PARACRINE MODULATION OF THE VASCULAR SMOOTH MUSCLE TONE BY ADIPOSE & RETINAL TISSUE

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List of abbreviations

20-HETE	20-hydroxyeicosatetraenoic acid
8-SPT	8-(p-sulfophenyl)theophylline
ADP	adenosine diphosphate
ADRF	adipocyte-derived relaxing factor
Ang	angiotensinogen
Ang II	angiotensin II
ANP	atrial natriuretic peptide
Apa	apamin
AT1	angiotensin type 1
ATP	adenosine triphosphate
BK_{Ca} channel	big (large) conductance Ca^{2+} activated K^{+} channel
BNP	brain natriuretic peptide
cAMP	cyclic adenosine 3´,5´-cyclic monophosphate
CETP	cholesteryl ester transfer protein
cGMP	cyclic guanosine 3´,5´-cyclic monophosphate
CGRP	calcitonin gene-related peptide
ChTX	charybdotoxin
CNP	C-type natriuretic peptide
CO	carbon monoxide
COX	cyclooxygenase
CRP	C-reactive protein
CSE	cystathionine γ-lyase
DL-APV	DL-aminophosphonovaleric acid
EC	endothelial cell
EDHF	endothelium-derived hyperpolarizing factor
EETs	epoxyeicosatreinoic acids
EFS	electrical field stimulation
endo	endothelium
ET-1	endothelin-1
GABA	γ-aminobutyric acid
glib	glibenclamide

H_2O_2	hydrogen peroxide
H_2S	hydrogen sulfide
HDL	high-density lipoprotein
HGF	hepatocyte growth factor
ICAM-1	intercellular adhesion molecule-1
ID	inside diameter
IK_{Ca} channel	intermediate conductance Ca^{2+} activated K^+ channel
IL-6	interleukin-6
indo	Indomethacin
K_{120}	Krebs-Ringer bicarbonate solution containing 120 mM potassium
K_{60}	Krebs-Ringer bicarbonate solution containing 60 mM potassium
K_{30}	Krebs-Ringer bicarbonate solution containing 30 mM potassium
K_{ATP} channel	ATP-sensitive K^+ channel
K_{Ca} channel	Ca^{2+} activated K^+ channel
KRB	Krebs-Ringer bicarbonate
K_v channel	voltage-dependent K^+ channel
L-NA	N^{ω}-nitro-L-arginine
MCP-1	monocyte chemoattractant protein-1
NaHS	sodium hydrosulfide
NANC	nonadrenergic noncholinergic
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOR	norepinephrine
OD	outside diameter
ODQ	1 H-[1, 2, 4]oxadiazolo[4,3-A]quinoxalin-1-one
PAI-1	plasminogen activator inhibitor-1
PAME	palmitic acid methyl ester
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostacyclin
PPARγ	peroxisome proliferator-activated receptor γ
PVAT	perivascular adipose tissue
ROS	reactive oxygen species
RRF	retinal relaxing factor

S.E.M.	standard error of the mean
SERCA	sarcoplasmic reticulum Ca^{2+}-ATPase
sGC	soluble guanylyl cyclase
SK_{Ca} channel	small conductance Ca^{2+} activated K^{+} channel
SNP	sodium nitroprusside
TEA	tetraethylammoniumchloride
TNF_{α}	tumor necrosis factor alpha
TP receptor	thromboxane/prostaglandin receptor
VCAM-1	vascular adhesion molecule-1
VEGF	vascular endothelial growth factor
ZnPPIX	zinc protoporphyrin IX

Introduction

Blood flow to different organs and tissues must adapt continuously to their metabolic needs. This is ensured by the heart pump creating appropriate blood pressure at the entrance of the tissues. Tissue perfusion depends on the vascular resistance within the tissue and vascular resistance depends on blood viscosity, the length and diameter of the blood vessels. This diameter is regulated by changes in tone of the vascular smooth muscle cells due to myogenic (i.e. changes in transmural pressure), neurogenic and metabolic (i.e. changes in the concentration of local metabolites) regulation. From the eighties of the previous century on, the identification of nitric oxide (NO) as an endothelium derived relaxing factor [1] clearly brought up the important concept that vascular smooth muscle cells are strongly influenced by substances from neighbouring cells. At present the endothelium, a monolayer of cells covering the luminal surface of all blood vessels, is considered to be an important tissue that regulates the homeostasis of the underlying layer of smooth muscle cells by controlling the balance between vasoconstriction and vasorelaxation. This is accomplished through the release of several vasoactive factors under basal conditions and in response to many chemical and mechanical stimuli. These vasoactive factors released by the vascular endothelium or by other neighbouring cells are called “paracrine modulators”. The term “paracrine” refers to a kind of hormone function in which the effects of the hormone are restricted to the local environment. Nowadays the endothelial signaling pathway is widely recognized, and its role in vascular physiology has been extensively studied. Besides NO, other endothelial vasorelaxing factors including prostacyclin (PGI_2), the endothelium-derived hyperpolarizing factor (EDHF), carbon monoxide (CO), hydrogen sulfide (H_2S), reactive oxygen species (ROS), epoxyeicosatreinoic acids (EETs) and contracting factors including endothelin-1 (ET-1), prostanoids, reactive oxygen species, 20-hydroxyeicosatetraenoic acid (20-HETE) have shown to participate in the control of vascular tone [2,3].

Actually the neurotransmitters released from vascular nerve endings can also be considered as paracrine modulators of vascular smooth muscle tone. In contrast to sympathetic adrenergic innervation, most human blood vessels are not parasympathetically innervated. With the advancement of immunohistochemical, electrophysiological and microscopal techniques it has become clear that the nervous control of the vascular system

comprises several transmitters other than the classical autonomic neurotransmitters noradrenaline, acting both as vasoconstrictor and dilator, and acetylcholine, acting as a vasodilator [4]. The discovery of parasympathetic nonadrenergic noncholinergic (NANC) innervations of vascular smooth muscle has led to a new understanding of the neurogenic control of vascular function. These nerve endings are able to release vasodilatory substances such as neuropeptides (e.g. calcitonin gene-related peptide (CGRP) [5]) and NO [6]. Therefore, NO has emerged as an important paracrine modulator to control vascular tone not only through the release from the endothelium, as mentioned above, but also from NANC nerves.

Furthermore, recent studies propose a paracrine role for perivascular adipose tissue in the regulation of arterial tone. For many years adipose tissue was viewed as playing a passive role in total body lipid and energy homeostasis. White adipose tissue was the site where excess energy was stored as triglycerides, and where that energy, when needed elsewhere in the body, was released as fatty acids. In contrast to the energy-storing white adipose tissue, brown adipose tissue was thought to play a major role in the regulation of body temperature. In the last decade it has become clear that adipose tissue is an active endocrine and paracrine organ secreting several mediators called adipokines. Adipokines include hormones (such as leptin, adiponectin), inflammatory cytokines (including tumor necrosis factor alpha (TNF_{α}), interleukin-6 (IL-6), omentin, visfatin) and other proteins (e.g. plasminogen activator inhibitor-1 (PAI-1), angiotensinogen, resistin, apelin) [7,8]. Furthermore, adipose tissue releases an “adipocyte-derived relaxing factor” (ADRF) [9], which relaxes arteries. So far the identity of the ADRF is not fully elucidated. Besides the ADRF, many other adipokines possess vasoactive properties which are discussed in a review in **chapter 1** (published in BMC Medicine 2011; 9(1): 25).

In addition, the retinal tissue is thought to influence retinal arterial tone by the release of several vasoactive substances. For example NO, which is not only released from the endothelium and perivascular nerves but also from retinal tissue [10]. Also prostaglandins [11] are released from retinal tissue and exert both vasorelaxing and contractile effects. Histamine [12] and lactate [13], both produced in retinal tissue, as well as adenosine [14], which is widely distributed within the retina of various species, possess vasorelaxing properties. Furthermore, retinal neurotransmitters can affect arterial tone, f.e. CGRP [15] and substance P [16] which induce vasorelaxation, while neuropeptide Y [17] causes vasoconstriction.

However there is evidence for the existence of another as yet unidentified retina-derived relaxing factor that might be important in the maintenance of retinal circulation, which is called the "retinal relaxing factor" (RRF) [18]. In **chapter 2** (published in Microcirculation 2007; 14(1): 39-48) of the present work, studies concerning characteristics of the retinal relaxing factor and its role in the regulation of arterial tone are reviewed.

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Aims of the work

The aim of our study was to further explore the paracrine role of perivascular retinal and adipose tissue in the regulation of arterial tone and more in particular possible alterations in the vasorelaxing effect exerted by these tissues. The results of the original studies are presented in chapter 3, 4 and 5.

Due to a compromised O_2 supply from the vasculature adipose tissue becomes hypoxic during the development of obesity. **Chapter 3** (based on a study published in Eur J Pharmacol 2010; 641(2-3): 207-12) investigates whether moderate hypoxia affects the vasorelaxing influence of perivascular adipose tissue in isolated mice thoracic aortas and whether the adipocyte-derived relaxing factor (ADRF) is involved. An enhanced vasorelaxing effect of perivascular adipose tissue could be a regulatory mechanism during hypoxia to adapt blood flow to the metabolic needs of the growing tissue and to prevent tissue damage.

Chapter 4 (based on a study accepted in Acta Physiol Scand) investigates whether hypoxia also influences the vasorelaxing effect of perivascular white adipose tissue surrounding mice mesenteric arteries. This study was conducted to test whether smaller blood vessels, which contribute to total peripheral vascular resistance, exert the same hypoxic response as larger (i.e. thoracic aorta) vessels, and secondly, whether there is a difference in hypoxic response between brown (surrounding mice thoracic aorta) and white (surrounding mice mesenteric arteries) perivascular adipose tissue.

Adenosine, an important neurotransmitter in the nervous system, is widely distributed within the retina and appears to subserve a number of physiological roles such as coupling of retinal blood flow to energy demand since adenosine is a known vasorelaxant. **Chapter 5** (based on a study published in Exp Eye Res 2009; 88(1): 71-8) investigates the effect of adenosine on the vasorelaxing influence of bovine, porcine and rat retinal tissue and the involvement of the retinal relaxing factor (RRF). This could be of clinical importance because an enhanced vasorelaxing effect of perivascular retinal tissue by adenosine may contribute to the increased retinal blood flow that occurs during tissue damage and inflammation.

Chapter 1

Regulation of vascular tone by adipocytes

Based on BMC Medicine 2011;9(1):25

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1.1. ABSTRACT

Recent studies show that adipose tissue is an active endocrine and paracrine organ secreting several mediators called adipokines. Adipokines include hormones, inflammatory cytokines and other proteins. In obesity, adipose tissue becomes dysfunctional resulting in an overproduction of proinflammatory adipokines and a lower production of anti-inflammatory adipokines. The pathological accumulation of dysfunctional adipose tissue that characterizes obesity is a major risk factor for many other diseases, including type 2 diabetes, cardiovascular disease and hypertension. Multiple physiological roles have been assigned to adipokines including the regulation of vascular tone. For example the unidentified “adipocyte-derived relaxing factor” (ADRF) released from adipose tissue has shown to relax arteries. Besides the ADRF, other adipokines like adiponectin, omentin and visfatin are vasorelaxants. On the other hand angiotensin II and resistin are vasoconstrictors released by adipocytes. Reactive oxygen species (ROS), leptin, tumor necrosis factor α (TNFα), interleukin-6 (IL-6) and apelin share both vasorelaxing and constricting properties. Dysregulated synthesis of the vasoactive and proinflammatory adipokines may underlie the compromised vascular reactivity in obesity and obesity-related disorders.

1.2. INTRODUCTION

For a long time adipose tissue or body fat was believed to be simply involved in total body lipid and overall energy homeostasis. White adipose tissue stores excess energy in the form of triglycerides, while brown adipose tissue is actively involved in the regulation of body temperature [1,2]. However, in the recent years it has become clear that adipose tissue is far more than a storage facility and thermoregulator, and is in fact an active secretory organ of multiple mediators, known as adipokines [3]. These adipokines include hormones (e.g. leptin, adiponectin), inflammatory cytokines (e.g. tumor necrosis factor alpha (TNF_{α}), interleukin-6 (IL-6), omentin, visfatin) and other proteins (e.g. plasminogen activator inhibitor-1 (PAI-1), angiotensinogen, resistin, apelin) [4,5]. Furthermore, adipose tissue is known to release an as yet unidentified "adipocyte-derived relaxing factor" (ADRF) [6], which relaxes several arteries. Here, we give an overview of the influence of different adipokines on vascular tone and on their potential role in obesity and obesity-related disorders.

1.3. WHITE VS. BROWN ADIPOSE TISSUE

Adipose tissue is predominantly located around blood vessels (perivascular), around internal organs (visceral or abdominal) or subcutaneously. Adipose tissue consists of a heterogenous mixture of cellular structures (i.e. adipocytes, precursor cells, macrophages, fibroblasts and endothelial cells) and tissue structures (i.e. small blood vessels, nerve tissue) [7]. The predominant cell type in adipose tissue is the adipocyte, which may be white or brown adipocytes. In accordance with the type of adipocytes, adipose tissue is subdivided into white and brown adipose tissue.

White adipose tissue comprises up to 20-25% of total body weight. In general, white adipose tissue acts mainly as energy store or reserve (i.e. lipid storage) and expands during obesity. It also provides thermal insulation (subcutaneous adipose tissue) and supports against mechanical shocks (e.g. skin, kidney) [1].

Brown adipose tissue regulates body temperature by lipid metabolism in newborn mammals and some hibernating animals [2]. Recent studies have shown that healthy adult humans still

possess a substantial fraction of metabolically active brown adipose tissue in the supraclavicular and the neck regions with some additional paravertebral, mediastinal, para-aortic and suprarenal locations [8,9]. Although the obesity-preventive role of brown adipose tissue has long been a matter of debate [10,11], more recent data clearly show an inverse correlation between body mass index and brown adipose tissue activity in man [8,12].

1.4. ADIPOKINES

Adipose tissue is known to produce and release numerous bioactive substances, known as adipokines, into its direct surroundings (auto- or paracrine) and the bloodstream (endocrine) [3]. Adipokines are involved in various physiological processes (table 1.1), including the regulation of arterial tone [4,13,14].

Physiological processes	Adipokines involved
Glucose metabolism	e.g. adiponectin, resistin
Lipid metabolism	e.g. CETP , retinol- binding protein
Immunity	e.g. adipsin
Inflammation	e.g. TNFα, IL-6
Coagulation	e.g. PAI-1
Maintaining normal reproduction	e.g. leptin, ghrelin
Pancreatic β cell function	e.g. interleukin-6, adiponectin, visfatin
Angiogenesis	e.g. leptin, VEGF, HGF
Feeding behaviour	e.g. leptin
Regulation vascular tone	e.g. ADRF, leptin, adiponectin

Table 1.1. Physiological processes in which adipokines are involved. CETP: cholesteryl ester transfer protein; TNFα: tumor necrosis factor alpha; IL-6: interleukin-6; PAI-1: plasminogen activator inhibitor-1; VEGF: vascular endothelial growth factor; HGF: hepatocyte growth factor; ADRF: adipocyte-derived relaxing factor

Therefore, adipose tissue does not only affect overall metabolism but also the functionality of many organs and tissues such as muscle, liver, brain and the vasculature. Total absence of adipose tissue has been reported to be associated with non-viability, which emphasizes the essential role of adipose tissue in human physiology [15]. Maintenance of a normal amount of adipose tissue is essential as imbalance can cause serious health problems, since dysregulated release of adipokines may lead to vascular disturbances and inflammation.

1.5. VASOACTIVE ADIPOKINES IN PHYSIOLOGY AND OBESITY

Under normal circumstances, vascular tone is influenced by adipokines (Fig. 1.1 & table 1.2).

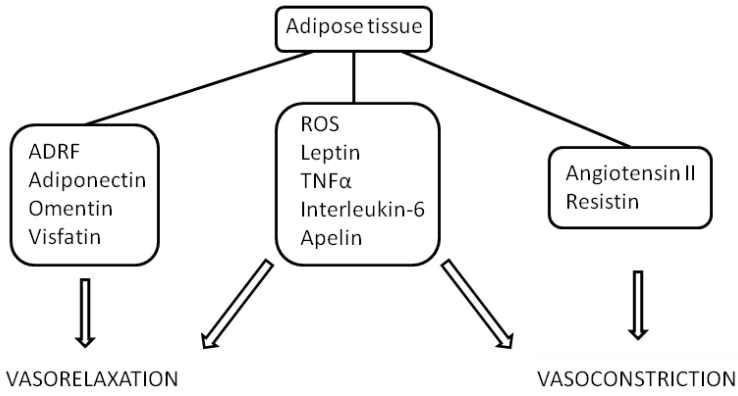

Fig. 1.1. Adipose tissue releases several adipokines. Some of them have vasorelaxing or vasocontractile properties, while others share both. ADRF: adipocyte-derived relaxing factor; ROS: reactive oxygen species; TNF_{α}: tumor necrosis factor alpha.

However, it is thought that vascular tone regulation is compromised in case of obesity and obesity-related disorders, in which the amount of adipose tissue has grown out of proportion. This eventually leads to a dysregulated synthesis of vasoactive adipokines by dysfunctional adipose tissue in favour of harmful proinflammatory adipokines (e.g. leptin) [14,16] (Fig. 1.2). The dysregulated synthesis/secretion of adipokines and the infiltration of macrophages into adipose tissue, possibly as a result of monocyte chemoattractant protein-1 (MCP-1) [17] and leptin [18] release from adipocytes, lead to a state of inflammation within adipose tissue.

A proinflammatory state in adipose tissue cannot only induce a dysregulation of vascular tone but also local insulin resistance, adhesion of monocytes, vascular remodelling, foam cell formation in the arterial wall and endothelial dysfunction. Endothelial dysfunction is reflected as a decrease in NO bioavailability, endothelium-dependent relaxation and impaired ability of the endothelium to respond to circulating hormones. All these changes clearly promote the development of cardiovascular diseases and type 2 diabetes [19].

It has been proposed that hypoxia underlies this inflammatory response, as hypoxia occurs in areas of the fat depots when the vascular oxygen supply is compromised due to tissue mass expansion [4]. Direct evidence that growing adipose tissue becomes hypoxic has recently been obtained in mice [20,21]. Furthermore, cell-culture studies using murine and human

adipocytes strongly support the modulatory role of hypoxia in the production of several proinflammatory adipokines [22,23].

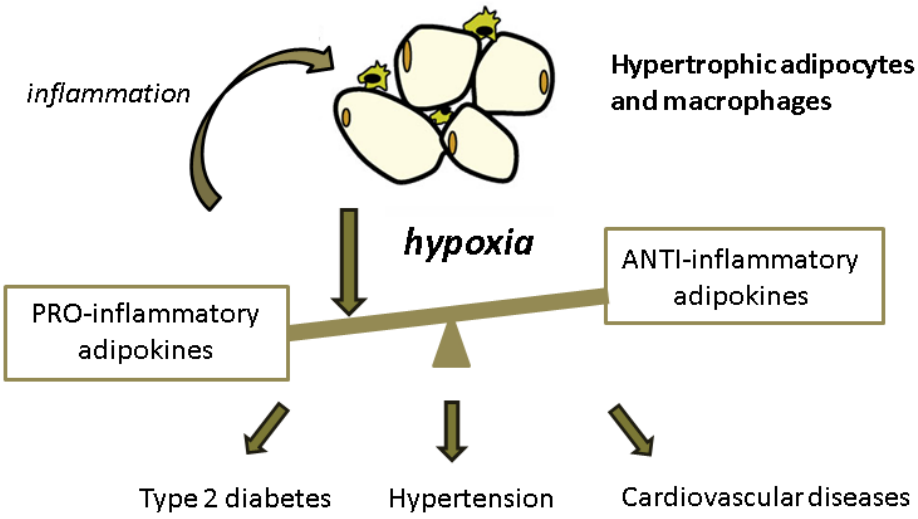

Fig. 1.2. Relationship between dysfunctional adipose tissue in obesity, inflammation, hypoxia, and obesity-related disorders. Adipose tissue mass increases during obesity which leads to a state in which the adipose tissue becomes hypoxic. There is a dysregulation in the synthesis of adipokines in favour of the proinflammatory ones. This might lead to obesity-related disorders and results in inflammation within adipose tissue. Hypoxia may underlie this inflammatory response by supporting the production of proinflammatory adipokines.

Furthermore, angiogenesis is promoted in response to hypoxia [24]. Novel vascularisation can be considered as an adaptive process to counter hypoxia and to ensure sufficient nutrient and oxygen supply to the different tissues. Hypoxia upregulates inducible transcription factors, which trigger the expression of angiogenic adipokines such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and plasminogen activator inhibitor-1 (PAI-1) [14], which promote vascular endothelial cell proliferation and the later stages of new vessel formation [25]. Also other adipokines such as leptin, basic fibroblast growth factor (bFGF) and IL-6 have shown to induce angiogenesis, while adiponectin and TNFα have pro- and anti-angiogenic properties [25].

Adipokines	Vasoactive effect	References
Superoxide anion	Vasoconstriction through Ca^{2+} sensitization; impairs EC-dependent relaxation by decreasing NO bioavailability; enhances vasoconstriction to perivascular nerve activation by electrical field stimulation	[26,27,38]
Hydrogen peroxide	EC-dependent and -independent vasorelaxation mediated by opening K_{Ca}, K_v and K_{ATP} channels; Ca^{2+} -dependent and -independent vasoconstriction	[28,29,31-36]
Leptin	Vasoconstriction due to sympathetic nervous system activation; EC-dependent and -independent vasorelaxation	[40-43,47,48]
TNFα	EC-dependent and -independent vasorelaxation; triggers ET-1 and Ang-induced vasoconstriction; impairs EC-dependent vasorelaxation due to decreased NO or increased ROS production; reduces vasorelaxing effect of PVAT due to increased ROS production	[59-62,66-69]
IL-6	EC-independent vasorelaxation; reduces vasorelaxing effect of PVAT due to increased ROS production; impairs endothelial function due to increased ROS and decreased NO production	[69,79,80,85]
Apelin	NO-dependent vasorelaxation; EC-independent vasoconstriction	[89-91,93]
Adiponectin	NO-dependent vasorelaxation mediated by opening K_v channels	[69,102-105]
Omentin	EC-dependent and -independent vasorelaxation	[116]
Visfatin	NO-dependent vasorelaxation	[123]
ADRF	Vasorelaxation through opening of K_{ATP}, KCNQ or K_{Ca} channels depending on the species	[6,106,129,131,132]
Ang II	Vasoconstriction via binding on AT1-receptors	[141]
Resistin	No effect on contractility of blood vessels; impairs endothelial function due to increased ET-1 and decreased NO production	[155,156,163,164]

Table 1.2. Vasoactive effect of adipokines. EC: endothelial cell; NO: nitric oxide; K_{Ca} channels: Ca^{2+} activated K^+ channels; K_v channels: voltage-dependent K^+ channels; K_{ATP} channels: ATP-sensitive K^+ channels; TNFα: tumor necrosis factor alpha; ET-1: endothelin-1; Ang,: angiotensinogen; ROS: reactive oxygen species; PVAT: perivascular adipose tissue; IL-6: interleukin-6; Ang II: angiotensin II; AT1: angiotensin type 1; ADRF: adipocyte-derived relaxing factor.

The vasoactive adipokines and their role in physiological conditions, in obesity and obesity-related disorders are described in more detail in the following subsection.

1.5.1. Adipokines with vasorelaxing and vasocontractile properties

1.5.1.1. Reactive oxygen species

Reactive oxygen species (ROS) are a class of oxygen-derived molecules including superoxide anion and hydrogen peroxide, both modulators of vascular tone. Vascular smooth muscle cells, endothelium and also perivascular adipose tissue are sources of ROS [26].
Superoxide anion can induce vasoconstriction through Ca^{2+} sensitization pathways, although it is not clear whether it is acting directly or via its conversion to hydrogen peroxide [27]. Furthermore, contraction in response to perivascular nerve activation by electrical field stimulation is enhanced by superoxide anion from perivascular adipose tissue [26].
Hydrogen peroxide is a more likely paracrine ROS because hydrogen peroxide is not a free radical and therefore more stable and less reactive with other tissue radicals [28]. Hydrogen peroxide is known to induce both vasorelaxation and –constriction depending on species, type of vascular bed, concentration, membrane potential and degree of obesity [28-30]. Vasorelaxation is possibly induced by endothelium-dependent mechanisms, involving the release of vasodilating cyclooxygenase metabolites [31] and NO [32], and endothelium-independent mechanisms [29,33] mediated by activation of different potassium channels on smooth muscle cells [31,34,35]. On the other hand, vasoconstriction by hydrogen peroxide is likely induced in a calcium-dependent way, although Ca^{2+} sensitization and calcium-independent pathways have also been reported [28,32,36]. Furthermore, hydroxyl radicals, cyclooxygenase metabolites, protein kinase C, phospholipase A2 and C, and tyrosine kinase appear to play a role in hydrogen peroxide-induced contractions [36].

Oxidative stress occurs when the production of ROS exceeds the cell's capacity to detoxify these potentially injurious oxidants using antioxidant defense systems [37]. In general, superoxide and hydrogen peroxide production in adipose tissue is increased in obese mice, which promotes endothelial dysfunction. Superoxide anion impairs endothelium-dependent vasorelaxation by decreasing NO bioavailability via formation of peroxynitrite, which is in turn another ROS [38]. Furthermore, ROS contributes to endothelial dysfunction by up-regulating the expression of adhesion and chemotactic molecules in endothelial cells, which promote monocyte adhesion and migration to the vessel wall [37]. The adhesion of these circulating blood cells to vascular endothelium is a key element in the development of

inflammation and thrombosis within the vasculature in vascular diseases associated with oxidative stress like atherosclerosis [37].

1.5.1.2. Leptin

This substance is almost exclusively secreted by white and brown adipocytes [39]. Under normal conditions, leptin contributes to a balanced blood pressure homeostasis by its vasorelaxing and vasocontractile effects [40,41]. While the contractile effect of leptin is attributed to sympathetic nervous system activation [40], various mechanisms seem to be responsible for the leptin-induced vasorelaxation. This latter effect can be endothelium-dependent either through the release of NO [42] or either by other mechanisms [41,43]. The involvement of the endothelium-derived hyperpolarizing factor (EDHF) in leptin-induced vasorelaxation remains controversial [41,44]. It has been postulated that epoxyeicosatrienoic acids (EETs)/EDHF-dependent vasorelaxation might act as a back-up in case of reduced NO availability *in vivo* [45]. On the other hand, EETs are able to activate endothelial NO synthase and subsequently release NO to influence arterial tone [46]. There is also evidence that leptin affects vascular tone without endothelial involvement [47]. A study on endothelium-denuded rat aortic rings showed that leptin attenuated angiotensin II-induced contraction by inhibiting Ca^{2+} release from the intracellular stores in vascular smooth muscle cells [48].

Leptin levels are markedly increased during obesity [30,49]. Hyperleptinemia in obesity is believed to dysregulate blood pressure, resulting in hypertension. Significant associations have been found between plasma leptin levels and hypertension in both males and females, which makes leptin a potential predictor of hypertension [50,51]. In obesity, endothelium-dependent vasorelaxation is likely to become less effective, as sustained hyperleptinemia leads to endothelial dysfunction [52]. This might be the result of a leptin-induced increase of vasoconstrictor endothelin-1 [53], a leptin-induced expression of endothelin type A receptors in vascular smooth muscle cells [54], a leptin-induced depletion of NO and increase of cytotoxic ROS [55]. Leptin also promotes smooth muscle cell proliferation contributing to the increased peripheral vascular resistance [56]. Furthermore, it stimulates the release of proinflammatory cytokines from macrophages which may further elevate blood pressure and exacerbate the inflammatory process [57].

1.5.1.3. Tumor necrosis factor alpha

The cytokine tumor necrosis factor alpha (TNFα) is a potent, time-dependent vasoconstrictor [58,59] and vasodilator [60-63]. Besides time-dependency it is unclear what underlies the differential regulation of arterial contractility by TNFα. Vasoregulatory actions of TNFα may be vascular bed specific. Also differences in experimental protocols used may explain the diversity of observations reported in various studies.
A source of TNFα that has recently been identified is perivascular adipose tissue [64]. This implies that TNFα is produced in the direct vicinity of the vascular endothelium. TNFα-mediated vasoregulation can occur through both endothelium-dependent [60,61] and endothelium-independent mechanisms [62]. Some studies suggested that TNFα promotes vasorelaxation by an increase of NO and prostaglandin production [60,61,63], while other suggested the involvement of hydrogen peroxide [65].
On the other hand, TNFα is able to induce vasoconstriction by increasing endothelin-1 [66] and angiotensinogen levels [67]. In addition, TNFα impairs endothelium-dependent vasorelaxation in various vascular beds as a result of a decrease in endothelial NO release [59,68] or an increase in NO-scavengers (ROS) [59]. Moreover, a recent study has shown a reduced vasorelaxing effect of perivascular adipose tissue in response to TNFα and interleukin-6 (IL-6), which upregulate ROS [69].

An increased adipose tissue expression of TNFα mRNA has been reported in different rodent models of obesity as well as in clinical studies involving obese patients [70,71]. TNFα is considered a molecule that links inflammation to obesity [71]. Moreover, the infiltration of macrophages in adipose tissue during obesity contributes to increased TNFα production [72]. The increase in TNFα expression induces the production of ROS, resulting in endothelial dysfunction in obesity and obesity-related disorders like hypertension, atherosclerosis and type 2 diabetes [73,74]. Furthermore, TNFα decreases adiponectin expression [75] and stimulates the secretion of proinflammatory proteins (e.g. interleukin-6) which contribute to the maintenance of the chronic inflammatory state of adipose tissue in obesity [76].

1.5.1.4. Interleukin-6

A sustained increase in proinflammatory cytokine interleukin-6 (IL-6) plasma levels is associated with high blood pressure [77,78]. On the other hand, acute exposure of IL-6 *in vitro* relaxes aortas [79]. This vasorelaxing effect is likely regulated by an endothelium-independent pathway involving an increase in prostacyclin in vascular smooth muscle cells. IL-6 also relaxes skeletal muscle resistance vessels. However, this occurs only *in vivo*, suggesting that IL-6 interacts with parenchymal or intravascular factors to elicit vasorelaxation [80].

