

ANGIOTENSIN II INDUCED INFLAMMATION AND VASCULAR DYSFUNCTION

Role of Oxidative Stress and Cyclooxygenase

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Academic Dissertation

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TABLE OF CONTENTS

- LIST OF ORIGINAL PUBLICATIONS 5
- ABBREVIATIONS 6
- ABSTRACT 8
- 1. INTRODUCTION..... 10
- 2. REVIEW OF THE LITERATURE 12
 - 2.1. Renin-angiotensin system (RAS) 12
 - 2.1.1. RAS in the kidney..... 15
 - 2.1.2. RAS in the heart..... 16
 - 2.1.3. RAS in the vasculature 18
 - 2.2. Arterial tone 20
 - 2.2.1. Vascular contractile responses..... 22
 - 2.2.1.1. Angiotensin II 22
 - 2.2.1.2. Endothelin-1..... 22
 - 2.2.1.3. Noradrenaline 24
 - 2.2.1.4. Potassium chloride 24
 - 2.2.2. Vascular relaxation responses..... 24
 - 2.2.2.1. Endothelium-dependent vascular relaxation..... 24
 - 2.2.2.2. Endothelium-independent vascular relaxation..... 27
 - 2.2.3. Arterial tone in angiotensin II-induced hypertension 28
 - 2.3. Vascular inflammatory response 30
 - 2.3.1. Recruitment of leukocytes 30
 - 2.3.1.1. Cellular adhesion molecules 31
 - 2.3.1.2. Cytokines 31
 - 2.3.1.3. Nuclear factor kappa-B..... 32
 - 2.3.1.4. Oxidative stress 32
 - 2.3.1.5. Nitric oxide 33
 - 2.3.1.6. Prostanoids..... 34
 - 2.3.2. Inflammation caused by angiotensin II 35
 - 2.4. General characteristics of experimental models used in this study 40
 - 2.5. General characteristics of drugs for prolonged treatment in this study 42
- 3. AIMS OF THE STUDY 46
- 4. MATERIALS AND METHODS 47
 - 4.1. Experimental animals 47

4.2. Drug treatments.....	47
4.3. Measurement of blood pressure and heart rate.....	49
4.4. Metabolic studies.....	50
4.5. Collection of blood and tissue samples.....	50
4.6. Arterial responses.....	51
4.7. Morphology	52
4.8. Immunohistochemistry.....	52
4.9. Autoradiography	52
4.10. Reverse transcription-polymerase chain reaction (RT-PCR)	52
4.11. Biochemical determinations	53
4.12. Compounds	55
4.13. Statistical analysis	55
4.14. Ethics	56
5. RESULTS.....	57
5.1. Blood pressure, cardiac hypertrophy and heart rate	57
5.2. Renal function.....	58
5.3. Arterial tone	59
5.4. Inflammation	61
5.5. Oxidative stress	63
5.6. Blood glucose and serum insulin	64
5.7. Plasma atrial natriuretic peptide and B-type natriuretic peptide	64
5.8. Serum lactate dehydrogenase	65
5.9. Angiotensin receptors, angiotensin-converting enzyme, neutral endo-peptidase, and endothelin receptors expression.....	65
5.10. Body weight, food intake, and drug dosages.....	65
6. DISCUSSION	68
6.1. Methodology	68
6.2. Increased RAS activity genetically and vascular dysfunction.....	71
6.3. Increased RAS activity in spontaneously diabetic Goto-Kakizaki rats	74
6.4. COX in the pathogenesis of Ang II-induced vascular inflammation	75
6.5. COX in the pathogenesis of Ang II-induced vascular dysfunction	79
7. SUMMARY AND CONCLUSIONS.....	81
ACKNOWLEDGEMENTS	82
REFERENCES.....	84
ORIGINAL PUBLICATIONS	103

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by the Roman numerals.

- I. Mervaala EMA, Cheng ZJ*, Tikkanen I, Lapatto R, Nurminen K, Vapaatalo H, Müller DN, Fiebeler A, Ganten U, Ganten D, Luft FC. Endothelial dysfunction and xanthine oxidoreductase activity in rats with human renin and angiotensinogen genes. *Hypertension* 2001;37:414-418 (*sharing the first authorship with EM).
- II. Cheng ZJ, Vaskonen T, Tikkanen I, Nurminen K, Ruskoaho H, Vapaatalo H, Müller DN, Park J-K, Luft FC. Mervaala EMA. Endothelial dysfunction and salt-sensitive hypertension in spontaneously diabetic Goto Kakizaki rats. *Hypertension* 2001;37:433-439.
- III. Cheng ZJ, Finckenberg P, Louhelainen M, Merasto S, Tikkanen I, Vapaatalo H, Mervaala EMA. Cardiovascular and renal effects of COX inhibition in transgenic rats harboring the mouse renin-2 gene (TGR(mREN2) rats) (submitted).
- IV. Cheng ZJ, Tikkanen I, Vapaatalo H, Mervaala EMA. Vascular effects of COX inhibition and AT₁ receptor blockade in transgenic rats harboring the mouse renin-2 gene. *J Physiol Pharmacol* (in press).

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ABBREVIATIONS

ACE	angiotensin-converting enzyme
ACh	acetylcholine
Ang	angiotensin
AT ₁	angiotensin II type 1 receptor
AT ₂	angiotensin II type 2 receptor
COX	cyclooxygenase
dTGR	double transgenic rat harboring human renin and angiotensinogen genes
ED ₁	monoclonal antibody recognizing rat monocytes and macrophages
EDHF	endothelium-derived hyperpolarizing factor
eNOS, NOS III	endothelial nitric oxide synthase
ET-1	endothelin-1
ET _A	endothelin receptor A
ET _B	endothelin receptor B
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GK	Goto-Kakizaki rat
HS	high-sodium
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
iNOS, NOS II	inducible nitric oxide synthase
L-NAME	N ^G -nitro-L-arginine methyl ester
LS	low-sodium
MCP-1	monocyte chemoattractant protein-1
mREN2	transgenic rat harboring the mouse renin-2 gene
mRNA	messenger ribonucleic acid
NA	noradrenaline
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa B
nNOS, NOS I	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NOx	nitrite and nitrate
O ₂ ⁻	superoxide
PG	prostaglandin

RAS	renin-angiotensin system
RT-PCR	reverse transcription-polymerase chain reaction
ROS	reactive oxygen species
SBP	systolic blood pressure
SD	Sprague-Dawley rat
SNP	sodium nitroprusside
SOD	superoxide dismutase
TNF	tumor necrosis factor
TXA ₂	thromboxane A ₂
VCAM-1	vascular cell adhesion molecule-1
VSMC	vascular smooth muscle cell
XOR	xanthine oxidoreductase

ABSTRACT

The renin-angiotensin system (RAS) plays a pivotal role in the pathogenesis of hypertension, myocardial infarction, stroke, renal failure, and diabetic vascular complications. Besides the classical regulatory effects on blood pressure, sodium excretion, and aldosterone secretion, evidence is now accumulating supporting the notion that RAS may also induce an inflammatory response and oxidative stress in the vascular wall by non-hemodynamic mechanisms. The present series of studies was conducted to investigate the mechanisms of angiotensin II (Ang II)-induced inflammation and vascular dysfunction, focusing especially on the role of oxidative stress and cyclooxygenase (COX) in the pathogenesis.

Two transgenic rats with increased local RAS activity: namely double transgenic rats harboring human renin and human angiotensinogen genes (dTGRs) (4 weeks old), and transgenic rats harboring the mouse renin-2 gene (mREN2 rats) (8 weeks old), as well as spontaneously diabetic Goto-Kakizaki (GK) rats (5-6 weeks old), were used as experimental animal models. The effects of prolonged treatment with the AT₁ receptor antagonists valsartan and losartan, a high-sodium (HS) diet, the selective cyclooxygenase-2 (COX-2) inhibitor MF-tricyclic, and the non-selective COX-1/COX-2 inhibitor sulindac were evaluated in the pathogenesis of Ang II-induced inflammation and vascular injury. Blood pressure was recorded either indirectly by the tail-cuff method or directly by radiotelemetry. Arterial contractile and relaxation responses were examined *ex vivo* by use of mesenteric, renal, or aortic rings. Inflammatory response in the kidney and heart was evaluated by immunohistochemistry. COX mRNA and protein expressions were quantified by RT-PCR and immunohistochemistry, respectively. As indirect markers of oxidative stress, serum 8-isoprostaglandin F_{2α} concentrations, as well as tissue xanthine oxidoreductase (XOR) activity were measured.

Ang II induced endothelial dysfunction via AT₁ receptor stimulation. This Ang II-induced endothelial dysfunction was associated with increased oxidative stress, which was partially mediated via XOR in dTGRs. In GK rats, increased RAS activity was also involved in the pathogenesis of endothelial dysfunction and vascular inflammation. An HS diet in these rats elevated blood pressure, and aggravated cardiac hypertrophy,

endothelial dysfunction, inflammatory response, vascular injury, and oxidative stress. The AT_1 receptor antagonist valsartan prevented the salt-induced inflammatory response and oxidative stress in GK rats by blood pressure-independent mechanisms. In mREN2 rats, renal COX-2 mRNA and protein expressions were down-regulated. Neither MF-tricyclic nor sulindac prevented an Ang II-induced inflammatory response, endothelial dysfunction, or vascular/tissue injury in these rats. In contrast, both drugs raised blood pressure and albuminuria to a similar extent in mREN2 rats. However, the therapeutic effects of the AT_1 receptor antagonist losartan on Ang II-induced hypertension, inflammatory response, endothelial dysfunction, and tissue morphology, were not interfered with by concurrent COX inhibition.

Taken together, our findings indicate that (1) oxidative stress is involved in the pathogenesis of Ang II-induced vascular dysfunction and inflammatory response, (2) the detrimental effects of Ang II on vascular function and tissue morphology are mediated via the AT_1 receptor, and (3) COX does not play a central role in the pathogenesis of Ang II-induced inflammation and vascular injury.

1. INTRODUCTION

Hypertension is one of the most important risk factors for arteriosclerosis, myocardial infarction, stroke, and renal failure (Collins *et al.*, 1990; MacMahon *et al.*, 1990; Perry *et al.*, 1995). Approximately 24% of the population in industrialized countries and more than half the population aged over 65 years suffers from hypertension (Ruoff, 1998). Despite the enormous amount of work carried out in past decades to explore the mechanisms involved in the pathogenesis of hypertension, the cause of high blood pressure in 95% of the patients remains unknown.

Some epidemiological evidence exists that hypertensive patients with activated RAS have a higher risk for myocardial infarction than other forms of hypertension (Alderman *et al.*, 1991; Meade *et al.*, 1993; Alderman, 1995; Laragh, 1995). Furthermore, treatment of hypertensive patients with angiotensin-converting enzyme (ACE) inhibitors and Ang II type I (AT₁) receptor antagonists reduces the incidence of myocardial infarctions and mortality (Yusuf *et al.*, 1992; Pitt, 1994; Timmermans & Smith, 1996; Gavras, 1997), indicating the central role of RAS in the pathogenesis of hypertension and in its complications.

Historically, Ang II, the key effector of RAS, was considered to be a circulating hormone that regulates blood pressure, aldosterone secretion, and salt reabsorption. At the present, it is generally accepted that Ang II is also formed locally in several tissues, such as vascular wall, heart, kidney, and brain (for review, see Bader *et al.*, 2001). Ang II regulates cellular growth, proliferation, and inflammatory responses. The production of Ang II within the arterial wall is also important in the regulation of arterial tone. The presence of an inflammatory response in the arterial wall has been described in different vascular diseases, including hypertension and atherosclerosis (Ross, 1999), and in hypertensive end-organ damage (Mervaala *et al.*, 1999b).

Non-insulin-dependent diabetes mellitus (NIDDM, type II DM) is also a prevalent disease in the western world, commonly accompanied by hypertension, dyslipidemia, and vascular complications (Grundy *et al.*, 1999). About 80% of the patients with NIDDM are hypertensive at the time of diagnosis (Grundy *et al.*, 1999). Most diabetic patients die

from macrovascular complications. The mechanisms of the pathogenesis of diabetic vascular disease, however, still remain unclear (for review, see Semenkovich & Heinecke, 1997). Recent clinical studies have revealed that both ACE inhibitors and AT₁ receptor antagonists prevent the development of diabetic complications, indicating the importance of RAS in the pathogenesis (Mogyorosi & Sonkodi, 1999; Brenner *et al.*, 2001).

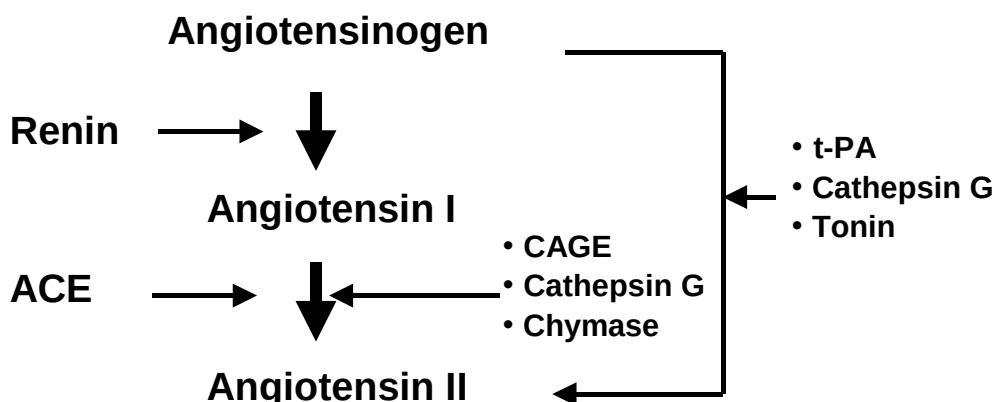
The present study was designed to explore the underlying mechanisms of Ang II-induced vascular dysfunction and inflammation in three animal models: double transgenic rats harboring human renin and human angiotensinogen genes (dTGRs), mouse transgenic rats harboring the mouse renin-2 gene (mREN2 rats), and spontaneously diabetic Goto-Kakizaki (GK) rats. Prolonged treatment with AT₁ receptor antagonists, or selective and non-selective COX inhibitors or both was used to clarify the pathophysiological role of Ang II and COX isoenzymes.

2. REVIEW OF THE LITERATURE

2.1. Renin-angiotensin system (RAS)

In past decades, RAS has been extensively studied as an important mediator of hypertension and hypertensive cardiac and renal damage. Originally, RAS was regarded as a circulating hormone system. Recent studies, however, have demonstrated the existence of local RAS in many tissues including the brain, kidney, adrenal cortex, heart, and blood vessel wall (for review, see Bader *et al.*, 2001). Here, RAS acts through paracrine-autocrine mechanisms (Rothermund & Paul, 1998). Tissue RAS is activated in pathophysiological situations, and local synthesis of Ang II appears to contribute to altered tissue function and morphology (Hirsch *et al.*, 1990). According to the original view, when the kidneys sense diminished effective arterial blood flow, renin is released from the macula densa of the juxtaglomerular apparatus. Once in the circulation, renin catalyzes the conversion of angiotensinogen which is synthesized in the liver, to angiotensin I (Ang I). Ang I is further converted to octapeptide Ang II, a key effector of RAS, by angiotensin-converting enzyme (ACE). ACE is primarily an endothelial-bound enzyme with particularly high concentrations in the lung (for review, see Bader *et al.*, 2001).

Non-ACE pathways have been suggested as catalyzing the production of Ang II within tissues, including cathepsin G, chymostatin-sensitive angiotensin II-generating enzyme (CAGE), chymase, tonin, and tissue plasminogen activator (t-PA) (Dzau, 1988; Dzau *et al.*, 1993; Gibbons, 1993; Gibbons & Dzau, 1994). Non-ACE pathways are functionally important in human blood vessels, as recently demonstrated in large and small resistance arteries from healthy human volunteers (Padmanabhan *et al.*, 1999). In humans, at least 40% of total Ang II is formed by non-ACE pathways (for review, see Hollenberg *et al.*, 1998), suggesting that full suppression of the RAS cannot be achieved by ACE inhibition alone (Petrie *et al.*, 2001). However, in rats, ACE seems to be the most important enzyme for Ang II formation (Okunishi *et al.*, 1993) (**Figure 1**).



ACE = angiotensin-converting enzyme

CAGE = chymostatin-sensitive angiotensin II-generating enzyme

t-PA = tissue plasminogen activator

Figure 1. Angiotensin II formation.

Ang II exerts its effects via binding to specific receptors on the cell surface. Ang II receptors of the cardiovascular system are divided into two main subtypes: Ang II type 1 (AT₁) and 2 (AT₂) receptors, which are specifically blocked by AT₁ receptor antagonists, e.g., losartan and valsartan (for review, see de Gasparo *et al.*, 2000). In rodents, AT₁ receptors are further divided into two subtypes, AT_{1a} and AT_{1b} (Kakar *et al.*, 1992). The AT₁ receptor is a G-protein-coupled receptor. The molecular and cellular actions of Ang II in cardiovascular and renal tissues are almost exclusively mediated by AT₁ receptors (Kim & Iwao, 2000). AT₂ is also a G-protein-coupled receptor (Hayashida *et al.*, 1996). Unlike AT₁ receptors, the role of the AT₂ receptor in cardiovascular and renal tissues is inconsistent and remains to be defined. Recent studies have suggested that the AT₂ receptor may play a counterregulatory role against the actions of Ang II mediated via the AT₁ receptor (Horiuchi *et al.*, 1999; Siragy *et al.*, 1999). Recently, the AT₄ receptor also has been identified (for review, see de Gasparo *et al.*, 2000).

The mechanism of RAS-induced hypertension has generally been attributed to the direct vasoconstrictor effects of Ang II and the mineralocorticoid effects of aldosterone. Ang II

enhances sodium reabsorption and water retention through stimulation of aldosterone release from the adrenal gland. Ang II can also elevate blood pressure by augmenting noradrenaline release from sympathetic nerve endings in the vasculature (Sealey & Laragh, 1990), by increasing secretion of potent vasoconstrictor ET-1 and antidiuretic hormone vasopressin, and by reducing baroreceptor activity in the vascular smooth muscle, by enhancing of the Ca^{2+} sensitivity of the contractile apparatus of the smooth muscle. Ang II is also the most potent dipsogen (Sealey & Laragh, 1990), which could induce an increase in blood volume.

Although the effectiveness of ACE inhibitors and AT_1 receptor antagonists in reducing blood pressure in experimental models and among hypertensive patients strongly suggests that RAS plays an important role in the pathogenesis of essential hypertension (Timmermans & Smith, 1996; Gavras, 1997), the mechanisms in the development and maintenance of hypertension are still incompletely understood. Increased plasma RAS activity is not a consistent feature in hypertensive rats and patients (Paran *et al.*, 1995). On the other hand, arterial wall ACE activity has been reported in rats to correlate positively with blood pressure (Nakata *et al.*, 1987; Cockcroft *et al.*, 1994), and elevated tissue levels of Ang II have been described in pathological conditions. The studies have demonstrated that local RAS is of importance in the pathogenesis of many diseases, including hypertension, other cardiovascular diseases, and kidney diseases, independent of the presence of hypertension (Egido, 1996).

It is important that Ang II is not only a vasoactive hormone which regulates blood pressure and electrolyte homeostasis by hemodynamic effects, but it is also a growth factor and immunomodulator influencing cell proliferation/apoptosis and fibrosis and participating in inflammatory responses in a non-hemodynamic manner (for review, see Wolf, 1998; Luft, 2001; Ruiz-Ortega *et al.*, 2001b). Evidence is accumulating that non-hemodynamic effects of Ang II are essential in hypertensive end-organ damages (for review, see Wolf, 1998; Luft, 2001; Ruiz-Ortega *et al.*, 2001b).

