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The importance of endothelin-1 for vascular function in patients with atherosclerosis and healthy controls

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Cover picture: *Atractaspis engaddensis* with single fang erected.
© Elazar Kochva. This israeli burowing asp killed Cleopatra VII with its venom sarofotoxin, a member of the endothelin family.

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ABSTRACT

Background: Atherosclerosis is associated with endothelial dysfunction, which is an early sign with prognostic implications in patients with coronary artery disease. Atherosclerosis is also associated with enhanced expression of the vasoconstrictor peptide endothelin-1 (ET-1). The enhanced production of ET-1 in atherosclerotic arteries may be related to increased activity of the endothelin converting enzyme (ECE) which converts big ET-1 to ET-1. ET-1 causes vasoconstriction via ET_A and ET_B receptors located on vascular smooth muscle cells and vasodilatation via ET_B receptors on endothelial cells. *In vitro* studies indicate that ET_B receptors are upregulated in atherosclerosis. The purpose of the thesis was to investigate the pathophysiological consequences of this altered ET-system in patients with atherosclerosis.

Study I: The ET_B receptor agonist sarafotoxin 6c evoked significantly larger reduction in forearm blood flow (FBF) in patients with atherosclerosis (n=7) than in age-matched healthy controls (n=6), whereas the non-selective ET_A and ET_B receptor agonist ET-1 induced a similar vasoconstrictor response in the two groups. These findings suggest an upregulation of vascular smooth muscle ET_B receptors in atherosclerosis.
Study II: ET_B receptor blockade evoked a significant increase in FBF in patients with atherosclerosis (n=10) whereas a small reduction was observed in age-matched controls (n=10). Combined ET_A and ET_B receptor antagonism evoked a marked increase in FBF in the patients whereas there was no effect in the controls. ET_A receptor blockade alone increased FBF to a similar degree in patients and in controls. These observations suggest an enhanced ET-1-mediated vascular tone in atherosclerotic patients, which at least partly is mediated via the ET_B receptors.
Study III: Intra-arterial infusion of ET-1 significantly blunted endothelium-dependent vasodilatation (EDV) in young healthy males (n=10). Selective ET_A receptor blockade significantly increased in patients with atherosclerosis (n=10), whereas no significant change was observed in healthy age-matched controls (n=9). These observations demonstrate that elevated levels of ET-1 impair EDV. Furthermore, ET_A receptor blockade improves EDV in patients with atherosclerosis indicating that ET-1 attenuates EDV via an ET_A receptor-mediated mechanism.
Study IV: Big ET-1 evoked a more pronounced reduction in FBF in patients with atherosclerosis (n=9) than in age-matched controls (n=9). The elevation of local venous plasma ET-1 and the net formation of ET-1 during administration of big ET-1 was more pronounced in the patients than in the controls. These findings suggest an increased ECE activity in the patients.
Study V: Systemic ET_A receptor blockade inhibited the increase in splanchnic and renal vascular resistance induced by ET-1 in healthy men (n=6). ET_B receptor blockade alone increased basal splanchnic and renal vascular resistance, and enhanced ET-1-induced vasoconstriction. Plasma ET-1 increased more following ET_B receptor blockade as compared to control conditions and following ET_A receptor blockade. These findings suggest that ET_A receptors mediate the splanchnic and renal vasoconstriction induced by ET-1 in healthy humans. The ET_B receptor seems to function as a clearance receptor and may modulate vascular tone by altering the plasma concentration of ET-1.

Conclusions: These findings suggest that ET-1 may play an important role in the enhanced vasoconstriction and endothelial dysfunction seen in patients with atherosclerosis. ET receptor blockade may be of therapeutic value by improving blood flow and endothelial function in these patients.

CONTENTS

ABBREVIATIONS

ACE	angiotensin converting enzyme
ACh	acetylcholine
ANOVA	analysis of variance
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DAG	diacylglycerol
ECE	endothelin converting enzyme
EDCF	endothelium-derived constricting factor
EDRF	endothelium-derived relaxing factor
EDV	endothelium-dependent vasodilatation
eNOS	endothelial nitric oxide synthase
ET	endothelin
ET-LI	endothelin-like immunoreactivity
FBF	forearm blood flow
HDL	high density lipoprotein
ICAM-1	intercellular adhesion molecule-1
iNOS	inducible nitric oxide synthase
IP_3	1,4,5-inositol trisphosphate
L-arg	L-arginine
LDL	low density lipoprotein
L-NMMA	N^G-monomethyl-L-arginine
MAP	mean arterial pressure
mRNA	messenger ribonucleic acid
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NFκB	nuclear factor kappa B
O_2^-	superoxide
$ONOO^-$	peroxynitrite anion
PA	pulmonary arterial
PAH	para-aminohippuric acid
PGI_2	prostacyclin
PKC	protein kinase C
RBF	renal blood flow
RVR	renal vascular resistance
SBF	splanchnic blood flow
SEM	standard error of mean
SNP	sodium nitroprusside
SplVR	splanchnic vascular resistance
SVR	systemic vascular resistance
TNF-α	tumour necrosis factor alpha
VCAM-1	vascular cell adhesion molecule-1

List of Original Papers

This thesis is based on the following studies, which will be referred to by their Roman numerals.

I

Pernow J, Böhm F, Johansson BL, Hedin U, Ryden L.

Enhanced vasoconstrictor response to endothelin-B-receptor stimulation in patients with atherosclerosis.

J Cardiovasc Pharmacol. 2000;36:S418-420.

II

Böhm F, Ahlborg G, Johansson BL, Hansson LO, Pernow J.

Combined endothelin receptor blockade evokes enhanced vasodilatation in patients with atherosclerosis.

Arterioscler Thromb Vasc Biol. 2002;22:674-679.

III

Böhm F, Ahlborg G, Pernow J.

Endothelin-1 inhibits endothelium-dependent vasodilatation in the human forearm: reversal by ET_A receptor blockade in patients with atherosclerosis.

Clin Sci (Lond). 2002;102:321-327.

IV

Böhm F, Johansson BL, Hedin U, Alving K, Pernow J.

Enhanced vasoconstrictor effect of big endothelin-1 in patients with atherosclerosis: relation to conversion to endothelin-1.

Atherosclerosis. 2002;160:215-222.

V

Böhm F, Pernow J, Lindström J, Ahlborg G.

ET_A receptors mediate vasoconstriction whereas ET_B receptors clear endothelin-1 in the splanchnic and renal circulation of healthy men.

Manuscript.

INTRODUCTION

Cardiovascular disease is the major cause of morbidity and mortality in Sweden as in other industrialized countries.[1] Coronary heart disease, stroke and peripheral arterial disease are the most prevalent manifestations of cardiovascular disease and the predominant underlying cause is atherosclerosis. In the past decades great advances have been made in the understanding of the pathogenesis of atherosclerosis and thrombosis, including the series of events that occur in the vessel wall during atherogenesis.[2-4] Atherosclerosis is associated with enhanced expression of ET-1 which may contribute to endothelial dysfunction, which in turn correlates with prognosis in patients with coronary artery disease. Although the use of aspirin, beta-blockers, statins and angiotensin converting enzyme (ACE) inhibitors has improved the outcome for atherosclerotic patients the prognosis is still dismal and a lot remains to be done to reduce the high morbidity and mortality in this disease. One way to further reduce the functional and thereby prognostic implications of atherosclerosis may be to interfere with the endothelin system.

The endothelium

Based on morphological characteristics, the anatomy of the arterial vessel is divided into three components: tunica intima, tunica media and tunica adventitia. The tunica intima consists of a single layer of endothelial cells that line the vessel lumen and the internal elastic lamina membrane. The tuncia media comprises the muscular portion of the blood vessel, and the tunica adventitia includes the external elastic lamina, terminal nerve fibers and surrounding connective tissue, which contains fibroblasts and tissue macrophages. Each of these three layers has specific properties and exerts different effects that are crucial for the regulation of vasomotor tone, protection against thrombosis and response to injury. The function of all the components of the vessel wall is altered during the progressive course of atherosclerotic vascular disease.[5]

Before 1980, studies investigating the regulation of vasomotor tone focused almost exclusively on the smooth muscle cell layer of the tunica media. The endothelium was considered a simple, passive barrier[6] and the adventitia simply a support structure of the vessel. In 1976, the discovery that prostacyclin (PGI_2) was derived from the vascular wall gave rise to an idea of the vessel wall as a local modulator of vascular function.[7] In 1980, Furchgott and Zawadzki were the first to demonstrate the necessity of the vascular endothelium for the vasdilator response to acetylcholine.[8] They had thereby discovered the first endothelium-derived relaxing factor (EDRF), subsequently identified as nitric oxide (NO).[9,10] This ushered a new era of vascular research, leading to the present concept of the endothelium as a synthetically highly active organ, a key player in regulation of vasomotor tone and also in the development of atherosclerotic changes in the vasculature. A multitude of studies have shown that EDRFs not only act on vasomotor tone but also inhibit platelet aggregation, coagulation and inflammatory and proliferative responses.[11] The healthy endothelium plays a central role in cardiovascular control, and endothelial damage may contribute to disease states characterized by vasoconstriction, inflammation, excessive thrombus formation, leukocyte adhesion to vessel walls, hypertension and atherosclerosis.[3,12]

In 1982, there was an unexpected finding of an endothelium-derived constricting factor (EDCF),[13] which was believed to be of a peptidergic nature.[14] The era of endothelin research began in 1988, when Yanagisawa and colleagues succeeded in isolating, purifying, sequencing and cloning this EDCF from the conditioned medium of cultured porcine aortic endothelial cells.[15] Yanagisawa named it endothelin (ET), and it turned out to be the most potent vasoconstrictor peptide known so far. This thesis aims

to evaluate the importance of ET-1, the predominant form of ET, for vascular function in patients with atherosclerosis and healthy controls.

Endothelin-1 (ET-1)

ET proved to be a family of at least four 21-amino acid peptides, ie, ET-1, ET-2, ET-3[16] and ET-4 (vasoactive intestinal constrictor).[17] In addition, 31-residue ETs have been identified.[18] ET-1, the predominant isoform in the cardiovascular system, has a striking similarity to sarafotoxin, the venom of snakes of the *Atractaspis* family.[19] In 30 B.C. the Egyptian queen Cleopatra VII became the most famous research subject in the exciting history of ET research when she exposed herself to this snake venom sarafotoxin in a dose that induced a lethal coronary vasoconstriction.[20] Like sarafotoxin, ETs are peptides with potent and characteristically long-lasting vasoconstrictor and vasopressor actions.[15] In addition to their cardiovascular effects, ETs are involved in embryonic development,[21] bronchoconstriction,[22] prostate growth,[23] carcinogenesis,[24] and gastrointestinal[25] and endocrine function.[26]

Regulation of ET-1 production

ET-1 is produced not only by the endothelium, but also by other cells involved in vascular disease, such as leukocytes,[27] macrophages,[28] smooth muscle cells,[29] cardiomyocytes[30] and mesangial cells.[31] The ET-1 synthesis is regulated by physicochemical factors such as pulsatile stretch,[32] shear stress[33] and pH.[34] Stimulators of ET-1 synthesis include procoagulant factors, cytokines and growth factors such as thrombin,[35] transforming growth factor-ß,[36] tumor necrosis factor-α,[37] interleukin-1,[38] epidermal growth factor,[39] platelet-derived growth factor,[29] insulin-like growth factor-I[40] and insulin.[40] In addition, ET-1 production is stimulated by vasoactive substances such as adrenaline,[15] angiotensin II,[41] arginine vasopressin,[41] and bradykinin.[42] Hypoxia is a strong stimulus for ET-1 synthesis,[43] an observation which may be important in ischemia. ET-1 biosynthesis is stimulated by cardiovascular risk factors such as elevated levels of oxidized and acetylated low density lipoprotein (LDL)[44] and glucose,[45] estrogen deficiency,[46] obesity,[47] cocaine use,[48] ageing[49] and low shear stress.[50] Phorbol esters [28], Ca^{2+} ionophores[15] and lipopolysaccharides[28] are other known stimulators of ET-1 synthesis. ET-1 has also been shown to increase its own mRNA expression by autoinduction.[51] On the other hand NO[52] (Fig. 1), prostacyclin (PGI_2),[53] atrial, brain and C-type natriuretic peptide,[54] adrenomedullin[55] and estrogens[56] inhibit ET-1 production.