In obesity an increase in cytokine IL-6 has been observed at mRNA and protein level in white adipose tissue [81,82]. IL-6 has been shown to be a predictor of future myocardial infarction [78] and is highly associated with cardiovascular mortality [83]. IL-6 induces the induction of hepatic C-reactive protein (CRP) production, which is now known to be an independent major risk factor of cardiovascular complications [71]. Some studies have suggested that IL-6 is rather an indirect marker of vascular dysfunction, while others have suggested a more active role in vascular dysfunction [84]. Long term elevation of IL-6 in mice has shown to impair endothelial function by increasing angiotensin II-stimulated production of ROS as well as by reducing endothelial NO synthase mRNA expression [85]. In addition, IL-6 enhances vascular smooth muscle cell proliferation [86], which is a key event in the genesis of atherosclerotic lesions.
Genetic deletion of IL-6 attenuates angiotensin II-induced hypertension in mice [77], suggesting that elevated IL-6 in obesity might contribute to hypertension via angiotensin II. In addition, IL-6 inhibits adiponectin gene expression in cultured adipocytes [81] which may exacerbate obesity-related hypertension.

1.5.1.5. Apelin

Apelin, of which different isoforms exist, acts through the binding to a specific G protein-coupled receptor named APJ [87] present on endothelial cells, vascular smooth muscle cells and cardiomyocytes [88]. Apelin causes NO-dependent vasorelaxation of human arteries both *in vitro* and *in vivo* [89,90]. *In vivo*, exogenous apelin administration causes a rapid NO-dependent fall in blood pressure in a rodent model, confirming its powerful vasorelaxing

effect [91]. However, some reports associate apelin with an increase in arterial pressure [92]. It has been proposed that apelin-induced changes in blood pressure (i.e. an increase or decrease) are both dose- and time-dependent [87]. Furthermore, it is also possible that observed bioactivity of apelin varies depending on species and/or vascular bed. Other data also suggest that apelin has vasoconstrictor potential by acting directly on vascular smooth muscle cells. In endothelium-denuded isolated human veins, apelin shows to be a potent vasoconstrictor with nanomolar potency and a maximum response comparable to that of angiotensin II [93]. In the presence of functional endothelium this vasoconstrictor effect may be counterbalanced or even masked by activation of APJ receptors on vascular endothelial cells, resulting in the release of endothelial vasodilator substances, such as NO [94]. All taken together, these data suggest a role for the apelin-APJ system as a regulator of vascular tone.

Apelin production in adipose tissue is strongly up-regulated by insulin, and plasma concentrations are increased in obese and hyperinsulinemic mice and humans [95]. In contrast to acute exposure, long term exposure of apelin does not affect blood pressure [96] which might be explained by resistance to its hypotensive effect. This is in contrast to a study in which high apelin levels increase blood pressure in obesity via stimulation of sympathetic outflow in the central nervous system when crossing the blood-brain barrier [97].
In atherosclerosis, apelin might have beneficial effects as apelin has shown to stimulate endothelial NO-production and antagonize the angiotensin II-induced formation of atherosclerotic lesions and aortic aneurysm in a mice model of atherosclerosis [98].

1.5.2. Vasorelaxing adipokines

1.5.2.1. Adiponectin

Adiponectin is mainly released by both brown [99] and white [82] adipocytes and is the most abundant adipokine in the circulation [100]. Adiponectin has been considered an anti-inflammatory and anti-oxidative adipokine that protects against cardiovascular disease [101]. Adiponectin inhibits TNFα production and other inflammatory pathways in adipocytes and macrophages [71,101]. Plasma adiponectin has been correlated with endothelium-dependent vasorelaxation in humans [102]. These results were confirmed as other studies showed an increase in NO production [103] and NO-mediated and potassium channel (i.e. voltage

dependent)-mediated vasorelaxation in rats by adiponectin [69,104,105]. NO-release from the endothelium is likely stimulated by adiponectin binding to either the adiponectin type 2 receptor or T-cadherin on the endothelial surface [69]. Increased NO-production inhibits platelet aggregation, leukocyte adhesion to endothelial cells and vascular smooth muscle cell proliferation. Furthermore, it reduces oxidative stress by decreasing ROS production in endothelial cells. All these effects protect the vascular system against endothelial dysfunction [101].

The use of an adiponectin receptor 1 blocking peptide abolished the vasorelaxing effect of human perivascular adipose tissue [69]. However, vasorelaxation induced by perivascular adipose tissue remained unchanged in adiponectin gene-deficient mice [105]. It is possible that this vasorelaxing effect of perivascular adipose tissue in the adiponectin gene-deficient mice might be the result of an endothelium-independent pathway [106]. Despite the latter findings, adiponectin remains an important vasoactive regulator.

Many studies on obesity-related diseases (e.g. type 2 diabetes, hypertension) [71,107,108], but not all [30,109], have reported an overall decrease in adiponectin levels. Hypoadiponectinemia causes endothelial dysfunction by increasing superoxide anion production [110], by promoting the production of adhesion molecules in endothelial cells and the proliferation of smooth muscle cells [111]. Low adiponectin levels have recently emerged as an independent predictor of early atherosclerosis in obese patients [111]. However, after the establishment of atherosclerosis, this association may become weaker, especially in the presence of conditions inducing a hyper-catabolic state (such as heart or renal failure) which are associated with increased plasma adiponectin, accelerated progression of atherosclerosis and worse clinical outcome [101]. In fact, several data show that high circulating adiponectin levels are associated with increased cardiovascular mortality in patients with coronary artery disease [101]. Therefore, hypoadiponectinemia may have a clinical value at the early stages of atherogenesis, but at more advanced disease stages its role as a meaningful biomarker is questioned.

Although it remains controversial whether low levels of adiponectin predict hypertension [51,112,113] or whether levels are decreased in hypertension [100,114], low adiponectin levels might contribute to the pathogenesis of obesity-related hypertension. Considering all the beneficial effects of adiponectin on vascular system, an antihypertensive therapy which increases adiponectin levels could be of great therapeutic value. It has already been demonstrated in obese adiponectin knock out mice with hypertension that adiponectin

replenishment lowers the elevated blood pressure [115]. Drugs such as peroxisome proliferator-activated receptor γ (PPARγ) agonists (thiazolidinediones), some angiotensin type 1 receptor blockers (telmisartan), angiotensin converting enzyme inhibitors and cannabinoid type 1 receptor blockers (rimonabant, taranabant) have been shown to increase circulating adiponectin levels [101]. However, future strategies should be focused on up-regulation of adiponectin's expression (and/or its receptors) or on targeting adiponectin's receptors through the development of specific agonists.

1.5.2.2. Omentin

Omentin is a recently identified adipose tissue-derived cytokine consisting of 313 amino acids and is mainly expressed in visceral rather than in subcutaneous adipose tissue [116]. Omentin consists of two isoforms in which omentin-1 appears to be the major isoform in human plasma [117]. Furthermore, higher plasma omentin 1 levels were detected in women compared with men [117]. In isolated rat aorta, omentin directly induces an endothelium-dependent relaxation which is mediated by NO. Omentin is even capable of inducing vasorelaxation in an endothelium-independent way. The omentin-induced relaxation is also observed in isolated rat mesenteric artery, indicating the effectiveness of omentin in resistance vessels [116]. Since only *in vitro* studies on isolated blood vessels have been performed, *in vivo* studies are necessary to explore the influence of omentin on blood pressure and its chronic influence on vascular reactivity.

Very little is known about omentin in obesity. What is known is that omentin plasma levels and the adipose tissue gene expression are decreased in obesity [117] and even more when overweight is combined with type 2 diabetes [118]. Furthermore, decreased omentin-1 levels are associated with low plasma adiponectin and high-density lipoprotein (HDL) levels. In addition, omentin-1 levels are negatively correlated with leptin levels, waist circumference, body mass index and insulin resistance [117]. Like adiponectin, circulating omentin-1 concentrations increase after weight loss-induced improvement of insulin sensitivity [119]. Although further research is necessary, elevating the omentin levels might be of therapeutic value in obesity and obesity-related disorders.

1.5.2.3. Visfatin

Visfatin is another recently identified cytokine, which is released from perivascular and visceral adipose tissue and which has an insulin-mimetic effect [120,121]. Visfatin has multiple functions in the vasculature. It stimulates growth of vascular smooth muscle cells [122] and endothelial angiogenesis via up-regulating vascular endothelial growth factor (VEGF) and matrix metalloproteinases [120]. Visfatin can also directly affect vascular contractility. Visfatin has been shown to induce endothelium-dependent vasorelaxation in rat isolated aorta through NO production. Also in mesenteric artery of rats visfatin induces relaxation, suggesting that visfatin is effective in resistance vessels [123]. Because only acute effects of visfatin have been demonstrated, further studies are necessary to explore the chronic influence of visfatin on vascular reactivity.

Most studies, but not all, showed an increase in visfatin levels in obesity [121,124,125]. A relationship of plasma visfatin levels was seen with body mass index and percentage of body fat but not with abdominal circumference or visceral fat estimated by computed tomography scan [125]. It has been reported that the expression of visfatin is high at plaque rupture sites in patients with coronary artery disease [126]. Visfatin accelerates monocyte adhesion to endothelial cells by up-regulating intercellular (ICAM-1) and vascular (VCAM-1) cell adhesion molecule-1 in vascular endothelial cells in response to ROS overproduction, suggesting a possible role for visfatin in the development of atherosclerosis [127]. Further studies are necessary to clarify the atherogenic and vasoactive effects of visfatin and its potential clinical relevance.

1.5.2.4. Adipocyte-derived relaxing factor (ADRF)

Vascular tone can also be regulated by an unidentified adipocyte-derived relaxing factor (ADRF) which is released from perivascular adipose tissue. Soltis and Cassis first described that the presence of perivascular adipose tissue reduced vascular contractions by norepinephrine in rat aorta [128], which was later confirmed by Löhn et al [6]. Also isolated adipose tissue and cultured rat adipocytes relaxed precontracted rat aorta previously cleaned of adherent adipose tissue. This modulatory effect was attributed to the ADRF which functions as regulator of arterial tone by active antagonism of contraction [6]. A similar

vasorelaxing effect of perivascular adipose tissue was observed in rat mesenteric arteries [129], in mice aortas [130] as well as in human internal thoracic arteries [131]. These data suggest a common pathway for arterial tone regulation in different species and different types of vascular structures. Verlohren et al. even showed a positive correlation between the vasorelaxing influence of ADRF and the amount of perivascular adipose tissue [129]. The observation that the resting membrane potential of vascular smooth muscle cells in arteries with adipose tissue is more hyperpolarized than in arteries without adipose tissue, further supports the idea that perivascular adipose tissue actively contributes to the basal arterial tone [129]. Whether nitric oxide (NO) formation and endothelium are involved in the vasorelaxation effect by ADRF is still a matter of debate [6,106,129]. On the other hand, the vasorelaxing effect of ADRF is likely mediated by opening of different K^+ channels in vascular smooth muscle cells, depending on the tissue and species studied [6,106,129,131,132]. These divergent observations could be explained by a different distribution of K^+ channels in different vessels and/or species or the existence of different ADRFs.

More and more evidence is accumulating in support of the existence of different ADRFs. Löhn et al. first suggested that ADRF is a protein [6]. Furthermore, analyses of adipose tissue secrete in a recent electrophoresis study resulted in the visualization of different protein bands with different molecular masses (13.8 to 74.0 kDa) which may include ADRF [133]. A possible candidate is peptide angiotensin (1-7) which is a vasodilator located within adipose tissue surrounding rat aorta [134]. Blocking of this particular peptide inhibits the vasorelaxing effect of perivascular adipose tissue surrounding rat aorta [134]. This hypothesis is, however, not in line with the fact that certain ADRF-related potassium channels (K_{ATP} or K_v) [6,132] are not involved in the observed vasorelaxing effect. In addition to proteins, hydrogen peroxide produced from the NAD(P)H oxidase in adipocytes has been described to be involved in the endothelium-independent pathway of the ADRF [106]. Also hydrogen sulfide has been proposed as a novel candidate of the ADRF or at least a mediator in the ADRF effect [132,135], which is consistent with inactivation of ADRF by heating (65°C, 10 min) [6]. Hydrogen sulfide has been recently described as a vasorelaxing gasotransmitter generated by cystathionine γ-lyase (CSE) in perivascular adipose tissue [136,137]. Blocking of CSE inhibits the vasorelaxing effect of perivascular adipose tissue in rat aorta and mice mesenteric arteries [132,135]. Moreover, hydrogen sulfide-induced vasorelaxation of rat aorta was inhibited by a particular ADRF-related potassium channel (KCNQ) blocker [132]. However,

hydrogen sulfide generation and CSE expression in perivascular adipose tissue of stenotic aortas (but not in aortic tissue) are shown to be increased in hypertensive rats induced by abdominal aortic banding [135], while the vasorelaxing effect of perivascular adipose tissue is shown to be impaired in spontaneously hypertensive rats [138]. This might indicate that other ADRF(s) besides hydrogen sulfide are impaired resulting in a reduced vasorelaxing effect of adipose tissue. On the other hand, it is difficult to compare both studies as different models of hypertension have been used. Furthermore, the up-regulation of CSE and hydrogen sulfide generation in perivascular adipose tissue of stenotic aortas may have been developed independently of hypertension as CSE knock-out mice are shown to be hypertensive [136].

Obesity is characterized by a decrease in vasorelaxing effect of perivascular adipose tissue leading to hypertension [30,69,105,139]. This might imply a decrease in ADRF release or an imbalance in adipose tissue derived relaxing and vasocontractile factors during obesity. On the other hand, hypoxia, which develops within adipose tissue during obesity [20], has recently been shown to enhance the release of (a) vasorelaxing factor(s) released from adipose tissue which might imply the ADRF [140]. So the release of ADRF in obesity warrants further research.

1.5.3. Vasocontractile adipokines

1.5.3.1. Angiotensinogen/Angiotensin II

Brown and white adipocytes are rich sources of angiotensinogen, the precursor protein of a major vasocontractile peptide called angiotensin II [141], and possess all the enzymes necessary to produce angiotensin II [142]. These findings suggest the existence of a local renin-angiotensin system in adipose tissue. Moreover, the amount of angiotensinogen mRNA in adipose tissue is 68% of that in liver, supporting an important role for adipocyte-derived angiotensinogen in angiotensin II production [143]. The importance of this angiotensinogen-source in the blood pressure regulation by the renin-angiotensin system was shown in wild type and angiotensinogen-deficient mice, in which adipocyte-derived angiotensinogen was overexpressed. When angiotensinogen expression was restricted to adipose tissue (on an angiotensinogen-deficient background), circulating angiotensinogen was detected and mice were normotensive. On the other hand, wild type mice were hypertensive, due to the

additional amount of angiotensinogen as a result of overexpression of adipocyte-derived angiotensinogen [144].

An important effect of angiotensin II is that this peptide enhances the metabolism of NO into oxygen free radicals, which damage the vascular tissue [145]. Therefore, an imbalance between angiotensin II and NO leads to endothelial dysfunction resulting in a loss of vasodilator capacity. This results in an increased expression of adhesion molecules and proinflammatory cytokines in endothelial cells, which promote monocyte and leukocyte adhesion and migration to the vessel wall [146]. Furthermore angiotensin II exerts detrimental effects on progression and destabilization of atherosclerotic plaque due to an increased release of plasminogen activator inhibitor (PAI-1) causing thrombosis and an increased expression of growth factors leading to smooth muscle cell proliferation and migration [146]. Most data support an elevation of angiotensinogen mRNA expression in adipose tissue during obesity [147]. Furthermore, several studies highlight a contribution of adipose tissue-derived angiotensinogen and/or angiotensin peptides to obesity-related hypertension [147-149]. High angiotensin II levels may deteriorate obesity-related hypertension due to an increased secretion of proinflammatory cytokines [150], decreased adiponectin secretion [151] and increased leptin production in adipocytes [152].

1.5.3.2. Resistin

Resistin, expressed in brown and white adipose tissue, is a member of the family of cysteine-rich proteins called resistin-like molecules [99,153,154]. Resistin is secreted into the medium by cultured adipocytes and circulates in plasma, indicating that it is a secretory product of adipose tissue. However, especially circulating monocytes and macrophages seem to be responsible for resistin production in humans [71]. Although resistin does not directly affect the contractility of isolated blood vessels [155], coronary blood flow, mean arterial pressure or heart rate [156], it has been associated with endothelial dysfunction and coronary heart disease [157].

Initial findings have reported an association between obesity and elevated resistin plasma levels [158,159]. However, this could not be confirmed by other investigators [160,161]. Resistin expression is stimulated by TNFα and IL-6, both increased in obesity [162], which

offers an explanation for an increased level of resistin in obesity. Resistin augments endothelin-1 release which causes endothelial dysfunction. Moreover, resistin impairs endothelial function with [163] or without [156] augmenting superoxide production resulting in a decreased expression of endothelial NO synthase and NO levels [164]. Resistin also augments the expression of vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1), both involved in early atherosclerotic lesion formation [165]. It has also been shown that high plasma resistin levels independently associate with an increased risk for hypertension among non-diabetic women [166].

1.6. CONCLUSION

Adipose tissue produces and secretes several adipokines. Some of these adipokines possess vasoactive properties (Fig. 1.1). Arterial tone can be controlled through the release of ROS, leptin, adiponectin, TNF_{α}, IL-6, Ang II, omentin, resistin, visfatin, apelin and ADRF. The regulation of arterial tone might be compromised in obesity and obesity-related disorders (e.g. type 2 diabetes, cardiovascular disease, hypertension) due to alterations in the secretion of vasoactive adipokines by dysfunctional adipose tissue. Circulating levels of adiponectin and omentin are decreased while levels of leptin, resistin, apelin and proinflammatory cytokines are increased. One therapeutic strategy to counter the progression of obesity-related vascular diseases is elevating adiponectin and omentin levels. Adiponectin levels can already been elevated through the use of existing drugs like thiazolidinediones, telmisartan, angiotensin converting enzyme inhibitors, rimonabant and taranabant [101]. On the other hand, development of specific agonists to target adiponectin and omentin receptors or inhibiting detrimental adipokines signaling pathways may be new and promising methods to attenuate the proinflammatory effects and ultimately to reduce the progression of obesity-related vascular diseases.

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Chapter 2

Control of retinal arterial tone by a paracrine retinal relaxing factor

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2.1. ABSTRACT

Retinal blood flow is regulated by local factors. In vitro bio-assay experiments give evidence that retinal tissue from different species (dogs, pigs, sheep, cows, rats and mice) continuously releases a factor lowering tone of isolated retinal arteries. This factor is a general relaxant as it was effective in relaxing different types of vascular as well as nonvascular smooth muscle preparations. This factor is called the “retinal relaxing factor” (RRF) and its characteristics do not correspond with those of the many well known vasorelaxants found in retina (f.e. NO, prostanoids, adenosine, ADP, ATP, lactate, glutamate, GABA, taurine, adrenomedullin, CGRP, ANP, BNP and CNP). This unknown RRF is transferable, hydrophilic and heat-stable. Its relaxing effect is independent of the presence of the vascular endothelium and of NO-synthase, adenylyl cyclase, guanylyl cyclase and cyclooxygenase activity. RRF might have a role in hypoxic vasodilation in retinal arteries since hypoxia induces relaxation only when retinal tissue is present. Thus, the RRF-pathway is sensitive to changes in oxygen tension and might be a sensitive mechanism for adjusting vascular diameter to retinal oxygen levels. Diminished RRF release might explain the decreased retinal circulation observed in disease with atrophic retina.

2.2. INTRODUCTION

The retina is a cardinal layer for vision as it transforms the incoming light into neural activity. This conversion requires energy and thus an adequate supply of nutrients to the retina. The oxygen consumption of the retina on a per gram basis has been described to be one of the highest in the body [1,2]. However, the vascularisation of the retina is limited by the need for a relatively unobstructed light path to the photoreceptors. An overly rich vascular network on the inner side of the retina would interfere with the transmission of light through the retina. Anatomically, the conflict between a sufficient blood supply to the retina and a minimal interference with the light path to the photoreceptors is solved by two supplies of blood nourishing the retina: the retinal and choroidal circulation [3]. The rich choroidal circulation supplies the outer part of the retina with nutrients passing the blood-retina barrier by passive diffusion or through specific transporter molecules [4,5]. The retinal vessels lying within the nerve fibre layer supply mainly the inner layers of the retina [5-7].

The regulation of blood flow differs substantially between the retinal and choroidal circulation. Whereas choroidal blood flow is considered to be mainly regulated by autonomic innervation, retinal vasculature has the unusual feature of lacking adrenergic, cholinergic or peptidergic innervation [3]. Therefore, retinal blood flow is thought to be mainly regulated by autoregulatory mechanisms and local factors released from neighbouring cells [8] such as NO [9], prostaglandins [10], endothelin-1 [11], histamine [12], adenosine [13], lactate [14-16], neuropeptides [17],… About 10 years ago we accidentally discovered that retinal tissue releases a transferable relaxing factor that also might be involved in the regulation of retinal blood flow. The characteristics of this factor did not correspond to those of the well known vasoactive molecules formed within the retina. It was coined the name “retinal relaxing factor (RRF)” [18].

2.3. DISCOVERY OF RRF

The discovery of RRF is based on difficulties met by a starting young scientist studying retinal circulation. When isolated bovine retinal arteries were mounted in a wire myograph for isometric tension recording, the contractile response in different ring segments was most

variable. At first sight, this variability seemed to be due to the inexperienced scientist damaging the small arteries during the isolation and mounting procedure. However, persistent experimentation led to the idea that the observed contractility of the retinal artery depended on the amount of surrounding retinal tissue. Therefore, the effects of vasocontractile agents on preparations with and without adhering retinal tissue were compared. In response to increasing concentrations of prostaglandin $F_{2\alpha}$, the retinal artery with adherent retinal tissue showed only a weak contraction. However, in ring segments that were completely cleaned of all surrounding retinal tissue, prostaglandin $F_{2\alpha}$ induced substantial, concentration-dependent contractions. Also contractions induced by serotonin, endothelin-1 and the thromboxane A_2 mimetic U-46619 were impaired in the presence of retinal tissue, indicating that the diminished contractile response was rather aspecific. On the other hand, preparations with and without retinal tissue contracted in a similar way in response to K^+ 120 mM. High K^+-induced contractions are considered to be due to a relatively simple mechanism. The high K^+-concentration causes membrane depolarisation, which opens the L-type voltage-dependent Ca^{2+} channels, increases the intracellular Ca^{2+}-concentration and induces contraction [19,20]. The observed resistance of high K^+-contractions suggests that the diminished (agonist-induced) contraction in preparations with adhering retinal tissue cannot be ascribed to mechanical hindrance by the presence of additional tissue [18].

2.4. BIO-ASSAY FOR RRF USING BOVINE TISSUES

Tone of vascular smooth muscle cells might be influenced by neighbouring cells either through a direct electrical coupling, through gap junctional communication or through a paracrine release of vasoactive substances. To investigate whether the inhibitory influence of adhering tissue was due to the release of a relaxing factor from retinal tissue, a bio-assay was developed. A ring segment of a bovine retinal artery, carefully cleaned of all retinal tissue (= detector of the bio-assay), was mounted in a wire myograph for isometric tension measurements and was contracted with prostaglandin $F_{2\alpha}$. Subsequently, a piece of retinal tissue (= donor of the bio-assay) was brought in close proximity of the ring segment (Fig. 2.1A). This elicited a complete and stable relaxation of the vessel. This relaxation was found to be reversible as removal of the retina resulted in a rapid recovery of vessel tone (Fig. 2.1B). This observation proved the involvement of a relaxing factor released from retinal tissue. The

same arteries contracted with K^+ 120 mM instead of $PGF_{2\alpha}$ relaxed significantly weaker when covered with retinal tissue, confirming the initial observation that the presence of retinal tissue has only a moderate influence on K^+ 120 mM induced contractions. Covering the detector tissue with a similar-sized piece of bovine choroidal tissue (BCT) failed to induce a pronounced relaxation in prostaglandin $F_{2\alpha}$-contracted retinal arteries (Fig. 2.1C). It is therefore unlikely that the observed relaxations are a mechanical or electrical artefact, due to positioning the retinal tissue on top of the ring segment [18]. Using a similar bio-assay set-up Takir et al. confirmed the complete relaxation of a bovine retinal artery when covered by a bovine retina. They described that this relaxation is mostly biphasic, suggesting a potential involvement of two different RRFs. In the presence of K^+ 120 mM the biphasic response became monophasic and much reduced [21].

2.5. BIO-ASSAY FOR RRF USING TISSUES FROM OTHER SPECIES

The RRF is not only released from bovine retina. Covering bovine retinal artery with retinal tissue from other animals (dogs, pigs and sheep) also induced complete relaxations (Fig. 2.1C). Further experiments revealed that the RRF also relaxes other vascular smooth muscle preparations such as the rat renal artery and the rat mesenteric artery. Even non-vascular smooth muscle cells can relax in response to the retinal relaxing factor as is evidenced by its relaxing influence on rat airway segments like the rat main bronchi (Fig. 2.1D). All taken together, these observations suggest that RRF is a more general relaxing substance. Anyhow, the retinal artery seemed to be the most responsive of all preparations tested [18].

A reliable bio-assay set-up for RRF has also been developed for small laboratory animals (rat and mouse). Boussery et al. [22] found that reproducible relaxations are obtained when a rat retina is put repetitively in close proximity of a precontracted rat carotid artery. A solution containing 30 mM K^+ and 30 µM $PGF_{2\alpha}$ was used to contract the carotid arteries because the presence of 30 mM K^+ had a stabilising effect on both the contractions and the relaxations. When a stable contraction was obtained, the rat retina was put on top of the artery. This elicited a profound and stable relaxation. Other tissues – for instance, samples of cornea, bladder wall or mesenterium of the rat – failed to relax the carotid artery under the same conditions. When the retina was removed, the relaxing influence disappeared and tone

returned to its original level. This protocol can be repeated several times, resulting in reproducible relaxation effects (Fig. 2.2A).

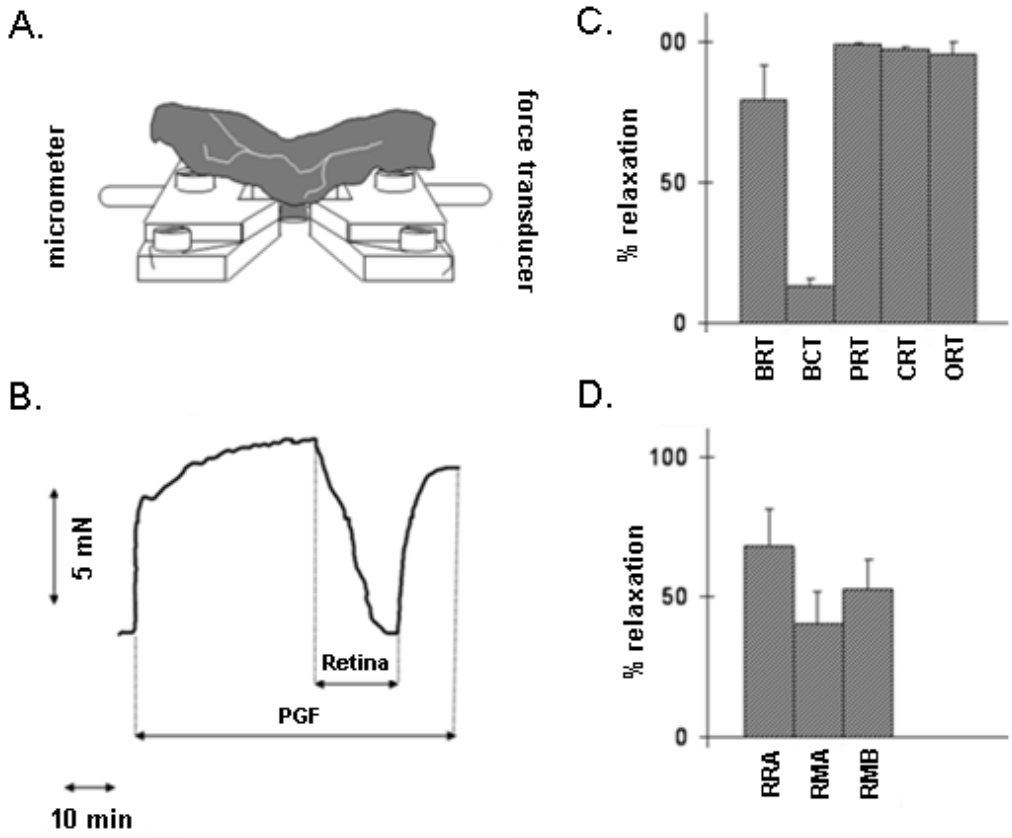

Fig. 2.1. Bio-assay experiments demonstrating the release of a relaxing factor from retinal tissue. A. Schematic representation of placing a piece of retinal tissue in proximity of an isolated bovine retinal artery mounted in a wire myograph; B. Representative tracing of the relaxation induced by placing a piece of bovine retinal tissue (+ Retina) in close proximity to an isolated bovine retinal artery contracted with prostaglandin $F_{2\alpha}$ (30 µM). Vertical scale shows the active force in mN; horizontal scale shows time; C. Relaxations (expressed as percent relaxation) of bovine retinal artery (contracted with prostaglandin $F_{2\alpha}$, 30 µM) induced by a piece of bovine retinal tissue (BRT), bovine choroidal tissue (BCT), porcine retinal tissue (PRT), canine retinal tissue (CRT) and ovine retinal tissue (ORT); D. Relaxation (expressed as percent relaxation) induced by placing a piece of bovine retinal tissue in close proximity which the rat renal artery (RRA) and the rat mesenteric artery (RMA) contracted with prostaglandin $F_{2\alpha}$ (30 µM) and the rat main bronchus (RMB) contracted with carbachol (0.1 mM) (based on Delaey and Van de Voorde, Circ Res 1998, 83: 714-720, with thanks to Lippincott Williams & Wilkins as copyright holder).