Ang II is metabolized enzymatically (aminopeptidase A and N) to Ang III and Ang IV. Ang III stimulates the release of aldosterone secretion. In addition, Ang III is also involved in the feeling of thirst via AT_1 - and AT_2 - receptor stimulation. Ang IV has distinct actions, probably via AT_4 , including release of plasminogen activator inhibitor-1 from the

endothelium (Kerins *et al.*, 1995; Mehta *et al.*, 2002). In addition, Ang (1-7) is generated from Ang II and Ang I. The biological functions of Ang III, Ang IV, and Ang (1-7) seem to be less important than those of Ang II (Riordan, 1995; Wright *et al.*, 1995; Ardaillou, 1997).

In summary, RAS plays an important role in the regulation of blood pressure. It appears that non-hemodynamic effects of locally formed Ang II are even more important than circulating Ang II in the pathogenesis of hypertension and other cardiovascular and renal diseases, yet the underlying mechanisms are still incompletely understood.

2.1.1. RAS in the kidney

Local renal RAS

The RAS is highly active locally in the kidney. In addition to the classical pathway, in the kidney Ang II is also formed locally (Erdos, 1990). Ang II is formed from systemically delivered angiotensinogen and Ang I as well as from locally formed angiotensinogen and Ang I. Angiotensinogen and its mRNA are localized in the proximal tubular cells (Gomez *et al.*, 1990; Ingelfinger *et al.*, 1990; Darby & Sernia, 1995), whereas ACE is found in endothelial cells of the renal vasculature and in proximal tubular brush-border membranes (Schulz *et al.*, 1988). AT₁ receptors are present on the luminal and basolateral membranes of tubules and in the blood vessels (Allen *et al.*, 1999). In various rat models of hypertension, including two-kidney/one-clip, Ang II infusion, mREN2 rat, and dTGR, total intrarenal Ang II concentration has been found to be much higher than circulating Ang II levels (Mullins *et al.*, 1990; Lippoldt *et al.*, 1996).

Effects of Ang II on the kidney

Ang II plays an important part in regulation of electrolyte homeostasis and fluid balance. It increases salt reabsorption both in proximal and distal tubules by increased secretion of aldosterone, and it regulates the glomerular filtration rate by the constriction of efferent arterioles, together with prostaglandin-induced vasodilation of the afferent

arterioles (for review, see Dzau *et al.*, 2001). Ang II likely plays a primary role in progressive deterioration of renal function in patients with hypertension, diabetes, or primary renal diseases (for review, see Weir & Dzau, 1999). Abnormalities of the systemic blood pressure, and glomerular hemodynamics influenced by Ang II involve a number of these processes and thereby influence the rate of progression of renal disease (Anderson *et al.*, 1986; Zatz *et al.*, 1986; Norman *et al.*, 1987; Nakamura *et al.*, 1995).

The accumulation of extracellular matrix (e.g., collagen IV, collagen I, laminin, fibronectin) in the mesangial region and collapse of the glomerular capillary are critical factors in progressive renal dysfunction (Ikoma *et al.*, 1991; Riser *et al.*, 1992; Arai *et al.*, 1995). Ang II not only upregulates extracellular matrix production, but it also inhibits its degradation (Norman *et al.*, 1987; Arai *et al.*, 1995; Nakamura *et al.*, 1995). In addition, Ang II-induced renal perivascular and peritubular inflammatory cell infiltration by blood pressure-independent mechanisms plays an important role in Ang II-induced glomerular injury and glomerulosclerosis (Kim & Iwao, 2000).

Taken together, It appears that besides the regulation of blood pressure, electrolyte homeostasis, and fluid balance, renal Ang II-induced accumulation of extracellular matrix as well as the inflammatory response both also play a critical role in hypertensive glomerular injury and glomerulosclerosis.

2.1.2. RAS in the heart

Local cardiac RAS

All components of the RAS are expressed in the heart (for review, see Bader *et al.*, 2001). Renin and its mRNA are present in atria, ventricles, and isolated cardiomyocytes of various species including humans, but the levels are very low (Dzau *et al.*, 1987a; Paul *et al.*, 1988; Endo-Mochizuki *et al.*, 1995; Zhang *et al.*, 1995). A recent study has suggested that circulating renin and prorenin are efficiently taken up by the cardiac circulation and may be the major source of Ang I-generating activity (van Kesteren *et al.*, 1997; Muller *et al.*, 1998). Angiotensinogen is expressed in all parts of the heart and in

cultured cardiac myocytes and fibroblasts (Campbell & Habener, 1986; Dzau *et al.*, 1987b; Hellmann *et al.*, 1988; Lindpaintner *et al.*, 1990; Dostal *et al.*, 1992; Sawa *et al.*, 1992; Sadoshima *et al.*, 1993). ACE is produced in cardiac tissue and is mainly localized in fibroblasts and in the endothelium (Hirsch *et al.*, 1990; Katwa *et al.*, 1995). In the human heart, chymase may also convert Ang I to Ang II (Urata *et al.*, 1990). AT₁ and AT₂, are about equally expressed in the heart and in isolated cardiomyocytes (Regitz-Zagrosek *et al.*, 1995; Booz & Baker, 1996; Rogg *et al.*, 1996). Cardiac fibroblasts express only AT₁ receptors under normal conditions (Crabos *et al.*, 1994; Matsubara *et al.*, 1994), but their number is increased in such conditions as heart failure (Haywood *et al.*, 1997; Ohkubo *et al.*, 1997).

Effects of Ang II on the heart

The role of the cardiac RAS differs from that of the renal RAS, which requires Ang II for normal kidney development (Esther *et al.*, 1996). Ang II is not required for the development or the maintenance of normal cardiac function (Holubarsch *et al.*, 1993). The highly important pathogenetic effect of Ang II on cardiomyocytes is a hypertrophic effect (for review, see Bader *et al.*, 2001). Ang II can also induce hyperplasia of cardiac fibroblasts, which thus can develop into cardiac fibrosis (Weber & Brilla, 1991). An intact cardiac RAS appears to be a requirement for local proliferation of fibroblasts and the consequent development of fibrosis (Matsusaka *et al.*, 1999). Another important pathogenetic effect of Ang II on cardiomyocytes is inotropic actions on the heart (Fukuta *et al.*, 1996). The inotropic effects of Ang II are apparently mediated by its influence on calcium homeostasis and transmembrane conductance. Ang II also has been shown to induce apoptosis of cardiac myocytes (Leri *et al.*, 1998).

Taken together, it seems that despite the fact that intact RAS exists in the heart, Ang II is not necessary for the maintenance of normal cardiac function; however, increased generation of cardiac Ang II plays an important role in the development of cardiac hypertrophy.

2.1.3. RAS in the vasculature

Local vascular RAS

The existence of renin, Ang II, ACE, and Ang II receptors in blood vessels has been well documented. Renin mRNA exists in the endothelial and smooth muscle cells, and renin can be locally synthesized in the vessel wall (Samani *et al.*, 1987) and in cultured smooth muscle cells (Re *et al.*, 1982). In addition, renin can be taken up directly from the circulating blood into the vessel wall (Loudon *et al.*, 1983). Northern blot and *in situ* hybridization studies have shown that angiotensinogen mRNA exists in the arteries (Campbell & Habener, 1987; Cassis *et al.*, 1988; Naftilan *et al.*, 1991). ACE exists in vascular endothelial cells and vascular smooth muscle cells (VSMCs), and also exists in inflammatory cells (for review, see Dzau *et al.*, 2001). Increased vascular ACE activity appears to be responsible for the increased vasoconstrictor response to Ang I infusion (Okamura *et al.*, 1986). Ang II receptors are present in the medial VSMCs as well as in endothelial cells. In the adult rat, the AT₁ receptor isoform is mainly expressed, while in fetal rats, the AT₂ isoform is the predominant receptor (Viswanathan *et al.*, 1991). AT₂ receptors may serve an important role during stages of rapid growth of the artery, and also have a significant function in the adult vasculature (Viswanathan *et al.*, 1991).

Effects of Ang II on the artery

Ang II has several effects on the vascular system (**Figure 2**). Its main is mediated through its binding to AT₁ receptors. One of the important effects of Ang II is its induction of vasoconstriction by stimulating phospholipase C and adrenergic nerves directly or indirectly, resulting in elevation of intracellular [Ca²⁺] (for review, see Palatini, 2001), thus increasing systemic and renal blood pressure and impairing vascular function.

Ang II induces vascular remodeling including growth, apoptosis, inflammation, and fibrosis (for review, see Intengan & Schiffrin, 2001), mainly by increasing activation of protein kinase C (Smith & Timmermans, 1994; Timmermans & Smith, 1994; Brasier *et al.*, 2000; Muller *et al.*, 2001c). Ang II increases the expression of several growth factors, e.g., fibroblast growth factor, transforming growth factor β -1, platelet-derived growth

factor, and insulin-like growth factor in the vascular wall, thus resulting in hypertrophy and fibrosis (Dzau, 1988; Daemen *et al.*, 1991; Gibbons, 1993; Gibbons & Dzau, 1994; Noble & Border, 1997). Ang II-induced VSMC growth has been seen in human vascular diseases and experimentally in rats (Dzau, 1988; Geisterfer *et al.*, 1988; Daemen *et al.*, 1991; Gibbons, 1993; Gibbons & Dzau, 1994).

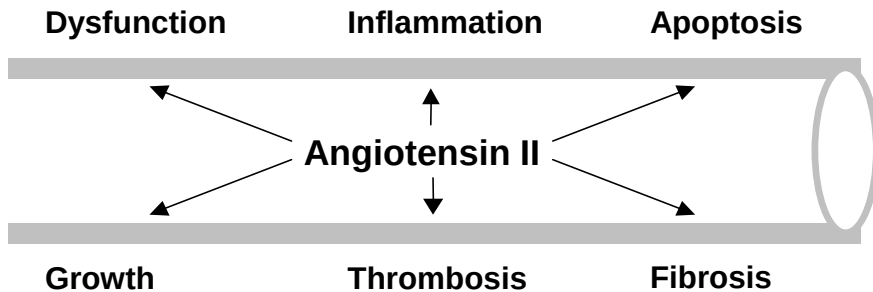


Figure 2. Effects of angiotensin II on an artery.

Ang II induces vascular inflammatory responses by activating macrophages and increasing their ability to attach to and invade into the vascular cells (Pratt, 1996). It is also a potential trigger of apoptosis (Yamada *et al.*, 1996; Tea *et al.*, 2000). Ang II induces thrombosis by increasing platelet aggregation, stimulating plasminogen activator inhibitor-1, and promoting endothelial dysfunction and oxidative stress of blood vessels (Dzau *et al.*, 1991; Griendling *et al.*, 1993; Feener *et al.*, 1995; Vaughan *et al.*, 1995). All these Ang II-induced vascular injurious effects can be explained as being closely related to hypertensive end-organ damage.

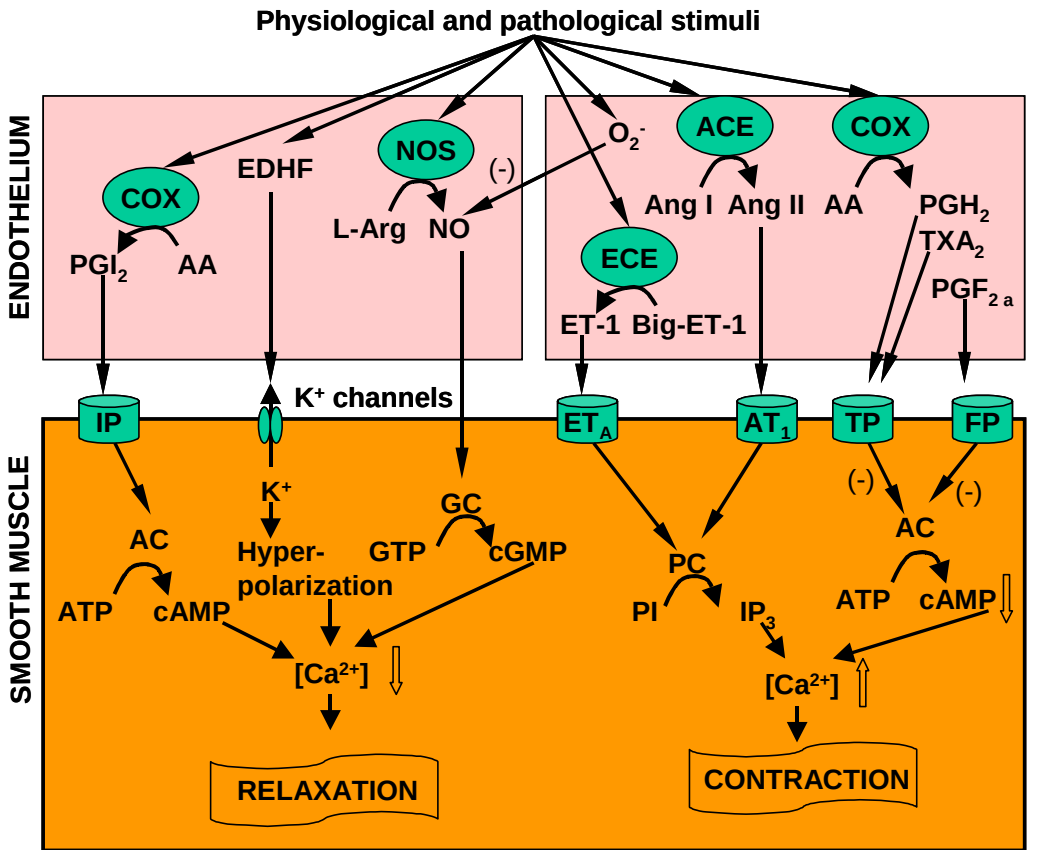
Taken together, Ang II-induced vasoconstriction plays a primary role in hypertension and vascular dysfunction. In addition, Ang II also induces vascular injuries, including vascular hypertrophy, apoptosis, inflammation, and fibrosis, which appear to be closely related to hypertensive end-organ damage.

2.2. Arterial tone

Arterial tone, one of the most important regulators of blood pressure in response to changes in transmural pressure (Meininger & Davis, 1992; Karibe *et al.*, 1997), is determined by both the endothelium and the smooth muscle. Human essential hypertension and animal models of hypertension are characterized by increased peripheral resistance (Shepherd, 1990). Since peripheral resistance can increase as a result of either enhanced contractility or impaired relaxation of resistance arteries, functional changes in arterial smooth muscle and endothelium are important in the pathogenesis of hypertension and its complications (Luscher & Vanhoutte, 1986; Moncada *et al.*, 1991; Cohen & Vanhoutte, 1995; Kahonen *et al.*, 1996; Harrison, 1997; Kojda & Harrison, 1999; McIntyre *et al.*, 1999).

The endothelium regulates vasomotor tone, local homeostasis, and proliferation processes by releasing vasodilator substances (e.g., nitric oxide, prostacyclin, or endothelium-derived hyperpolarizing factor) and vasoconstrictor substances (e.g., angiotensin II, endothelin-1, prostaglandin $F_{2\alpha}$, prostaglandin H_2 , thromboxane A_2 , or free radicals) (**Figure 3**) in response to physiological or pathophysiological stimuli (Luscher & Vanhoutte, 1986; Moncada *et al.*, 1991; Cohen & Vanhoutte, 1995; Kahonen *et al.*, 1996; Harrison, 1997; Kojda & Harrison, 1999; McIntyre *et al.*, 1999).

Endothelial dysfunction has been associated with several cardiovascular diseases or risk factors including hypertension, hypercholesterolemia, smoking, and congestive heart failure (Kojda & Harrison, 1999). Maintaining endothelial integrity is critical for the preservation of blood flow and the prevention of thrombosis. An imbalance between endothelium-derived constricting and relaxing factors contributes to increased peripheral resistance, as well as to hypertension-related end-organ damage. Endothelial dysfunction in hypertension may be due to impaired NO synthesis and/or inactivation of endothelium-derived NO by reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and peroxynitrite (Harrison, 1997; Kojda & Harrison, 1999; McIntyre *et al.*, 1999). Recent studies support the notion that the balance between nitric oxide and superoxide may be even more important than the absolute levels of either alone



Ang I = angiotensin I
 Ang II = angiotensin II
 AT1 = angiotensin II type I receptor
 ATP = adenosine triphosphate
 cAMP = cyclic adenosine 3',5'-monophosphate
 cGMP = cyclic guanosine 3',5'-monophosphate
 COX = cyclooxygenase
 ECE = endothelin-converting enzyme
 EDHF = endothelium-derived hyperpolarizing factor
 ET = endothelin
 ET_A = endothelin-1 receptor A
 FP = prostaglandin F_{2a} receptor

GC = guanylate cyclase
 IP = prostaglandin I₂ receptor
 IP₃ = inositol triphosphate
 L-Arg = L-arginine
 NO = nitric oxide
 NOS = nitric oxide synthase
 O₂⁻ = superoxide
 PC = phospholipase C
 PG = prostaglandin
 PGI₂ = prostacyclin
 PI = phosphoinositol
 TP = thromboxane A₂-prostaglandin H₂ receptor
 TXA₂ = thromboxane A₂

Figure 3. Endothelium-derived vasodilatory factors and contractile factors (Luscher & Vanhoutte, 1986; Moncada *et al.*, 1991; Cohen & Vanhoutte, 1995; Kahonen *et al.*, 1996; Harrison, 1997; Kojda & Harrison, 1999; McIntyre *et al.*, 1999).

(McIntyre *et al.*, 1999). The other vasodilator products are probably of less importance than NO.

2.2.1. Vascular contractile responses

2.2.1.1. Angiotensin II

The vascular contractile response to Ang II is mediated via AT₁ receptors. The stimulation of AT₁ receptors in VSMCs leads to the activation of phospholipase A₂, C, and D, activation of voltage-dependent Ca²⁺ channels, inhibition of adenylate cyclase, and subsequently to an increase in intracellular [Ca²⁺] (de Gasparo *et al.*, 1995; Orlov *et al.*, 1993; Timmermans *et al.*, 1993). Ang II induces VSMC contraction also by stimulating the sympathetic nervous system centrally and peripherally through activation of the presynaptic AT₁ receptors (Palatini, 2001). In addition, because Ang II is a powerful stimulator of the potent vasoconstrictor endothelin-1 (ET-1) formation in VSMCs and endothelial cells (Imai *et al.*, 1992; Crawford *et al.*, 1994; Sung *et al.*, 1994), it can further increase vascular contractile response to Ang II.

2.2.1.2. Endothelin-1

Endothelins (ETs), including ET-1, ET-2, and ET-3, are peptides that possess characteristically sustained vasoconstrictor properties (for review, see Schiffrin, 2001). The predominant member of the ET family appears to be ET-1. ET-1 is a peptide containing 21 amino acids; it is synthesized from larger precursor, preproendothelin, via the formation of proendothelin, which is cleaved to big endothelin and then catalyzed by endothelin-converting enzyme, and then further converted to an active peptide (for review, see Schiffrin, 2001). Ang II is a powerful stimulator of endothelin-1 (ET-1) formation in VSMCs and endothelial cells (Imai *et al.*, 1992; Crawford *et al.*, 1994; Sung *et al.*, 1994). Moreover, production and release of ET-1 also occurs in response to many other stimuli, including adrenaline, cytokines, free radical, and physical factors such as stretch, hypoxia, and low shear stress (Kuchan & Frangos, 1993; Luscher & Noll, 1995; Haynes & Webb, 1998; Rothermund & Paul, 1998). Eighty percent of ET-1 is secreted

on the basal side of endothelial cells, resulting in concentrations within the vascular wall 100-fold more than the levels in plasma (Wagner *et al.*, 1992). ET-1 is one of the most active pressor substances discovered (for review, see Masaki, 1995). The role of ET-1 in the regulation of blood pressure in Ang II-induced hypertension is still unclear (Li *et al.*, 1996; d'Uscio *et al.*, 1997; Rajagopalan *et al.*, 1997; Hocher *et al.*, 1999; Andreis *et al.*, 2000). However, clinically, the fact that the endothelin receptor antagonist bosentan significantly lowers blood pressure in patients with essential hypertension suggests that endothelin may contribute to elevated blood pressure in such patients (Krum *et al.*, 1998). Endothelin antagonists even provide a therapeutic approach beyond mere blood pressure lowering in hypertensive animals with cardiovascular diseases (for review, see Schiffrin, 2001).

