected than of knocking out NOS-1 and cGKI. Indeed, in-vitro, nitrergic relaxation in the pylorus only displayed a tendency towards

reduction in male sGCα_1 KO compared to WT mice suggesting that nitrergic relaxation of pyloric rings can occur via activation of sGC$\alpha_2\beta_1$ in male sGCα_1 KO mice.

Small intestine

In jejunal circular muscle strips of male WT mice, exogenous NO induces relaxation through activation of sGC as the sGC inhibitor ODQ clearly reduced these nitrergic relaxations. Furthermore, exogenous NO induced a 10-fold increase in cGMP levels in jejunum strips of male WT mice further supporting the importance of sGC activation in nitrergic relaxation. In male sGCα_1 KO mice, responses evoked by exogenous NO were almost absent and the increase in cGMP levels by exogenous NO was clearly reduced indicating that the major sGC isoform involved in nitrergic relaxation of male jejunum is sGC$\alpha_1\beta_1$. Still, the small NO-induced responses were sensitive to ODQ, and exogenous NO induced a 2.5-fold increase in cGMP levels in sGCα_1 KO mice indicating that nitrergic relaxation can occur via activation of sGC$\alpha_2\beta_1$.

In WT jejunal circular muscle strips, L-NAME abolished the EFS-induced relaxations indicating the release of endogenous NO upon EFS. These nitrergic relaxations are clearly reduced by ODQ suggesting that NO induces relaxation by sGC activation. As with relaxations induced by exogenous NO, responses evoked by endogenous NO were almost absent in male sGCα_1 KO mice indicating that sGC$\alpha_1\beta_1$ is the most important sGC isoform involved in nitrergic relaxation of jejunum of male mice. The small EFS-induced responses were sensitive to ODQ indicating that also endogenous NO can relax jejunal strips by activation of sGC$\alpha_2\beta_1$.

In male WT jejunum, application of L-NAME induced an increase of the NO-evoked responses suggesting desensitization of sGC by tonically released NO (Shirasaki & Su, 1985; Busse *et al.*, 1989; Luscher *et al.*, 1989). This is corroborated by the finding that the relaxant response to 8-Br-cGMP, which activates the relaxing pathway down-stream of sGC, was increased by ODQ suggesting that the relaxant pathway downstream of sGC is also desensitized. An additional argument for sGC desensitization is that the response to 8-Br-cGMP was increased in male KO compared to male WT mice indicating that in jejunum strips, the loss of sGCα_1 leads to increased responsiveness in the down-stream pathway of sGC. These observations differ from observations in the gastric fundus and vascular tissue of sGCα_1 KO mice with an exon 6 (5) deletion but are in agreement with observations in vascular tissue of sGCα_1 KO mice with an exon 4 deletion (Mergia *et al.*, 2006). Still, activation of the down-stream pathway via sGC$\alpha_2\beta_1$ also induces some desensitization as ODQ still increased the 8-Br-cGMP-induced response in jejunum strips of sGCα_1 KO mice.

We have no obvious explanation why sGC and the down-stream pathway are desensitized in jejunum and not in gastric fundus.

sGC functioning in female mice

At the level of the pylorus, the decrease in responses to exogenous and endogenous NO were much more pronounced in female KO tissues compared to male KO tissues. This will lead to a more pronounced resistance at the pylorus and probably explains the gender-dependent influence of knocking out sGCα_1 on gastric emptying. Indeed, in female KO mice, gastric emptying was not increased as in male KO mice because liquid gastric emptying is more opposed at the level of the pylorus in female mice. The more pronounced decrease in nitrergic relaxation in the pylorus thus influences the balance between the 2 opposite forces controlling liquid emptying (enhanced fundic pressure increasing emptying and increased resistance at the pylorus slowing emptying).

In jejunum strips of female sGCα_1 KO mice, a clear-cut relaxation after application of exogenous NO and upon EFS was observed. The clearly remaining responses to exogenous NO and EFS (2-8 Hz) were sensitive to ODQ, suggesting that sGC$\alpha_2\beta_1$ plays a more important role in nitrergic relaxation of jejunum strips of female sGCα_1 KO mice than in male sGCα_1 KO mice.