Endothelin-forming enzymes

Human ET-1 is synthesized from a larger preproform, called prepro ET-1, consisting of 212 amino acid residues[15] (Fig. 2). Human prepro ET-1 is cleaved by a furin convertase[57] to pro ET-1, also called big ET-1, consisting of 38 amino acid residues.[58] Once formed, big ET-1 is processed to $ET\text{-}1_{(1\text{-}21)}$ through cleavage of the Trp_{21}-Val_{22} bond

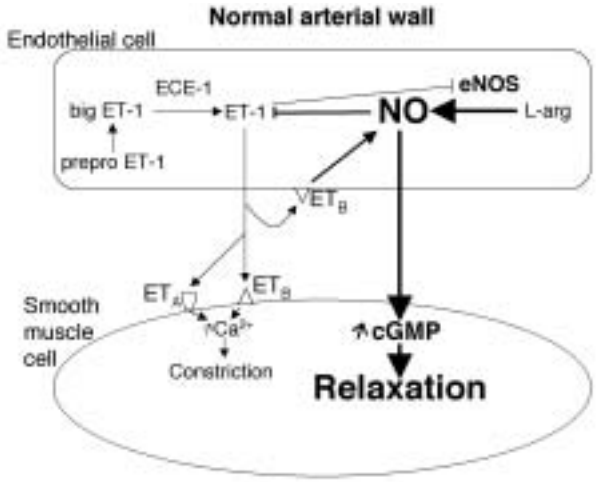

Figure 1. Schematic illustration of the biosynthesis of endothelin-1 (ET-1) and nitric oxide (NO) and their effects on the endothelial cells and vascular smooth muscle cells in the normal arterial wall. ET-1 is initially synthesized as a prepropeptide which is hydrolyzed to big ET-1. Big ET-1 is then converted to mature ET-1 by different isoforms of ET converting enzyme-1 (ECE-1). ET-1 exerts its effects via the two subtypes of ET receptors, ET_A and ET_B. ET_A receptors mediate vasoconstriction of smooth muscle cells via an increase in intracellular calcium. ET_B receptors located on smooth muscle cells mediate vasoconstriction, whereas ET_B receptors located on the endothelium mediate vasorelaxation via the release of NO. NO is produced from L-arginine (L-arg) by the constitutively expressed enzyme endothelial NO synthase (eNOS). This enzyme can be inhibited by ET-1. NO causes relaxation of smooth muscle cells via an increase in cyclic guanosine monophosphate (cGMP) and inhibits the production of ET-1. There is a balance between the vasoconstrictor ET-1 and the vasodilator NO in the vessel wall. In healthy vessels the production of ET-1 is small and the effect of NO results in a vasodilator tone.

by ET-converting enzyme-1 (ECE-1), which exists in 4 isoforms (a, b, c and d),[59] and by ECE-2 (Fig. 2).[60] The chemical nature and tissue distribution of ECE-1 and ECE-2 suggest that ECE-1 is responsible for the conversion of big ET-1 to ET-1 in the vascular bed. ECE-1 mRNA has a striking expression in vascular endothelial cells, but is also expressed in ovary, testis, adrenal gland, lung, liver, heart, brain, kidney, spleen, pancreas and skeletal muscle.[61] In addition, human chymase cleaves big ET-1 at the Tyr_{31}-Gly_{32} bond, resulting in the formation of $ET\text{-}1_{(1\text{-}31)}$ (Fig. 2).[62] ECE-1 belongs to the metalloprotease family[63] and may also hydrolyze peptides such as bradykinin, substance P and insulin.[64] ECE-1 expression is regulated through protein kinase C-dependent mechanisms,[65] ET_B receptors,[66] the transcription factor Ets-1[67] and cytokines.[68] Recent studies have shown that both the expression[69,70] and the activity of ECE-1 are enhanced in models of vascular injury and atherosclerosis.[71,72]

Endothelin receptors

ET-1, as well as other ET isopeptides, exert their actions through binding to specific ET receptors, which belong to the rhodopsin superfamily of transmembrane G-protein-coupled receptors. Five receptors have been cloned in different species. Humans possess ET_A[73] and ET_B receptors.[74] In the vasculature, ET_A receptors are found in smooth muscle cells, whereas ET_B receptors are localized not only on smooth muscle cells, but also on endothelial cells[75] and macrophages.[76] The affinity of ET_A receptors for ET-1 and ET-2 is >100-fold higher than for ET-3, whereas ET_B receptors bind ET isopeptides with a similar affinity.[77] Cross-talk between ET_A and ET_B receptors has been reported.[78,79] This may affect receptor function such that if only one receptor is blocked, the other receptor can compensate for the loss of activity.[80,81] Blockade of one of the receptors may attenuate an inhibitory action on the other receptor.[82]

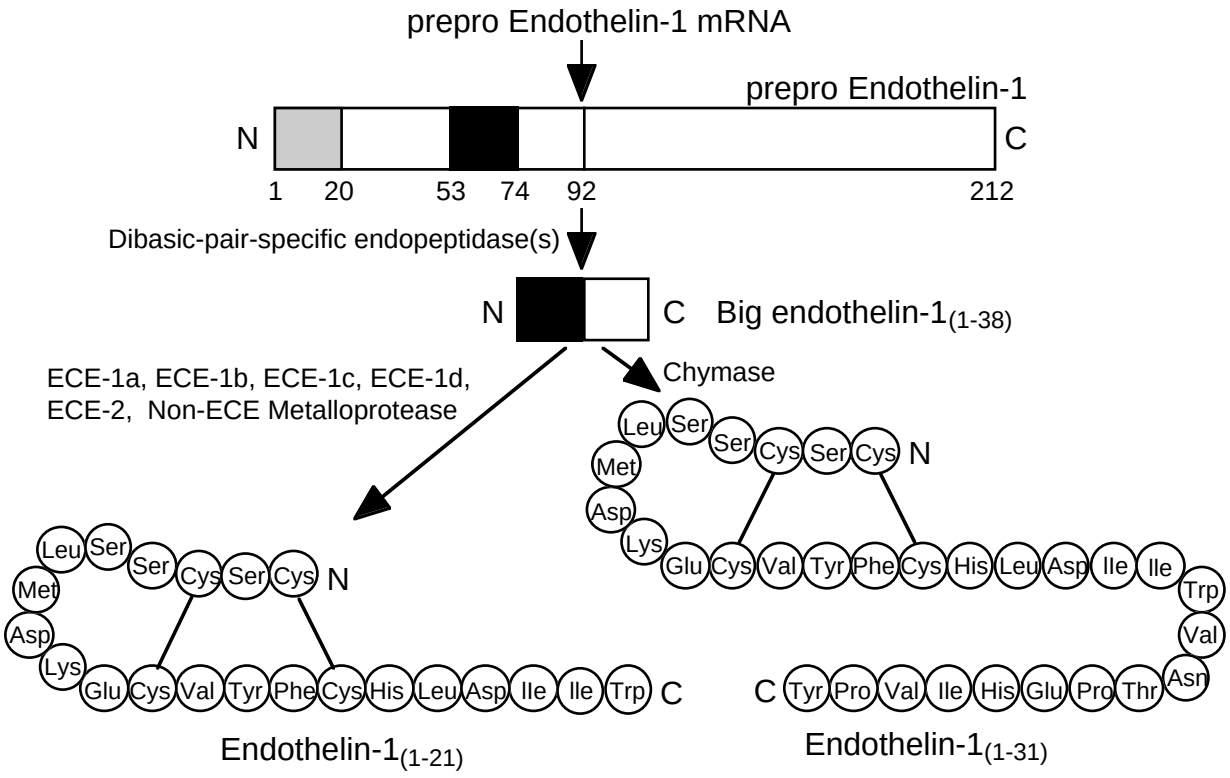

Figure 2. Biosynthesis of $ET\text{-}1_{(1\text{-}21)}$ and $ET\text{-}1_{(1\text{-}31)}$ peptides.
Prepro ET-1 mRNA is translated into prepro ET-1 protein, a 212-amino acid peptide, which is cleaved by furin convertase to the 38-amino acid precursor big $ET\text{-}1_{(1\text{-}38)}$. Big ET-1 is processed into $ET\text{-}1_{(1\text{-}21)}$ by endothelin converting enzymes (ECEs) and non-ECE metalloprotease (left). By an alternative pathway involving chymase, a 31-amino acid, $ET\text{-}1_{(1\text{-}31)}$ is formed (right). Modified from[15] and.[120]

Stimulation of ET_A and ET_B receptors on vascular smooth muscle cells elicits a vasoconstrictor response, whereas stimulation of endothelial ET_B receptors evokes vasodilatation (Fig. 1). ET receptor binding activates various signal transduction pathways in which several intracellular second messengers are involved.[83] The binding of ET-1 to ET_A and ET_B receptors on vascular smooth muscle cells activates phospholipase C, which leads to an accumulation of the second messegers inositol trisphosphate (IP_3) and diacylglycerol (DAG).[84] ET-1 also activates phospholipase D which gives rise to a sustained accumulation of DAG. IP_3 then mobilizes Ca^{2+} from intracellular stores. There may also be direct Ca^{2+} influx through the cell membrane caused by activation of voltage-operated Ca^{2+} channels[84] secondary to ET-1-induced membrane depolarization.[85] DAG activates protein kinase C, which probably contributes to the increase in Ca^{2+} influx[86] and mediates sensitization of the contractile apparatus to Ca^{2+}.[87] This increase in cytosolic free Ca^{2+} finally results in long-lasting vasoconstriction.[14,15,88] The prolonged effect may be due to recycling back to the plasma membrane of the ET_A receptor after it has been internalized by binding of ET-1. This recycling may provide the basis for sustained activation of signal-transducing G-proteins.[89,90] Besides causing vasoconstriction, ET receptor binding also elicits stimulation of cellular growth and proliferation via activation of protein kinase C and other signal transduction pathways.[83] In addition, ET-1 has been shown to activate phospholipase A_2 to release arachidonic acid from membrane phospholipids.[91] Free arachidonate is then converted to eicosanoids, particularly PGI_2 and thromboxane A_2.

In contrast, the activation of endothelial ET_B receptors stimulates the release of NO and PGI_2[92,93] which in turn increase intracellular levels of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP), respectively, causing relaxation of vascular smooth muscle cells. Stimulation of endothelial ET_B receptors also prevents apoptosis[94] and inhibits ECE-1 expression in endothelial cells.[66] In addition, endothelial ET_B receptors mediate the pulmonary clearance of circulating ET-1[95] and the reuptake of ET-1 by endothelial cells.[96]

Vasoactive effects of endothelin-1

ET-1 is one of the most potent vasoconstrictors known, as was demonstrated already in the original report by Yanagisawa and co-workers.[15] In the endothelium, ET-1 is predominantly released abluminally towards the vascular smooth muscle, suggesting a paracrine role.[97] In healthy humans, intra-venous administration of ET-1 increases mean arterial blood pressure, reduces heart rate, cardiac output and stroke volume and causes vasoconstriction in the pulmonary,[98] renal, splanchnic,[99] myocardial[100] and skeletal muscle[101] vasculature. Haynes and Webb demonstrated that the selective ET_A receptor antagonist BQ123 and the inhibitor of ECE, phosphoramidon, evoke increases in forearm blood flow (FBF).[102,103] These findings strongly suggest that endogenous ET-1 plays a fundamental physiological role in the maintenance of vascular tone in healthy humans.

