Catecholamines, angiotensin II and the neointima

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CATECHOLAMINES, ANGIOTENSIN II AND THE NEOINTIMA

A search for the cause of the relative insensitivity
of the neointima for α_1 -adrenoceptor blockade

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CATECHOLAMINES, ANGIOTENSIN II AND THE NEOINTIMA

A search for the cause of the relative insensitivity
of the neointima for α_1 -adrenoceptor blockade

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof.dr. A.C. Nieuwenhuijzen Kruseman
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen op
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Het nieuwe ontstaat nooit eenvoudigweg door interpolatie van het oude...

-Michel Houellebecq-

Aan mijn ouders

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List of Abbreviations

ACE	angiotensin I converting enzyme
AngI	angiotensin I
AngII	angiotensin II
AT ₁ receptor	angiotensin type 1 receptor
AT ₂ receptor	angiotensin type 2 receptor
AV	arteriovenous
BrdU	5-bromo-2'-deoxyuridine
CEC	chloroethylclonidine
COMT	catechol- <i>O</i> -methyl transferase
CSA	cross-sectional area
DAG	diacylglycerol
FGF	fibroblast growth factor
G protein	guanine nucleotide-binding regulatory protein
IP ₃	inositol 1,4,5-triphosphate
ISH	in situ hybridization
LA	lumen area
LF	labeling fraction
MAO	monoamine oxidase
MAPK	mitogen-activated protein kinase
NET	norepinephrine transporter
NF	neurofilament
NO	nitric oxide
NPY	neuropeptide tyrosine
<i>O.D.</i>	optical density
PDGF	platelet derived growth factor
PGP-9.5	protein gene product-9.5
PiP ₂	phosphatidylinositol 4,5-biphosphate
PKC	protein kinase C
PLA	phospholipase A
PLC	phospholipase C
PLD	phospholipase D
PTCA	percutaneous transluminal coronary angioplasty
PTFE	polytetrafluoroethylene
RAS	renin angiotensin system
RT-PCR	reverse transcription-polymerase chain reaction
SMC	smooth muscle cell
SNS	sympathetic nervous system
TGF- β_1	transforming growth factor- β_1
TH	tyrosine hydroxylase

CHAPTER 1

General Introduction

1.1 Introduction

Percutaneous transluminal coronary angioplasty (PTCA) has become a successful and widely used treatment for patients with coronary artery disease since its first clinical application by Andreas Grüntzig in 1977.(Grüntzig *et al.*, 1977) Despite the increase in procedure and case complexity, primary success rates have improved. However, late restenosis, which constitutes the most important problem after successful angioplasty, continues to occur in 30-40% of patients within 3-6 months. Restenosis after PTCA involves a fibroproliferative response to the vascular injury in the setting of mural thrombosis with platelet activation, thrombin generation, and the release of mitogens.(Ip *et al.*, 1991)

The balloon injury model in the rat carotid artery has often been used as a model to study the fibroproliferative response after injury and has been critical in elucidating the key regulators of the response. The present study focuses on two of these regulators, angiotensin II (AngII) and the sympathetic nervous system (SNS).

1.2 Vascular Layers

The connective tissue sheath around the vascular lumen consists of three layers. The innermost layer, called 'tunica intima' is defined as the combination of endothelium and connective tissue on the luminal side of the internal elastic lamina. The tunica intima of the aorta and other large arteries contains a matrix of collagen, proteoglycans, and small amounts of elastin. The major cell type in the tunica intima is the intimal smooth muscle cell (SMC). Other cells that occasionally reside include lymphocytes, macrophages and other inflammatory cells derived from the blood. The tunica intima is particularly important in atherosclerosis and restenosis after angioplasty, since lipid and SMC accumulation primarily affect this layer of the vessel wall.(Schwartz *et al.*, 1986) Proceeding outward, the vessel wall displays layers of SMCs collectively called the 'tunica media'. Breakdown of the media, particularly its elastic layers, leads to formation of aneurysms.(Schwartz *et al.*, 1986) The outermost layer of the vessel wall is the 'tunica adventitia'. This layer is a collagen-rich connective tissue sheath, containing numerous fibroblasts, small vessels which give rise to the vasa vasorum, and nerves. The 'vasa vasorum' located in the connective tissue sheath surrounding large vessels develop small branches and penetrate into the tunica media from the exterior of the vessel wall, the adventitia, into the media.(Wolinsky and Glagov, 1967)

1.2.1 Response to Injury

Formation of an intima may occur by proliferation of cells already trapped in the intima during formation of the internal elastic lamina, migration of cells from the media with their subsequent proliferation or by proliferation of entrapped cells derived from the blood stream. The vessel wall responds to angioplasty by forming a new layer in the intima called the 'neointima'.

Neointimal formation after injury is studied in vessels like the rat carotid artery that normally have little or no intima. The injury to a rat carotid artery by a balloon catheter completely removes the endothelium and injures the underlying media, causing death of ~30% of SMCs.(Clowes *et al.*, 1983a) Shortly after injury, a monolayer of platelets adhere to the denuded surface and thrombin is activated.(Ross, 1986) Subsequent cellular events can be divided into three partially overlapping 'waves': (1) medial smooth muscle proliferation; (2) migration of medial SMCs to the intima; and (3) proliferation of intimal SMCs. Medial DNA synthesis takes place approximately 30 hours after injury and is largely complete within 48 hours. As many as 40% of SMCs remaining after balloon injury enter the cell cycle and synchronously progress into S phase within 33 hours of wounding.(Majesky *et al.*, 1987) DNA synthesis is followed by cell division and SMC

numbers lost by balloon injury induced apoptosis begin to be restored in the damaged media within 48 hours of injury.(Perlman *et al.*, 1997)

1.2.2 Mediators in the Response to Injury

A great number of polypeptide growth factors have been demonstrated to be induced after balloon injury (table 1.1). Possible mitogenic signals include: (1) platelet adherence and release of peptide growth factors (e.g. platelet derived growth factor (PDGF), transforming growth factor (TGF)- β 1) into the damaged vessel wall(Ross *et al.*, 1986) (2) activation of preformed, inactive or sequestered growth factors present in the artery wall prior to the injury event, by SMC death and release of intracellular mitogens,(Gajdusek and Carbon, 1989) or by proteases of for instance the coagulation cascade,(Gajdusek *et al.*, 1986; Carlos and Harlan, 1990) (3) formation of AngII(Gibbons *et al.*, 1992) and (4) release from local growth inhibition due to loss of endothelium(Castellot *et al.*, 1982) or to inactivation of SMC growth inhibitors present in the arterial media.(Clowes and Clowes, 1985; Fritze *et al.*, 1985)

In the carotid balloon injury model the 'first wave' of medial DNA synthesis is the result of the release of endogenous fibroblast growth factor (FGF) from traumatized SMCs.(Lindner and Reidy, 1991) AngII is also an endogenous SMC mitogen since antagonists of the angiotensin type 1 receptor (AT₁) block the first wave(Prescott *et al.*, 1991) and infused AngII is mitogenic for the intact medial SMCs.(Daemen *et al.*, 1991) The process of SMC migration appears to be separable from SMC proliferation per se, since cells that have not undergone DNA replication are found among those that have migrated into the intima.(Clowes and Schwartz, 1985) Of particular interest to the acquisition of a motile phenotype(Gabbiani *et al.*, 1982; Kocher *et al.*, 1991) may be the decrease in expression of smooth muscle α -actin,(Gabbiani *et al.*, 1984) loss of intermediate filament protein desmin,(Gabbiani *et al.*, 1982; Kocher *et al.*, 1991) changes in the splicing pattern of vinculin,(Glukhova *et al.*, 1988) a protein involved in cell-cell and cell-substrate adhesion, and changes in the splicing pattern of fibronectin, an adhesive cell matrix protein.(Glukhova *et al.*, 1989)

Table 1.1 Mediators of Neointimal Formation(Schwartz *et al.*, 1995)

	Mediators	Inhibition		Stimulation
		Antibody	Antagonist	Agonist
1st wave; medial SMC replication	FGF	+	NA	+
	PDGF	±	NA	-
	TGF- β	NA	ND	±
	AngII	ND	+	ND*
2nd wave; SMC migration	PDGF	+	NA	+
	AngII	ND	+	ND
	FGF	+	NA	+
3rd wave; intimal SMC replication	FGF	-	NA	±
	PDGF	-	NA	-
	TGF- β	NA	ND	+ §
	AngII	ND	-	+ §

+ Indicates supporting evidence; - evidence against hypothesis; ± weak response; NA not available; ND not determined. * AngII does stimulate medial smooth muscle. § Restimulation experiments.

A number of molecules in the platelets, including PDGF, are chemotactic for SMCs.(Grotendorst *et al.*, 1981) Tissue-type plasminogen activator and urokinase also

increase SMC migration.(Jackson *et al.*, 1993; Kenagy *et al.*, 1996) Once formed, the neointima continues to grow by cell replication. A relatively high percentage of intimal SMC nuclei continue to replicate for weeks, particularly at the luminal surface.(Schwartz *et al.*, 1975) When animals were infused chronically with AngII, the neointima produced a three- to five-fold increase in replication as compared with the underlying vessel wall.(Daemen *et al.*, 1991)

Regeneration of the endothelium inhibits proliferation of the underlying neointimal cells.(Haudenschild and Schwartz, 1979; Clowes *et al.*, 1983a; Clowes *et al.*, 1986) Suppression of neointimal cell proliferation might be due to endothelial production of nitric oxide (NO). Administration of large doses of L-arginine aimed at chronically raising NO levels in animals immediately after balloon catheter-induced endothelial denudation has resulted in significant inhibition of neointimal thickening.(McNamara *et al.*, 1993; Hamon *et al.*, 1994; Tarry and Makhoul, 1994) Hansson *et al.*(Hansson *et al.*, 1994) have reported that the cytokine-inducible isoform of NO synthase is chronically expressed at high levels by SMCs at the denuded surface of the neointimal lesion in the rat injured carotid artery.(Hansson *et al.*, 1994) an area in which smooth muscle DNA synthesis remains chronically elevated.(Clowes *et al.*, 1986)

1.3 Vascular Remodeling

The response to vascular injury not only includes growth of the intima, but also remodeling of the vessel wall. Vascular remodeling is defined as the vascular wall changes from dynamic and trophic stimuli resulting in vascular hypertrophy or rearrangement of vascular wall material without growth.(Heagerty *et al.*, 1993) Several types of vascular remodeling have recently been described by Mulvany *et al.*(Mulvany *et al.*, 1996) and are depicted in figure 1.1. Inward remodeling describes a decrease in lumen diameter, whereas outward remodeling refers to an increase in lumen diameter. Hypotrophic remodeling describes a decrease in the amount of vascular wall material independent of changes in the lumen diameter, whereas eutrophic remodeling refers to an absence of change in the amount and properties of wall material regardless of changes in lumen diameter. Hypertrophic inward vascular remodeling is the predominant of these processes and is associated with an increase in wall thickness, an increase in wall-to-lumen ratio, and a decrease in lumen diameter.(Korsgaard and Mulvany, 1988) In contrast, hypertrophic outward remodeling is rare; it has been seen in the esophageal veins of rabbits after partial occlusion of the portal vein(Juhl *et al.*, 1989) and during restenosis in human atherosclerotic lesions.(Glagov *et al.*, 1987; Post *et al.*, 1995) In restenosis, the two contributors to lumen renarrowing, i.e. intimal hyperplasia and vascular remodeling, have been identified. Plaque accumulation or neointima formation may progress without reducing the lumen or even with increasing lumen size if the artery enlarges. If compensation is incomplete or if the artery does not remodel, intimal hyperplasia will narrow the lumen. A subset of arteries shrink thus aggravating lumen narrowing by intimal hyperplasia.(Post *et al.*, 1995; Pasterkamp *et al.*, 1998; Pasterkamp *et al.*, 1999)

Figure 1.1 Various Types of Vascular Remodeling. Inward hypotrophic, eutrophic, and hypertrophic are associated with decreased lumen diameter and decreased, unchanged, and increased wall thickness, respectively. In contrast, outward hypotrophic, eutrophic, and hypertrophic vascular remodeling are associated with increased lumen diameter and decreased, unchanged, and increased wall thickness, respectively.(Mulvany *et al.*, 1996)

1.4 Renin Angiotensin System

1.4.1 Production and Actions of Angiotensin II

The renin angiotensin system (RAS) plays an important role in blood pressure regulation. Reduced sodium delivery at the macula densa, decreased renal perfusion pressure, and sympathetic activation all stimulate secretion of renin by the juxtaglomerular cell.(Johnston, 1990) Renin cleaves the inactive decapeptide angiotensin I (AngI) from the prohormone angiotensinogen (figure 1.2). AngII is then cleaved from AngI by the action of angiotensin I converting enzyme (ACE).(Erdos, 1977) AngII is a potent vasoconstrictor, acting directly on vascular SMCs.(Folkow *et al.*, 1961) In addition, AngII interacts with the SNS both peripherally and centrally to increase vascular tone.(Zimmerman *et al.*, 1984) AngII causes volume expansion through sodium retention (via aldosteron(Biron *et al.*, 1961) and renal vasoconstriction) and fluid retention (via antidiuretic hormone).(Padfield and Morton, 1977) At the cellular level, AngII promotes migration, proliferation, and hypertrophy of SMCs.(Bell and Madri, 1990; Daemen *et al.*, 1991; Itoh *et al.*, 1993; Huckle and Earp, 1994; Dostal *et al.*, 1996)

In addition to catalyzing the formation of AngII, ACE catalyzes the degradation of bradykinin.(Erdos, 1977) Bradykinin promotes vasodilatation by stimulating the production of arachidonic acid metabolites, NO and endothelium derived hyperpolarizing factor in vascular endothelium.(Vanhoutte, 1989)

Figure 1.2 Schematic of RAS and Kallikrein-Kinin System. ACE is strategically positioned to regulate the balance between AngII and bradykinin.(Brown and Vaughan, 1998)

1.4.2 Angiotensin II as a Growth Factor

AngII is a multifunctional hormone with pleiotrophic effects on vascular smooth muscle. It was originally identified as an acute regulator of vasomotor tone(Elliott and Peart, 1956) and has since been shown to promote vascular hypertrophy(Geisterfer *et al.*, 1988; Berk *et al.*, 1989) and in some cases, hyperplasia.(Campbell and Robertson, 1981) It also affects vascular cell migration(Dubey *et al.*, 1995) and extracellular matrix production.(Kato *et al.*, 1991)

In vivo AngII has been shown to induce SMC proliferation, measured by incorporation of bromodeoxyuridine in the balloon injured rat carotid artery.(Daemen *et al.*, 1991) In that same study the effect of AngII was shown to be more marked on neointimal cells. Medial SMC proliferation in response to AngII was shown to be transient in uninjured regions of the vessel compared with the sustained mitogenic action of AngII on the neointima.(deBlois *et al.*, 1996b)

In contrast, AngII was reported to only increase protein, but not DNA synthesis in isolated rat aorta studied using an organ culture technique.(Holycross *et al.*, 1993) This may imply that AngII requires cofactors for expression of its mitogenic potential within the intact blood vessel.

1.4.3 Angiotensin II Receptors

The tissue response to AngII is mediated by specific receptors located on the cell surface. As defined by their pharmacological profile, there are two main subtypes of AngII receptors: AT₁ and AT₂, blocked by specific antagonists.(de Gasparo *et al.*, 1995) Both AT₁ and AT₂ receptors contain a seven transmembrane domain and are coupled to G proteins. The AT₁ receptor subtype, predominant in the vascular system, mediates most of the actions of AngII such as secretion of aldosterone and catecholamines from the adrenal gland, central nervous responses, release of catecholamines from sympathetic nerve endings, and the regulation of vascular tone by vasoconstriction.(Whitebread *et al.*, 1989; Wong *et al.*, 1990; Gehlert *et al.*, 1991; Timmermans *et al.*, 1991) Treatment with suppressor doses of AngII enhanced the growth of the neointima in wildtype mice but not in AT_{1a} knockout mice after injury.(Harada *et al.*, 1999)

The AT_2 receptor has an antiproliferative effect on neointimal formation after vascular injury (Nakajima *et al.*, 1995) and on coronary endothelial cells (Stoll *et al.*, 1995). Recent studies have indicated that AT_2 associates with G_i proteins (Zhang and Pratt, 1996) and mediates inhibitory effects on mitogen-activated protein kinase (MAPK) activation in vascular SMCs (Nakajima *et al.*, 1995), PC12W cells (Yamada *et al.*, 1996) and neuronal cultures (Huang *et al.*, 1996). The AT_2 receptor subtype also has proapoptotic properties (Stoll *et al.*, 1995; Yamada *et al.*, 1996). The ability of a tissue to change its expression of AT_1 to AT_2 receptors has been described in experimentally-induced vascular injury (Dzau *et al.*, 1991; Janiak *et al.*, 1992) suggesting that the AT_2 receptor may also play a role in SMC differentiation, proliferation and apoptosis. Indeed, transfection of an AT_2 receptor expression vector into the balloon injured rat carotid artery attenuated neointimal formation (Nakajima *et al.*, 1995).

1.4.4 Angiotensin II Signaling

AngII rapidly stimulates multiple phospholipases to hydrolyze membrane phospholipids and generate signaling molecules (figure 1.3). In cultured vascular SMCs, phospholipase C (PLC) is activated within 5 seconds, hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Alexander *et al.*, 1985; Griendling *et al.*, 1986). This response is transient, returning toward baseline levels by 2 minutes. These second messengers in turn release calcium from internal stores and activate protein kinase C (PKC), leading to a cascade of protein phosphorylations that direct the subsequent response of the cell. PKC also serves as a negative-feedback regulator of PLC, since it is apparently responsible, at least in part, for terminating the PLC response (Brock *et al.*, 1985).

Phospholipases

While PLC- β is generally considered to be involved in mediating responses to AngII in cultured vascular SMCs (Schelling *et al.*, 1997), recent data has also implicated PLC- γ in mediating AngII-induced PIP_2 hydrolysis in cultured SMCs (Marrero *et al.*, 1995a). However, a major role of PLC- γ seems inconsistent with reports that tyrosine kinase inhibitors, which would be anticipated to inhibit activation of PLC- γ , fail to inhibit the rise in IP_3 or release of intracellular Ca^{2+} in response to AngII in cultured rat (Schelling *et al.*, 1997) or human vascular SMCs (Erdo, 1977).

In addition to PLC, phospholipase D (PLD) may also play a role in activation of PKC by AngII in vascular SMCs (Ford and Gross, 1989). Activation of PLD is not associated with a rise in IP_3 but has been reported to be the major source of DAG in cultured rat aortic SMCs (Lassegue *et al.*, 1993). DAG produced by PLC or PLD stimulates PKC. Activation of PKC by AngII has been reported to be required for AngII-induced growth in several studies using cultured vascular SMCs (Langan *et al.*, 1994; Malarkey *et al.*, 1996; Liao *et al.*, 1996; Liao *et al.*, 1997).

Figure 1.3 Schematic Presentation of the Two Pathways That Couple the AT₁ Receptor to PKC. ? represents unidentified tyrosine kinases(van Bilsen, 1997)

Tyrosine Phosphorylation

AngII induces tyrosine phosphorylation of multiple proteins in cultured rat aortic vascular SMCs.(Butcher *et al.*, 1993) Several studies have shown that inhibition of tyrosine kinases blocks protein synthesis(Leduc *et al.*, 1995) or DNA synthesis(Kobayashi *et al.*, 1994) in response to AngII. There is also evidence that a Ca²⁺-dependent tyrosine kinase may contribute to AngII-induced tyrosine phosphorylation, possibly in conjunction with PKC.(Tsuda *et al.*, 1991) Another class of tyrosine kinases linked to AT₁ receptor activation is the Janus kinase (JAK) family. The JAK/STAT pathway is a signaling pathway comprising a number of tyrosine kinases and transcription factors. Stimulation of AT₁ receptors has recently been shown to induce activation of JAK2 and to a lesser extent TYK2 in rat aortic SMCs and in the same studies the AT₁ receptor was shown to co-immunoprecipitate with JAK2, but not with JAK1 or TYK2.(Marrero *et al.*, 1995b; Berk and Corson, 1997)

Intracellular Ca²⁺

A rise in [Ca²⁺]_i is the primary signal initiating force production in vascular smooth muscle.(Somlyo and Somlyo, 1994) An increase in [Ca²⁺]_i activates calmodulin (CaM) which in turn activates myosin light chain kinase allowing the actin-myosin interaction to proceed with resultant ATP hydrolysis, crossbridge formation and force generation.(Stull *et al.*, 1991) The sensitivity of the contractile process to [Ca²⁺]_i can also be influenced by contractile agonists, probably through modulation of myosin phosphatase activity(Kureishi *et al.*, 1997) or by MAPK dependent phosphorylation of thin filament proteins caldesmon and calponin.(Horowitz *et al.*, 1996) There is also evidence that AngII can enhance α₁-adrenoceptor mediated contraction in rabbit aorta by activation of PKC.(Henrion *et al.*, 1992a; Henrion *et al.*, 1992b)

1.5 The Sympathetic Nervous System and the Vascular Response to Injury

Activation of neurohumoral systems has been implicated in the process of vascular remodeling.(Daemen and De Mey, 1995) Experimental evidence suggests a role for the SNS and circulating catecholamines in the development of vascular remodeling. It has been demonstrated that catecholamines induce proliferation and protein synthesis of cultured vascular SMCs through stimulation of α_1 -adrenoceptors.(Blaes and Boissel, 1983; Yamori *et al.*, 1987; Nakaki *et al.*, 1990; Chen *et al.*, 1995; Siwik and Dale-Brown, 1996) Prolonged infusions of norepinephrine or phenylephrine resulted in significant increases in the cross-sectional area (CSA) of rat carotid arteries.(deBlois *et al.*, 1996a) In spontaneously hypertensive rats, hypertrophic inward vascular remodeling could partially be prevented by neonatal sympathectomy or treatment with an antibody against nerve growth factor.(Lee *et al.*, 1987) This reduction of vascular remodeling was even more pronounced after adrenal demedullectomy or chronic α_1 -adrenoceptor blockade in neonatal rats(Lee *et al.*, 1991; Korner *et al.*, 1993) supporting a role for catecholamines derived from the SNS as well as from the adrenal medulla in vascular remodeling. Also, surgical or chemical denervation results in thickening of the vessel wall, due to vascular SMC hypertrophy. Furthermore, vascular SMCs show important morphological and ultrastructural alterations after sympathectomy including, amongst others, an irregular shape, an increase in the number of organelles and large indented nuclei rich in euchromatin.(Fronck, 1983; Branco *et al.*, 1984; Dimitriadou *et al.*, 1988) These findings suggest that the SNS plays an important role in the regulation of vascular SMC differentiation in neonates, while in the adult it may significantly contribute to the maintenance of the contractile phenotype of vascular SMCs.(Daemen and De Mey, 1995) It remains to be established which neurotransmitter is the actual trophic factor of sympathetic innervation since norepinephrine as well as the sympathetic cotransmitters ATP and NPY have been shown to exert mitogenic effects on vascular SMCs.(Erlinge *et al.*, 1993; Zukowska-Grojec *et al.*, 1993) There is also evidence suggesting adenosine, derived from neurogenically released ATP, as the trophic factor of the SNS.(Osswald, 1991; Osswald and Azevedo, 1991)

1.5.1 Norepinephrine Transporter

The biosynthetic pathway of norepinephrine synthesis is shown in figure 1.4. The metabolic precursor of norepinephrine is L-tyrosine, an aromatic amino acid present in body fluids, which is taken up by adrenergic neurons. Tyrosine hydroxylase represents the rate limiting step in the biosynthesis of norepinephrine. Most of the synthesized norepinephrine is contained in vesicles. Depolarization of the nerve terminal membrane promotes the fusion and discharge of synaptic vesicles. Released norepinephrine is metabolized by two enzymes, monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT). MAO occurs within cells, bound to the surface membrane of mitochondria. COMT occurs in both neuronal and non-neuronal tissues and is mainly involved in the metabolism of catecholamines released in the circulation and in the inactivation of norepinephrine in tissues with sparse adrenergic innervation.(Rang *et al.*, 1991) However, degradation of norepinephrine by the catabolizing enzymes MAO and COMT plays only a minor role in comparison to the uptake.