In a similar manner, Boussery et al. [23] demonstrated the release of a retinal relaxing factor from the retina of mice. Instead of a carotid artery, a mouse aorta contracted with 30 mM K^+ and 30 µM $PGF_{2\alpha}$, was used as detector of the bio-assay. Also with this technique reproducible relaxations were obtained on repeated application of retinal tissue.

Besides cows, dogs, sheep and pigs [18], small laboratory animals like rats and mice can also be used for research on the RRF. The use of rats and mice offers the possibility to perform in vivo interventions and to use pathological animal models (e.g. animal models of diabetic retinopathy). Furthermore, experiments with transgenic mice or rats could help to unravel the identity, mechanisms of action and physiological relevance of the RRF [22,23].

2.6. RRF IN HYPOXIC VASODILATION

Hypoxia is a well known vasodilatory stimulus in retinal arterial vascular bed [24], reflecting a physiological regulatory process that adapts blood flow to the metabolic needs of the tissue. Hypoxia-induced vasodilation may result from direct effects on the blood vessel wall, as well as from indirect effects, such as the release of metabolic factors from the surrounding tissue [25]. Considering that surrounding retinal tissue exerts a pronounced vasorelaxing influence on retinal arteries, it was studied whether the surrounding retinal tissue could have a role in hypoxia-induced retinal vasodilation of isolated bovine arteries by using an in vitro setup.

Acute hypoxia was induced by abruptly switching the gas mixture used to bubble the organ bath solution from an oxygenating composition (95 % O_2/ 5 % CO_2) to a composition without oxygen (95 % N_2/ 5 % CO_2). Hypoxia induced a pronounced relaxation of preparations with some adherent retinal tissue. In contrast, hypoxia had no effect on retinal arteries without surrounding retinal tissue. This innovative technique of incomplete removal of the perivascular tissue clearly demonstrated the essential role of the presence of retinal tissue in retinal hypoxic vasodilation [26]. The relaxing influence is related to the pO_2 in the organ bath solution as is clear from experiments in which preparations were exposed to gas mixtures with different percentage of O_2 resulting in different pO_2 values (Fig. 2.3). Fig. 2.3 also illustrates that in normoxic conditions (94 mmHg) retina-induced relaxation is about half maximum. Hyperoxia and hypoxia thus lead respectively to less or more relaxing influence of

the retinal tissue. Such changes may be a fundamental mechanism for matching blood flow to the metabolic needs of the retina and corresponds with in vivo observations of vasoconstriction in hyperoxia and vasodilation in hypoxia.

The retina-mediated hypoxic vasodilation does not require direct contact between the vascular and retinal cells. This is clear from experiments in which detached bovine retinal tissue is brought in close apposition with a completely cleaned retinal artery. While the cleaned retinal artery on itself does not relax in response to hypoxia, it does relax when retinal tissue is in close proximity. This study reinforces the view that a transferable factor released from retinal tissue participates in the hypoxic response. Neither NO, prostanoids, adenosine, lactate or changes in pH seemed to be involved in this hypoxic response. Furthermore, hypoxia does not induce a marked relaxation of the retinal artery contracted with K^+ 120 mM, suggesting that RRF might be the mediator of the hypoxic response [26].

The release or the effect of this retinal factor is possibly triggered by reduced ATP levels rather than by hypoxia itself. This is suggested by the fact that under hyperoxic conditions in the organ bath, inhibition of ATP formation with iodoacetate or sodium-cyanide induces a complete relaxation of the bovine retinal arteries with adherent retinal tissue in contrast to the partial relaxation seen in preparations without retinal tissue [26].

Also in the rat model, the RRF-response was largely enhanced in hypoxic conditions. This enhanced relaxation cannot be attributed to a direct effect of hypoxia on precontracted rat carotid artery, because acute hypoxia applied in the absence of retina had only a small relaxing influence (Fig. 2.2B). Hydrogen ions, lactic acid, K^+, prostaglandins, adenosine, GABA and excitatory amino acids (as glutamic acid, aspartic acid, glycine and taurine) have all been suggested as potential mediators of hypoxia-induced vasodilation [10,14,25,27-30]. However, they could all be excluded from mediating the enhanced response to RRF in the rat bio-assay [22].

The enhanced retina-induced relaxation during hypoxia was also observed in the mice model of bio-assay for RRF, suggesting that also in mice RRF might be the mediator of the hypoxic response [23].

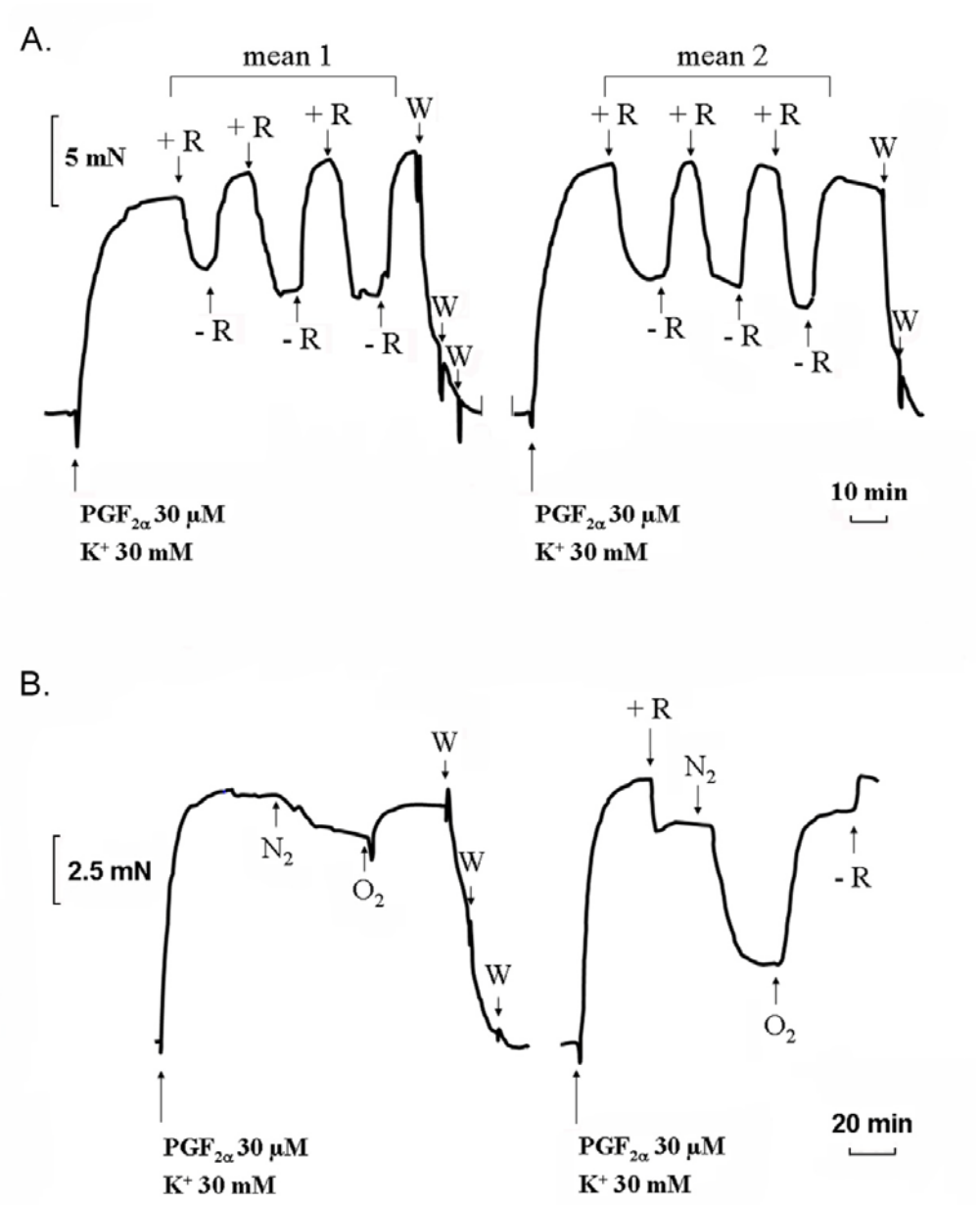

Fig. 2.2. Original tracings of experiments in which a rat carotid artery was contracted with a 30 µM prostaglandin $F_{2\alpha}$ and 30 mM potassium. A. When a stable contraction was obtained, the rat retina was put in close proximity with the artery (+R). When a stable relaxation was elicited, the retina was removed (-R). This was repeated 3 times, and thereafter the organ bath was washed (W). Then the entire procedure was repeated. Without intervention between the two procedures the means of the two series of relaxations were comparable. B. In the absence of rat retinal tissue, rat carotid artery only shows a minimal relaxation in response to hypoxia (N_2). Addition of rat retinal tissue (+R) elicits a relaxation which is drastically enhanced when changing to hypoxic conditions. On reoxygenation (O_2) the relaxation is greatly diminished and completely disappears after removal of the retinal tissue (-R) (based on Boussery et al., Invest Ophthalm Vis Sci 2002, 43: 3279-3286, with thanks to the Association for Research in Vision and Ophthalmology as copyright holder).

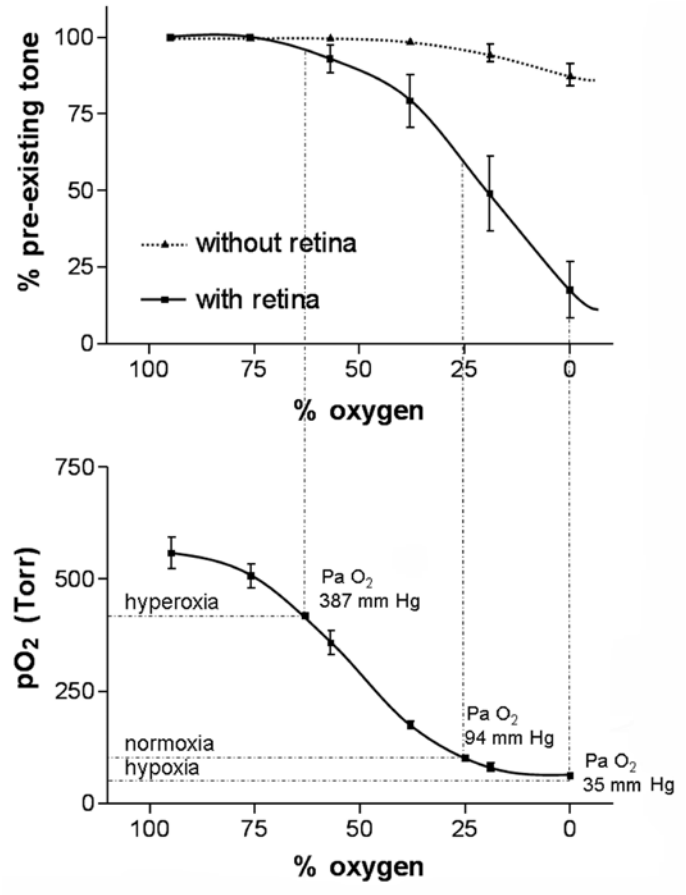

Fig. 2.3. Relation between pO_2 (expressed in Torr), percent oxygen in bubbling solution and the relaxing influence on precontracted (prostaglandin $F_{2\alpha}$, 30 µM) bovine retinal artery with and without adhering retinal tissue.

2.7. RRF IN SOLUTION

Having established that the relaxing effect of the retinal tissue was caused by a relaxing factor released by the retina, another bio-assay technique was developed to analyze some characteristics of this RRF. In this technique bovine retinas were incubated for 6 hours in Krebs-Ringer bicarbonate solution and the effect of the incubation solution was investigated on precontracted isolated bovine retinal arteries completely cleaned from retinal tissue. Changing the superfusion from an incubation solution without retinas to an incubation solution with retinas elicited a relaxation of the retinal artery, suggesting that RRF can indeed be obtained as a stable molecule in solution [18].

The relaxing influence of the RRF-containing solution persisted after extraction with hexane. Furthermore, heating the solution to 70°C for 1 hour did not alter the relaxing effect. The relaxations induced by the RRF-containing solution could not be attributed to changes in pH,

because the pH-changes alone were not sufficient to substantially influence the retinal arterial tone. These data suggest that the RRF is a hydrophilic and thermostable molecule [18].

2.8. MECHANISMS OF RRF-INDUCED RELAXATION

Many stimuli are known to relax isolated blood vessels in an endothelium-dependent way [31-33]. Delaey et al. [18] investigated whether the presence of the endothelium was required for the retina-induced relaxations of bovine retinal arteries. Retinal arteries without retinal tissue were first contracted with prostaglandin $F_{2\alpha}$ (30 µM). When the contraction reached a steady state, the retinal artery was covered with a piece of retinal tissue, resulting in a pronounced relaxation. This procedure was then repeated after removal of the vascular endothelium. This did not alter the relaxations induced by the retinal tissue, suggesting that bovine RRF is not mediated by the endothelium.

Also, removal of the endothelium of the rat carotid artery did not significantly decrease the relaxing influence of a rat retina placed nearby. In both the bovine and the rat model, the efficacy of endothelium removal was demonstrated by the fact that acetylcholine-induced relaxations (well known to be endothelium-dependent) were significantly decreased after endothelium removal [22]. From all these data it is clear that RRF elicits its relaxation in an endothelium-independent way.

Both cGMP and cAMP are important second messengers eliciting relaxation in vascular smooth muscle cells. The involvement of cGMP in the RRF-response is very unlikely considering that the cGMP-pathway is a relatively weak stimulator of relaxation in bovine retinal artery. Both NO (activator of sGC) and 8-Br-cGMP (analogue of cGMP) elicit a weaker relaxation than the RRF [34]. Moreover, the RRF-induced relaxation is not influenced by ODQ, an inhibitor of sGC [22,23]. In addition, RRF induced relaxation can still be demonstrated in aorta from $sGC_{\alpha 1}$-knock out mice, which lack the most important isoform of sGC in vascular smooth muscle (own unpublished observations).
The involvement of cAMP-dependent signal transduction pathways in the RRF-response is also unlikely. In bio-assay experiments using rat carotid artery as detector it was found that total cAMP levels are not increased in artery rings that are relaxed by the RRF released from

retinal tissue. On the other hand cAMP levels were found to increase in response to forskolin, used in a concentration eliciting a similar degree of relaxation as the RRF (own unpublished observations).

The potential involvement of several $[Ca]_i$-reducing mechanisms in the RRF-response was also evaluated using the rat model [22]. Inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) with thapsigargin and cyclopiazonic acid did not influence the retina-induced relaxations, suggesting that SERCA-activation is not involved in the relaxing effect of the RRF. The involvement of the Na^+-Ca^{2+} exchanger was assessed with two inhibitors, DMTU and amiloride. Neither DMTU nor amiloride was able to block the retina-induced relaxations, suggesting that it is very unlikely that the Na^+-Ca^{2+} exchanger plays an important role in the RRF-induced relaxation. In the presence of L-type Ca^{2+} channel blocker nifedipine, application of the retina still induced relaxation of the carotid artery, suggesting that blockade of the L-type Ca^{2+} channels does not play an important role in the RRF-response. On the other hand, inhibition of the plasma membrane Ca^{2+}-ATPase with vanadate resulted in a significantly reduced retina-induced relaxation, suggesting that Ca^{2+}-extrusion by the plasma membrane Ca^{2+}-ATPase may play a role in the RRF-induced relaxation of the rat carotid artery. It should however be noted that the effect of vanadate is not very specific since vanadate is known to influence other mechanisms [35].

2.9. IDENTITY OF RRF

It has been suggested before that the retinal arterial tone may be controlled by mediators released from the surrounding retinal tissue. Both NO [9] and prostaglandin E_1 [10] have been proposed as possible mediators. However, both can be excluded from being the RRF.

NO has a short half-life and would be destroyed after 1 hour at 70°C, whereas the RRF showed to be thermostable. In addition, NO induces only a moderate relaxation of the bovine retinal artery compared to the response of the RRF [34]. In experiments in which concentration-response curves to prostaglandin $F_{2\alpha}$ were made in the presence or absence of NO-synthase inhibitor nitro-L-arginine (L-NA) on bovine retinal arteries with and without adherent retinal tissue, L-NA was unable to abolish the inhibitory influence of the adherent retinal tissue. Methylene blue, a blocker of guanylyl cyclase that mediates the relaxant

influence of NO, had no effect on the relaxation induced by the application of a piece of retinal tissue on a bovine retinal artery contracted with prostaglandin $F_{2\alpha}$ [18]. L-NA and guanylyl cyclase inhibitor ODQ were also used to test whether NO could be involved in the RRF-induced relaxations in the rat and mouse bio-assay for RRF. L-NA was unable to abolish the relaxing influence of a rat or mouse retina placed on a rat carotid artery or mouse aorta respectively. The acetylcholine-induced relaxation, in contrast, was completely blocked by L-NA. Treatment with ODQ caused a small but not significant decrease in rat RRF-response and did not diminish the mouse retina-induced relaxation [22,23]. However, the relaxation in response to SNP was significantly decreased by ODQ in both species. These observations provide evidence that RRF can not be identified as NO.

To exclude the possibility of prostaglandin E_1 or another cyclooxygenase metabolite being the RRF, indomethacin (10 µM), a cyclooxygenase inhibitor, was tested on bovine retinal arteries with and without adherent retinal tissue. Indomethacin did not alter the concentration-response curves to prostaglandin $F_{2\alpha}$ when used on bovine preparations with adherent retinal tissue [18]. To investigate the potential role of prostaglandins in RRF-response in rat and mouse, both the retina and the rat carotid artery/mouse aorta were treated with indomethacin or sodium diclofenac. These treatments did not decrease, but in contrast even increased, the relaxations induced by a retina placed nearby [22,23]. All these experiments show that it is also very unlikely that the RRF is a prostanoid or that its effect is prostanoid-dependent.

Because the RRF did not correspond with previously proposed relaxing factors from the retina, the potential role of other substances present in the retina was investigated. The neural retina consists of 6 types of neurons: photoreceptor, horizontal, bipolar, amacrine, interplexiform and ganglion cells. Müller cells and retinal pigment epithelium are the two principal non-neuronal cells of the retina. Astrocytes are present in the nerve fiber layer but are absent from the deeper retinal laminae. Each of these cells releases various molecules - some with vasoactive properties and thus potential candidates for being the RRF. These include glutamate, glycine, dopamine and GABA. However, these molecules do not induce a relaxation of isolated bovine retinal arteries contracted with prostaglandin $F_{2\alpha}$. Melatonin, which is also released by the retina, is incapable of relaxing these arteries as well. Since the relaxation induced by the retinal tissue occurs independently of the endothelium, acetylcholine and other endothelium-dependent vasodilators (e.g. histamine [12]) too are unlikely to be the RRF [18].

Adenosine is a stable, hydrophilic molecule and is capable of relaxing the isolated retinal artery contracted with prostaglandin $F_{2\alpha}$. The presence of 8-phenyltheophylline (10 µM), a non-specific adenosine receptor blocker, did not alter the relaxations induced by application of the bovine retinal tissue. Furthermore, adenosine is unable to relax isolated rat main bronchi contracted with carbachol, whereas the retinal tissue induces a relaxation of this same preparation. Adenosine even slightly contracts isolated rat main bronchi [18] and only induces a small and transient relaxation of rat carotid artery in a high concentration [22]. All these data thus exclude adenosine from being the RRF.

Several studies reported a potential role for adrenomedullin in the normal physiology of the eye [36-39], and in the pathophysiology of some ocular diseases [40,41]. We demonstrated that adrenomedullin relaxes isolated bovine retinal arteries. However, this relaxation is partly endothelium-dependent and antagonised by $CGRP_1$ receptor antagonist CGRP 8-37 [42]. Therefore, it is rather unlikely that adrenomedullin is the as yet unidentified RRF, because the RRF response in bovine retinal arteries showed to be endothelium-independent [18] and insensitive to CGRP 8-37 [42].

Calcitonin gene-related peptide (CGRP) and natriuretic peptides, which are expressed in the retina [43] induce a marked vasodilation in different vascular beds [44-46]. A role for neuropeptides such as CGRP in the local regulation of retinal blood flow has been introduced before [17]. CGRP has a powerful relaxing effect on bovine retinal arterial smooth muscle, but this effect is mediated by endothelial NO [47]. Since the RRF-response is completely unaffected by removal of the endothelium or by treatment with L-NA [18], the hypothesis of CGRP being the unidentified RRF can be rejected.

The possible involvement of a local natriuretic peptide system in retinal blood flow regulation has recently been mentioned as well [43]. However, the natriuretic peptides ANP (atrial natriuretic peptide), BNP (brain natriuretic peptide) and CNP (C-type natriuretic peptide) can be excluded as possible candidates for being the RRF since none of these peptides induces a substantial relaxation in $PGF_{2\alpha}$-contracted isolated bovine retinal arteries [47].

2.10. CONCLUSION

The data presented provide evidence that retinovascular tone and hence retinal blood flow is modulated by (a) relaxing factor(s) released from perivascular retinal tissue, one of them being the so called RRF. The cell type releasing the RRF remains unknown. The presence of tetrodotoxin, which inhibits the release of neurotransmitters from retinal neurons, did not significantly alter the inhibitory effect of the retinal tissue, which suggests that glial rather than neuronal cells release this factor [18]. The RRF seems to be released continuously from the isolated retina without requiring a specific stimulus, because the inhibitory influence exerted by the retinal tissue does not diminish during prolonged experiments. Continuously released RRF might have a physiological role in regulating retinal blood flow, even more so because in different pathologies associated with atrophy of the retina also the retinal vessel diameter is decreased in retinal circulation (f.e. retinitis pigmentosa, panretinal photocoagulation and descending optic atrophy) [48].

The RRF-pathway is most probably linked to the oxygen tension in the retina. High oxygen levels inhibit the relaxing influence of the retina (enhancing vasoconstriction) whereas low oxygen levels stimulate it (inducing vasodilation). Increased retinal metabolism (e.g. dark adaptation or flickstimulation) [49] or reduced blood flow (e.g. due to hypotension) result in lower retinal oxygen levels and induce metabolic vasodilation. It remains to be determined whether this vasodilation is due to enhanced release of RRF or another mechanism (e.g. increased sensitivity of the artery to RRF, concomitant release of potentiating vasodilator) [22]. Reduced retinal metabolism (e.g. during light adaptation) or increased blood flow (e.g. due to isometric exercise) would in contrast result in higher oxygen levels and would diminish the release of this factor, effectively inhibiting metabolic vasodilation. Thus, the release of the RRF from the retina could provide a sensitive mechanism that appropriately adjusts vascular diameters to changes in retinal oxygen levels. However, the exact physiological role of the RRF in the retinal circulation can only be established after the RRF is identified and selective antagonists become available.

So far, the RRF is only studied with the intention of establishing its identity and its role in the physiological regulatory process of hypoxic retinal vasodilation. However, characterisation of the RRF and other local regulatory mechanisms in the retinal circulation could not only offer a better understanding of normal retinal blood flow regulation. These local mechanisms could

also form a target for future treatment options in retinal pathologies with a vascular component. In glaucoma treatment for example, it has been suggested that besides lowering the intraocular pressure, any vasoactive effect on the retinal vasculature may be particularly relevant [8].

Some interesting information on the RRF has been collected so far, although many questions remain to be answered. It seems logical that in future research priority should be given to exploring the identity and the mechanism of action of the RRF. Understanding the mechanism of action of the RRF could offer the opportunity of selectively antagonising the relaxing effect of the RRF. This would on its turn facilitate the evaluation of the physiological and pathophysiological role of the RRF and potential pharmacological applications.

2.11. FUNDING

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Chapter 3

Hypoxia enhances the relaxing influence of perivascular adipose tissue in isolated mice aorta

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3.1. ABSTRACT

Adipose tissue releases an “adipocyte-derived relaxing factor” (ADRF) lowering tone of isolated arteries. The potential influence of hypoxia on the vasorelaxing properties of adipose tissue was investigated. Aortas from male Swiss mice with or without adherent adipose tissue were mounted in a wire myograph for isometric tension recording. Hypoxia (bubbling with 95% N_2, 5% CO_2) relaxed precontracted (norephinephrine, 5 µM) aorta with adipose tissue while only a minimal vasorelaxing effect was observed in arteries without adipose tissue. This effect was also seen after precontraction with prostaglandin $F_{2\alpha}$ (30 µM) or U-46619 (10 nM). Precontraction with 60 or 120 mM K^+, incubation with tetraethylammoniumchloride (3 mM) or glibenclamide (30 µM) significantly impaired the hypoxic response. Glibenclamide (30 µM) enhanced the vasorelaxing effect of NaHS (except at high concentrations of NaHS). Lactate (10 nM to 1 mM) had no effect on preparations with or without adipose tissue. 8-(p-sulfophenyl)theophylline (0.1 mM), zinc protoporphyrin IX (10 µM), 1 H-[1, 2, 4]oxadiazolo[4,3-A]quinoxalin-1-one (10 µM) and removal of the endothelium did not influence the hypoxic relaxation. Our findings indicate that hypoxia has a relaxing influence on mice aorta that is dependent on the presence of adherent adipose tissue. This relaxation is partly mediated by opening K_{ATP} channels and independent of the endothelium and soluble guanylyl cyclase. Neither lactate, adenosine, CO nor H_2S seems to be involved in this hypoxic response. However, the involvement of the as yet unidentified "adipocyte-derived relaxing factor" (ADRF) cannot be excluded.

3.2. INTRODUCTION

Virtually all blood vessels are surrounded by variable amounts of adventitial adipose tissue. Perivascular adipose tissue is widely assumed to serve as structural support. However recent studies also uncovered a paracrine role for perivascular adipose tissue in the regulation of vascular tone [1]. Adipose tissue produces and secretes a variety of bioactive molecules called adipokines, which are hormones and inflammatory cytokines. Several studies demonstrated that perivascular fat significantly attenuates vascular responsiveness of aortic [2,3] and mesenteric arteries [4] in response to a variety of vasoconstrictors. The vasorelaxing influence is due to the unknown "adipocyte-derived relaxing factor" (ADRF) released from adipose tissue [2].

In obesity a dysregulation of the synthesis and secretion of adipokines leads to a state of inflammation within adipose tissue. As hypoxia occurs in areas of the fat depots as the tissue mass increases during the progressive development of obesity, it has been proposed that hypoxia may underlie this inflammatory response. Hypoxia in adipose tissue has been demonstrated in several obese mice models [5,6]. Furthermore, cell-culture studies using murine [7] and human [8] adipocytes have demonstrated that hypoxia influences the expression of inflammation-related adipokine genes. Therefore, the present study was designed to test the hypothesis that hypoxia affects the relaxing properties of adipose tissue. This was done using *in vitro* tension measurements of mice aortas with or without adherent adipose tissue.

3.3. MATERIALS AND METHODS

3.3.1. Aortic ring segments

Thoracic aortas (n=98) surrounded by adipose tissue from male Swiss mice (age: 7-10 weeks, supplier: Janvier) were isolated and kept in cooled and oxygenated (5% CO_2 in O_2) Krebs-Ringer bicarbonate solution. Approval was granted by the university ethics review board of the Faculty of Medicine and Health Sciences.

3.3.2. Tension measurements

The aortas were transferred to a wire myograph for isometric tension recording (Fig. 3.1). Segments carefully cleaned of adipose tissue served as control. The segments with or without adipose tissue were equilibrated for approximately 30 min in oxygenated (5% CO_2 in O_2) Krebs-Ringer bicarbonate solution at 37°C (pH 7.4) and gradually stretched until a stable preload of 0.5 g was obtained.

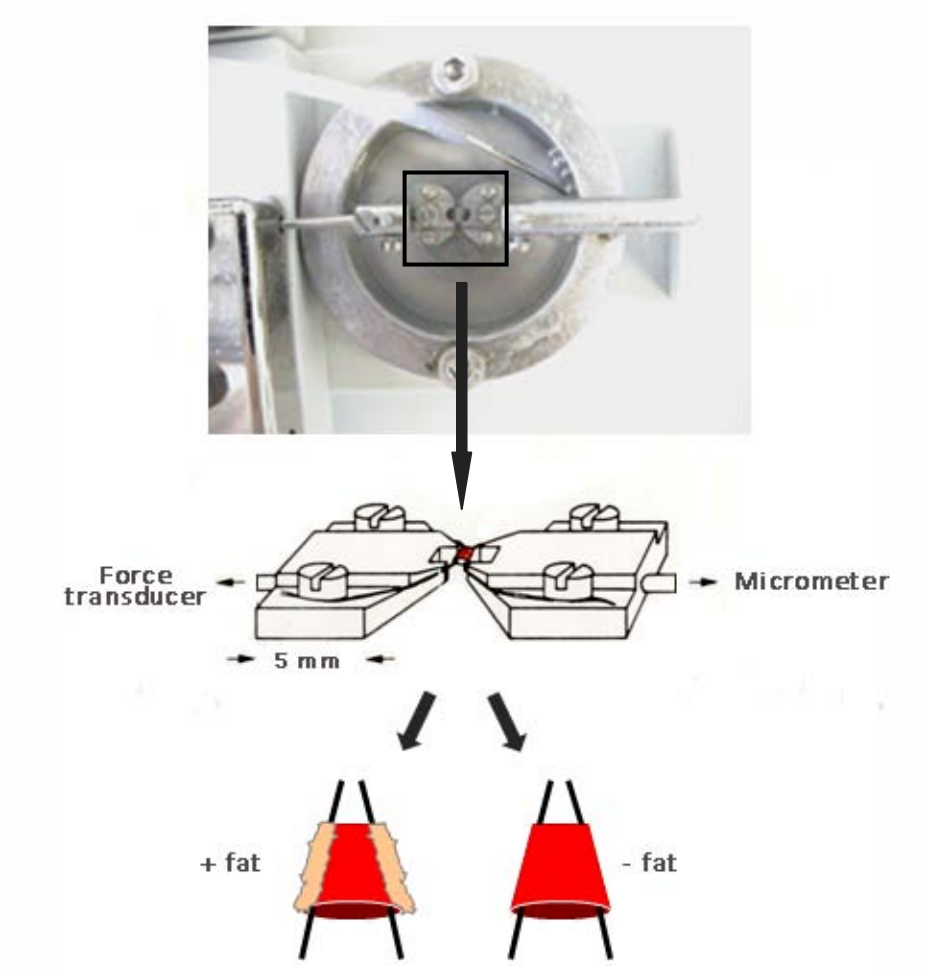

Fig. 3.1. A photograph of the organ bath of the wire myograph and a schematic detail of a pair of holders and vessel segments with and without adherent adipose tissue. Vessel segments are mounted on two stainless steel wires (40 µm), fixed on the two holders in the organ bath. One holder is connected to a micrometer which is used to change the distance between the wires. The other holder is connected to a force transducer which measures isometric tension changes in the vessel segment.