Thus at the level of the pylorus, the influence of knocking out sGCα_1 on nitrergic relaxation is more pronounced in female than in male mice, while the opposite is found at the level of the jejunum. This gender-related difference is not caused by gender-dependent changes in the sGC$\alpha_2\beta_1$ isoform in the female mice, as we found no up-regulation of sGCα_2 and sGCβ_1 mRNA in jejunal tissue of male as well as female sGCα_1 KO mice. We do thus not have an evident explanation for this gender-specific alteration and for the tissue specific difference in female KO mice. Still, when studying the cardiovascular consequences in the sGCα_1 KO mice with an exon 6 (5) deletion, Buys et al. (2008) also observed a gender-specific phenotype with male sGCα_1 KO mice developing hypertension, whereas female sGCα_1 KO mice did not. Ovariectomy did not influence this gender-specific phenotype indicating that the female-specific compensatory mechanism is oestrogen independent. However, orchidectomy and treatment with an androgen receptor antagonist prevented the male-specific hypertension indicating that the hypertension observed in male KO mice is testosteron-dependent.

IX.2.2 Role of sGC in postoperative jejunal motility

We examined the possible role of sGC in POI by measuring cGMP levels in plasma and jejunum and by evaluating the influence of treatment with sGC inhibitors on alterations induced by laparotomy and manipulation in inhibitory neurotransmission.

We showed that 24 hours postoperatively, plasma cGMP levels were decreased in comparison to pre-operative levels in operated animals, while plasma cGMP levels were well-maintained in control animals. Although intestinally produced cGMP contributes to plasmatic cGMP (Ito *et al.*, 1988), cGMP levels in jejunal tissues were not decreased 24 hours after abdominal operation. These findings differ from results obtained in a study in a rat model of DSS-induced colitis, in which both plasmatic and colonic cGMP levels were reported to be reduced 7 days after the start of colitis induction (Van Crombruggen *et al.*, 2007).

Intestinal manipulation causes a pan-enteric inflammation (Schwarz *et al.*, 2004; Turler *et al.*, 2007) and the lower postoperative plasmatic cGMP levels might thus be caused by the high levels of pan-enteric NOS-2-derived NO that causes decreased responsiveness to sGC. Decreased sGC sensitivity might represent an adaptive mechanism to prevent excessive cGMP levels. Indeed, Papapetropoulos et al. (1996) suggested that decreased sGC sensitivity might represent an important homeostatic mechanism to offset extensive vasodilatation in sepsis as they observed decreased responsiveness of sGC to NO stimulation following cytokine or LPS challenge. The relaxant response to exogenous NO in the jejunum was not influenced in operated animals, arguing against a decrease in jejunal sGC sensitivity and corresponding with the maintained jejunal cGMP levels. Still, it cannot be excluded that other parts of the manipulated small intestine such as the ileum have decreased sGC activity.

In vivo treatment with the sGC inhibitors methylene blue and ODQ had no influence on plasmatic and jejunal cGMP levels, nor on the reduced EFS-induced relaxation. The doses used (i.e., a total dose of 20 mg/kg for ODQ and a total dose of 40 mg/kg for methylene blue) were based on literature data where they reversed endotoxin-induced delay in murine gastric emptying measured 18 hours after exposure to endotoxin (De Winter *et al.*, 2002) and attenuated the cardiac cGMP rise and the protective effect of CO on myocardial ischemia-reperfusion injury (Fujimoto *et al.*, 2004). But these doses do thus not allow to assess whether in vivo sGC inhibition and cGMP decrease might be beneficial on POI within our experimental conditions.

IX.3 Future perspectives and clinical implications

It is unclear whether inhibition of sGC might be a therapeutic option to treat POI as our results with sGC inhibitors are inconclusive. No ideal sGC inhibitors for in-vivo use are

currently available. However, as we observed a decrease in plasmatic cGMP levels 24 hours after laparotomy and intestinal manipulation, it might be useful to create a study design in which the consequences of prevention of cGMP reduction is examined in a 24 hour POI model in rat by treatment with a PDE-5 inhibitor. PDE-5 preferentially hydrolyzes cGMP and is reported to be the most important cGMP-regulated PDE in smooth muscle (Wallis *et al.*, 1999). In addition, inhibition of this PDE was reported to induce cGMP accumulation in gastrointestinal smooth muscle cells (Kaneda *et al.*, 1997; Murthy, 2001). If cGMP is indeed involved in anti-inflammatory pathways as proposed by Dal Secco et al. (2006) and Glynos et al. (2007), then manipulation-induced hypomotility should be prevented. Conversely, if sGC activation is involved in endotoxin-induced gastrointestinal motility disturbances as proposed by De Winter et al. (2002) and Quintana et al. (2004) and/or if decreased sGC sensitivity indeed represents an adaptive mechanism to prevent excessive cGMP levels, then the motility symptoms of POI will aggravate. Another possibility to further investigate the role of sGC in physiological and pathophysiological conditions is the use of $sGC\beta_1$ knock-in mice. In these mice, NO can no longer stimulate sGC (i.e., both $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$). Surely, these animals will show disturbed gastrointestinal motility responses under physiological conditions, but once established the animals can also be studied to investigate the pro-/anti-inflammatory effect of sGC in inflammatory models such as POI.