The vascular effects of ET-1 are mediated through two G-protein receptor subtypes, namely ET_A and ET_B. The ET_A receptor has a high affinity for ET-1, is present in smooth muscle cells and cardiomyocytes, and predominantly mediates contraction (Rothermund & Paul, 1998). Activation of ET_A receptors on VSMCs increases intracellular calcium levels by activation of phospholipase C, leading to prolonged vasoconstriction. The ET_B receptor is equally sensitive to the three ET isoforms, and is present both in the endothelial cells, where it induces the release of NO, PGI₂, and perhaps other vasodilators—resulting in vasodilatation, and in VSMCs, where it mediates vasoconstriction (Clozel *et al.*, 1992; Luscher *et al.*, 1993a; Masaki, 1998). The majority of endothelin receptors are of the ET_A subtype and are localized in the VSMCs.

Besides its direct vasocontractile properties, ET-1 can also potentiate the vasocontractile response to other vasoconstrictors such as NA, and it may also provoke the release of free radicals and COX-derived vasoconstrictor factors (Luscher *et al.*, 1993b; De Artinano & Gonzalez, 1999). In addition, ET-1 also has inotropic, trophic, and mitogenic properties, influences the homeostasis of salt and water, alters central and peripheral sympathetic activity, and stimulates the RAS and the sympathetic nervous system (Yamamoto *et al.*, 1992; Haynes & Webb, 1998; Moreau, 1998).

In short, ET-1 appears to be a predominant member of the ET family, and it has a potent direct or indirect vasoconstrictive property via the ET_A receptor in VSMCs. Ang II is a powerful stimulator of ET-1 formation in the vascular wall.

2.2.1.3. Noradrenaline

Noradrenaline (NA) is often used as a precontracting agent in the study of endothelial-dependent and -independent vascular relaxation responses. NA induces smooth muscle cell contraction by stimulating α_1 -adrenoceptors, which causes a rapid calcium release from the sarcoplasmic reticulum and prevents calcium reuptake. NA also depolarizes the arterial smooth muscle cells and consequently activates voltage-operated Ca^{2+} channels in the plasma membrane of the smooth muscle, leading to an increased influx of Ca^{2+} (Nelson *et al.*, 1990). The sustained contractions result from calcium influx through the receptor-operated calcium channels (Johns *et al.*, 1987; Karaki & Weiss, 1988).

2.2.1.4. Potassium chloride

Any increase in extracellular potassium chloride (KCl) causes vascular contraction mainly by inhibition of Na^+ , K^+ -ATPase, leading to sodium leakage to the smooth muscle cells, which in turn causes membrane depolarization with influx of Ca^{2+} via the voltage-dependent Ca^{2+} channels (Mulvany, 1985; Karaki & Weiss, 1988). Thus the inconsistent arterial contraction in response to KCl in different forms in experimental hypertension (Bohr & Webb, 1984) may reflect the functional changes in Na^+ , K^+ -ATPase. Furthermore, VSMC depolarization liberates endogenous noradrenaline from vascular sympathetic nerve endings, which also participates in the contractile response (Fouda *et al.*, 1991).

2.2.2. Vascular relaxation responses

2.2.2.1. Endothelium-dependent vascular relaxation

Acetylcholine (ACh) is the endogenous neurotransmitter at cholinergic synapses and neuroeffector junctions in the central and peripheral nervous system. Its actions are mediated through nicotinic and muscarinic cholinergic receptors, which transduce signals via distinct mechanisms. Acetylcholine dilates vascular beds by stimulation of muscarinic receptors, primarily of the M_3 subtype (Bruning *et al.*, 1994; Caulfield &

Birdsall, 1998), despite the lack of apparent cholinergic innervation of most arteries. The muscarinic receptors responsible for relaxation are located on the endothelial cells of the vasculature. When these receptors are stimulated, the endothelial cells release several endothelium-derived factors, the most prominent of these being NO, prostacyclin (PGI₂), and the endothelium-derived hyperpolarizing factor (EDHF) (Busse R & Fleming I, 1993).

Nitric oxide

In 1980, Furchgott and Zawadzki first reported the endothelium-derived relaxing factor. Later, endothelium-derived relaxing factor was recognized as NO (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). NO is a small, gaseous, easily diffusible, biologically extremely active compound which plays a critical role in control of vascular tone. A normal endothelium constantly releases small amounts of NO. Extra NO is released in response to physiological stimuli such as increased shear stress and oxidative stress, and to substances such as ACh, bradykinin, histamine, thrombin, ADP, ATP, and the substance P.

NO is constantly synthesized from the endothelial cells by NO synthases (NOS) in response to ACh via muscarinic-receptor stimulation (for review, see Stuehr, 1999). Three isoforms of NOS have been isolated: constitutive NOS, which includes endothelial NOS (eNOS, also called NOS III) and neuronal NOS (nNOS, also called NOS I), and inducible NOS (iNOS, called NOS II). eNOS plays a central role in the regulation of blood pressure (Ohashi *et al.*, 1998). NO is mainly synthesized from L-arginine by eNOS, which is competitively inhibited by L-arginine analogues such as N^G-nitro-L-arginine methyl ester (L-NAME) (for review, see Hobbs *et al.*, 1999). However, in the absence of the substrate L-arginine or the cofactor tetrahydrobiopterin, eNOS and iNOS may produce O₂⁻ instead of NO and thereby directly contribute to oxidative stress (Vasquez-Vivar *et al.*, 1998; Xia *et al.*, 1998; Vasquez-Vivar & Kalyanaraman, 2000).

NO is unstable but can form more stable nitrosothiols, particularly with a cysteine residue in globin; this allows red cells to perform in the same way as endothelial cells. NO is inactivated by combination with the heme of hemoglobin or by oxidation to nitrite

and nitrate, which are excreted in the urine. Under oxidative stress, NO rapidly reacts with O_2^- to produce the toxic peroxynitrite anion ($ONOO^-$) and other reactive nitrogen species. The reaction between NO and O_2^- occurs more rapidly than does O_2^- neutralization by superoxide dismutase (SOD) (Pryor & Squadrito, 1995). NO relaxes vascular smooth muscle by activation of guanylate cyclase, which leads to the increased production of cyclic guanosine 3',5'-monophosphate (cGMP), and further reduces concentration of intracellular calcium. *In vivo*, NO is destroyed mainly by superoxide anion, and extremely reactive peroxynitrite is formed (for review, see Stuehr, 1999).

In summary, NO plays a critical role in the regulation of vascular tone. Endothelial dysfunction in hypertension is mainly due to impaired NO synthesis and/or inactivation of endothelium-derived NO by ROS. It appears that the balance between nitric oxide and superoxide is even more important than the absolute levels of either alone.

Prostacyclin and other prostanoids

Prostacyclin (PGI_2), the major vasodilatory prostanoid produced in endothelial cells, is released in response to shear stress, hypoxia, or to substances that stimulate NO formation (Gryglewski, 1995; Luscher & Noll, 1995). Prostacyclin relaxes vascular smooth muscle by activation of adenylate cyclase, and thus leads to increased production of cyclic adenosine monophosphate (cAMP). Prostacyclin is synthesized from arachidonic acid by COX. Recently, it has been argued that in endothelium COX-2 is mainly responsible for prostacyclin production (Kosonen *et al.*, 1998; FitzGerald & Patrono, 2001). The synthesis of PGI_2 is inhibited by non-steroidal anti-inflammatory drugs (Smith & Willis, 1971; Vane, 1971; Hawkey, 2001).

In addition to the vasodilatory prostanoids, endothelial cells also produce vasoconstrictive prostanoids such as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin H_2 (PGH_2), or thromboxane A_2 (TXA_2) (Matrougui *et al.*, 1997; Kagota *et al.*, 1999; Zhou *et al.*, 1999). It has been proposed that in hypertensive rat endothelium, the production of a lipoxygenase can inhibit the conversion of vasoconstrictor PGH_2 to PGI_2 , resulting in the accumulation of vasoconstrictor PGH_2 (Cohen, 1995). Hypertension therefore seems also to be associated with an imbalance in the endothelial production of COX-derived

vasodilators and vasoconstrictors in favor of vasoconstriction. Interestingly, in contrast to the inhibition of NO synthase, the blockade of COX by non-steroidal anti-inflammatory drugs did not lower but even increased systolic blood pressure (Busse R & Fleming I, 1993; Muscara *et al.*, 2000). Therefore, contractile prostanoids synthesized by COX do not appear to participate in the development of hypertension (Taddei *et al.*, 1997).

Taken together, It appears that hypertension involves an imbalance in the endothelial production of vasodilatory and contractile prostanoids in favor of contractile prostanoids.

Endothelium-derived hyperpolarizing factor (EDHF)

The endothelium-mediated relaxation that remains resistant to NOS and COX inhibition is thought to be mediated by EDHF (Cohen & Vanhoutte, 1995). The nature of EDHF remains, however, unclear. It may be a cytochrome P450-derived arachidonic acid epoxide, e.g., 11,12 EET (Fisslthaler *et al.*, 1999). Current data support the concept that the vascular relaxation elicited by EDHF is mediated by the opening of Ca^{2+} -regulated K^{+} channels in the VSMCs (for review, see Feletou & Vanhoutte, 1999). The action of EDHF can be inhibited by the K^{+} -channel blocker tetraethylammonium (TEA) or by a family of venom-derived peptides such as charybdotoxin and iberiotoxin, or by depolarization of the smooth muscle cell membrane with high potassium concentrations (Adeagbo & Triggle, 1993).

NO has been shown to inhibit both the production and action of EDHF (Bauersachs *et al.*, 1996; McCulloch *et al.*, 1997). In physiological conditions, the contribution of EDHF to endothelium-dependent vasodilation is difficult to assess; EDHF-mediated dilatation can be observed only after inhibition of NO synthase (Bauersachs *et al.*, 1996). Therefore, in the presence of impaired NO availability, a hyperpolarizing factor seems to act as a compensatory pathway to sustain endothelium-dependent relaxation (for review, see Taddei *et al.*, 2001).

2.2.2.2. Endothelium-independent vascular relaxation

NO donors, e.g., nitroglycerin, sodium nitroprusside (SNP), S-nitroso-N-acetyl-D,L-penicillamine (SNAP), 3-morpholinosydnonimine (SIN-1), mesoionic 3-aryl substituted oxatriazole-5-imine derivatives (GEA compounds), share a common feature in producing NO-related activity when applied to biological systems. They mimic an endogenous NO response by generating NO or related N-oxide species in a controlled manner and thus offer a useful tool for pharmacological studies related to NO (for review, see Feelisch, 1998). Clinically, NO donors are used in hypertensive emergencies and angina. They induce vascular smooth muscle relaxation by stimulating soluble guanylate cyclase to produce cGMP in the smooth muscle cells—even when endothelium is absent—or in the presence of L-NAME, an eNOS inhibitor. NO donors also show the capacity to hyperpolarize vascular smooth muscle of the guinea pig or rat (Tare *et al.*, 1990). Endothelium-independent relaxation by SNP is used to reflect the sensitivity of vascular smooth muscle to exogenous NO.

2.2.3. Arterial tone in angiotensin II-induced hypertension

In Ang II-induced hypertension, impaired endothelial function is a major change in vascular tone (Harrison, 1997). Ang II-induced endothelial dysfunction has been shown to be primarily related to increased superoxide formation within the endothelium, the media, and/or adventitial layer (Harrison, 1997; Kojda & Harrison, 1999; McIntyre *et al.*, 1999). Impaired endothelium-dependent vascular relaxation in Ang II-induced hypertension has been partially improved by preincubation with SOD (Rajagopalan *et al.*, 1996; Wattanapitayakul *et al.*, 2000). Reaction between NO and superoxide (O_2^-), resulting in highly active peroxynitrate ($OONO^-$) anions, may be one of the major causes of impaired endothelium-dependent relaxation in Ang II-induced hypertension.

Endothelium (Suzuki *et al.*, 1995; White *et al.*, 1996; Swei *et al.*, 1997) and the smooth muscle cells (Rajagopalan *et al.*, 1996) are the predominant sources of vascular O_2^- production in several disease states. The serum level of 8-isoprostaglandin $F_{2\alpha}$, a potent vasoconstrictor and antinatriuretic arachidonic acid metabolite, provides the index of oxidative stress *in vivo* (for review, see Romero & Reckelhoff, 1999). The O_2^- formation induced by Ang II is mainly mediated by NADPH oxidase (Laursen *et al.*, 1997; Di Wang *et al.*, 1999). Ang II induces the components of NADPH oxidase at mRNA level. Ang II

stimulates superoxide generation by enhancing the activity of the NADPH oxidase *in vitro* (Griendling *et al.*, 1994) and *in vivo* (Rajagopalan *et al.*, 1996).

Besides NADPH oxidase, uncoupled NOS is also involved in Ang II-induced oxidative stress. It is clear that in both eNOS and nNOS, in the absence of the substrate L-arginine or the cofactor tetrahydrobiopterin, heme reduction in the enzyme uncouples NOS, thereby producing O_2^- rather than NO (Vasquez-Vivar *et al.*, 1998; Xia *et al.*, 1998). In atherosclerosis (Oelze *et al.*, 2000) and diabetes (Hink *et al.*, 2001), increased oxidative stress and endothelial dysfunction are associated with upregulated but dysfunctional uncoupled eNOS. NADPH oxidase-induced O_2^- by Ang II may trigger eNOS uncoupling, leading to impaired NO/cGMP signaling and to endothelial dysfunction (Mollnau *et al.*, 2002).

ROS generated by the inflammatory cells are also able to reduce the bioactivity of endothelium-derived NO (McIntyre *et al.*, 1999; Romero & Reckelhoff, 1999). Ang II-induced vascular inflammatory response is therefore one of the explanations for Ang II-induced endothelial dysfunction. Endothelial dysfunction is an important factor in the relationship between inflammation and cardiovascular diseases (for review, see Blake & Ridker, 2001).

In addition, evidence is accumulating to indicate that increased vasocontractile prostaglandins, e.g., prostaglandin H_2 , is involved in Ang II-induced impaired endothelium-dependent vascular relaxation by ACh (Didion *et al.*, 2000). However, whether or not impaired EDHF is involved in Ang II-induced endothelial dysfunction remains to be clarified. Ang II induces endothelial dysfunction differently in different vessels, species, and under differing experimental conditions.

Endothelium-independent relaxation by SNP in Ang II-induced hypertension is normally unaltered, indicating that the sensitivity of VSMCs to exogenous NO is not impaired (Arnet *et al.*, 1999). The vascular responses to Ang II, ET-1, and NA in Ang II-induced hypertensive rats are inconsistent and depend on experimental conditions, and on the animal model and the vascular bed studied. In general, attenuated responses to Ang II and ET-1 have been reported in aortic arteries of mREN2 rats and Ang II-infused

hypertensive rats (d'Uscio *et al.*, 1997; Arnet *et al.*, 1999), suggesting downregulation of AT₁ and ET_A receptors expression or of their signal transduction mechanisms or of both.

It seems, therefore, that endothelial dysfunction resulting predominantly from the reaction between NO and O₂⁻ plays an important role in Ang II-related vascular dysfunction. The increased O₂⁻ caused by Ang II is mainly produced by NADPH oxidase and by uncoupled eNOS. In addition, ROS generated by the inflammatory cells and increased contractile prostaglandins are also involved. However, the contractile responses to Ang II, ET-1, and NA are inconsistent in Ang II-induced hypertension.

2.3. Vascular inflammatory response

The Inflammatory process is a reaction of blood vessels leading to the accumulation of fluid and leukocytes in extravascular tissues. It has been divided into acute and prolonged phases. Acute inflammation is a stereotypical response to all forms of injury, and prolonged inflammation is due to persistent stimuli such as repeated infection or self-perpetuating immunological responses. Inflammation universally occurs in three steps: 1) increase in vascular permeability, 2) infiltration of blood cells, 3) tissue reconstitution and cell growth. The persistent recruitment of leukocytes is critical for the pathogenesis of the inflammatory response, which is considered to contribute to the vascular and tissue injury.

2.3.1. Recruitment of leukocytes

Leukocytes, e.g., granulocytes, monocytes/macrophages, and lymphocytes, are bone-marrow-derived cells, which circulate in the blood in a low adhesive state before migrating into tissues. Leukocytes are important in the defense against invading microbes and participate in immune functions and wound repair. Adhesion to other cells and to the extracellular matrix is a fundamental feature of leukocyte physiology. An important prerequisite for an inflammatory response is the trafficking of leukocytes from the blood to the site of inflammation, including three steps; rolling, adhesion, and

transmigration. Many factors responsible for leukocyte adhesion and concurrent extravasation have been characterized, e.g., cellular adhesion molecules, cytokines, transcription factor NF- κ B, and oxidative stress.

2.3.1.1. Cellular adhesion molecules

Leukocyte adhesion molecules play a critical role in facilitating migration of leukocytes from the blood to extravascular sites of inflammation. Three major families of leukocyte adhesion molecules exist, namely selectins, immunoglobulin superfamilies, and integrins. Other adhesion molecules are also involved in this process, e.g., cadherins and mucin-like molecules (Carlos & Harlan, 1994; D'Amico & Malavasi, 1995).

The migration of leukocytes into an inflammatory site involves a series of interdependent and closely regulated adhesive interactions between leukocytes and the vascular endothelium (Ley & Tedder, 1995). The first of these interactions is mediated predominantly by the selectins, which are specialized in capturing free-flowing leukocytes from the blood (Tedder *et al.*, 1995). The integrins are expressed in leukocytes and bind to the immunoglobulin superfamily, e.g., intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1), which are located on endothelial cells and thus mediate leukocyte firm adherence and also transduce signals from the extracellular matrix and the cell membrane to the inside of the cell (for review, see Springer, 1990). Leukocytes adhere tightly but reversibly to endothelium and transmigrate through the endothelium to the sites of inflammation.

2.3.1.2. Cytokines

Cytokines are peptides released from inflammatory tissue, connective tissue, and immune system cells; they act by autocrine and paracrine mechanisms. More than 50 cytokines have been identified. The cytokine superfamily includes interleukins, chemokines, colony-stimulating factors, growth factors, interferons, and the transforming growth factor (TGF) and tumor necrosis factor (TNF) families. The biologic responses to

cytokines result from cytokine ligation on specific cytokine receptors in target cells (Rang *et al.*, 1999).

Cytokines are important in recruiting leukocytes to sites of inflammation. They can activate and stimulate the generation of leukocyte adhesion molecules in endothelial cells. The primary pro-inflammatory cytokines are TNF- α and interleukin (IL)-1, which are released from macrophages and many other cells. IL-1 synergizes with TNF- α for many of the latter's actions, and its synthesis is stimulated by TNF- α . In addition, chemokine IL-8 is predominantly involved in an acute inflammatory response. The chemokine monocyte chemoattractant protein-1 (MCP-1) is involved predominantly in prolonged inflammatory responses (Rang *et al.*, 1999).