The norepinephrine transporter (NET) is an integral membrane Na^+ /cotransporter protein that can rapidly move norepinephrine (and Na^+) back inside synaptic terminals. In that way it can terminate synaptic transmission and recycle neurotransmitters.(Axelrod, 1971) Recent work by Lu *et al.* showed that chronic stimulation of norepinephrine uptake by AngII involves stimulation of NET mRNA production in the brain of rats.(Lu *et al.*, 1996) The data presented in that study suggests that AngII binds to the neuronal AT_1 -receptor subtype and stimulates the

phospholipase C-phosphoinositol hydrolysis system (figure 1.3). This increases the activity of PKC and the transcription of the NET gene. The result is an increase in NET-activity and an increased norepinephrine uptake.(Lu *et al.*, 1996)

Figure 1.4 Biosynthetic Pathway of Norepinephrine Synthesis at a Varicosity of the Sympathetic Nerve Terminal. Abbreviations are explained in the text.

Analysis of the structure of the NET cDNAs predicts highly hydrophobic, integral membrane proteins with twelve transmembrane domains and cytoplasmatic NH₂ and COOH termini.(Melikian *et al.*, 1994; Brüss *et al.*, 1995)

Interestingly, possible NET coding variants are apparent from divergent bovine NET isolates of adrenal gland,(Jursky *et al.*, 1994; Lingen *et al.*, 1994) involving differential splicing of coding sequences in the COOH terminus.(Pörzgen *et al.*, 1995) At present it is not known whether the vascular NET system is also activated by AngII.

1.6 α₁-Adrenergic Receptors

The work of Ahlquist in 1948 using epinephrine and its metabolites defined the concept of α- and β-adrenoceptors.(Ahlquist, 1948) Ahlquist stated that the receptor mediating vasoconstriction should be termed the α-adrenotropic and the receptor mediating vasodilatation and myocardial contraction be termed the β-adrenoceptor.(Ahlquist, 1948) In 1957, Brown and Gillespie noted that α-adrenoceptor blockers increased the outflow of norepinephrine following nerve stimulation of the cat spleen.(Brown and Gillespie, 1957) This led to the hypothesis of presynaptic α-adrenoceptor that modulates the release of neurotransmitter from the synaptic nerve terminal. The significant difference in the potency of different phenoxybenzamine concentrations for inhibiting vascular effects and transmitter release led Dubocovich and Langer(Dubocovich and Langer, 1974) to suggest that the pre- and postjunctional

receptors were not identical. Langer (Langer, 1974) suggested that the prejunctional α -adrenoceptors be called α_2 , while the postjunctional α -adrenoceptors be referred to as α_1 .

However, the concept that the receptors could be classified based on synaptic location proved to be incorrect. With the development of selective ligands for both α -adrenoceptor subtypes, the nature of pre- and postjunctional α -adrenoceptors could be evaluated. Drew and Whiting (Drew and Whiting, 1979) identified two types of vascular α -adrenoceptors, the prazosin sensitive α_1 - and the prazosin insensitive α_2 -adrenoceptor. Shortly after the subclassification of α_1 - and α_2 -adrenoceptors, evidence began to emerge that was inconsistent with a single postjunctional vascular α_1 -adrenoceptor. (Ruffolo *et al.*, 1977; Bevan, 1981) Colucci *et al.* (Colucci *et al.*, 1985) provided evidence of receptor heterogeneity in cultured aortic SMCs. Johnson and Minneman (Johnson and Minneman, 1987) showed that an alkylating analogue of clonidine, chloroethylclonidine (CEC) could only reduce the α_1 -adrenoceptor population of rat brain by approximately 50%. Furthermore, Scatchard plots indicating high- and low-affinity [3 H] prazosin sites in rabbit aorta were made by Piascik *et al.* (Piascik *et al.*, 1988). Radioligand binding studies provided clear and compelling evidence of α_1 -adrenoceptor subtypes. (Morrow and Creese, 1986) The site at which such ligands as WB 4101, phentolamine, epinephrine, norepinephrine, and phenylephrine interacted with high affinity was termed the α_{1A} -adrenoceptor. The low-affinity site for these ligands was termed the α_{1B} -adrenoceptor.

1.6.1 The α_1 -Adrenergic Receptor Subtypes

In discussing α_1 -adrenoceptor subtypes, the lower case letters a, b, c, and d indicate cloned receptors. When speaking of pharmacologically defined receptors, the upper case letters A, B, and D are used. The first α_1 -adrenoceptor was cloned in 1988. (Cotecchia *et al.*, 1988) The cDNA encoded a single polypeptide of 515 amino acids with an apparent molecular weight of approximately 80 kD (table 1.2). This receptor has pharmacological characteristics similar to the α_{1B} -adrenoceptor. (Morrow and Creese, 1986) The cloned α_{1B} -adrenoceptor has the very familiar structural motif common to the superfamily of G protein coupled seven transmembrane receptors. It possesses seven hydrophobic domains consisting of 20-28 amino acids, which comprise the membrane spanning regions of the receptor. The N-terminus is located in the space. The cytoplasmic carboxyl-terminus and intracellular loops are important for coupling to G proteins and intracellular signaling pathways. The cloned α_{1B} -adrenoceptor binds norepinephrine and epinephrine in the micromolar range and exhibits the appropriate stereochemistry for an adrenergic receptor. The α_{1B} -adrenoceptor was further shown to possess low affinity for ligands such as WB 4101, phentolamine, 5-methylurapidil and phenylephrine. (Cotecchia *et al.*, 1988; Lomasney *et al.*, 1991a; Perez *et al.*, 1991) This receptor is sensitive to CEC inactivation. In situ hybridization (ISH), histochemistry and ribonuclease protection assays have also localized mRNA for the α_{1B} -adrenoceptor in a variety of peripheral blood vessels. (Piascik *et al.*, 1994; Guarino *et al.*, 1996)

Table 1.2 Cloning and Characteristics of α_1 -Adrenoceptor Subtypes (Piascik *et al.*, 1996)

Current Nomenclature	Gene Product	Amino Acids	Selective Agonists or Antagonist	CEC Sensitivity
α_{1A} (Schwinn <i>et al.</i> , 1990)	α_{1c}	466	5-methylurapidil, (+)-niguldipine, oxymetazoline, A61603, cirazoline	Low
α_{1B} (Cotecchia <i>et al.</i> , 1988)	α_{1b}	515	AH11110A	High (90% inactivated)
α_{1D} (Perez <i>et al.</i> , 1991)	α_{1d}	560	BMY 7378	Moderate to high (70% inactivated)

In the early 90's cDNA isolated from bovine brain encoded a novel peptide of 466 amino acids.(Schwinn *et al.*, 1990) This receptor was 72% homologous to the α_{1B} -adrenoceptor in the putative membrane spanning regions, and was designated the α_{1c} -adrenoceptor.(Schwinn *et al.*, 1990) Recent reports have suggested that the cloned α_{1c} -adrenoceptor in fact may be the pharmacological α_{1A} -adrenoceptor.(Forray *et al.*, 1994; Laz *et al.*, 1994; Perez *et al.*, 1994; Price *et al.*, 1994)

In 1991, Perez *et al.*(Perez *et al.*, 1991) cloned and expressed a novel cDNA. The deduced amino acid sequence from this cDNA was a single polypeptide chain of 560 amino acids, the topology of which was similar to that of G protein coupled receptors. They showed, furthermore, that this receptor was sensitive to CEC inactivation and therefore suggested that this novel receptor represented a new α_1 -adrenoceptor subtype, the α_{1d} -adrenoceptor.(Perez *et al.*, 1991) All subtypes activate PLC and PLA₂, increase cAMP levels, mediate an IP₃-dependent increase in intracellular Ca²⁺ and stimulate Ca²⁺ influx through L-type channels.(Piascik *et al.*, 1996)

1.6.2 Coupling to G Proteins

The G proteins coupling α_1 -adrenoceptors to their intracellular effectors and the selectivity of the various α_1 -adrenoceptors subtypes for different G proteins have not been clearly defined. α_1 -Adrenoceptors predominantly couple to pertussis toxin-insensitive G proteins of the G_{q/11} family, and there is evidence for selectivity among the various α_1 subtypes for coupling to the different members of this family.(Wu *et al.*, 1992) For example, G_q, G₁₁, G₁₄, and G₁₆ can mediate inositol-phosphate turnover by the α_{1B} -adrenoceptor, but the α_{1D} -adrenoceptor couples only via G_q or G₁₁.(Wu *et al.*, 1992) It has also been demonstrated that the various cloned α_1 -adrenoceptor subtypes can activate multiple effectors via coupling to both pertussis toxin-sensitive (G_i or G_o family) and -insensitive G proteins.(Nebigil and Malik, 1992) Moreover, both the α and the $\beta\gamma$ subunits of the pertussis toxin-insensitive G proteins may mediate activation of specific receptor-coupled effectors, such as the β isoform of PLC.(Blank *et al.*, 1992)

In addition to activation of PLC- β via G_{q/11}, there is evidence that the α_{1B} -adrenoceptor activates a 69-kD PLC via coupling to a high molecular mass (74-kD) class of pertussis toxin-insensitive G proteins, termed G_h, that are distinct from the heterotrimeric G proteins.(Nakaoka *et al.*, 1994) These G_h proteins are multifunctional proteins with both transglutaminase and receptor signaling functions and are expressed in various tissues, including brain, heart, and liver.(Nakaoka *et al.*, 1994) Interestingly, binding of GTP by G_h inhibits its transglutaminase activity, whereas binding of Ca²⁺, which is required for transglutaminase activity, prevents GTP binding.

It is interesting to note that in model cell systems, all three α_1 -adrenoceptor subtypes generate the same diverse array of intracellular second messengers by activating

similar signaling pathways. If this also occurs in the vasculature, there appears to be redundant control of smooth muscle function by the α_1 -adrenoceptors, with each subtype capable of activating contraction via nearly identical pathways. What may occur *in vivo* is that receptors may preferentially activate one component of these diverse signaling pathways. Also, there may be differences in G protein expression that could promote a certain type of differential second messenger coupling. (Neer, 1995; Chidiac, 1998)

1.6.3 Genomic Organization, Promoters and Regulatory Elements

α_1 -Adrenoceptors all contain a single intron.(Ramarao *et al.*, 1992; Weinberg *et al.*, 1994) Thus, each of the genes for the three α_1 -adrenoceptor subtypes consists of two exons and a single large intron (14 to 20 kb) that interrupts the coding region at the end of the putative sixth transmembrane domain. In all cases, the exon/intron boundary is situated after the first base of the codon, indicating a type-1 splice phase. This genomic organization is unique among those G protein coupled receptor genes that do contain introns. Given a genomic organization of two exons and only one intron, splicing of nascent transcripts cannot account for different α_1 -adrenoceptor subtypes unless cryptic splice sites are used or unless there is transsplicing of exons. Transsplicing has, as yet, not been convincingly demonstrated for mammalian genes. However, there is evidence for the use of a cryptic splice site in transcription of the α_{1c} -adrenoceptor.(Hirasawa *et al.*, 1995) This gives rise to two alternate splice variants, which differ in length and sequences of their encoded C-terminal domains.

The 5' untranslated region of the rat α_{1b} -adrenoceptor gene has also been sequenced and evaluated for transcriptional control elements.(Gao and Kunos, 1994; Kanasaki *et al.*, 1994; Gao and Kunos, 1993; Gao *et al.*, 1995) Like the human α_{1b} -adrenoceptor gene, a cAMP response element has been identified in the rat gene.(Gao and Kunos, 1993; Kanasaki *et al.*, 1994) The 5' untranslated region (1621 bp) of the rat α_{1b} -adrenoceptor gene has a high degree of similarity (87%) with the nucleotide sequence of the human α_{1b} -adrenoceptor gene located 500 bp upstream from the translation start site.(Kanasaki *et al.*, 1994) Independently, Gao and Kunos(Gao and Kunos, 1993) found that the overall structure of the rat α_{1b} -adrenoceptor gene is highly conserved with that of the human gene. They identified additional consensus sites for AP-1 and AP-2 binding sites and glucocorticoid and thyroid response elements in the 5' flanking region, as well as a putative polyadenylation signal (ATTAAA) at the same location in the 3' flanking region as in the human α_{1b} -adrenoceptor gene.

Although the gene for the α_{1c} -adrenoceptor has been isolated,(Perez *et al.*, 1994) neither its regulatory elements nor those of the α_{1d} -adrenoceptor gene have been defined. It is possible that transcription of these genes is also under the control of different promoters that are differentially activated in various tissues and species or in response to developmental or hormonal stimuli, since three α_{1c} -transcripts (5.0, 3.9, 3.0 kb) and two α_{1d} -transcripts (3.0 and 1.7 kb) have been identified in the human prostate with the use of cDNA probes that clearly do not cross-hybridize,(Faure *et al.*, 1994) whereas only a single α_{1c} -and α_{1d} -adrenoceptor transcript was observed in rat heart(Perez *et al.*, 1994) and in rat brain,(Perez *et al.*, 1991; Lomasney *et al.*, 1991a) respectively.

It should be mentioned, in this regard, that analysis of α_1 -adrenoceptor regulatory elements and transcripts is particularly problematic, since (1) the 5' flanking regions are very high in GC content, making primer extension, ribonuclease protection assays, and polymerase chain reaction analyses of those regions difficult, (2) there is a high degree of homology between α_1 -adrenoceptor subtypes, even at the nucleotide level, and (3) in

many tissues, the abundance of α_1 -adrenoceptor transcripts is extremely low. As a result, relatively large amounts of RNA are required for evaluation of α_1 -adrenoceptor transcripts. This could potentially lead to the identification of unrelated cross-hybridizing species, which may account, in part, for some of the marked variations (e.g., from 2.2 to 11 kb for the α_{1c} , from 2.1 kb to 5.0 kb for the α_{1b} -adrenoceptor, and from 1.7 to 3.0 kb for the α_{1a} -adrenoceptor) in reported transcript sizes.(Perez *et al.*, 1991; Lomasney *et al.*, 1991a; Schwinn *et al.*, 1991; Esbenshade *et al.*, 1993; Hu and Hoffman, 1993; Faure *et al.*, 1994; Stewart *et al.*, 1994)

1.6.4 Receptor Coupled Effectors

α_1 -Adrenoceptor-mediated activation of PLC results in the phosphodiesteratic cleavage of a minor component of the membrane phospholipid pool, PiP_2 , to yield the second messengers, IP_3 and DAG (figure 1.5). IP_3 , thus generated, interacts with specific receptors on intracellular organelles, such as sarcoplasmic reticulum, to release stored Ca^{2+} . DAG activates PKC, which then phosphorylates a variety of cellular substrates, including Ca^{2+} channels, that may regulate intracellular Ca^{2+} or activate transcription of various genes. Blockade of Ca^{2+} channels with dihydropyridines or removal of extracellular Ca^{2+} was found to inhibit α_{1A} - but not α_{1B} -mediated contractile responses in isolated vascular smooth muscle or Ca^{2+} influx in hepatocytes.(Minneman, 1988)

Studies with expressed cloned α_1 -adrenoceptor subtypes demonstrate that all three subtypes can both mobilize intracellular Ca^{2+} and increase Ca^{2+} influx via voltage-operated Ca^{2+} channels.(Schwinn *et al.*, 1991; Perez *et al.*, 1993) These studies also demonstrate that all three subtypes can couple not only to PLC activation but also to activation of PLA_2 and PLD and can promote cAMP accumulation.(Schwinn *et al.*, 1991; Perez *et al.*, 1993) This latter effect probably occurs via a PKC-mediated potentiation of adenylyl cyclase activity,(Perez *et al.*, 1993) although there is also evidence for α_1 -adrenoceptor mediated inhibition of cAMP degradation.(Buxton and Brunton, 1985)

Figure 1.5 ERK1/2 (extracellular signal-regulated kinase) Activation by G Protein-Coupled Receptors Coupled to Pertussis Toxin-Insensitive G Proteins. MEK is MAPK kinase.(Vanbiesen *et al.*, 1996)

1.7 The Sympathetic Nervous System of Response to AngII

Several laboratories have demonstrated that AngII interacts with the SNS at various levels. AngII facilitates the release of norepinephrine from the synaptic sympathetic varicosities via specific AngII-receptors (AT_1 receptor) and of epinephrine from the chromaffin cells of the adrenal medulla.(Feldberg and Lewis, 1964; Starke, 1977) It also increases smooth vascular tone using the same intracellular second messengers, IP_3 and DAG, as does norepinephrine, thereby enhancing the effects of that catecholamine on vascular SMCs.(Berridge, 1993; Griendling *et al.*, 1997) AngII is known to stimulate the activity of the SNS, not only centrally, but also at the level of the synapse and through stimulation of the adrenal release of catecholamines. These effects are probably mediated by AT_1 receptors.(Wong *et al.*, 1990) Furthermore, it remains to be established whether the effect of AngII is mediated by a direct effect of the peptide on the vascular wall or by its interaction with the SNS since the mitogenic response of the rat aorta, induced by chronic low-dose AngII infusion, can be blocked by the α_1 -adrenoceptor antagonist prazosin.(van Kleef *et al.*, 1992)

Increased mRNA transcription, without changes in mRNA stability, has also been observed for the α_{1b} -adrenoceptor in response to AngII treatment of cultured rat aortic SMCs. This effect of AngII was associated with increased α_1 -adrenoceptor expression and α_{1d} -adrenoceptor mRNA levels, as well as an increase in α_{1b} -adrenoceptor mRNA, and involved a PKC-dependent, but Ca^{2+} -independent, mechanism.(Hu *et al.*, 1995) However, in rabbit aortic SMCs, activation of PKC as a result of α_{1b} -adrenoceptor stimulation by norepinephrine, although having a permissive effect on α_{1b} -adrenoceptor gene transcription, had a greater effect in destabilizing α_{1b} -adrenoceptor mRNA.(Izzo, Jr. *et al.*, 1994)

1.8 Hypothesis

Angiotensin II and α_1 -adrenoceptors have prominent effects on vascular growth and particularly on the vascular response after injury.(Jackson *et al.*, 1988; O'Malley *et al.*, 1989; Fingerle *et al.*, 1991; Daemen *et al.*, 1991; Vashisht *et al.*, 1992) However, this response after injury is heterogeneous i.e. medial SMCs are sensitive and the neointimal SMCs are insensitive.(van Kleef *et al.*, 1996a) Also, the precise mechanisms and interactions are still unknown. We therefore further investigated the role of the α_1 -adrenoceptors in the growth regulation in the balloon injured rat carotid artery after stimulation with AngII.

We hypothesized that AngII induces an increase in the release of norepinephrine from the sympathetic nerve endings (neurogenic). This neurogenic release is modulated by the NET, which is also regulated by AngII. Furthermore, we hypothesized that AngII induces an increase in the systemic (humoral) release of (nor)epinephrine from the adrenal medulla. Since both pathways are able to stimulate α_1 -adrenoceptors located on the SMC surface, its difficult to distinguish between them. Therefore, we further hypothesize that the differences in AngII induced growth responses between the uninjured media and the injured neointima comes from differences in sympathetic innervation and/or differences in the presence of α_1 -adrenoceptor subtypes between the media and the neointima in the rat carotid artery.

1.9 This Thesis

In the first experimental chapter (chapter 2), mRNA localization and mRNA amount of two α_1 -adrenoceptor subtypes in the balloon injured rat carotid artery was investigated at different time points after injury. In chapter 3, the possible upregulation of the NET in the balloon injured rat carotid artery after AngII-stimulation was investigated. In chapter 4, the effects of AngII on SMC growth in the injured rat carotid artery after the blockade

of the release of neuronal norepinephrine were investigated. The release of neuronal norepinephrine from the SNS, was blocked by chemical sympathectomy with 6-OHDA. In chapter 5, the possible interactions between adrenal medulla derived catecholamines and α_1 -adrenoceptors on AngII induced neointimal growth in the balloon injured rat carotid artery model, were investigated by removal of both adrenal glands and infusion of the α_1 -adrenoceptor antagonist prazosin. Chapter 6 deals with the involvement of the SNS on the regulation of intimal thickening in humans. In that chapter, we investigated sympathetic innervation patterns in human stenotic arteriovenous fistulas. The results of the studies described in the previous chapters are discussed in chapter 7.

CHAPTER 2

Distribution of the mRNAs of the α_1 -Adrenoceptor Subtypes
in the Rat Carotid Artery After Balloon Catheter Induced
Injury

Abstract

To investigate the distribution of the mRNAs of the α_1 -adrenoceptor subtypes in the injured rat carotid artery, a balloon injury of the left carotid artery was performed on male Wistar rats. Subsequently, the mRNAs of the α_{1b} - and α_{1d} -adrenoceptor genes were detected by in situ hybridization (ISH) at 4 and 12 weeks after injury, while the amount of the mRNAs of the α_{1b} - and α_{1d} -adrenoceptors were quantified by a reverse transcription-polymerase chain reaction (RT-PCR) on total RNA isolated from carotid arteries at 1, 4, and 12 weeks after balloon injury.

4 Weeks after injury the mRNA levels for both the α_1 -adrenoceptor subtypes was increased (α_{1b} : 147.5 ± 39.1 fg/250 ng total RNA; α_{1d} : 390.7 ± 61.9 fg/250 ng total RNA) as compared to 12 weeks after injury (α_{1b} : 41.0 ± 9.3 fg/250 ng total RNA; α_{1d} : 52.3 ± 11.4 fg/250 ng total RNA). The amount of mRNA of the α_{1b} - and α_{1d} -adrenoceptor of the uninjured rat carotid artery did not change in time after injury.