Besides its vasoconstrictive effects, ET-1 has been suggested to be of pathophysiological importance in atherogenesis.[104] Thus, ET-1 may regulate cellular growth and proliferation by stimulating DNA synthesis in vascular smooth muscle cells[105] and mesangial cells. ET-1 also stimulates matrix gene expression and thereby promotes formation of fibrous tissue.[106,107] The production of cytokines[108,109] and growth factors such as vascular endodothelial growth factor,[110] basic fibroblast growth factor-2[111] and epiregulin[112] is stimulated by ET-1. Moreover, ET-1 induces the formation of extracellular matrix proteins[113] and fibronectin.[114] There are also reports suggesting that ET-1 induces a procoagulatory state since it stimulates neutrophil adhesion[115] and platelet aggregation[116] and may act as an antifibrinolytic factor.[117-119] Furthermore, ET-1 promotes cell-cycle progression in an autocrine fashion.[120] These effects are mediated predominantly via the ET_A receptor.[121] ET-1 may contribute to the progression of several cardiovascular disorders like congestive heart failure, hypertension, ischemic heart disease and atherosclerosis.[104,122] It has also been speculated that ET-1 is of importance in patients with renal failure [123-125] and in portal hypertension.[126,127] Sustained afferent and efferent arteriolar vasoconstriction induced by ET-1 may contribute to ischemia in acute renal failure.[128,129] Furthermore, patients with primary pulmonary hypertension

have enhanced expression,[130] as well as increased plasma levels of ET-1[131] which correlate with the severity of primary pulmonary hypertension.[132]

Besides evoking vasoconstriction, ET-1 stimulates the development of glomerulosclerosis and interstitial fibrosis as demonstrated in transgenic mice expressing human ET-1.[133] The expression of ET receptors is increased in portal hypertension,[126] and consequently predisposes the liver to microcirculatory dysfunction,[134] and plasma ET-1 levels correlate with the severity of cirrhosis and portal hypertension in biliary atresia.[127] Therefore ET-1 may be an interesting target for therapy aiming at preserving renal and splanchnic function. This is further supported by the fact that the sensitivity to ET-1 is preserved in renal insufficiency[135] and even enhanced in hypertension.[136] In a recent study, selective ET_A receptor blockade was found to attenuate renal effects of ET-1 in humans.[137] Apart from that, the effects of ET-1 on the different receptors in the renal and splanchnic vasculature in humans are not known.

Endothelin-1 and atherosclerosis

An early step in the pathogenesis of atherosclerosis is an imbalance between the numerous vasoactive and inflammatory factors. The imbalance is caused by cardiovascular risk factors such as dyslipidemia, diabetes, hypertension and smoking. This results in endothelial dysfunction, before any morphological changes are detectable. The first microscopic indication of atherosclerosis is accumulation of lipid in the intima of susceptible arteries, the so-called fatty streak, which can even be detected in young children. The lesions are charactherized by extracellular and intracellular lipid material in the intima beneath apparently intact endothelium. Apart from the deposition of amorphous and membranous lipids,[138] the fatty streak consists of monocyte-derived macrophages and T lymphocytes.[139] Continued exposure to risk factors and inflammation results in increased numbers of macrophages and lymphocytes, which both emigrate from the blood and multiply within the lesion. Activation of these cells leads to the release of hydrolytic enzymes, cytokines, chemokines and growth factors, which can induce further damage and eventually lead to focal necrosis. Thus, cycles of accumulation of mononuclear cells, migration and proliferation of smooth muscle cells, and formation of fibrous tissue lead to further enlargement and restructuring of the lesion, so that it becomes covered by a fibrous cap that overlies a core of lipid and necrotic tissue, a so-called advanced complicated lesion.[140]

Soon after its discovery, ET-1 was implicated in the pathophysiology of various cardiovascular diseases,[141] including atherosclerosis.[104] Raised plasma levels of ET-1 have been described in patients with coronary artery disease.[142] Elevated plasma ET-1 concentrations have also been demonstrated in patients with increased cardiovascular risk factors such as hypercholesterolemia, who do not have symptomatic atherosclerosis.[143] However, plasma levels in patients with atherosclerosis are lower than those in patients with acute coronary events.[144] Raised plasma ET-1 levels have also been described in patients with hypertension, diabetes mellitus[145,146] and in cigarette smokers.[143] In patients with established atherosclerosis, a significant correlation between plasma ET-1 levels and the number of atherosclerotic lesions has been shown.[142] The increased production of ET-1 in atherosclerotic arteries may be due to enhanced expression and activity of ECE-1 in the vascular wall.[69,72] Accordingly, ECE-1 activity is enhanced in isolated endothelium-denuded human atherosclerotic coronary arteries[71] as well as in rabbit atherosclerotic arteries[72] *in vitro*. The *in vivo* vasoconstrictor effect evoked by local conversion of big ET-1 to ET-1 in patients with atherosclerosis has not been investigated previously.

Upregulation of ET-1 and ET receptors has been demonstrated in atherosclerotic lesions in humans and in experimental animal models.[147] ET-1-like immunoreactivity has been found overlying and within regions of atherosclerotic plaques.[148] ET-1 immunostaining has also been identified in microvascular endothelial cells at regions of neovascularization and recanalization of plaques in atherosclerotic human coronary arteries.[149]

Using *in vitro* receptor autoradiography, binding sites for [^{125}I]-ET-1 on atherosclerotic human blood vessels have been identified

predominantly on smooth muscle cells of the tunica media, which is the principal site of ET-1 mediated vasoconstriction.[150] In displacement binding studies, the receptors in the tunica media were identified to be predominantly ET_A receptors.[151] Although the ET_A receptor seems to be the major one mediating vasoconstriction in healthy humans,[102] the situation may be different in atherosclerosis. *In situ* hybridization studies localized both ET_A and ET_B receptors in endothelial cells, smooth muscle cells and macrophages in atherosclerotic plaques in hyperlipidemic hamsters.[152] Moreover, using immunohistochemical techniques to study ET receptor distribution in both atherosclerotic humans and apolipoprotein E knock-out mice, increased number of ET_B receptors were found in macrophages, T-lymphocytes and medial smooth muscle cells.[147,153] In addition, smooth muscle cells located beneath foamy macrophages exhibited higher ET_B receptor immunoreactivity than those lying beneath a normal intima. These results suggest that the expression of ET receptor subtypes changes and the accumulation of foamy macrophages in atherosclerosis may cause a shift of receptor subtype expression from ET_A to ET_B, which may then become the principal receptor involved in the progression of atherosclerosis.[154]

ET-1 has also been implicated as an inflammatory mediator that potentiates interactions between circulating platelets and leukocytes and the vascular endothelium.[115,155] Moreover, ET-1 strongly stimulates macrophages to synthesize monocyte chemoattractant protein-1[156] allowing monocyte invasion into the arterial wall, which is an essential step in atherogenesis.[157] By activating monocytes and stimulating release of cytokines such as interleukin-6,[158,159] chemokines, prostaglandins[160] and upregulating adhesion molecule expression,[161] ET-1 is capable of promoting the inflammatory response. The pro-atherogenic factor C-reactive protein (CRP) has recently been shown to increase the ET-1 release.[162] ET-1 release is also stimulated by factors such as TNF-a, interleukin-ß and interleukin-2.[163] These latter effects appear to be ET_B receptor-mediated.[154] However, stimulation of this receptor subtype located on the endothelium also results in NO release, which has an anti-inflammatory effect. Finally, the expression of ET-1 is enhanced in smooth muscle cells and macrophages of human atherosclerotic plaques.[164]

Further observations supporting a role of ET-1 in various proliferative and inflammatory aspects of coronary artery disease have been made *in vivo*, including studies using the rat model of neointima formation in balloon-injured carotid arteries as a model of vascular injury. Infusion of exogenous ET-1 potentiates the development of intimal hyperplasia following ballon catheter injury[165] whilst mixed ET_A/ET_B receptor antagonists reduce angioplasty-induced neointima formation.[166,167] Furthermore, in the ischemia/reperfusion injury model, ET receptor blockade limits infarct size and protects the myocardium from neutrophil injury.[168,169]

Based on the effects evoked by ET-1 mentioned above, inhibition of the ET-1 pathway may be a promising target for cardiovascular therapy. The importance of endogenous ET-1 in blood flow regulation via the different ET receptors in atherosclerotic patients has so far not been investigated, however. It also remains to be established which receptors should be blocked to achieve the best perfusion of atherosclerotic vascular beds in humans. Although the ET_A receptor seems to be the major subtype mediating vasoconstriction in healthy humans, the situation may be different in atherosclerosis due to an upregulation of ET_B receptors in atherosclerotic arteries.[170]

Nitric oxide (NO)

The discovery by Furchgott and Zawadski[8] of the role played by the endothelial cells and EDRF for relaxation of isolated arteries in response to acetylcholine initiated a major scientific inquiry into the pivotal role of the endothelium for the normal physiological function of the vascular wall. This EDRF was subsequently shown to be NO.[9,10] In endothelial cells NO is produced from the substrate L-arginine by the endothelial isoform of the enzyme NO synthase (eNOS or NOS III).[171] Two other isoforms of the enzyme are neuronal NOS (nNOS or NOS I) and inducible NOS (iNOS or NOS II). In basal conditions NO is continuously secreted by endothelial cells, leading to a constant state of vasodilatation (Fig. 1). NO is one of the most important substances in preserving a normal endothelial function. NO

inhibits expression of adhesion molecules (selectins, vascular cell adhesion molecule-1, intercellular adhesion molecule-1), cytokines and the potentially harmful iNOS.[172-176] These effects are mediated by inhibition of the transcription factor nuclear factor kappa B (NFκB), through the induction and stabilization of NFκB inhibitor. Thus, NO may tonically inhibit the expression of NFκB-dependent proinflammatory genes. NO also possesses antithrombotic properties, one of its mechanisms of actions is inhibition of the expression of tissue factor.[177] Furthermore, NO inhibits proliferation and migration of endothelial and smooth muscle cells, and diminished eNOS gene expression in atherosclerosis leads to increased proliferation and remodeling of the vessel wall. In addition, NO may protect from apoptosis via upregulation of several protective proteins (heat shock proteins, cyclooxygenase-2).[177] Finally, NO can act as a scavenger of the superoxide anion (O_2^-) by reacting with it to form peroxynitrite anion ($ONOO^-$).[178]

Endothelial dysfunction

Endothelial dysfunction is characterized by an imbalance between relaxing and contracting factors, between anticoagulant and procoagulant mediators, between anti-inflammatory and pro-inflammatory mediators, and between growth-inhibiting and promoting factors. This imbalance results in a change towards a vasoconstrictive, pro-thrombotic, pro-inflammatory and growth-promoting dysfunctional state. Such dysfunction can result from mechanical or biochemical injury to the endothelium.[179] Endothelial dysfunction, in the form of reduced NO-mediated vasodilatation, is seen in a multitude of cardiovascular and metabolic diseases.[180] All traditional risk factors for atherosclerosis are associated with endothelial dysfunction. The impaired endothelium-dependent vasodilatation (EDV) is the result of reduced bioavailability of NO in the vessel wall.[181] This reduction in NO availability may occur through several potential mechanisms at many sites, including (a) impairment of membrane receptors that interact with agonists or physical stimuli (i.e. shear stress) capable of generating NO; (b) reduced concentrations or impaired utilization of the NO-precursor L-arginine; (c) reduction in expression or activity of eNOS; (d) impaired release of NO from the damaged atherosclerotic endothelium; (e) impaired NO diffusion from the endothelium to the vascular smooth muscle cells followed by decreased sensitivity to its vasodilator action; (f) enhanced degradation of NO by increased generation of free radicals and/or oxidation-sensitive mechanisms; and (g) impaired interaction of NO with guanylate cyclase and consequently limitation of cGMP production.[182]

Endothelin-1 and nitric oxide interactions

There seems to be close interactions between NO and ET-1 in the vascular wall. Thus, NO inhibits the production of ET-1 in endothelial cells[52] and ET-1 is known to stimulate release of NO from healthy arteries via activation of the ET_B receptor located on endothelial cells.[183] However, ET-1 may reduce the bioavailability of NO in the vessel wall. Accordingly, ET-1 has been demonstrated to increase superoxide production in the vascular wall[184,185] via an effect that seems to be coupled to the ET_A receptor.[186] Superoxide will react with NO released from the endothelium and thereby reduce its bioavailability. Another mechanism may be that ET-1 reduces iNOS activity,[187] an effect that can be restored in dogs by dual ET_A/ET_B receptor blockade.[188] Moreover, dual ET_A/ET_B receptor blockade enhances calcium-dependent eNOS activity more than selective ET_A receptor blockade in pigs with hypercholesterolemia.[189]

Endothelial dysfunction in the coronary and forearm vascular beds is associated with increased risk for cardiovascular events.[190,191] The enhanced expression of ET-1 and its receptors in atherosclerosis may contribute to endothelial dysfunction by reducing the bioavailability of NO in the vessel wall. ET receptor antagonism has been reported to improve endothelial function in isolated arteries from apolipoprotein E-deficient mice[192] and in isolated internal mammary arteries from patients with coronary artery disease.[193] Apart from these observations, little is known about the functional consequences of the altered ET system in atherosclerosis on blood flow and endothelial function in humans. Restoration of NO-mediated signaling pathways may

represent an important therapeutic target for new drugs intended for improved vascular function in patients with atherosclerosis. Such novel therapeutic strategies may include administration of L-arginine, antioxidants, gene-transfer approaches, angiotensin converting enzyme (ACE) inhibitors, lipid lowering drugs and ET receptor antagonists. To further explore the rationale behind such therapeutic concepts the following hypothesis and aims for this thesis was formulated:

Hypothesis and Aims

Hypothesis

The main hypothesis of the present research project is that ET-1 exerts negative effects on endothelial function and blood flow in patients with atherosclerosis.