2.3.1.3. Nuclear factor kappa-B

Nuclear factor kappa-B (NF- κ B), a transcription factor responsible for the regulation of inflammatory genes, resides as inactive and binds to the inhibitory protein I- κ B in the cytoplasm of T lymphocytes, monocytes, macrophages, endothelial cells, and smooth muscle cells (Barnes & Karin, 1997).

NF- κ B is a pivotal transcription factor in prolonged immune responses and inflammatory disease (Barnes & Karin, 1997). NF- κ B activation has been associated with vascular inflammation and cell survival (Baichwal & Baeuerle, 1997). Increased NF- κ B activation is associated with vascular inflammation and cell survival (Baichwal & Baeuerle, 1997; Muller *et al.*, 2000c). NF- κ B is activated by numerous stimuli including cytokines, protein kinase C activators, viruses, immune stimuli, and above all, ROS. The activation of NF- κ B involves the phosphorylation and subsequent proteolytic degradation of the inhibitory protein I- κ B by specific I- κ B kinases. The free NF- κ B, a heterodimer consisting of 2 proteins, p50 and p65, passes into the nucleus, where it binds to κ B sites in the promoter region of numerous genes involved in inflammation.

2.3.1.4. Oxidative stress

In addition to the modulation of vascular tone mentioned above, oxidative stress is also related to cellular growth, hypertrophy, remodeling, lipid oxidation, and inflammation (Alexander, 1995; Kunsch & Medford, 1999; Griendling *et al.*, 2000a). Reactive oxygen species (ROS) can act as intracellular signaling molecules in vascular cells controlling growth, survival, and apoptosis (Mervaala *et al.*, 2000b). The specific response is dictated by the intracellular targets involved. The potential targets of ROS in endothelial and smooth muscle cells are extracellular signal-related kinases, stress-activated protein kinases, caspases, NF- κ B, and Akt kinase.

2.3.1.5. Nitric oxide

Nitric oxide (NO) is not only a main regulator of vascular tone, but also an important autoregulatory inhibitor of vascular inflammation (Collins, 1993; Peng *et al.*, 1998). It is a potent inhibitor of leukocyte adhesion to the microvascular endothelium and of thrombocyte aggregation (Gaboury *et al.*, 1993; Kosonen *et al.*, 1999; Kosonen *et al.*, 2000). The NO released by exogenous NO donors can reduce cytokine-induced endothelial cell activation (De Caterina *et al.*, 1995; Khan *et al.*, 1996), inhibit endothelial-leukocyte interaction (Kubes *et al.*, 1991; Gauthier *et al.*, 1994; Tsao *et al.*, 1997), and attenuate vascular inflammation (Marks *et al.*, 1995). NO inhibition of NF- κ B occurs, in part, via the induction and nuclear translocation of the NF- κ B inhibitor I κ B (Spiecker *et al.*, 1997). NO inhibits oxidative stress in the microvasculature, through its ability to scavenge O_2^- (Miles *et al.*, 1996). However, under oxidative stress, excess generation of NO contributes to the inflammatory response, in part by reacting with O_2^- to form a potent oxidant, peroxynitrite (ONOO $^-$)/peroxynitric acid (ONOOH) (Beckman *et al.*, 1990).

Endothelial-derived endogenous NO attenuates inflammatory processes (Garg & Hassid, 1989; Radomski *et al.*, 1990; Moncada *et al.*, 1991). Overexpression of eNOS attenuates smooth muscle proliferation and vascular inflammation in the injured rat carotid artery (von der *et al.*, 1995). In eNOS- and nNOS-deficient mice, basal leukocyte rolling and adhesion were elevated (Lefer *et al.*, 1999; Hickey *et al.*, 2001), suggesting that eNOS and nNOS has beneficial effects in ameliorating vascular inflammatory responses.

The precise role of iNOS in the vascular inflammatory response is still poorly understood. Mainly, iNOS increases leukocyte recruitment by modulating leukocyte function but not by regulating endothelial adhesion molecules (for review, see Hickey *et al.*, 2001). NO attenuates iNOS expression in macrophages, and VCAM-1 expression in endothelial cells and inhibits monocyte adhesion to endothelial cells (Peng *et al.*, 1998).

2.3.1.6. Prostanoids

Eicosanoids are implicated in the control of many physiological processes and are among the most important mediators and modulators of the inflammatory reaction. The principal eicosanoids are the prostanoids, e.g., prostaglandins and thromboxanes, and the leukotrienes. Eicosanoids are synthesized mainly from arachidonic acid, which is released from cell membrane phospholipids through the action of phospholipase A₂ and phospholipase C (Rang *et al.*, 1999). The free arachidonic acid is metabolized by several pathways—by one of two fatty acid cyclooxygenases which initiate the biosynthesis of the prostanoids, and by various lipoxygenases which initiate the synthesis of the leukotrienes, and by other pathways, such as the cyp 450 catalyzed pathway which initiates the synthesis of other compounds, for example, 11,12-, 14,15-epoxyeicosatetraenoic acids (EETs) (for review, see Capdevila *et al.*, 1992).

Prostanoids are local mediators of inflammation and modulators of physiological functions, including the maintenance of gastric mucosal integrity, the regulation of renal microvascular hemodynamics, renin release, and tubular electrolyte and water reabsorption (Moncada *et al.*, 1973; Velo *et al.*, 1973; Harris, 2000; Breyer & Harris, 2001). PGI₂ and TXA₂ are the major prostanoids in vascular endothelium and in platelets, respectively (Hamberg & Samuelsson, 1974; Gryglewski, 1995). The balance between PGI₂ and TXA₂ regulates interactions between platelets and vessel wall *in vivo* (Moncada *et al.*, 1976), whereas an imbalance between PGI₂ and TXA₂ may lead to vascular injury and thrombotic complications.

Two isoforms of COX exist, namely COX-1 and COX-2. COX-1 is a constitutive isoform constantly expressed in most tissues under physiological conditions. COX-1-derived prostanoids regulate mucosal integrity of the gastrointestinal tract, arterial tone, platelet

aggregation, and maintenance of perfusion in the kidneys (Eberhart & DuBois, 1995; DuBois *et al.*, 1998; Vane *et al.*, 1998). The other isoform, COX-2, is not constitutively expressed in most tissues but can be induced in endothelial cells, fibroblasts, smooth muscle cells, and macrophages by proinflammatory cytokines, endotoxin, tumor promoters, mitogens, and hypoxia (Mitchell *et al.*, 1995; Wu, 1995). However, COX-2 may be expressed constitutively in some tissues, e.g., the kidneys and central nervous system, vascular endothelium, and the female reproductive organs. At these sites, COX-2 activity and abundant prostanoids production is required for homeostatic and physiological processes (DuBois *et al.*, 1998; Harris, 2000; Breyer & Harris, 2001; Funk, 2001; Hinz & Brune, 2002). It still remains to be established whether COX-2 overexpression in the vasculature is deleterious or protective. As mentioned previously, induction of COX-2 in macrophages and fibroblasts may contribute significantly to vascular inflammation, injury, and development of fibrosis, whereas COX-2 overexpression in endothelial cells, leading to increased synthesis of vasoprotective prostacyclin, may represent a compensatory mechanism to defend against vascular injury (Wu, 1998a; FitzGerald *et al.*, 2001). It is suggested that in the early stage of inflammatory response, prostanoids are mainly produced by the constitutive COX-1 (Smith *et al.*, 2000). Later on, the induced COX-2 is the principle contributor to the production of inflammatory prostanoids (DuBois *et al.*, 1998; Herschman, 1999). The effects of prostanoids are mediated through stimulation of their respective receptors. The balance between different prostanoids is crucial for their physiological and pathological effects.

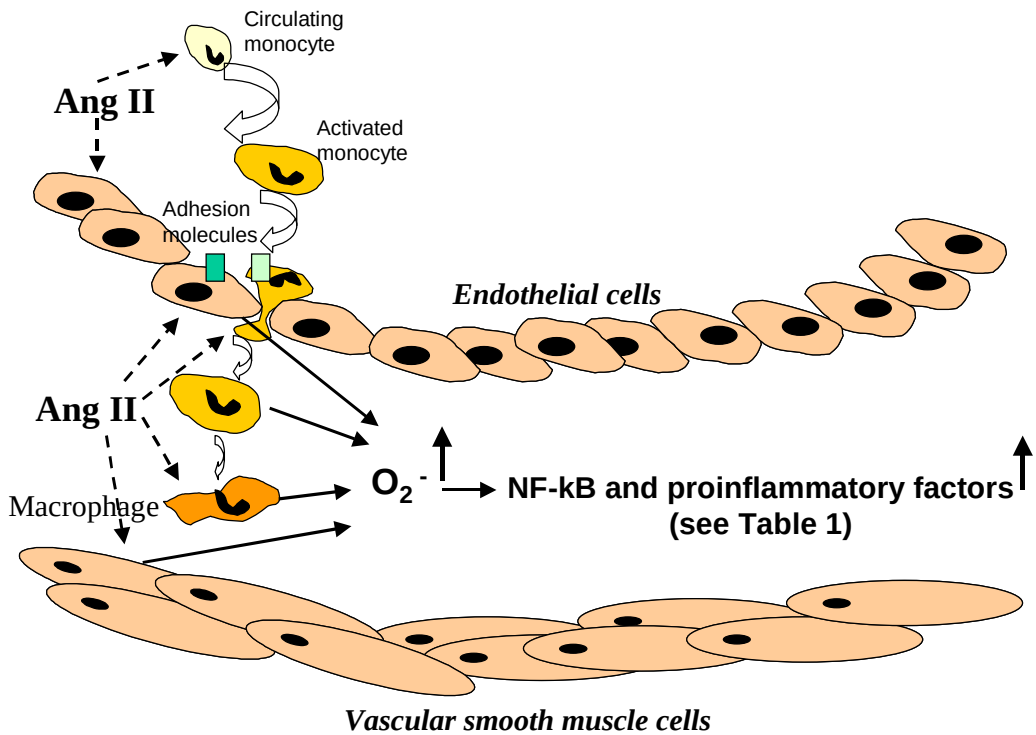
2.3.2. Inflammation caused by angiotensin II

Hypertension, smoking, diabetes, or elevated levels of low-density lipoprotein cholesterol are risk factors for vascular endothelial cell injury, and this injury initiates the inflammatory process (for review, see Ismail *et al.*, 1999). Vascular injury occurring during inflammation is a major contributing factor in the development of organ dysfunction.

The mechanisms of Ang II-induced vascular inflammation are not completely understood. Recent studies have demonstrated that Ang II may participate in the

inflammatory response by a direct activation of inflammatory cells or by the regulation of adhesion molecule and cytokine expression, via redox mechanisms and the NF- κ B pathway, contributing to the recruitment of inflammatory cells into the lesion (for review, see Ruiz-Ortega *et al.*, 2001b) (**Figure 4**).

Ang II induces the adhesion of monocytes and neutrophils to endothelial cells (Kim *et al.*, 1996; Krejcy *et al.*, 1996) through upregulation of P-selectin, ICAM-1, and VCAM-1 in vascular endothelial cells and through VSMCs *in vivo* and *in vitro* (Kim *et al.*, 1996; Tayeh & Scicli, 1998; Pastore *et al.*, 1999; Tummala *et al.*, 1999). Infusion of Ang II into rats causes hypertension accompanied by marked monocyte infiltration as well as VCAM-1 and MCP-1 expression in the aorta (Capers *et al.*, 1997; Tummala *et al.*, 1999). In VSMCs and in endothelial and mononuclear cells, Ang II increases MCP-1 (Hernandez-Presa *et al.*, 1997; Chen *et al.*, 1998; Ruiz-Ortega *et al.*, 1998), the main chemokine for monocyte/macrophages and interleukin-8 (IL-8) (Hernandez-Presa *et al.*, 1998; Ortego *et al.*, 1999), which are potent chemoattractants and activators of neutrophils. Ang II increases IL-6 production in vascular cells and in macrophages, and Ang II upregulates TNF- α and IL-6 gene expression (Han *et al.*, 1999; Nakamura *et al.*, 1999). ACE inhibitor and AT₁ antagonists diminish inflammatory cells, adhesion molecules, and also cytokine expression (Ruiz-Ortega *et al.*, 2001d). These data show that Ang II activates vascular and inflammatory cells to secrete proinflammatory mediators that help to recruit new mononuclear cells and can thus result in additional inflammatory response contributing to the progression of vascular damage.



Ang II = angiotensin II
 NF- κ B = nuclear factor kappa B
 O_2^- = superoxide

Figure 4. Mechanisms of angiotensin II-induced vascular inflammation (modified from Ruiz-Ortega *et al.*, 2001b).

Ang II-induced vascular inflammation is associated with increased formation of ROS in the VSMCs and myocytes (Romero & Reckelhoff, 1999; Griending *et al.*, 2000b). In VSMCs, the induction of MCP-1 and IL-6 by Ang II is dependent on the activation of NADPH (Chen *et al.*, 1998; Kranzhofer *et al.*, 1999b). Ang II increases VCAM-1 and MCP-1 expression by redox-related mechanisms. ROS contribute to TNF- α production and release in part via activation of COX (Guo *et al.*, 2001). ROS may act as signal transduction messengers for several important transcription factors, including NF- κ B and activator protein-1 (Sen & Packer, 1996).

NF- κ B, one of the most important targets of ROS in endothelial and VSMCs, is associated with vascular inflammation and cell survival (Baichwal & Baeuerle, 1997).

Emerging data suggest the potential role of NF- κ B as a mediator of the Ang II-induced inflammatory process. Earlier, Brasier *et al* (1990, 1994, 1996) showed that the angiotensinogen gene was transcriptionally activated during activation of NF- κ B. A later study showed that Ang II is responsible for directing monocyte infiltration by promoting NF- κ B activation and MCP-1 expression in a model of accelerated atherosclerosis in rabbits (Hernandez-Presa *et al.*, 1997). Infusion of Ang II into normal rats increases NF- κ B activity in the vessels and kidneys (Ruiz-Ortega *et al.*, 2000b). Ang II activates NF- κ B in several cell types, including VSMCs, and in endothelial, glomerular, tubular, and mononuclear cells (Ruiz-Ortega *et al.*, 2001a; Ruiz-Ortega *et al.*, 2001d). Ang II enhances MCP-1 mRNA expression and NF- κ B activity (Ruiz-Ortega *et al.*, 2000a). Several reports have indicated that ACE-inhibition and AT₁ receptor blockage reduce NF- κ B activity (Sen & Packer, 1996; Ruiz-Ortega *et al.*, 1998). The Ang II degradation product Ang III is also capable of activating NF- κ B in renal and mononuclear cells (Ruiz-Ortega *et al.*, 2000a; Ruiz-Ortega *et al.*, 2000b). Furthermore, Ang II activates NF- κ B through both the AT₁ and AT₂ receptors in VSMCs (Ruiz-Ortega *et al.*, 2000b; Wolf *et al.*, 2002), implying that Ang II-mediated proinflammatory renal and cardiovascular effects may not be totally antagonized by the currently increased clinical use of AT₁ receptor antagonists. Possible factors of Ang II-induced inflammatory responses are summarized in **Table 1**.

The interaction between Ang II and prostanoids still remains unclear. Ang II elevates the production of prostanoids in the kidney (Navar *et al.*, 1996; Purdy & Arendshorst, 1999), which buffer the Ang II-mediated decrease in renal blood flow (Chatziantoniou & Arendshorst, 1992a; Chatziantoniou & Arendshorst, 1992b; Jackson & Herzer, 1993). In contrast, ACE inhibitors and AT₁ receptor antagonists enhance the expression of COX-2 in animals kept on a low-salt diet (Wolf *et al.*, 1999). Ang II downregulates COX-2 expression in macula densa (Harris *et al.*, 2000).

Ang II also induces generation of iNOS (Mervaala *et al.*, 2000a; Park *et al.*, 2000). However, the role of iNOS in the Ang II-induced inflammatory response is still not completely understood.

Table 1. Potential proinflammatory factors of angiotensin II -induced vascular inflammation.

Proinflammatory factors	References	
ICAM-1, VCAM-1, and P-selectin	(Kim <i>et al.</i> , 1996; Grafe <i>et al.</i> , 1997; Mervaala <i>et al.</i> , 1999b; Pastore <i>et al.</i> , 1999; Tummala <i>et al.</i> , 1999; Park <i>et al.</i> , 2000; Piqueras <i>et al.</i> , 2000; Muller <i>et al.</i> , 2000c; Muller <i>et al.</i> , 2001b; Theuer <i>et al.</i> , 2002)	
$\alpha_5\beta_2$ and $\alpha_4\beta_1$ integrins	(Mervaala <i>et al.</i> , 1999b)	
MCP-1	(Hernandez-Presa <i>et al.</i> , 1997; Chen <i>et al.</i> , 1998; Hernandez-Presa <i>et al.</i> , 1998; Ruiz-Ortega <i>et al.</i> , 1998; Ortego <i>et al.</i> , 1999; Wu <i>et al.</i> , 2001; Theuer <i>et al.</i> , 2002)	
IL-1	(Wu <i>et al.</i> , 2001)	
IL-6	(Han <i>et al.</i> , 1999; Kranzhofer <i>et al.</i> , 1999b; Nakamura <i>et al.</i> , 1999; Mervaala <i>et al.</i> , 2000a; Schieffer <i>et al.</i> , 2000; Wu <i>et al.</i> , 2001)	
LTA (4)	(Ishizaka <i>et al.</i> , 1999)	
TNF- α , TNF- β	(Ruiz-Ortega <i>et al.</i> , 1998; Nakamura <i>et al.</i> , 1999; Wu <i>et al.</i> , 2001)	
PAI-1	(Theuer <i>et al.</i> , 2002)	
RhoA	(Piqueras <i>et al.</i> , 2000)	
NF- κ B, p65	(Kranzhofer <i>et al.</i> , 1999a; Kranzhofer <i>et al.</i> , 1999b; Muller <i>et al.</i> , 2000a; Park <i>et al.</i> , 2000; Dechend <i>et al.</i> , 2001; Ruiz-Ortega <i>et al.</i> , 2001a; Ruiz-Ortega <i>et al.</i> , 2001c; Theuer <i>et al.</i> , 2002)	
AP-1	(Park <i>et al.</i> , 2000; Piqueras <i>et al.</i> , 2000; Dechend <i>et al.</i> , 2001; Haxsen <i>et al.</i> , 2001; Ruiz-Ortega <i>et al.</i> , 2001a)	
p-ERK	(Park <i>et al.</i> , 2000)	
I κ B	(Muller <i>et al.</i> , 2001b)	
c-fos and c-jun	(Muller <i>et al.</i> , 2000a)	
ROS	(Griendling <i>et al.</i> , 1994)	
iNOS	(Mervaala <i>et al.</i> , 2000a)	
AP-1 = activator protein-1	p65 = NF- κ B subunit	TNF- β = necrosis factor-beta
ICAM-1 = intracellular adhesion molecule-1	PAI-1 = plasminogen activator inhibitor-1	VCAM-1= vascular cellular adhesion molecule
IL = interleukin	p-ERK = phosphorylated extracellular signal-regulated kinase	
I κ B = inhibitory protein of kappa B	Rho = small GTPase	
LTA (4) = leukotriene A (4)	ROS = reactive oxygen species	
MCP-1 = monocyte chemoattractant protein	TNF- α = tumor necrosis factor-alpha	
NF- κ B = nuclear factor kappa B		

To date, several compounds have been shown to be effective in inhibiting Ang II-induced inflammation and vascular injury (**Table 2**).