At the start of each experiment, the preparations were repeatedly activated with 120 mM K^+ and 5 µM norepinephrine. To exclude the influence of basal activity of cyclooxygenase metabolites and NO, all experiments were performed on preparations incubated with the cyclooxygenase inhibitor indomethacin (10 µM, 20 min) and the NO-synthase inhibitor N^{ω}-nitro-L-arginine (0.1 mM, 10 min). Subsequently, the preparations were contracted by adding 5 µM norepinephrine to the standard Krebs-Ringer bicarbonate solution in the organ bath.

3.3.3. Hypoxia

Moderate hypoxia in the organ baths was induced for 30 min by switching the gas mixture used to bubble the organ bath solution from 95% O_2/5% CO_2 to 95% N_2/5% CO_2. This reduces pO_2 levels to about 60 mmHg (own observations).

3.3.4. Removal of the endothelium

Arteries were unstretched in the myograph and the endothelium removed by rubbing the lumen with a PE10 tube (ID 0.28 mm, OD 0.61 mm). Thereafter the wires were reset to their original positions and the vessel was allowed to reequilibrate for half an hour.

3.3.5. Statistical methods

The data were computed as means ± S.E.M. and evaluated statistically with Student's t-test for paired or unpaired observations. Two groups of data were considered significantly different when $P<0.05$. Relaxations are expressed as % decrease in pre-existing tone. Measurements were made 30 min after inducing hypoxia. N is the number of animals used.

3.3.6. Drugs

The experiments were performed in a Krebs-Ringer bicarbonate solution of the following composition (mmol/L): NaCl 135, KCl 5, $NaHCO_3$ 20, glucose 10, $CaCl_2$ 2.5, $MgSO_4$ 1.3,

KH_2PO_4 1.2 and EDTA 0.026 in H_2O. Krebs-Ringer bicarbonate solutions containing 60 mM (K_{60}) and 120 mM K^+ (K_{120}) were prepared by equimolar replacement of NaCl by KCl. N^{ω}-nitro-L-arginine, indomethacin, norepinephrine, sodium cyanide (NaCN), sodium DL-lactate, 8-(p-sulfophenyl)-theophylline, tetraethylammoniumchloride, glibenclamide, 4-aminopyridine, apamin, 1 H-[1, 2, 4]oxadiazolo[4,3- A]quinoxalin-1-one (ODQ), acetylcholine, sodium hydrosulfide hydrate (NaHS) and zinc protoporphyrin IX were obtained from Sigma (St. Louis, MO), prostaglandin $F_{2\alpha}$ (Dinolytic®) from Pfizer (Puurs, Belgium), $BaCl_2$ from Merck (Darmstadt, Germany) and U-46619 from Calbiochem (San Diego, US). Stock solutions were made in water, except for indomethacin (dissolved in ethanol), U-46619 (dissolved in methylacetate), apamin (dissolved in 0.05 mol/L acetic acid), glibenclamide and ODQ (both dissolved in dimethylsulfoxide), zinc protoporphyrin IX (dissolved in 0.1 mol/L NaOH) and acetylcholine (dissolved in 50 mmol/L potassium hydrogen phthalate buffer, pH 4.0). The final concentration of ethanol, methylacetate, acetic acid, dimethylsulfoxide and NaOH in the organ bath never surpassed 0.1 %.

3.4. RESULTS

3.4.1. Effect of hypoxia on aortas with and without adherent adipose tissue

An original recording is shown in Fig. 3.2A. Hypoxia resulted in a pronounced vasorelaxation of preparations with adipose tissue while only a minimal vasorelaxation was observed during hypoxia on preparations without adipose tissue. Also in the absence of the cyclooxygenase inhibitor indomethacin and the NO-synthase inhibitor N^{ω}-nitro-L-arginine, hypoxia resulted in a pronounced vasorelaxation of preparations with adipose tissue. In contrast, preparations without adipose tissue were not affected by the change in oxygenation (Fig. 3.2B).

Precontractile tone was found to be less pronounced in the presence of adipose tissue. Therefore, the influence of a similar lowering of contractile tone (by reducing the norepinephrine concentration) was studied on the hypoxia induced relaxation. A similar precontraction level was obtained in preparations without adipose tissue (10.54 ± 1.07 mN) precontracted with norepinephrine (< 5 µM) and preparations with adipose tissue (10.64 ± 0.43 mN) precontracted with norepinephrine 5 µM (n=6, P>0.05). Reducing the

norepinephrine concentration (< 5 µM) on preparations without adipose tissue, results in a significantly lower contractile tone (10.54 ± 1.07 mN) vs. tone with norepinephrine 5 µM (16.15 ± 1.11 mN, n=6, P<0.05). However, the hypoxic relaxing response of these preparations was similar (24.91 ± 8.39 % relaxation when precontracted with norepinephrine < 5 µM vs. 15.16 ± 4.84 % relaxation when precontracted with norepinephrine 5 µM, n=6, P>0.05). Furthermore, there is still a significantly stronger increase in hypoxic response in the presence of adipose tissue (56.45 ± 1.49 % relaxation) than after lowering tone (24.91 ± 8.39 % relaxation, n=6, P<0.05).

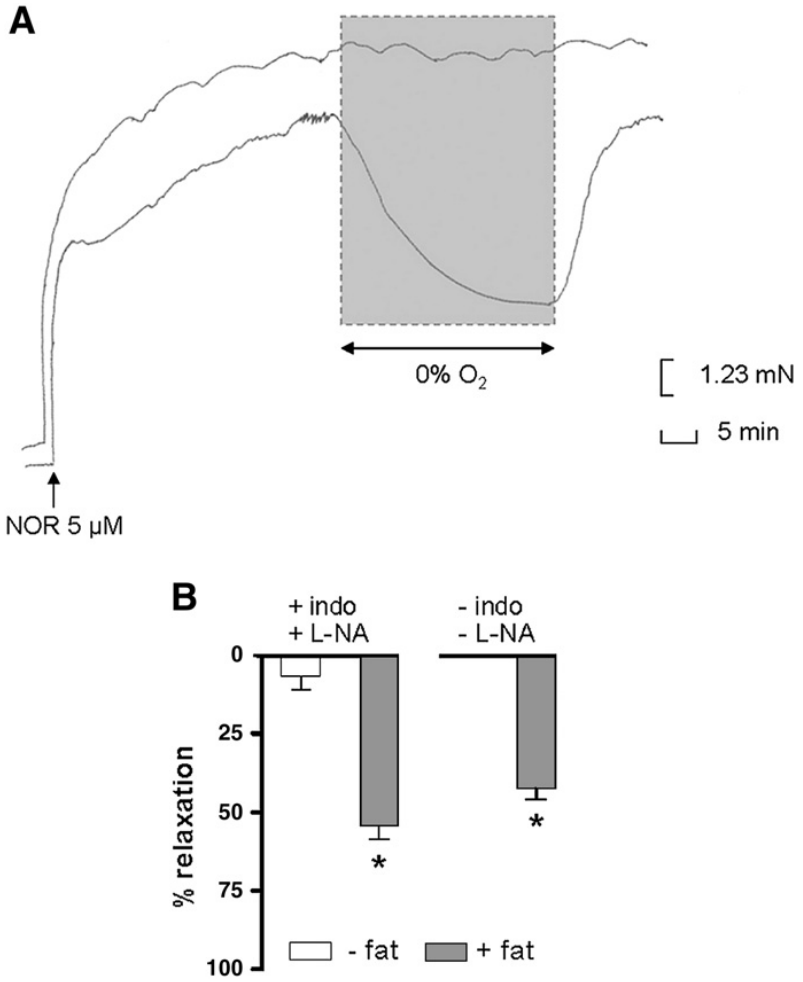

Fig. 3.2. A. Original tracing showing a response curve to hypoxia in a norepinephrine (NOR, 5 µM) precontracted mice aorta with and without adipose tissue. B. Effect of hypoxia on precontracted norepinephrine (NOR, 5 µM) mice aorta with (▬) and without (▭) adipose tissue in the presence (n=7, *P<0.05) and absence (n=6, *P<0.05) of indomethacin (indo, 10 µM) and N^{ω}-nitro-L-arginine (L-NA, 0.1 mM).

3.4.2. Effect of hypoxia on contractions induced by different agonists

The relaxing response of hypoxia was studied on arteries with and without adipose tissue, precontracted with 30 µM prostaglandin $F_{2\alpha}$ and 5 µM norepinephrine in changing order. A similar adipose tissue dependent hypoxic response was observed when precontracted with norepinephrine (59.57 ± 6.26 % relaxation) and prostaglandin $F_{2\alpha}$ (63.87 ± 6.42 % relaxation, n=6, P>0.05).

After precontraction with the thromboxane A2 mimetic, U-46619 (10 nM), hypoxia elicited a pronounced vasorelaxation of preparations with adipose tissue (80.23 ± 5.05 % relaxation, n=6). This relaxation was even stronger than after precontraction with 5 µM norepinephrine (60.37 ± 3.87 % relaxation, n=6, P<0.05).

Hypoxia only induced a small vasorelaxing effect on preparations without adipose tissue precontracted with 30 µM prostaglandin $F_{2\alpha}$ (21.70 ± 6.13 % relaxation, n=6) and 10 nM U-46619 (23.80 ± 10.56, n=6).

3.4.3. Influence of K^+ channel blocking on the hypoxic response

Experiments were performed in which the K^+ concentration in the organ bath solution was increased to 60 and 120 mM. The hypoxic response of preparations with adipose tissue was significantly diminished in the presence of 60 (Fig. 3.3A) and 120 mM K^+ (Fig. 3.3B) compared with the effect after precontraction with 5 µM norepinephrine. Furthermore, experiments were performed before and after addition of the non-selective potassium channel blocker tetraethylammoniumchloride (3 mM, 15 min). Treatment of the aortas surrounded by adipose tissue with tetraethylammoniumchloride significantly reduced the hypoxic response (Fig. 3.3C).

The adipocyte dependent hypoxic vasorelaxation remained similar in the presence (69.57 ± 3.79 % relaxation) and absence (73.11 ± 4.12 % relaxation, n=4, P>0.05) of the inward rectifier potassium channel blocker Ba^{2+} (0.1 mM, 10 min), in the presence (44.21 ± 6.45 % relaxation) and absence (52.32 ± 7.31 % relaxation, n=5, P>0.05) of the large-conductance Ca^{2+} activated potassium channel blocker tetraethylammoniumchloride (1 mM, 15 min), the presence (62.75 ± 5.41 % relaxation) and absence (65.88 ± 3.59 % relaxation, n=6, P>0.05) of

the small-conductance Ca^{2+} activated potassium channel blocker apamin (1 µM, 30 min) and in the presence (53.29 ± 3.15 % relaxation) and absence (57.77 ± 2.32 % relaxation, n=4, P>0.05) of the voltage-dependent potassium channel blocker 4-aminopyridine (0.5 mM, 10 min). Treatment with the ATP-sensitive potassium channel blocker glibenclamide (30 µM, 10 min) significantly reduced the hypoxic response of preparations with adipose tissue (Fig. 3.3D).

To exclude potential time-dependent influences in all these experiments, two consecutive hypoxia induced response curves were constructed in the ring segments with adipose tissue. No difference in hypoxic response was observed in these control experiments (1st curve: 56.00 ± 6.08 % relaxation, 2nd curve: 56.45 ± 1.49 % relaxation, n=6, P>0.05).

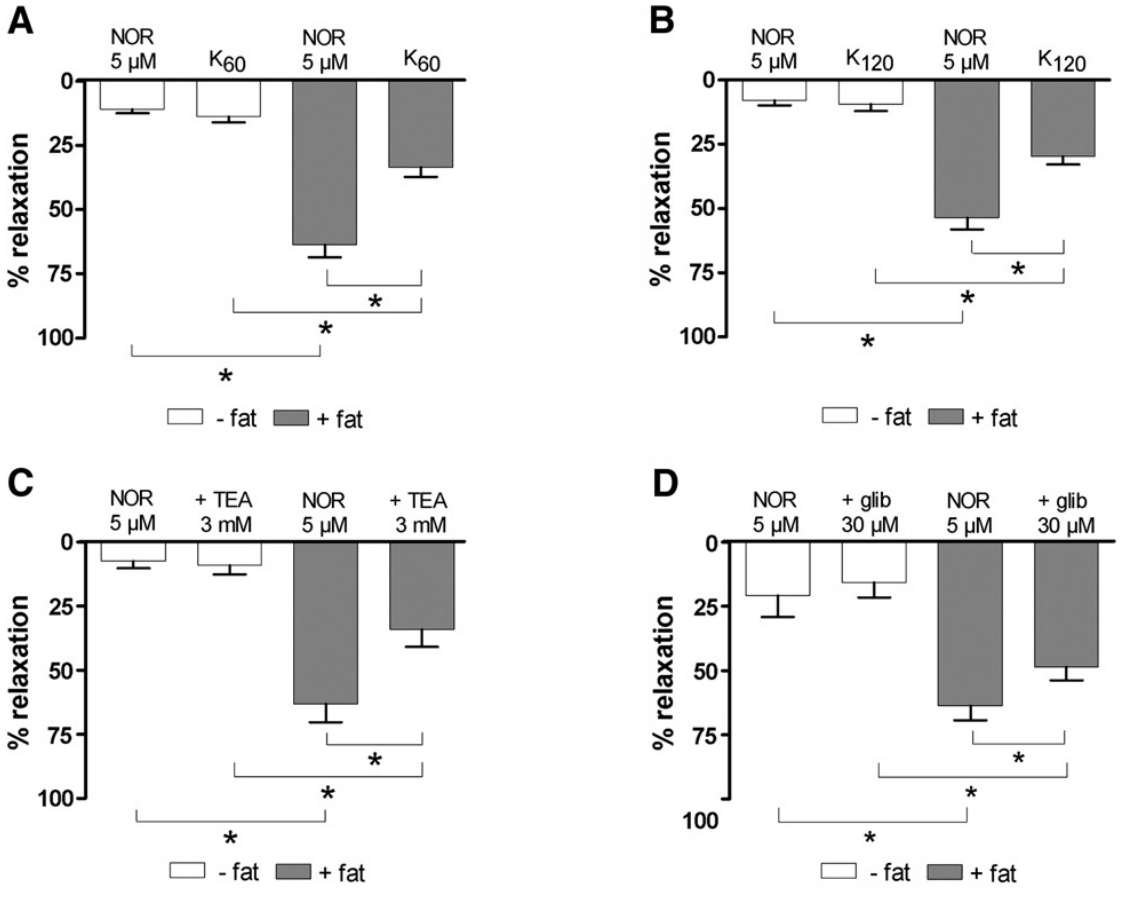

Fig. 3.3. Effect of hypoxia on preparations with (▬) and without (▭) adipose tissue precontracted with A. 5 µM norepinephrine (NOR) or 60 mM K^+ (K_{60}) (n=5, *P<0.05); B. 5 µM norepinephrine or 120 mM K^+ (K_{120}) (n=8, *P<0.05); C. 5 µM norepinephrine in the absence and presence of 3 mM tetraethylammoniumchloride (TEA) (n=4, *P<0.05); D. 5 µM norepinephrine in the absence and presence of 30 µM glibenclamide (glib) (n=6, *P<0.05).

3.4.4. Role of the endothelium and soluble guanylyl cyclase

The absence of functional endothelium after removal was confirmed by the lack of relaxation induced by 10 µM acetylcholine (8.70 ± 7.06 % relaxation after removal, n=4, vs. 52.23 ± 11.56 % relaxation before removal, n=5, $P<0.05$). A similar adipose tissue dependent hypoxic response was observed before (40.49 ± 7.91 % relaxation, n=5) and after (55.82 ± 2.08 % relaxation, n=4, $P>0.05$) removal of the endothelium.

The adipocyte dependent hypoxic response was significantly enhanced in the presence of the soluble guanylyl cyclase inhibitor ODQ (10 µM, 20 min) (56.55 ± 9.11 % relaxation, n=4) compared to treatment without ODQ (40.49 ± 7.91 % relaxation, n=5, $P<0.05$).

3.4.5. Involvement of lactate on arterial tone

As lactate production is known to be increased in hypoxic conditions, we investigated the effect of lactate on aortic arterial tone. Cumulative additions of DL-lactate (10 nM to 1 mM) to precontracted (norepinephrine, 5 µM) aortic rings failed to relax preparations with or without adipose tissue (n=4).

3.4.6. Possible mediators of the hypoxic response

We tested the hypothesis that a relaxing factor is continuously released from adipose tissue under basal normoxic conditions. Norepinephrine concentration response curves were constructed on preparations with and without adherent adipose tissue in the presence and absence of the K_{ATP} channel blocker glibenclamide (30 µM, 10 min). In the presence of glibenclamide, the norepinephrine concentration response curve is shifted upwards in preparations with adipose tissue but not in preparations without adipose tissue, indicating that a basal vasorelaxing effect of adipose tissue is blocked. Removal of adipose tissue caused a higher leftward shift of the norepinephrine response curve under basal conditions, indicating that only a part of the basally inhibitory effect of adipose tissue occurs through opening of K_{ATP} channels (Fig. 3.4A and B).

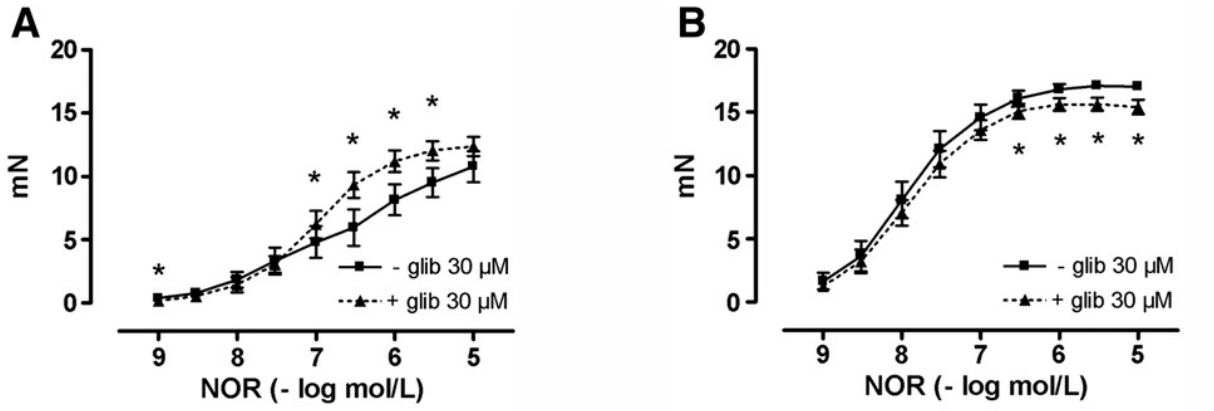

Fig. 3.4. Concentration-response curves for norepinephrine (NOR) in preparations with A. or without B. adipose tissue in the absence (■—) and presence (▲···) of 30 µM glibenclamide (glib) (n=6, *P<0.05).

Treatment of the aortas for 10 min with the adenosine receptor antagonist 8-(p-sulfophenyl)-theophylline (0.1 mM) did not affect the hypoxic response of preparations with adipose tissue (Fig. 3.5A).

Treatment of the aortas for 60 min with the heme oxygenase inhibitor zinc protoporphyrin IX (10 µM) did not affect the hypoxic response of preparations with adipose tissue (Fig. 3.5B).

Experiments were performed using the H_2S donor NaHS (10 µM to 3 mM) in the presence and absence of glibenclamide (30 µM). Treatment of the aortas surrounded by adipose tissue for 10 min with glibenclamide, enhanced the NaHS induced vasorelaxation and showed only a small inhibitory effect at high concentrations (Fig. 3.5C).

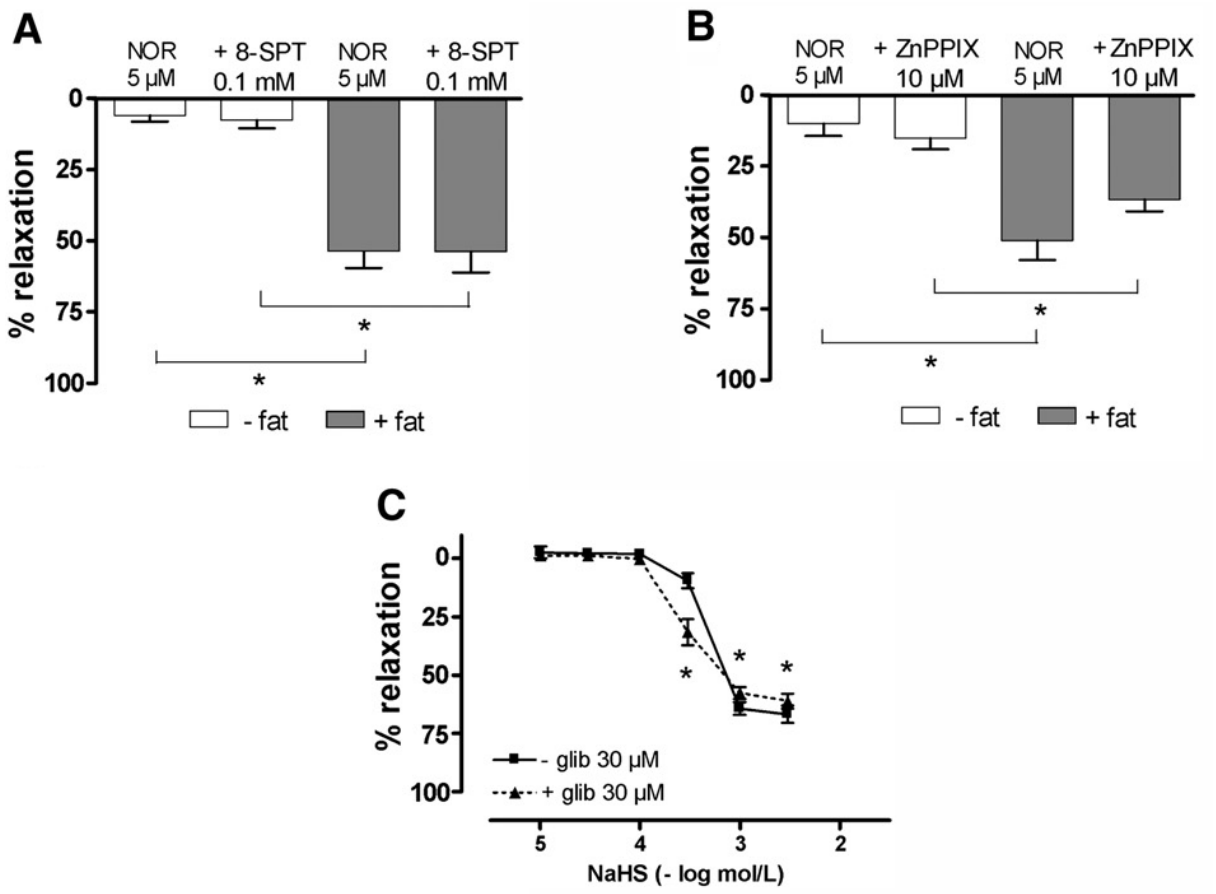

Fig. 3.5. A. Effect of hypoxia on preparations with (▬) and without (▭) adipose tissue precontracted with 5 µM norepinephrine in the absence and presence of 0.1 mM 8-(p-sulfophenyl)-theophylline (8-SPT, n=5, *P<0.05) and B. in the absence and presence of 10 µM zinc protoporphyrin IX (ZnPPIX, n=6, *P<0.05).
C. Concentration-response curves for NaHS in preparations with adipose tissue in the absence (–■–) and presence (··▲··) of 30 µM glibenclamide (glib, n=5, *P<0.05).

3.5. DISCUSSION

The main original finding of the present study is that hypoxia - as occurs in areas of growing adipose tissue - enhances the vasorelaxing effect of perivascular adipose tissue in mice aorta, suggesting that hypoxia enhances the effect of (a) vasorelaxing factor(s) released from adipose tissue. This conclusion is based on the observation that an isolated mice aorta, precontracted with norepinephrine, prostaglandin $F_{2\alpha}$ or U-46619 relaxes much stronger in response to hypoxia when the preparation is surrounded by adipose tissue.

As known from literature [2-4] the presence of adipose tissue reduces precontractile tone. To investigate whether a decrease in contractile tone as such explains the enhanced hypoxic relaxation in preparations with adherent adipose tissue, experiments were performed in which contractile tone was lowered to a similar level as the tone induced when adipose tissue is present. The fact that there is still a significantly stronger increase in hypoxic response in the presence of adipose tissue than after lowering tone demonstrates that the enhanced effect during hypoxia must be attributed to the presence of adipose tissue and not to a diminished contractile tone as such.

The presence of 60 and 120 mM of K^+ significantly diminished the hypoxic response of preparations surrounded by adipose tissue. In addition, the non-selective K^+ channel blocker tetraethylammoniumchloride (3 mM) significantly blocked this response to hypoxia. This indicates that K^+ channels are involved in the enhanced vasorelaxing effect of adipose tissue during hypoxia. However, other mechanisms cannot be excluded.
Tetraethylammoniumchloride at a concentration of 3 mM blocks K_{ATP} channels (own unpublished observations). Furthermore, the K_{ATP} channel blocker glibenclamide had a small but significant inhibitory effect on the hypoxic vasorelaxation, pointing to the involvement of K_{ATP} channels at least in part of the hypoxic response. This is in line with the activation of vascular K_{ATP} channels by numerous endogenous substances released under conditions of increased blood demand or hypoxia [9]. As known in literature, adipocytes express sulfonylurea receptors and exhibit a glibenclamide dose-responsive increase in $[Ca^{2+}]_i$ [10]. K_{ATP} channels are known to be comprised of four Kir6.x subunits associated with four regulatory sulfonylurea receptors [11]. Therefore, besides vascular K_{ATP} channels also K_{ATP} channels on adipocytes could be involved in the adipocyte dependent hypoxic response.
Removal of adipose tissue caused a leftward shift of the norepinephrine response curve under basal conditions. In addition, a small but significant leftward shift of the norepinephrine response curve was seen on preparations with adipose tissue in the presence of glibenclamide. This suggests that i) a basally released mediator might be involved in the adipocyte dependent hypoxic vasorelaxation and ii) only a small proportion of the basally inhibitory effect of adipose tissue occurs through opening of K_{ATP} channels.

Hypoxia alters energy metabolism which leads to an increased lactate concentration in tissue. The release or the effect of the mediator(s) released by adipose tissue is triggered by hypoxia itself, rather than by elevated lactate concentration. This is suggested by the fact that lactate

itself does not relax preparations with or without adipose tissue. Thus lactate can be excluded as mediator of the hypoxic response.

Other mediators have been proposed in relation to the effects of hypoxia such as cyclooxygenase metabolites [12], NO [13] or adenosine [14,15]. In our experiments indomethacin, a cyclooxygenase inhibitor, failed to inhibit the hypoxic vasorelaxation, excluding the involvement of cyclooxygenase metabolites released from the hypoxic adipose tissue. To investigate the involvement of NO, experiments were performed using the NO-synthase inhibitor N^{ω}-nitro-L-arginine. Addition of N^{ω}-nitro-L-arginine failed to inhibit the hypoxic relaxation. These results suggest that dilatory responses to hypoxia occur independent of NO synthesis or an NO-dependent vasorelaxant such as leptin [16]. Also adiponectin, another adipokine, can be excluded from being the mediator since adiponectin induces NO-mediated vasorelaxation [17] and adiponectin levels diminish during hypoxia [18]. In addition, the hypoxia induced relaxation occurs independently of the endothelium, therefore other endothelium-dependent vasodilators (e.g. endothelium-derived hyperpolarizing factor, epoxyeicosatrienoic acids) are unlikely to be the mediator. Our results also do not support the involvement of adenosine since the adenosine receptor antagonist 8-(p-sulfophenyl)-theophylline did not affect the hypoxic relaxation.

CO, produced from the degradation of heme by heme oxygenases has been described as a potential mediator in hypoxic vasorelaxation [19]. It is known that the heme oxygenase pathway diminishes vascular oxidative stress which occurs for example during hypoxia [20]. To investigate the involvement of CO in the hypoxic response, experiments were performed using the heme oxygenase inhibitor zinc protoporphyrin IX. However, treatment of the aortas with zinc protoporphyrin IX did not significantly affect the hypoxic response, suggesting that CO is not a mediator of the hypoxic response. Furthermore, the soluble guanylyl cyclase inhibitor ODQ – in combination with indomethacin and N^{ω}-nitro-L-arginine - did not influence the hypoxic response. This indicates that the hypoxic response is independent of soluble guanylyl cyclase and therefore unlikely to be mediated by CO [21].

H_2S is another gaseous molecule that recently attracted much interest as a potent vasorelaxing substance [22]. Furthermore, Olson et al. [23] suggested that H_2S may serve as an O_2 sensor/transducer in the vascular responses to hypoxia. The K_{ATP} channel blocker glibenclamide enhanced the vasorelaxation in response to the H_2S donor NaHS and only had a

small inhibitory effect at high concentrations. This suggests that H_2S can be excluded as a mediator of the hypoxic response.

Evidence for the existence of an unknown adipocyte derived relaxing factor (ADRF) has been reported previously. It is known that the ADRF induces vasorelaxation without the requirement of functional adenosine receptors and the cyclooxygenase pathway. Furthermore, this factor induces vasorelaxation by opening K^+ channels (i.e. at least in part by K_{ATP} channels) [2]. All these properties of the ADRF are in line with our observations, suggesting the involvement of the ADRF in the adipocyte dependent hypoxic response, f. e. by an increased release of the ADRF from adipocytes or an enhanced sensitivity of vascular smooth muscle cells to the ADRF. On the other hand, the presence of the endothelium and nitric oxide formation were not needed in the hypoxic relaxation, while it is not clear whether these are required for the ADRF induced vasorelaxation [2,4,24].