Pharmacological options for the treatment of POI include the use of prokinetic agents, laxatives, non-steroidal anti-inflammatory drugs and selective μ_2-opioid receptor antagonists; however, no generally applicable pharmacologic therapy currently exists (Bauer & Boeckxstaens, 2004; Person & Wexner, 2006). We observed a manipulation-induced reduction of the nitrergic neuronal activity caused by a reduced efficiency of the NOS-1 enzyme, possibly induced by increased quantities of NOS-2-derived NO in the second prolonged phase of POI. As normal NOS-1 activity is required for optimal intestinal transit, it might be useful to treat patients suffering from POI with selective NOS-2 inhibitors which will prevent the increase of NOS-2-derived NO and subsequently result in the prevention of the inhibition of NOS-1 activity. Thus, maintaining normal NOS-1 activity by NOS-2 inhibition might prevent postoperative impairment of intestinal motility. Highly selective NOS-2 inhibitors are 1400W and GW274150 of which only GW274150 is still in clinical development as the development of 1400W was stopped because of acute toxicity (Chatterjee *et al.*, 2003).

IX.4 Conclusion

Nitric oxide is the main inhibitory neurotransmitter in circular muscle of Wistar rat jejunum; it induces inhibition via a rise in cGMP levels and the activation of SK_{Ca}-channels in a serial

way. Manipulation-induced inflammation of rat small intestine induces an impairment of the nitrergic neurotransmission caused by decreased NOS-1 activity in the nitrergic nerves possibly in reaction to increased quantities of NO produced by NOS-2. The possible positive versus negative role of sGC in manipulation-induced intestinal inflammation remains ambiguous as in-vivo treatment with sGC inhibitors was not able to change cGMP levels.
In murine gastric and small intestinal tissues, $sGC\alpha_1\beta_1$ plays an important role in nitrergic relaxation in-vitro, but some degree of nitrergic relaxation can occur via $sGC\alpha_2\beta_1$ activation in $sGC\alpha_1$ KO mice, which contributes to the moderate in-vivo consequence on gastric emptying. In the small intestine, relaxation via activation of $sGC\alpha_2\beta_1$ is more pronounced in female than in male $sGC\alpha_1$ KO mice while the opposite was found in the pylorus.

IX.5 References

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Chapter X

SUMMARY

Chapter X Summary

Gastrointestinal motility is regulated by hormonal and neuronal control mechanisms. Besides contractile cholinergic and inhibitory adrenergic pathways, also non-adrenergic, non-cholinergic (NANC) pathways are involved in the neuronal control of gastrointestinal motility. The main NANC pathway evoking relaxations is the nitrergic pathway, which uses nitric oxide (NO) as inhibitory neurotransmitter. NO is produced by neuronal NO synthase 1 (NOS-1) and induces relaxations by activating soluble guanylate cyclase (sGC) in the smooth muscle cells. Still, NO has sGC-independent effects such as nitration and S-nitrosylation of proteins and sGC can be stimulated by other stimuli than NO such as carbon monoxide (CO). Depending upon species and region of the gastrointestinal tract, also other inhibitory NANC neurotransmitters such as vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating polypeptide (PACAP), adenosine triphosphate (ATP) and CO are involved in gastrointestinal inhibitory neurotransmission.