The ISH data show that transcription of the two α_1 -adrenoceptor genes at 4 weeks after injury, occurs throughout the adventitia, media and neointima of injured carotid arteries. Both the smooth muscle cells in the media and the neointima, as the vasa vasorum and nerves in the adventitia, showed positivity for the α_1 -adrenoceptor subtypes mRNAs. This positive signal showed a gradient increasing from the adventitia towards the lumen of the vessel and was higher as compared to the ISH data 12 weeks after injury, which showed almost no signal for the α_1 -adrenoceptor subtypes mRNAs. With ISH no obvious differences between the distribution of the α_1 -adrenoceptor subtypes could be detected.

These data show that the amount and distribution of the mRNA for the α_1 -adrenoceptor subtypes changes in time after balloon injury of the rat carotid artery.

Introduction

α_1 -Adrenergic receptors in vascular smooth muscle exert trophic actions to regulate the phenotype of vascular smooth muscle cells, in addition to their dynamic regulation of vascular contractility in response to stimulation of the sympathetic nervous system. While sympathetic denervation of the rabbit ear artery inhibits normal vessel wall development in young animals, (Bevan, 1984) acute injection of the α_1 -selective adrenergic agonist phenylephrine or the infusion of catecholamines increases protein, mRNA synthesis and cellular polyploidy, but not cellular proliferation, in rat aorta. (Yamori *et al.*, 1987; Majesky *et al.*, 1990) Conversely, α_1 -adrenoceptor blockade with prazosin inhibits neointimal thickening in models of vascular injury in the rat and rabbit. (O'Malley *et al.*, 1989; Fingerle *et al.*, 1991) The α_1 -adrenoceptor antagonist doxazosin however, did not reduce SMC DNA synthesis in the media and neointima of the injured rat carotid artery when infused together with AngII two weeks after balloon injury. It did, however, decrease SMC DNA synthesis in the media of the uninjured carotid artery. (van Kleef *et al.*, 1996a)

One possible explanation for this relative insensitivity of the neointima to α_1 -adrenergic blockade is a relative loss of α_1 -adrenoceptors in the neointima. An alternative explanation is a shift in α_1 -adrenoceptor subtypes which may result in different effects.

Although there are some studies about the tissue and vascular distribution of the α_1 -adrenoceptor subtypes in different species, (Piascik *et al.*, 1994; Kohno *et al.*, 1994; Alonso-Llamazares *et al.*, 1995; Piascik *et al.*, 1997; Suzuki *et al.*, 1997) there is no data on the distribution of α_1 -adrenoceptors subtypes in the rat carotid artery after balloon injury.

We report here the analysis of the mRNA amount in the injured and uninjured rat carotid artery at 1, 4, and 12 weeks after injury. The quantitative RT-PCR, of total RNA isolated from injured rat carotid artery, revealed that the amount of both the α_{1b} - and α_{1d} -adrenoceptor mRNAs were increased at 4 weeks after balloon injury of the rat carotid artery as compared to 12 weeks after injury. The mRNA levels in the uninjured carotid artery remained unchanged.

In situ hybridization showed that both receptor subtypes were present throughout the entire vessel wall, with a gradient from the adventitia to the lumen. No obvious changes in receptor distribution pattern over time were found after injury.

These data show that the neointima, that develops after balloon injury of the rat carotid artery, contains α_1 -adrenoceptor mRNAs. Their quantities, but not subtype or distribution changes over time.

Materials & Methods

Animals

Male Wistar rats obtained from Iffa Credo (Someren, the Netherlands) were used (body weight, 275-325 g; n=15). They had free access to standard rat chow (Hope Farms, Woerden, the Netherlands) and tap water. The experiments were performed according to institutional guidelines.

Balloon Injury

All rats were anaesthetized with pentobarbital (60 mg/kg body weight) by intra-peritoneal (*i.p.*) injection. The left common carotid artery was injured by introduction of a 2F balloon catheter (Baxter, Maarsen, the Netherlands) into the left external carotid artery. After inflation, the catheter was withdrawn through the common carotid artery. This procedure was repeated three times after which the catheter was removed and the

external carotid artery ligated. The right carotid artery was not traumatized and served as a control.(Clowes *et al.*, 1983a; Clowes *et al.*, 1983b)

Tissue Processing

After 1, 4, and 12 weeks after balloon injury 3 animals per time-point were anaesthetized with sodium pentobarbital (80 mg/kg *i.p.*) and the left and right carotid artery, the vas deferens and the heart were rapidly removed and dissected in ice-cold saline (0.9% NaCl) containing 2 units/ml of recombinant ribonuclease inhibitor (RNasin, Promega Corp., Madison, WI). The tissues were frozen in liquid nitrogen and then stored at -70°C until extraction of RNA.

After 4 (n=3) and 12 weeks (n=3) after injury of the left carotid artery rats received 0.5 ml 0.5% Evans blue (Chroma Gesellschaft, Stuttgart, Germany) in 0.9% NaCl by intravenous (*i.v.*) injection, to stain non-endothelialized tissue in the left common carotid artery. One hour later the animals were killed by pentobarbital overdose (80 mg/kg body weight *i.p.*) and the arterial system was perfused with phosphate buffered saline (PBS) followed by perfusion fixation with 4% phosphate buffered paraformaldehyde (pH 7.4), at a pressure of 100 mmHg. Maximal vasodilatation was ensured by adding nitroprusside (0.1 mg/ml, Sigma Chemical Co., St. Louis, Mo., USA) to the perfusate.

One circular segment (2-3 mm long) was taken from the center of the blue stained non-endothelialized part of the left carotid artery. Heart (α_{1b}) and vas deferens (α_{1d}) served as positive controls for the ISH.(Scofield *et al.*, 1995) All tissues were fixed overnight in 4% phosphate buffered paraformaldehyde, routinely processed and embedded in paraffin (Para Clean, Klinipath, Duiven, the Netherlands).

Total RNA Isolation

Injured left and uninjured right carotid arteries were homogenized in 4 M guanidine isothiocyanate solution using a Polytron P1200 homogenizer (Kinematica AG). The homogenized samples were loaded onto 5.7 M cesium chloride containing ultracentrifuge tubes. Total-RNA was pelleted by centrifugation at 35,000 rounds per minute for 18 hours at room temperature. After removal of the supernatant the RNA pellet was resuspended in 0.3 M sodium acetate, pH 6.0 and ethanol. Precipitation was performed for 1 hour at -70°C. After centrifugation the pellet was washed with 70% ethanol, centrifuged again, and the resulting pellet was dissolved in 15 μ l H₂O. The integrity of the RNA was checked by agarose gel electrophoresis, while the total RNA content of each individual sample was determined by optical density at 260 nm.

Polymerase Chain Reaction Primer Selection

Primers used in the PCR reaction were designed in such a way that the PCR product would span the nucleotide sequence of the third intracellular loop, specifically amplifying transmembrane region 5 to transmembrane region 6 of the rat α_{1b} -(Voigt *et al.*, 1990) and α_{1d} -adrenergic receptor.(Lomasney *et al.*, 1991a; Perez *et al.*, 1991) Previous results indicated that these two α_1 -adrenoceptor subtypes are present in rat tissues.(Lomasney *et al.*, 1991b) The α_{1A} -adrenoceptor could not be detected in large muscular arteries(Stassen *et al.*, 1997a; Muramatsu, 1991) and was therefore not investigated. For the rat α_{1b} -adrenoceptor cDNA, a 300 bp PCR product was formed using the sense, primer 5'-GCTCCTTCTACATCCCGCTCG-3' and the corresponding anti-sense primer 5'-AGGGGAGCCAAACATAAGATGA-3'. For the α_{1d} -adrenoceptor the sense primer was 5'-CGTGTGCTCCTTCTACCTACC-3', and the corresponding anti-sense primer was 5'-GCACAGGACGAAGACACCCAC-3'. The predicted cDNA amplification product of the α_{1d} -adrenergic receptor was 304 bp long.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was transcribed to cDNA with the use of the 'Ready-To-Go You-Prime First-Strand Beads' (Pharmacia, Uppsala, Sweden). Briefly, 250 ng of total RNA and 100 pmol of random hexanucleotide primer were denaturated at 65°C for 10 minutes, then chilled on ice for 2 minutes. The RNA solution was transferred to the tube of 'First-Strand Reaction Mix Beads' and brought to a final volume of 33 μ l with DEPC-treated water. After gentle mixture of the tube, cDNA was transcribed at 37°C for 60 minutes. Of this RT solution, 3.3 μ l was subjected to PCR with the use of the above described specific primers for the α_{1b} - and α_{1d} -adrenergic receptor cDNA.

The PCR reaction mix contained 20 mM Tris-HCl, pH 8.3, 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgSO_4 , 50 μ M of each dNTP, 20 pmol of sense and anti-sense primers, and 2U *Taq* DNA polymerase in a total volume of 25 μ l. PCR was performed during 35 cycli, with each cycle at 94°C for 30 seconds, 58°C for 1 minute, and 72°C for 1 minute.

Agarose Gel Electrophoresis of RT-PCR Products

10 μ l Of the PCR product was mixed with 2 μ l 6 $\times$ gel loading buffer (0.25% Orange G, 40% (w/v) sucrose) and loaded on a 3% agarose gel prepared in 1 $\times$ TBE buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, pH 8.0) and containing 0.5 μ g/ml ethidium bromide. PCR products were electrophoresed for 45 minutes at 100 V in a Bio-Rad Mini-Gel System (Bio-Rad Laboratories, Hercules, CA). After electrophoresis, fragments were visualized by UV light.

In Situ Hybridization

The PCR fragments were ligated in the pCR2.1-vector (Invitrogene, molar ratio 3:1, 1:1 and 1:3), containing a 3' thymidine overhang and a T7 and Sp6 RNA polymerase site. The specificity of these products were checked by sequencing (Sanger method). A ^{35}S -UTP-labeled anti-sense riboprobe was created by transcription using T7 RNA polymerase (Riboprobe System II, Promega). Plasmid DNA was removed by RQ1 DNase (Promega) treatment and the probe was precipitated after phenol/chloroform extraction. Sections (4 μ m) of paraformaldehyde fixed, paraffin embedded tissues (see *Tissue Processing* section) were cut, mounted on silane-coated slides and dried for 48 hours at 37°C. Pre-treatment consisted of 10 minutes fixation in 4% paraformaldehyde, followed by proteinase K (2 μ g/ml) treatment for 10 minutes at room temperature. Hereafter, the sections were incubated for 1 hour in a prehybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris pH 8.0, 5mM EDTA, 0.02% polyvinyl-pyrrolidone, 0.02% Ficoll, 0.02% BSA, 10% dextran sulphate, and 10 mM DTT) at 37°C. Hybridization was performed overnight at 55°C after addition of 600.000 cpm of the ^{35}S labeled probe per slide. After hybridization the slides were washed in 2 X SSC (2 X SSC= 0.3 M NaCl, 30 mM sodium citrate, pH 7.0), 1mM EDTA and 10 mM β -mercaptoethanol for 10 minutes (2X), RNase treatment and washed for 2 hours in 0.1 X SSC at 55°C. This was followed by a wash in 0.5 X SSC without EDTA/ β -mercaptoethanol and dehydration by immersion in increasing alcohol grades. After dehydration and drying, the slides were dipped in G5 photo emulsion (Ilford, Leiden, The Netherlands) at 42°C, and exposed at 4°C. Depending on message abundance the exposure time varied between 2-6 weeks. The slides were developed with Kodak Dektol developer (Eastman Kodak Company, Rochester, USA) and fixed in 0.8 M sodium thiosulphate and 0.09 M potassium disulphate. The sections were counterstained with hematoxylin and examined by bright- and dark-field microscopy.

Quantitative Reverse Transcription-Polymerase Chain Reaction

The technique of quantitative RT-PCR with the use of internal standards was performed as described previously. (Passier *et al.*, 1995; Passier *et al.*, 1996)

Internal Standards Formation

The pCR2.1-plasmid containing the α_{1b} and α_{1d} PCR-fragments used for the ISH, were digested with specific restriction enzymes. The α_{1b} -adrenoceptor PCR fragment (300 bp) was digested by BspH1 (New England Biolabs) and the α_{1d} -adrenoceptor PCR fragment (304 bp) was digested by DpnII (New England Biolabs). Digestion of the PCR fragments resulted in three fragments of different lengths. The 5' and 3' fragments of each PCR fragment were separately isolated by gel electroelution and consequently ligated with 1 U of T4 DNA ligase (Promega) at 15°C overnight. After amplification (35 cycles), the deletion fragment was checked by agarose gel electrophoresis for length and purity. Subsequently, the deletion fragment was ligated for 15 hours at 15°C with 2 U T4 DNA ligase in the presence of the pCR2.1-vector (Invitrogene, San Diego, USA). After linearizing the plasmid with SacI (Promega), RNA was transcribed with 1 U T7 RNA polymerase (Promega), 1 hour at 37°C. Plasmid DNA was removed by electroelution, followed by DNase treatment with 5U of RQ1 DNase (Promega), 30 minutes at 37°C. The concentration of the internal standard RNA was measured by spectrophotometry at 260 nm and integrity was checked on a 1% agarose gel. Possible plasmid DNA contamination was ruled out by amplification of the RNA. For stabilizing the standard RNA, 30 µg/ml tRNA was added. The lengths of the internal standards for the α_{1b} -adrenoceptor and the α_{1d} -adrenoceptor were 242 bp and 196 bp, respectively.

Reverse Transcription

In the same tube total RNA isolated from the carotid artery was mixed together with increasing amounts of the standard RNA (5 fg to 20 pg α_{1b} -adrenoceptor or 5 fg to 20 pg α_{1d} -adrenoceptor standard RNA per 250 ng total RNA, 12 concentrations/total RNA sample) and transcribed to cDNA with the use of the 'Ready-To-Go You-Prime First-Strand Beads' (Pharmacia, Uppsala, Sweden). Briefly, tubes containing total RNA and increasing amounts of the standard RNA and 100 pmol of random hexanucleotide primer were denatured at 65°C for 10 minutes, then chilled on ice for 2 minutes. The RNA solution was transferred to the tube of 'First-Strand Reaction Mix Beads' and brought to a final volume of 33 µl with DEPC-treated water. After gentle mixture of the tube, cDNA was transcribed at 37°C for 60 minutes.

Polymerase Chain Reaction

The cDNA fragments from each reverse transcription reaction were amplified in duplicate. 5 µl Of the reverse transcription mixture was added to 20 µl of a PCR-mix, containing 20mM Tris-HCl, pH 8.3, 50 mM KCl, 20 pmol of both the sense and anti-sense α_{1b} -adrenoceptor primers and 1.25 U AmpliTaq DNA polymerase (Perkin & Elmer). For the α_{1d} -adrenoceptor PCR, 10 µl of the reverse transcription mixture was added to 15 µl of a PCR-mix, containing 20 mM Tris-HCl, pH 8.3, 50 mM KCl, 20 pmol of both the sense and anti-sense α_{1d} -adrenoceptor primers and 1.25 U AmpliTaq DNA polymerase. The final MgCl₂ concentration in the PCR-mix was 2.0 mM.

The primers used were:

α_{1b} -adrenoceptor sense: 5'-GCTCCTTCTACATCCCGCTCG-3'

α_{1b} -adrenoceptor anti-sense: 5'-AGGGGAGCCAACATAAGATGA-3'

α_{1d} -adrenoceptor sense: 5'-CGTGTGCTCCTTCTACCTACC-3'

α_{1d} -adrenoceptor anti-sense: 5'-GCACAGGACGAAGACACCCAC-3'

The PCR reaction for both the α_{1b} - and the α_{1d} -adrenoceptor cDNA was performed during 40 cycles, with each cycle at 94°C for 30 seconds, 58°C for 1 minute, and 72°C for 1 minute.

Quantification of the PCR Fragments

After amplification, 10 μ l of the PCR fragments were separated by electrophoresis on a 3% agarose gel, containing ethidium bromide. Two fragments were detected by UV light and the image was captured by a digital camera. The optical density of the two fragments was measured by the Gelpro Analyzer software (Version 2.0, Media Cybernetics). The value of the optical density of the internal standard was multiplied by the ratio of target DNA bp/standard bp, thereby correcting for the difference in ethidium bromide density, caused by the difference in length between the target fragment and the internal standard. The mean values of the duplicate samples were plotted as the logarithm of the ratio of the internal standard to the target fragment against the logarithm of the known amount of internal standard RNA in each tube. The line of best fit and the correlation coefficient (r) were calculated. If r^2 was below 0.95 data were discarded. The logarithm value of zero on the y-axis represents the equivalence point, indicating the equal amount of internal standard RNA and target RNA.

Statistics

The mean values of the RNA samples were related to the mean of the standard total RNA. The mRNA amount is expressed in femto (f)g/250 ng total RNA. The mean mRNA amounts ($\pm$ SEM) of 3 animals are given. The mRNA amounts in the injured and the non-injured rat carotid artery at the different time points after injury are evaluated. Statistical differences were calculated using one way ANOVA followed by the Newman-Keuls test for multiple comparisons. Statistical significance was assumed at $p < 0.05$.

Results

α_{1b} -Adrenoceptor mRNA Levels in the Injured and Uninjured Rat Carotid Artery

Quantitative RT-PCR revealed that the mRNA amount of the α_{1b} -adrenoceptor in the injured rat left carotid artery was 67.9 ± 22.8 fg/250 ng total RNA at 1 week, and 147.5 ± 39.1 fg/250 ng total RNA at 4 weeks after balloon injury. This difference however, was not statistically significant ($p=0.45$). The mRNA concentration in the injured left carotid artery at 12 weeks after injury decreased to 41.0 ± 9.3 fg/250 ng total RNA as compared to 4 weeks after injury ($p=0.04$; table 2.1). There was no difference between the α_{1b} -adrenoceptor mRNA amount of the injured left carotid artery at 1 week as compared to 12 weeks after injury.

No differences were detected between the mRNA amounts of the α_{1b} -adrenoceptor in the uninjured right carotid artery. Basal levels were 249.1 ± 53.2 fg/250 ng total RNA and remained unchanged over time.

α_{1d} -Adrenoceptor mRNA Levels in the Injured and Uninjured Rat Carotid Artery

The mRNA level of the α_{1d} -adrenoceptor in the injured rat left carotid artery was 155.1 ± 59.0 fg/250 ng total RNA at 1 week and 390.7 ± 61.9 fg/250 ng total RNA at 4 weeks after balloon injury ($p=0.39$). Quantitative RT-PCR showed that the mRNA concentrations in the injured left carotid artery at 12 weeks after injury decreased to 52.3 ± 11.4 fg/250 ng total RNA as compared to 4 weeks after injury ($p=0.03$; table 2.1). There was no statistical difference between the α_{1d} -adrenoceptor mRNA amount of the injured left carotid artery at 1 week after injury as compared to the α_{1d} -adrenoceptor mRNA amount of the injured left carotid artery at 12 weeks after injury.

In the uninjured rat carotid artery the levels of α_{1d} -adrenoceptor mRNA did not differ throughout the experiment and was 413.5 ± 57.8 fg/250 ng total RNA.

Table 2.1 The Amount of mRNA For the α_1 -Adrenoceptor Subtypes (α_{1b} and α_{1d}) in the Injured Left Carotid (LC) and the Uninjured Right Carotid (RC) Artery at Different Time Points After Balloon Injury

Time After Injury	α_{1b}		α_{1d}	
	LC	RC	LC	RC
7 Days	67.9 $\pm$ 22.8	124.8 $\pm$ 48.0	155.1 $\pm$ 59.0	397.5 $\pm$ 65.4
28 Days	147.5 $\pm$ 39.1	328.5 $\pm$ 56.4	390.7 $\pm$ 61.9	469.8 $\pm$ 28.0
12 Weeks	41.0 $\pm$ 9.3*	257.3 $\pm$ 43.2	52.3 $\pm$ 11.4*	385.4 $\pm$ 47.6

The mRNA amount is expressed in femto (f)g/250 ng of total RNA. Data represent mean $\pm$ SEM.

*) Statistically different from 28 days.

In Situ Hybridization

With the use of mRNA riboprobes designed to recognize specific regions of the α_{1b} - and α_{1d} -adrenoceptors, we were able to localize the mRNAs of both adrenoceptor subtypes in the injured carotid artery at 4 weeks after balloon injury. The mRNAs of both α_1 -adrenoceptor subtypes were located on SMCs in the media and neointima, and on the vasa vasorum and nerves in the adventitia (figure 2.1). Furthermore, the mRNA levels of both α_1 -adrenoceptor subtypes seemed to gradually increase from the adventitia towards the lumen of the vessel (figure 2.1).

On the other hand, no increased mRNA signals for the α_1 -adrenoceptor subtypes could be detected in the injured carotid artery at 12 weeks after injury. This lack of specific signal was, however, not due to sampling error or technical difficulties since both the vas deferens (α_{1d}) and the heart (α_{1b}) showed a positive signal (data not shown).

Discussion

Adrenergic receptors are a family of G protein-linked signaling proteins that can be functionally and structurally classified into three subfamilies, designated α_1 , α_2 and β . Three subtypes of α_1 -adrenergic receptors (α_{1A} , α_{1B} and α_{1D}) have been identified and characterized pharmacologically, (Minneman and Esbenshade, 1994; Minneman, 1988; Minneman *et al.*, 1988) whereas four subtypes (α_{1A} , α_{1B} , α_{1C} and α_{1D}) have been identified through molecular cloning, (Cotecchia *et al.*, 1988; Schwinn *et al.*, 1990; Voigt *et al.*, 1990; Perez *et al.*, 1991; Lomasney *et al.*, 1991a; Forray *et al.*, 1994; Laz *et al.*, 1994; Perez *et al.*, 1994) However, the α_{1A} and α_{1D} cDNA appeared to be the same and are now simply called α_{1d} -adrenoceptor, (Hieble *et al.*, 1995)

Recent studies have led several investigators to conclude that the cloned α_{1d} - and α_{1b} -adrenergic receptors are identical to the pharmacologically defined α_{1D} - and α_{1B} -adrenergic receptors, respectively, and that the cloned α_{1c} -adrenergic receptor is the α_{1A} -adrenoceptor, (Ford *et al.*, 1994; Forray *et al.*, 1994; Price *et al.*, 1994; Perez *et al.*, 1994; Laz *et al.*, 1994) Evidence for additional subtypes has been reported from radio ligand binding studies, (Abel *et al.*, 1995; Ford *et al.*, 1994; Muramatsu *et al.*, 1990) but it remains uncertain whether these novel binding sites represents distinct gene products.

Figure 2.1 Photomicrograph (200x) of a cross-section of a balloon injured left carotid artery hybridized with [³⁵S]-labeled α_{1b} - (A and C) and α_{1d} -adrenoceptor probe (B and D). Upper panels represent 4 weeks and lower panels 12 weeks after balloon injury. L= lumen, I= intima, M= media, A= adventitia.