The present study puts forward the view that vasorelaxation occurs during hypoxia when adipose tissue is present. However, this observation is not in line with the very recent study of Greenstein et al. [25] reporting that hypoxia significantly attenuates the relaxing effect of perivascular tissue surrounding rat mesenteric arteries. Potential species and regional differences might explain these divergent observations and need to be further explored.
In conclusion, it was observed that hypoxia enhances the vasorelaxing influence of adipose tissue. This cannot be explained by a direct effect of hypoxia on precontracted mice aorta, since hypoxia had only a small relaxing influence on precontracted mice aorta in the absence of adipose tissue. Our findings are in line with the involvement of the as yet unidentified ADRF as being the mediator of the adipocyte dependent hypoxic vasorelaxation. Our observations bring up the concept that blood flow in adipose tissue adapts to the metabolic needs of growth by an indirect effect mediated by a vasodilator released from adipose tissue, acting partly through opening of K_{ATP} channels.

3.6. ACKNOWLEDGEMENTS

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Chapter 4

Effect of hypoxia in mice mesenteric arteries surrounded by adipose tissue

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4.1. ABSTRACT

Aim: To investigate the influence of hypoxia on the vasoactive effect of perivascular white adipose tissue.

Methods: Isometric tension recordings were performed on mesenteric arteries from Swiss male mice with or without adherent adipose tissue.

Results: Hypoxia (bubbling with 95% N_2, 5% CO_2) induced a biphasic response, i.e. vasoconstriction followed by vasorelaxation, in precontracted (NOR, 10 µM) mesenteric arteries with adipose tissue in the presence of indomethacin (10 µM) and N^{ω}-nitro-L-arginine (0.1 mM). Only a small vasorelaxation was observed in arteries without adipose tissue. Precontraction with 60 or 120 mM K^+, incubation with tetraethylammoniumchloride (1 and 3 mM), apamin (1 µM) combined with charybdotoxin (0.1 µM) or 1-[(2-chlorophenyl) diphenylmethyl]-1H-pyrazole (TRAM-34, 10 µM) significantly impaired the hypoxic vasorelaxation. Removal of the endothelium only reduced the hypoxic vasorelaxation. Apamin (1 µM) and charybdotoxin (0.1 µM) did not influence the vasorelaxation of NaHS. Zinc protoporphyrin IX (10 µM), miconazole (10 µM), 8-(p-sulfophenyl)theophylline (0.1 mM), 1 H-[1, 2, 4]oxadiazolo[4,3- A]quinoxalin-1-one (10 µM), apocynin (0.3 mM), diphenyliodonium (1 µM), catalase (2500 U/ml) and tempol (0.1 mM) did not influence the hypoxic vasorelaxation. In contrast to losartan (0.1 mM), indomethacin (10 µM) and SQ-29548 (10 µM) significantly reduced the hypoxic vasoconstriction.

Conclusions: Moderate hypoxia induces a biphasic vasomotor response in mice mesenteric arteries surrounded by adipose tissue. The hypoxic vasoconstriction is endothelium independent, whereas the vasodilation is endothelium dependent, soluble guanylyl cyclase independent and in part mediated by opening Ca^{2+} activated K^+ channels. Cyclooxygenase metabolites mediate the hypoxic vasoconstriction, while EDHF plays a small role in the hypoxic vasorelaxation.

4.2. INTRODUCTION

The view on the biological role of white adipose tissue has been dramatically changed over the last years. Since the discovery in 1994 of the hormone leptin [1], white adipose tissue is recognised to be a major endocrine and paracrine organ producing and releasing several biologically active substances collectively termed adipokines [2,3]. Both vasodilating, for example the unknown "adipocyte derived relaxing factor" (ADRF) [4,5], and vasocontractile substances for example angiotensin II [6] are secreted by adipocytes. The mesenteric vascular bed, which significantly contributes to total peripheral vascular resistance, is surrounded by a considerable amount of white adipose tissue [7,8]. Furthermore, it has been suggested that the release of the ADRF from perivascular white adipose tissue contributes to the maintenance of basal mesenteric arterial tone [5]. This is based on the observation that the vasorelaxing effect of white adipose tissue depends on the amount of adipose tissue and that the resting membrane potential of mesenteric vascular smooth muscle cells in preparations with adipose tissue is more hyperpolarized than in preparations without adipose tissue.

From obese mice models it is known that hypoxia develops within adipose tissue as tissue mass increases [9,10]. Furthermore, in obesity there is a dysregulation in the synthesis of adipokines in favor of the pro-inflammatory ones resulting in inflammation within adipose tissue [11,12]. Cell-culture studies using murine [13] and human [14] adipocytes strongly support the role of hypoxia in this inflammatory response since hypoxia has shown to influence the expression of pro-inflammatory adipokine genes. Recently, we demonstrated in mice aorta that hypoxia enhances the vasorelaxing effect of perivascular adipose tissue, suggesting that hypoxia enhances the effect of (a) vasorelaxing factor(s) released from adipose tissue [15]. This conclusion was based on the observation that an isolated mice aorta relaxes much stronger in response to hypoxia when the preparation is surrounded by adipose tissue. In that study, the involvement of the ADRF was suggested as the adipocyte dependent hypoxic response occurs through opening of K_{ATP} channels and occurs independent of the cyclooxygenase pathway and functional adenosine receptors. In another study on rats, Greenstein et al. reported a vasocontractile response during hypoxia of mesenteric arteries of which the mediators seem to be TNF_{α} and interleukin-6 [16]. In the present study we investigated on mice whether hypoxia also influences the vasoactive effect of adipose tissue in mesenteric arteries.

4.3. MATERIALS AND METHODS

4.3.1. Mesenteric ring segments

Mesenteric arteries (1^{st} and 2^{nd} order) (334.05 ± 4.74 µm, n=123) surrounded by adipose tissue from healthy Swiss male wild-type mice (age: 7 - 10 weeks, supplier: Janvier, Saint-Berthevin, France) were isolated and kept in cooled and oxygenated (5% CO_2 in O_2) Krebs-Ringer bicarbonate solution. The mice were fed ad libitum and killed by cervical dislocation. Experiments were approved by the ethical committee on animal research of the Faculty of Medicine and Health Sciences and are conform to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

4.3.2. Tension measurements

The mesenteric arteries were transferred to a wire myograph for isometric tension recording and cut into segments of about 3 mm in length. Segments carefully cleaned of adipose tissue served as control. The segments with or without adipose tissue were equilibrated for approximately 30 min in oxygenated (5% CO_2 in O_2) Krebs-Ringer bicarbonate solution at 37°C (pH 7.4) before the vessels were stretched to their optimal lumen diameter that gives a maximum response, as calculated on the basis of the passive wall tension-internal circumferences relationship [17]. At the start of each experiment the arteries were repeatedly activated with 120 mM K^+ and 10 µM norepinephrine. To exclude the influence of basal influence of cyclooxygenase metabolites and NO, all experiments were performed on preparations continuously incubated with the cyclooxygenase inhibitor indomethacin (10 µM, 20 min) and the NO-synthase inhibitor N^{ω}-nitro-L-arginine (0.1 mM, 10 min). Subsequently, the preparations were contracted by adding 10 µM norepinephrine to the Krebs-Ringer bicarbonate solution.

4.3.3. Hypoxia

Moderate hypoxia in the organ baths was induced for 30 min by switching the gas mixture used to bubble the organ bath solution from 95% O_2/5% CO_2 to 95% N_2/5% CO_2. This reduces pO_2 levels to about 60 mmHg (own observations).

4.3.4. Removal of the endothelium

Arteries were unstretched in the myograph and the endothelium was gently removed by rubbing the lumen with a human hair. Thereafter the wires were reset to their original positions and the vessel was allowed to reequilibrate for 30 min.

4.3.5. Statistical methods

The data were computed as means ± S.E.M. and evaluated statistically with Student's t-test and non-parametric test for paired or unpaired observations. Two groups of data were considered significantly different when $P<0.05$. Contractions and relaxations are expressed as % decrease in pre-existing tone. Measurements were made when reaching a maximum vasocontractile or vasodilating response during hypoxia. N is the number of animals used.

4.3.6. Drugs

The experiments were performed in a Krebs-Ringer bicarbonate solution of the following composition (mmol L-1): NaCl 135, KCl 5, $NaHCO_3$ 20, glucose 10, $CaCl_2$ 2.5, $MgSO_4$ 1.3, KH_2PO_4 1.2 and EDTA 0.026 in H_2O. Krebs-Ringer bicarbonate solutions containing 60 mM (K_{60}) and 120 mM K^+ (K_{120}) were prepared by equimolar replacement of NaCl by KCl. N^{ω}-nitro-L-arginine, indomethacin, norepinephrine, sodium DL-lactate, 8-(p-sulfophenyl)-theophylline, tetraethylammoniumchloride, glibenclamide, 4-aminopyridine, apamin, 1 H-[1, 2, 4]oxadiazolo[4,3- A]quinoxalin-1-one (ODQ), acetylcholine, sodium hydrosulfide hydrate (NaHS), zinc protoporphyrin IX, catalase from bovine liver, losartan potassium, 4-hydroxy-tempo (tempol), hydrogen peroxide solution, diphenyliodonium chloride, TRAM-34,

charybdotoxin and miconazole were obtained from Sigma (St. Louis, MO), prostaglandin $F_{2\alpha}$ (Dinolytic®) from Pfizer (Puurs, Belgium), $BaCl_2$ from Merck (Darmstadt, Germany), apocynin (acetovanillone) from SAFC supply solutions (St. Louis, MO), U-46619 from Calbiochem (San Diego, CA, USA) and SQ-29548 from Enzo Life Sciences (Lausen, Switzerland). Stock solutions were made in water, except for indomethacin (dissolved in ethanol), U-46619 (dissolved in methylacetate), apamin (dissolved in 0.05 mol/L acetic acid), glibenclamide, ODQ, apocynin, diphenyliodonium, TRAM-34, miconazole and SQ-29548 (dissolved in dimethylsulfoxide), zinc protoporphyrin IX (dissolved in 0.1 mol/L NaOH) and acetylcholine (dissolved in 50 mmol/L potassium hydrogen phthalate buffer, pH 4.0). The final concentration of ethanol, methylacetate, acetic acid, dimethylsulfoxide and NaOH in the organ bath never surpassed 0.1 %.

4.4. RESULTS

4.4.1. Influence of hypoxia in preparations with and without adherent adipose tissue

An original recording is shown in Fig. 4.1A. A biphasic hypoxic response i.e. vasoconstriction followed by vasorelaxation was observed in preparations with adipose tissue. On the other hand, hypoxia elicited only a small vasorelaxation in preparations without adipose tissue (Fig. 4.1B). Precontractile tone with norepinephrine was less pronounced in the presence vs. absence of adipose tissue at concentrations lower than 10 µM (results not shown, n=7, $P<0.05$). However, precontraction with 10 µM norepinephrine induced a similar tone in the presence (8.04 ± 1.09 mN) and absence (9.10 ± 1.40 mN, n=8, $p>0.05$) of adherent adipose tissue.

In the absence of the cyclooxygenase inhibitor indomethacin (10 µM) and the NO-synthase inhibitor N^{ω}-nitro-L-arginine (0.1 mM), a pronounced vasoconstriction was observed during hypoxia in preparations with adipose tissue, while almost no effect was seen in preparations without adipose tissue (Fig. 4.1C). However, precontractile tone was significantly lower in the absence (2.57 ± 0.42 mN, n=12) vs. in the presence (8.04 ± 1.09 mN, n=8, $P<0.05$) of indomethacin and N^{ω}-nitro-L-arginine.

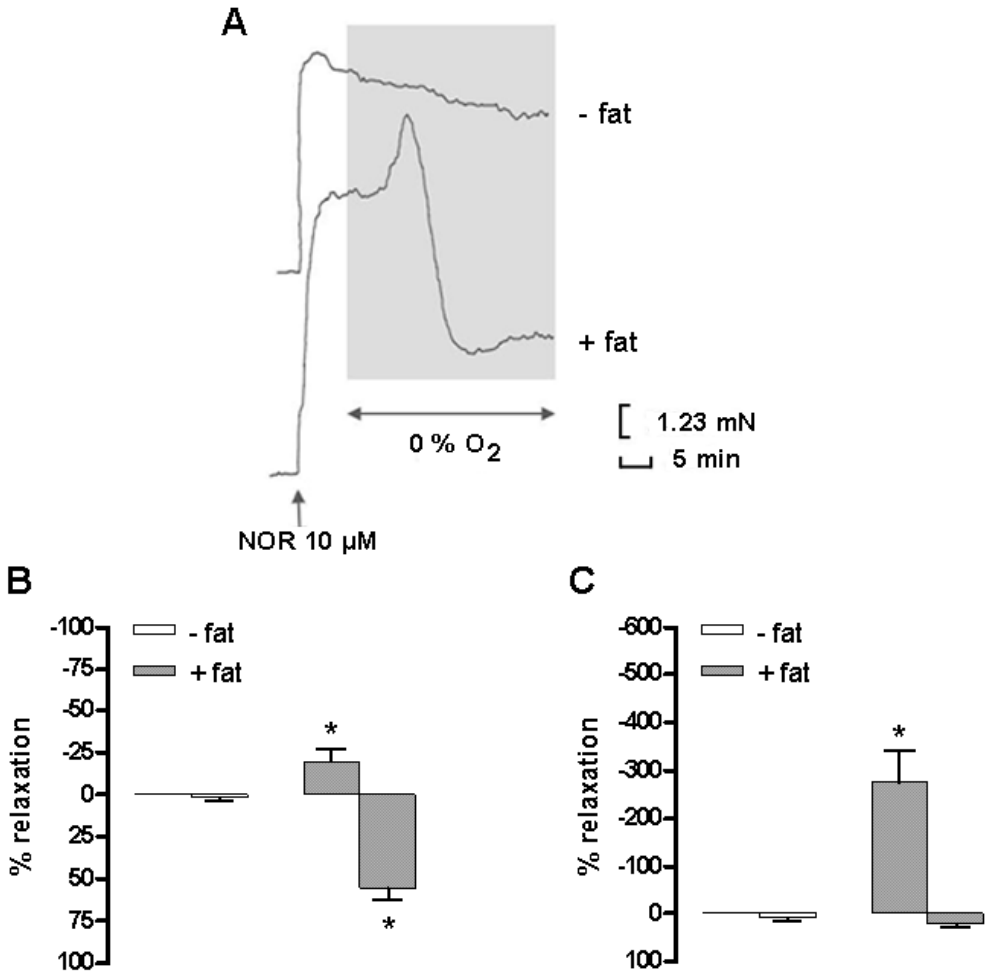

Fig. 4.1. A. Original tracing showing a response curve to hypoxia in norepinephrine (NOR, 10 µM) precontracted mice mesenteric arteries with and without adipose tissue in the presence of the endothelium, indomethacin (10 µM) and N^{ω}-nitro-L-arginine (0.1 mM). B. Effect of hypoxia on precontracted norepinephrine (NOR, 10 µM) mice mesenteric arteries in the presence of the endothelium with (▬) and without (▭) adipose tissue in the presence (n=8, *P<0.05) and C. absence (n=3: – fat, n=12: + fat, *P<0.05) of indomethacin (10 µM) and N^{ω}-nitro-L-arginine (0.1 mM).

When only applying N^{ω}-nitro-L-arginine (0.1 mM), the hypoxic vasoconstriction was significantly reduced (36.00 ± 6.78 % contraction, n=13) while the hypoxic vasorelaxation was significantly enhanced (40.92 ± 3.82 % relaxation, n=13) compared to treatment without indomethacin and N^{ω}-nitro-L-arginine (275.79 ± 66.59 % contraction followed by 19.27 ± 8.17 % relaxation, n=12, P<0.05). However, precontractile tone was significantly enhanced in the presence of N^{ω}-nitro-L-arginine (7.81 ± 0.68 mN, n=13) vs. in the absence of indomethacin and N^{ω}-nitro-L-arginine (2.57 ± 0.42 mN, n=12, P<0.05).

Similarly, when only applying indomethacin (10 µM), the hypoxic vasoconstriction was significantly reduced (63.81 ± 24.13 % contraction, n=6) while the hypoxic vasorelaxation was significantly enhanced (63.01 ± 4.56 % relaxation, n=6) compared to treatment without indomethacin and N^{ω}-nitro-L-arginine ($P<0.05$). However, precontractile tone was significantly enhanced in the presence of indomethacin (6.41 ± 0.70 mN, n=6) vs. in the absence of indomethacin and N^{ω}-nitro-L-arginine (2.57 ± 0.42 mN, n=12, $P<0.05$).

To test whether thromboxane/prostaglandin (TP) receptors are involved in the hypoxic vasoconstriction, the TP receptor blocker SQ-29548 (10 µM, 30 min) was used in preparations surrounded by adipose tissue in the absence of indomethacin and N^{ω}-nitro-L-arginine. This treatment significantly reduced the hypoxic vasoconstriction (115.79 ± 36.90 % contraction in the presence vs. 199.96 ± 37.72 % contraction in the absence of SQ-29548, n=7, $P<0.05$), whereas the hypoxic vasorelaxation was not altered (30.18 ± 13.94 % relaxation in the presence vs. 27.99 ± 12.48 % relaxation in the absence of SQ-29548, n=7, $P>0.05$). Precontractile tone was similar in the absence (2.77 ± 0.50 mN, n=7) vs. in the presence (2.91 ± 0.54 mN, n=7, $P>0.05$) of SQ-29548.

4.4.2. Influence of hypoxia when precontracting with different agonists

Hypoxia was induced after precontraction with prostaglandin $F_{2\alpha}$ (30 µM), the thromboxane A2 mimetic U-46619 (10 or 100 nM) and norepinephrine (10 µM) in changing order in arteries surrounded by adipose tissue. The hypoxic vasoconstriction was significantly diminished when precontracting with prostaglandin $F_{2\alpha}$ (0.78 ± 0.78 % constriction, n=9) or U-46619 (4.23 ± 2.82 % constriction, n=8) vs. norepinephrine (16.47 ± 4.85 % constriction, n=8, $P<0.05$). In contrast, a similar hypoxic vasorelaxation was observed when precontracting with prostaglandin $F_{2\alpha}$ (53.07 ± 7.70 % relaxation, n=9), U-46619 (46.74 ± 10.12 % relaxation, n=8) and norepinephrine (56.95 ± 5.01 % relaxation, n=8, $P>0.05$).

4.4.3. Influence of K^+ channel blocking

When increasing the K^+ concentration in the organ bath solution to 60 or 120 mM, the hypoxic vasorelaxing response was significantly diminished (Fig. 4.2A). Experiments using

the non-selective potassium channel blocker tetraethylammoniumchloride (3 mM, 15 min) showed a significantly reduced hypoxic constriction as well as dilation (Fig. 4.2B).

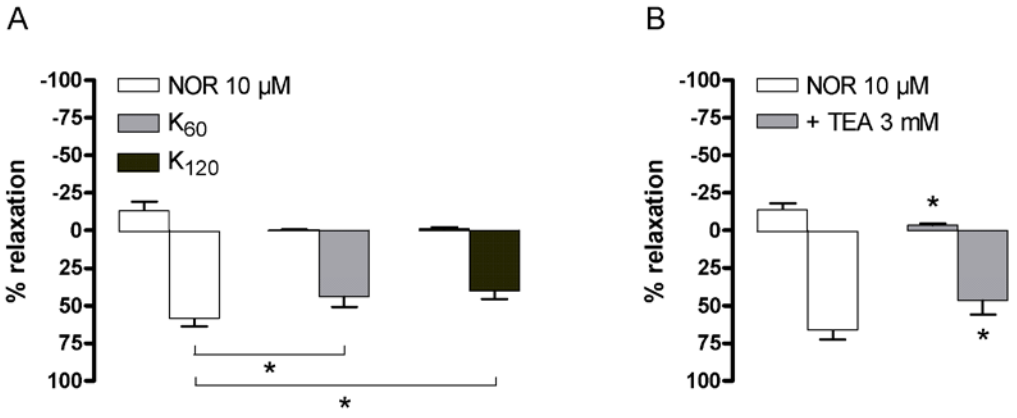

Fig. 4.2. Effect of hypoxia on preparations with adipose tissue in the presence of the endothelium, indomethacin (10 µM) and N^{ω}-nitro-L-arginine (0.1 mM) precontracted with A. 10 µM norepinephrine (NOR) (▭, n=10), 60 mM K^+ (K_{60}) (▭, n=10) or 120 mM K^+ (K_{120}) (▬, n=9, *P<0.05) and B. 10 µM norepinephrine (NOR) in the absence (▭) and presence (▭) of 3 mM tetraethylammoniumchloride (TEA) (n=7, *P<0.05).

The adipocyte dependent hypoxic response remained similar in the presence (2.37 ± 1.40 % contraction followed by 51.98 ± 9.37 % relaxation) and absence (7.96 ± 5.13 % contraction followed by 54.86 ± 7.45 % relaxation, n=4, P>0.05) of the inward rectifier potassium channel blocker Ba^{2+} (0.1 mM, 10 min), in the presence (0 % contraction followed by 29.25 ± 10.31 % relaxation) and absence (4.21 ± 2.76 % contraction followed by 40.25 ± 6.71 % relaxation, n=4, P>0.05) of the voltage-dependent potassium channel blocker 4-aminopyridine (0.5 mM, 10 min) and in the presence (31.05 ± 16.24 % contraction followed by 38.14 ± 8.73 % relaxation) and absence (20.28 ± 6.38 % contraction followed by 55.40 ± 6.31 % relaxation, n=11, P>0.05) of the ATP-sensitive potassium channel blocker glibenclamide (30 µM, 10 min). Treatment with the large-conductance Ca^{2+} activated potassium channel blocker tetraethylammoniumchloride (1 mM, 15 min) (Fig. 4.3A) and the small-conductance Ca^{2+} activated potassium channel blocker apamin (1 µM, 30 min) combined with the intermediate and large-conductance Ca^{2+} activated potassium channel blocker charybdotoxin (0.1 µM, 30 min) (Fig. 4.3B) significantly reduced the hypoxic vasorelaxation of preparations with adipose tissue. In the presence of apamin (1 µM, 30 min) and the intermediate-conductance Ca^{2+} activated potassium channel blocker TRAM-34 (10 µM, 30 min), the hypoxic

vasoconstriction as well as vasorelaxation was significantly reduced (Fig 4.3C).

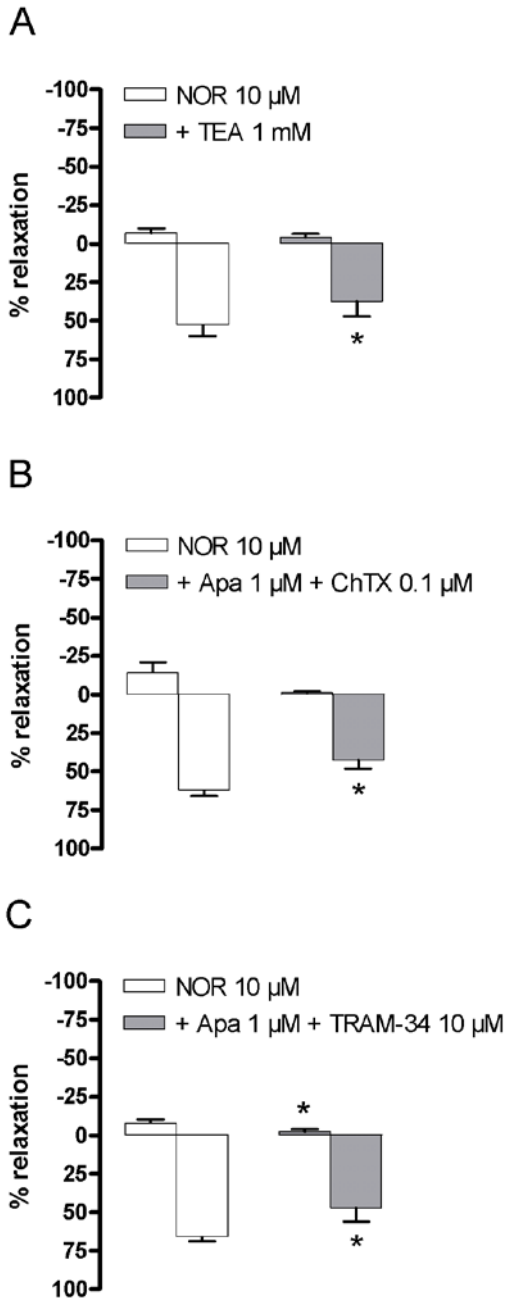

Fig. 4.3. Effect of hypoxia on preparations with adipose tissue in the presence of the endothelium, indomethacin (10 µM) and N^{ω}-nitro-L-arginine (0.1 mM) precontracted with 10 µM norepinephrine in the absence (□) and presence (■) of A. 1 mM tetraethylammoniumchloride (TEA) (n=5, *P<0.05), B. 1 µM apamin (Apa) with 0.1 µM charybdotoxin (ChTX) (n=7, *P<0.05) and C. 1 µM apamin (Apa) with 10 µM TRAM-34 (n=8, *P<0.05).

To exclude potential time-dependent influences in all these experiments, two consecutive hypoxic response curves were constructed in preparations with adipose tissue. No difference

in hypoxic response was observed in these control experiments (1st curve: 19.96 ± 5.08 % contraction followed by 70.85 ± 3.04 % relaxation, 2nd curve: 17.29 ± 5.75 % contraction followed by 66.13 ± 5.40 % relaxation, n=5, P>0.05).

4.4.4. Role of the endothelium and soluble guanylyl cyclase

The role of the endothelium in the adipocyte dependent hypoxic response was studied by comparing the hypoxic responses before and after removal of the endothelium of the preparations. The relaxing effect of acetylcholine (10 µM) was assessed to evaluate the effectiveness of endothelium removal. After removal of the endothelium, the acetylcholine induced relaxation was significantly decreased (10.25 ± 2.53 % relaxation after vs. 77.31 ± 5.42 % relaxation before removal, n=8, P<0.05). While the adipocyte dependent hypoxic vasoconstriction was not influenced, the hypoxic vasorelaxation was significantly impaired after removal of the endothelium (Fig. 4.4).

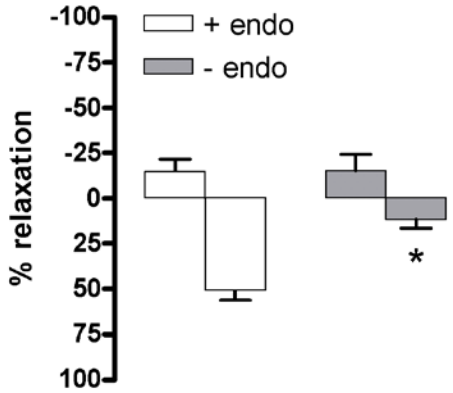

Fig. 4.4. Effect of hypoxia on preparations with adipose tissue precontracted with 10 µM norepinephrine before (□) and after (■) removal of the endothelium (endo) in the presence of indomethacin (10 µM) and N^{ω}-nitro-L-arginine (0.1 mM) (n=8, *P<0.05).

The adipocyte dependent hypoxic response remained similar in the absence (19.71 ± 8.10 % contraction followed by 38.86 ± 9.58 % relaxation) and presence (12.76 ± 9.29 % contraction, followed by 58.69 ± 13.54 % relaxation, n=6, P>0.05) of the soluble guanylyl cyclase inhibitor ODQ (10 µM, 20 min).

4.4.5. Involvement of lactate

It is known that hypoxia increases lactate production. Therefore we performed concentration-response curves of DL-lactate (10 nM to 1 mM) on precontracted (norepinephrine, 10 µM) preparations with and without adherent adipose tissue. No contraction or relaxation was seen in preparations with or without adipose tissue (n=4).

4.4.6. Possible mediators of the hypoxic response

4.4.6.1. Vasodilating mediators

The adenosine receptor blocker 8-(p-sulfophenyl)-theophylline (0.1 mM, 10 min) was used to investigate the involvement of adenosine in the hypoxic vasorelaxation. The adipocyte dependent hypoxic response was not significantly different in the presence (17.75 ± 6.30 % contraction followed by 68.30 ± 6.71 % relaxation) and absence (3.36 ± 0.71 % contraction followed by 56.85 ± 5.40 % relaxation, n=4, $P>0.05$) of 8-(p-sulfophenyl)-theophylline.

The heme oxygenase inhibitor zinc protoporphyrin IX (10 µM, 60 min) was used to investigate the involvement of CO in the hypoxic vasorelaxation. The adipocyte dependent hypoxic response was not significantly different in the presence (17.16 ± 5.55 % contraction followed by 47.84 ± 9.59 % relaxation) and absence (25.73 ± 12.45 % contraction followed by 61.36 ± 2.30 % relaxation, n=8, $P>0.05$) of zinc protoporphyrin IX.

To test the involvement of H_2S, experiments were performed using the H_2S donor NaHS (10 µM to 3 mM) in the presence and absence of apamin (1 µM, 30 min) and charybdotoxin (0.1 µM, 30 min). Treatment of the preparations surrounded by adipose tissue with these blockers did not alter the NaHS induced vasorelaxation (at 3 mM: 67.24 ± 5.52 % relaxation in the absence vs. 59.77 ± 3.56 % relaxation in the presence of apamin and charybdotoxin, n=8, $P>0.05$).

The epoxygenase inhibitor miconazole (10 µM, 15 min) was used to investigate the involvement of epoxyeicosatrienoic acids in the hypoxic vasorelaxation. The adipocyte dependent hypoxic response was not significantly reduced in the presence (10.58 ± 3.58 %

contraction followed by 49.85 ± 8.06 % relaxation) vs. absence (20.35 ± 4.23 % contraction followed by 57.59 ± 4.23 % relaxation, n=8, P>0.05) of miconazole.