Upon inflammation of the gastrointestinal tract, NOS-1-derived NO was reported to induce beneficial effects, while inducible NOS-2-derived NO was originally thought to only induce detrimental effects. However, several reports suggested that NOS-2-derived NO might also be beneficial and that NOS-1-derived NO has detrimental effects. In the acute phase of postoperative ileus (POI), hypomotility is indeed thought to be caused by an increase of neuronal nitrergic activity and also adrenergic activity as these pathways are activated by an important manipulation-induced spinal reflex. In the second more prolonged inflammatory phase of POI, hypomotility is caused by secretion of NO and prostaglandins by activated resident macrophages and recruited leukocytes with up-regulated NOS-2 and cyclo-oxygenase-2 (COX-2). sGC activation was reported to have a protective role during inflammation. However, also detrimental effects during gastrointestinal inflammation were suggested to be mediated by sGC activation.

The first part of this work focused on the inhibitory NANC neurotransmitter NO and its principal mechanism of action, i.e., activation of sGC, and investigated the role of NO and sGC in jejunal motility under physiological and pathophysiological conditions using Wistar rats under normal conditions and 24 hours after intestinal manipulation (a model for POI).

In a first study, we investigated the involvement of NO, ATP, VIP and PACAP in rat jejunal inhibitory neurotransmission and studied their mechanism of action concentrating on the role

of sGC and small conductance Ca^{2+}-activated K^+-channels (SK_{Ca}-channels) (**Chapter III**). We showed that NO is the main inhibitory neurotransmitter in circular muscle of Wistar rat jejunum and that NO induces relaxation by activation of sGC and SK_{Ca}-channels. For the lower concentrations of NO, there is a serial link between the rise of guanosine 3',5'-cyclic monophosphate (cGMP) and the activation of SK_{Ca}-channels but direct activation of SK_{Ca}-channels by higher concentrations of NO released at higher stimulation frequencies could not be excluded. Exogenous ATP, VIP and PACAP induced relaxation but no evidence was obtained for a role in jejunal inhibitory neurotransmission.

In a second series of experiments, we studied the role of NO and the effect of sGC inhibition on inflammation and NANC neurotransmission in jejunal tissue of a Wistar rat model of POI (**Chapter IV**). We observed that both electrical field stimulation (EFS)-induced NO release and basal tonic NO release were reduced during inflammation in the second prolonged phase of POI. The relaxations induced by exogenous NO were not impaired; the amount of NOS-1 messenger ribonucleic acid (mRNA) and the number of submucosal and myenteric nitrergic neurons did not differ between control and operated animals. Therefore, the decreased release of NO is probably caused by a reduced efficiency of the NOS-1 enzyme within the nitrergic neurons, possibly induced by increased quantities of NOS-2-derived NO. Plasmatic cGMP levels were decreased 24 hours after intestinal manipulation, while jejunal cGMP levels were maintained. To investigate whether a decrease in cGMP levels by pharmacological means would ameliorate or aggravate the POI-induced alterations, in-vivo treatment with sGC inhibitors was studied. Although doses based on the literature were used, this treatment did not influence plasmatic or jejunal cGMP levels so that our hypothesis that sGC inhibition might be beneficial for POI could not be assessed.
As an increased number of α_2-receptors has been reported on infiltrating monocytes in the intestinal muscularis during the second prolonged phase of POI, playing a role in the regulation of NOS-2 induction, we also assessed whether the presynaptic α_2-receptor population on the cholinergic nerve endings is influenced during POI. We found no change in the presynaptic α_2-receptor mediated control of intestinal cholinergic nerve activity.

sGC exists in 2 isoforms i.e., sGC$\alpha_1\beta_1$ and sGC$\alpha_2\beta_1$ of which the $\alpha_1\beta_1$-isoform predominates in the gastrointestinal tract. Therefore, in part 2 of the thesis, the consequences of knocking out the α_1-subunit of sGC were studied at the level of the stomach (**Chapter V**), where NO is involved in the control of gastric emptying, and at the level of the jejunum (**Chapter VI**), a tissue displaying phasic activity where NO is involved in peristalsis.