2.4. General characteristics of experimental models used in this study

A double transgenic rat (dTGR) harboring human renin and human angiotensinogen genes was generated by Ganten and Murakami in the early 1990's (Ganten *et al.*, 1992). In this model, the human RAS components do not interact with the endogenous rat RAS *in vivo* (Ganten *et al.*, 1992). These animals are very suitable for studying human renin inhibitors that do not influence rat renin. Hypertension and vascular damage in this model are dependent on local Ang II formation (Ganten *et al.*, 1992; Bohlender *et al.*, 1997). Endothelial adhesion molecule activation and perivascular leukocyte infiltration play a central role in the pathogenesis of Ang II-induced myocardial and renal damage in dTGRs (Luft *et al.*, 1999; Mervaala *et al.*, 1999b). Ang II induces perivascular inflammation in dTGRs even by blood pressure-independent mechanisms (Mervaala *et al.*, 1999b). Heterozygous dTGRs die of end-organ cardiac and renal damage by week 7 (Luft *et al.*, 1999).

The mREN2 rat, which harbors the mouse renin-2 gene, was developed in 1990 by Mullins *et al.* (1990). It is the first animal model of hypertension with the advantage of a defined genetic basis. In this model, mouse renin converts rat angiotensinogen with a 10-fold higher facility than does rat renin (Bohlender *et al.*, 1998).

Unlike dTGRs, the survival rate of heterozygous mREN2 rats is almost 100% at the age of 25 weeks. Fulminating hypertension in the presence of suppressed plasma RAS and high renin expression in extrarenal tissues suggests that activated extrarenal RAS may be responsible for the hypertensive phenotype (for review, see Lee *et al.*, 1996). The recruitment and infiltration of leukocytes into the subendothelium in mREN2 rats are in part mediated by pressure-independent AT₁ receptor pathways (Strawn *et al.*, 1999).

Table 2. Some drugs protecting against Ang II-induced inflammatory response.

Drugs	Pharmacological class	Animal model or cell type where tested	Reference
RO-65-7219	Human renin inhibitor	dTGRs	(Mervaala <i>et al.</i> , 1999b)
Cilazapril	ACE inhibitor	dTGRs	(Mervaala <i>et al.</i> , 1999b)
Losartan	AT ₁ receptor antagonist	SD rats infused by Ang II, human monocytic cells, human endothelial cells. dTGRs	(Kranzhofer <i>et al.</i> , 1999b; Mervaala <i>et al.</i> , 1999a; Kiarash <i>et al.</i> , 2001; Kramer <i>et al.</i> , 2002)
Valsartan	AT ₁ receptor antagonist	dTGRs, Ang II infused rats, VSMCs of mouse, two-kidney, 1-clip rats	(Mervaala <i>et al.</i> , 1999a; Muller <i>et al.</i> , 2000b; Hilgers <i>et al.</i> , 2001; Wu <i>et al.</i> , 2001; Wolf <i>et al.</i> , 2002)
Spironolactone	Aldosterone receptor antagonist	dTGRs	(Fiebeler <i>et al.</i> , 2001)
Bosentan	ET _A /ET _B receptor antagonist	dTGRs	(Muller <i>et al.</i> , 2000c)
Acetylsalicylic acid	COX-1/COX-2 inhibitor	dTGRs	(Muller <i>et al.</i> , 2001b)
NS-398	COX-2 inhibitor	Human cardiac fibroblasts	(Scheuren <i>et al.</i> , 2002)
PDTC	NF-κB inhibitor	dTGRs	(Muller <i>et al.</i> , 2000a; Theuer <i>et al.</i> , 2002)
Cyclosporin	Immunosuppressive agent	dTGRs	(Mervaala <i>et al.</i> , 2000a)
Cerivastatin	HMG-CoA inhibitor	dTGRs	(Park <i>et al.</i> , 2000; Dechend <i>et al.</i> , 2001)
Pioglitazone and rosiglitazone	PPARγ agonist	SD rats infused by Ang II	(Diep <i>et al.</i> , 2002)
Retinoids	RXR agonist	VSMCs of rats	(Haxsen <i>et al.</i> , 2001)
ACE = angiotensin-converting enzyme Ang II = angiotensin II AT ₁ = angiotensin II type 1 receptor COX = cyclooxygenase dTGR = double transgenic rat harboring human renin and human angiotensinogen genes ET _A = endothelin receptor A ET _B = endothelin receptor B HMG-CoA = 3-hydroxy-3-methylglutaryl-CoA NF-κB = nuclear factor kappa B PDTC = pyrrolidine dithiocarbamate			PPARγ = peroxisome proliferator-activated receptor gamma RXR = retinoid X receptor SD = Sprague-Dawley rat VSMCs = vascular smooth muscle cells

In 1976, the Goto-Kakizaki (GK) rat, a non-obese type II diabetic rat model, was developed by repeated inbreeding of glucose-intolerant Wistar rats (Goto *et al.*, 1976). It is an extensively studied model for type II diabetes, but not considered as a model for hypertension. The phenotype of GK is well characterized and exhibits several features typical of types II diabetes, such as impaired ontogenetic development of islet cells, abnormal insulin release after a glucose load, insulin resistance, a basal hyperinsulinemia, and abnormal glucose metabolism (Galli *et al.*, 1999; Janssen *et al.*, 1999). Recent studies have demonstrated that GK rats show several structural changes in the kidney similar to those observed in type II diabetes patients without overt kidney disease. These changes include thickening of the glomerular and tubular basement membranes, glomerular hypertrophy, and early podocyte damage, but not glomerulosclerosis or interstitial fibrosis (Galli *et al.*, 1999; Janssen *et al.*, 1999; Riley *et al.*, 1999).

2.5. General characteristics of drugs for prolonged treatment in this study

Angiotensin receptor antagonists

Losartan and valsartan are highly selective and potent AT₁ antagonists. Losartan is the prototype of this class of drugs, and was synthesized in 1990 (Nicholls *et al.*, 1994). Since then, numerous mainly pharmacokinetically different AT₁ receptor antagonists have been developed, and the literature about the cardiovascular effects of these compounds has expanded rapidly.

AT₁ receptor antagonists dose-dependently attenuate the pressor response to intravenous Ang II (Fierens *et al.*, 1999) and reduce blood pressure in animal models of hypertension. They also reduce cardiac hypertrophy and improve hemodynamics in animal models of heart failure (Mizuno *et al.*, 1994; Goa & Wagstaff, 1996; Hayashi *et al.*, 1997; Spinale *et al.*, 1997). They induce diuresis, increase salt excretion, reduce blood pressure, and reduce glomerulosclerosis in rats with prolonged renal failure

(Mackenzie *et al.*, 1997; Remuzzi *et al.*, 1998). Preliminary studies suggest that AT₁ receptor antagonists are at least as effective as ACE inhibitors but with a more favorable adverse-effect profile. AT₁ receptor antagonists reduce filtration and urinary albumin excretion (Gansevoort *et al.*, 1994; Remuzzi *et al.*, 1999). AT₁ receptor antagonists ameliorate Ang II-induced inflammatory response and end-organ damage by blood pressure-independent mechanisms (Mervaala *et al.*, 1999a; Mervaala *et al.*, 2000a).

The beneficial effect of AT₁ receptor antagonists is mainly attributed to the inhibition of the binding of Ang II to AT₁ receptor. Furthermore, any interference with the negative feedback control of renin release by AT₁ receptor antagonists results in the accumulation of Ang II (Bunkenburg *et al.*, 1991; Goldberg *et al.*, 1993). This elevates the stimulation of unopposed Ang II receptors such as the AT₂ receptor (Chung *et al.*, 1996). The stimulation of AT₂ receptors has been suggested to enhance the vascular production of NO (Wiemer *et al.*, 1993; Seyedi *et al.*, 1995). In addition, Ang (1-7), which is generated endogenously from Ang II and Ang I, has been found to release NO and vasodilatory PGs from the endothelium (Jaiswal *et al.*, 1992; Porsti *et al.*, 1994; Brosnihan *et al.*, 1996). The anti-inflammatory action of AT₁ receptor antagonists may be mediated via the NF- κ B pathway (Muller *et al.*, 2000c).

Cyclooxygenase inhibitors

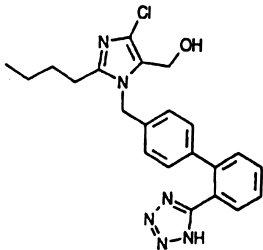
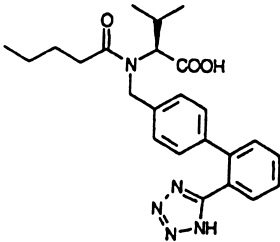
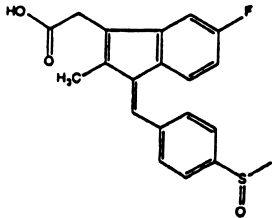
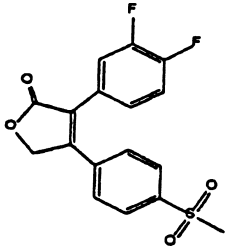
Sulindac, a non-selective COX-1/COX-2 inhibitor, and MF-tricyclic (3-(3,4-difluorophenyl)-4-(4-(methylsulfonyl)phenyl)-2(5H)-furanone), a selective COX-2 inhibitor, are non-steroidal anti-inflammatory agents. Sulindac is a pro-drug whose metabolite, sulindac sulfide, is a fairly potent inhibitor of both COX-1 and COX-2. (Myers *et al.*, 2001).

Unlike other COX-2 inhibitors, MF-tricyclic, a novel selective COX-2 inhibitor, does not affect renal blood flow in volume-depleted dogs (Black *et al.*, 1998). Selective COX-2 inhibitors have been shown to cause fewer gastrointestinal adverse events than do the previous traditional non-selective COX inhibitors (FitzGerald & Patrono, 2001). However, only limited information is available on the cardiovascular effects and the mechanism of these compounds and their interference with cardiovascular drugs at the

pharmacodynamic level. In fact, it has been claimed that the use of selective COX-2 inhibitors may be associated even with increased risk for cardiovascular events (myocardial infarction, stroke, death) (Mukherjee *et al.*, 2001). A recent study suggests that combination treatment with a COX-2 inhibitor and an AT₁ receptor antagonist may reduce COX-2 inhibitor-induced renal adverse effects (Kistler & Ambuhl, 2001). To date, few studies have been published on the effect of COX inhibitors in Ang II-induced inflammation. The non-selective COX-1/COX-2 inhibitor acetylsalicylic acid reduces Ang II-induced inflammatory response in dTGRs via the NF- κ B pathway (Muller *et al.*, 2001b).

The pharmacological classes and structural formulae of the drugs used for prolonged treatment in the present study are presented in **Table 3**.

Table 3. Pharmacological classes and structural formulas of drugs used for prolonged treatment.

Drug	Pharmacological class	Structural formula
Losartan	AT ₁ receptor antagonist	
Valsartan	AT ₁ receptor antagonist	
Sulindac	Non-selective COX-1/COX-2 inhibitor	
MF-tricyclic	Selective COX-2 inhibitor	

AT₁ =angiotensin II type I receptor
COX =cyclooxygenase

3. AIMS OF THE STUDY

Originally, Ang II, the key effector of RAS, was considered to be a circulating vasoactive hormone playing a central role in the regulation of blood pressure, aldosterone secretion, and salt reabsorption. In the past few years, this view has been modified; Ang II is now considered to be a growth factor that can also regulate cellular growth and cell proliferation. Recently, studies support the notion that Ang II may act as an immunomodulator capable of inducing inflammatory responses and vascular injury even by non-hemodynamic mechanisms.

In the present study, poorly understood mechanisms of Ang II-induced inflammation and vascular dysfunction were investigated, with concentration specifically on investigation of the following:

1. The mechanisms of Ang II-induced vascular dysfunction.
2. The involvement of increased RAS activity in the pathogenesis of hypertension, endothelial dysfunction, and vascular inflammation in spontaneously diabetic Goto-Kakizaki rats.
3. The role of COX-1/COX-2 in the pathogenesis of Ang II-induced inflammation and vascular dysfunction.

4. MATERIALS AND METHODS

4.1. Experimental animals

In Study I, 40 double transgenic rats harboring human renin and human angiotensinogen genes (dTGRs) and 10 age-matched normotensive Sprague-Dawley (SD) rats (Max Delbrück Center for Molecular Medicine, Berlin, Germany) were used.

Study II included 50 spontaneously diabetic Goto-Kakizaki (GK) rats and 20 age-matched nondiabetic Wistar rats purchased from Møllegaards Breeding Centre (Ejby, Lille Skensved, Denmark).

For Studies III and IV, 60 heterozygous transgenic rats harboring the mouse renin-2 gene (mREN2) rats and 10 age-matched normotensive SD rats were purchased from Møllegaards Breeding Centre (Ejby).

A summary of experimental animals and the design of the studies is presented in **Table 4**.

The rats were housed five animals per cage in a standard experimental animal laboratory (illuminated from 6.30 a.m. until 6.30 p.m.; temperature $22\pm1^{\circ}\text{C}$). They had free access to tap water and rat chow during the experiment.

4.2. Drug treatments

Commercially available standard rat chow served as the control diet (I-IV). In Study I, the AT_1 receptor antagonist valsartan was dissolved in 0.5% carboxymethyl cellulose and administered by gavage once a day for 3 weeks; control dTGRs and SD rats received the same volume of vehicle.

Table 4. Experimental animals and experimental design of the studies.

Study	Strain and gender	Age at the beginning (weeks)	Experimental group and number of animals in each group	Treatment period (weeks)	Major measurements
I	dTGRs SD rats Male	4	dTGRs (n=25) dTGRs+valsartan (n=15) SD rats (n=10)	3	SBP, vascular function in the renal artery, cardiac hypertrophy, proteinuria, renal XOR activity, serum prostaglandin F _{2α} , urinary NO _x
II	GK rats Wistar rats Male	8	GK rats (n=20) GK rats+6% NaCl (n=10) GK rats+valsartan (n=10) GK rats+6% NaCl+valsartan (n=10) Wistar rats (n=20)	8	SBP, DBP, MAP, oral blood glucose test, cardiac hypertrophy endothelial function in the mesenteric artery, albuminuria, serum prostaglandin F _{2α} , urinary NO _x , ICAM-1 and ED ₁ -positive cell expression, and morphological changes in the kidneys
III	mREN2 rats SD rats Male	5-6	mREN2 rats (n=10) mREN2 rats+MF-tricyclic (n=10) mREN2 rats+sulindac (n=10) mREN2 rats+losartan (n=10) mREN2 rats+MF-tricyclic+losartan (n=10) mREN2 rats+sulindac+losartan (n=10) SD rats (n=10)	8	SBP, cardiac hypertrophy, serum LDH, albuminuria, ED ₁ -positive cell and COX-1 and COX-2 expression, and morphological changes in the heart and the kidneys
IV	mREN2 SD rats Male	5-6	mREN2 rats (n=10) mREN2 rats+MF-tricyclic (n=10) mREN2 rats+sulindac (n=10) mREN2 rats+losartan (n=10) SD rats (n=10)	8	SBP, endothelial function in the aortic and renal arteries, urinary NO _x , ET _A , and ET _B receptors expression in the aorta
COX	= cyclooxygenase		ET _B	= endothelin-1 receptor B	
dTGR	= double transgenic rat harboring human renin and human angiotensinogen genes		GK	= Goto-Kakizaki rat	
DBP	= diastolic blood pressure		ICAM-1	= intracellular adhesion molecule-1	
ED ₁	= monoclonal antibody recognizing rat monocytes and macrophages		LDH	= serum lactate dehydrogenase	
ET _A	= endothelin-1 receptor A		mREN2	= mouse transgenic rat harboring the mouse renin-2 gene	
			MAP	= mean arterial blood pressure	
			NO _x	= nitrite plus nitrate	
			SBP	= systolic blood pressure	
			SD	= Sprague-Dawley rat	
			XOR	= xanthine oxidoreductase	

In Study II-IV, compounds for oral administration were mixed directly with food. An HS diet (NaCl 6%) (II) was made by adding sodium chloride (Sigma-Aldrich Corp. St. Louis, MO, USA) to the control diet. Drug concentration in the rat chow was calculated based on the fact that food consumption in adult rats is usually 20-25 g/day. The route of administration and doses of drugs are given in **Table 5**.

Table 5. Route of administration and doses of drugs.

Study	Drug	Administration	Concentration in the rat chow (mg/kg)	Estimated daily dose (mg/kg)
I	Valsartan	Gavage	0	30
II	Valsartan	Mixed with diet	117	10
III-IV	Losartan	Mixed with diet	467	40
III-IV	MF-tricyclic	Mixed with diet	163	14
III-IV	Sulindac	Mixed with diet	163	14

4.3. Measurement of blood pressure and heart rate

Tail-cuff method

Systolic blood pressure and heart rate of the pretrained rats were measured with a tail-cuff analyzer (I-IV) (Apollo-2AB, Blood Pressure Analyzer, Model 179-2AB, IITC Life Science, Woodland Hills, CA, USA). Before measurement, each rat was warmed at 29°C for 30 minutes to get detectable pulsations from its tail artery. Systolic blood pressure was determined by the arithmetic mean of the three consecutive results without disturbances of the signal. The heart rate was obtained during blood pressure measurement.

Radiotelemetric method

In Study II, systolic blood pressure, diastolic blood pressure, and mean arterial blood pressure of control rats were measured by radiotelemetry also (Dataquest IV telemetry system, Data Sciences International, St. Paul, MN, USA), as described previously (Vaskonen *et al.*, 2002). Briefly, 12-week-old rats were anesthetized with midazolam (5 mg/kg i.p.), fentanyl (0.3 mg/kg, i.p.), and fluanisone (10 mg/kg i.p.), and the flexible catheter of the transmitter was surgically secured in the abdominal aorta just below the renal arteries, aimed against the blood flow. The transmitter was sutured to the abdominal wall. The rats were given buprenorfin (0.1 mg/kg s.c.) for the first two postoperative days. They were housed individually after the implantation and were allowed to stabilize for 2 weeks, unrestrained and free to move in their cages. The data were collected every 5 minutes for 10 seconds for each rat. The values were calculated with the Dataquest software.

4.4. Metabolic studies

The rats (during the last week of Study I, and every second week of Study II-IV) were housed individually in metabolic cages where they had free access to tap water and chow. Food intake and consumption of water were recorded, and urine was collected over 24-h periods. Urine volumes were measured and samples stored at -80 °C until the biochemical determinations were performed.

4.5. Collection of blood and tissue samples

At the end of the experiment, the rats were anesthetized with CO₂/O₂ (70/30%) (AGA, Riihimäki, Finland) and then decapitated. Blood samples were taken into chilled tubes with or without ethylenediaminetetraacetic acid (EDTA) (4.5 mM) as anticoagulant and centrifuged at +4°C for 15 minutes; then plasma and serum were stored at -80°C. The heart and kidneys were removed, washed with ice-cold saline, blotted dry, and weighed. The ratio of the heart weight to body weight was calculated as an index of cardiac hypertrophy. Tissue samples were snap-frozen in liquid nitrogen, and samples for

immunohistochemistry (III) and autoradiography (I, IV) in isopentane (-35°C), then all were stored at -80°C until assayed. Tissue samples for morphology were fixed in 4% buffered paraformaldehyde (I, II) or 10% formaline (III) at room temperature.