The non-selective NAD(P)H oxidase blockers apocynin (0.3 mM, 30 min) and diphenyliodonium (1 µM, 30 min) were used to test the involvement of reactive oxygen species (ROS) in the adipocyte dependent hypoxic response. The hypoxic response was not significantly different in the presence (1.10 ± 1.10 % contraction followed by 60.30 ± 3.96 % relaxation) vs. absence (8.88 ± 2.93 % contraction followed by 63.38 ± 4.72 % relaxation, n=6, P>0.05) of apocynin and in the presence (0.32 ± 0.32 % contraction followed by 54.54 ± 13.89 % relaxation) vs. absence (3.01 ± 1.95 % contraction followed by 40.45 ± 8.32 % relaxation, n=5, P>0.05) of diphenyliodonium. The superoxide dismutase mimetic tempol (0.1 mM, 20 min) was used to test whether superoxide anions are involved in the hypoxic response. The adipocyte dependent hypoxic response remained similar before (7.48 ± 1.70 % contraction followed by 63.16 ± 7.85 % relaxation) vs. after (5.92 ± 3.77 % contraction followed by 70.73 ± 7.81 % relaxation, n=7, P>0.05) treatment of the preparations with tempol. To test the involvement of H_2O_2, concentration-response curves of H_2O_2 (1 µM to 1 mM) were performed on preparations with and without adherent adipose tissue. Only at a concentration of 1 mM H_2O_2 induced a pronounced vasorelaxation in preparations with (35.90 ± 15.49 % relaxation) and without (73.23 ± 6.50 % relaxation, n=5, P>0.05) adipose tissue. The H_2O_2 scavenger catalase (2500 U/ml, 30 min) was used to test the involvement of H_2O_2 in the adipocyte dependent hypoxic response. The hypoxic response was not significantly different before (7.95 ± 2.41 % contraction followed by 46.65 ± 4.05 % relaxation) vs. after (21.31 ± 6.11 % contraction followed by 35.36 ± 9.85 % relaxation, n=10, P>0.05) treatment of the preparations with catalase.

4.4.6.2. Vasocontractile mediators

The involvement of angiotensin II was investigated using the angiotensin I receptor blocker losartan (0.1 mM, 30 min). Treatment of the preparations with losartan did not significantly reduce the adipocyte dependent hypoxic vasocontraction (17.45 ± 7.42 % contraction in the presence vs. 29.04 ± 13.37 % contraction in the absence of losartan, n=6, P>0.05) nor vasorelaxation (37.78 ± 14.53 % relaxation in the presence vs. 32.68 ± 12.15 % relaxation in the absence of losartan, n=6, P>0.05).

The involvement of cyclooxygenase metabolites was investigated in the adipocyte dependent hypoxic contraction. Therefore, experiments were performed in the presence of the NO-synthase inhibitor N^{ω}-nitro-L-arginine (0.1 mM, 10 min) with or without the cyclooxygenase inhibitor indomethacin (10 µM, 20 min) in preparations surrounded by adipose tissue. The hypoxic vasocontraction was significantly reduced in the presence of indomethacin, whereas the hypoxic vasorelaxation was significantly enhanced (Fig. 4.5A). No significant difference was observed in time control experiments in which two consecutive hypoxic response curves were constructed only in the presence of N^{ω}-nitro-L-arginine (1^{st} curve: 31.31 ± 7.06 % contraction followed by 40.36 ± 5.77 % relaxation; 2^{nd} curve: 33.73 ± 11.61 % contraction followed by 40.97 ± 6.26 % relaxation, n=13, P>0.05).

In addition, experiments were performed in the presence of N^{ω}-nitro-L-arginine (0.1 mM, 10 min) with or without the thromboxane/prostaglandin (TP) receptor blocker SQ-29548 (10 µM, 30 min) in preparations surrounded by adipose tissue. The hypoxic vasoconstriction was significantly reduced in the presence of SQ-29548, whereas the hypoxic vasorelaxation was not altered (Fig. 4.5B).

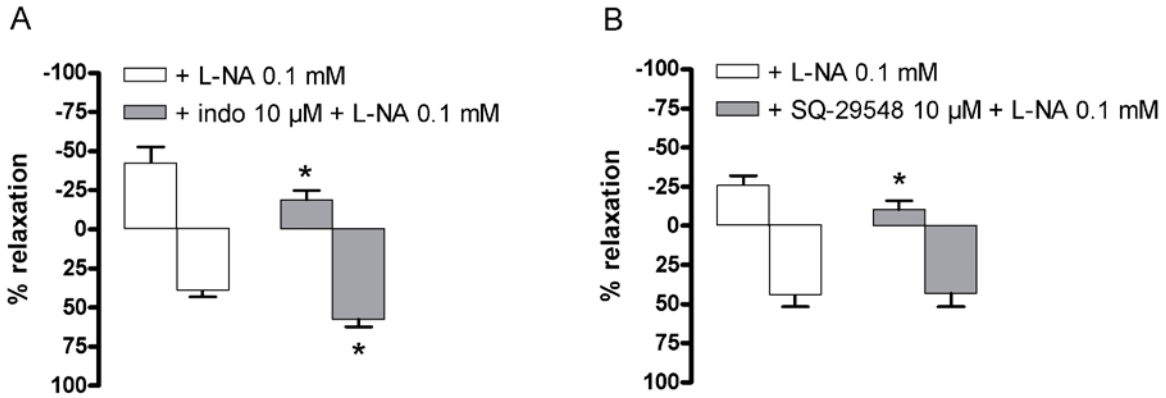

Fig. 4.5. Effect of hypoxia on preparations with adipose tissue precontracted with 10 µM norepinephrine A. in the presence of the endothelium and 0.1 mM N^{ω}-nitro-L-arginine (L-NA) with (▭) or without (▭)10 µM indomethacin (n=8, *P<0.05) and B. with or without 10 µM SQ-29548 (n=5, *P<0.05).

4.4.7. Summarizing table

Δ conditions	% contraction	P<0.05?	% relaxation	P<0.05?	N
K^+ 60 mM	0.45 ± 0.31	No	**44.31 ± 6.80**	**Yes**	10
Control	13.06 ± 5.97		**58.56 ± 5.42**		
K^+ 120 mM	1.33 ± 0.80	No	**40.39 ± 5.16**	**Yes**	9
Control	13.06 ± 5.97		**58.56 ± 5.42**		
TEA 3 mM	**3.41 ± 1.18**	**Yes**	**46.71 ± 9.37**	**Yes**	7
Control	**13.50 ± 4.62**		**66.32 ± 6.35**		
Ba^{2+} 0.1 mM	2.37 ± 1.40	No	51.98 ± 9.37	No	4
Control	7.96 ± 5.13		54.86 ± 7.45		
4-AP 0.5 mM	0	No	29.25 ± 10.31	No	4
Control	4.21 ± 2.76		40.25 ± 6.71		
Glib 30 µM	31.05 ± 16.24	No	38.14 ± 8.73	No	11
Control	20.28 ± 6.38		55.40 ± 6.31		
TEA 1 mM	3.84 ± 2.57	No	**37.77 ± 9.61**	**Yes**	5
Control	6.96 ± 2.74		**52.36 ± 7.80**		
Apa 1 µM + ChTX 0.1 µM	1.05 ± 0.96	No	**42.38 ± 6.02**	**Yes**	7
Control	14.28 ± 6.53		**62.27 ± 3.99**		
Apa 1 µM + TRAM-34 10 µM	**2.25 ± 1.38**	**Yes**	**47.19 ± 8.99**	**Yes**	8
Control	**7.81 ± 2.51**		**65.69 ± 3.34**		
- Endo	15.36 ± 8.91	No	**11.62 ± 5.04**	**Yes**	8
Control	14.78 ± 6.75		**50.87 ± 5.45**		
+ ODQ 10 µM	12.76 ± 9.29	No	58.69 ± 13.54	No	6
Control	19.71 ± 8.10		38.86 ± 9.58		
8-SPT 0.1 mM	17.75 ± 6.30	No	68.30 ± 6.71	No	4
Control	3.36 ± 0.71		56.85 ± 5.40		
ZnPPIX 10 µM	17.16 ± 5.55	No	47.84 ± 9.59	No	8
Control	25.73 ± 12.45		61.36 ± 2.30		
Miconazole 10 µM	10.58 ± 3.58	No	49.85 ± 8.06	No	8
Control	20.35 ± 4.23		57.59 ± 4.23		
Apocynin 0.3 mM	1.10 ± 1.10	No	60.30 ± 3.96	No	6
Control	8.88 ± 2.93		63.38 ± 4.72		
DPI 1 µM	0.32 ± 0.32	No	54.54 ± 13.89	No	5
Control	3.01 ± 1.95		40.45 ± 8.32		
Tempol 0.1 mM	5.92 ± 3.77	No	70.73 ± 7.81	No	7
Control	7.48 ± 1.70		63.16 ± 7.85		
Catalase 2500 U/ml	21.31 ± 6.11	No	35.36 ± 9.85	No	10
Control	7.95 ± 2.41		46.65 ± 4.05		
Losartan 0.1 mM	17.45 ± 7.42	No	37.78 ± 14.53	No	6
Control	29.04 ± 13.37		32.68 ± 12.15		
Indomethacin 10 µM	**18.82 ± 5.82**	**Yes**	**57.53 ± 4.90**	**Yes**	8
Control	**42.43 ± 9.93**		**39.00 ± 4.19**		
SQ-29548 10 µM	**10.47 ± 5.37**	**Yes**	43.13 ± 8.77	No	5
Control	**25.71 ± 6.29**		44.00 ± 7.79		

Biphasic hypoxic responses of mesenteric artery with adipose tissue in the absence (control) and presence of different blockers/conditions. All experiments were performed in the presence of indomethacin 10 µM and N^{ω}-nitro-L-arginine 0.1 mM, except the last three (only in N^{ω}-nitro-L-arginine 0.1 mM). Hypoxic responses were obtained after precontraction with norepinephrine 10 µM. N: number of animals used; TEA: tetraethylammoniumchloride; 4-AP: 4-aminopyridine; Glib: glibenclamide; Apa: apamin; ChTX: charybdotoxin; Endo: endothelium; ODQ: 1 H-[1, 2, 4]oxadiazolo[4,3-A]quinoxalin-1-one; 8-SPT: 8-(p-sulfophenyl)-theophylline; ZnPPIX: zinc protoporphyrin IX; DPI: diphenyliodonium.

4.5. DISCUSSION

This study clearly demonstrates a biphasic vasoactive effect of hypoxic white adipose tissue in mice mesenteric arteries through the release of (a) vasocontractile and vasorelaxing mediator(s). The release or the effect of the vasoactive adipokines is triggered by hypoxia itself, rather than by elevated lactate concentration since lactate has shown no influence on precontractile tone of preparations with or without adherent adipose tissue.

In the present study almost no effect on arterial tone was seen during moderate hypoxia in the absence of adherent adipose tissue, which is consistent with recent studies in mice aortas [15] and rat mesenteric arteries [16]. However, in the presence of adherent adipose tissue a pronounced biphasic response to hypoxia was observed. A vasocontractile response was followed by vasorelaxation, whereas only vasorelaxation was seen in mice aortas [15] or only vasoconstriction in rat mesenteric arteries [16]. Besides species and regional differences, variation in adipose tissue type might explain these divergent observations. Mesenteric arteries are surrounded by white adipose tissue [7,8] and aortas by brown adipose tissue [18]. Furthermore, in contrast to Greenstein et al. (2009) this study was performed on preparations incubated with the cyclooxygenase blocker indomethacin and NO-synthase blocker N^{ω}-nitro-L-arginine. In the absence of these blockers we also observed a pronounced vasocontractile hypoxic response. However, in the absence of indomethacin and N^{ω}-nitro-L-arginine precontractile tone is low, which could provide an explanation for the observed vasoconstriction rather than a biphasic response since vasorelaxation is less obvious to occur. Similarly, the reduced hypoxic vasocontractile response in the presence of either indomethacin or N^{ω}-nitro-L-arginine might be attributed to an enhanced precontractile tone. Experiments were performed in the presence of indomethacin and N^{ω}-nitro-L-arginine as this mimic endothelial dysfunction due to a decreased bioavailability of endothelial vasodilators. Since endothelial dysfunction is associated with obesity [19], the observed biphasic response to hypoxia might be physiological relevant when adipose tissue dramatically increases. Whatsoever, our experiments suggest the involvement of cyclooxygenase metabolites acting on thromboxane/prostaglandin (TP) receptors in the vasocontractile hypoxic response since the TP receptor blocker SQ-29548 significantly reduced this contraction.

K^+ channels seem to be involved in the adipocyte dependent hypoxic vasorelaxation since this response was significantly reduced in the presence of the non-specific K^+ channel blockers 60, 120 mM of K^+ and tetraethylammoniumchloride (3 mM). However, other mechanisms cannot be excluded. In addition, the hypoxic vasocontractile responses were reduced. This surprising effect might be explained by blocking K^+ channels on adipocytes [20-22] resulting in a reduced extracellular K^+ concentration and depolarization of the vascular smooth muscle cells. In our experiments, Ca^{2+} activated (K_{Ca}) K^+ channels seem to be involved in the adipocyte dependent hypoxic vasorelaxation since K_{Ca} channel blockers tetraethylammoniumchloride (1 mM), apamin and charybdotoxin, or combined with TRAM-34 significantly reduced the hypoxic response.

Mainly endothelial vasorelaxing factors play a role in the hypoxic vasorelaxation since removal of the endothelium significantly impaired this response. As the relaxation occurs in the presence of blockers of NO and cyclooxygenase metabolites, the role of the endothelium-derived hyperpolarizing factor (EDHF) was investigated in the hypoxic vasorelaxation. The well-known EDHF blockers apamin and charybdotoxin, respectively a small (SK_{Ca}) and intermediate & large (IK_{Ca} & BK_{Ca}) conductance Ca^{2+} activated K^+ channel blocker [23], partly reduced the hypoxic vasorelaxation, suggesting a small role for EDHF in the hypoxic vasorelaxation. Furthermore, the partial involvement of EDHF was confirmed using apamin and the selective IK_{Ca} channel blocker TRAM-34 showing a reduced hypoxic vasorelaxation since only SK_{Ca} and IK_{Ca} (not BK_{Ca}) channels are known to be pivotal in the EDHF response [24,25]. Also the vasocontractile hypoxic response was reduced in the presence of these K_{Ca} blockers, which we unfortunately cannot explain since only BK_{Ca} channels and no SK_{Ca} and IK_{Ca} have been identified so far on adipocytes [22]. On the other hand, epoxyeicosatrienoic acids (EETs), considered as an EDHF [26], were excluded as mediators of the hypoxic vasorelaxation, based on the observation that miconazole, an epoxygenase inhibitor, failed to inhibit this hypoxic response.

Our experiments also excluded the involvement of gaseous mediator CO [27] in the hypoxic vasorelaxation, in contrast to previous findings [28]. This is based on the observation that treatment of the preparations with zinc protoporphyrin IX, a heme oxygenase inhibitor which blocks CO generation from heme, did not significantly reduce the hypoxic response. In addition, soluble guanylyl cyclase, which can be activated by CO [29], was not involved in the hypoxic vasorelaxation since the soluble guanylyl cyclase inhibitor ODQ did not reduce

this response. Another gaseous vasodilator, H_2S [30,31] was not involved in the hypoxic vasorelaxation, although H_2S has been proposed to serve as an O_2 sensor/transducer in hypoxic vascular responses [32]. This is based on the observation that apamin and charybdotoxin, which reduced the hypoxic response, did not alter the vasorelaxation of the H_2S donor NaHS in preparations with adherent adipose tissue.

Reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide, can be excluded from being involved in the adipocyte dependent hypoxic response, despite the abundant presence of ROS in the endothelium [33] and perivascular adipose tissue [34]. Moreover, hydrogen peroxide has been previously described as mediator in endothelium dependent relaxations in mesenteric arteries [35,36]. In our experiments, the hypoxic vasorelaxation was not attenuated by apocynin and diphenyliodonium. These are both inhibitors of NAD(P)H oxidase, an enzyme responsible for ROS production in adipocytes [37]. In addition, hydrogen peroxide was only able to relax mesenteric arteries with and without adherent adipose tissue at a high concentration. Also catalyzing the formation of hydrogen peroxide from superoxide by the superoxide dismutase mimetic tempol or metabolizing hydrogen peroxide into water by catalase did not significantly alter the hypoxic vasorelaxation. An extra argument to exclude endothelial ROS as mediators of the hypoxic vasorelaxation is because N^{ω}-nitro-L-arginine blocks NO-synthase, which is considered as the main source of hydrogen peroxide in the endothelium in mice mesenteric arteries [38].

Adenosine has shown to be involved in hypoxic vasorelaxation [39]. However, adenosine can be excluded as being a mediator in our study since the adenosine receptor antagonist 8-(p-sulfophenyl)-theophylline did not affect the hypoxic response.

In a previous study, the ADRF has been proposed to be a mediator of hypoxic vasorelaxation [15]. The only argument against the involvement of the ADRF in this study is that voltage-dependent K^+ (K_v) channels were not required in the hypoxic response, whereas these are believed to be associated with the ADRF induced vasorelaxation in mesenteric arteries [5].

Non-endothelial vasoconstrictor(s) seem(s) to be involved in the hypoxic vasoconstriction since removal of the endothelium did not reduce the hypoxia induced vasocontractile response. Angiotensin II producing enzymes have been located in adipose tissue [6] and can be activated during hypoxia [40]. However, angiotensin II can be excluded from playing a role in

the hypoxic vasoconstriction since the angiotensin I receptor blocker losartan did not significantly reduce the hypoxic response.
On the other hand, cyclooxygenase metabolites acting on thromboxane/prostaglandin (TP) receptors of vascular smooth muscle cells, seem to play a role in the adipocyte dependent hypoxic vasoconstriction since the cyclooxygenase blocker indomethacin and TP receptor blocker SQ-29548 inhibited this hypoxic response. This is in line with the study of Yang et al. (2002) showing an enhanced release of cyclooxygenase metabolites in response to hypoxia in vascular smooth muscle cells [41]. The involvement of cyclooxygenase metabolites in the hypoxic vasoconstriction could offer an explanation for the reduced vasoconstrictions during hypoxia when precontracting with prostaglandin $F_{2\alpha}$ and the thromboxane analogue U-46619. Prostaglandin $F_{2\alpha}$ and U-46619 may occupy the TP receptor for cyclooxygenase metabolites resulting in a smaller effect of the cyclooxygenase metabolites released during hypoxia.

In conclusion, hypoxia induces a biphasic vasoactive response in mice mesenteric arteries surrounded by white adipose tissue i.e. an endothelium independent vasoconstriction followed by a mainly endothelium dependent vasorelaxation. The hypoxic response is attributed to the presence of adipose tissue, since hypoxia had only a small relaxing influence on precontracted preparations in the absence of adipose tissue. Our results suggest the involvement of two types of mediators: vasocontractile and vasorelaxing mediator(s). Cyclooxygenase metabolites seem to be involved in the hypoxic vasocontraction, whereas at least in part the EDHF is involved in the hypoxic vasorelaxation. Our results put forward the view that during hypoxia - which occurs in obesity - blood flow in white adipose tissue is diminished due to vasocontractile cyclooxygenase metabolites. This is followed by a mechanism in which blood flow is enhanced to adapt to the metabolic needs of growth by the release of vasorelaxing adipokine(s), acting through the endothelium and opening K_{Ca} channels.

4.6. ACKNOWLEDGEMENTS

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Chapter 5

Adenosine enhances the relaxing influence of retinal tissue

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5.1. ABSTRACT

Retinal tissue from different species continuously releases an as yet unidentified retinal relaxing factor (RRF) lowering tone of isolated arteries. The potential influence of adenosine on this relaxing influence was investigated using isometric tension recording of different isolated arteries. The presence of bovine retinal tissue or rat retinal tissue enhanced the vasorelaxing effect of adenosine on isolated bovine retinal artery. In isolated rat carotid artery adenosine elicited no relaxation. However, a small relaxation is observed in the presence of rat retinal tissue, but not in the presence of porcine retina. The fact that adenosine potentiates the effect of rat retinal tissue but not that of a similar piece of porcine retinal tissue indicates species differences.

Neither a NO-synthase inhibitor (nitro-L-arginine, 0.1 mM), a cyclooxygenase inhibitor (indomethacin, 10 µM) or an epoxygenase inhibitor (miconazole, 10 µM) influenced the enhanced vasodilating effect of adenosine on bovine retinal arteries in the presence of bovine retinal tissue. On the other hand, when the retinal arteries were contracted with 120 mM K^+, adenosine no longer induced relaxation of the preparation with bovine retinal tissue. This is in line with the concept that adenosine enhances the influence of RRF. Also, the fact that rat carotid artery is less sensitive to RRF than bovine retinal artery - corresponding with a less enhanced adenosine response in rat carotid artery - is in line with the potential involvement of the RRF in the enhanced adenosine response. However, experiments using a bioassay setup for RRF gave no evidence for an increased RRF-release from the retina, nor for an increased RRF-sensitivity of the retinal artery in the presence of adenosine.

In conclusion, our findings indicate that adenosine potentiates the relaxing influence of bovine and rat retinal tissue. This effect is species dependent as it is not seen with porcine retinal tissue. Neither NO, cyclooxygenase metabolites or epoxyeicosatrienoic acids seem to be involved in this enhanced vasorelaxing response. The involvement of the RRF cannot be excluded.

5.2. INTRODUCTION

Adenosine, an important neurotransmitter in the nervous system, is widely distributed in the retina within the ganglion cell layer and the inner plexiform layer in proximity to the retinal vessels [1,2]. This purine nucleoside is released from metabolic active cells by catabolism of adenosine triphosphate (ATP) and appears to subserve a number of physiological roles such as coupling of retinal blood flow to energy demand. Many of the effects of adenosine are more prominent in pathological situations such as hypoxia and ischemia and in these situations adenosine becomes neuroprotective [3-5].

Previous studies have shown a relaxing effect of adenosine on isolated retinal arterioles from different species [6-9]. Recently, Holmgaard et al. (2007) reported that the presence of adherent perivascular retinal tissue influences the ATP- and adenosine-induced relaxing effect in isolated porcine retinal arterioles. It was found that the ATP-induced relaxation (i) is almost completely dependent on the presence of perivascular retinal tissue, and (ii) is blocked by an adenosine receptor antagonist. Furthermore, based on the observation that cyclooxygenase (COX) inhibitor ibuprofen had no effect on the response of adenosine in the presence of retinal tissue, the authors concluded that COX-metabolites are not involved. However, not only COX-metabolites can be considered as retinal mediators of adenosine-induced vasorelaxation of retinal arteries [10]. Also nitric oxide (NO) [11], epoxyeicosatrienoic acids (EETs) [12,13], the retinal relaxing factor (RRF) [6,14] and other vasorelaxing mediators are released from retina. In the present study we aimed to investigate the potential involvement of these retinal relaxing molecules in the adenosine-induced vasorelaxation. Furthermore, we investigated potential species differences in the role of retinal tissue in the relaxation effect of ATP and adenosine.

5.3. MATERIALS AND METHODS

5.3.1. Bovine and porcine tissues

Bovine and porcine eyes, obtained from local abattoirs, were enucleated within half an hour after the animals died and were transported to the laboratory in ice-cold Krebs-Ringer

bicarbonate (KRB) solution. The anterior segment and the vitreous were removed and the eyecup was placed in cold KRB solution for further preparation. A segment of the bovine retinal artery (259 ± 2 µm, n=102) located between the optic disc and the first branch of the most prominent artery was carefully excised with surrounding retinal tissue. The bovine or porcine retina was gently teased free from the choroid and cut loose at the optic nerve. The detached retina was then placed in oxygenated (5% CO_2 in O_2) KRB solution at 37°C (pH 7.4) either for use in the experiment or for incubation (see below).

5.3.2. Rat tissues

Rat carotid arteries and rat retinas were taken from female Wistar rats (230 ± 7 g, n=26) that were killed by cervical dislocation in accordance with a protocol approved by the local ethical committee. The arteries and the retinas were isolated from the surrounding tissues in cooled KRB solution. Isolated retinas were kept in oxygenated (5% CO_2 in O_2) KRB solution at 37°C (pH 7.4) before use in the experiment.

5.3.3. Tension measurements

The arterial segments were transferred to a wire myograph (constructed by the technical department of the research unit) for isometric tension recording of small vessels containing 10 ml KRB solution. Two stainless steel wires (40 µm) were guided through the lumen of the vessels. One wire was fixed on a holder connected to a force-displacement transducer, and the other was fixed on a holder connected to a micrometer. After the first wire was fixed, the adhering retinal tissue was completely removed from the bovine retinal artery. The segments were equilibrated for approximately 30 minutes in oxygenated (5% CO_2 in O_2) KRB solution at 37°C (pH 7.4). In the experiments with retinal arteries, the passive wall tension-internal circumference characteristics of the retinal vessels were then determined. On the basis of this relationship, the circumference was set to a normalized internal circumference that gives a maximum response [15,16]. In the experiments on rat carotid arteries, the distance between the two stainless steel wires was gradually increased with the micrometer until a stable preload of 0.5 g was obtained.

At the start of each experiment, the bovine retinal arteries were repeatedly activated with 120 mM K^+. Maximal contractility was assessed by stimulating the arteries with a 120 mM K^+ solution to which 30 µM $PGF_{2\alpha}$ was added. The rat carotid arteries were repeatedly contracted with 120 mM K^+ and 10 µM norepinephrine (NOR). Subsequently, the preparations were contracted by adding 30 µM $PGF_{2\alpha}$ to the standard KRB-solution in the organ bath in case of bovine retinal arteries, or by replacing the standard KRB-solution by a KRB-solution containing 30 mM K^+ and 30 µM $PGF_{2\alpha}$ when using rat carotid arteries. Retina-induced relaxation was obtained by bringing a piece of bovine, porcine, or rat retina in close proximity to the precontracted artery.

5.3.4. Bioassay

The arteries were mounted in an organ bath, normalized and activated, all as described above. After these procedures, the vessels were superfused at a rate of 0.5 ml/min with warmed (37 °C) and bubbled (95% O_2 / 5% CO_2) KRB solutions. The control KRB superfusion solution was changed for 2 minutes to different test solutions obtained by incubation.

5.3.5. Solutions obtained by incubation

Detached bovine retinas were incubated in pairs for 6 hours in 10 ml oxygenated KRB solution at 37 °C with or without 30 µM adenosine using a Warburg apparatus. After incubation, the retinas were removed from the flask and the remaining solution was centrifuged at 2000 rpm for 5 minutes. After centrifugation, the supernatant was collected (= solution containing RRF, with or without adenosine). KRB solutions without retinas with or without 30 µM adenosine, were treated in the same way as solutions with retinas and served as control solutions.

5.3.6. Statistical methods

The data were computed as mean ± S.E.M. and evaluated statistically with Student's t-test for paired or unpaired observations. Two groups of data were considered significantly different

when $P<0.05$. Decrease of pre-existing tone is expressed as % relaxation. N is the number of animals used.

5.3.7. Drugs

The experiments were performed in a Krebs-Ringer bicarbonate (KRB) solution of the following composition (mmol/L): NaCl 135, KCl 5, $NaHCO_3$ 20, glucose 10, $CaCl_2$ 2.5, $MgSO_4$ 1.3, KH_2PO_4 1.2 and EDTA 0.026 in H_2O. KRB solutions containing 30 mM K^+ (K_{30}) and 120 mM K^+ (K_{120}) were prepared by equimolar replacement of NaCl by KCl. Adenosine, adenosine 5'-triphosphate (ATP), N^{ω}-nitro-L-arginine (L-NA), indomethacin, miconazole, norepinephrine (NOR), 8-(p-sulfophenyl)theofylline (8-SPT) were obtained from Sigma (St. Louis, MO) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Dinolytic®) from Pfizer (Puurs, Belgium). Stock solutions were made in water, except for indomethacin (dissolved in ethanol) and miconazole (dissolved in dimethylsulfoxide). The final concentration of both ethanol and dimethylsulfoxide in the organ bath never surpassed 0.1 %.

5.4. RESULTS

5.4.1. Effect of adenosine on bovine retinal arteries

The effect of adenosine was studied on isolated bovine retinal arteries, both in the presence and absence of bovine retinal tissue. An original recording is shown in Fig. 5.1A. After $PGF_{2\alpha}$ (30 µM) induced a stable contraction, concentration-response curves were generated by cumulative addition of adenosine (0.1 µM to 0.1 mM) to the organ bath. This experiment was repeated on the same preparation in the presence of a piece of retinal tissue. The size of this piece (varying from 35 to 50 mm^2) was adapted to elicit only a limited relaxation (about 10 % of pre-existing tone). In the presence of retinal tissue, adenosine elicited a significantly enhanced concentration-dependent relaxation of bovine retinal arteries (Fig. 5.1B).

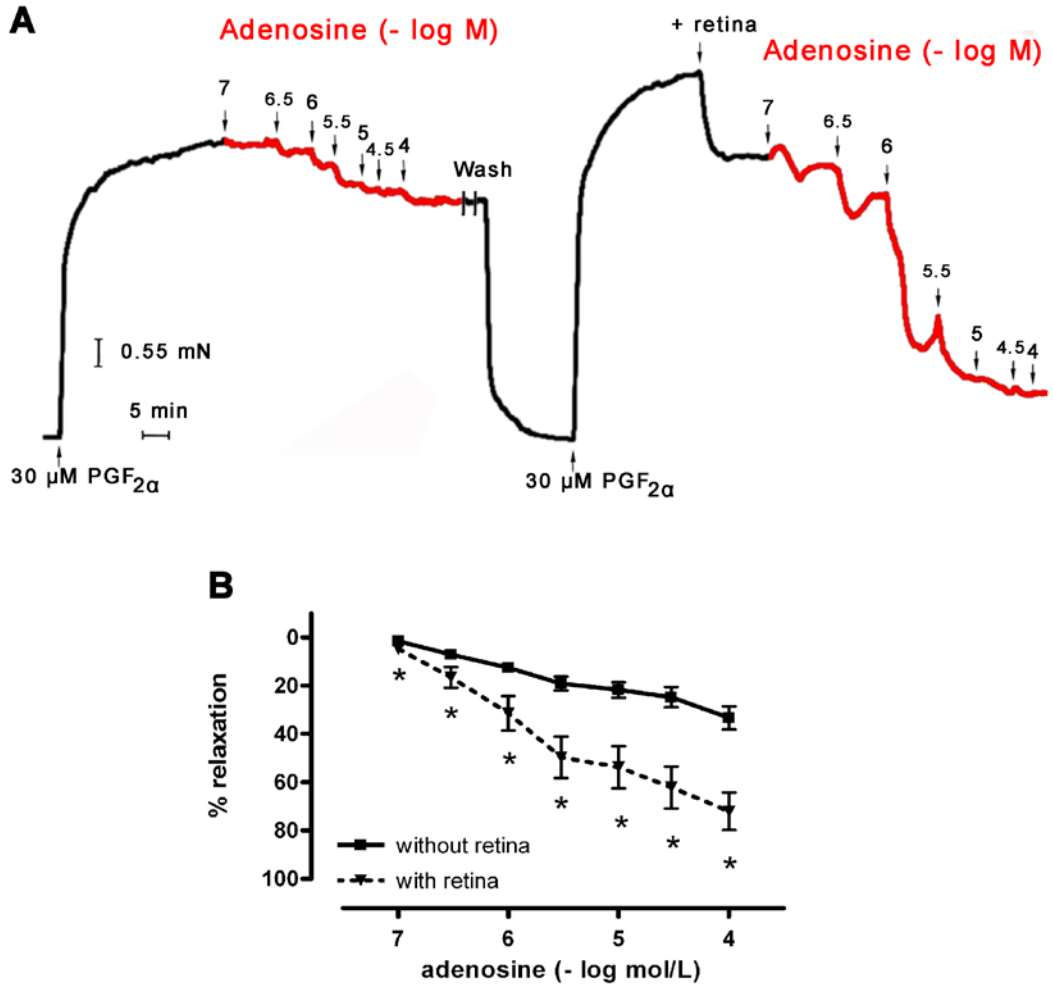

Fig. 5.1. A. Original tracing showing a response curve to adenosine (0.1 µM to 0.1 mM) in a bovine retinal artery in the absence and presence of retinal tissue. B. Effect of adenosine on precontracted (30 µM $PGF_{2\alpha}$) bovine retinal arteries in the absence (—■—) and presence (··▼··) of a piece of bovine retinal tissue (n=10, *P<0.05).