In the gastric fundus, we observed that exogenous NO can induce relaxation through activation of $sGC\alpha_2\beta_1$ in knock-out (KO) mice. The results with endogenous NO indicate that $sGC\alpha_1\beta_1$ is essential for a fast nitrergic response but that more prolonged release of endogenous NO can also lead to activation of $sGC\alpha_2\beta_1$ in gastric fundus strips of $sGC\alpha_1$ KO mice. In male $sGC\alpha_1$ KO mice, liquid gastric emptying was accelerated 15 min after gavage, which is probably due to reduction of the nitrergic storage capacity in the fundus. Gastric liquid emptying in female $sGC\alpha_1$ KO mice was not increased as in male $sGC\alpha_1$ KO mice because gastric liquid emptying was more opposed at the level of the pylorus. Indeed, pyloric nitrergic relaxation was clearly reduced in female $sGC\alpha_1$ KO mice indicating that $sGC\alpha_1\beta_1$ is the most important sGC isoform involved in nitrergic relaxation of pylorus of female mice. In contrast, nitrergic relaxation occurs via $sGC\alpha_2\beta_1$ in pylorus of male $sGC\alpha_1$ KO mice.
In the jejunum, we observed that both exogenous and endogenous NO mainly acts through activation of $sGC\alpha_1\beta_1$ in male mice. Still, moderate nitrergic relaxations can occur via activation of $sGC\alpha_2\beta_1$ in male $sGC\alpha_1$ KO mice. In contrast, in female mice, exogenous and endogenous NO mainly acts via activation of $sGC\alpha_2\beta_1$ in $sGC\alpha_1$ KO mice. Thus in comparison to male mice, the maintenance of nitrergic relaxation through $sGC\alpha_2\beta_1$ activation is more pronounced in the jejunum of female $sGC\alpha_1$ KO mice, while this mechanism is less pronounced in the pylorus.

In conclusion, NO is the main inhibitory neurotransmitter in circular muscle of Wistar rat jejunum; it induces inhibition via a rise in cGMP levels and the activation of SK_{Ca}-channels in a serial way. Manipulation-induced inflammation of rat small intestine induces an impairment of the nitrergic neurotransmission caused by decreased NOS-1 activity in the nitrergic nerves possibly in reaction to increased quantities of NO produced by NOS-2. The possible role of sGC in manipulation-induced inflammation remains ambiguous as in-vivo treatment with sGC inhibitors did not allow to change plasmatic and jejunal cGMP levels within the experimental conditions used.
In murine gastric and small intestinal tissues, $sGC\alpha_1\beta_1$ plays an important role in nitrergic relaxation in-vitro. However, some degree of nitrergic relaxation can occur via $sGC\alpha_2\beta_1$ activation in $sGC\alpha_1$ KO mice in a gender and tissue specific way.

Chapter X

SAMENVATTING

Chapter XI Samenvatting

Gastro-intestinale motiliteit wordt geregeld door hormonale en neuronale controle-mechanismen. Behalve contractiele cholinerge en relaxerende noradrenerge transductiewegen, zijn ook niet-adrenerge niet-cholinerge (NANC) transductiewegen betrokken in de neuronale controle van de gastro-intestinale motiliteit. De belangrijkste relaxerende NANC transductieweg is de nitrerge weg die relaxaties veroorzaakt door de vrijstelling van de neurotransmitter stikstofmonoxide (NO). NO wordt geproduceerd door neuronaal NO synthase-1 (NOS-1) en veroorzaakt relaxatie van de gladde spiercellen door activering van oplosbaar guanylaat cyclase (sGC). Niettemin heeft NO ook sGC-onafhankelijke effecten zoals nitratie en S-nitrosylatie van eiwitten en kan sGC worden gestimuleerd door andere neurotransmitters dan NO, zoals koolstofmonoxide (CO). Behalve NO zijn ook andere relaxerende neurotransmitters (zoals vasoactief intestinaal peptide (VIP), hypofysair adenylaat cyclase activerend peptide (PACAP), adenosine-trifosfaat (ATP) en CO) betrokken in gastro-intestinale relaxerende neurontransmissie.

Er werd aangenomen dat NO aangemaakt door NOS-1 enkel voordelige effecten zou hebben tijdens ontsteking van de gastro-intestinale tractus, terwijl NO aangemaakt door induceerbaar NOS-2 enkel nadelige gevolgen zou veroorzaken. Verschillende studies meldden echter dat activering van NOS-2 ook heilzaam kan zijn en dat activering van NOS-1 mogelijk ook schadelijke effecten kan veroorzaken. De verminderde motiliteit waargenomen tijdens de acute fase van postoperatieve ileus (POI) wordt veroorzaakt door een verhoging van de neuronale nitrerge en noradrenerge activiteit aangezien deze transductiewegen geactiveerd worden door een belangrijke spinale reflex die geactiveerd wordt door manipulatie van de darm. De verminderde motiliteit waargenomen tijdens de tweede langdurige fase van POI wordt veroorzaakt door secretie van NO, aangemaakt door NOS-2, en prostaglandines, aangemaakt door induceerbaar cyclo-oxygenase-2 (COX-2), door geactiveerde reeds aanwezige macrofagen en aangetrokken leukocyten. De voordelige effecten van NO tijdens inflammatie worden toegeschreven aan activering van sGC. Niettemin werden ook negatieve effecten van NO tijdens gastro-intestinale ontsteking toegeschreven aan activering van sGC.