Aims

-to evaluate the effect of ET receptor stimulation on forearm blood flow in patients with atherosclerosis.

-to elucidate the forearm vasodilator response to selective ET_A receptor and combined ET_A and ET_B receptor inhibition in patients with atherosclerosis.

-to investigate whether elevated levels of ET-1 exert negative effects on endothelial function and to assess whether ET_A receptor antagonism improves endothelial function in patients with atherosclerosis.

-to evaluate whether the vasoconstrictor effect of big ET-1 is enhanced in patients with atherosclerosis due to increased conversion to ET-1.

-to characterize the effect of ET-1 on renal and splanchnic blood flow mediated via the ET_A or ET_B receptors in healthy humans.

Material and Methods

Subjects

For studies I-IV, patients with atherosclerosis were recruited from the Departments of Cardiology and Vascular Surgery, Karolinska Hospital and the Department of Vascular Surgery, Huddinge Hospital. All patients had symptoms of intermittent claudication with significant atherosclerotic lesions and flow obstructions in the large arteries of the legs as determined by ultrasound scanning and/or angiography. Some patients (all in studies II and III) also had coronary artery disease (previous myocardial infarction or angina pectoris that had required coronary revascularization). None of the patients had any history of congestive heart failure or insulin-dependent diabetes mellitus. Two of the patients in study II-III and one of the patients in study IV were being treated for hypertension. Some basal characteristics of the study population are presented in Table 1. For studies I-IV, age-matched healthy controls were recruited from prior studies at the Department of Cardiology, Karolinska Hospital. The age-matched control subjects were normotensive, without history of cardiovascular disease and with normal ankle/brachial pressure index and pulse curves in the dorsalis pedis artery. None of the subjects in studies II-IV had any evidence of atherosclerotic plaques and all had normal flow profiles in the brachial artery as determined by ultrasound scanning. The patients were on regular medication as presented in Table 2. All medication with vasodilator properties (i.e. nitrates, calcium channel blockers and angiotensin II inhibitors) was withheld for at least four half-lives prior to the investigation. On the morning of the investigation, the patients did not take any of their regular medication. None of the age-matched control subjects was on any regular medication. Both patients and control subjects were given 320 mg acetylsalicylic acid the day before and on the day of the examination to block a possible ET-induced release of cyclooxygenase products. For studies II, III and V, young, healthy, non-smoking males were recruited. Some basal characteristics of these volunteers are presented in Table 3. Informed consent was obtained from all subjects. The investigations were carried out in accordance with the Declaration of Helsinki and were approved by the ethics committee of the Karolinska Hospital (studies I-IV) or the regional ethical committee of the Karolinska Institute (study V).

Table 1. Basal characteristics of patients with atherosclerosis and age-matched controls. Means±SEM

	Study I		**Study II**		**Study III**		**Study IV**	
	Patients (n=7)	Controls (n=6)	Patients (n=11)	Controls (n=12)	Patients (n=10)	Controls (n=9)	Patients (n=9)	Controls (n=9)
Age (years)	65±4	64±3	64±3	63±2	63±3	62±2	67±2	67±2
BMI (weight/height^2)	27±1	25±1	26±1	26±1	26±1	26±1	26±1	27±1
MAP (mmHg)	95±4	88±3	93±5	93±5	98±4	93±4	96±6	102±5
Total cholesterol (mmol/l)	6.0±0.4*	4.9±0.2	4.9±0.3	5.3±0.3	4.9±0.4	5.3±0.4	5.0±0.4	5.1±0.3
LDL cholesterol (mmol/l)	3.9±0.3*	2.7±0.3	3.2±0.3	3.2±0.3	3.2±0.4	3.3±0.4	3.4±0.3	2.9±0.4
Triglycerides (mmol/l)	2.0±0.4	1.7±0.4	1.8±0.2	1.8±0.2	1.9±0.3	1.7±0.2	1.3±0.2	1.9±0.3
Smokers (no.)	2	0	4	2	4	2	3	0
Ex-smokers (>1 year; no.)	4	1	7	3	6	1	6	4
Non-smokers (no.)	1	5	0	7	0	6	0	5

*P<0.05 vs. Controls.
BMI: Body Mass Index, MAP: Mean Arterial Pressure.

Table 2. Regular medication of patients (I-IV)

	Study I (n=7)	Study II (n=11)	Study III (n=10)	Study IV (n=9)
Acetylsalisylic acid	6	9	8	8
ß-blockers	1	8	6	5
Statins	0	3	2	0
Long acting nitrates	2	4	3	4
Calcium channel blockers	1	4	3	2
ACE-inhibitors	2	0	0	1
Angiotensin II receptor antagonists	0	2	2	1
Dipyridamol	0	0	0	1

Forearm blood flow measurements (I-IV)

The investigations were performed with the subjects in the supine position in a quiet laboratory with controlled temperature (Fig. 3A). The subjects were allowed a light breakfast without caffeine-containing drinks or alcohol and were instructed not to smoke on the day of the study. Under local anesthesia a percutaneous catheter was inserted in the proximal direction into the brachial artery of the non-dominant arm for infusions, measurement of blood pressure (Senso Nor 840, Senso Nor A.S. Horten, Norway) and blood sampling (Fig. 3B). Another catheter was inserted in the distal direction into a deep cubital vein for collection of blood samples. Forearm blood flow (FBF) was measured in the infused arm (I and IV) or simultaneously in both arms (II-III) by venous occlusion plethysmography using an airfilled cuff (I, II, IV and on patients and age-matched controls in study III) or a mercury-in-silastic strain gauge (on the young healthy volunteers in III) applied around the widest part of the forearm (Fig. 3B).[194] A cuff placed around the upper arm was inflated to 50 mmHg for 10 sec in order to obstruct the venous outflow during the recording of FBF. The circulation of the hands was occluded by inflating a wrist cuff to at least 30 mmHg above systolic blood pressure. Thirty min after the arterial cannulation basal FBF was determined during an infusion of saline.

Renal and splanchnic blood flow measurements (V)

The subjects were studied in the supine position, after an overnight fast, in a quiet laboratory with controlled temperature. A thin catheter was inserted percutaneously into an antecubital vein for infusion of cardiogreen, para-amino hippuric acid (PAH), ET-1 and BQ123 or BQ788. Cardiogreen and PAH were infused intra-venously at constant rates for determination of splanchnic (SBF) and renal blood flows (RBF) as previously described.[99] Another catheter was inserted into the brachial artery for blood sampling and measurement of blood pressure. A balloon-tipped catheter was inserted percutaneously into an antecubital vein and advanced under fluoroscopic guidance to a branch of the pulmonary artery for blood sampling and measurement of cardiac output. Cardiac output was determined by Fick's principle, based on the pulmonary oxygen uptake divided by the systemic arterial-pulmonary arterial oxygen difference. In a sub-

Table 3. Basal characteristics of young healthy controls

	Study II	Study III			Study V
Intervention	BQ788 (n=5)	ET-1 (n=10)	NA (n=6)	L-NMMA (n=7)	ET-1/BQ788/BQ123 (n=6)
Age (years)	29±1	29±2	26±1	24±1	25±1
Body mass index (weight/height^2)	23±2	24±1	22±1	23±1	23±1
Mean arterial pressure (mmHg)	90±6	90±2	92±1	94±3	91±4
Non-smokers (no.)	5	10	6	7	6

ET-1; endothelin-1, NA; noradrenaline, L-NMMA; N^G-mono-methyl-L-arginine

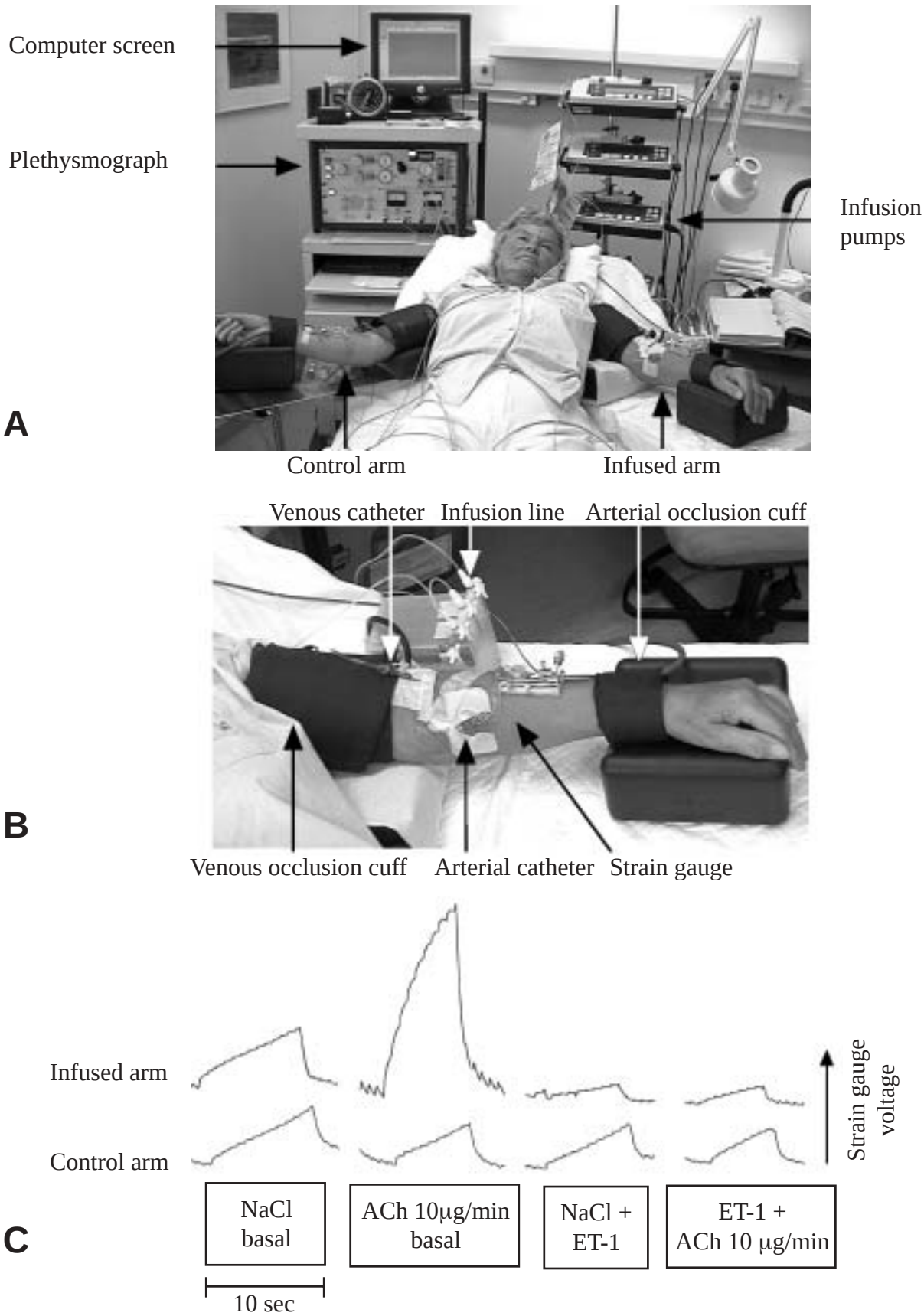

Figure 3. Venous occlusion plethysmography. (A): Assessment of forearm blood flow using mercury-in-silastic strain gauge venous occlusion plethysmography simultaneously in both arms. (B): Strain gauge, arterial and venous catheters in the infused arm. (C): Effect of intra-arterial ET-1 on endothelium-dependent vasodilation in a healthy control subject in study III. Acetylcholine (ACh; 10 μg/min) produces a marked increase in blood flow in the infused arm, as illustrated by the steep increase in the slope of the tracing. A 60 min infusion of ET-1 (10 pmol/min) reduces blood flow in the infused arm. The endothelium-dependent vasodilatation induced by acetylcholine is blocked during infusion of ET-1.