In the presence of the adenosine receptor antagonist 8-(p-sulphophenyl)theofylline (8-SPT, 0.1 mM), added 10 min before precontraction with $PGF_{2\alpha}$ (30 µM), the effect of adenosine in the presence of a piece of bovine retinal tissue was significantly diminished (Fig. 5.2).

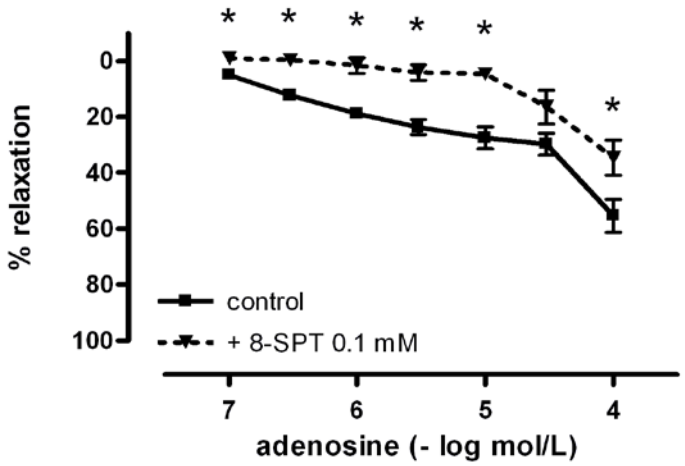

Fig. 5.2. Concentration-response curves for adenosine in bovine retinal arteries with bovine retinal tissue in the absence (control (-■-) and presence (·▼·) of the adenosine receptor antagonist 8-(p-sulfophenyl)theophylline (8-SPT, 0.1 mM) (n=7, *P<0.05).

As application of retinal tissue as such lowers tone, also the influence of similar lowering of contractile tone (by reducing the $PGF_{2\alpha}$ concentration) (Fig. 5.3A) on the adenosine-induced relaxation was studied. Only a small increase in adenosine response was observed when precontraction level was lowered (Fig. 5.3B). There is a significantly stronger increase in adenosine response (at a concentration of 0.1 µM and from 3 µM to 0.1 mM) in the presence of retinal tissue than after lowering of tone.

5.4.2. Effect of ATP on bovine retinal arteries

After $PGF_{2\alpha}$ (30 µM) induced a stable contraction, administration of ATP (0.01 to 10 µM) resulted only in a small concentration-dependent relaxation of bovine retinal arteries. This relaxation was significantly more pronounced in the presence of a piece of bovine retinal tissue (Fig. 5.4A). When the retinal arteries were precontracted with a lower concentration of $PGF_{2\alpha}$ (10 µM), only a very small increase in ATP response was observed (Fig. 5.4B). There is a significantly stronger increase of ATP response (from 0.3 till 3 µM) due to the presence of retinal tissue than due to the lowering of tone.

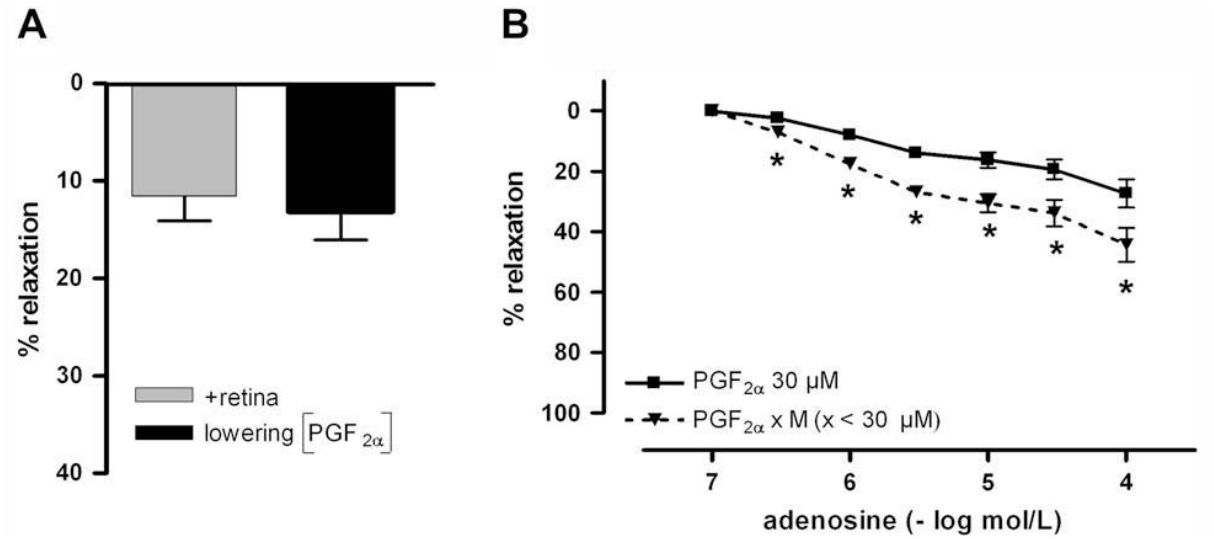

Fig. 5.3. A. Decreased tone (expressed as % relaxation) when placing a piece of bovine retinal tissue in close proximity to a retinal artery precontracted with 30 µM of $PGF_{2\alpha}$ (▭) and when contracted with lower concentration of $PGF_{2\alpha}$ (< 30 µM) (▬) (n=7). B. Vasorelaxing effect of adenosine on isolated bovine retinal arteries in the absence of retinal tissue precontracted with 30 µM of $PGF_{2\alpha}$ (–■–) vs. precontracted with a lower concentration of $PGF_{2\alpha}$ (< 30 µM) (··▼··) (n=7, *P<0.05).

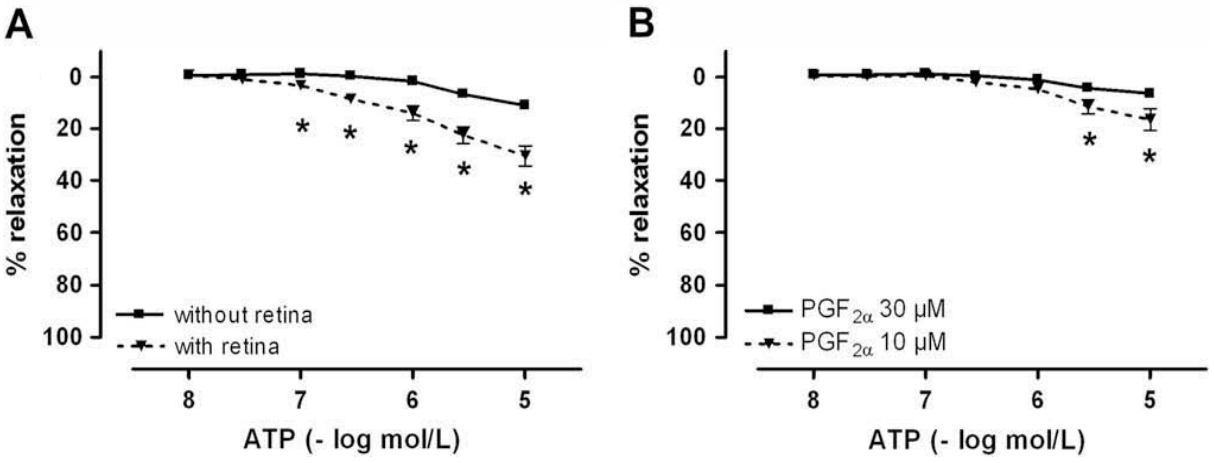

Fig. 5.4. A. Relaxation effect of ATP on precontracted (30 µM $PGF_{2\alpha}$) bovine retinal arteries in the absence (–■–) and presence (··▼··) of bovine retinal tissue (n=18, *P<0.05). B. Concentration-response curves for ATP in bovine retinal arteries in the absence of retinal tissue when precontracted with 30 µM $PGF_{2\alpha}$ (–■–) vs. 10 µM $PGF_{2\alpha}$ (··▼··) (n=8, *P<0.05).

5.4.3. Involvement of NO, cyclooxygenase metabolites and epoxyeicosatrienoic acids

The potential role of vasodilators released from the retina, such as NO [11], COX-metabolites [10] and EETs [12,13], on the enhanced adenosine response was investigated using specific inhibitors of their synthesis. The presence of the cyclooxygenase inhibitor indomethacin (10 µM, added 20 min before inducing contraction) and the NO-synthase inhibitor L-NA (0.1 mM, added 10 min before inducing contraction) did not influence the vasorelaxing effect of adenosine in bovine retinal arteries in the presence of bovine retinal tissue (Fig. 5.5A). Also miconazole (10 µM), an inhibitor of epoxyeicosatrienoic acid synthesis, added 15 min before precontraction with $PGF_{2\alpha}$ (30 µM) had no effect on the adenosine response (Fig. 5.5B).

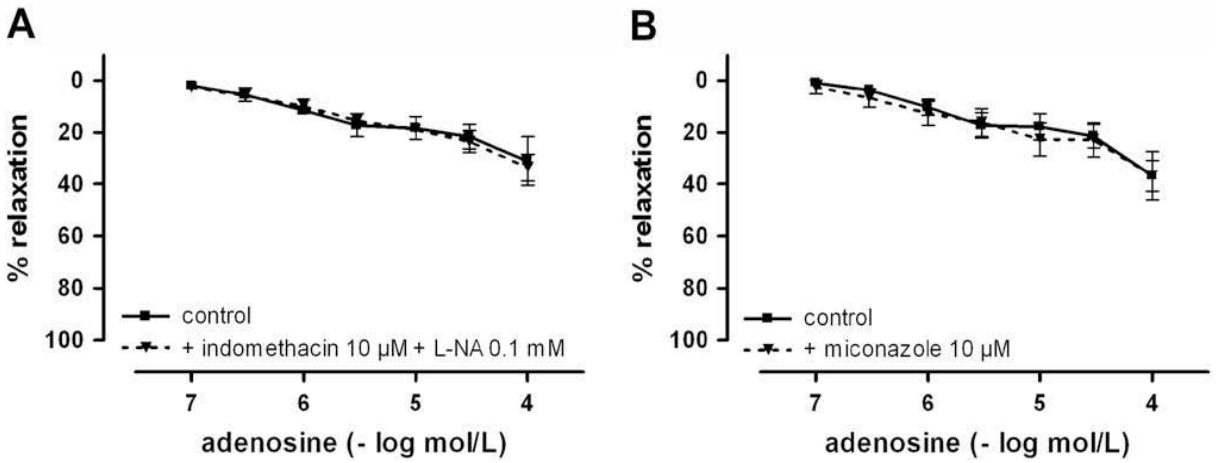

Fig. 5.5. Concentration-response curves for adenosine in bovine retinal arteries with bovine retinal tissue in the absence (control ■) and presence (▼) of A. indomethacin (10 µM) and L-NA (0.1 mM) (n=4); B. miconazole (10 µM) (n=6).

To exclude potential time-dependent influences in these experiments, two consecutive adenosine concentration-response curves were constructed in the ring segments with retinal tissue. No difference in adenosine-induced response was observed in these control experiments (data not shown).

5.4.4. Involvement of the retinal relaxing factor

This series of experiments was performed to find out whether the enhanced adenosine response is mediated by the as yet unidentified retinal relaxing factor (RRF) [14]. In the presence of K^+ 120 mM, a situation in which the influence of RRF is blocked [6,17-19], the enhancement of adenosine relaxation in the presence of retina is also blocked (Fig. 5.6).

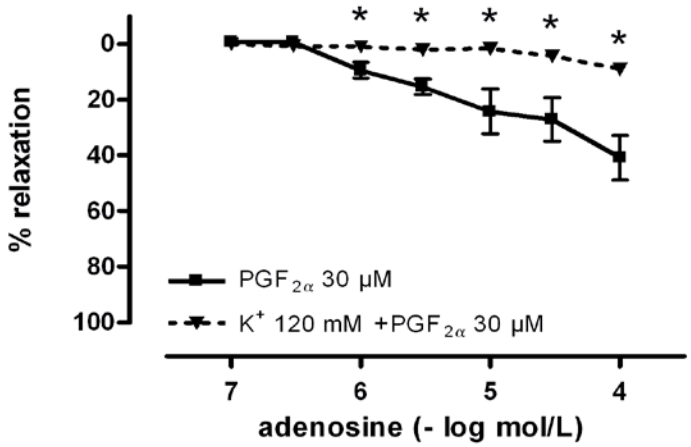

Fig. 5.6. Concentration-response curves for adenosine in bovine retinal arteries with bovine retinal tissue when precontracted with $PGF_{2\alpha}$ 30 µM (–■–) vs. K^+ 120 mM + $PGF_{2\alpha}$ 30 µM (··▼··) (n=11, *P<0.05).

Considering the potential involvement of RRF in the enhanced adenosine response, a bioassay setup was developed to determine whether adenosine might enhance the sensitivity of the retinal artery to the RRF, or whether adenosine might enhance the release of RRF from the retina. In both experiments, an isolated bovine retinal artery was mounted for isometric tension recording. The artery was continuously superfused with drops of warmed and bubbled Krebs-Ringer bicarbonate (KRB) solution to which prostaglandin $F_{2\alpha}$ (30µM) was added to precontract the retinal artery (= KRB + $PGF_{2\alpha}$ superfusion solution).

To test the hypothesis of enhanced blood vessel sensitivity to RRF (Fig. 5.7A), the non-incubated KRB + $PGF_{2\alpha}$ superfusion solution was changed for 2 min to an incubated KRB + $PGF_{2\alpha}$ solution with RRF. This solution induced a pronounced vasorelaxation. After washout of the RRF, the superfusion solution was changed to a KRB + $PGF_{2\alpha}$ solution with adenosine (30 µM). This solution elicited a small relaxation of the retinal artery. Then, the superfusion

solution was switched to the incubated KRB + $PGF_{2\alpha}$ + RRF solution to which adenosine (30 µM) was added. This solution elicited a relaxation of the retinal artery, which is similar to the relaxation induced by the same incubated KRB + $PGF_{2\alpha}$ + RRF solution in the absence of adenosine (Fig. 5.7B). These results are not in line with the hypothesis that adenosine enhances the sensitivity of retinal artery to RRF.

As already mentioned, the increased response of adenosine in the presence of retinal tissue might also be due to the fact that adenosine enhances the release of RRF from retina (Fig. 5.8A). To test this hypothesis in our bioassay setup, a non-incubated KRB + $PGF_{2\alpha}$ (30 µM) superfusion solution was changed for 2 min to an incubated KRB + $PGF_{2\alpha}$ solution without RRF (control(1)). This solution elicited a small relaxation. After washout of control(1), the superfusion solution was changed to an incubated KRB + $PGF_{2\alpha}$ solution with RRF (RRF(1)). This solution elicited a significantly stronger relaxation compared to the control(1) solution. After washout of RRF(1) solution with pure KRB solution, the retinal vessel was continuously superfused with a non-incubated KRB + $PGF_{2\alpha}$ solution to which adenosine (30 µM) was added. When precontraction was stabilized, the superfusion solution was changed for 2 min to an incubated KRB-adenosine + $PGF_{2\alpha}$ solution without RRF (control(2)). This solution induced a small increase in tone, which is significantly different from the response induced by control(1). After washout of control(2), the solution was changed to an incubated KRB-adenosine + $PGF_{2\alpha}$ solution with RRF (RRF(2)). This solution elicited only a small relaxation, which was even significantly smaller compared to the relaxation induced by RRF(1). (Fig. 5.8B). These results are certainly not in line with the hypothesis that adenosine enhances RRF formation in retina.

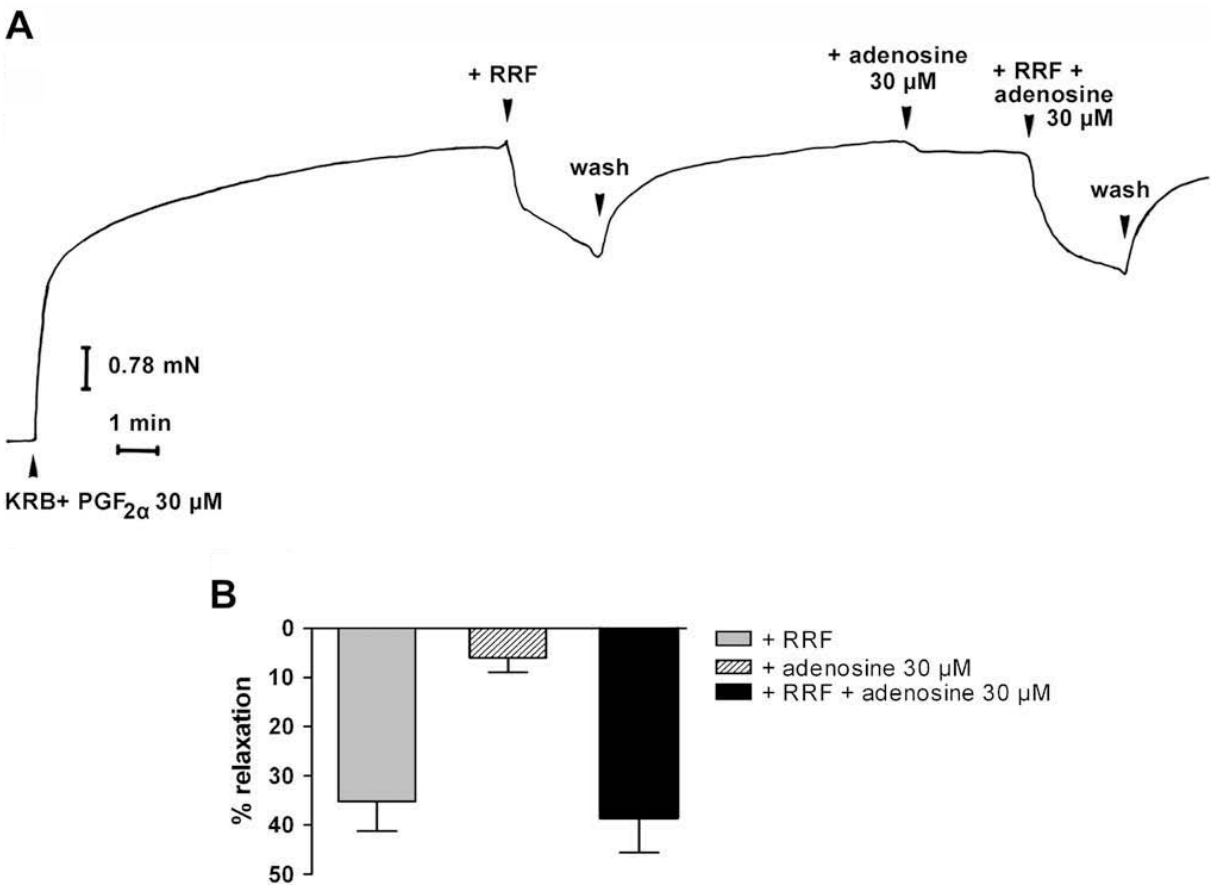

Fig. 5.7. A. Protocol of a bio-assay experiment. Bovine retinal artery is continuously superfused with KRB + $PGF_{2\alpha}$ (30 µM) solution. The superfusion solution is changed for 2 minutes to three different solutions. RRF = 1 ml of a KRB solution with RRF (= KRB solution 6 hours incubated with 2 bovine retinas) + $PGF_{2\alpha}$ (30 µM); Adenosine (30 µM) = 1 ml of a KRB solution with adenosine (30 µM) + $PGF_{2\alpha}$ (30 µM); RRF + adenosine (30 µM) = 1 ml of a KRB solution with RRF (from the same pool as RRF used in control conditions) + $PGF_{2\alpha}$ (30 µM) to which adenosine (30 µM) was added. B. Relaxation effects of RRF, adenosine 30 µM and RRF + adenosine 30 µM on precontracted ($PGF_{2\alpha}$ 30 µM) retinal arteries (n=6).

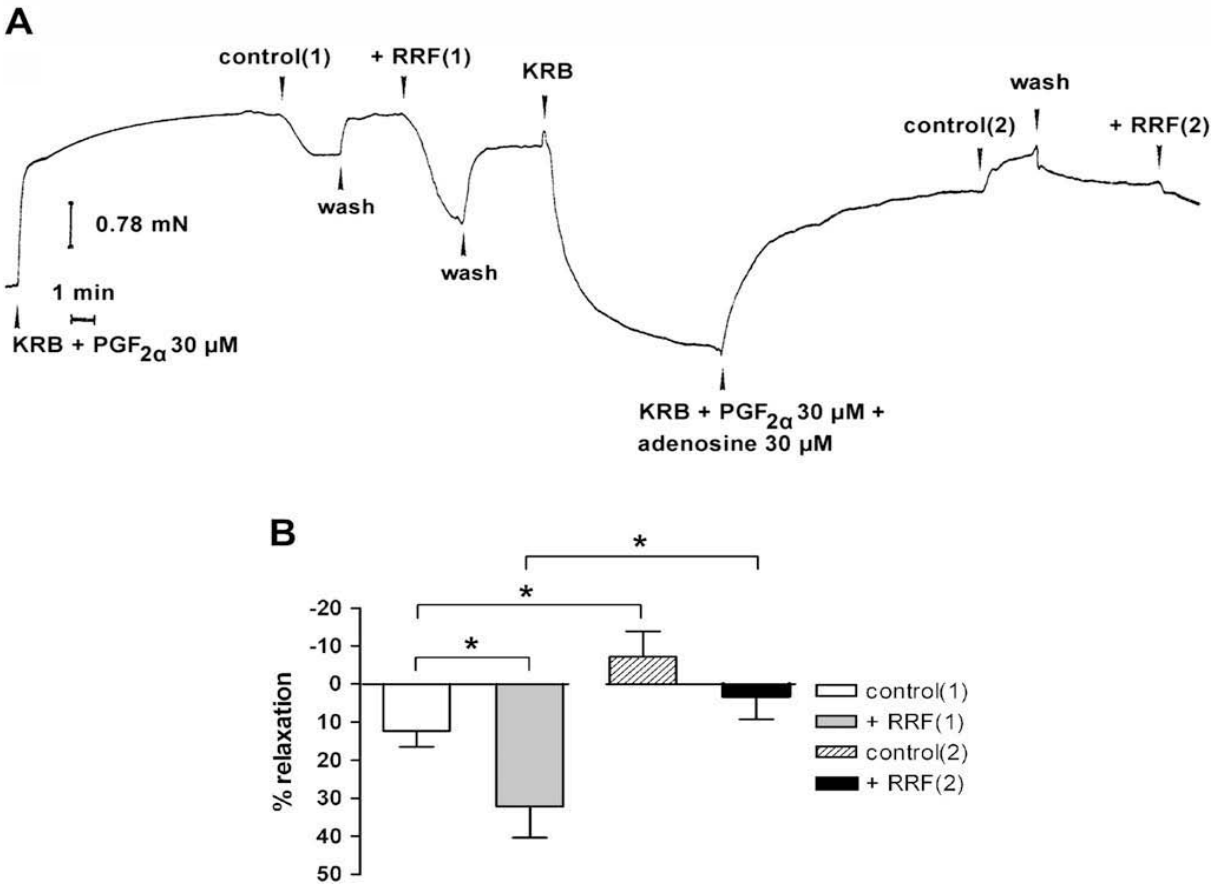

Fig. 5.8. A. Protocol of one series of experiments. Bovine retinal artery is continuously superfused with KRB + $PGF_{2\alpha}$ (30 µM) solution with or without adenosine (30 µM). Superfusion solution was changed for 2 minutes to four different solutions. Control(1) = 1 ml of a KRB solution 6 hours incubated without bovine retinas + $PGF_{2\alpha}$ (30 µM); RRF(1) = 1 ml of a KRB solution 6 hours incubated with 2 bovine retinas + $PGF_{2\alpha}$ (30 µM); control(2) = 1 ml of a KRB solution with adenosine (30 µM) 6 hours incubated without bovine retinas + $PGF_{2\alpha}$ (30 µM); RRF(2) = 1 ml of a KRB solution with adenosine (30 µM) 6 hours incubated with 2 bovine retinas + $PGF_{2\alpha}$ (30 µM). B. Relaxation effects of control and RRF-containing solutions on precontracted ($PGF_{2\alpha}$ 30 µM) retinal arteries, in the presence or absence of adenosine (30µM), incubated with (control(2) and RRF(2)) and without (control(1) and RRF(1)) adenosine 30 µM (n=8, *P<0.05).

5.4.5. Species differences

In this series of experiments we investigated whether the enhanced adenosine response in the presence of retinal tissue can also be demonstrated with porcine or rat retinal tissue and using different arteries as detector.

In a first series of experiments, the effect of adenosine was studied on bovine retinal arteries in the presence and absence of a piece of rat (varying from 16 to 25 mm^2) or porcine retinal tissue (varying from 56 to 86 mm^2). The size of the retina was adapted to elicit a limited relaxation (about 10 % of pre-existing tone), similar to that in the experiments with bovine retinal tissue. Cumulative addition of adenosine (0.1 µM to 0.1 mM) to precontracted ($PGF_{2\alpha}$ 30 µM) bovine retinal artery elicited a significantly enhanced concentration-dependent relaxation in the presence of a piece of rat (Fig. 5.9A) and porcine retinal tissue (Fig. 5.9B). As application of retinal tissue as such lowers tone, also the influence of similar lowering of contractile tone (by reducing the $PGF_{2\alpha}$ concentration) on the adenosine-induced relaxation was studied. Only a small increase in adenosine response was observed when precontraction level was lowered (Fig. 5.3B). The increase in adenosine response (at a concentration of 3 µM and from 30 µM to 0.1 mM) in the presence of rat retinal tissue is significantly higher than the corresponding increase in adenosine response after lowering the precontraction level. On the contrary, only at a concentration of 0.1 µM is the adenosine response more pronounced in the presence of porcine retinal tissue than after lowering the precontraction level.

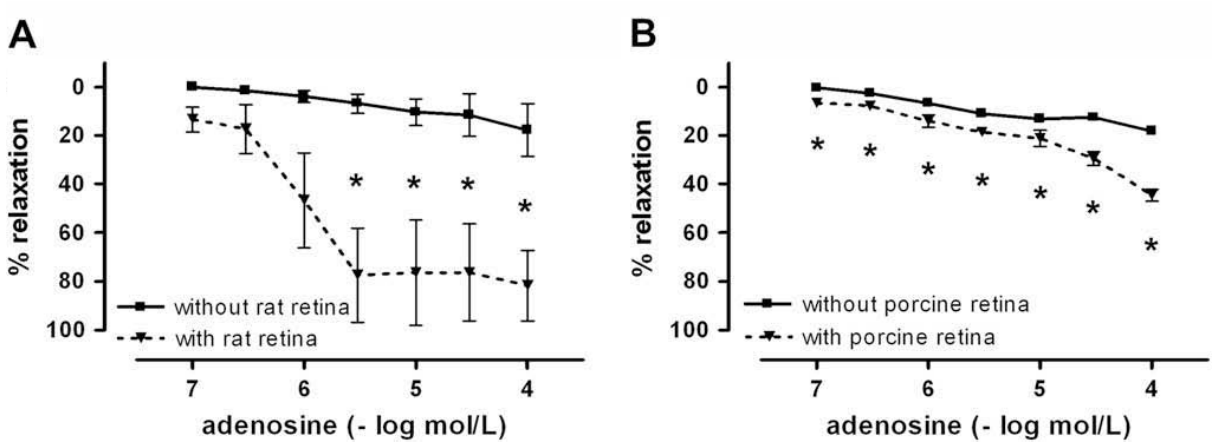

Fig. 5.9. Effect of adenosine on precontracted (30 µM $PGF_{2\alpha}$) bovine retinal arteries in the absence (-■-) and presence (·▼·) of A. a piece of rat retinal tissue (n=4, *P<0.05); B. a piece of porcine retinal tissue (n=6, *P<0.05).

In the next series of experiments, the effect of adenosine was studied using rat carotid arteries as detector of the bio-assay. These experiments were done in the presence and absence of rat and porcine retinal tissue. Cumulative addition of adenosine (0.1 µM to 0.1 mM) to precontracted ($PGF_{2\alpha}$ 30 µM and K^+ 30 mM) rat carotid artery elicited a small relaxation (3.91±1.08% at 0.1 mM, n=7) in the absence of rat retinal tissue. In the presence of rat retinal

tissue the response to adenosine was significantly enhanced (Fig. 5.10). On the other hand, in the presence of porcine retinal tissue no significant difference was found (3.79±1.13% (n=5) without and 6.07±3.26% (n=5) with retina at the highest concentration (0.1 mM)).