In het eerste deel van deze studie concentreerden we ons op de relaxerende NANC neurotransmitter NO en zijn voornaamste werkingsmechanisme, nl. activering van sGC. De rol van NO en sGC in de motiliteit van de dunne darm tijdens fysiologische en

pathofysiologische omstandigheden werd onderzocht met behulp van normale Wistar ratten en Wistar ratten die 24 uren voordien een abdominale operatie ondergingen (een model voor POI).

In een eerste studie onderzochten we de betrokkenheid van NO, ATP, VIP en PACAP in relaxerende neurotransmissie in het jejunum van de rat. Daarenboven werd ook hun werkingsmechanisme, concentrerend op de rol van sGC en Ca^{2+}-afhankelijke K^+-kanalen met lage geleiding (SK_{Ca}-kanalen), bestudeerd (**Hoofdstuk III**). Er werd aangetoond dat NO de belangrijkste relaxerende neurotransmitter is in de circulaire spierlaag van het jejunum van de Wistar rat en dat NO relaxaties veroorzaakt door activering van sGC en SK_{Ca}-kanalen. Voor de lagere NO concentraties bestaat er een seriële band tussen de verhoging van de spiegels van cyclisch guanosinemonofosfaat (cGMP) en de activering van SK_{Ca}-kanalen maar directe activering van SK_{Ca}-kanalen door hogere concentraties van NO vrijgesteld bij hogere stimulatiefrequenties kan niet worden uitgesloten. Exogeen toegediend ATP, VIP en PACAP veroorzaakten een relaxatie maar er werden geen argumenten voor hun betrokkenheid in relaxerende neurotransmissie van het jejunum van de rat gevonden.

In een tweede serie van experimenten onderzochten we de rol van NO en het effect van inhibitie van sGC op ontsteking en NANC neurotransmissie in het jejunum van Wistar ratten waarvan de darm 24 uren voordien was gemanipuleerd na laparotomie (een model voor POI) (**Hoofdstuk IV**). We toonden aan dat zowel vrijstelling van NO door elektrische veldstimulatie (EFS) als basale tonische vrijstelling van NO verminderd waren tijdens de ontsteking in de tweede langdurige fase van POI. De relaxaties veroorzaakt door exogeen NO waren niet aangetast. Daarenboven was de hoeveelheid boodschapper ribonucleïnezuur (mRNA) voor NOS-1 en het aantal submucosale en myenterische nitrerge neuronen niet verschillend tussen controles en geopereerde dieren. Bijgevolg wordt de verminderde vrijstelling van NO waarschijnlijk veroorzaakt door een verminderde efficiëntie van het NOS-1 enzym in de nitrerge neuronen. Deze verminderde efficiëntie wordt mogelijk veroorzaakt door de grote hoeveelheden NO aangemaakt door NOS-2. De gehaltes van cGMP in het plasma waren verlaagd 24 uren na de abdominale operatie, terwijl de gehaltes van cGMP in het jejunale weefsel behouden bleven. Om na te gaan of een verlaging van de gehaltes van cGMP door middel van farmacologische middelen de wijzigingen veroorzaakt door POI zou verbeteren of verergeren, werden de gevolgen van in-vivo behandeling met remmers van sGC bestudeerd. Alhoewel de gebruikte dosissen gebaseerd waren op de literatuur had deze behandeling geen invloed op de spiegels van cGMP in plasma en jejunum, zodat onze hypothese, dat inhibitie van sGC heilzaam zou zijn tijdens POI, niet beoordeeld kon worden.