Although there are only two endogenous ligands, epinephrine and norepinephrine, the physiological responses to catecholamines vary widely. One level of control that may be important in mediating different tissue responses is at the level of the receptors, where different receptor subtypes may regulate very different responses.(Chen *et al.*, 1995) Therefore, tissue or organ differences in receptor subtype expression may represent an important factor in determining adrenoceptor physiology.(Schofield *et al.*, 1995) Furthermore, we do not know whether α_1 -adrenoceptors are present in the neointima of the rat carotid artery. We do know, however, that the neointima is insensitive to blockade with α_1 -adrenoceptor antagonists(van Kleef *et al.*, 1996a) and that there are less α_1 -adrenoceptors present in the vascular wall after injury (see chapter 4). This may suggest that the lack of an adequate number of α_1 -adrenoceptors in the neointima is responsible for the insensitivity for α_1 -adrenoceptor blockade.

This study shows, with the use of competitive RT-PCR, that the mRNA amount of the α_{1b} - and α_{1d} -adrenoceptors in the injured rat carotid artery were significantly decreased at 12 weeks as compared to 4 weeks after balloon injury. Furthermore, there were no differences detected between the α_1 -adrenoceptor subtypes mRNA levels of the uninjured carotid artery at 1, 4, and 12 weeks after injury. α_1 -Adrenoceptor mRNA levels in the injured carotid artery were lower than in the control vessel. Since mRNA levels are expressed per 250 ng total RNA, one explanation is an increase in total RNA levels after injury. Indeed, mRNA levels of for instance proelastin and α_1 procollagen (type I) are dramatically increased after balloon injury.(Majesky *et al.*, 1992)

This study further showed that ISH, with complementary RNA probes capable of differentiating between the α_{1b} - and α_{1d} -adrenoceptor subtypes, detected mRNA for α_1 -adrenoceptors in injured rat carotid arteries at 4 weeks, but not at 12 weeks after balloon injury. The α_1 -adrenoceptor subtypes were present throughout the adventitial, medial and neointimal layer of the carotid arteries and showed an increase in mRNA expression from the adventitia towards the lumen of the vessels. Therefore, it can be concluded that the neointima does contain at least the mRNA of two α_1 -adrenoceptor subtypes. The presence of the α_{1b} - and α_{1d} -adrenoceptor mRNAs have been reported earlier in cultured rat vascular smooth muscle cells.(Hu *et al.*, 1995) Furthermore, ISH on the intact rat aorta clearly showed the presence of mRNA for the α_{1b} - and α_{1d} -adrenoceptor in the media.(Piascik *et al.*, 1994) This is in accordance with the data presented in this study which also indicates that mRNA for the α_{1b} - and α_{1d} -adrenoceptor can be found in the media of the carotid artery.

Infusion of phenylephrine during the 3rd and 4th week after injury showed no increase in DNA labeling fraction in the neointima of injured rat carotid artery.(deBlois *et al.*, 1996a) This is in accordance with the receptor binding studies showing less specific binding of [3 H]-prazosin at two weeks after injury (chapter 4). Furthermore, this study shows that α_1 -adrenoceptor mRNA amounts decrease after injury as compared with uninjured vessels. This is in accordance with the data on receptor binding presented in chapter 4 and may explain the relative insensitivity of α_1 -adrenoceptors for stimulation(deBlois *et al.*, 1996a) and blockade(van Kleef *et al.*, 1996a) on DNA synthesis.

When we review the data at 12 weeks after injury there was almost no mRNA production for the α_1 -adrenoceptors in the injured vessels. However, infusion of phenylephrine at this time point significantly increased the neointimal SMC DNA synthesis.(deBlois *et al.*, 1996a) This indicates that at 12 weeks after injury enough functional α_1 -adrenoceptors may be present on the vessel wall. The low levels of the α_{1b} - and α_{1d} -adrenoceptor mRNA in the injured vessels measured at 12 weeks after injury may be explained by the normalized protein levels with subsequent downregulation of the mRNA.

In summary this study shows i) that the amount of α_1 -adrenoceptor mRNA decreases after carotid injury when compared to the uninjured carotid artery ii) that the injured carotid artery shows a transient increase in α_1 -adrenoceptor mRNA iii) that at least the mRNAs of two α_1 -adrenoceptor subtypes are present in the injured and uninjured rat carotid artery iv) that the neointima contains the mRNA of at least two subtypes v) that there is no clear switch in subtype after injury.

We conclude that the relative insensitivity of the rat carotid artery to α_1 -adrenoceptor stimulation and blockade is not due to an absence of α_1 -adrenoceptors in the neointima, not due to a subtype switch, but may be due to a lack of sufficient α_1 -adrenoceptors after injury.

CHAPTER 3

Detection of Norepinephrine Transporter mRNA in the
Balloon Injured Rat Carotid Artery After Stimulation With
Angiotensin II

Abstract

Previous results indicated that the α_1 -adrenoceptor plays an important role in the angiotensin II (AngII) induced increase in medial, but not in neointimal vascular smooth muscle cell (SMC) DNA synthesis. One of the possible explanations for this difference in growth response between medial and neointimal SMCs during AngII infusion is the amount of norepinephrine in the synaptic cleft, which depends, amongst others, on the balance of release and uptake of the neurotransmitter. The norepinephrine transporter (NET) is a transmembrane protein mediating the uptake of norepinephrine from the synaptic cleft into the sympathetic nerves endings. In doing so it terminates synaptic transmission. In the brain stimulation of norepinephrine uptake by AngII involves NET gene transcription.

To investigate a possible role of the NET in the vascular wall, we have used the reverse transcription-polymerase chain reaction (RT-PCR) technique to detect the mRNA of the NET gene in both balloon-injured and uninjured rat carotid arteries after chronic AngII infusions. In the injured rat carotid artery NET mRNA levels could be detected at eight and twenty-four hours after continuous infusion with AngII (2.81 ± 0.08 and 2.66 ± 0.10 optical density (*O.D.*), respectively). In the uninjured carotid artery however, NET mRNA levels were only detected at twenty-four hours (7.37 ± 0.48 *O.D.*) after chronic AngII infusion. NET mRNA could neither be detected in the injured nor in the uninjured rat carotid artery after NaCl infusion.

These data show i) that the NET gene transcription is upregulated in the vascular wall after AngII infusion and ii) that injured vessels are more susceptible to this AngII-induced increase in NET gene transcription than uninjured vessels. These data suggest that the NET could play an important role in the difference in growth response between injured and uninjured rat carotid arteries upon stimulation with AngII.

Introduction

Although the involvement of the sympathetic nervous system in the mitogenic response of vascular smooth muscle cells (SMC)s to angiotensin II (AngII) is well established the mechanism remains to be elucidated. Previous data from our lab showed that the AngII induced increase in medial SMC DNA synthesis of the carotid artery was decreased, not only after co-infusion with the α_1 -adrenoceptor antagonist doxazosin, (van Kleef *et al.*, 1996a) but also after chemical sympathectomy with 6-OHDA (chapter 4). However, the AngII induced increase in neointimal SMC DNA synthesis two weeks after balloon injury of the left carotid artery was insensitive to pharmacological α_1 -adrenoceptor blockade (van Kleef *et al.*, 1996a) or chemical sympathectomy with 6-OHDA (chapter 4). Thus, the AngII induced increase in rat carotid SMC DNA synthesis is mediated by the sympathetic nervous system in the media, but not in the neointima formed after balloon catheter induced injury. This suggests a differential regulation of AngII induced SMC growth in injured and uninjured vessels, at least as far as the involvement of the sympathetic nervous system is concerned.

One of the explanations for the difference in AngII induced SMC growth in injured and uninjured vessels may be the differential involvement of the norepinephrine transporter (NET). The NET is an integral membrane Na^+ /cotransporter protein that can rapidly move norepinephrine (and Na^+) back inside sympathetic nerve endings (figure 1.4, chapter 1) which ends synaptic transmission. (Axelrod, 1971)

Recent work by Lu *et al.* showed that chronic stimulation of norepinephrine uptake by AngII involves stimulation of NET mRNA production in the rat brain. (Lu *et al.*, 1996) The data presented in that study suggests that AngII binds to the neuronal angiotensin type 1 (AT_1) receptor and stimulates the phospholipase C-phosphoinositol hydrolysis system (figure 1.3, chapter 1). This increases the activity of phosphokinase C and the transcription of the NET gene which results in a chronic increase in NET-activity and an increase in norepinephrine uptake. (Lu *et al.*, 1996)

In analogy with the findings in the rat brain, we hypothesized that AngII infusion would also increase the transcription of the NET gene in the vessel wall. Furthermore, we hypothesized that elevated transcription is only apparent in the balloon injured artery and not in the uninjured artery. This difference in NET mediated uptake of norepinephrine would therefore explain the difference in growth response between injured and uninjured rat carotid arteries upon stimulation with AngII.

Materials & Methods

Animals

Male Wistar rats obtained from Iffa Credo (Someren, the Netherlands) were used (body weight, 275-325 g). They had free access to standard rat chow (Hope Farms, Woerden, the Netherlands) and tap water. Rats were randomly divided into two groups. Group A (n=15) received AngII ten weeks after balloon injury while group N (n=15) received NaCl. All experiments were performed according to institutional guidelines.

Balloon Denudation

All rats were anaesthetized with pentobarbital (60 mg/kg bodyweight) by intra-peritoneal (i.p.) injection. The left common carotid artery was injured by introduction of a 2F balloon catheter (Baxter, Maarsen, the Netherlands) into the left external carotid artery. After inflation, the catheter was withdrawn through the common carotid artery. This procedure was repeated three times after which the catheter was removed and the external carotid artery ligated. The right carotid artery served as a control and was not traumatized. (Clowes *et al.*, 1983a)

Drug Infusion

Human [Val⁵]-AngII (Saxon Biochemicals GMBH, Hannover, Germany) was continuously infused using osmotic minipumps (Alzet, model 2001, Alza Corp., Palo Alto, Ca., USA) implanted subcutaneously between the shoulder blades under ether anaesthesia. AngII was dissolved in 0.9% NaCl and infused at a rate of 250 ng/kg/min. Previous results in rat brain neuronal cultures showed an upregulation of NET mRNA between 2-8 hours after AngII stimulation, with peak levels at 8 hours, which remained elevated for up to 24 hours.(Lu *et al.*, 1996) Since phenylephrine infusion in the first few weeks after balloon injury has no effect on neointimal SMC growth, but increases neointimal SMC growth when infused at ten weeks after balloon injury,(deBlois *et al.*, 1996a) all infusions were started ten weeks after balloon injury. Thus, infusions with AngII or NaCl started ten weeks after balloon denudation and were given for 8 hours (group A8(n=5), N8(n=5)), 24 hours (group A24(n=5), N24(n=5)), and 7 days (group A7(n=5), N7(n=5)).

Tissue Processing

At the end of the infusion period animals were killed by ether anaesthesia. From all animals both the injured left and the uninjured right carotid artery as well as the brain and adrenal glands, which served as positive controls for the NET primer set used (see RT-PCR section), were quickly excised, snap-frozen in liquid nitrogen and stored at -70°C until they were used for total RNA isolation.

Total RNA Isolation

Tissue samples were homogenized in 4 M guanidine isothiocyanate solution using a Polytron P1200 homogenizer (Kinematica AG). The homogenized samples were loaded onto 5.7 M cesium chloride containing ultracentrifuge tubes. Total-RNA was pelleted by centrifugation at 35,000 rounds per minute for 18 hours at room temperature. After removal of the supernatant the RNA pellet was resuspended in 0.3 M sodium acetate, pH 6.0 and ethanol. Precipitation was performed for 1 hour at -70°C. After centrifugation the pellet was washed with 70% ethanol, centrifuged again, and the resulting pellet was dissolved in 15 µl H₂O. The integrity of the RNA was checked by agarose gel electrophoresis, while the total RNA content of each individual sample was determined by optical density at 260 nm.

Reverse Transcription-Polymerase Chain Reaction

Since it is not known whether the NET gene is present in the vessel wall of rats, a reverse transcription-polymerase chain reaction (RT-PCR), with a primer set selected from the human NET cDNA sequence(Pacholczyk *et al.*, 1991) was performed. This primer set was also used by Cubells *et al.*(Cubells *et al.*, 1995a) to detect the NET mRNA levels in the rat adrenal medulla and locus ceruleus in the rat brain.

Total RNA was transcribed to cDNA with the use of the 'Ready-To-Go You-Prime First-Strand Beads' (Pharmacia, Uppsala, Sweden). Briefly, 250 ng of total RNA and 100 pmol of random hexanucleotide primer were denatured at 65°C for 10 minutes, then chilled on ice for 2 minutes. The RNA solution was transferred to the tube of 'First-Strand Reaction Mix Beads' and brought to a final volume of 33 µl with DEPC-treated water. After gentle mixture of the tube, cDNA was transcribed at 37°C for 60 minutes. Of this RT solution, 3.3 µl was subjected to PCR with the use of the NET primers. Equal sample loading was checked by PCR with GAPDH-primers.

The primers used were:

NET-sense: 5'-CAGTTACCAACAAATTTGACAACAACTGT-3'

NET-anti-sense: 5'-GTCGTTTCGGAACCTGTCCACTCC-3'

GAPDH-sense: 5'-TCCGCCCCCTTCGCTGATGCC-3'

GAPDH-anti-sense: 5'-TAGTGGGCCCCCTCGGCCGCCTG-3'

The PCR reaction contained 20 mM Tris-HCl, pH 8.75, 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgSO_4 , 50 μM of each dNTP, 20 pmol of sense and anti-sense primers, and 2U *Taq* DNA polymerase in a total volume of 25 μl . PCR was performed during 35 cycles, with each cycle at 94°C for 30 seconds, 54°C for 1 minute, and 72°C for 1 minute.

Agarose Gel Electrophoresis of RT-PCR Products.

After PCR, 10 μl of samples were mixed with 2 μl 6× gel loading buffer (0.25% Orange G, 40% (w/v) sucrose) and loaded on a 3% agarose gel prepared in 1 × TBE buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, pH 8.0) and containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. PCR products were electrophoresed for 45 minutes at 100 V in a Bio-Rad Mini-Gel System (Bio-Rad Laboratories, Hercules, CA). After electrophoresis, fragments were visualized by UV light.

Quantification of RT-PCR products

After amplification, 10 μl of the PCR fragments were separated by electrophoresis on a 3% agarose gel, containing ethidium bromide. The fragment was detected by UV light and the image was captured by a digital camera. The optical density of the fragment was measured by the Gelpro Analyzer software (Version 2.0, Media Cybernetics). The value of the optical density of the sample was multiplied by the GAPDH-correction factor of the same sample to correct for equal sample loading. The mRNA amounts in the injured and the non-injured rat carotid artery at the different time points after injury are evaluated.

Statistics

Data are presented as mean $\pm$ SEM. Statistical differences were calculated using one way ANOVA followed by the Newman-Keuls test for multiple comparisons. Statistical significance was assumed at $p < 0.05$.

Results

Effects of NaCl Infusion on the NET mRNA Levels of the Carotid Arteries

mRNA signals for the NET could neither be detected in the injured nor in the uninjured rat carotid artery after all three NaCl infusion periods (table 3.1). However, RT-PCR performed on total RNA isolated from the brain and the adrenal glands of rats after seven days of chronic NaCl-infusion, showed a signal for the NET mRNA in both tissues and validated the RT-PCR procedure used (data not shown).

Table 3.1 The Amount of mRNA For the Norepinephrine Transporter in the Injured Left Carotid (LC) and the Uninjured Right Carotid (RC) Artery at Different Time Points After Infusion

Time After Infusion	LC		RC	
	NaCl	AngII	NaCl	AngII
8 hours	ND	2.81 ± 0.08	ND	ND
24 hours	ND	2.66 ± 0.10	ND	7.37 ± 0.48
7 days	ND	ND	ND	ND

The mRNA amount is expressed in optical density. Data represent mean $\pm$ SEM. ND; Non Detectable.

Effects of AngII Infusion on the NET mRNA Levels of the Carotid Arteries

Rat NET mRNA was detected in the injured left carotid artery after AngII infusion (table 3.1). The NET mRNA level in the injured left carotid artery was 2.81 ± 0.08 optical density (O.D.) at eight hours after AngII infusion and 2.66 ± 0.10 O.D. at twenty-four hours after AngII-infusion and was not significantly different ($p=0.45$).

In the uninjured right carotid artery, no NET mRNA could be detected eight hours after AngII infusion (table 3.1). However, NET mRNA levels of the uninjured right carotid artery increased to 7.37 ± 0.48 O.D. at twenty-four hours after chronic AngII infusion.

NET mRNA could neither be detected in the injured nor in the uninjured rat carotid artery after chronic AngII infusion for seven days (table 3.1).

Discussion

In the present study, mRNA expression of the NET in both the injured and the uninjured rat carotid arteries was observed after chronic AngII infusion, while no NET mRNA could be detected after NaCl infusion. These findings do support our hypothesis that AngII infusion may increase the uptake of norepinephrine into the sympathetic nerve endings through the NET, which will result in decreased levels of norepinephrine in the synapse. However, the NET mRNA not only increased in the injured but also in the uninjured rat carotid artery after AngII infusion, albeit that the time course was different. While NET mRNA levels in the injured arteries were elevated at eight and twenty-four hours after AngII infusion, we found a peak at twenty-four hours in uninjured arteries. The question therefore arises whether the observed shift in the time course of NET mRNA upregulation can explain the difference in the mitogenic response between injured and uninjured rat carotid arteries upon AngII stimulation, since previous data from our laboratory indicate that sympathetic innervation or more exactly α_1 -adrenoceptors (van Kleef et al., 1992; van Kleef et al., 1996a) mediate the AngII-induced replication of SMCs, but only in the uninjured rat carotid artery. Therefore, it could be possible that the increase in NET mRNA in uninjured arteries may not be sufficient to decrease the amount of norepinephrine, leaving them susceptible to α_1 -adrenoceptor blockade, whereas the early increase of NET mRNA in injured arteries may have been sufficient to make them insensitive to α_1 -adrenoceptor blockade.

Another possibility is that not only the uptake of norepinephrine in the carotid artery is increased after AngII infusion, but that enhanced metabolic degradation of norepinephrine may explain the presumed lowering of norepinephrine concentrations in the synaptic cleft. However, the effect of injury and AngII infusion on the catecholamines metabolizing enzymes monoamine oxidase and catechol-O-methyl transferase is not known.

NET cDNAs predict highly hydrophobic, integral membrane proteins with 12 transmembrane domains and cytoplasmic NH_2 and COOH termini (Melikian et al., 1994; Brüss et al., 1995). Recently, Pörzgen and co-workers (Pörzgen et al., 1995) cloned and characterized the human NET gene, revealing a highly dispersed locus with ~2 kb of coding exons spread across 45 kb of genomic DNA. The gene is single copy in humans and located at chromosome 16q12.2 (Brüss et al., 1993). Although sites of NET gene expression (Pacholczyk et al., 1991; Ramamoorthy et al., 1993; Lorang et al., 1994; Cubells et al., 1995a) match expectations from norepinephrine biosynthesis and uptake studies in the rat brain and adrenal gland, full-length rodent cDNAs suitable for regulatory studies *in vitro* and *in vivo* have yet to be reported. However, all amino acid residues that are presently considered essential for the function and specificity in the mono-amino transporters (Borowsky and Hoffman, 1995) are conserved between the mouse, human, and bovine NET species variants.

The recent cloning and sequencing of the human NET gene allowed investigation of factors that modulate NET gene expression. The data presented here indicate that AngII is one of these factors and that AngII also modulates the NET in the vessel wall. In general, agents that increase sympathetic activity, such as reserpine or insulin, decrease NET mRNA levels.(Figlewicz *et al.*, 1993; Cubells *et al.*, 1995b; Cubells *et al.*, 1995a) In contrast, desipramine, which decreases neuronal firing rates, increases NET mRNA.(Shores *et al.*, 1994) AngII, which facilitates sympathetic activity, also seems to increase NET mRNA levels.

The data presented in this study further show that the NET gene transcription is differentially upregulated by AngII, i.e. injured vessels are more susceptible to this AngII-induced increase in NET gene transcription than uninjured vessels. The data suggest that the NET could play an important role in the regulation of the mitogenic responses of injured and uninjured rat carotid arteries as seen after AngII stimulation. To our knowledge this is the first study which detects the presence of mRNA of the NET in the vessel wall.

CHAPTER 4

Effects of Chemical Sympathectomy on Angiotensin II-Induced Neointimal Growth in the Balloon Injured Rat Carotid Artery

Abstract

To investigate the role of the sympathetic nervous system in angiotensin II (AngII) stimulated medial and neointimal smooth muscle cell (SMC) replication, we sympathectomized rats with 6-hydroxydopamine (6-OHDA) in which the left carotid artery was injured by a balloon catheter. AngII (250 ng/kg/min), infused two weeks after balloon injury of the rat left carotid artery, increased systolic blood pressure by approximately 70 mmHg. There was no effect of 6-OHDA on this pressor response. AngII increased the cumulative 5-bromo-2'-deoxyuridine (BrdU) labeling fraction (LF) in the uninjured right carotid media and the injured left carotid neointima as compared to controls ($5.7 \pm 1.6\%$ vs. $0.4 \pm 0.1\%$, $p < 0.05$; $10.6 \pm 0.9\%$ vs. $5.0 \pm 0.8\%$, $p < 0.05$; respectively). 6-OHDA decreased the AngII induced increase in LF in the media of the uninjured right carotid artery (AngII/6-OHDA: $0.9 \pm 0.2\%$ vs. AngII: $5.7 \pm 1.6\%$, $p < 0.05$). 6-OHDA did not decrease the AngII induced increase in LF in both the injured left carotid media and neointima at 4 weeks after balloon injury. The effects of chemical sympathectomy were comparable with those obtained 12 weeks after balloon injury. Thus, data show that the sympathetic nervous system mediates the AngII induced increase in SMC DNA synthesis, but only in the uninjured carotid media. This indicates a differential regulation of AngII induced SMC replication in injured and uninjured vessels.