4.6. Arterial responses

The superior mesenteric artery, abdominal aorta, and renal artery were carefully excised and cleaned of adherent connective tissue. Three-mm sections of mesenteric (II) and abdominal aortic (IV), and 2 to 3-mm section of renal arteries (I, IV) were used. The endothelium-intact arterial rings were stretched on stainless steel hooks and mounted in an organ bath chamber in Krebs-Ringer buffer (pH 7.4) of the following composition (mM): NaCl (119.0), NaHCO₃ (25.0), glucose (11.1), CaCl₂·2H₂O (1.6), KCl (4.7), KH₂PO₄ (1.2), MgSO₄·7H₂O (1.2), which was aerated with 95% O₂ and 5% CO₂. The rings were equilibrated for one hour at +37°C at a resting tension of 1.5 g. The force of contraction was measured with an isometric force-displacement transducer and registered with a polygraph (FTO3C transducer, Model 7C8 Polygraph; Grass Instrument Co., Quincy, MA, USA).

The presence of intact endothelium in the vascular preparation was confirmed by observing the relaxation response to 1 µM acetylcholine (ACh) in rings precontracted by 1 µM noradrenaline (NA) or by 0.1µM NA (renal artery in Study I). Cumulative concentration contractile response curves were determined for NA and endothelin-1 (ET-1). Because of the rapid development of tachyphylaxis (Khairallah *et al.*, 1966), Ang II was given only as a single concentration (0.1 µM). Cumulative relaxation responses to ACh and sodium nitroprusside (SNP) were examined after precontraction with 1 µM NA for mesenteric and aortic arteries, and 0.1µM NA for the renal artery. To analyze the components of endothelium-dependent vascular relaxation, COX and /or NOS were inhibited with diclofenac (3µM) and/or L-NAME (0.1mM), respectively.

4.7. Morphology

The samples for histology were fixed with 4% buffered paraformaldehyde (I, II) or 10% formaline (III) for 24 hours then dehydrated and embedded in paraffin. Sections (2-3 μm) were cut with a microtome (Leitz 1512). The sections were deparaffinized and rehydrated before being stained with Masson's trichrome. The slides were examined in a blinded fashion.

4.8. Immunohistochemistry

For tissue ED₁ (II, III) and ICAM-1 (II) expression, frozen kidneys (II), or formalin-fixed kidneys and hearts (6 μm) were processed and semiquantitative scoring was performed as described in detail previously (Mervaala *et al.*, 1999b). Monoclonal antibodies: anti- ED₁ (Serotec, Oxford, England) and ICAM-1 (1A29, R&D Systems, Inc. Minneapolis, MN, USA) were used. The sections were counterstained with hematoxylin-eosin before being examined under a light microscope.

4.9. Autoradiography

Frozen kidney sections (I, II) or aorta sections (IV) (20 μm thick) were cut with a cryostat at -17°C, thaw-mounted onto Super Frost Plus slides, dried in a desiccator under reduced pressure at 4°C overnight, and stored at -80°C in silica gel until further processing for autoradiographic studies. ACE, angiotensin receptors (AT₁ and AT₂), neutral endopeptidase (NEP), and ET_A and ET_B receptors were quantified by *in vitro* autoradiography as described previously (Kohzuki *et al.*, 1991).

4.10. Reverse transcription-polymerase chain reaction (RT-PCR)

Frozen hearts and kidneys were used for the measurement of gene expression. Renal AT₁ receptor mRNA (I) was measured by real time-RT-PCR (ABI's Prism 7700 Sequence Detection System, Perkin Elmer, Corporation, Wellesley, MA, USA) according to the instructions of the TaqMan EZ RT-PCR TaqMan kit protocol. The following RT-PCR primers and TaqMan-probe for GAPDH (housekeeping gene) and AT₁ receptor were used: GAPDH forward, AAGCTGGTCATCAATGGGAAAC; GAPDH reverse, ACCCCATTTGATGTTAGCGG; GAPDH probe, CATCACCATCTTCCAGGAGCGCGC-GAT, FAM (6-carboxytetrafluorescein) and TAMRA (quencher) labeled; AT₁ receptor forward, CCATCGTCCACCCAATGAAG; AT₁ receptor reverse, TGCAGGTG-ACTTTGGCCAC; and AT₁ receptor probe, FAM-TCGCCTTCGCCGCACGATG-TAMRA.

COX-1 and COX-2 mRNA in the heart and kidneys (III) were also measured by RT-PCR. Total RNA was extracted from the kidneys and heart with Trizol (Gibco Life Technologies, Invitrogen Corporation, Paisley, UK) reagent, according to the instructions of the manufacture. After extraction and quantitation with spectrophotometry, a 2-µg aliquot of total RNA was reverse-transcribed to cDNA by incubation for 50 min at 45°C in the presence of reverse transcriptase (Enhanced Avian RT-PCR kit, Sigma Chemical Co., USA). Subsequently, 4 µl of cDNA was subjected to PCR for the detection of COX-1 mRNA, COX-2 mRNA, and the GAPDH mRNA. The following RT-PCR primers for GAPDH, COX-1, and COX-2 were used: GAPDH forward, TGGG-GCAGCCCAGAACATCA; GAPDH reverse, GCCGCCTGCTTCACCACCTT; COX-1 forward, CCCATCTGTTCCCCAGAGTA; COX-1 reverse, GTGAGACCCCCAAGTTCC-AAA; COX-2 forward, AGTGCCTCCCACTCCAGACT; COX-2 reverse, CACT-GGCTTATGCCGAAAAA (Sigma Genosys, London, UK). The reaction products were run simultaneously on a 2% agarose gel. The intensity of the resulting bands was measured by a computer-based imaging analysis system (Genetools, Syngene, London, UK). COX-1 and COX-2 densities of COX-1 and COX-2 bands were expressed as relative to the density obtained for GAPDH. The intensity of the resulting bands was measured by a computer-based imaging analysis system (Syngene).

4.11. Biochemical determinations

Albuminuria

Urinary albumin (albuminuria) was measured by ELISA with rat albumin as a standard (Celltrend, GmbH, Lückenwalde, Germany).

Serum 8-isoprostaglandin F_{2α}

Serum 8-isoprostaglandin F_{2α} concentration was determined by ELISA according to the instructions of the manufacture (Cayman Chemical, Ann Arbor, MI, USA).

Urinary nitrate plus nitrite

Urinary nitrate and nitrite (NO_x) concentrations were assessed with a commercially available NO_x colorimetric assay kit (Cayman).

Renal xanthine oxidoreductase activity

Renal xanthine oxidoreductase (XOR) activity was measured fluorometrically as described in detail previously (Laakso *et al.*, 1998).

Plasma atrial natriuretic peptide and B-type natriuretic peptide

Plasma atrial natriuretic peptide and B-type natriuretic peptide levels were measured by radioimmunoassay as described in detail earlier (Kinnunen *et al.*, 1993).

Blood glucose and serum insulin

Blood glucose was measured with an automatic analyzer (Reflotron, Boehringer-Mannheim, Mannheim, Germany). Serum insulin was determined by radioimmunoassay (Linco Research Inc. St. Charles, MO, USA) according the instructions of the manufacture.

Serum lactate dehydrogenase

Serum lactate dehydrogenase (LDH) concentration was measured enzymatically (United Laboratories, Helsinki, Finland).

4.12. Compounds

The following compounds were used: sodium chloride, potassium chloride (Sigma-Aldrich Co.), acetylcholine chloride, angiotensin II acetate, N^G-nitro-L-arginine methyl ester hydrochloride, diclofenac, oxypurinol, superoxide dismutase, noradrenaline bitartrate, endothelin-1, sodium nitroprusside dihydrate, carboxymethyl cellulose (Sigma), losartan, MF-tricyclic and sulindac (Merck & Co. Inc., Rahway, NJ, USA), valsartan (Novartis Ltd, Basel, Switzerland), buprenorphin (Temgesic[®], Reckitt & Colman, Hull, UK), fentanyl-fluanisone (Hypnorm[®], Janssen Pharmaceutica, Beerse, Belgium), and midazolam (Dormicum[®], Hoffmann-La Roche, Basel, Switzerland).

Compounds to be given to animals intraperitoneally were dissolved in physiological saline (0.9% NaCl). Compounds for *in vitro* vascular response studies were dissolved in distilled water, except oxypurinol, which was dissolved in dimethylsulfoxide (DMSO) (96%); the final concentration of DMSO in the buffer liquid was limited to less than 5%. All solutions were freshly prepared before use and protected from light.

4.13. Statistical analysis

Statistical analysis was carried out by one-way analysis of variance (ANOVA) supported by Tukey's (I) and LSD's (II-IV) test. ANOVA for repeated measurements was applied for data consisting of repeated observations at successive time-points. The difference was considered significant at $p < 0.05$. The sensitivity of the artery to each cumulative relaxation response is presented as pD_2 values, which are calculated as the negative log of the dose required to produce the 50% maximal response (IV). Data are presented as means $\pm$ SEM.

4.14. Ethics

These studies were approved by the Animal Experimentation Committee of the University of Helsinki, Finland.

5. RESULTS

5.1. Blood pressure, cardiac hypertrophy and heart rate

In dTGRs, systolic blood pressure and the heart weight-to-body weight were higher than those of age-matched normotensive SD rats. Valsartan treatment completely normalized blood pressure, cardiac hypertrophy in dTGRs (I).

In GK rats, systolic blood pressure measured by both tail cuff and radiotelemetry methods were higher than in Wistar control rats. By radiotelemetry diastolic blood pressure was similar between GK rats and Wistar controls (**Figure 5**). The heart weight-to-body weight ratio was higher in GK rats than in Wistar rats. Valsartan significantly reduced blood pressure in GK rats on a low-sodium (LS) diet. The HS diet for 8 weeks raised systolic blood pressure by 24 mmHg, and induced cardiac hypertrophy. Valsartan did not reduce blood pressure or prevent the development of cardiac hypertrophy in GK rats during the HS diet (II).

In mREN2 rats, systolic blood pressure was markedly higher than in normotensive SD rats. These rats showed cardiac hypertrophy, estimated as absolute heart weights or heart weight-to-body weight ratios. Both the selective COX-2 inhibitor MF-tricyclic and the non-selective COX-1/COX-2 inhibitor sulindac further increased blood pressure and the absolute heart weight similarly. Losartan completely normalized blood pressure and prevented the development of cardiac hypertrophy, both in the presence and in the absence of COX inhibitors. There was a close correlation between systolic blood pressure and degree of cardiac hypertrophy ($p<0.001$, $R=0.881$, $n=60$) (III, IV).

No differences appeared in the heart rate between the experimental rats and control rats. None of the treatments influenced the heart rate of the rats (I-IV).

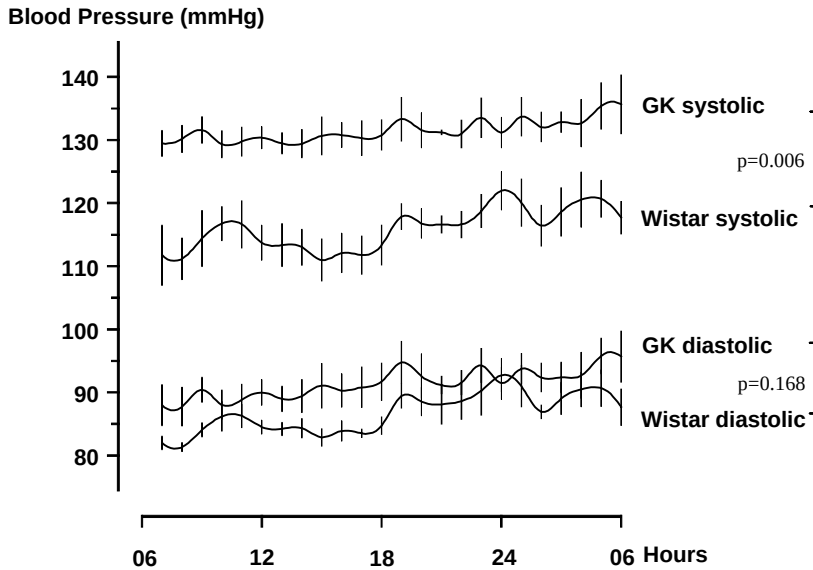


Figure 5. 24-hour blood pressure profile of Goto-Kakizaki (GK) rats and Wistar rats. Values are presented as means $\pm$ SEM, n=10 in each group.

5.2. Renal function

Proteinuria (I) or albuminuria (II, III) was measured as an index of renal dysfunction in the present study. In dTGRs, proteinuria was 14-fold higher than in SD control rats. Valsartan treatment completely normalized renal function in dTGRs (I).

In GK rats, albuminuria was markedly higher than in Wistar rats. The HS diet increased albuminuria by 350% in GK rats. Valsartan treatment did not significantly change albuminuria during the LS diet, but it markedly reduced high-salt-induced albuminuria (II).

In mREN2 rats, 24-hour urine volume and urinary albumin excretion rate were markedly higher than in SD rats. MF-tricyclic and sulindac further increased albuminuria in mREN2 rats, but did not significantly influence urine volume. Losartan, both in the absence and presence of COX inhibitors, completely prevented the development of albuminuria in mREN2 rats, as well as any Ang II-induced increase in urinary output (III).

5.3. Arterial tone

In dTGRs, the endothelium-dependent vascular relaxation by ACh was markedly impaired compared with that in SD controls. Preincubation of the dTGR renal arteries with SOD and oxypurinol markedly improved endothelium-dependent vascular relaxation by ACh. Endothelial-independent vascular relaxation by SNP was similar between dTGRs and SD rats. Contractions following a single dose of Ang II (0.1 $\mu\text{mol/L}$) and a cumulative dose of ET-1, or NA were markedly impaired in dTGRs. Valsartan treatment completely normalized vascular tone in these rats (I). Detailed data on maximal relaxations, contractions, and PD_2 -values are summarized in **Table 6**.

In GK rats, endothelium-mediated vascular relaxation of noradrenaline-precontracted mesenteric arterial rings in response to ACh was markedly impaired compared with Wistar rats, but the endothelium-independent relaxations to SNP were similar in both strains. Preincubation of the arterial rings with the NOS inhibitor L-NAME and the cyclooxygenase inhibitor diclofenac abolished relaxation by ACh almost completely in GK rats but not in Wistar rats. The HS diet impaired vascular relaxation in response to ACh and also to SNP. Valsartan treatment improved endothelium-dependent vascular relaxation during the LS diet, but had no effect on endothelium-dependent and - independent vascular relaxation responses during the HS diet. The improvement of endothelium-dependent vascular relaxation by valsartan treatment during low salt intake was also found after preincubation of the arterial rings with the combination of L-NAME and diclofenc (II). Detailed data on maximal relaxations and PD_2 -values are summarized in **Table 7**.

RESULTS

Table 6. Maximal relaxation and contraction responses (%) and PD₂ (-log EC₅₀) in isolated renal arteries from double transgenic rats harboring human renin and angiotensinogen genes (dTGRs), from valsartan-treated dTGRs, and from Sprague-Dawley (SD) rats, all aged 7 weeks.

	dTGRs	dTGRs+Valsartan	SD rats	ANOVA (p-value)
Relaxation responses to:				
Acetylcholine				
PD ₂		7.61±0.19	7.38±0.10	<0.001
Maximal relaxation (%)	43.07±13.43 ^{##}	93.92±2.01	93.46±2.16	<0.001
Sodium nitroprusside				
PD ₂	7.85±0.26 [#]	9.04±0.16	8.47±0.28	0.005
Maximal relaxation (%)	91.38±2.38 ^{##}	98.24±0.37	97.90±0.49	0.003
Contraction responses to:				
Endothelin-1				
PD ₂	8.62±0.18	8.83±0.11	8.59±0.26	0.835
Maximal contraction (g)	0.81±0.33 ^{##}	1.86±0.16	2.00±0.17	0.006
Noradrenaline				
PD ₂	6.40±0.13 [*]	7.12±0.17	7.28±0.11	0.018
Maximal contraction (g)	1.28±0.13 ^{##}	1.87±0.24	1.93±0.23	0.021

Values are means±s.e.; n=8 in each group; EC₅₀ values are presented as the negative logarithm (PD₂) of concentration of the agonist. ^{*}p<0.05 versus SD rats group, [#]p<0.05 versus valsartan-treated dTGRs group.

Table 7. Maximal relaxation responses (%) and PD₂ (-log EC₅₀) in isolated mesenteric arteries of 16-week-old variously treated spontaneously diabetic Goto-kakizaki (GK) rats and age-matched non-diabetic Wistar rats.

	GK	GK+salt	GK+valsartan	GK+valsartan +salt	Wistar rats	ANOVA (p-value)
Acetylcholine						
PD ₂	8.0±0.2	7.4±0.3 ^{##*}	8.0±0.2	7.3±0.3 ^{##*}	8.0±0.1	0.006
Maximal relaxation (%)	79.4±4.6 [*]	62.2±5.4 ^{##*}	83.3±6.0 ^{##*}	55.1±5.1 ^{##*}	97.0±1.1	<0.001
Acetylcholine preincubated with L-NAME+diclofenac						
PD ₂	-	-	-	-	6.2±0.2	
Maximal relaxation (%)	23.1±6.0 ^{##*}	18.2±9.6 ^{##*}	48.2±11.2 [*]	1.2±3.2 ^{##*}	67.4±4.6	<0.001
Sodium nitroprusside						
PD ₂	8.4±0.0	7.5±0.2 ^{##*}	8.1±0.1	7.3±0.3 ^{##*}	8.1±0.3	0.003
Maximal relaxation (%)	97.3±0.6	92.0±1.8 ^{##*}	97.8±1.0	86.2±3.7 ^{##*}	96.2±0.7	0.003

Values are means ± s.e.; n=8 in each group. EC₅₀ values are presented as the negative logarithm (PD₂) of concentration of the agonist. ^{*}p<0.05 vs. Wistar rats group, [#]p<0.05 vs. GK controls group, ^{##}p<0.05 vs. valsartan-treated GK rats group.

In mREN2 rats, the endothelium-dependent vascular relaxation by ACh and endothelium-independent vascular relaxation by SNP were markedly impaired in the aorta but not in the renal arteries when compared to SD controls. The endothelium-dependent relaxation by ACh was improved by preincubation of the aortic rings with diclofenac, and was completely abolished by preincubation with either L-NAME alone or the combination of diclofenac and L-NAME. The tendency toward contractile responses with the highest ACh concentration (10 μ M) disappeared totally in the presence of diclofenac. Both in the aorta and renal arteries, the contractions caused by a single dose of Ang II (0.1 μ mol/L) were slightly more pronounced in mREN2 rats than in SD controls. The contractile responses to ET-1 were attenuated in the aorta, but not in the renal arteries. No differences occurred in contractile response to NA in either strain. Losartan treatment of mREN2 rats completely normalized endothelium-dependent vascular relaxation by ACh and endothelium-independent vascular relaxation by SNP. In losartan-treated mREN2 rats, 20% relaxation by ACh remained in the presence of the combination of diclofenac and L-NAME, indicating that losartan improves endothelial function in mREN2 rats through the EDHF pathway. In addition, losartan treatment attenuated Ang II-induced contractions both in the aorta and in the renal arteries, and normalized ET-1-induced contractions in the aorta. Neither *In vivo* treatment with MF-tricyclic nor sulindac showed any clearly protective effect on vascular tone in mREN2 rats (IV).

5.4. Inflammation

Tissue morphology

In dTGRs, the media-to-lumen ratio of mesenteric arteries was slightly increased compared to that of SD rats (0.20 $\pm$ 0.04 versus 0.14 $\pm$ 0.09, $p < 0.05$) (I).

In GK rats, the kidneys on the HS diet showed some mesangial thickening and slight thickening of the media of the afferent arteriols, but with no clear signs of glomerulosclerosis or interstitial fibrosis (II).