Tijdens de tweede langdurige fase van POI werd op infiltrerende monocyten in de intestinale spierlaag een verhoogd aantal α_2-receptoren gevonden, die een rol spelen in de regulatie van NOS-2 inductie. Daarom werd ook nagegaan of de populatie van de presynaptische α_2-receptoren op de cholinerge zenuwuiteinden beïnvloed wordt tijdens POI. Er werd geen verschil gevonden in de beïnvloeding van intestinale cholinerge zenuwactiviteit via presynaptische α_2-receptoren tussen controle dieren en geopereerde dieren.

sGC bestaat in 2 isovormen nl, sGC$\alpha_1\beta_1$ en sGC$\alpha_2\beta_1$ waarvan de $\alpha_1\beta_1$-isovorm de belangrijkste is in de gastro-intestinale tractus. Daarom werden in het tweede deel van dit proefschrift de gevolgen van het elimineren van de α_1-subeenheid van het sGC enzym bestudeerd ter hoogte van de maag (**Hoofdstuk V**), waar NO een rol speelt in de controle van de maaglediging, en ter hoogte van het jejunum (**Hoofdstuk VI**), waar NO betrokken is in peristaltiek.
We vonden dat, in de maagfundus, exogeen NO relaxaties kan veroorzaken door activering van sGC$\alpha_2\beta_1$ in sGCα_1 knock-out (KO) muizen. De resultaten met endogeen NO gaven aan dat sGC$\alpha_1\beta_1$ essentieel is voor snelle nitrerge antwoorden maar dat langdurige vrijstelling van endogeen NO ook kan leiden tot activering van sGC$\alpha_2\beta_1$ in de maagfundus van sGCα_1 KO muizen. De maaglediging van een vloeibare maaltijd was versneld 15 min na toediening bij mannelijke sGCα_1 KO muizen. Dit werd waarschijnlijk veroorzaakt door de vermindering van de nitrerge opslagcapaciteit in de fundus. Maaglediging bij vrouwelijke sGCα_1 KO muizen was niet zoveel versneld als in mannelijke sGCα_1 KO muizen omdat deze tegengewerkt wordt ter hoogte van de pylorus. De nitrerge relaxatie in de pylorus was inderdaad duidelijk verminderd bij vrouwelijke sGCα_1 KO muizen wat wijst op een belangrijke rol voor sGC$\alpha_1\beta_1$ in nitrerge relaxatie van de pylorus. In tegenstelling tot vrouwelijke muizen, gebeurt de nitrerge relaxatie in de pylorus van mannelijke sGCα_1 KO muizen vooral via activering van sGC$\alpha_2\beta_1$.
We stellen vast dat in het jejunum, exogeen en endogeen NO vooral werken via activering van sGC$\alpha_1\beta_1$ bij mannelijke muizen. Niettemin kan een matige nitrerge relaxatie zich voordoen via activering van sGC$\alpha_2\beta_1$ bij mannelijke sGCα_1 KO muizen. In vrouwelijke sGCα_1 KO muizen werkt exogeen en endogeen NO vooral via activering van sGC$\alpha_2\beta_1$. Het behoud van nitrerge relaxatie door activering van sGC$\alpha_2\beta_1$ is dus meer uitgesproken in het jejunum van vrouwelijke sGCα_1 KO muizen in vergelijking met mannelijke muizen, terwijl het omgekeerde geldt in de pylorus.

Samenvattend tonen de resultaten van dit werk aan dat NO de belangrijkste relaxerende neurotransmitter is in de circulaire spierlaag van het jejunum van de Wistar rat. NO

veroorzaakt relaxaties door middel van een stijging van de gehaltes van cGMP en de activering van SK_{Ca}-kanalen via een serieel verband. Ontsteking veroorzaakt door manipulatie van de dunne darm leidt tot een minder efficiënte werking van de nitrerge neurotransmisie. Dit werd veroorzaakt door een verminderde NOS-1 activiteit in de nitrerge zenuwen mogelijk in reactie op verhoogde hoeveelheden NO geproduceerd door NOS-2. De mogelijke rol van sGC in de ontsteking veroorzaakt door manipulatie van de darm blijft onduidelijk omdat de in-vivo behandeling met remmers van sGC niet in staat was om de gehaltes van cGMP in plasma en jejunum te verlagen binnen de gebruikte experimentele omstandigheden.

$sGC\alpha_1\beta_1$ speelt een belangrijke rol in de nitrerge relaxatie van de maag en de dunne darm van de muis. Er kan zich echter een zekere mate van nitrerge relaxatie voordoen via activering van $sGC\alpha_2\beta_1$ in $sGC\alpha_1$ KO muizen, dit op een geslachts- en weefselafhankelijke manier.

Chapter XI

Dankwoord

Dankwoord

Kan nog wat aangepast worden

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