Introduction

The sympathetic nervous system plays an important role in the regulation of vascular smooth muscle cell (SMC) DNA synthesis. For instance, surgical sympathetic denervation of the rabbit ear artery reduces SMC proliferation,(Bevan, 1975) and chemical sympathectomy with 6-OHDA, which destroys the sympathetic nerve endings leaving vessels denervated,(Aprigliano and Hermismeyer, 1976) reduces the number of SMCs in the growing aortic media.(Fronek *et al.*, 1978) In contrast, stimulation of α_1 -adrenoceptors by phenylephrine increases the expression of growth related factors such as PDGF-A,(Majesky *et al.*, 1990) an effect which may be mediated via mitogen activated protein kinases.(Yu *et al.*, 1996) α_1 -Adrenoceptor antagonists, when administered immediately after balloon injury, reduce SMC DNA synthesis and neointima formation in the rat carotid artery and rabbit abdominal aorta.(Jackson *et al.*, 1988; O'Malley *et al.*, 1989; Fingerle *et al.*, 1991; Vashisht *et al.*, 1992) AngII also stimulates SMC DNA synthesis in the media and the neointima after balloon injury,(Daemen *et al.*, 1991) an effect which may be partly mediated through α_1 -adrenoceptors since AngII facilitates sympathetic transmission by potentiating neurotransmitter release and inhibiting norepinephrine uptake,(Starke, 1977) and stimulates the humoral release of catecholamines from the adrenal medulla.(Feldberg and Lewis, 1964) The α_1 -adrenoceptor antagonist doxazosin however, did not reduce SMC DNA synthesis in the media and neointima of the injured rat carotid artery when infused together with AngII two weeks after balloon injury. It did however, decrease SMC DNA synthesis in the media of the uninjured carotid artery.(van Kleef *et al.*, 1996a) Furthermore, if the α_1 -adrenoceptor agonist phenylephrine was infused two weeks after balloon injury of the rat common carotid artery SMC DNA synthesis in the neointima was not affected.(deBlois *et al.*, 1996a) If, however, phenylephrine was infused ten weeks after injury neointimal SMC DNA synthesis did increase.(deBlois *et al.*, 1996a) These results indicate that in the uninjured vessel the mitogenic effects of AngII are mediated by α_1 -adrenoceptors. The role of α_1 -adrenoceptors in the growth regulation of injured vessels appears to be limited and restricted to the late phase of neointimal growth.(deBlois *et al.*, 1996a)

A limitation of the α_1 -adrenoceptor antagonists studies is that it is not possible to distinguish between the AngII induced increase in release of norepinephrine from the sympathetic nerve endings (neurogenic) or the AngII induced increase in systemic (humoral) release of (nor)epinephrine from the adrenal medulla. Therefore we decided to remove neuronal norepinephrine but not adrenal medulla norepinephrine by chemical sympathectomy using 6-OHDA(Aprigliano and Hermismeyer, 1976; Yamaguchi and Nakada, 1992) at 4 and at 12 weeks after balloon injury of the left carotid artery. Based on the lack of effect of phenylephrine infusion on neointima SMC growth in the first few weeks after balloon injury(deBlois *et al.*, 1996a) we hypothesized that removal of the neurogenic input during AngII infusion would not decrease neointimal SMC growth 4 weeks after balloon denudation, but would decrease AngII induced neointimal SMC growth at 12 weeks after balloon denudation as phenylephrine infusion during that period established an increase in neointimal SMC growth.(deBlois *et al.*, 1996a)

Materials & Methods

Animals

Male Wistar-Kyoto rats obtained from a local inbred strain (University of Maastricht), were used (body weight, 275-325 g; n=74). They had free access to standard rat chow (Hope Farms, Woerden, the Netherlands) and tap water.

Rats were randomly divided into eight groups: group A (4 weeks: n=9) received 0.9% NaCl; group B (4 weeks: n=9) was treated with AngII; group C (4 weeks: n=9) received

AngII and was sympathectomized with 6-OHDA, and group D (4 weeks: n=10) received only 6-OHDA. Group E (12 weeks: n=8) received 0.9% NaCl; group F (12 weeks: n=9) received AngII; group G (12 weeks: n=11) received AngII and 6-OHDA, and group H (12 weeks: n=9) received only 6-OHDA. The experiments were performed according to institutional guidelines.

Balloon Injury

All rats were anaesthetized with pentobarbital (60 mg/kg body weight) by intra-peritoneal (*i.p.*) injection. The left common carotid artery was injured by introduction of a 2F balloon catheter (Baxter, Maarsen, the Netherlands) into the left external carotid artery. After inflation, the catheter was withdrawn through the common carotid artery. This procedure was repeated three times after which the catheter was removed and the external carotid artery ligated. The right carotid artery was not traumatized and served as a control. (Clowes *et al.*, 1983a; Clowes *et al.*, 1983b)

Blood Pressure Measurement

Systolic blood pressures were measured by tail cuff plethysmography (ITTC inc. Woodland Hills. Ca., USA) in slightly restrained, conscious rats. After a training period of seven days, blood pressure measurements started two days before 6-OHDA treatment and were performed three times a week.

Chemical Sympathectomy

6-OHDA (Sigma Chemical Co., St. Louis, Mo., USA) was dissolved in 0.9% NaCl (50 mg/kg) and brought to pH 4.7 with glutathione (Merck, Darmstadt, Germany). 6-OHDA was injected 2x *i.p.* with 2 hours intervals. This procedure results in complete sympatholysis for 9-10 days. (Aprigliano and Hermesmeyer, 1977) To establish complete sympatholysis during the entire two weeks of AngII infusions, 6-OHDA was repeated eight days after the first treatment. Controls received only vehicle.

Drug Infusion

Human [Val⁶]-AngII (Saxon Biochemicals GMBH, Hannover, Germany) and BrdU (Serva, Heidelberg, Germany) were continuously infused using osmotic minipumps (Alzet model 2002, Alza Corp., Palo Alto, Ca., USA) implanted subcutaneously between the shoulder blades under ether anaesthesia. AngII was dissolved in 0.9% NaCl and infused at a rate of 250 ng/kg/min. BrdU was also dissolved in 0.9% NaCl (30 mg/ml) and infused at a rate of 714 ng/kg/min. All infusions started two (groups A,B,C,D) or ten (groups E,F,G,H) weeks after balloon injury and lasted for two weeks.

Tissue Processing

At the end of the experiment all rats received 0.5 ml 0.5% Evans blue (Chroma Gesellschaft, Stuttgart, Germany) in 0.9% NaCl by intravenous (*i.v.*) injection, to stain non-endothelialized tissue in the left common carotid artery. One hour later the animals were killed by pentobarbital overdose (80 mg/kg body weight *i.p.*) and the arterial bed was perfused during 5 minutes with phosphate buffered saline (PBS) followed by perfusion fixation with 4% phosphate buffered paraformaldehyde (pH 7.4) during 5 minutes, at a pressure of 100 mmHg, by a catheter advanced retrogradely in the abdominal aorta. Maximal vasodilatation was ensured by the addition of nitroprusside (0.1 mg/ml, Sigma Chemical Co.) to the perfusate.

One circular segment (2-3 mm long) was taken from the center of the blue stained non-endothelialized part of the left carotid artery. For comparison, one segment was taken at a similar location from the noninjured right carotid artery.

Hearts were excised, blotted dry and weighed after removal of the atria. All tissues were fixed overnight in 4% phosphate buffered paraformaldehyde, routinely processed and embedded in paraffin (Para Clean, Klinipath, Duiven, the Netherlands).

Evaluation of Innervation

Prior to perfusion fixation the right femoral artery was ligated, excised, and placed in freshly prepared 2% glyoxylic acid (monohydrate, ICN Biomedicals Inc., Aurora, Ohio, USA) in 0.1 M phosphate buffer at pH 7.2 supplemented with 10% sucrose. After 30 minutes vessels were stretched on slides and dried for 30 minutes under an airstream at room temperature. Subsequently, the slides were heated for 4 minutes at 100°C, mounted on entellan, and stored in the dark. The preparations were evaluated with a Leitz Dialux 20 microscope (Rijswijk, the Netherlands) equipped with orthomat camera and filterblock D by an observer blinded for the different groups. With this method nerves that contain norepinephrine stain bright green whereas norepinephrine depleted nerves show no staining. (Cowen and Burnstock, 1980)

Since femoral arteries are densely innervated they served as a control to assess sympatholysis by 6-OHDA. (Rosenblum and Chen, 1976) We assumed that if the femoral artery was denervated by 6-OHDA, the lesser innervated carotid artery would also be denervated. Femoral arteries of groups A, B, E, and F (not-6-OHDA treated groups) showed strong staining with glyoxylic acid indicating intact sympathetic innervation (data not shown). Femoral arteries of 6-OHDA treated rats (groups C, D, G and H, 6-OHDA treated groups) showed no staining with glyoxylic acid at the end of the experimental period indicating effective denervation.

Morphometric Analysis

Cross-sectional areas of the media, defined as the area between the external and internal elastic lamina, and the neointima, defined as the area between the internal elastic lamina and the lumen of the artery, were measured using a computerized morphometric system (Quantimet 570, Leica), on 4 µm Lawson (Boom B.V., Meppel, the Netherlands) stained cross-sections of the carotid arteries. Measurements were performed on two cross-sections taken from each ring.

Immunohistochemistry

The cumulative labeling fractions of BrdU positive cells in the media of both carotid arteries and in the neointima of the left carotid artery were determined after immunostaining of 4 µm cross-sections with a monoclonal antibody directed against BrdU using methods published earlier. (van Kleef *et al.*, 1992; van Kleef *et al.*, 1996a) The cumulative labeling fraction ($[\text{BrdU-positive cells}/\text{total number of cells}] \times 100\%$) was determined by one observer, blinded for the different groups, who counted the number of labeled cells and the total number of cells in the media or neointima.

Ligand Binding

Analysis of specific binding of α_1 -adrenoceptors in the presence of 0.3 nM [^3H]-prazosin in injured left carotid arteries and uninjured right carotid control arteries was performed essentially as described by Stassen *et al.* (Stassen *et al.*, 1997a) Segments (5-10 mm long) of carotid artery were incubated for 60 minutes at 37°C in 250 µl 50 mM Tris-HCl, 5 mM MgCl_2 (pH 7.4) (incubation buffer) containing 0.3 nM [^3H]-prazosin. Non-specific binding was determined in parallel incubations in the continuous presence of phentolamine (25 µM). After incubation the arterial segments were gently blotted and rinsed during vortexing for 30 seconds with 1 ml incubation buffer at 37°C. They were then filtered over Whatman filters (GF/C) 5 times with 5 ml ice-cold incubation buffer.

Arterial segments were recovered from the filters and solubilized in 200 μ l 1 N NaOH. To 100 μ l of this, 5 ml scintillation solution (Formula 989, Packard) was added and the radioactivity determined in a liquid scintillation counter (Beckman). To the remaining 100 μ l, 900 μ l H₂O was added and the protein and DNA content were determined as described by Bradford (Bradford, 1976) and by Labarca and Paigan, (Labarca and Paigan, 1980) using bovine serum albumin and calf thymus DNA as internal standards, respectively.

Statistics

Data are presented as mean $\pm$ SEM. Comparisons between the groups were made by non-parametric multiple comparison using the Kruskal-Wallis H-test. Statistical significance was assumed at $p < 0.05$.

Results

Effects of AngII and 6-OHDA on Body Weight and Heart Weight

The body weights of the animals in the different groups did not differ neither at the start of the experiment nor at the start of the infusions/injections. In the 4 weeks experiment (group A-D) body weights decreased during infusion with AngII (group B: -10 ± 3 g), whereas animals in the control group increased their body weight (group A: 20 ± 3 g, table 4.1). When 6-OHDA was given together with AngII (group C) there was no further decrease in body weight as compared to the group that received AngII alone. The increase in body weight during administration of 6-OHDA alone did not differ from the increase in the NaCl alone group. The heart-to-body-weight ratio in the AngII group (group B: 3.3 ± 0.1 g/kg) was increased as compared to the NaCl group (group A: 2.8 ± 0.1 g/kg), which was mainly due to the loss in body weight during the 2 weeks infusion period (table 4.1). There was no effect of the addition of 6-OHDA on the heart-to-body-weight ratio neither when given alone nor when given together with AngII.

Body weights of the animals in the different groups in the 12 weeks experiment (group E-H) did not differ neither at the start of the experiment nor at the start of the infusions/injections. Due to normal growth, the body weight at the start of the infusions/injections had increased with approximately 50 g as compared to the animals in the 4 weeks experiment. The changes in body weight after the infusions/injections were comparable to those described above for the 4 weeks experiment (table 4.1).

Table 4.1 Effects of Ang II and 6-OHDA on Body Weight and Heart Weight at 4 and 12 Weeks After Balloon Injury of the Left Carotid Artery.

4 WEEKS				
Groups	A NaCl	B AngII	C AngII/6-OHDA	D NaCl/6-OHDA
Change in body weight (g)	$+20 \pm 3$	$-10 \pm 3^*$	-9 ± 5	$+19 \pm 4$
Heart weight (mg)	993 ± 25	1101 ± 23	1066 ± 36	959 ± 23
Heart-to-body- weight (10^{-3})	2.8 ± 0.1	$3.3 \pm 0.1^*$	3.4 ± 0.1	2.9 ± 0.1

12 WEEKS				
Groups	E NaCl	F AngII	G AngII/6-OHDA	H NaCl/6-OHDA
Change in body weight (g)	+7 ± 3	-16 ± 6*	-35 ± 7	-5 ± 3
Heart weight (mg)	1217 ± 66	1388 ± 30	1162 ± 39	1158 ± 55
Heart-to-body-weight (10 ⁻³)	3.1 ± 0.1	3.8 ± 0.1*	3.4 ± 0.1	3.0 ± 0.2

Changes in body weight and heart weight are given over the infusion period. Data represent mean ± S.E.M. *) Significantly different from NaCl, $p < 0.05$.

Effects of AngII and 6-OHDA on Systolic Blood Pressure

The systolic blood pressure (SBP) of AngII infused animals increased from 136 ± 11 mmHg five days prior to infusion of AngII to 201 ± 19 mmHg at the end of the two weeks infusion period (figure 4.1A). Injection of 6-OHDA (groups C & D) had a small but transient blood pressure lowering effect. As compared to the NaCl and AngII groups, SBPs in the 6-OHDA groups were not different.

The rise in SBP of AngII infused animals in the 12 weeks experiment was comparable with the 4 weeks experiment (figure 4.1B). Injections of 6-OHDA (groups G & H) had, as in the 4 weeks experiment, no effect on the final outcome of blood pressure as compared to the NaCl and AngII groups (figure 4.1B).

Figure 4.1 The Development of Systolic Blood Pressure (mmHg) During the A) 4 Weeks Experiment and B) During the 12 Weeks Experiment. Data represent mean ± S.E.M. *) Significantly different from NaCl over time, $p < 0.05$. #) Significantly different from 6-OHDA over time, $p < 0.05$. Arrows indicate days of injections with 6-OHDA.

Effects of AngII and 6-OHDA on Uninjured Right Carotid Artery Medial Structure and Lumen

In the 4 weeks experiment AngII infusion increased the LF of SMCs (figure 4.2A) in the media of the non-injured right carotid arteries from $0.4 \pm 0.1\%$ in the NaCl control group to $5.7 \pm 1.6\%$ in the AngII group. Addition of 6-OHDA reduced the AngII induced increase in medial LF as compared to the group which received AngII alone ($0.9 \pm 0.2\%$ vs. $5.7 \pm 1.6\%$). AngII also increased the medial CSA (table 4.2). When 6-OHDA was given together with AngII (group C) there was no decrease in medial CSA as compared

to animals receiving AngII alone. Also 6-OHDA alone (group D) did not affect the medial CSA. The lumen area after AngII infusion was comparable to the lumen area of animals receiving NaCl (table 4.2). Neither administration of 6-OHDA during AngII infusion nor 6-OHDA alone had an effect on lumen area as compared to administration of AngII alone or NaCl.

In the 12 weeks experiment largely similar findings were obtained (figure 4.2B and table 4.2) as compared to the 4 weeks experiment.

Effects of AngII and 6-OHDA on Injured Left Carotid Artery Medial Structure

In the 4 weeks experiment, the labeling fraction (LF) in the media of the injured left carotid artery was slightly increased during AngII infusion from $0.8 \pm 0.3\%$ in group A to $1.8 \pm 0.4\%$ ($p=0.10$) in group B (figure 4.2C). Addition of 6-OHDA did not affect the LF during AngII infusion as compared to the group receiving AngII alone. When 6-OHDA was given alone (group D) the LF was $0.3 \pm 0.1\%$. This result was not significantly different from the NaCl group (group A). During the 2 weeks infusion with AngII the CSA of the media did not increase (table 4.2). There was no effect of 6-OHDA when given alone, nor when given during AngII infusion.

In the 12 weeks experiment largely similar findings were obtained (figure 4.2D and table 4.2) as compared to the 4 weeks experiment.

Table 4.2 Effects of AngII and 6-OHDA on Cross-Sectional Area (CSA) and Lumen Area (LA) at 4 and 12 Weeks After Balloon Injury of the Left Carotid Artery

4 WEEKS				
Groups	A NaCl	B AngII	C AngII/6-OHDA	D NaCl/6-OHDA
CSA (mm ²)				
right media	0.09 ± 0.01	$0.12 \pm 0.01^*$	0.11 ± 0.01	0.09 ± 0.01
left media	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.11 ± 0.01
left neointima	0.11 ± 0.01	$0.16 \pm 0.01^*$	0.15 ± 0.01	0.10 ± 0.01
LA (mm ²)				
right artery	0.29 ± 0.02	0.29 ± 0.01	0.30 ± 0.02	0.28 ± 0.01
left artery	0.19 ± 0.02	0.16 ± 0.01	0.17 ± 0.01	0.18 ± 0.01
12 WEEKS				
Groups	E NaCl	F AngII	G AngII/6-OHDA	H NaCl/6-OHDA
CSA (mm ²)				
right media	0.10 ± 0.01	$0.12 \pm 0.01^*$	0.12 ± 0.01	0.10 ± 0.01
left media	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.11 ± 0.01
left neointima	0.13 ± 0.01	$0.17 \pm 0.01^*$	0.18 ± 0.02	0.11 ± 0.01
LA (mm ²)				
right artery	0.23 ± 0.03	0.24 ± 0.02	0.22 ± 0.02	0.22 ± 0.02
left artery	0.19 ± 0.02	0.16 ± 0.01	0.16 ± 0.01	0.20 ± 0.02

Data represent mean $\pm$ S.E.M. *) Significantly different from NaCl, $p < 0.05$.

Figure 4.2 Effects of AngII and 6-OHDA on labeling fraction of uninjured right carotid media A) 4 weeks after balloon denudation and B) 12 weeks after balloon denudation. C) represents the effects of AngII and 6-OHDA on labeling fraction of injured left carotid media and neointima 4 weeks after balloon denudation and D) 12 weeks after balloon denudation. Data represent mean $\pm$ S.E.M. *) Significantly different from NaCl, $p < 0.05$. #) Significantly different from AngII, $p < 0.05$.

Effects of AngII and 6-OHDA on Injured Left Carotid Artery Neointimal Structure and Lumen

In the neointima of the injured left carotid artery the LF increased during infusion with AngII as compared to the group receiving NaCl alone (figure 4.2C; $10.6 \pm 0.9\%$ vs. $5.0 \pm 0.8\%$). Most labeling was found at the luminal side of the vessel. Sympathectomy did not affect the LFs in saline (group A and D) and AngII (group B and C) treated animals. AngII caused a significant increase in neointimal CSA as compared to animals receiving NaCl (0.16 ± 0.01 vs. 0.11 ± 0.01 mm², table 4.2). Addition of 6-OHDA did not affect the AngII induced increase in neointimal CSA. Also 6-OHDA alone had no effect on CSA. Balloon injury decreased the lumen area. Addition of AngII further decreased this parameter ($p = 0.09$, table 4.2). Administration of 6-OHDA together with AngII infusion had no effect on lumen area as compared to administration of AngII alone. 6-OHDA alone had no effect on lumen area as compared to the NaCl group.

In the 12 weeks experiment largely similar findings were obtained (figure 4.2D and table 4.2) as compared to the 4 weeks experiment.

[³H]-Prazosin Binding

To evaluate whether the lack in response to AngII was due to a reduction of α_1 -adrenoceptor number, the amount of α_1 -adrenoceptors in the carotid artery at several time points after balloon injury was measured with 0.3 nM [³H]-prazosin. Balloon injury decreased the specific α_1 -adrenoceptor binding from day 3 up to 10 weeks after injury when expressed relative to the total protein content (figure 4.3A) as compared to

controls, and from day 3 up to 20 weeks after injury when expressed relative to the total DNA content (figure 4.3B).

Figure 4.3 Specific binding of α_1 -adrenoceptors in the presence of 0.3 nmol l^{-1} [^3H]-prazosin in injured left carotid arteries and uninjured right carotid control arteries at different time points after balloon denudation and A) expressed relative to total protein content and B) relative to DNA content. Data represent mean $\pm$ S.E.M. *) Significantly different from control, $p < 0.05$. **) Significantly different from control, $p < 0.01$.

Discussion

The main findings of this study are that balloon injury modifies the effects of chemical sympathectomy with 6-OHDA on AngII induced SMC DNA synthesis. While 6-OHDA did reduce the AngII induced SMC DNA synthesis in the media of uninjured carotid arteries, it had no effects on AngII induced SMC DNA synthesis in injured carotid arteries neither at 4 nor at 12 weeks after injury. Moreover, 6-OHDA had no effects on AngII induced changes in CSA or lumen area.

Our observation that 6-OHDA induced sympathectomy inhibits the mitogenic response of AngII in uninjured vessels is in agreement with earlier findings that α_1 -antagonists blunt the proliferative effect of AngII.(van Kleef *et al.*, 1992; van Kleef *et al.*, 1996a) Indeed, AngII is known to stimulate the activity of the sympathetic nervous system, not only centrally, but also at the level of the synapse and through stimulation of the adrenal release of catecholamines. These effects are probably mediated by AT_1 receptors.(Wong *et al.*, 1990)

The fact that 6-OHDA treatment did not reduce blood pressure suggests that effects of 6-OHDA on arterial mitogenic responses are not due to an effect on blood pressure. Since all AngII infused animals became hypertensive it is possible that the stimulatory effect of AngII on smooth muscle cell replication is due at least in part to its hypertensive effect or to a direct effect of AngII on the SMC. The argument against an indirect blood pressure mediated effect of AngII is found in *in vitro* data where AngII has been shown to stimulate SMC protein and DNA synthesis in cell culture,(Geisterfer *et al.*, 1988; Schelling *et al.*, 1991) and by *in vivo* data where AngII has been shown to stimulate vascular hypertrophy through a non-pressor pathway.(Griffin *et al.*, 1991) Moreover, also in the present study 6-OHDA was able to reduce the AngII-induced increase in DNA synthesis in the media of the carotid artery without an effect on the blood pressure.

Figure 4.4 Schematic Drawing of the Systemic and Sympathetic Actions on α_1 -Adrenoceptor After Ang II Stimulation. AngII binds to the AT_1 receptors on the sympathetic nervous endings triggering the release of norepinephrine (NE) from the synapses. NE binds to the α_1 -adrenoceptors which increases the SMC DNA synthesis. The same events can be triggered by binding of AngII at AT_1 receptors located on the adrenals. This binding will trigger the release of NE directly in the system where it can bind to the α_1 -adrenoceptors located on SMCs and trigger the increase in SMC DNA synthesis.