In mREN2 rats, the myocardium showed severe histopathological changes. In the larger epicardial arteries appeared notable adventitial fibrosis and medial hypertrophy. There was an increase in inflammatory cells in the perivascular area. Smaller, mostly intramuscular arteries were even more severely damaged, and showed concentric hypertrophy occluding the vessel lumens. Neither MF-tricyclic nor sulindac had any clearly beneficial or detrimental effects on cardiac morphology. In contrast, losartan completely prevented the Ang II-induced morphological changes in mREN2 rats, both in the presence and in the absence of COX inhibitors (III).

In mREN2 rats, the kidneys showed focal glomerulosclerosis with mesangial expansion, and apparent tubular atrophy with mild peritubular/interstitial fibrosis. The mid-sized arteries showed notable adventitial and medial thickening, whereas the smaller arteries also had intimal hyperplasia, leading to narrowing of the luminal space. Inflammatory cells appeared in periarterial and peritubular areas. Neither MF-tricyclic nor sulindac was able to prevent Ang II-induced changes in renal morphology. In contrast, some samples of the selective COX-2 or non-selective COX-1/COX-2 inhibitor groups showed necrosis of the tubular epithelium. Renal morphology in mREN2 rats treated with losartan was indistinguishable from that of SD rats (III).

Leukocyte accumulation and adhesion molecule expression

GK rats during the LS diet already had significant monocyte/macrophage infiltration (ED₁-positive cells) in the renal perivascular space and increased expression of ICAM-1 in the interstitium, intima, and adventitia of the small renal vessels. The HS diet aggravated their inflammatory response. Valsartan treatment reduced the number of ED₁-positive cells and ICAM-1 expression during both LS and HS diets (II).

In mREN2 rats, ED₁-positive cells appeared both in the heart and kidneys. Neither MF-tricyclic nor sulindac ameliorated the inflammatory response in these mREN2 rats, and losartan completely prevented leukocyte infiltration (III).

COX expression

In mREN2 and SD rats, only minimal COX-2 protein expression appeared in endothelial cells in the heart (data not shown). Weak COX-2 immunopositive staining was also found in the cortex and medullary interstitial cells. Renal COX-2 expression was clearly lower in mREN2 rats than in SD rats (III).

COX-2 mRNA expression in the heart and kidneys was also lower compared that into SD rats. MF-tricyclic, sulindac, and losartan significantly increased myocardial and renal COX-2 mRNA expression in mREN2 rats. No difference existed in COX-1 mRNA expression in the heart or kidneys between mREN2 and SD rats. Sulindac enhanced COX-1 mRNA expression, but not MF-tricyclic. Losartan did not influence COX-1 gene expression when given alone or in combination with COX inhibitors (III).

Excretion of NO metabolites

Urinary nitrate plus nitrite (NOx), the main NO metabolites, was measured in the present study. **In dTGRs**, urinary NOx excretion was reduced by 85% compared to SD control rats. Valsartan treatment markedly improved urinary NOx excretion in these rats (I).

In GK rats, urinary NOx excretion was similar to that in Wistar controls, but was markedly increased during the HS diet. Increased NOx excretion in GK rats during the HS diet was normalized by prolonged treatment with valsartan (II).

In mREN2 rats, urinary NOx excretion was lower than in SD controls, and was completely normalized by losartan treatment. In contrast, both MF-tricyclic and sulindac slightly reduced ($p < 0.05$) or tended to reduce their urinary NOx excretion (IV).

5.5. Oxidative stress

In the present study, serum 8-isoprostaglandin $F_{2\alpha}$ level and renal xanthine oxidoreductase activity, the indirect markers of oxidative stress *in vivo* (for review, see Romero & Reckelhoff, 1999), were measured.

Serum 8-isoprostaglandin F_{2α}

In dTGRs, serum 8-isoprostaglandin F_{2α} levels were 100% higher than in SD control rats. Valsartan treatment completely normalized serum 8-isoprostaglandin F_{2α} levels (I).

In GK rats, serum 8-isoprostaglandin F_{2α} levels tended to be higher than in Wistar rats, but not significantly. The HS diet raised serum 8-isoprostaglandin F_{2α} levels by 400% in GK rats; this was prevented by prolonged treatment with valsartan (II).

Renal xanthine oxidoreductase activity

In dTGRs, the activity of XOR in the kidney was 40% higher than in SD control rats. Valsartan treatment completely normalized their renal XOR activity (I).

5.6. Blood glucose and serum insulin

In GK rats, blood glucose and serum insulin levels were higher than in Wistar rats, but were not markedly influenced by the HS diet or by valsartan treatment (II).

5.7. Plasma atrial natriuretic peptide and B-type natriuretic peptide

In GK rats, plasma atrial natriuretic peptide and B-type natriuretic peptide concentrations were similar to those in Wistar controls. Neither the HS diet nor valsartan treatment affected plasma atrial natriuretic peptide concentrations. Plasma B-type natriuretic peptide levels were elevated by the HS diet both in the presence and in the absence of valsartan treatment (II).

5.8. Serum lactate dehydrogenase

In mREN2 rats, serum LDH concentrations were similar to those in SD controls, and no drugs used had any influence on this (III).

5.9. Angiotensin receptors, angiotensin-converting enzyme, neutral endopeptidase, and endothelin receptors expression

In dTGRs, renal AT₁ mRNA expression was markedly lower than in SD rats. Valsartan treatment raised renal AT₁ mRNA expression to the levels found in SD rats. **In dTGRs**, AT₁ and AT₂ receptor expression in the cortex and medulla were also markedly reduced, whereas ACE expression remained unchanged. Valsartan slightly elevated cortical AT₁ receptor expression but did not influence AT₁ receptor expression in the medulla; similarly, AT₂ receptor and ACE expression were both unchanged (I).

In GK rats, AT₁ receptor expression in the medulla was lower than in Wistar rats, whereas cortical AT₁ receptor expression, medullary and cortical AT₂ receptor expression, and renal ACE and NEP expressions were unchanged. The HS diet did not influence renal AT₁, AT₂, ACE, or NEP expression. In valsartan-treated GK rats, the cortical and medullary AT₁ receptor expression were lower in the presence and in the absence of the HS diet (II).

In mREN2 rats, ET_A and ET_B receptor expressions in the aorta did not differ from those in SD rats. None of the drugs significantly influenced ET_A and ET_B receptor expressions. In the aorta, ET_A receptor expression was 7 to 20 times as high as ET_B receptor expression (IV).

5.10. Body weight, food intake, and drug dosages

In Studies III and IV, final body weight in untreated mREN2 and SD rats (412±8 vs. 392±14 g, $p=0.27$) was similar. At the end of the experimental period body weight of

sulindac-treated (455 ± 17 g), losartan-treated (452 ± 19 g), and MF-tricyclic+losartan-treated (457 ± 15 g) rats were slightly, but significantly higher than of untreated mREN2 rats ($p<0.05$). No difference existed between the treatment groups in the 24-hour food intake at 2, 4, 6, and 8 weeks.

During the 8-week experimental period, the daily dosage of selective COX-2 inhibitor MF-tricyclic varied from 22 to 9 mg/kg, that of non-selective COX-1/COX-2 inhibitor sulindac from 25 to 9 mg/kg, and that of losartan from 67 to 27 mg/kg during the 8 weeks' experiment (III, IV).

The main findings in the animals and the effects of compounds in corresponding animals are summarized in **Table 8**.

Table 8. Main findings in the rats and effects of prolonged treatment with different compounds in corresponding animals (I-IV).

Study	Strain	Treatment	SBP	Cardiac hypertrophy	Proteinuria	NOx	Serum 8-isoprostaglandin F _{2α}	Renal XOR activity
I	SD	Vehicle	—	—	—	—	—	—
I	dTGR	Vehicle	↑ ^a	↑ ^a	↑ ^a	↓ ^a	↑ ^a	↑ ^a
I	dTGR	Valsartan	↓ ^b	↓ ^b	↓ ^b	↑ ^b	↓ ^b	↓ ^b

Study	Strain	Treatment	SBP	Cardiac hypertrophy	Albuminuria	NOx	Serum 8-isoprostaglandin F _{2α}	Renal ED ₁ -positive cells	Renal ICAM-1
II	Wistar	Vehicle	—	—	—	—	—	—	—
II	GK	Vehicle	↑ ^c	↑ ^c	↑ ^c	↔ ^c	↔ ^c	↑ ^c	↑ ^c
II	GK	High salt	↑ ^d	↑ ^d	↑ ^d	↑ ^d	↑ ^d	↑ ^d	↑ ^d
II	GK	Valsartan	↓ ^d	↔ ^d	↔ ^d	↔ ^d	↔ ^d	↓ ^d	↓ ^d
II	GK	High salt+valsartan	↑ ^d	↑ ^d	↑ ^d , ↓ [#]	↔ ^d , ↓ [#]	↔ ^d , ↓ [#]	↓ ^d , ↓ [#]	↓ ^d , ↓ [#]

Study	Strain	Treatment	SBP	Cardiac hypertrophy	Albuminuria	NOx	Cardiac ED ₁ -positive cells	Renal ED ₁ -positive cells
III, IV	SD	Vehicle	—	—	—	—	—	—
III, IV	mREN2	Vehicle	↑ ^e	↑ ^e	↑ ^e	↓ ^e	↑ ^e	↑ ^e
III, IV	mREN2	MF-tricyclic	↑ ^g	↑ ^g	↑ ^g	↓ ^g	↔ ^g	↔ ^g
III, IV	mREN2	Sulindac	↑ ^g	↑ ^g	↑ ^g	↔ ^g	↔ ^g	↔ ^g
III, IV	mREN2	Losartan	↓ ^g	↓ ^g	↓ ^g	↑ ^g	↓ ^g	↓ ^g
III, IV	mREN2	MF-tricyclic+losartan	↓ ^{g*}	↓ ^{g*}	↓ ^{g*}	↓ ^{g*}	↓ ^{g*}	↓ ^{g*}
III, IV	mREN2	Sulindac+losartan	↓ ^{g*}	↓ ^{g*}	↓ ^{g*}	↓ ^{g*}	↓ ^{g*}	↓ ^{g*}

— normal; ↑ increased; ↓ decreased; ↔ no changes; **a** compared to SD rats in Study I (I); **b** compared to untreated dTGRs (I); **c** compared to Wistar rats (II); **d** compared to untreated GK rats (II); **#** compared to GK rats with high-sodium diet (II); **e** compared to SD rats in Studies III and IV; **g** compared to untreated mREN2 rats (III-IV); ***** compared to MF-tricyclic- and sulindac-treated mREN2 rats.

dTGR = double transgenic rat harboring human renin and human angiotensinogen genes
ED₁ = monoclonal antibody recognizing rat monocytes and macrophages
GK = Goto-Kakizaki rat
ICAM-1 = intracellular adhesion molecule-1

mREN2 = mouse transgenic rat harboring the mouse renin-2 gene
NOx = nitrite plus nitrate
SBP = systolic blood pressure
SD = Sprague-Dawley rat
XOR = xanthine oxidoreductase

6. DISCUSSION

RAS plays a central role in the regulation of blood pressure, sodium excretion, and aldosterone secretion. Ang II is a key effector of RAS, and recent studies have demonstrated that Ang II-induced inflammation and vascular injury are involved in the pathogenesis of Ang II-induced hypertension and other cardiovascular diseases. The exact mechanisms, however, still remain unclear. In the present study, the role of oxidative stress and COX isoenzymes in the Ang II-induced inflammatory response and vascular dysfunction were evaluated by the use of two transgenic models with increased local RAS: dTGRs and mREN2 rats, and spontaneously diabetic GK rats.

6.1. Methodology

Rat strains

The three models chosen, i.e., the dTGR, and the mREN2 and GK rat, were especially suitable for answering the questions concerning the mechanisms and prevention of Ang II-induced vascular injury and inflammatory response. In dTGRs, the rat and human RAS do not interact, so the pathogenesis in this rat is the result of the overexpression of the human RAS (Muller *et al.*, 2000a; Muller *et al.*, 2000b), thus, the dTGR can be regarded as a good animal model for studying the mechanisms of Ang II-induced pathogenesis and the effect of ACE inhibitors and AT₁ antagonists in man. As compared to that of dTGRs, the pathogenesis of heterozygous mREN2 rats is moderate, and the survival rate of heterozygous mREN2 rats is also much longer. Therefore, the effects of 3 weeks' treatment of dTGRs and 8 weeks' treatment of mREN2 rats could be determined without loss of animals.

In the present study, that spontaneously diabetic GK rats are salt- sensitive and show several structural changes in the kidney similar to those observed in type II diabetic patients, implies that these rats may be a good animal model for studying the pathogenesis of type II diabetes in man.

Blood pressure measurements

In the present study, blood pressure was measured by the tail cuff method. In Study II, blood pressure was also measured by the radiotelemetry method. Blood pressure measured by the tail cuff method has shown a good correlation with direct recordings by using intra-arterial catheters (Bunag & Butterfield, 1982). The advantages of this method are its low cost and simplicity. The limitations, on the other hand, are overestimation of blood pressure by stressful stimuli from the environment, e.g., people, noise, high temperatures, tail-cuffs, and holders. Stress-induced influences can be minimized by pre-training the animals, and by having the same person regularly perform the measurements in a peaceful environment.

The radiotelemetry method offers the possibility for providing accurate, reliable, and continuous recordings in unrestrained, untethered animals for a relatively long time-period. The main limitations of the method are the process of telemeter setting and the expensive devices operation for telemeter setting and the expensive device.

In the present study, systolic blood pressure measured by the tail cuff and radiotelemetry method in GK rats was similar. In accordance with our previous pilot study in diabetic Zucker rats (data not shown), the present results indicate that the telemetric and tail cuff values are also comparable in GK rats.

Arterial ring preparations

The major complications of hypertension result from injury to relatively large arteries (Safar *et al.*, 1998). Regional differences in the vascular effects of increased RAS have also been described in spontaneously hypertensive rats (Clozel *et al.*, 1991). In the present study, we used the superior mesenteric artery, renal artery, and aorta in different experiments. The mesenteric artery served as a model of a resistance artery and is suitable for the experimental setting, which required stable precontractions and reproducible relaxation responses. The aorta does not contribute to peripheral vascular resistance, but may serve as a model of conduit arteries, which are most prone to vascular diseases and responsible for the clinical consequences of

hypertension. Renal failure is a common complication of hypertension. Functional changes in renal arteries may play an important role in the regulation of renal hemodynamics and renal function. It is noteworthy that Ang II may affect arterial function differently in different parts of the vasculature.

Dosage selection of COX inhibitors

Dosage choices for the selective COX-2 inhibitor MF-tricyclic and non-selective COX-1/COX-2 inhibitor sulindac were based on *in vivo* studies performed in mice (Oshima et al., 1996), and male SD rats (Buttar et al., 2002). Using a mouse model of human familial adenomatous polyposis, Oshima et al. (1996) reported that treating the mice with MF-tricyclic suppresses polyp formation in a dose-dependent manner. MF-tricyclic, when given at daily dosages of 14 mg/kg and 3.5 mg/kg (mixed in the chow), reduced the number of polyps by 62% and 50%, respectively. In contrast, sulindac (12 mg/kg mixed in chow) did not influence polyp formation. In that experiment, the drug concentration in mice receiving MF-tricyclic at a daily dosage of 14 mg/kg was 0.45 µg/ml (corresponding approximately to 1.3 µmol/l), and that of sulindac sulfide, the active form of sulindac, in mice receiving sulindac (12 mg/kg) 0.54 µg/ml (corresponding approximately to 1-2 µmol/l).

Buttar et al. (2002) reported recently that MF-tricyclic, when given orally to SD rats for 28 weeks at a daily dosage of 10 mg/kg (mixed in the chow), inhibits inflammation, COX-2 activity, and the development of adenocarcinoma. In that study, high-dose sulindac (30 mg/kg mixed in the chow) also exerted a pronounced anti-inflammatory effect and protected against development of adenocarcinoma. The serum drug concentration in rats receiving MF-tricyclic at a daily dose of 10 mg/kg was about 0.8 µg/ml, which, according to the authors, is approximately double the amount that can be achieved with 25 mg of rofecoxib (a MF-tricyclic derivative) in humans.

Studies in male SD rats have revealed that MF-tricyclic exerts the following *in vivo* activities after drug administration: rat paw edema inhibitory dose (ID₅₀): 2.8 mg/kg, antipyretic ID₅₀: 0.22 mg/kg, rat paw hyperalgesia ID₅₀: 1.3 mg/kg, adjuvant arthritis:

about 1 mg/kg. The bioavailability of this drug is high (Pauline Luk, Merck Frost Canada, personal communication).

Cell culture studies using recombinant human COX-1 and COX-2 proteins have revealed that MF-tricyclic inhibits COX-2 at nanomolar concentration (IC_{50} : 16.4 nmol/l) and COX-1 at high micromolar concentration (IC_{50} > 50 000 nmol/l) (Oshima et al., 1996). In contrast, sulindac sulfide inhibits both COX-1 and COX-2 at nanomolar concentration (IC_{50} for COX-1 47.9 nmol/l; IC_{50} for COX-2 1.2 nmol/l) (Oshima et al., 1996). In human whole blood assays, MF-tricyclic inhibits COX-2 at low micromolar concentration (IC_{50} : 0.82 μ mol/l), and COX-1 at high micromolar concentration (IC_{50} : 35 μ mol/l) (Pauline Luk, Merck Frost Canada, personal communication).

In the present study, food consumption in mREN2 rats was recorded every second week by using metabolic cages, and the average drug dosages of MF-tricyclic and sulindac from the metabolic data were calculated. In our 8-week experiment, the daily dosage of MF-tricyclic varied from 22 to 9 mg/kg, and that of sulindac from 25 to 9 mg/kg. Taking into consideration the data from these *in vivo* and *in vitro* studies, it is likely that MF tricyclic, as well as sulindac, produced COX inhibition also in our experiment. The drug-induced increases in blood pressure and the 24-hour albuminuria, as well as the drug-induced changes in renal COX mRNA and protein expressions, also support this notion.

6.2. Increased RAS activity genetically and vascular dysfunction

In the present study, as an indication of endothelial dysfunction, endothelium-dependent relaxation responses to ACh were impaired in dTGRs and mREN2 rats (I, IV). However, the endothelium-independent relaxation responses to SNP and the vascular contractile responses to Ang II, ET-1, and NA were inconsistent. These results suggest that Ang II-induced vascular dysfunction is mainly due to endothelial dysfunction.

Ang II, when infused into normotensive rats, induces pronounced endothelial dysfunction (Rajagopalan *et al.*, 1996; Wattanapitayakul *et al.*, 2000). Ang II-induced endothelial dysfunction is secondary to increased superoxide formation within the endothelium, the media, and/or adventitial layer (Harrison, 1997; Kojda & Harrison, 1999; McIntyre *et al.*, 1999). Endothelial dysfunction in Ang II-infused rats can be corrected by SOD and by AT₁ receptor blockade (Rajagopalan *et al.*, 1996). Even a subpressor dose of Ang II causes oxidative stress, as measured by the production of serum 8-isoprostaglandin F_{2α} level, a noncyclooxygenase-produced metabolite of arachidonic acid (Romero & Reckelhoff, 1999). In agreement with this, the present study showed that the serum 8-isoprostaglandin F_{2α} level was about 100% higher in untreated dTGRs, and that preincubation of the renal arteries with SOD normalized ACh-mediated, endothelium-dependent vascular relaxation completely. Furthermore, urinary NOx, a marker of total body NO generation, was decreased in dTGRs and mREN2 rats. These support the previous finding that Ang II selectively reduces vascular NO bioactivity (Wattanapitayakul *et al.*, 2000) by increased oxidative stress (Romero & Reckelhoff, 1999; Haas *et al.*, 1999). However, whether or not endothelial dysfunction in dTGRs and mREN2 rats is due to the direct effects of Ang II on endothelial cells or whether Ang II-induced hypertension is capable of impairing endothelial function indirectly, or both, remains to be determined.