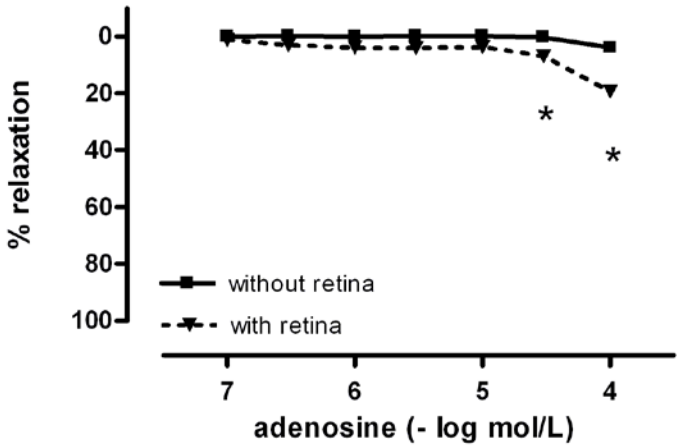

Fig. 5.10. Concentration-response curves for adenosine in rat carotid artery precontracted with 30 mM K^+ and 30 µM $PGF_{2\alpha}$ in the absence (-■-) and presence (·▼·) of rat retinal tissue (n=7, *P<0.05).

Additionally, we tested whether rat carotid artery is less sensitive to the RRF compared to bovine retinal artery. In these experiments, bovine retinal artery and rat carotid artery were precontracted with respectively $PGF_{2\alpha}$ 30 µM and $PGF_{2\alpha}$ 30 µM with K^+ 30 mM. When a stable contraction was obtained, a bovine retina was brought in close proximity to the artery. When a stable relaxation was elicited, the retina was removed and placed in close on the other artery. This procedure was repeated 3 times. The mean relaxation of the bovine retinal artery and the rat carotid artery was respectively 98.40± 0.73% and 70.20±3.46% (n=5, P<0.05).

Finally, the effect of ATP was studied on rat carotid artery in the presence and absence of porcine retinal tissue. Cumulative addition of ATP (0.01 µM - 10 µM), after precontraction with $PGF_{2\alpha}$ 30 µM and K^+ 30 mM, caused almost no relaxation of the rat carotid artery in the absence and in the presence of porcine retinal tissue, respectively 1.18±0.49% (n=5) and 5.56±5.56% (n=5) at the highest concentration (10 µM).

5.5. DISCUSSION

The main finding of the present study is that the presence of bovine retinal tissue enhances the vasorelaxing effect of adenosine in bovine retinal arteries, suggesting that adenosine enhances the effect of (a) vasorelaxing factor(s) released from bovine retina. This conclusion is based on the observation that an isolated bovine retinal artery, precontracted with $PGF_{2\alpha}$, relaxes much stronger in response to adenosine when a piece of retinal tissue is brought in close proximity to the artery. Additionally, experiments in which the non-selective adenosine receptor antagonist 8-(p-sulfophenyl)theophylline was used, showed a significant blocking effect of adenosine on bovine retinal arteries in the presence of retinal tissue. The observed rightward shift of the concentration-response curve of adenosine in the presence of 8-SPT indicates that the action of this antagonist was competitive.

However, bringing retinal tissue in close proximity to a bovine retinal artery reduces contractile tone of the artery precontracted with $PGF_{2\alpha}$. To investigate whether a decrease in contractile tone as such explains the enhanced adenosine response, experiments were performed in which contractile tone was lowered to the same level as during application of retinal tissue. A small increase in adenosine response was observed at lower precontraction level. However, there is a significantly greater increase of adenosine response when bovine retinal tissue is present compared to the increase of adenosine response when only lowering contractile tone. This shows that the enhanced vasorelaxing effect of adenosine is mainly due to the presence of bovine retinal tissue and only for a limited part due to the diminished contractile tone.

Also the effect of ATP was tested on bovine retinal arteries in the presence and absence of bovine retinal tissue. These experiments also showed a significantly - but much less extensively - enhanced vasorelaxing effect of ATP on bovine retinal arteries in the presence of bovine retinal tissue. Experiments in which a lower tone was induced, showed only a small increased ATP response, again demonstrating that the enhanced vasorelaxing effect of ATP is mainly due to the presence of bovine retinal tissue and only in part to the diminished contractile tone.

Our findings with bovine retinal tissues are in line with the observation in the study of Holmgaard et al. (2007) performed on porcine tissues. They showed that adenosine- and ATP-induced relaxation of porcine retinal arteries was much stronger in the presence of perivascular porcine retinal tissue. This study showed that retinal tissue mainly enhances the relaxing effect of ATP rather than that of adenosine. However, in our study on bovine retinal arteries, mainly an enhanced adenosine response and a less enhanced ATP response is observed. In order to evaluate this difference, we extended our experiments to rat and porcine retina and to other arteries as detectors of the bio-assay. In a first series of experiments, the effect of adenosine was studied on bovine retinal arteries in the presence and absence of a piece of rat and porcine retinal tissue. These experiments showed that an isolated bovine retinal artery relaxes stronger in response to adenosine, especially in the presence of a piece of rat retinal tissue. There is a significantly stronger increase in adenosine response in the presence of rat retinal tissue than after lowering of tone. On the contrary, the much smaller enhanced vasorelaxing effect of adenosine in the presence of porcine retina can be attributed to the diminished contractile tone. This indicates that species differences are involved in this effect.

In the next series of experiments, adenosine was tested using the rat carotid artery as detector. Experiments were performed in the presence and absence of rat or porcine retinal tissue. These experiments showed only a small, but significantly enhanced vasorelaxing effect to adenosine in the presence of rat retinal tissue, but not with porcine retinal tissue, again indicating species differences. In another series of experiments using rat carotid artery, no relaxation is seen with ATP in the presence of porcine retinal tissue, although such a relaxation has been described in porcine retinal artery [8]. This may also be related to species differences.

The enhanced adenosine response in the presence of retinal tissue could be explained by accepting that adenosine interferes with a paracrine relaxing factor released from the retina. In our experiments, NO and cyclooxygenase (COX) metabolites, both described as retinal vasodilators [10,11], were excluded as being involved in the enhanced adenosine reponse. A combination of indomethacin (a cyclooxygenase inhibitor) and L-NA (a NO-synthase inhibitor), was unable to block the enhanced vasorelaxing effect of adenosine on bovine retinal arteries in the presence of bovine retinal tissue. These results exclude the involvement of COX-metabolites - which is in line with the results of Holmgaard et al. (2007) - and of NO

in the enhanced adenosine response. We also evaluated the potential involvement of epoxyeicosatrienoic acids (EETs), also known as vasodilators [12], by using miconazole, a blocker of EET synthesis by epoxygenases. Also miconazole was unable to block the enhanced adenosine response, suggesting that EETs can be excluded as mediators of the enhanced adenosine response. It should be noted that no time-effect is involved, since experiments in which two consecutive adenosine concentration-response curves were constructed showed identical responses of adenosine in bovine retinal arteries in the presence of retinal tissue.

Furthermore, it was tested whether the retinal relaxing factor (RRF) could be involved in the enhanced response of adenosine. The RRF is a transferable relaxing factor that is continuously released from the retina and that might be involved in the regulation of retinal blood flow [6,17,18]. However, the tools to test this hypothesis are limited, because the identity of this factor remains unknown. It is known, however, that the influence of the RRF is strongly diminished in the presence of K^+ 120 mM [6,17-19]. Adenosine-induced vasorelaxation of bovine retinal arteries in the presence of bovine retina was significantly less pronounced in the presence of K^+ 120 mM. Thus, this observation is in line with the possible involvement of the RRF, although the involvement of other relaxant agents cannot be excluded on the basis of only this rather aspecific observation. The fact that rat carotid artery is less sensitive to RRF than bovine retinal artery – corresponding with a less enhanced adenosine response in rat carotid artery – is also in line with the potential involvement of the RRF in the enhanced adenosine response.

Considering that RRF may be involved we wondered whether the increased adenosine response in the presence of retinal tissue could be due to an enhanced release of RRF from retinal tissue or to an increased sensitivity of the retinal vessel to RRF. To investigate this, a bioassay setup for RRF was developed. These experiments gave no evidence that adenosine enhances the RRF release from retina, nor that there is an increase in the sensitivity for RRF in the presence of adenosine. The involvement in the enhanced adenosine response of an unstable molecule that decomposes during the 6 hours of incubation could offer an explanation for the rather unexpected results.

It can be concluded that ATP and especially adenosine enhances the vasorelaxing influence of bovine retinal tissue on bovine retinal arteries. Furthermore, adenosine enhances the

vasorelaxing influence of rat retinal tissue on rat carotid arteries and even more prominent on bovine retinal arteries. However, the exact mechanism of the enhanced adenosine response in the presence of retinal tissue remains to be elucidated. This could be of clinical importance because the enhanced vasorelaxing effect of adenosine may contribute to the increased retinal blood flow that occurs during tissue damage and inflammation. For example, in situations such as diabetic retinopathy and retinal vein occlusion, it is hypothesized that the ischemic retina releases diffusible factors that increase vascular permeability, endothelial cell proliferation, and angiogenesis. The release of adenosine by ischemic retina may be an initial signal in the development of ischemic macular edema and neovascularisation [20].

5.6. ACKNOWLEDGEMENTS

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General discussion

The regulation of blood flow is necessary to meet the metabolic needs of organs and tissues. Blood vessels are able to regulate blood flow by altering their diameter accomplished by changing tone of vascular smooth muscle cells. Vascular smooth muscle tone can be controlled by the release of vasoactive factors from neighbouring tissues (i.e. paracrine modulators) including the endothelium, nerve endings, perivascular adipose and retinal tissue. Perivascular adipose and retinal tissue have shown to exert a vasorelaxing effect due to the release of respectively the adipocyte-derived relaxing factor (ADRF) [1] and the retinal relaxing factor (RRF) [2]. In this thesis we focused on possible alterations in the vasorelaxing effect of both tissues. Secondly we aimed to investigate the involvement of the ADRF and RRF in these responses.

During obesity adipose tissue becomes hypoxic due to a diminished oxygen supply from the vasculature [3,4]. Hypoxia promotes the syntheses of proinflammatory adipokines in adipose tissue during obesity [5-7]. Some of these adipokines possess vasoactive properties (described in detail in chapter 1). We found that hypoxia induces a different vasoactive response in isolated mice thoracic aortas (chapter 3) and mesenteric arteries (chapter 4) surrounded by adipose tissue. In aorta, a pronounced vasorelaxation was seen [8], while in mesenteric arteries, a vasoconstriction was observed followed by a pronounced vasorelaxation (i.e. biphasic response) during hypoxia in the presence of adherent adipose tissue. A hypoxic vasoconstriction has also been described in rat mesenteric arteries [9]. The different responses to hypoxia can be explained by experimental set-up, species and regional differences but also differences in type of adipose tissue. It is known that thoracic aortas are surrounded by brown adipose tissue [10], while mesenteric arteries are surrounded by white adipose tissue [11,12]. These findings were also confirmed in our laboratory using hematoxiline and eosin stainings. An interesting approach in future research is testing whether hypoxia induces the same vasomotor response of brown and white adipose tissue when switching the detector vessels i.e. mesenteric arteries in the presence of brown and aortas in the presence of white adipose tissue. Therefore, coating adipose tissue with an inert polydimethylsiloxane (e.g. Sylgard^{TM}) can provide a handy way to by-pass the floating properties of adipose tissue when bringing this tissue into the organ bath.

From our experiments it was concluded that hypoxia enhances the effect of (a) vasodilator(s) from perivascular brown adipose tissue (chapter 3) [8]. Several potential candidates linked to hypoxia were excluded from being the mediator in the hypoxic response f.e. lactate, cyclooxygenase metabolites, nitric oxide, adenosine, carbon monoxide and hydrogen sulfide [13-19]. However, the ADRF might be involved since the hypoxic relaxation of aorta as well as the ADRF response act partially through opening of ATP sensitive potassium channels and act independently of the cyclooxygenase pathway and adenosine receptors [1,8]. The presence of the endothelium and nitric oxide formation are not needed in the hypoxic relaxation of aorta [8]. However, whether these are required for the ADRF response is a matter of debate [1,20,21]. To further exclude the requirement of nitric oxide in the ADRF response and thereby favouring the ADRF being the hypoxic mediator, it would be interesting to test whether the ADRF response is unaltered in β soluble guanylyl cyclase (sGC) knock-out or knock-in mice, since relaxations elicited by nitric oxide requires the activation of the β subunit of sGC [22].

The identity of the ADRF is until now not fully elucidated, which makes it difficult to point the ADRF as being the mediator in the hypoxic response (see review chapter 1). To clarify the identity of the ADRF, it would be appropriate to use advanced chromatographic techniques on supernatans collected from cultured purified adipocytes, such as two-dimensional liquid chromatography combined with mass spectrometry. Today, there is more and more evidence supporting the existence of different ADRFs, including angiotensin (1-7) [23]. However, the fact that ATP sensitive or voltage dependent potassium channels are not involved in the vasorelaxing effect of angiotensin (1-7) in rat aorta [23] is not in line with angiotensin (1-7) being the ADRF [1,24]. Also in our experiments, angiotensin (1-7) can be excluded from being the mediator in the hypoxic response because angiotensin (1-7) causes NO-dependent vasorelaxation [23]. Furthermore, H_2S has recently been described as a possible ADRF or mediator in the ADRF response [24]. However in our experiments, H_2S was excluded as being the mediator in the hypoxic response [8]. Also hydrogen peroxide produced from the NAD(P)H oxidase in adipocytes has been described to be involved in the ADRF response [20]. However, scavenging hydrogen peroxide (catalase, 2500 U/ml) had no influence on the aortic relaxation induced by hypoxia (own unpublished results). This indicates that hydrogen peroxide can be excluded from being the hypoxic mediator in aorta, despite the fact that brown adipose tissue harbors plenty of mitochondria which is a major source of reactive oxygen species under pathological conditions [25].

In contrast to brown adipose tissue, perivascular white adipose tissue releases two types of mediators during hypoxia i.e. (a) vasoconstrictor(s) and dilator(s) (chapter 4). The vasoconstrictor seems to be a cyclooxygenase metabolite acting on thromboxane/ prostaglandin (TP) receptors, independently of the presence of the endothelium. The dilator however, acts through the endothelium and opening of Ca^{2+} sensitive potassium channels. Besides the endothelium-derived hyperpolarizing factor (EDHF) also (an)other endothelium-derived relaxing factor(s) might be involved, excluding nitric oxide, carbon monoxide, hydrogen sulfide, cyclooxygenase metabolites, reactive oxygen species and epoxyeicosatrienoic acids. This hypothesis was based on the observation that blockers of small and intermediate conductance Ca^{2+} active potassium channels, which virtually abolish the EDHF response [26,27], only partially reduced the hypoxic response. Furthermore, the hypoxic vasorelaxation was more attenuated after endothelium removal than after blocking EDHF. We cannot exclude the involvement of the ADRF completely. However, the fact that voltage-dependent potassium channels were not required in the hypoxic vasorelaxation of mesenteric arteries is not in line with the ADRF being involved [1,21].

Besides adipose tissue, also retinal tissue from different species continuously releases an as yet unidentified relaxing factor, called the retinal relaxing factor (RRF) [2]. The RRF shares some similarities with the ADRF. Both molecules are transferable [1,2], might play a role in hypoxic vasorelaxation [8,28-30] and act through opening of potassium channels [1,2,20,21,28,29,31]. Recently, palmitic acid methyl ester (PAME or methyl palmitate) has been suggested as being the RRF in rat model [32]. According to the authors, the RRF and PAME share similar biochemical and pharmacological properties. PAME appears to be (i) spontaneously released from rat retina in a calcium-dependent way, (ii) a transferable endothelium-independent vasodilator of rat aorta acting through voltage-dependent K^+ (K_v) channels on smooth muscle cells, which is at least 400 times more potent than NO, (iii) thermostable (after heat treatment of 70°C for 1 hour) and (iv) hydrophobic [32]. However, we could not confirm the hypothesis of PAME being the RRF in preliminary studies using rat and bovine model. In our setup, PAME did not relax rat aorta and bovine retinal arteries, even at a high concentration (0.1 mM). Furthermore, treatment of both retina and artery with a K_v channel blocker (4-aminopyridine, 2 mM) did not block the retina induced vasorelaxation in rat and bovine model (own unpublished observations). Finally, in bovine model, the RRF seems to be hydrophilic instead of hydrophobic since the relaxing influence of RRF-solution persisted after extraction of hexane [2]. Therefore, these divergent observations about PAME

being the RRF requires more research in the near future, in terms of using different species and vascular preparations to test whether PAME is a general relaxing factor like the RRF.
Besides PAME, also N-methyl-D-aspartate (NMDA) and gamma-amino butyric acid (GABA) mimetic substances have been recently suggested being involved in the RRF induced vasorelaxation in porcine model [33]. However NMDA or GABA are unlikely candidates for the RRF since cyclooxygenase inhibition, which blocks NMDA and GABA induced vasodilation [33], does not block the RRF response [2,28,29,32]. In addition, NMDA was only able to induce a small relaxation of bovine retinal arteries with or without adherent retinal tissue at a high concentration (1 mM). Furthermore, preliminary studies showed that the bovine retina induced relaxation of retinal arteries was not inhibited when blocking NMDA receptors (DL-APV, 50 µM) (own unpublished observations).

We attempted to identify the RRF by chemical analysis in collaboration with the Charité Campus Benjamin Franklin in Berlin. Incubation solutions with RRF were fractionized by gel filtration, reversed phase, anion and cation exchange chromatography. Surprisingly, either all fractions relaxed isolated bovine retinal arteries or no effect on vascular tone was seen. In order to assess the polarity of the RRF, liquid-liquid extraction and solid-phase extraction on incubation solutions with RRF were performed in collaboration with prof. Sandra of the University of Ghent. However, some polar, non-polar as well as some control fractions induced vasorelaxation. When these fractions were desalted, no vasorelaxation was observed (preliminary studies). So the identification of the RRF by chemical analysis remains extremely challenging. While these several attempts to identify the RRF were not successful, we were able to obtain additional information on the molecular size of this factor. Therefore dialyses membranes with varying cutoff values (Microcon^{TM}) were used to filter incubation solutions with RRF. Incubation solutions obtained after filtration with a dialysis membrane with cutoff 3000 g/mol were still able to relax bovine retinal arteries (own unpublished observations). From these experiments it was clear that the molecular size of the RRF is not exceeding 3000 g/mol.

In order to gain information of the cell type releasing the RRF, electrical field stimulation (EFS) was used to stimulate retinal neurons. Furthermore, EFS could offer a new model for investigating neurovascular coupling in retina, meaning that blood flow adapts to neuronal activity by altering arterial tone. EFS (train duration 20 sec; frequency 0.125 – 8 Hz; pulse duration 5 msec; 80 V) was applied by a stimulator via electrodes placed on each side of

a retinal artery with adherent retinal tissue. EFS only elicited a rapid and reversible frequency dependent relaxation when the arteries were surrounded by retinal tissue. Unfortunately these promising results could no longer be reproduced systematically due to unexplained problems. Besides neurons, also glial cells can be involved in the release the RRF. Therefore, it would be interesting to test whether supernatans of purified Müller cell cultures, which are the most abundant non-neuronal cells in the vertebrate retina [34], is able to relax isolated arteries.

In the final part of this project, we assessed the influence of adenosine on the vasorelaxing effect of retinal tissue (chapter 4) [35]. Adenosine, widely distributed within the retina [36], is a known vasodilator in retinal arterial beds [2,37-39]. The findings in our work point to a significant species-dependent role of adenosine in enhancing the vasorelaxing effect of retinal tissue. We suggested that adenosine enhances the effect of (a) retinal derived vasorelaxing factor(s), excluding cyclooxygenase metabolites, nitric oxide and epoxyeicosatrienoic acids. The involvement of the RRF was difficult to test since the identity of this factor is not known. However two reasons pointed to the involvement of this fascinating factor. First the fact that 120 mM of potassium, known to suppress the RRF induced vasorelaxation [2,28,29,40], strongly diminished the enhanced vasorelaxing effect of retinal tissue. Secondly, the RRF was less effective to relax rat carotid artery than bovine retinal artery which corresponds with a less enhanced adenosine response in rat carotid artery compared to bovine retinal artery. On the other hand, we were unable to conclude whether an enhanced release of the RRF or an enhanced sensitivity of the blood vessel for the RRF occurs.

In conclusion, this work provides new insight about the role of perivascular adipose tissue in the vascular response to hypoxia and the role of retinal tissue in the vascular response to adenosine. Hypoxia and adenosine adjust vascular diameters appropriately by enhancing the effect of (a) vasorelaxing factor(s) from adipose or retinal tissue. This mechanism might lead to an increased blood flow which could be important to prevent tissue damage due to deprived oxygen supply.

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Summary

Blood flow regulation is pivotal in order to meet the metabolic needs of organs and tissues. Vasoactive factors released from neighbouring cells (i.e. paracrine modulators), including adipose and retinal tissue, play a substantial role in the regulation of blood flow. Two of these factors are the adipocyte-derived relaxing factor (ADRF) and retinal relaxing factor (RRF), which provoke the vasorelaxing effect of respectively perivascular adipose and retinal tissue described in **chapter 1** and **2**. The main focus of this thesis was to explore possible alterations in the vasorelaxing effect of both tissues and to investigate the involvement of possible mediators such as the ADRF and RRF. Therefore, we used an in vitro set-up based on isometric tension measurements in arteries mounted in a wire myograph.

Hypoxia develops within adipose tissue during tissue mass expansion. In **chapter 3** we demonstrated that moderate hypoxia enhances the vasorelaxing effect of perivascular brown adipose tissue in mice thoracic aortas due to a possible enhanced effect of the ADRF. The enhanced release of the ADRF or increased sensitivity of the blood vessel to the ADRF could be part of a regulatory mechanism during hypoxia to adapt blood flow to the metabolic needs of the growing tissue which prevents tissue damage due to deprived oxygen supply.

In **chapter 4** we extended our study towards perivascular white adipose tissue surrounding mice mesenteric arteries. This study demonstrated that regional and/or differences in adipose tissue (white vs. brown) influences the hypoxic response as hypoxia induced a biphasic vasomotor effect (i.e. a vasoconstriction followed by a vasorelaxation) in the presence of white adipose tissue. Non-endothelial cyclooxygenase metabolites mediate the hypoxic vasoconstriction, whereas endothelial vasodilators, including the endothelium-derived hyperpolarizing factor (EDHF), are involved in the hypoxic vasorelaxation. In contrast to brown adipose tissue, the ADRF is less likely to be involved in the hypoxic response in arteries surrounded by white adipose tissue.

Adenosine, widely distributed within the retina, is a known vasorelaxant. In **chapter 5** we demonstrated that adenosine enhances the vasorelaxing effect of bovine and rat retinal tissue in which the involvement of the RRF was questioned. This study supports the idea that the enhanced vasorelaxing effect of retinal tissue by adenosine serves as a protective

mechanism during tissue damage and inflammation by contributing to an increased retinal blood flow.

In conclusion, this work demonstrates that adipose and retinal tissue exerts an enhanced vasorelaxing effect during hypoxia or in the presence of adenosine. This is accomplished by an enhanced effect of (a) vasorelaxing factor(s) released from adipose or retinal tissue. This mechanism might lead to an increased blood flow which could be important to prevent tissue damage due to deprived oxygen supply.

Samenvatting

De regeling van de doorbloeding is noodzakelijk om aan de metabole noden van organen en weefsels te voldoen. Vasoactieve substanties vrijgesteld uit naburige weefsels van de bloedvaten, waaronder het vetweefsel en netvlies (retina), spelen hierin een belangrijke rol. Twee van deze substanties, ook wel paracriene modulatoren genoemd, zijn de van de vetcel afkomstige relaxerende factor (VARF) en de retinale relaxerende factor (RRF). De VARF en RRF lokken het vasorelaxerend effect uit van respectievelijk het vetweefsel en de retina, beschreven in **hoofdstuk 1** en **2**. De belangrijkste doelstelling van het onderzoek was nagaan in welke omstandigheden het vasorelaxerend vermogen van beide weefsels versterkt wordt en welke mediatoren, o.a. de VARF en RRF, hierbij mogelijks betrokken zijn. Hiervoor werd gebruik gemaakt van een in vitro techniek die gebaseerd is op het meten van isometrische spanningsveranderingen in arteriën opgespannen in een draadmyograaf.

Naarmate vetweefsel groeit, krijgt dit weefsel een tekort aan zuurstof (m.a.w. het weefsel wordt hypoxisch). In **hoofdstuk 3** hebben we aangetoond dat matige hypoxie het vasorelaxerend effect van bruin vetweefsel rond thoracale aorta's van de muis versterkt. Mogelijks komt dit door een verhoogde vrijstelling van de VARF of door een toegenomen gevoeligheid van de bloedvaten voor deze factor. De ontstane verhoogde doorbloeding kan het groeiend vetweefsel beschermen tegen zuurstoftekort en weefselschade.

In **hoofdstuk 4** hebben we ons onderzoek uitgebreid naar wit vetweefsel rond mesenterische arteriën van de muis. Hier hebben we aangetoond dat verschillen in bloedvattype of in type van vetweefsel de respons op hypoxie kan beïnvloeden. Bij deze experimenten vonden we namelijk dat hypoxie een bifasisch vasomotorisch effect (namelijk een vasoconstrictie gevolgd door een vasorelaxatie) uitlokt. Niet-endotheel afhankelijke cyclooxygenase metabolieten zijn verantwoordelijk voor de vasoconstrictie tijdens hypoxie, terwijl endotheliale vasodilatoren, waaronder de endotheliale hyperpolarizerende factor, betrokken zijn in de vasorelaxatie tijdens hypoxie. Het is minder waarschijnlijk dat de VARF betrokken is in de hypoxierespons van wit vetweefsel, terwijl dat wel het geval is met bruin vetweefsel.

Adenosine is een bekende vasodilator die sterk verspreid is in de retina. In **hoofdstuk 5** hebben we aangetoond dat adenosine het relaxerend effect van de retina van het rund en de rat versterkt, zonder de betrokkenheid van de RRF uit te sluiten. Dit effect kan een beschermend mechanisme zijn tijdens weefselschade en inflammatie, door bij te dragen aan een verhoogde retinale doorbloeding.

Samenvattend tonen de resultaten van deze studies aan dat vetweefsel en retina een versterkend vasorelaxerend effect induceren tijdens hypoxie of in aanwezigheid van adenosine. Dit berust op een versterkt effect van (een) vasorelaxerende factor(en) vrijgesteld uit deze weefsels. Dit mechanisme kan leiden tot een verhoogde doorbloeding, waardoor weefselschade door een tekort aan zuurstof kan voorkomen worden.

Curriculum vitae

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A_1-publications

- Maenhaut N, Boussery K, Delaey C and Van de Voorde J. Control of retinal arterial tone by a paracrine retinal relaxing factor. *Microcirculation* 2007;14:39-48

- Maenhaut N, Boussery K, Delaey C and Van de Voorde J. Adenosine enhances the relaxing influence of retinal tissue. *Exp Eye Res* 2009;88(1):71-8

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Abstracts & presentations

- Maenhaut N, Boussery K, Delaey C and Van de Voorde J. Adenosine enhances the relaxing influence of rat and bovine retinal tissue. Poster presentation on the Autumn Meeting of the Belgian Society for Fundamental and Clinical Physiology and Pharmacology. Leuven (Belgium), 17 Nov 2007. *Acta Physiologica* 2008;192,S662:P-21.

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- Maenhaut N, Boydens C and Van de Voorde J. Hypoxia enhances the vasorelaxing influence of adipose tissue. Oral presentation on the FWO Vascular Biology Meeting. Antwerp (Belgium), 27 Nov 2009.

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- Maenhaut N and Van de Voorde J. Different vasoactive effect of adherent adipose tissue in mice aorta and mesenteric arteries. Poster presentation (bullet session) on the Spring Meeting of the Belgian Society for Fundamental and Clinical Physiology and Pharmacology. Namur (Belgium), 27 Mar 2010. *Acta Physiologica* 2010;199,S678:P-02.

- Maenhaut N, Boydens C and Van de Voorde J. Hypoxia enhances the vasorelaxing influence of mice adipose tissue. Oral and poster presentation on the 12th Symposium on Vascular Neuroeffector Mechanisms. Odense (Denmark), 24-26 July 2010. *Acta Physiologica* 2010;199,S680:10.

- Maenhaut N, Boussery K, Delaey C and Van de Voorde J. Adenosine enhances the relaxing influence of rat and bovine retinal tissue. Poster presentation on the 12th Symposium on Vascular Neuroeffector Mechanisms. Odense (Denmark), 24-26 July 2010. *Acta Physiologica* 2010;199,S680:02.

- Maenhaut N, Boydens C and Van de Voorde J. Different vasoactive effect of adherent adipose tissue during hypoxia in mice aorta and mesenteric arteries. Oral presentation on the 15th Annual Meeting of the European Council for Cardiovascular Research (ECCR). Nice (France), 8-10 Oct 2010. *Hypertension* 2010;56,1165.

- Maenhaut N and Van de Voorde J. Different vasoactive effect of adherent adipose tissue during hypoxia in mice aorta and mesenteric arteries. Oral presentation on the FWO Vascular Biology Meeting. Namur (Belgium), 26 Nov 2010.

- Maenhaut N, Boydens C and Van de Voorde J. Different vasoactive effect of adherent adipose tissue during hypoxia in mice aorta and mesenteric arteries. Oral presentation on the 1st Benelux congress on Physiology and Pharmacology. Liège (Belgium), 18-19 March 2011.

Teaching & supervising students

- Lab sessions Physiology for students
  2nd bachelor of Medicine, Dentistry, Pharmacy, Biomedical Sciences
  1st bachelor of Biomedical Sciences, Logopedics & Audiology

- "Role of endothelin in cardiovascular system" Literature study Lies Verheuen (student 2nd bachelor of Medicine 2007-2008)

- "Neurovascular coupling in retina" Literature study Emmanuel Annaert (student 2nd bachelor of Medicine 2008-2009)

- "Influence of perivascular adipose tissue on arterial tone" Graduation thesis Charlotte Boydens (student Biomedical Sciences 2008-2010)

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