Since 6-OHDA is known to leave the catecholamine content of the adrenal medulla intact (Mueller *et al.*, 1969) the data presented here suggest that the adrenal medulla may not be an important factor in the pathway that controls medial SMC replication in response to AngII. As indicated in figure 4.4, this leaves open the possibility of an interaction at the level of the central nervous system or at the level of the synapse. (Starke *et al.*, 1969; Zimmerman and Whitmore, 1967; Wong *et al.*, 1990; Lu *et al.*, 1996)

In the uninjured media, where 6-OHDA reduced SMC replication, no effect on lumen area or CSA were found, indicating no effect of 6-OHDA on vessel growth or remodeling. This is in accordance with previous results which showed that α_1 -adrenoceptor blockade with doxazosin decreased AngII induced DNA synthesis but had no effect on CSA. (van Kleef *et al.*, 1996a) When, however, the AT_1 receptor blocker losartan was infused together with AngII the CSA decreased. (van Kleef *et al.*, 1996b) AngII stimulates collagen synthesis in cultured rat thoracic SMCs. (Kato *et al.*, 1991) Furthermore, treatment of young (4 or 10 weeks old) normotensive rats with the ACE inhibitor enalapril caused a marked decrease in both large (aorta, renal and carotid artery) and smaller arteries (mesenteric) in elastin and collagen protein content (Keeley *et al.*, 1992) suggesting stimulatory effects of AngII on elastin and collagen protein deposition. The lack of effect of 6-OHDA on medial CSA, but not on SMC replication, may suggest that the α_1 -adrenoceptors are involved in the regulation of SMC replication but not in (AngII stimulated) matrix growth.

Balloon injury increased the CSA and decreased the lumen area of the carotid artery indicating hypertrophic inward remodeling (table 4.2; figure 1.1, chapter 1). (Mulvany *et al.*, 1996) Interestingly, no such relation between an increased neointimal area and a smaller lumen area was found in a pig coronary artery injury model. (Andersen *et al.*, 1996) Administration of AngII further increased the vessel CSA, without an effect on lumen area, supporting the idea that AngII stimulates vessel growth in the rat carotid neointima. (Daemen *et al.*, 1991)

Although infusion of the α_1 -agonist phenylephrine 10 weeks after injury increased neointimal SMC DNA synthesis,(deBlois *et al.*, 1996a) sympathetic denervation 10 weeks after injury did not reduce the AngII induced neointimal SMC replication. An explanation for this apparent discrepancy is that we might not have succeeded to completely denervate the injured vessel for the entire 2 weeks. This possibility is, however, very unlikely since glyoxylic acid fluorescence staining was still absent in the highly innervated femoral artery (data not shown) indicating successful catecholamine depletion(Cowen and Burnstock, 1980) at the end of the experiment.

The AngII induced SMC replication in the neointima was not only insensitive to sympathetic denervation (this study), but also to pharmacological α_1 -adrenoceptor blockade.(van Kleef *et al.*, 1996a) One possibility that may explain this phenomenon is a loss of α_1 -adrenoceptors after balloon injury. To test this hypothesis, we measured the amount of α_1 -adrenoceptors in the carotid artery at several time points after balloon injury. As is shown in this study, balloon injury is associated with a loss of specific [3 H]-prazosin binding. Even at 20 weeks after balloon injury the amount of α_1 -adrenoceptors is lower in the injured than in the uninjured carotid artery. The loss of α_1 -adrenoceptors after injury may also explain why a two weeks infusion of the α_1 -adrenoceptor agonist phenylephrine failed to increase SMC replication in the rat carotid neointima.(deBlois *et al.*, 1996a) It is not clear why the density of α_1 -adrenoceptors is decreased after injury. One possible explanation may be the increased exposure of the vessel to neurogenic and circulating catecholamines after balloon injury that may down regulate α_1 -adrenoceptors. Indirect evidence for this possibility is provided in the rabbit iliac artery injury model where the increase in local facilitation of adrenergic neurotransmission after injury resulted in decreased contractile responses to exogenous catecholamines.(Candipan *et al.*, 1994) However, in contrast to β_2 -adrenoceptors, α_1 -adrenoceptors have been reported to be rather resistant to homologous downregulation.(Colucci *et al.*, 1981) Other possibilities include heterologous downregulation of α_1 -adrenoceptors as a result of increased intra-arterial levels of AngII.(Clements and Faber, 1997)

The most likely explanation for the apparent paradox between the stimulatory effect of phenylephrine(deBlois *et al.*, 1996a) and the lack of effect of sympathetic denervation is the humoral release of catecholamines from the adrenal medulla which is stimulated by AngII,(Feldberg and Lewis, 1964) and not affected by 6-OHDA.(Mueller *et al.*, 1969) Previous results from our laboratory(Stassen *et al.*, 1997b) and from Henegar *et al.*(Henegar *et al.*, 1995) indicated a rise in norepinephrine plasma levels after chronic AngII infusion. Thus, the release of catecholamines from the adrenal medulla may have overruled the effect of sympathetic denervation on SMC replication by stimulation of non-innervated α_1 -adrenoceptors on the vascular SMCs.

In conclusion, the data presented here help to unravel the pathways that control SMC replication *in vivo* in response to AngII, in which the AT₁ receptor(van Kleef *et al.*, 1996b) and factors like IGF(DelaFontaine and Lou, 1993) have been shown to be involved. Our data indicate that sympathetic innervation or more exactly α_1 -adrenoceptors(van Kleef *et al.*, 1992; van Kleef *et al.*, 1996a) mediate the AngII induced replication, but only in the uninjured vessel.

CHAPTER 5

Angiotensin II Modifies the Contribution of Adrenal Medulla
Derived Catecholamines to the Regulation of Neointimal
Growth After Balloon Injury of the Rat Carotid Artery

Abstract

In this study, we investigated the possible interactions between adrenal medulla derived catecholamines on angiotensin II (AngII) induced neointimal growth in the balloon injured rat carotid artery model. To this purpose, the left carotid artery of male Wistar rats, from which both adrenal medullas were removed, was injured by a balloon catheter. From 10-12 weeks after injury animals received AngII to stimulate intimal smooth muscle cell growth and/or the α_1 -adrenoceptor antagonist prazosin to block α_1 -adrenoceptors in the vascular wall.

Body weights and systolic blood pressures in the demedullation-prazosin groups were not different from those in the demedullation groups. Demedullation decreased adrenal medulla derived plasma epinephrine concentrations, but did not change neuronal plasma norepinephrine concentrations. Addition of prazosin did not affect these plasma catecholamine concentrations. In the AngII infused groups, coinfusion of prazosin after demedullation had no effect on neointimal or lumen CSA as compared to the demedullation alone group. Also, prazosin by itself did not affect neointimal CSA. However, demedullation tended to increase neointimal growth by sensitization of the α_1 -adrenoceptors since this increase in neointimal growth was affected by prazosin.

These data show that neointimal growth after a balloon catheter induced injury is α_1 -adrenoceptor regulated, but that neither the adrenal medulla derived catecholamines nor the α_1 -adrenoceptors contribute to the AngII-induced neointimal growth.

Introduction

The sympathetic nervous system plays an important role in the regulation of vascular smooth muscle cell (SMC) growth. Chronic administration of catecholamines not only accelerates the progression of atherosclerosis in several animal species, (Kukreja *et al.*, 1981; Majesky *et al.*, 1985) but also induces significant DNA replication in SMCs in both the normal and injured rat arterial wall. (deBlois *et al.*, 1996a) α_1 -Adrenoceptor antagonists, when administered immediately after balloon injury, reduce SMC DNA synthesis and neointima formation in the rat carotid artery and rabbit abdominal aorta. (Jackson *et al.*, 1988; O'Malley *et al.*, 1989; Fingerle *et al.*, 1991; Vashisht *et al.*, 1992) AngII also stimulates SMC DNA synthesis in the media and the neointima after balloon injury. (Daemen *et al.*, 1991) This effect may be partly mediated through α_1 -adrenoceptors, since AngII facilitates sympathetic transmission by potentiating neurotransmitter release and inhibiting norepinephrine uptake. (Starke, 1977) and stimulates the humoral release of catecholamines from the adrenal medulla. (Feldberg and Lewis, 1964) The α_1 -adrenoceptor antagonist doxazosin however, did not reduce SMC DNA synthesis in the media and neointima of the injured rat carotid artery, when infused together with AngII two weeks after balloon injury. On the other hand, it did however, decrease SMC DNA synthesis in the media of the uninjured carotid artery. (van Kleef *et al.*, 1996a) Furthermore, when the α_1 -adrenoceptor agonist phenylephrine was infused two weeks after balloon injury of the rat common carotid artery, SMC DNA synthesis in the neointima was not affected. (deBlois *et al.*, 1996a) In contrast, if phenylephrine was infused ten weeks after injury, neointimal SMC DNA synthesis did increase. (deBlois *et al.*, 1996a)

When neuronal norepinephrine, but not adrenal medulla derived (nor)epinephrine, was removed by chemical sympathectomy using 6-OHDA (Aprigliano and Hermsmeyer, 1976; Yamaguchi and Nakada, 1992) it became apparent that balloon injury modifies the effects of chemical sympathectomy with 6-OHDA on AngII induced SMC DNA synthesis (chapter 4). While 6-OHDA did prevent the AngII induced SMC DNA synthesis in the media of uninjured carotid arteries, it had no effects on AngII induced SMC DNA synthesis in injured carotid arteries neither at 4 nor at 12 weeks after injury. Moreover, 6-OHDA had no effects on AngII induced changes in intimal cross-sectional area or lumen area (chapter 4). One explanation for the apparent paradox between the stimulatory effect of phenylephrine (deBlois *et al.*, 1996a) and the lack of effect of sympathetic denervation is the humoral release of catecholamines from the adrenal medulla, which is stimulated by AngII. (Feldberg and Lewis, 1964; Henegar *et al.*, 1995; Stassen *et al.*, 1997b) but not affected by 6-OHDA. (Mueller *et al.*, 1969) This AngII stimulated release of humoral catecholamines may have overruled the effects of sympathetic denervation on neointimal growth and in that way may have obscured a possible inhibiting effect of sympathetic denervation on neointimal growth (figure 4.4, chapter 4).

In the present investigation, we have studied the role of the adrenal medulla in the control of neointimal growth of balloon catheter induced vascular injury in more detail. Moreover, we investigated the effects of possible modifiers such as AngII and α_1 -adrenoceptor blockade.

Materials & Methods

Animals

Male Wistar rats obtained from Iffa Credo (Someren, the Netherlands) were used (body weight, 275-325 g; n=46). They had free access to standard rat chow (Hope Farms, Woerden, the Netherlands) and tap water. The experiments were performed according to institutional guidelines.

Rats in which the left common carotid artery had been injured by a balloon catheter (see below) were randomly divided into six groups (figure 5.1): group A (n=8) was treated only with AngII. Group B (n=8) received AngII and a bilateral adrenal demedullation; group C (n=8) received AngII, prazosin (α_1 -adrenoceptor antagonist) and a bilateral adrenal demedullation. Group D (n=7) received 0.9% NaCl. Group E (n=7) received 0.9% NaCl, and a bilateral adrenal demedullation; group F (n=8) received NaCl, prazosin and a bilateral adrenal demedullation.

Balloon Injury

All rats were anaesthetized with pentobarbital (60 mg/kg body weight) by intra-peritoneal (*i.p.*) injection. The left common carotid artery was injured by introduction of a 2F balloon catheter (Baxter, Maarsen, the Netherlands) into the left external carotid artery. After inflation, the catheter was withdrawn through the common carotid artery. This procedure was repeated three times after which the catheter was removed and the external carotid artery ligated. The right carotid artery was not traumatized and served as a control. (Clowes *et al.*, 1983a; Clowes *et al.*, 1983b)

Figure 5.1 Outline of the Research Protocol With Randomization Scheme.

Bilateral Adrenal Demedullation

Bilateral adrenal demedullation was performed under ether anaesthesia via flank incisions one week before the start of the infusions (figure 5.1). After exposing the adrenal gland, a small incision was made in the adrenal cortex. Enucleation of the adrenal medulla was performed by gentle squeezing with a forceps. A sham operation, consisting of the same procedure described above without the enucleation of the adrenal medulla, was performed on control animals (group C and F).

Blood Pressure Measurement

Systolic blood pressures were measured by tail cuff plethysmography (ITTC inc. Woodland Hills, Ca., USA) in slightly restrained, conscious rats. After a training period of

seven days, blood pressure measurements started five days before drug infusions and were performed three times a week until sacrifice.

Drug Infusion

Human [Val⁵]-AngII (Saxon Biochemicals GMBH, Hannover, Germany) was continuously infused using osmotic minipumps (Alzet model 2002, Alza Corp., Palo Alto, Ca., USA) implanted subcutaneously between the shoulder blades under ether anaesthesia. AngII was dissolved in 0.9% NaCl and infused at a rate of 250 ng/kg/min. Prazosin (Sigma Chemical Co., St. Louis, Mo., USA) was dissolved in 50% dimethyl sulphoxide (7.3 mg/ml) and infused at a rate of 1.23 µg/min (0.35 µg/100 g per minute) in an Alzet model 2ml1 osmotic minipump. (Theeuwes and Yum, 1976) Since phenylephrine infusion in the first few weeks after balloon injury has no effect on neointima SMC growth, but increases neointimal SMC growth when infused at ten weeks after balloon injury, (deBlois *et al.*, 1996a) all infusions were started ten weeks after balloon injury and lasted for two weeks.

Catecholamine Analysis

Two days before sacrifice, rats were anaesthetized with pentobarbital (60 mg/kg *i.p.*). A polyethylene (PE 10) catheter was implanted under aseptic conditions into the femoral artery and advanced into the abdominal artery. The saline-filled catheter was guided subcutaneously to the base of the neck, exteriorized, and sealed with a metal plug.

Two hours before sacrifice arterial blood samples were obtained for determination of catecholamine plasma levels. Blood (1.2 ml) was withdrawn from quietly resting conscious rats and put into chilled tubes containing 1 mg glutathion (Merck, Darmstadt, Germany) and 10 µl heparin (Leo Pharmaceutical Products, 5000 u/ml). The blood samples were centrifuged at 3000 g for 15 minutes, and the plasma was stored at -20°C. Catecholamines were determined by fluorescence high performance liquid chromatography as described earlier. (van der Hoorn *et al.*, 1989)

Tissue Processing

At the end of the experiment all rats received 0.5 ml 0.5% Evans blue (Chroma Gesellschaft, Stuttgart, Germany) in 0.9% NaCl by intravenous (*i.v.*) injection, to stain non-endothelialized tissue in the left common carotid artery. One hour later the animals were killed by pentobarbital overdose (80 mg/kg body weight *i.p.*) and the arterial system was perfused with phosphate buffered saline (PBS) followed by perfusion fixation with 4% phosphate buffered paraformaldehyde (pH 7.4), at a pressure of 100 mmHg. Maximal vasodilatation was ensured by adding nitroprusside (0.1 mg/ml, Sigma Chemical Co., St. Louis, Mo., USA) to the perfusate.

One circular segment (2-3 mm long) was taken from the center of the blue stained non-endothelialized part of the left carotid artery. For comparison one segment was taken at a similar location from the uninjured right carotid artery. All tissues were fixed overnight in 4% phosphate buffered paraformaldehyde, routinely processed and embedded in paraffin (Para Clean, Klinipath, Duiven, the Netherlands).

Morphometric Analysis

Cross-sectional areas of the media, defined as the area between the external and internal elastic lamina, and the neointima, defined as the area between the internal elastic lamina and the lumen of the artery, and the lumen area were measured using a computerized morphometric system (Quantimet 570, Leica), on 4 µm Lawson (Boom B.V., Meppel, the Netherlands) stained cross-sections of the carotid artery rings. Measurements were performed on two cross-sections taken from each ring.

Statistics

Data are presented as mean $\pm$ SEM. Comparisons between groups for blood pressure and body weight were made by two-way analysis of variance (ANOVA). Comparisons between groups for catecholamine content and morphometry were made by Kruskal-Wallis H-test. Statistical significance was assumed at $p < 0.05$.

Results

Body Weight

Body weights of the animals in the different groups did not differ, neither at the start of the experiment nor at the start of the infusions. Body weight decreased during infusion with AngII (table 5.1) and animals in the NaCl group increased their body weight. Neither demedullation nor prazosin did affect body weight changes in the AngII groups or the NaCl infused groups.

Table 5.1 Effects of Angiotensin II, Demedullation and Prazosin on Body Weight and Systolic Blood Pressure 12 Weeks After Balloon Injury of the Left Carotid Artery

Treatment	body weight, g		blood pressure, mmHg	
	start	change	start	change
AngII	425 $\pm$ 8	-41 $\pm$ 8*	109 $\pm$ 3	+78 $\pm$ 6*
AngII/demedullation	411 $\pm$ 7	-42 $\pm$ 8#	111 $\pm$ 7	+94 $\pm$ 7#
AngII/demedullation/prazosin	436 $\pm$ 12	-20 $\pm$ 7\$	118 $\pm$ 6	+90 $\pm$ 10\$
NaCl	428 $\pm$ 9	+11 $\pm$ 3	115 $\pm$ 5	-10 $\pm$ 3
NaCl/demedullation	409 $\pm$ 7	0 $\pm$ 6	114 $\pm$ 2	-9 $\pm$ 5
NaCl/demedullation/prazosin	418 $\pm$ 4	+11 $\pm$ 2	112 $\pm$ 4	+2 $\pm$ 3

Starting values of body weight Changes in body weight and systolic blood pressure are given over the infusion period. Data represent mean $\pm$ SEM. *) Significantly different from NaCl: $p < 0.05$. #) Significantly different from NaCl/demedullation: $p < 0.05$. \$) Significantly different from NaCl/demedullation/prazosin: $p < 0.05$.

Systolic Blood Pressure

The systolic blood pressures (SBP) of the animals in the different groups were comparable at the start of the infusions. AngII infusion increased the SBP regardless of the interventions performed (table 5.1). Also in NaCl infused animals neither demedullation nor prazosin did affect SBP.

Plasma Epinephrine Concentrations

Demedullation decreased the plasma epinephrine concentration in both NaCl- and AngII infused animals to almost zero, indicating the efficacy of the procedure. In both the NaCl- and the AngII infused groups prazosin infusion had no effect on the plasma epinephrine concentration of demedullated animals (table 5.2).

Table 5.2 Effects of Angiotensin II, Demedullation and Prazosin on Plasma Epinephrine and Norepinephrine Levels 12 Weeks After Balloon Injury of the Left Carotid Artery

Treatment	epinephrine, nmol/l	norepinephrine, nmol/l
AngII	1.02 ± 0.65	2.38 ± 0.83
AngII/demedullation	0.02 ± 0.01#	1.97 ± 0.43
AngII/demedullation/prazosin	0.03 ± 0.01#	1.62 ± 0.27
NaCl	0.85 ± 0.42	0.89 ± 0.18
NaCl/demedullation	0.09 ± 0.03*	0.87 ± 0.09
NaCl/demedullation/prazosin	0.06 ± 0.02*	1.82 ± 0.38*

Plasma epinephrine and norepinephrine levels after the infusion period. Data represent mean ± SEM.

*) Significantly different from NaCl: $p < 0.05$. #) Significantly different from AngII: $p < 0.05$.

Plasma Norepinephrine Concentrations

No significant differences in plasma norepinephrine concentrations were found between the three AngII infused groups (A-C). In the NaCl groups (D-F) prazosin increased the plasma norepinephrine concentration in the prazosin-demedullated animals (group F) to 1.82 ± 0.38 nM as compared to 0.87 ± 0.09 nM in the demedullated animals (Group E, $p = 0.04$, table 5.2).

MORPHOMETRY

Uninjured Right Carotid Artery

Medial CSA

Removal of both adrenal medullas before infusion of AngII did not change medial cross-sectional area (CSA) as compared to infusion of AngII alone. Infusion of prazosin together with AngII in demedullated animals (group C) did not change the medial CSA of the right carotid artery as compared to AngII infusion after demedullation alone (group B). The medial CSA of the right carotid artery however, increased from 0.10 ± 0.01 mm² in the control NaCl group to 0.13 ± 0.01 mm² in the AngII group ($p = 0.03$, table 5.3). There were no significant differences in the medial CSA of the right carotid artery between the different NaCl infused groups (D-F).

Lumen Area

The lumen area of the right carotid artery in demedullated animals receiving AngII did not differ from the lumen area of the right carotid artery in animals infused with AngII. There were no effects of prazosin in combination with demedullation on lumen area, neither in the AngII- nor in the NaCl infused groups (table 5.3). In fact, lumen area was not changed in any of the experimental groups.

Injured Left Carotid Artery

Medial CSA

Demedullation had no effect on the medial CSA of the left carotid artery in AngII infused animals. Coinfusion of prazosin and AngII after demedullation (group C) had also no effect on the medial CSA of the left carotid artery. Infusion of AngII did increase the medial CSA of the left carotid artery from 0.10 ± 0.01 mm² after NaCl infusion to 0.12 ± 0.01 mm² ($p = 0.04$, table 5.4). In the NaCl infused groups (D-F) the CSA of the media of the left carotid artery was not affected, neither by demedullation nor by prazosin (table 5.4).

Table 5.3 Effects of Angiotensin II, Demedullation and Prazosin on Uninjured Medial CSA and Lumen Area 12 Weeks After Balloon Injury of the Left Carotid Artery

Treatment	media CSA, mm ²	LA, mm ²
AngII	0.13 ± 0.01	0.38 ± 0.04
AngII/demedullation	0.12 ± 0.01	0.36 ± 0.03
AngII/demedullation/prazosin	0.12 ± 0.01	0.38 ± 0.02
NaCl	0.10 ± 0.01	0.35 ± 0.02
NaCl/demedullation	0.10 ± 0.01	0.35 ± 0.04
NaCl/demedullation/prazosin	0.10 ± 0.01	0.44 ± 0.04

Cross-sectional area (CSA) and lumen area (LA) of the uninjured right carotid artery after the infusion period. Data represent mean ± SEM.

Neointimal CSA

Demedullation of animals receiving AngII did not change neointimal CSA of the left carotid artery. In the AngII infused groups, coinfusion of prazosin after demedullation had no effect on neointimal CSA as compared to the demedullation group. The neointimal CSA of the injured left carotid artery tended to increase during infusion with AngII as compared to the group receiving NaCl alone (0.20 ± 0.04 mm² vs. 0.17 ± 0.02 mm², $p=0.15$, table 5.4). The neointimal CSA of the left carotid artery of demedullated animals receiving NaCl (group E) decreased from 0.22 ± 0.02 mm² to 0.15 ± 0.01 mm² after infusion of prazosin ($p=0.04$, table 5.4). Demedullation alone increased neointimal CSA from 0.17 ± 0.02 mm² in NaCl infused control animals to 0.22 ± 0.02 mm² after demedullation. However, this increase was not significant ($p=0.13$, table 5.4). In other words, without additional AngII present, adrenal demedullation sensitizes α_1 -adrenoceptors which promotes vessel growth.