group of the subjects (n=5) a Cournand catheter no. 7 was inserted in a femoral vein and positioned in either the right renal vein or a central hepatic vein under fluoroscopic guidance. This was done to ascertain that the fractional extraction of cardiogreen and PAH was uninfluenced by the ET receptor blockers or ET-1. We found that neither ET-1, BQ123 nor BQ788 induced any change in the fractional extraction of cardiogreen or PAH. Moreover, previous studies have demonstrated that ET-1 does not affect the fractional extraction of cardiogreen.[99,195]

Study protocols (Fig. 4)

Study I – role of ET_B receptors in normal and atherosclerotic subjects

This study was performed on seven patients with atherosclerosis and six age-matched controls (Table 1). After 30 min of supine rest 0.9% NaCl was infused into the brachial artery for 5 min at a rate of 2 ml/min. Basal FBF was calculated as mean blood flow during the saline infusion. Sarafotoxin 6c (3, 10 and 30 pmol/min) to stimulate only ET_B receptors, and ET -1 (3, 10 and 30 pmol/min) to stimulate ET_A and ET_B receptors were then administered; each dose was administered for 5 min at a rate of 2 ml/min (Fig. 4A). Inflow curves (4 per min) were recorded during and for 2 min after each infusion.[101] Sarafotoxin 6c and ET-1 were administered in random order with a recovery period of 60 min between the different agonists to make sure the blood flow had returned to basal values. Saline was infused as above and flow measurements were made at 10 min intervals during the recovery period. The doses of ET-1 and sarafotoxin 6c used here were calculated to result in local arterial plasma concentrations in the range of 200-2000 pmol/l based on an estimated plasma flow of 15 ml/min in the brachial artery. In this concentration range ET-1 is likely to stimulate both ET_A and ET_B receptors, given that the *K*i for ET-1 at human ET_A and ET_B receptors is 0.58 and 0.12 nmol/l, respectively.[196] The *K*i for sarafotoxin 6c at ET_B receptors is 0.29 nmol/l, whereas it is 2800 nmol/l at ET_A receptors,[196] which indicates that sarafotoxin 6c is highly selective for the ET_B receptor at the presently used doses.

Study II – effect of selective ET_A and combined ET_A and ET_B receptor inhibition in patients with atherosclerosis

This study was performed on 10 patients with atherosclerosis and 10 age-matched controls (Table 1). After 30 min of supine rest 0.9% NaCl was infused into the brachial artery for five min at a rate of 0.5 ml/min. Basal FBF was calculated as the mean blood flow during the last two min of the saline infusion.

In protocol 1 (Fig. 4B) the selective ET_B receptor antagonist BQ788 [197] (10 nmol/min) was infused into the brachial artery for 110 min at a rate of 0.5 ml/min. After 40 min the selective ET_A receptor antagonist BQ123 [198] was co-infused at the same concentration and flow rate, along with BQ788 for the remainder of the protocol. The doses of BQ788 and BQ123 were based on a previous report.[199] To further evaluate the receptor blockade, the ET-1 precursor big ET-1 (15 pmol/min) was infused at a rate of 1 ml/min during the last 30 min of the BQ788 and BQ123 co-administration. On a separate occasion (study IV), big ET-1 (15 pmol/min) was administered in the absence of ET receptor blockade on a subgroup of patients (n=4) and controls (n=7).

In protocol 2 BQ123 (10 nmol/min) was infused into the brachial artery at a rate of 0.5 ml/min for 80 min (Fig. 4C). Inflow curves were recorded for 2 min every 10 min during the infusions. At the end of the protocol, sodium nitroprusside (SNP; 3 μg/min) was infused during 2 min to test the forearm vasodilator capacity of the subjects. SNP increased FBF to a similar degree in patients and controls (241±52% and 225±49%, respectively).

The ET_B receptor blockade achieved by BQ788 was evaluated in a separate study on five healthy control subjects (Table 3) as follows: the selective ET_B receptor agonist sarafotoxin 6c (10 pmol/min) was infused before and during co-infusion of BQ788 (10 nmol/min). Sarafotoxin 6c reduced FBF by 29±8% in the absence as compared to 3±7% in the presence of BQ788 ($P<0.05$) demonstrating a high degree of receptor blockade.

Study III – role of ET-1 and ET_A receptor antagonism in endothelial function

Protocol 1 was performed on young, healthy,

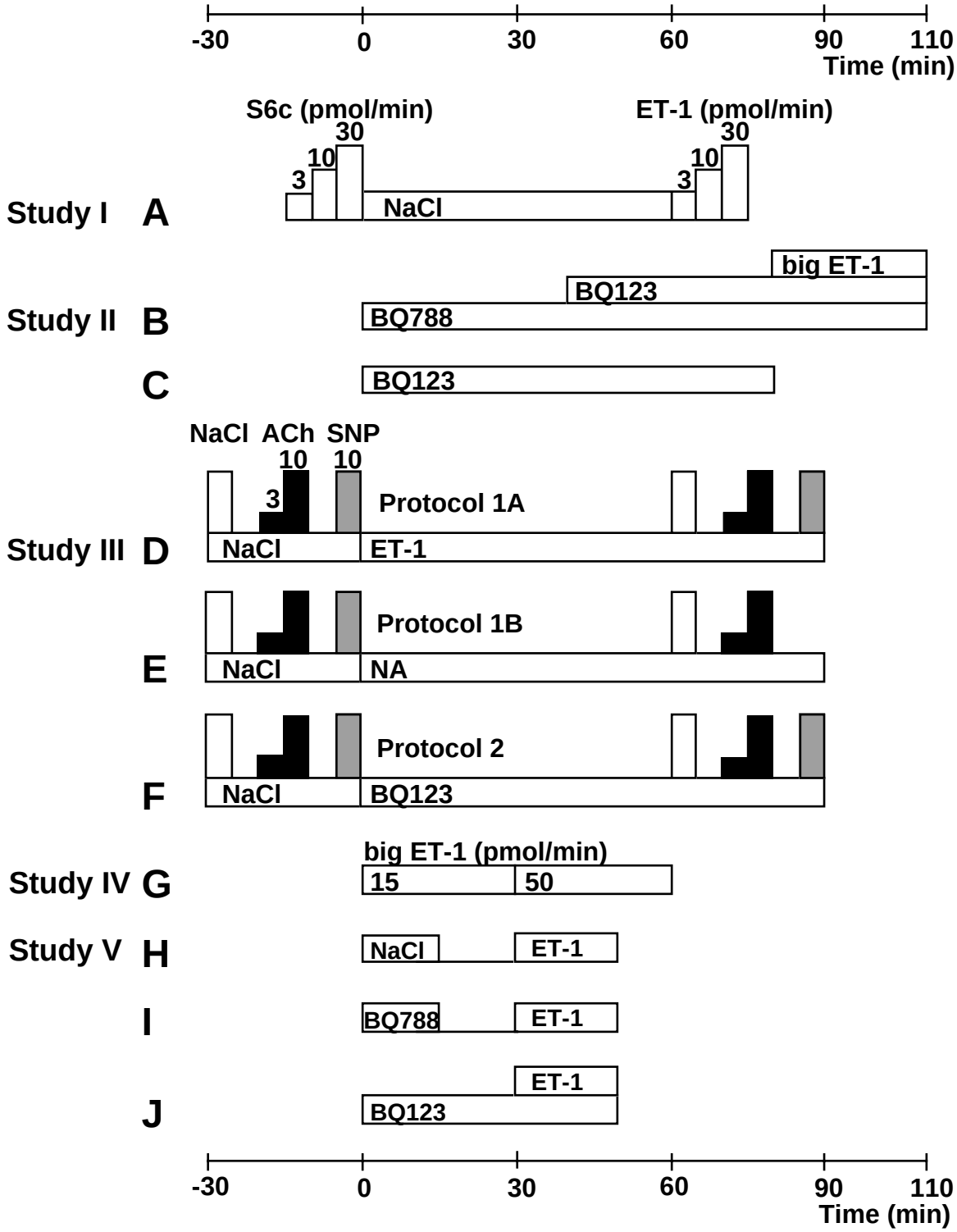

Figure 4. **Study protocols. Study I** (A): Intra-arterial infusion of sarafotoxin 6c (S6c; 3, 10 and 30 pmol/min) to patients with atherosclerosis (n=7) and age-matched controls (n=6) for 5 min/dose. After 60 min of NaCl infusion, ET-1 (3, 10 and 30 pmol/min) was given for 5 min/dose. **Study II** (B): Intra-arterial infusion of the ET_B receptor antagonist BQ788 followed by co-infusion with the ET_A receptor antagonist BQ123 (both 10 nmol/min) in patients with atherosclerosis (n=10) and healthy controls (n=10). Big ET-1 (15 pmol/min) was given together with the antagonists at the end. (C): Infusion of BQ123 (10 nmol/min) alone in the same subjects on another occasion. **Study III** (D): Intra-arterial infusion of saline, two doses of acetylcholine (ACh; 10 and 30 μg/min) and one dose of sodium nitroprusside (SNP; 10 μg/min) during saline and following a 60 min infusion of ET-1 to 10 healthy subjects. In another 6 individuals (E), saline and ACh were given during saline and following a 60 min infusion of noradrenaline (NA). (F): Intra-arterial infusion of saline, ACh and SNP during saline and following a 60 min infusion of the ET_A receptor antagonist BQ123 (10 nmol/min) to patients with atherosclerosis (n=10) and age-matched controls (n=9). **Study IV** (G): Intra-arterial infusion of big ET-1 (15 and 50 pmol/min) for 30 min/dose in patients with atherosclerosis (n=9) and age-matched controls (n=9). **Study V** (H): Intra-venous infusion of NaCl (n=6) for 15 min. (I): Intra-venous infusion of the selective ET_B receptor antagonist BQ788 (4 nmol/kg/min; n=6) for 15 min. (J): Intra-venous infusion of the selective ET_A receptor antagonist BQ123 (2.5 nmol/kg/min, n=2; or 5 nmol/kg/min, n=4) for 50 min. In all three protocols ET-1 (4 pmol/kg/min) was infused for 20 min starting at time 30 min.

male subjects who received infusions of either ET-1 (protocol 1A ; n=10) or noradrenaline (protocol 1B; n=6). In this protocol (Fig. 4D-E; Table 3) EDV was determined by infusion of acetylcholine (10 and 30 μg/min) at a rate of 2.5 ml/min into the brachial artery. Each dose was given during 2 min. ET-1 (10 pmol/min; Fig. 4D) or noradrenaline (80 ng/min; Fig. 4E) was then infused for 80 min at a rate of 0.5 ml/min. After 60 min the infusions of acetylcholine were repeated. In the subjects receiving ET-1, the NO donor SNP (10 μg/min) was also infused before and during infusion of ET-1 for the determination of EDV. Blood pressure was measured before and after the infusions.

Protocol 2 was performed on 10 male patients with atherosclerosis and on nine male, healthy, age-matched controls (Table 1). Nine of the patients and seven of the controls also took part in study II. In this protocol (Fig. 4F) acetylcholine (10 and 30 μg/min) and SNP (10 μg/min) were infused as above before and following 60 min infusion of the selective ET_A receptor antagonist BQ123 (10 nmol/min; 0.5 ml/min) into the brachial artery. Blood pressure and heart rate were recorded before and after the infusions.

Study IV - effect of big ET-1-induced vasoconstriction in patients with atherosclerosis

This study was performed on nine patients with atherosclerosis and nine age-matched controls (Table 1). After 30 min of supine rest 0.9% NaCl was infused into the brachial artery for 5 min at a rate of 1 ml/min. Basal FBF was calculated as the mean blood flow during the saline infusion. Big ET-1 was then infused into the brachial artery at two doses, 15 and 50 pmol/min based on a previous report[200] (Fig. 4G). Each dose was administered for 30 min at a rate of 1 ml/min. Inflow curves were recorded during 2 min every 10 min during the infusion of big ET-1. Intra-arterial pressure was recorded before and immediately after each infusion.