Previous studies by us and others have shown that spontaneously hypertensive rats and Dahl salt-sensitive rats have higher XOR activities in the kidney and other tissues than Wistar controls (Suzuki *et al.*, 1998; Laakso *et al.*, 1998), and that prolonged inhibition of NOS by L-NAME induces renal XOR in spontaneously hypertensive rats (Laakso *et al.*, 1999). The increased renal XOR activity in dTGRs speaks for the involvement of XOR in the renal pathogenesis induced by Ang II. The moderate improvement, however, of the endothelium-dependent relaxation response by preincubation of the vascular rings with the XOR inhibitor oxypurinol in dTGRs suggests only partial involvement of XOR in ROS generation. It is therefore very likely that, compared to XOR, other enzyme systems capable of producing ROS (e.g., NADH/NADPH oxidase and uncoupled endothelial NO synthase) play a more important role in the onset of Ang II-induced vascular dysfunction in dTGRs (Fukui *et al.*, 1997; Laursen *et al.*, 1997).

In the present study, prolonged treatment with AT₁ receptor antagonists effectively normalized endothelium-dependent vascular relaxation responses to ACh and improved endothelial-independent vascular relaxation responses to SNP both in dTGRs and mREN2 rats, indicating that Ang II impairs endothelial function and sensitivity of VSMCs to NO via stimulation of the AT₁ receptor.

Ang II induces a vascular contractile response via the AT₁ receptor in VSMCs. In dTGRs, the contractile response to Ang II was less prominent than in SD rats. Previously we have demonstrated that Ang II concentrations in the plasma and kidney are 4- to 5-fold higher in dTGRs than in SD rats (Mervaala *et al.*, 2000b). In the present study, AT₁ receptor protein expression and AT₁ mRNA were both lower in dTGR kidneys. The present findings clearly indicate that high circulating Ang II and tissue Ang II concentrations induce agonist-dependent AT₁ receptor downregulation in dTGRs. Furthermore, vascular responses to ET-1 and NA were also attenuated and normalized by valsartan treatment in dTGRs, suggesting that down-regulated ET_A receptors and α -adrenoceptors may also be involved in Ang II-induced vascular dysfunction in dTGRs.

In contrast to dTGRs, vascular contractile responses to Ang II were slightly more prominent in aortic and renal arteries in mREN2 rats than in SD rats. Noll et al (1997) have also demonstrated an enhanced contractile response to Ang II in the mesenteric artery of mREN2 rats. These results indicate that high tissue Ang II concentrations do not induce agonist-dependent down-regulation of the AT₁ receptor in mREN rats. In the present study, the vascular contractile responses to ET-1 in mREN2 rats were attenuated in the aorta, but not in the renal arteries. However, ET_A and ET_B receptors were not down-regulated in mREN2 rats. In contrast to our findings in dTGRs, the vascular contractile responses to NA in mREN2 rats were not attenuated, neither in the aorta nor in the renal artery, suggesting that hypertension in mREN2 rats is not associated with agonist-dependent down-regulation of the α -adrenoceptors. The AT₁ receptor antagonist valsartan completely normalized vascular responses in mREN2 rats.

In summary, endothelial dysfunction is the major change in Ang II-induced vascular dysfunction which is mainly related to Ang II-induced oxidative stress. Increased XOR

activity is partially involved in the generation of O_2^- by Ang II. The vascular contractile response to Ang II, ET-1, and NA depends upon differences in animal species and vasculature. AT_1 receptor antagonists are able to normalize Ang II-induced oxidative stress and vascular dysfunction completely, indicating Ang II-induced vascular dysfunction via stimulation of the AT_1 receptor.

6.3. Increased RAS activity in spontaneously diabetic Goto-Kakizaki rats

In the present study, spontaneously diabetic GK rats showed higher blood glucose and serum insulin levels, and impaired blood glucose tolerance, than did Wistar controls. GK rats were also moderately hypertensive and showed markedly impaired endothelial function, pronounced perivascular monocyte/macrophage infiltration, and overexpression of adhesion molecules in the kidneys.

Long-term treatment with the AT_1 receptor antagonist valsartan normalized blood pressure and improved endothelial dysfunction and perivascular inflammatory response in GK rats during the LS diet, indicating the involvement of increased RAS activity. The improvement of endothelium-dependent vascular relaxation by valsartan treatment also occurred when production of NO and prostacyclin were blocked, suggesting that endothelial dysfunction in GK rats may be attributed, at least in part, to reduced relaxation via EDHF in the vascular smooth muscle (Cohen & Vanhoutte, 1995). However, the improvement in endothelium-dependent vascular relaxation by valsartan may also have been, at least in part, due to stimulation of the AT_2 receptor, leading to increased production of bradykinin and further, of NO (Siragy *et al.*, 2000). No difference appeared in NOx excretion between GK and Wistar controls, suggesting that endothelial NO production in GK rats is unaltered. Because the serum 8-isoprostaglandin $F_{2\alpha}$ level only tended to be increased in GK rats on the LS diet, the increased oxidative stress in contrast to the case of dTGRs and mREN2 rats was probably not the crucial cause of the endothelial dysfunction in spontaneously diabetic GK rats.

A long-term HS diet in GK rats caused a marked increase in blood pressure associated with the development of cardiac hypertrophy. The HS diet also induced renal damage and aggravated inflammation and endothelial dysfunction, indicating that GK rats are sensitive to the deleterious effect of salt. This agrees with the studies in human beings which have demonstrated that hypertension in diabetic patients is often volume-dependent and salt-sensitive (Ritz & Orth, 1999). The increased urinary 8-isoprostaglandin $F_{2\alpha}$ excretion in GK rats during the HS diet supports the theory that in GK rats the detrimental effects of high salt are associated with increased oxidative stress. Valsartan normalized salt-induced oxidative stress in these rats, suggesting that Ang II is involved in salt-induced oxidative stress in GK rats. Valsartan also effectively prevented a salt-induced inflammatory response by blood-pressure-independent mechanisms. Surprisingly, valsartan did not abolish high-salt-induced hypertension, endothelial dysfunction, or cardiac hypertrophy. The present findings suggest that the harmful effect of the HS diet in this animal model of type II diabetes may be, at least partially, due to increased RAS activity.

In summary, increased RAS activity is involved in the pathogenesis of hypertension and renal failure in the spontaneously diabetic GK rat. Hypertension in this rat is salt-sensitive. The deleterious effects of salt in GK rats are related to Ang II-induced oxidative stress. Valsartan prevented the salt-induced inflammatory response and oxidative stress in GK rats by blood-pressure-independent mechanisms.

6.4. COX in the pathogenesis of Ang II-induced vascular inflammation

The mREN2 rat with increased local RAS activity is characterized by hypertension and Ang II-induced tissue injury in the brain, heart, kidney, and vasculature (Mullins *et al.*, 1990; Bohm *et al.*, 1995; Lee *et al.*, 1996). Evidence is accumulating to indicate that the inflammatory response plays an important role in the pathogenesis of Ang II-induced vascular/tissue damage. Prostanoids are major mediators of the inflammatory response and are synthesized by COX from arachidonic acid. However, whether COX-synthesized prostanoids are involved in Ang II-induced inflammation and vascular injury is still

unclear. In the present study, neither the selective COX-2 inhibitor MF-tricyclic nor the non-selective COX-1/COX-2 inhibitor sulindac was capable of preventing Ang II-induced inflammatory responses or tissue damage. In contrast, both compounds showed clear blood-pressure-increasing effects and further aggravated Ang II-induced renal damage. Furthermore, RT-PCR gene-expression analysis revealed that COX-2 mRNA expression was down-regulated by Ang II both in the kidney and heart. Although COX inhibitors exerted detrimental cardiovascular and renal effects in mREN2 rats, they did not attenuate the therapeutic effects of the AT₁ receptor antagonist losartan.

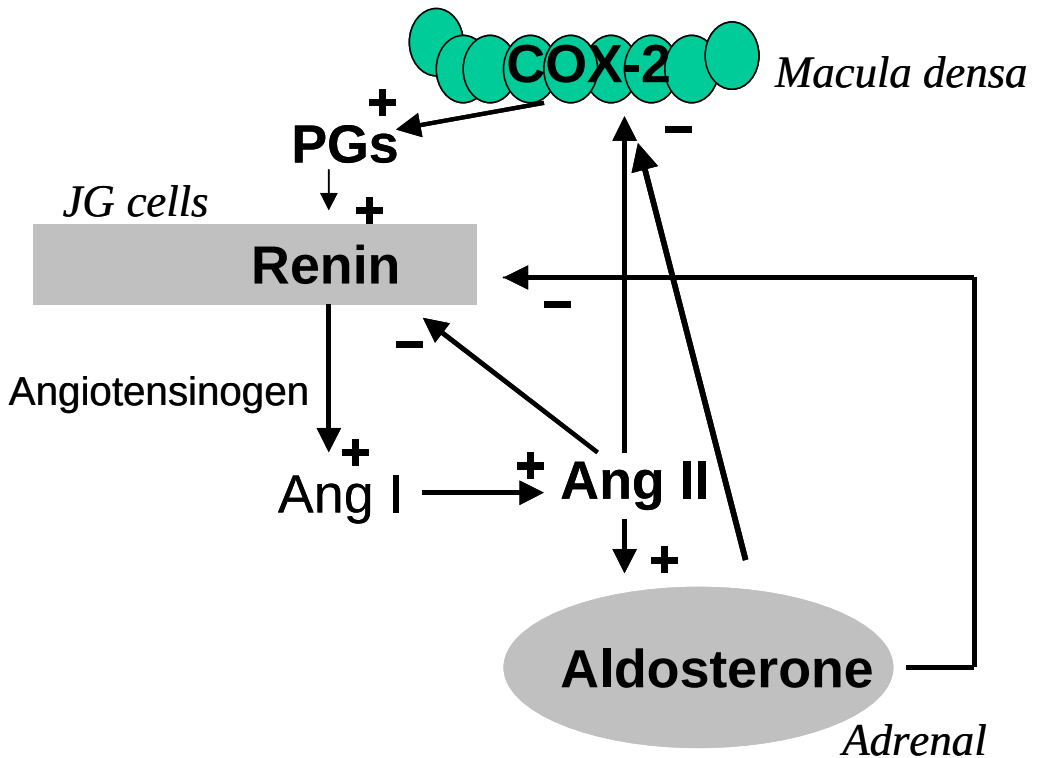
The present study supports the recent clinical finding that COX-2 inhibitors may increase risk for cardiovascular events, for instance, through increased blood pressure (FitzGerald & Patrono, 2001; Mukherjee *et al.*, 2001). An important finding of the present study was the close correlation between systolic blood pressure and degree of cardiac hypertrophy in mREN2 rats, suggesting that the development of cardiac hypertrophy in these rats is primarily mediated by increased pressure overload. However, the present study could provide no evidence for further aggravation of Ang II-induced myocardial or vascular lesions by COX inhibition.

Although the kidneys exhibit abundant constitutive expression of COX-2 (Harris, 2000; Breyer & Harris, 2001), the physiological role of COX-2 in the kidney is not fully understood. Several studies support the notion that the renal COX-2 may regulate renin release as well as renal microcirculation in response to environmental factors such as salt and water depletion (Harris, 2000; Breyer & Harris, 2001). Recent studies have provided compelling evidence that endothelial- and VSMC-derived vasodilatory prostanoids attenuate the renal vascular response to Ang II (Imig, 2000). In the present study, the blood-pressure-increasing effect of COX inhibitors in mREN2 rats is likely to be due to derangement in the renal microcirculation and to tissue damage.

COX-2 is almost exclusively expressed in the renal macula densa cells (Harris *et al.*, 1994; Vio *et al.*, 1997; Hartner *et al.*, 1998). The macula densa is directly adjoined to the renin-producing cells of the afferent arterioles. Macula densa-derived prostaglandins (Gerber *et al.*, 1981; Greenberg *et al.*, 1993) are well-known stimulators of renin synthesis and of renin secretion. However, to the best of our knowledge, the influence of the RAS on COX-2 expression is still unclear. In the present study, COX-2 mRNA

expression both in the heart and kidneys were down-regulated, whereas AT₁ receptor blockade by losartan increased COX-2 gene expression. Regulation of COX-2 protein expression by RAS was also noted with COX-2 immunohistochemistry. Our findings are thus in good agreement with those of previous studies (Cheng *et al.*, 1999; Harris, 2000; Breyer & Harris, 2001) and further support the suggestion that inhibition of COX-2 by Ang II could form novel indirect negative feedback control of the renin system (Wolf *et al.*, 1999; Zhang *et al.*, 1999) (**Figure 6**). The mechanisms by which Ang II inhibits COX-2 expression should be investigated in further experiments.

Due to increased Ang II formation in tissues, mREN2 rats develop hypertension, cardiac hypertrophy, and renal damage (Mullins *et al.*, 1990; Bohm *et al.*, 1995; Lee *et al.*, 1996). We showed recently that Ang II induces tissue damage even by blood pressure-independent mechanisms (Mervaala *et al.*, 2000b). Ang II generates ROS, activates the redox-sensitive transcription factors NF- κ B and activator protein-1, and induces severe perivascular inflammation through AT₁ receptor stimulation (Luft *et al.*, 1999). Both COX-1 and COX-2-derived prostanoids play important roles in mediating and terminating the inflammatory response (Mitchell *et al.*, 1995; Harris, 2000; Imig, 2000; Breyer & Harris, 2001). COX-2 can be induced in endothelial cells, fibroblasts, smooth muscle cells, and macrophages by proinflammatory cytokines, endotoxin, prostanoids, tumor promoters, mitogens, and hypoxia (Mitchell *et al.*, 1995; Wu, 1995). Intriguingly, treatment with COX-2 selective inhibitors like NS398 and celecoxib, but not with the non-selective COX-1/COX-2 inhibitor indomethacin, severely diminished early and late events of T cell activation, partly through NF- κ B and NF-AT-transcription pathways, supporting the notion that selective COX-2 inhibitors may act as modulators of the immune system (Iniguez *et al.*, 1999). Anti-inflammatory effects of COX-2-derived prostanoids have, however, also been described (Gilroy *et al.*, 1999). It has also been claimed that COX-2 induction in endothelial cells may represent an important compensatory mechanism to defend against vascular injury (Wu, 1998b). In the present study, Ang II induced a marked monocyte/macrophage infiltration both in the heart and the kidneys. That the COX inhibitors had no detectable anti-inflammatory effects suggests that COX does not play a central role in the pathogenesis of the Ang II-induced inflammatory response.



ACE = angiotensin II converting enzyme
 COX-2 = cyclooxygenase-2
 JC = juxtaglomerular
 PG = prostaglandin

Figure 6. Possible interaction between renin-angiotensin system, cyclo-oxygenase and aldosterone (modified from Wolf *et al.*, 1999).

In conclusion, in mREN2 rats, COX-2 mRNA and protein expression in the heart and the kidneys were downregulated. Neither the selective COX-2 inhibitor MF-tricyclic nor the non-selective COX-1/COX-2 inhibitor sulindac had a beneficial effect on Ang II-induced inflammation. In fact, they even further increased blood pressure and aggravated renal damage; however, neither COX inhibitor influenced the therapeutic effects of losartan in mREN2 rats.

6.5. COX in the pathogenesis of Ang II-induced vascular dysfunction

Prostanoids converted by COX from arachidonic acid are also critical modulators of vascular tone in both physiological and pathophysiological conditions. Under normal physiological conditions, prostanoids produced by the COX pathway generally induce vasorelaxation. However, in vascular pathologies, there may be an imbalance in which where COX-dependent vasoconstrictors become more predominant (for review, see Davidge, 2001).

The present study investigated the role of COX-synthesized eicosanoids in Ang II-induced vascular dysfunction in mREN2 rats. An important finding of the present study was that hypertension in mREN2 rats is associated with impaired vascular endothelium-dependent and endothelium-independent vascular relaxation in the aorta, whereas the renal arterial function remained largely unchanged. Urinary NO_x excretion, a commonly used marker of total body NO generation, was markedly decreased in mREN2 rats. Both MF-tricyclic and sulindac slightly reduced urinary NO_x excretion in mREN2 rats. However, neither treatment with the COX-2 selective MF-tricyclic nor with the non-selective COX-1/COX-2 inhibitor sulindac was capable of preventing Ang II-induced vascular dysfunction in mREN2 rats. Previous study demonstrated that contractile prostanoids synthesized by COX does not play a role in the development of hypertension (Taddei *et al.*, 1997). These results further indicate that COX also does not appear to participate in the development of Ang II-induced vascular dysfunction in mREN2 rats.

COX-2-deficient mice developed ductus arteriosus, and 35% of the mice have died within 48 hours after birth (Loftin *et al.*, 2001). Moreover, 100% of the mice deficient in both COX-1 and COX-2 have died with a patent ductus arteriosus within 12 hours of birth, indicating that both COX-2 and COX-1 have physiological roles in vessel development. Moreover, a recent study demonstrated that COX-2 induction in endothelial cells may represent an important compensatory mechanism to defend against vascular injury (Wu, 1998b). In mREN2 rats, both COX-2 protein and mRNA expression were downregulated in the heart and the kidneys. Neither MF-tricyclic nor sulindac provides beneficial effects in Ang II-induced pathogenesis in mTRN2 rats. The

present study supports the above findings and suggests that COX is not involved in Ang II-induced vascular injury in mREN2 rats.

The relationship between increased blood pressure and vascular dysfunction in different forms of hypertension has been widely argued. In the present study, treatment with both the selective COX-2 inhibitor MF-tricyclic and the non-selective COX-1/COX-2 inhibitor sulindac elevated blood pressure similarly, but did not affect endothelial function in mREN2 rats. These results support the previous suggestion that endothelial dysfunction may be secondary to the hypertension (McIntyre *et al.*, 1999; Vapaatalo *et al.*, 2000). The present study also supports the recent notion that the role of COX-dependent modulation of vascular function in hypertension may depend on animal models and type of vessels (for review, see Davidge, 2001).

In summary, neither prolonged treatment with the selective COX-2 inhibitor MF-tricyclic nor with the non-selective COX-1/COX-2 inhibitor sulindac affected Ang II-induced vascular dysfunction in mREN2 rats.

7. SUMMARY AND CONCLUSIONS

The present study aimed at exploring the mechanisms of Ang II-induced inflammatory response and vascular injury, and especially the role of oxidative stress and COX-1/COX-2 in this pathogenesis. Two transgenic strains with increased local RAS activity (dTGRs, mREN2 rats) as well as spontaneously diabetic Goto-Kakizaki rats served as experimental animals.

The major conclusions are as follows:

1. Ang II induces vascular dysfunction via AT₁ receptor stimulation. Ang II-induced vascular dysfunction is mainly due to endothelial dysfunction and is associated with increased oxidative stress. XOR is responsible, at least in part, for Ang II-induced ROS formation.
2. In spontaneously diabetic Goto-Kakizaki rats, increased RAS activity is involved in the pathogenesis of endothelial dysfunction and vascular inflammation.
3. The pathological changes in GK rats are salt-sensitive. AT₁ receptor blockade prevents the salt-induced inflammatory response and oxidative stress in GK rats by blood pressure-independent mechanisms.
4. Ang II downregulates COX-2 mRNA and protein expression. COX does not play a central role in the pathogenesis of Ang II-induced inflammatory response and vascular injury.
5. Prolonged treatment with the selective COX-2 inhibitor MF-tricyclic and the non-selective COX-1/COX-2 inhibitor sulindac aggravates Ang II-induced hypertension and renal damage.

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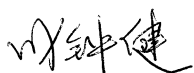
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