Table 5.4 Effects of Angiotensin II, Demedullation and Prazosin on Injured Medial and Neointimal CSA and Lumen Area 12 Weeks After Balloon Injury of the Left Carotid Artery

treatment	CSA, mm ²		
	media	neointima	LA, mm ²
AngII	0.12 ± 0.01	0.20 ± 0.04	0.25 ± 0.03
AngII/demedullation	0.11 ± 0.01	0.20 ± 0.01	0.27 ± 0.04
AngII/demedullation/prazosin	0.10 ± 0.01#	0.21 ± 0.03	0.27 ± 0.04
NaCl	0.10 ± 0.01	0.17 ± 0.02	0.16 ± 0.01
NaCl/demedullation	0.11 ± 0.01	0.22 ± 0.02	0.27 ± 0.03*
NaCl/demedullation/prazosin	0.10 ± 0.01	0.15 ± 0.01\$	0.27 ± 0.02*

Cross-sectional area (CSA) and lumen area (LA) of the injured left carotid artery after the infusion period. Data represent mean ± SEM. Significantly different from NaCl: * $p<0.05$. Significantly different from AngII: # $p<0.05$. Significantly different from NaCl/demedullation: \$ $p<0.05$.

Lumen Area

Infusion of AngII increased the lumen area from 0.16 ± 0.01 mm² in the NaCl group to 0.25 ± 0.03 mm² in the AngII group ($p=0.01$, table 5.4). There were no significant differences between the three AngII infusion groups with regard to lumen area of the injured left carotid artery. Demedullation in the NaCl groups increased lumen area as

compared to the NaCl infusion alone group indicating hypertrophic outward remodeling. This increase in lumen area of the left carotid artery by demedullation was not affected by infusion of prazosin (table 5.4).

Discussion

The studies described here further dissect the mechanism of AngII-induced neointimal growth in the injured rat carotid artery. The major findings are that i) removal of the adrenal medulla increases the response of the neointima to balloon catheter induced vascular injury, ii) this effect is sensitive to α_1 -adrenoceptor blockade and iii) this effect is absent during infusion with AngII.

Since there is no effect of demedullation on AngII stimulated neointimal growth, the data presented in this study indicate that the AngII-induced increase in neointimal growth in the balloon injured rat carotid artery is not mediated by the release of catecholamines from the adrenal medulla. Previous results from our laboratory have shown that this increase is also not mediated by stimulation of α_1 -adrenoceptors on the neointimal SMCs through the release of catecholamines from the sympathetic nervous system (chapter 4). Thus, the data suggest that AngII-induced SMC growth in the neointima of the rat carotid artery is mediated by a direct effect of AngII on the AT₁ receptor and not by indirect effects involving catecholamines. Indeed, coinfusion of the AT₁ receptor antagonist losartan and AngII after balloon injury in the rat showed a significant decrease in neointimal CSA as compared to controls.(van Kleef *et al.*, 1996b) This decrease in neointimal CSA after AT₁ receptor blockade,(van Kleef *et al.*, 1996b) but not after α_1 -adrenoceptor blockade or demedullation (this study) of animals receiving AngII, also suggests that there is a lack of cross-talk between the α_1 -adrenoceptors and the AT₁ receptors on neointimal SMCs as observed in neuronal cultures of Wistar-Kyoto rats.(Yang *et al.*, 1996) In those cells stimulation of α_1 -adrenoceptors by norepinephrine causes a down regulation of AT₁ receptors. Interestingly, norepinephrine does not decrease AT₁ receptors in neuronal cultures of spontaneously hypertensive rat brains.(Yang *et al.*, 1996)

The fact that demedullation, and the combination of prazosin and demedullation had no effect on the AngII-induced increase in systolic blood pressure (SBP), suggests that also the increase in SBP after AngII is not α_1 -adrenoceptor mediated. These results are in agreement with previous results showing that the AngII-induced increase in SBP is an AT₁ receptor mediated phenomenon.(van Kleef *et al.*, 1996b) Furthermore, infusion of prazosin (this study) and doxazosin(van Kleef *et al.*, 1996a) do not decrease the AngII-induced increase in SBP. Bilateral adrenal demedullation did also not affect vascular smooth muscle reactivity since the pressor responses to bolus injections of phenylephrine were remarkably similar in demedullated and sham-operated animals.(Borkowski and Quinn, 1983)

Demedullation increases the turnover of norepinephrine in peripheral sympathetic nerves.(Micalizzi and Pals, 1979; de Champlain and van Ameringen, 1975) However, in the present study bilateral adrenal demedullation depleted plasma epinephrine concentrations, but did not reduce norepinephrine concentrations, as might be expected if a facilitatory stimulus is removed. We can however, not exclude that an increased neuronal uptake of norepinephrine prevented plasma norepinephrine concentrations from rising. The implications of changes in plasma norepinephrine levels related to peripheral sympathetic nervous system activity must be interpreted with caution. Absolute levels of norepinephrine may not reflect true body and organ turnover or activity, especially when release and clearance of norepinephrine are unknown.

In the present studies the most striking results were found in the NaCl infused animals, where i) demedullation increased neointimal CSA after injury and ii) addition of

prazosin to NaCl animals prevented this increase in neointimal CSA. Since, demedullation increases sympathetic activity, (de Champlain and van Ameringen, 1975; Micalizzi and Pals, 1979) one explanation could be that more norepinephrine is released from the sympathetic nerve terminals which could increase stimulation of the α_1 -adrenoceptors present on the neointimal SMCs (figure 4.4, chapter 4). The suggestion that α_1 -adrenoceptors were sensitized is supported by our findings that blockade of the α_1 -adrenoceptors prevented this effect of demedullectomy on neointimal CSA. As can be concluded from the data presented in table 5.1, this effect of α_1 -adrenoceptor blockade is not mediated through the blood pressure. These data are in contrast with data published earlier that show that the rat carotid neointima was insensitive to infusion of the α_1 -adrenoceptor agonist phenylephrine during the ninth and tenth week after injury.(deBlois *et al.*, 1996a)

Not only the neointimal CSA of NaCl animals increases after demedullation of the injured rat carotid artery, but also the lumen area, indicating hypertrophic outward remodeling (figure 1.1, chapter 1).(Mulvany *et al.*, 1996) However, previous data from our laboratory showed no effects of 6-OHDA on arterial remodeling (chapter 4). An explanation for the apparent discrepancy between the present study and the previous chapter, is a regain of responses of neointimal SMCs to α_1 -adrenoceptor agonists in the eleventh and twelfth week after injury. This explanation is supported by data presented in the previous chapter, where we showed that balloon injury decreased the specific α_1 -adrenoceptor binding capacity from day 3 up to 10 weeks after injury when expressed relative to the total protein content, and from day 3 up to 20 weeks after injury when expressed relative to the total DNA content (chapter 4). However, α_1 -adrenoceptor specific binding started to increase from week 10 after injury (see figure 4.3). We propose therefore, that in the present study, in which infusions were performed during the eleventh and twelfth week after injury, some neointimal SMCs could have regained enough α_1 -adrenoceptors to respond to α_1 -adrenoceptor stimulation.(Orlandi *et al.*, 1994; Kocher *et al.*, 1991)

In conclusion, this study shows that neointimal growth after balloon catheter induced injury is α_1 -adrenoceptor sensitive and that there seems to be no apparent role for catecholamines and α_1 -adrenoceptors in this response to injury during AngII infusion.

CHAPTER 6

Anastomotic Innervation in Peripheral Bypasses and Graft Arteriovenous Fistulas in Humans

Abstract

Vascular interventions are often complicated by the development of intimal thickening, leading to stenosis. Vascular smooth muscle cell (SMC) proliferation and extracellular matrix deposition is considered to be one of the key events in the development of intimal thickening. One of the regulators of intimal thickening, at least in animal studies, is the sympathetic nervous system. However, information on the importance of the sympathetic nervous system on the regulation of intimal thickening in humans is scarce. To determine a possible correlation between sympathetic (re)innervation and the development of vascular stenosis we determined the immunoreactivity of the anastomotic region of 10 human stenotic vessels, derived from either peripheral bypasses (n=5) or haemodialysis arteriovenous (AV)-fistulas (n=5) with the use of antibodies directed against antigens in sympathetic nerves. The antibodies used were directed against S100, protein gene product 9.5 (PGP-9.5), tyrosine hydroxylase (TH), neurofilament (NF) and neuropeptide tyrosine (NPY). In addition, 5 saphenous vein control specimens were stained with the same antibodies

S100 and PGP-9.5 containing nerve bundles were present in the adventitia of normal saphenous veins, peripheral bypasses and AV-fistulas. Nerve fibers positive for TH were located around small blood vessels in the adventitia of all vessels examined. Neither the media nor the intima stained positive with the forementioned antibodies. This was true for control vessels, peripheral bypasses and AV-fistulas. Nerve fibers positive for NF or NPY were not detected.

We conclude that the adventitia of human peripheral bypasses and AV-fistulas contain S100, PGP-9.5 and TH positive, but NF and NPY negative sympathetic nerves. Both the media and intima of stenosed human peripheral bypass grafts, AV-shunts and the media of non-diseased saphenous veins lack sympathetic nerves. The data thus suggests that (re)innervation does not contribute to intimal growth and vascular stenosis in human stenotic tissue derived from AV-fistulas and peripheral bypass grafts.

Introduction

The replacement or bypass of sections of diseased arteries with autologous vein segments has become firmly established. The transplanted vein segment shows some degree of endothelial denudation, but rapidly becomes 'arterialized' by the establishment of an endothelial lining derived from arterial sources and the development of a new smooth muscle layer in the wall. (Dilley *et al.*, 1988) However, this muscle layer is unlike the media of the normal muscular artery. It develops subendothelially in the intimal region, without a clear anatomic distinction from the pre-existing media. Thus the terms neointima, intimal thickening, and intimal hyperplasia have been used to describe this new muscle layer in a vein graft. (Fuchs *et al.*, 1978) In some cases the arterialized vein develops an excessively thick intimal muscle layer that may ultimately obliterate the lumen and cause graft failure. (Motwani and Topol, 1998) Indeed, the long-term patency of arteriovenous (AV)-fistulas created for haemodialysis treatment of patients with end-stage renal-failure is limited. In particular, those fistulas created with prosthetic graft materials such as polytetrafluoroethylene (PTFE) have patency rates that are usually measured in months only. (Swedberg *et al.*, 1989) Although some failures are due to acute perioperative thrombosis, a significant proportion of graft stenosis is due to the development of an intimal hyperplastic lesion in the anastomosis of the graft with the vein. (Swedberg *et al.*, 1989; Sottiurai, 1990)

The sympathetic nervous system may be one of the regulators of this intimal hyperplasia. The importance of the sympathetic nervous system in the regulation of smooth muscle cell (SMC) growth is underlined by the following examples which are derived from animal studies. Surgical sympathetic denervation of the growing rabbit ear artery reduces SMC proliferation, (Bevan, 1975) and chemical sympathectomy with 6-hydroxydopamine (6-OHDA), which destroys the sympathetic nerve endings leaving vessels denervated, (Aprigliano and Hermesmeyer, 1976) reduces the number of SMCs in the growing aortic media. (Fronek *et al.*, 1978) Stimulation of α_1 -adrenoceptors by phenylephrine increases the expression of growth related factors such as platelet derived growth factor-A. (Majesky *et al.*, 1990) Previous results from our laboratory indicate that angiotensin II induces SMC proliferation in both the media and neointima of rat carotid arteries. This response could be blocked by both α_1 -adrenoceptor blockers and by sympathetic denervation with 6-hydroxydopamine, but only in the media (chapter 4). (van Kleef *et al.*, 1992; van Kleef *et al.*, 1996a) The intima was insensitive to both interventions. This indicates that the sympathetic nervous system may act as a trophic factor in the media, but does not seem to be important in the regulation of neointimal growth. Also, in arteries of adult animals, surgical denervation and 6-OHDA induced an increase in SMC size and ultrastructural changes compatible with increased synthetic activity. (Fronek *et al.*, 1978; Branco *et al.*, 1984; Bevan and Tsuru, 1981; Dimitriadou *et al.*, 1988; Fronek, 1983) Thus, although sympathetic nerves seem to exert a trophic action on vascular SMCs during development and disease, they may help stabilize the contractile phenotype of the SMC in the non-diseased adult vessel.

Although we know a lot of the contribution of the sympathetic nervous system in animals, the extent of sympathetic (re)innervation and the role of the sympathetic nervous system in the regulation of intimal growth in human vein grafts has received scant attention. Human saphenous veins have a dense plexus network at the adventitia-media border consisting of catecholamine and cholinergic nerves. (Herbst *et al.*, 1992; Amenta *et al.*, 1983) Prior to grafting, human saphenous veins are cut and stripped of their adventitia, which renders them denervated. (Kraiss and Clowes, 1997; Holme *et al.*, 1990) Reinnervation of the vasa vasorum of vein grafts does not become established until two to four weeks after grafting. (Waris *et al.*, 1984; Meagher *et al.*, 1984) Also, neuromodulin positivity, a phosphoprotein transported anterogradely from the somata to

the axon terminals of neurons, that is present in neurons that are capable of regenerative responses, (Benowitz and Perrone-Bizzozero, 1991) was strongly decreased or had disappeared almost completely in areas where intimal thickening was prominent. (De Meyer *et al.*, 1997) Furthermore, in regions of the human carotid artery where intimal thickening occurred, the perivascular nerve network was degenerated. (Fuchs *et al.*, 1978)

Thus, although several animal studies implicate that sympathetic nerves may act as a trophic factor of vascular growth, the few data in human vein grafts suggests a lack of re-(innervation), that may contribute to the development of intimal hyperplasia. To gain further insights in the innervation of human vein grafts and to determine the possible role of sympathetic nerves in the regulation of intimal growth, we evaluated the innervation status of the anastomotic region of stenosed human vein graft using a panel of antibodies directed against different epitopes present in sympathetic nerves.

Materials & Methods

Patient Clinical Profile

Patients With AV Fistulas

This group consisted of 3 men and 2 women, with a mean age of 52 years (range, 40 to 66 years). The AV-fistula was repaired after an average time of 14.2 months (range, 8 to 20 months). The diagnosis of the stenosis was based on duplex ultrasound and confirmed by fistulography. (Tordoir *et al.*, 1987) In all patients, the occurrence of a stenosis was associated with an increase in venous pressure and/or a decrease in flow of the fistula during haemodialysis necessitated reintervention. The stenotic sample was derived from the venous anastomosis of a PTFE interposition graft.

Patients With a Peripheral Bypass

This group of patients consisted of 2 men and 3 women, with a mean age of 66 years (range, 40 to 87 years). Reintervention was performed after an average time of 5.2 months (range, 1 to 21 months). The diagnosis of a stenosis was based on generally accepted duplex criteria and confirmed by angiography. (Buth *et al.*, 1991) Patients with a graft at risk underwent repair of the stenotic segment. The stenotic tissue was derived from the proximal anastomosis of reversed saphenous vein grafts.

Control Vessels

This group of patients consisted of 2 men and 3 women whose mean age was 64 years (range, 50 to 78 years). Saphenous veins, which were left over after coronary artery bypass grafting was established, officiated as control vessels in this study.

Immunohistochemistry

All tissue specimens were fixed overnight in 10% phosphate buffered formalin, processed according to routine histological procedures and paraffin-embedded. The distribution of the innervation markers used (TH: Chemicon, U.S.A. (1:5000); PGP-9.5: Ultraclone, U.K. (1:500); NPY: Boehringer Ingelheim, Germany (1:250); S100: Dako, Denmark (1:5000); NF: Euro-diagnostics, the Netherlands (1:250), table 6.1) was visualized in both AV-fistulas, peripheral bypass grafts and in control vessels. Four μ m thick sections were deparaffinized and incubated in 0.3% H_2O_2 in methanol to block endogenous peroxidase activity. After rinsing in tris-buffered saline (TBS) sections were incubated with each respective primary antibody, followed by incubation with biotinylated rabbit anti mouse IgG (Amersham, U.K.) for a monoclonal antibody (NF) or with biotinylated sheep anti rabbit IgG (Amersham, U.K.) if a polyclonal antibody (S100, PGP-9.5, NPY, TH) was used. Both incubations lasted 45 minutes and were performed

at room temperature. Sections stained with the antibody against neurofilaments (NF) and S100 were pretreated with pepsin (1 mg/ml) for 30 minutes at room temperature or with trypsin (1 mg/ml) for 15 minutes at 37°C, respectively. Subsequently, the sections were labeled for 30 minutes at room temperature with an avidin/biotin-peroxidase complex (Vectastain-ABC kit, Vector Labs, Burlingame, CA, USA) and 3,3'-diaminobenzidine was used as the chromogen. Omission of the primary antibody served as negative control. The sections were counterstained with haematoxylin, dehydrated through graded series of alcohols, cleared in xylene and mounted in entellan (Merck).

Table 6.1. Immunohistochemical innervation markers used.

ANTIBODY	MONO-/POLYCLONAL	LOCALIZATION	SOURCE
Neurofilament	Monoclonal	Neurofilament protein	Euro-Diagnostics
Protein gene product 9.5	Polyclonal	Nerve endings	Ultraclone
Tyrosine hydroxylase	Polyclonal	Nerve endings	Chemicon
S100	Polyclonal	Glial cells, Schwann cells, Melanocytes	Dako
Neuropeptide tyrosine	Polyclonal	Nerve endings	Boehringer Ingelheim

Results

Control Vessels

Control, non-diseased human saphenous veins containing sympathetic nerves, were positive for S100, PGP-9.5, and TH. S100 and PGP-9.5 positive nerve bundles were present in the adventitia (figure 6.1a, b), but not in the media or intima. TH (figure 6.1c) stained nerve endings around small blood vessels in the adventitia. No S100, PGP-9.5 or TH positive fibers were found in the media and intima. NF or NPY positive nerve fibers could not be found, neither in the adventitia, nor in the media or intima.

Peripheral Vein Grafts and AV-Shunts

S100, PGP-9.5, and TH positive nerves were also found in peripheral bypass grafts, especially in the adventitia. S100 and PGP-9.5 positive nerve bundles were present in the adventitia (figure 6.1d, e), but not in the media or intima. TH (figure 6.1f) stained nerve endings around small blood vessels in the adventitia. No S100, PGP-9.5, or TH positive fibers were found in the media and intima. NF or NPY positive nerve fibers could not be found, neither in the adventitia, nor in the media or the intima. The innervation pattern of AV-shunts was identical to that observed in the peripheral vein grafts. Thus, sympathetic nerves were only found in the adventitia. Large bundles were S100 and PGP-9.5 positive, while small fibers that were present around small blood vessels were positive for TH (data not shown).

Figure 6.1 Photomicrographs of Non-Diseased Human Saphenous Veins (a-c) and Human Peripheral Bypass Grafts (d-f). Sections stained with antisera to S100 (a, d), PGP-9.5 (b, e) or TH (c, f). Haematoxylin counterstain, magnification x400. Arrowheads indicate positivity.

Discussion

In this study we have investigated whether the sympathetic nervous system may play a role in the control of intimal growth in human vascular stenotic tissues by staining human vascular stenotic tissue and control vessels with a panel of innervation markers. The data show that the innervation pattern of control human saphenous veins, peripheral bypass grafts and AV-fistula shows remarkable similarities. In the adventitia, relative large nerve fibers positive for S100 and PGP-9.5 are present as well as small TH positive nerve fibers around small vessels. Nerve fibers positive for NF or NPY were not found. Also, the media and intima was devoid of markers of sympathetic nerves, suggesting that sympathetic nerves do not play a major role in the regulation of intimal growth in vein grafts and AV-shunts.

Amenta *et al.* (Amenta *et al.*, 1983) were able to show, using glyoxylic acid histofluorescence and acetylcholinesterase histochemistry in specimens of the non-

diseased human saphenous vein, a dual catecholamine and cholinergic innervation, that was organized in a plexus network at the adventitia-media border. Interestingly, no fibers were found in the media.(Amenta *et al.*, 1983) In addition, peptidergic innervation of the human saphenous vein could be demonstrated by immunohistochemistry. Herbst *et al.*(Herbst *et al.*, 1992) were able to show, in resected human saphenous vein, that the peptidergic fibers were organized along the vasa vasorum, whereas no immunoreactivity of neuronal fibers was found in the inner layers of the tunica media or in the intima.(Herbst *et al.*, 1992) The present immunohistochemical study confirms the results obtained by Amenta and Herbst and shows that this pattern does not change in specimens of stenosed saphenous vein grafts and AV-shunts.

During the vein graft procedure sympathetic nerves in blood vessels are damaged by clamping or by removing of superficial connective tissue from the vessel wall.(Cowen *et al.*, 1982) This renders the vessels denervated.(Holme *et al.*, 1990) Studies, using the anterior chamber of the eye as an experimental model for studies on adrenergic nerve regeneration,(Todd, 1986) have shown that tissue transplants taken from organs normally without adrenergic innervation fail to become adrenergically innervated, whereas transplants taken from normally richly adrenergically innervated structures will become reinnervated. Hence, adrenergic nerves do possess the ability to regenerate.(Waris *et al.*, 1984)

From animal studies it became clear that surgical denervation and 6-OHDA induced an increase in SMC size and ultrastructural changes compatible with increased synthetic activity.(Fronek *et al.*, 1978; Branco *et al.*, 1984; Bevan and Tsuru, 1981; Dimitriadou *et al.*, 1988) Furthermore, a study in rabbits shows that intimal thickening in the carotid artery was associated with degeneration of the perivascular network,(Scott *et al.*, 1992) whereas α_1 -adrenoceptors are present in the intima after balloon injury of the rat carotid artery (chapter 4). Thus, one may hypothesize that loss of sympathetic innervation may trigger intimal growth. This might also explain why patency rates of *innervated* internal mammary artery grafts(He *et al.*, 1993) are better compared to *denervated* saphenous vein grafts.(Holme *et al.*, 1990; Zeff *et al.*, 1988; Pragliola *et al.*, 1994; Gibson and Loop, 1986)

The data presented here, however, do not support a major role of sympathetic nerves in the regulation of intimal growth in human vein graft, since the intima and media lack sympathetic nerves, whereas the pattern of sympathetic innervation in the adventitia of stenosed grafts is identical to that found in control, undiseased saphenous veins.

CHAPTER 7

General Discussion

7.1 Introduction

In this thesis we have further elucidated the role of AngII in neointima formation after balloon catheter induced injury of the rat carotid artery and the role of the sympathetic nervous system herein. By now it is quite evident that the AngII-induced responses in the media and the neointima after a balloon catheter induced injury of the rat carotid artery are differentially regulated (table 7.1). The findings that the AngII-induced increase in *neointimal* SMC DNA synthesis could not be blocked by α_1 -adrenoceptor antagonists and that direct stimulation of the α_1 -adrenoceptor with phenylephrine did not result in an increase in *neointimal* SMC DNA synthesis after injury are exactly opposite of the situation in the media. In the media the AngII-induced increase in SMC DNA synthesis can be blocked by an α_1 -adrenoceptor antagonist and phenylephrine increases *medial* SMC DNA synthesis (table 7.1). The studies described in this thesis were designed to further dissect the mechanisms of this differential responses of the media and neointima. We hypothesized that the differential responses of the media and the neointima to α_1 -adrenoceptor blockade/stimulation are due to differences in sympathetic innervation and/or differences in the presence of α_1 -adrenoceptor subtypes. Furthermore, we hypothesized that the neurogenic release of norepinephrine in the vasculature is under the control of AngII and the norepinephrine transporter and that AngII increases the systemic (humoral) release of (nor)epinephrine from the adrenal medulla and that this vascular NET is differentially upregulated in the media and neointima.