Study V – role of ET_A and ET_B receptors in renal and splanchnic circulation

This study was performed on six young, healthy, male volunteers (Table 3). Each subject participated in three different study protocols (Fig. 4H-J) performed on different occasions. In all three protocols there was an initial resting period of one hour following the catheterization, whereafter the infusions began (time 0). In protocol 1, NaCl was administered intravenously for 15 min starting at time 0 (Fig. 4H). In protocol 2, the selective ET_B receptor antagonist BQ788 (4 nmol/kg/min) was infused for 15 min (Fig. 4I). In protocol 3, the selective ET_A receptor antagonist BQ123 (2.5 nmol/kg/min, n=2; or 5 nmol/kg/min n=4) was infused intra-venously for 50 min starting at time 0 (Fig. 4J). In protocol 3 we did not observe complete blockade of renal vasoconstriction in the first two subjects receiving the low dose BQ123. Therefore, in the remainder of the study the dose of BQ123 was doubled in order to obtain a greater antagonism of the ET_A receptor. However, in these four subjects the vasoconstrictor response was similar in magnitude to that observed in the first two subjects. Therefore, the data from all six subjects are pooled. ET-1 (4 pmol/kg/min) was infused intra-venously in all three protocols for 20 min starting 30 min after the start of infusions of NaCl, BQ788 or BQ123. The doses and the duration of the infusions of the ET receptor antagonists were selected on the basis of previous reports.[201,202] Heart rate and MAP were recorded continuously. Pulmonary oxygen uptake was determined in the basal state and at 20 min of the ET-1 infusion in each protocol.

Biochemical analyses

ET-like immunoreactivity (I-V)

In all studies deep venous blood and arterial blood samples were put into pre-chilled test tubes containing EDTA (10 mmol/l final concentration) on ice. After centrifugation (4°C, 2000 g for 15 min), plasma aliqouts of 1 ml were pipetted off and stored at -80°C until analyzed. After ethanol extraction ET-like immunoreactivity was analyzed by radioimmunoassay using the commercially available antiserum rabbit anti-ET-1 6901 (Peninsula, Merseyside, UK; study I-III and V) or the antiserum E1 (study IV) as described previously.[203] The cross-reactivity of the anti-

serum 6901 is 7% with ET-2, 7% with ET-3 and 17% with big ET-1 when cross-reactivity with ET-1 is set at 100%. The intra- and inter-assay variations are 3 and 6%, respectively. The cross-reactivity of antiserum E1 with human big ET-1 is 0.03%. To determine the cross-reactivity of E1 with the fragment ET-$1_{(1-31)}$, the fragment was tested in the radioimmunoassay in concentrations ranging from 1 pmol/l to 1 mmol/l.

Lipoprotein lipids (I-IV)

Concentrations of cholesterol and triglycerides in LDL and HDL fractions were determined in fresh plasma by a combination of preparative ultracentrifugation and precipitation of apo B-containing lipoproteins followed by lipid analyses.[204] Cholesterol and triglyceride concentrations were determined by chemical methods after extraction.[205]

Ultrasound examinations (II-IV)

In study II-IV, the lumen diameter, and the posterior and anterior wall thickness of the brachial artery were measured in the arm in which the blood flow studies were performed by means of a non-invasive high-resolution ultrasound technique prior to the plethysmography. The first 10 cm of the brachial artery above the elbow was scanned longitudinally for the detection of atherosclerotic plaques. Flow velocity curves were measured by pulsed Doppler recordings at an angle correction of 55 degrees. These ultrasound scans and Doppler measurements were performed with an Acuson 128 XP/10 apparatus with a 7 MHz ART linear array transducer.

As a basal characterization in study II, flow-mediated vasodilatation in the brachial artery was determined in a subgroup of the patients (n=8) and the controls (n=8) on a separate occasion. Flow-mediated dilatation of the brachial artery was measured during reactive hyperemia according to a previously described method[204] using an Acuson 128 XP/10 apparatus with a 6 MHz ART linear array transducer. Endothelium-independent dilatation of the brachial artery was determined following sublingual administration of nitroglycerin (0.4 mg).

Immunohistochemistry (IV)

Samples of the radial artery were obtained from a separate group of four patients undergoing coronary artery by-pass surgery. The samples were fixed in 3% buffered formaldehyde overnight, stored in phosphate buffer solution (PBS)/ 0.02% sodium azide, and embedded in paraffin. Sections of 5 μm thickness were cut on a standard microtome, routinely stained with hematoxylin-eosin, and studied in a Nikon Labophot microscope (Nikon, Tokyo, Japan). Immunostaining was performed in normal and atheromatous radial artery samples. Sections were rehydrated in xylene and graded ethanol (100-70%) and were rinsed in water and 0.3% hydrogen peroxide in water for 5 min. The sections were blocked for 60 min in 1% BSA in PBS and then incubated with a monoclonal antibody against the extracellular epitope of ECE-1[207,208] (B61/104; BASF Ag, Ludwigshafen, Germany) diluted at 1:200 in 0.1% BSA in PBS overnight at 4°C. The sections were thereafter incubated with secondary antibody for 60 min (1:400 in PBS, 0.1% BSA), followed by ABC Elite avidin-biotin amplification (Vector Laboratories, Burlingame, CA) for 30 min and incubation with 3',3'-diaminobenzidine, 0.02% hydrogen peroxide for 8 min producing a brown reaction product. Sections were counterstained in hematoxylin-eosin for 3 min and mounted. The antibody detects all forms of human ECE-1 (ECE-1 a, b and c).

Drugs (I-V)

ET-1 (Alexis Corporation, Läufelfingen, Switzerland or Peninsula Laboratories, Merseyside, UK), sarafotoxin 6c, N^G-monomethyl-L-arginine (L-NMMA; Alexis Corporation) and big ET-1 (Peninsula Laboratories) were dissolved in sterile water containing 0.5% albumin and thereafter injected through a Millipore sterile filter. The substances were then stored at -80°C until use. In study IV, ET-$1_{(1-21)}$ and ET-$1_{(1-31)}$ were purchased from Peptide Institute (Osaka, Japan) to be used in the radioimmunoassay. [^{125}I]-ET-1 was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). BQ123, BQ788 (Clinalfa AG, Läufelfingen, Switzerland) and acetylcholine (CIBA Vision, Roskilde, Denmark) were dissolved in sterile 0.9 % NaCl and stored

frozen at -80°C. SNP was purchased from Abbot, Chicago, USA. On the day of the experients all substances were diluted to the proper concentrations in sterile 0.9% NaCl.

Calculations (I-V)

FBF was calculated each minute as the mean of four to eight inflow recordings. In studies I and IV FBF during drug administration is expressed as per cent change in FBF from basal blood flow during saline infusion. In study II-III the ratio between flows in the infused and noninfused arms at each time point is expressed as percentage change from baseline (Fig. 3C). Forearm vascular resistance was calculated as MAP divided by FBF. The ultrasound examinations of brachial artery wall thickness were performed by one of the investigators blinded to which group the subjects belonged to. The local net formation of ET-1 in the forearm vascular bed (study IV) was calculated according to the following formula: veno-arterial difference in plasma concentration x forearm blood flow x (1-hematocrit). In study V renal vascular resistance (RVR) was calculated as MAP/RBF and splanchnic vascular resistance (SplVR) as MAP/SBF. All data are given as mean values and standard error of the mean (SEM). Statistical differences were calculated using Student's unpaired t-test for between groups comparisons (I, II and IV) or the nonparametric Wilcoxon test for between groups comparisons or ANOVA and Kruskal Wallis for repeated measures (III) or ANOVA followed by Fischer's protected least-significant difference test for repeated measures (V). A $P<0.05$ was regarded as significant.

Results and Comments

Study subject characteristics (I-V)

The basal characteristics of the patients and control subjects are summarized in Table 1-3. In study I total and LDL cholesterol were higher among the patients. In study II HDL cholesterol was lower in the patients than in the age-matched controls. Apart from these, there were no significant differences in age, body mass index, MAP, total and LDL cholesterol or triglycerides between patients and controls within each study (I-IV; Table 1). The young control subjects had normal body mass index and MAP and were non-smokers (Table 3).

Blood flow changes during stimulation and blockade of ET receptors

Effects of selective ET_B receptor agonism (I)

Infusion of sarafotoxin 6c resulted in an initial but transient increase in FBF in patients with atherosclerosis as well as in controls. This increase was significant at all doses of sarafotoxin 6c. The largest increase in the patient group was evoked by the intermediate dose (10 pmol/min; 39±11%). The vasodilator response was somewhat but not significantly larger than that obtained in healthy subjects (22±10%). Following the initial vasodilator response, sarafotoxin 6c caused a dose-dependent and progressive reduction in FBF (Fig. 5A). The reduction evoked by the two lower doses of sarafotoxin 6c was significantly larger in patients than in controls, whereas the highest dose evoked similar reductions in FBF in the two groups. The per cent reduction in FBF induced by the two lower doses of sarafotoxin 6c (3 and 10 pmol/min) correlated significantly with LDL cholesterol levels (r=0.47; $P<0.05$). There was no significant correlation between the reduction in FBF and total cholesterol, mean arterial blood pressure or basal FBF. These findings suggest an upregulation of vascular smooth muscle ET_B receptors in atherosclerosis.

Effects of non-selective ET receptor agonism (I)

Infusion of ET-1 resulted in a slight initial increase in FBF in some subjects although this was not a consistent finding. It was followed by a dose-dependent reduction in FBF in both groups without any significant difference between patients and controls (Fig. 5B). On a molar basis ET-1 evoked significantly greater vasoconstriction than sarafotoxin 6c in the control subjects. Thus, FBF was reduced by 44±5% in response to 10 pmol/min ET-1 compared to 27±6% in response to 10 pmol/min sarafotoxin 6c ($P<0.01$). In the patients, ET-1 and sarafotoxin 6c (10 pmol/min) reduced FBF by similar magnitudes (42±9% vs. 42±2%; Fig. 5B). Administration of sarafotoxin 6c or ET-1 did not affect MAP. Accordingly, forearm vascular resistance changed in parallel with the changes in FBF. These findings suggest that the sensitivity to the non-selective ET_A/ET_B agonist ET-1 does not differ between patients and controls.

Effects of ET_B receptor blockade alone and in combination with ET_A receptor blockade (II)

There were no significant differences in baseline hemodynamics between patients and controls. Furthermore, there were no significant changes in blood flow in the noninfused forearm or in systemic arterial blood pressure during the course of the two protocols. Infusion of the selective ET_B receptor antagonist BQ788 evoked a slight but significant decrease in FBF in the controls (Fig. 5C). The maximum reduction was 20±9% at 30 min. In contrast, BQ788 increased FBF by 31±13% at 10 min in the patients. FBF was significantly higher in the patients than in controls during the entire infusion of BQ788 alone (Fig. 5C), suggesting that vasoconstrictor ET_B