Table 7.1 Effect of AngII, AT₁-R antagonist and α_1 -Adrenoceptor Agonist/Antagonist on SMC DNA synthesis

Infusion	Injured neointima	Uninjured media	Reference
AngII	↑↑	↑	(Daemen et al., 1991)
AngII + AT ₁ -receptor antagonist	=	=	(van Kleef et al., 1996b)
α_1 -agonist	=	↑	(deBlois et al., 1996a)
AngII + α_1 -antagonist	↑	=	(van Kleef et al., 1996a)

Table shows changes in SMC DNA synthesis in the injured neointima and uninjured media of the rat carotid artery. ↑ indicates increase; = indicates no change compared to the correct control

7.2 The Media

7.2.1 Role For α_1 -Adrenoceptors and Catecholamines

The data in chapter 2 show that α_1 -adrenoceptor mRNAs of at least two subtypes are present in the media of uninjured rat carotid arteries which is supported by findings that the rat carotid media is sensitive to α_1 -adrenoceptor stimulation (deBlois et al., 1996a) and blockade (van Kleef et al., 1996a). Furthermore, the data in chapter 3 show that the transcription of the NET gene is upregulated several hours after the start of AngII infusions which confirms our hypothesis that AngII regulates the NET in the vascular wall.

7.2.2 Neurogenic or Systemic?

The data in chapters 4 and 5 furthermore show that the AngII-induced medial SMC replication is sensitive to chemical sympathectomy by 6-OHDA and that the medial response to AngII is not influenced by removal of the adrenal medulla. This implies that the AngII-induced SMC replication is stimulated by norepinephrine released from the sympathetic nerve endings which are able to bind and stimulate the α_1 -adrenoceptors

located on the SMC. Furthermore it implies that there is no important contribution of humoral derived catecholamines in this response (figure 7.1).

Peripheral Vein Grafts and AV-Shunts

S100, PGP-9.5, and TH positive nerves were also found in peripheral bypass grafts, especially in the adventitia. S100 and PGP-9.5 positive nerve bundles were present in the adventitia (figure 6.1d, e), but not in the media or intima. TH (figure 6.1f) stained nerve endings around small blood vessels in the adventitia. No S100, PGP-9.5, or TH positive fibers were found in the media and intima. NF or NPY positive nerve fibers could not be found, neither in the adventitia, nor in the media or the intima. The innervation pattern of AV-shunts was identical to that observed in the peripheral vein grafts. Thus, sympathetic nerves were only found in the adventitia. Large bundles were S100 and PGP-9.5 positive, while small fibers that were present around small blood vessels were positive for TH (data not shown).

7.3 The Neointima

7.3.1 Role For α_1 -Adrenoceptors and Catecholamines

The data presented in chapters 2 and 3 show that in the neointima formed after balloon catheter induced injury of the rat carotid artery i) the number of α_1 -adrenoceptors decreases, ii) there is no apparent shift in α_1 -adrenoceptor subtypes and iii) AngII increases the NET mRNA.

From that data it can be concluded that the relative insensitivity of the rat carotid artery neointima to α_1 -adrenoceptor stimulation (deBlois et al., 1996a) and blockade (van Kleef et al., 1996a) is not due to an absence of α_1 -adrenoceptors in the neointima, not due to a subtype switch, but may be due to a lack of a sufficient amount of α_1 -adrenoceptors in the neointima. Furthermore, the transcription of the NET gene, which is primarily responsible for the reuptake of released catecholamines into the synaptic nerve endings, (Axelrod, 1971) is increased in the injured rat carotid artery within hours after AngII infusion. This suggests that norepinephrine released from the sympathetic nerve endings upon AngII stimulation (Starke, 1977; Zimmerman, 1978) is rapidly removed to prevent further stimulation of α_1 -adrenoceptors in the neointimal SMCs.

7.3.2 Neurogenic or Systemic?

The data in chapters 4 and 5 furthermore show i) that the AngII-induced increase in neointimal SMC replication after balloon catheter induced injury of the rat carotid artery is insensitive to sympathetic denervation and ii) that the AngII-induced increase in neointimal growth response is insensitive to bilateral adrenal demedullectomy.

From these data it can be concluded that the release of adrenal medulla derived catecholamines, which is also stimulated by AngII (Feldberg and Lewis, 1964) and not affected by chemical sympathetic denervation, (Mueller et al., 1969) does not overrule the effect of sympathetic denervation on SMC replication (figure 7.1).

Figure 7.1 Proposed mechanisms of AngII-induced increase in SMC growth in the uninjured media (A) and the neointima (B) after a balloon catheter induced injury of the rat carotid artery with regard to the SNS. X indicates non-contributing pathway.

7.4 Presence of α_1 -Adrenoceptors

Thus, the relative insensitivity of rat carotid artery growth responses to α_1 -adrenoceptor stimulation and blockade is not due to an absence of α_1 -adrenoceptors in the neointima, not due to a subtype switch, but may be due to a decrease in the total amount of α_1 -adrenoceptors present in the injured rat carotid artery as compared to the uninjured rat carotid artery. However, it is not clear why the density of α_1 -adrenoceptors is decreased after injury. One possible explanation may be the increased exposure of the vessel to neurogenic and circulating catecholamines after balloon injury that may downregulate α_1 -adrenoceptors. Indirect evidence for this possibility is provided in the rabbit iliac artery injury model where the increase in local facilitation of adrenergic neurotransmission after injury resulted in decreased contractile responses to exogenous catecholamines.(Candipan et al., 1994) However, α_1 -adrenoceptors have been reported to be rather resistant to homologous downregulation,(Colucci et al., 1981) i.e. downregulation of α_1 -adrenoceptors to an increased catecholamine release by AngII. Other possibilities include heterologous downregulation of α_1 -adrenoceptors as a result of increased intra-arterial levels of AngII,(Clements and Faber, 1997) i.e. downregulation of α_1 -adrenoceptors by an upregulation of AT_1 receptors.

7.5 Proposed Mechanisms of AngII-Induced Increase in SMC Growth of the Rat Carotid Artery

Thus our working hypothesis is that in the uninjured media of the rat carotid artery, AngII stimulates the AT_1 receptors located on the sympathetic nerve endings and facilitates the release of norepinephrine (figure 7.1a). The released norepinephrine can either stimulate α_1 -adrenoceptors mediated processes in the SMC, be taken up in the sympathetic nerves via the NET (uptake 1) or taken up in extraneuronally organs and degraded by COMT and MAO (uptake 2). There is no involvement of the adrenal medulla in this response.

In the neointima of the injured rat carotid artery, however, neither norepinephrine released from the SNS, nor catecholamines released from the adrenal medulla are involved in the AngII-induced response (figure 7.1b). On the contrary, NET gene transcription is increased by AngII which indicates that even if norepinephrine is released into the synaptic cleft it will be rapidly removed by the NET. Direct activation of the AT_1 receptors by AngII is most likely the mechanism by which DNA synthesis is increased in the neointima of injured rat carotid artery (figure 7.1B),(van Kleef et al., 1996b) whereas indirect activation of the α_1 -adrenoceptors by AngII seems to be the mechanism via which DNA synthesis is increased in the media of uninjured rat carotid artery (*chapter 4*).

7.6 Neointimal Innervation in Humans

All the animal experiments discussed hitherto show that innervation does not seem to be important in the development of a neointima in the rat common carotid artery after injury. Alternatively, it might be that this lack of innervation contributes to the development of intimal hyperplasia. Prior to grafting human saphenous veins are cut and stripped of their adventitia which renders them denervated.(Waris et al., 1984; Meagher et al., 1984) Interestingly, replacement or bypass of sections of human diseased arteries with denervated autologous vein segments have low patency rates(Swedberg et al., 1989; Sottirai, 1990) which may suggest that lack of innervation may contribute to intimal hyperplasia. However, immunohistochemical data presented in *chapter 6* do not support this hypothesis since the pattern of sympathetic innervation in the adventitia of stenosed grafts is identical to that found in the control, undiseased autologous vein segments prior to grafting. No staining could be detected in the neointima of all vessels studied. These data leaves us to believe that innervation is not a necessity for neointima formation after

injury. Alternatively one may conclude that the lack of sympathetic innervation may reduce neointima formation.

7.7 Cross-talk Between Receptors

As mentioned previously, direct activation of the AT_1 receptors by AngII is most likely the mechanism by which DNA synthesis is increased in the neointima of injured rat carotid artery (figure 7.1B), (van Kleef et al., 1996b) whereas indirect activation of the α_1 -adrenoceptors by AngII seems to be the mechanism via which DNA synthesis is increased in the media of the uninjured rat carotid artery (*chapter 4*). Remarkably, the signal transduction pathways of both receptors are very similar, which opens the possibility of cross-talk between the AT_1 - and the α_1 -adrenoceptor.

Both the α_1 -adrenoceptors and the AT_1 receptors are members of the 7-transmembrane spanning domain group of receptors which produce intracellular changes through their coupling to guanine nucleotide-binding regulatory proteins. The G protein consists of 3 subunits, α , β and γ . Receptor activation causes replacement of the GDP bound to the α -subunit with GTP and the dissociation of $G\alpha$ from $G\beta\gamma$ (figure 7.2). The potential complexity of the signaling mechanisms is increased by the finding of at least 16 $G\alpha$ subunits, 5 $G\beta$ subunits and 11 $G\gamma$ subunits. While many α_1 -adrenoceptors and AT_1 receptors have been reported to couple to phospholipase C, additional intracellular signaling effectors have been described including phospholipase D, and activation of Ca^{2+} and K^+ channels. In addition there are many different forms of DAG including the production of polyunsaturated 1,2-diacylglycerols by PLC specific phospholipase C and mono-unsaturated and saturated phosphatidates by the breakdown of phosphatidylcholine by phospholipase D.

Figure 7.2 Activated G_i - and G_q coupled receptors stimulate PLC to release IP_3 and DAG, which cause the release of intracellular Ca^{2+} and activate PKC, respectively. Co-stimulation of G_i - and G_q coupled receptors could result in augmentation of PLC and PKC activity. (Selbie et al., 1995)

A model for cross-talk of two G protein coupled receptors requires in the simplest sense that the signal transduction of one receptor can be modified by that of the other receptor (figure 7.2). G_i - (AT_1 receptor) and G_q - (α_1 -adrenoceptor) coupled receptors have been shown to stimulate MAPK activity, as well as other kinases, leading to changes in gene expression. (Vanbiesen et al., 1996; Okuda et al., 1996) In the case of G_i coupled effects on G_q coupled receptor stimulated smooth muscle proliferation, receptor co-stimulation could result in augmentation of intracellular events that precede and/or modulate the final response. In studies using tissue preparations, stable cell lines and

cells transfected with different G protein coupled receptors, G_i coupled receptor stimulation results in augmentation of G_q coupled receptor mediated PLC activity and PKC activity.(Selbie et al., 1995) However, AngII induced medial SMC DNA synthesis could not only be inhibited by 6-OHDA (chapter 4) but also by the AT_1 receptor antagonist losartan.(van Kleef et al., 1996b) Both receptor ligands can stimulate growth in the uninjured rat carotid artery and therefore cross-talk is not a necessity, but cannot be ruled out. Not only cross-talk but also cooperativity, i.e. the same signal transduction pathway (e.g. G_q coupled) can be activated by different receptors, between the AT_1 - and the α_1 -adrenoceptor is possible, since AT_1 receptors in uninjured medial SMCs are not only coupled to G_i - but also to G_q proteins.(Schmitz and Berk, 1997) However cooperativity seems unlikely, since the AngII-induced increase in the SMC DNA synthesis in the uninjured media is blocked both by an AT_1 receptor antagonist and an α_1 -adrenoceptor antagonist (table 7.1). Furthermore, it seems that there is no need for cross-talk or cooperativity in the injured neointima since the AngII-induced effect on the neointima is insensitive for the blockade of α_1 -adrenoceptor mediated signal transduction pathway which seems to be lacking from the injured neointimal SMCs.

In conclusion, there are several different signal transduction pathways associated with α_1 -adrenoceptor subtypes in cells and tissues. One subtype does not couple uniquely to one transduction pathway and the determining factors are more likely to be tissue dependent in terms of the availability of particular G proteins and effector enzymes. In some cells α_1 -adrenoceptors may couple to more than one pathway to produce a variety of responses. Measurement of a change in a second messenger does not necessarily imply that this is involved in mediating the observed response. Furthermore, there are different time scales of response following activation of α_1 -adrenoceptors; short term changes in e.g. vascular resistance and longer term increases in cell growth in the heart and vasculature. Cross-talk and cooperativity between receptors further complexes the signal transduction networks and renders the outcome of interventions in these signal transduction networks less certain.

7.8 Limitations of the Study

In this thesis we have used the balloon catheter induced injury model to study the differential effects of AngII on the media and neointima formation in the rat carotid artery. The rat carotid artery has provided perhaps the most widely used experimental preparation for the study of the response to acute injury.(Clowes et al., 1989) However, there were some unforeseen difficulties during the course of the studies and some insights which we had not anticipated.

7.8.1 Proliferation and Apoptosis

In all but one study, we have used male Wistar rats. With the Wistar rats we encountered rather tedious problems in trying to visualize the BrdU. The BrdU concentration used was 30 mg/ml and was infused at a rate of 714 ng/kg/min. Although this concentration was sufficient to obtain labeling fractions in the male Wistar-Kyoto rats (chapter 4), no BrdU could be detected in the male Wistar rats (chapter 5). Whether this was due to strain differences or other factors involved is not known.

In recent years it became apparent that not only SMC proliferation but also SMC apoptosis is involved in the pathogenesis of vascular remodeling. Apoptosis is increased in the intima thickening at 15 days after a balloon catheter induced injury in the rat thoracic aorta.(Bochaton-Piallat et al., 1995) In cardiovascular disease, apoptosis of SMCs has been identified in atherosclerotic and restenotic lesions after PTCA.(Kearney et al., 1997; Malik et al., 1998) Many regulatory factors including humoral growth factors, cytokines and matrix components are well characterized in atherosclerotic and restenotic

lesions.(Schwartz et al., 1995) Characterization of the role of e.g. anti-apoptotic genes in the pathogenesis of vascular diseases may provide a therapeutic opportunity. The induction of apoptosis by balloon injury of the rat carotid artery coincides with a marked downregulation of *bcl-x* expression.(Perlman et al., 1997) Recently, downregulation of intimal cell *bcl-x* expression with the use of oligonucleotides induced apoptosis and acute regression of vascular lesions in cholesterol-fed rabbits.(Pollman et al., 1998) AngII antagonizes nitric oxide-induced apoptosis and inhibits apoptosis in vascular SMCs.(Pollman et al., 1996) However, the relative number of BrdU positive nuclei did not change throughout our experimental model (chapter 4), which may suggest that apoptosis does not play an important regulatory role in the AngII mediated neointima formation in the rat carotid artery after injury.

7.8.2 Norepinephrine Content and Carotid Artery Innervation

Data in this thesis shows that there seems to be a balance between norepinephrine release stimulated by AngII and reuptake by the NET in blood vessels (chapter 3). Plasma levels of norepinephrine do not reflect the amounts locally present in the vessels. Therefore, without knowledge of the exact amount of norepinephrine molecules which reside in the synaptic cleft during AngII infusion, it remains difficult to explain the differences in vascular growth between injured and uninjured vessels with regard to the SNS and the α_1 -adrenoceptors.

One possible method to detect innervation of naive rat carotid arteries is with glyoxylic acid (data not shown).(Cowen and Burnstock, 1980) We did not evaluate the innervation of the rat carotid artery after balloon catheter induced injury. Furthermore, we were not able to stain the naive and balloon injured rat carotid arteries with the panel of antibodies used to evaluate the innervation of human AV-fistulas and peripheral bypass grafts (chapter 6) due to the lack of cross-reactivity between those human antibodies and the rat tissues. Recently, an antibody against the rat α_{1B} -adrenoceptor came available.(Piascik et al., 1997) It would be very interesting to see if the mRNA distribution and localization matches that of its protein.

7.9 Future Outlook

The findings in this thesis further dissect the role of AngII and the SNS in the regulation of neointimal growth after injury.

First, it has become apparent that neointimal SMCs and medial SMCs behave and react differently to AngII and catecholamine stimulation. It seems therefore imperative to study the effects of therapies in experimental models where a neointima is already formed, since this may alter the outcome.

Second, we expect that sympathetic innervation does not seem to be important in the AngII-induced increase in neointimal growth. Since data, presented in this thesis, also show that the neointima of human vessels is not innervated, we will not propose to use this sympathetic innervation as a potential target for the treatment of neointimal thickening in humans.

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Samenvatting

Het proces van vasculaire remodellering na vaatwandschade is zeer complex. In dit proefschrift is gekeken naar het effect van de groeifactor angiotensine II (AngII) op de neointimale groei na beschadiging van de arteria carotis van de rat. In het bijzonder is gekeken naar de rol van de α_1 -adrenoreceptoren en het sympathische zenuwstelsel (figuur 4.4).

Uit eerder onderzoek is gebleken dat de AngII respons na ballondenudatie van de arteria carotis van de rat verschilt tussen mediale en neointimale gladde spiercellen. De mediale gladde spiercellen zijn gevoelig voor α_1 -adrenoreceptoren blokkade en de neointimale gladde spiercellen niet. Dit proefschrift probeert een verklaring te vinden voor de relatieve ongevoeligheid van de neointima voor α_1 -adrenoreceptoren blokkade.

In het eerste experimentele hoofdstuk (hoofdstuk 2) is gekeken naar de hoeveelheid en locatie van de boodschapper RNA (mRNA) bij verschillende tijdstippen na ballondenudatie van de arteria carotis van de rat. Uit de resultaten blijkt dat er α_1 -adrenoreceptoren aanwezig zijn in de gevormde neointima en dat de hoeveelheid, maar niet het subtype of de distributie van de α_1 -adrenoreceptoren verandert in de tijd. Dat de hoeveelheid receptoren veranderd is na ballondenudatie kan tevens worden aangetoond via een receptor bindingsstudie (hoofdstuk 4).

Een andere verklaring voor het gebrek aan gevoeligheid van de beschadigde neointima in de arteria carotis van de rat voor α_1 -adrenoreceptoren blokkade na infusie van AngII is de hoeveelheid catecholamine die vrij gemaakt wordt na stimulatie met AngII. Resultaten uit hoofdstuk 3 tonen aan dat de aanmaak van het mRNA voor het norepinephrine transporter (NET) gen opgereguleerd is en dat beschadigde vaten ontvankelijker zijn voor de door AngII geïnduceerde toename in het NET mRNA. Aangezien het NET zorg draagt voor de opname van vrijgekomen catecholamines is het zeer goed mogelijk dat het NET belangrijk is voor de regulatie van de groeirespons van zowel beschadigde als intacte bloedvaten na stimulatie met AngII.

Om een onderscheid te kunnen maken tussen catecholamines vrijgemaakt uit de sympathische zenuwuiteinden (neuronaal), die de arteria carotis van de rat omgeven, en de catecholamines vrijgemaakt uit de bijnier, die via het bloed de arteria carotis bereiken (systemisch), is in hoofdstuk 4 enkel de neuronale catecholamines verwijderd met behulp van een chemische sympathectomie met 6-OHDA. Deze resultaten bevestigen eerdere studies dat de α_1 -adrenoreceptoren verantwoordelijk zijn voor de door AngII geïnduceerde groeirespons in onbeschadigde bloedvaten. Nieuw in deze studie is de bevinding dat die respons neuronaal geregeld is en niet systemisch. Echter de neointima blijft ongevoelig voor sympathische denervatie. Wellicht wordt de neointimale groeirespons na AngII stimulatie systemisch gereguleerd, dit in tegenstelling tot de neuronale regulatie van de onbeschadigde media. Resultaten uit hoofdstuk 5 tonen echter aan dat noch de catecholamines afkomstig van de bijnier, noch de α_1 -adrenoreceptoren bijdragen aan de AngII geïnduceerde neointimale groei.

In hoofdstuk 6 is gekeken of innervatie, of beter het gebrek aan innervatie, belangrijk is voor intimale groei in humane vaten. Er is gekeken naar de innervatie status van de anastomose regio van gestenoseerde getransplanteerde menselijke bloedvaten en naar dezelfde bloedvaten vóór transplantatie met een panel van antilichamen gericht tegen verschillende epitopen aanwezig in sympathische zenuwen. De resultaten tonen dat er geen bewijs is voor een bijdrage van de sympathische zenuwen in de intimale groei in getransplanteerde humane bloedvaten.

In conclusie kan gesteld worden dat de resultaten in dit proefschrift een verdere verklaring geven voor de rol van AngII en het sympathische zenuwstelsel in de neointimale groei van beschadigde bloedvaten. Vooraleer is het duidelijk geworden dat neointimale en mediale gladde spiercellen verschillend reageren op AngII en

catecholamine stimulatie. Het is daarom zeer belangrijk om het effect van bepaalde therapieën ter voorkomen van restenose ook daadwerkelijk te onderzoeken in experimentele modellen waarbij al een intima aanwezig is. Voorts zijn wij van mening dat sympathische innervatie niet belangrijk is in de door AngII-geïnduceerde toename in neointimale groei. Aangezien resultaten in dit proefschrift aantonen dat de neointima van humane vaten niet geïnnerveerd is lijkt het ons weinig zinvol om de sympathische innervatie als aangrijppunt voor een potentiële behandelingsmethode tegen restenose te gebruiken.

Curriculum Vitae

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1981-1987	HAVO Scholengemeenschap Nijmegen-West te Nijmegen
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List of Publications

- Bruijns R, van Kleef E, Smits J, Daemen M: Heterogeneous effects of chemical sympathectomy on neointimal and medial growth after stimulation with angiotensin II. (abstract). *J. Vasc. Res.* 1996;33(suppl. 1):12.
- Bruijns RHJ, van Kleef EM, Smits JFM, De Mey JGR, Daemen MJAP: Effects of chemical sympathectomy on angiotensin II-induced neointimal growth in the balloon injured rat carotid artery. (abstract). *Hypertension.* 1997;30:999.
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- Bruijns RHJ, Van den Bossche RMA, Bult H: Effect of cytochalasin D on vascular reactivity and cellular outgrowth in the rabbit carotid artery. (abstract). *Br. J. Pharmacol. (suppl.)*. 2000:(in press).

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