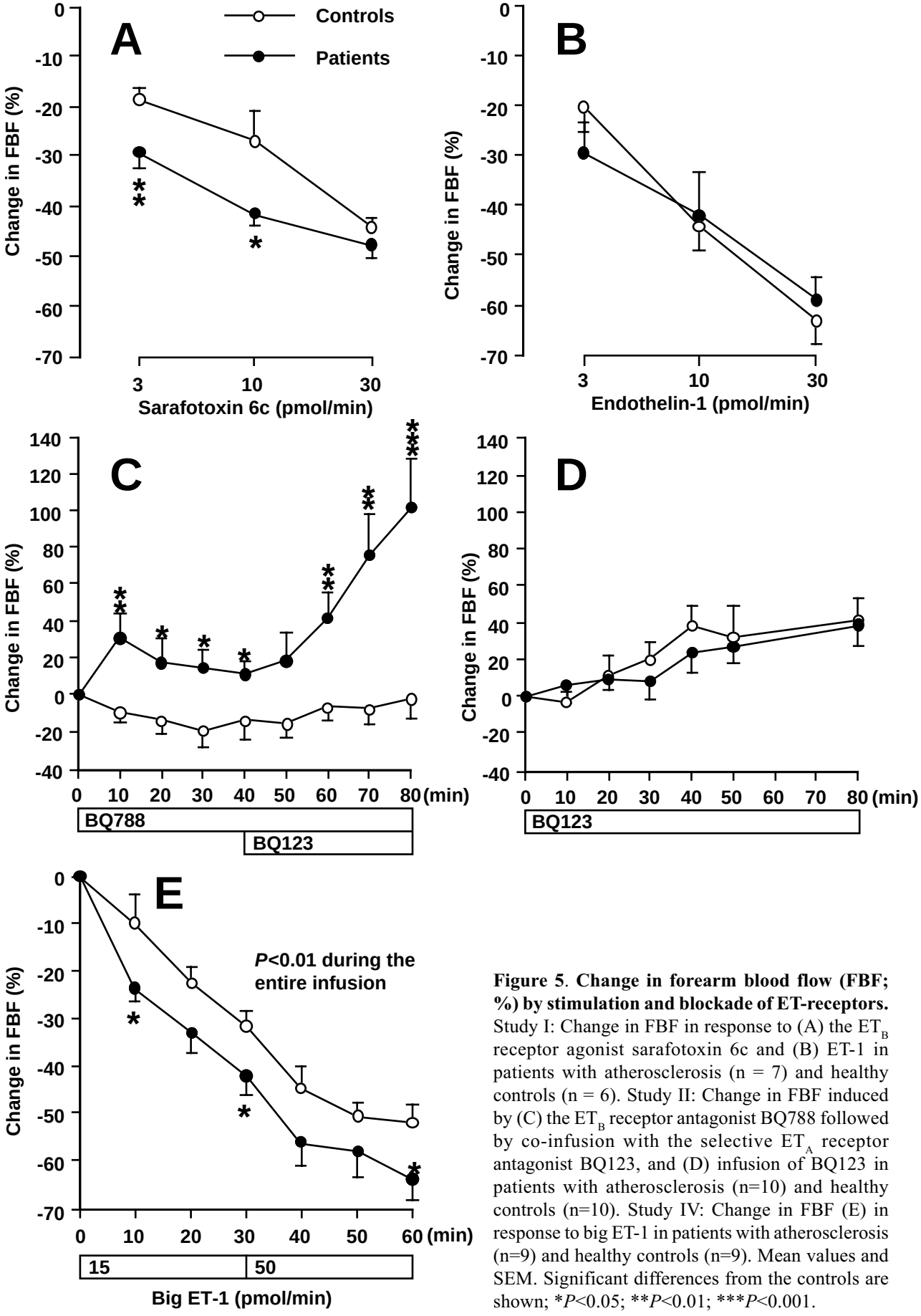

Figure 5. **Change in forearm blood flow (FBF; %) by stimulation and blockade of ET-receptors.** Study I: Change in FBF in response to (A) the ET_B receptor agonist sarafotoxin 6c and (B) ET-1 in patients with atherosclerosis (n = 7) and healthy controls (n = 6). Study II: Change in FBF induced by (C) the ET_B receptor antagonist BQ788 followed by co-infusion with the selective ET_A receptor antagonist BQ123, and (D) infusion of BQ123 in patients with atherosclerosis (n=10) and healthy controls (n=10). Study IV: Change in FBF (E) in response to big ET-1 in patients with atherosclerosis (n=9) and healthy controls (n=9). Mean values and SEM. Significant differences from the controls are shown; **P*<0.05; ***P*<0.01; ****P*<0.001.

receptors are upregulated in the patients.

Addition of BQ123 to the infusion of BQ788 evoked no significant change in FBF in the controls (Fig. 5C). In contrast, addition of BQ123 evoked pronounced vasodilatation in the patients. The maximal increase in FBF in response to combined infusion of BQ123 and BQ788 in the patients was 102±25% at 80 min. The corresponding change in FBF in the controls was –3±9% (P<0.001 vs. patients). These findings suggest an enhanced ET-1-mediated vascular tone in the patients and that combined ET_A and ET_B receptor blockade evokes enhanced vasodilation in patients with atherosclerosis compared to healthy controls.

Administration of big ET-1 for 30 min in the presence of combined ET_A and ET_B receptor antagonism did not significantly affect forearm blood flow in the controls (+14±13%) or in the patients (-14±11%). In the absence of ET receptor blockade (study IV), big ET-1 reduced FBF by 47±5% (patients; n=4) and 32±4% (controls; n=7) in the same patients and controls that were included in study II. These observations suggest a high degree of ET receptor blockade.

Effects of selective ET_A receptor blockade (II)

Selective ET_A receptor antagonism with BQ123 caused a slowly developing increase in FBF in both patients and controls (P<0.05; Fig. 5D). There were no significant differences between the groups during the course of the infusion. The increase in FBF in the patients during selective ET_A antagonism (39±11%) was significantly smaller than that seen during combined ET_A and ET_B receptor antagonism in protocol 1 (102±25%; P<0.05). In the controls, on the other hand, BQ123 alone evoked significantly greater vasodilatation than the combination of BQ123 and BQ788 (41±11% vs. –3±9% at 80 min, P<0.01). These findings suggest that combined ET_A and ET_B receptor blockade evokes a significantly more pronounced vasodilator response in patients with atherosclerosis as compared to selective ET_A receptor blockade and compared to healthy controls.

Effects of big ET-1 (IV)

Local intra-arterial infusion of big ET-1 caused a dose-dependent and progressive reduction in FBF in both patients and controls. The reduction in FBF during the entire infusion was significantly larger in the patients than in the controls (P<0.01; Fig. 5E). A significant difference between the two groups was apparent already after 10 min infusion of the low dose of big ET-1 (P<0.05). The high dose (50 pmol/min) reduced FBF by a maximum of 64±5% in the patients compared to 52±4% in controls and a plateau appeared to be reached at 50 min in the control group (Fig. 5E). In addition, big ET-1 increased forearm vascular resistance significantly more in the patients than in the controls. There was no significant difference in MAP between patients and controls before or during the infusions of big ET-1. No significant correlation was found between the per cent reduction in FBF and basal MAP, basal forearm vascular resistance, total cholesterol levels or LDL cholesterol levels. Since the vasoconstrictor response to ET-1 was similar in atherosclerotic patients and controls (study I), the present results indicate enhanced conversion from big ET-1 to ET-1 in atherosclerotic patients.

Effects of ET-1, ET_A and ET_B receptor blockade on splanchnic and renal circulation (V)

There were no significant differences in basal hemodynamic parameters between the three study protocols (Table 4). Administration of ET-1 increased MAP in the control and BQ788 protocols, but not in the BQ123 protocol. There were significant differences in SBF, SplVR, RBF and RVR between the three protocols as measured by ANOVA (P<0.0001; Fig. 6). In the control protocol, administration of ET-1 reduced SBF by 31±5% (P<0.01) and increased SplVR by 64±18% (P<0.0001). SBF was significantly reduced by BQ788 alone (24±5%; P<0.01). Moreover, the increase in SplVR in response to ET-1 was enhanced following administration of BQ788 (140±37 vs. 64±18% in the control protocol; P<0.05; Fig. 6A). Although BQ123 did not affect SBF or SplVR *per se*, it completely blocked the effect of ET-1. In the control protocol ET-1 induced a significant reduction in RBF and increase in RVR (Fig. 6B). RBF was significantly reduced and RVR was increased by BQ788 alone (P<0.01). The increase in RVR induced by ET-1 was significantly greater in the BQ788 protocol

Table 4. Hemodynamic changes during the infusions

Time (min)	Basal	30	**50**
Intervention	NaCl	NaCl/ antagonist	**ET-1**
HR (beats/min)			
Control (n=6)	57±4	61±4	**56±3**
BQ123 (n=6)	56±2	63±3	**60±3***
BQ788 (n=6)	57±2	59±1	**56±4**
MAP (mmHg)			
Control (n=6)	91±4	93±6	**97±6††**
BQ123 (n=6)	88±4	88±5	**89±5****
BQ788 (n=6)	91±5	94±4	**100±5†††**
CO (l/min)			
Control (n=4)	7.0±0.6	7.5±1.0	**6.2±0.5**
BQ123 (n=5)	7.7±0.4	8.2±0.4	**9.1±0.6**†**
BQ788 (n=4)	7.4±0.8	7.7±1.5	**6.9±1.1**
SVR (mmHg/min/l)			
Control (n=4)	13.2±0.8	13.0±1.6	**16.3±1.6†**
BQ123 (n=5)	11.7±0.9	11.0±0.5*	**10.1±1.1**†**
BQ788 (n=4)	12.8±1.5	13.0±2.6	**16.0±2.5†**

Heart rate (HR), mean arterial blood pressure (MAP), cardiac output (CO) and systemic vascular resistance (SVR) before, during and up to 60 min after a 20 min infusion of ET-1 (4 pmol/kg/min). **Bold figures represent values during the ET-1 infusion**. *P<0.05; **P<0.01 represent significant differences compared to the control protocol at each time point and †P<0.05; ††P<0.01; †††P<0.001 represent differences compared with the basal value within each protocol. Mean values and SEM.

than in the control protocol (67±14 vs. 36±6%; P<0.01). ET-1 evoked a slight increase in RVR also in the presence of BQ123 (12±3%; P<0.05), but this increase was significantly smaller than that obtained in the control protocol (P<0.01, Fig. 6B). The differences in RVR between the three protocols were maintained even 60 min after the ET-1 infusion. ET-1 induced a significant increase in systemic vascular resistance (SVR) in the control protocol and a reduction in cardiac output in all subjects, although the latter effect was not statistically significant. In contrast ET-1 induced an increase in cardiac output and a reduction in SVR in the BQ123 protocol (P<0.05). These responses to ET-1 were also significantly different from those seen in the control protocol (P<0.01). ET-1 induced an increase in SVR in the BQ788 protocol (P<0.05). The changes in cardiac output and SVR in the BQ788 protocol did not differ from the changes in the control protocol. These findings suggest that ET_A receptors mediate the splanchnic and renal vasoconstriction induced by ET-1 in healthy humans.

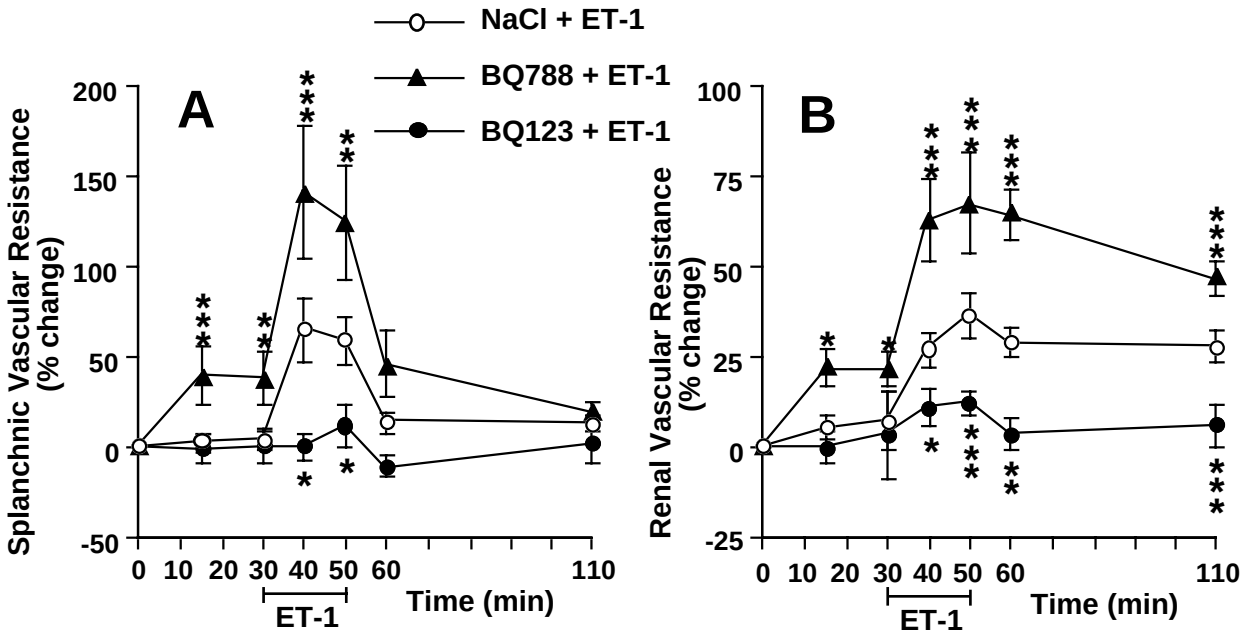

Figure 6. Effect of ET receptor blockade on splanchnic and renal vascular resistance. Per cent change in (A) splanchnic vascular resistance and (B) renal vascular resistance induced by ET-1 in the presence of NaCl, in the presence of ET_B receptor blockade (BQ788) and in the presence of ET_A receptor blockade (BQ123). Mean values and SEM, n=6 in all protocols. Significant differences from the NaCl protocol are shown; *P<0.05; **P<0.01; ***P<0.001.

Effects of stimulation and blockade of ET receptors on endothelial function

Characterization of EDV (III)

The degree of NO-dependent vasodilatation evoked by acetylcholine was evaluated prior to the two study protocols on seven healthy, non-smoking control subjects (Table 3) as follows: acetylcholine (10 and 30 μg/min) was infused into the brachial artery at a rate of 2.5 ml/min (2 min per dose) together with a continuous infusion of saline (0.5 ml/min). The NOS inhibitor L-NMMA (1 mg/min) was then infused for 45 min. L-NMMA decreased FBF by 19±5% (P<0.01). Following 15 min infusion of L-NMMA, SNP (0.1-0.3 μg/min) was co-infused during five min to restore basal FBF before the infusions of acetylcholine were repeated. The vasodilator response to both doses of acetylcholine was significantly blunted during as compared to before co-infusion of L-NMMA and SNP (367±115 vs. 493±132% and 223±98 vs. 501±126% increase in FBF by acetylcholine at 10 and 30 μg/min, respectively; P<0.05). These findings support the concept that the vasodilator response induced by acetylcholine to a large part is mediated by NO.

Effects of ET-1 and noradrenaline on EDV (III)

Neither the ET-1 nor the noradrenaline infusion evoked any changes in heart rate, blood pressure or FBF in the non-infused arm.

Both doses of acetylcholine caused marked increases in FBF during saline before the ET-1 infusion (P<0.001; Fig. 7A-B). The vasodilator effect of both doses of acetylcholine was significantly blunted after 60 min infusion of ET-1 (Fig. 7A-B). The per cent increase in FBF evoked by SNP was enhanced during infusion of ET-1 (P<0.05; Fig. 9A). However, the absolute increase in FBF in response to SNP was reduced during infusion of ET-1 (100±10 vs. 174±18 ml/min x 1000 ml; P<0.01). These findings strongly suggest that elevated levels of ET-1 impair endothelial function in the human forearm.

The absolute increase in FBF evoked by acetylcholine was not affected by noradrenaline (Fig. 7D), but due to the reduction in baseline FBF, the per cent increase in FBF evoked by acetylcholine was significantly greater during infusion of noradrenaline compared to during saline infusion (Fig. 7C). This observation demonstrates that the decreased vasodilator response to acetylcholine during ET-1 administration is not due to reduced FBF *per se*.

Effect of ET_A receptor blockade on EDV (III)

There were no significant changes in heart rate, blood pressure or FBF in the non-infused arm in either the patients or the controls. Both doses of acetylcholine increased FBF in patients and controls (Fig. 8). The vasodilator response to acetylcholine during saline infusion was slightly but non-significantly greater in the age-matched controls than in the patients, but significantly smaller than that obtained in the younger controls included in protocol 1, study III (P<0.01). Infusion of the selective ET_A receptor antagonist BQ123 increased FBF to a similar extent in both patients and controls (39±11% and 41±11%, respectively; P<0.05 for both). In the patients, the vasodilator responses to the two doses of acetylcholine were significantly enhanced during infusion of BQ123 (Fig. 8). In the controls, on the other hand, there were no significant differences in vasodilatation induced by acetylcholine before and during infusion of BQ123 (Fig. 8). The vasodilator response to SNP was not affected by administration of BQ123 in the patients or controls (Fig. 9B). These findings demonstrate that ET_A receptor blockade improves EDV in patients with atherosclerosis, indicating that endogenous ET-1 attenuates EDV via an ET_A receptor-mediated mechanism.

Effect of stimulation and blockade of ET-receptors on ET-levels

Effect of ET-1 on ET-levels (I)

Attempts were made to collect deep venous plasma samples for the determination of ET-1 during the last min of each ET-1 infusion. Due to the reductions in FBF this was only possible in some of the subjects (n=2-4). In these subjects the infusions elevated ET-1 to similar levels in patients and healthy controls.

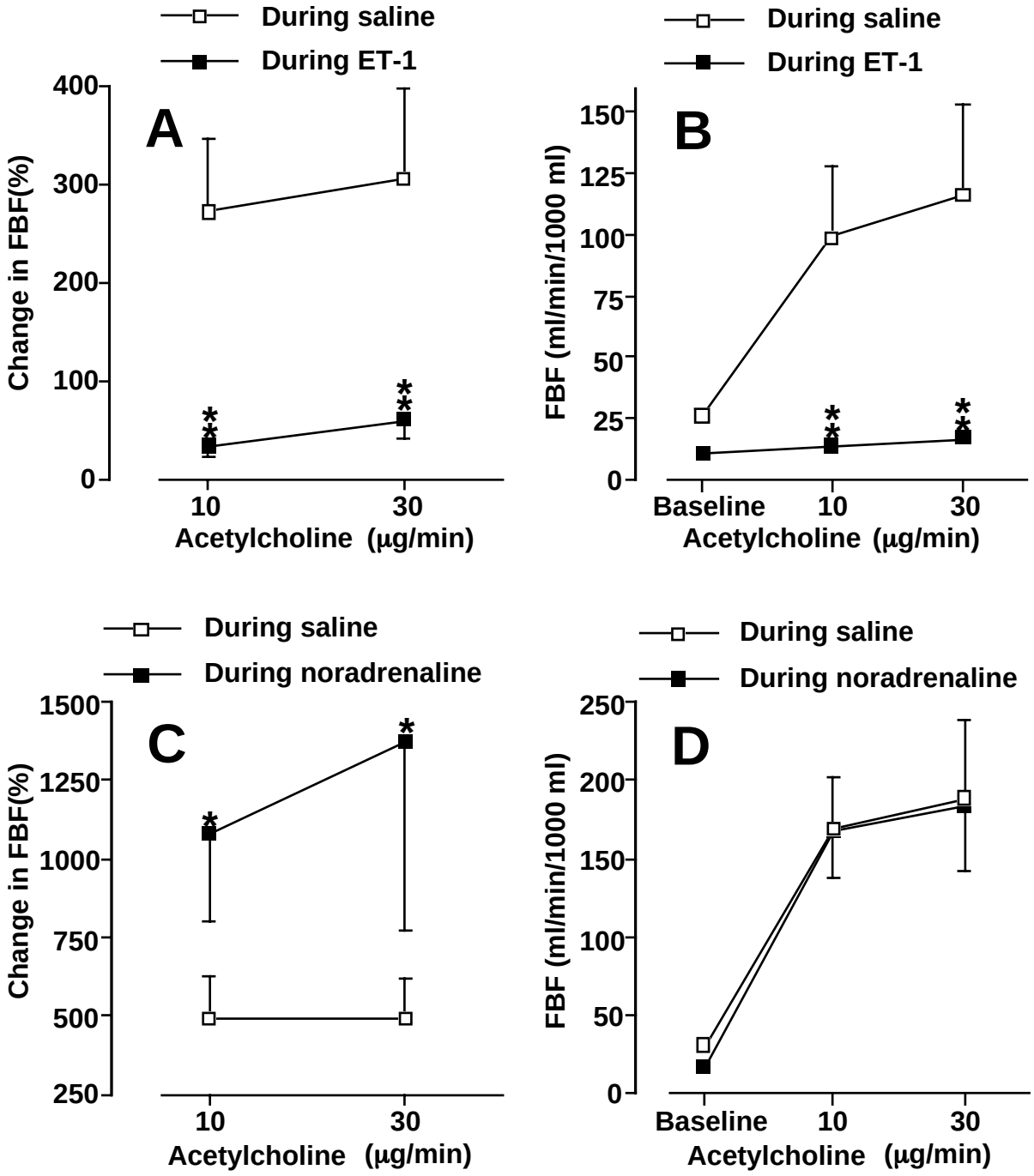

Figure 7. **Effect of ET-1 on endothelium-dependent vasodilatation.** Effect of acetylcholine on (A, C) per cent change in forearm blood flow (FBF) and (B, D) absolute FBF during saline infusion and following a 60 min infusion of ET-1 (A,B; n=10) or noradrenaline (C,D; n=6) in healthy controls. Mean values and SEM. Significant differences from the response during saline are shown; * $P<0.05$; ** $P<0.01$.

Effect of ET receptor blockade on ET-levels in patients with atherosclerosis and age-matched controls (II)

Deep venous plasma levels of ET were significantly elevated at 40 min infusion of BQ788 and remained increased after another 40 min co-infusion of BQ123 in study II (Fig. 10A). Venous ET levels did not change during infusion of BQ123 alone (Fig. 10B). Arterial ET levels were not changed by either antagonist. These findings suggest that ET_B, but not ET_A, receptor blockade increases plasma ET-levels.

Effect of big ET-1 on ET-levels (IV)

In order to determine local conversion of big ET-1 to ET-1, arterial and venous plasma samples were

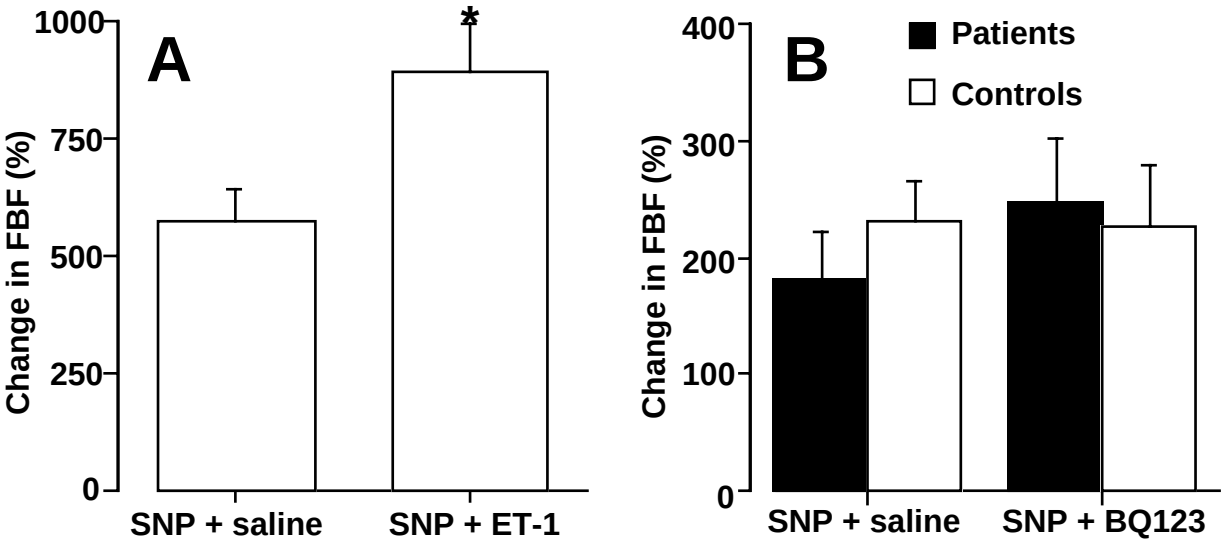

Figure 8. ET-1 and endothelium-independent vasodilatation. Effect of sodium nitroprusside (SNP;10 μg/min) during (A) saline and following a 60 min ET-1 infusion in healthy controls (n=9) and (B) during saline infusion and following a 60 min infusion of the ET_A receptor antagonist BQ123 in patients with atherosclerosis (n=9) and age-matched controls (n=10). Mean values and SEM. Significant differences from the response during saline are shown; * P<0.05.

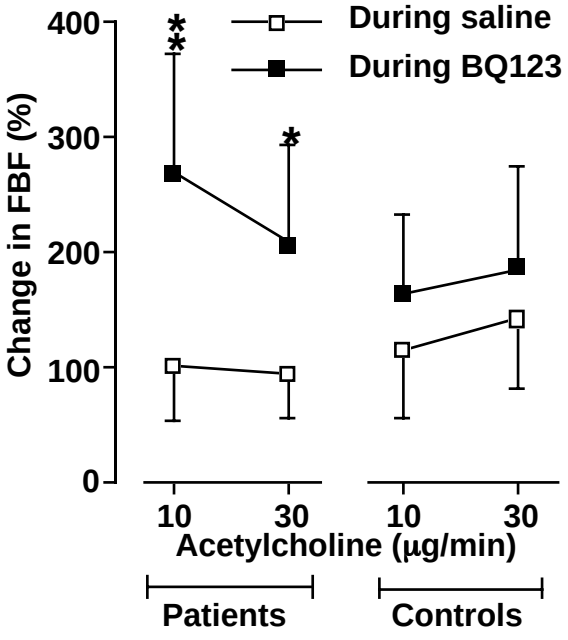

Figure 9. ET_A receptor blockade and endothelium-dependent vasodilatation. Effect of acetylcholine on per cent change in forearm blood flow (FBF) during saline infusion and following a 60 min infusion of the ET_A receptor antagonist BQ123 in patients with atherosclerosis (n=9) and age-matched controls (n=10). Mean values and SEM. Significant differences from the response during saline are shown; * P<0.05; ** P<0.01.

collected before and during the intra-arterial infusions of big ET-1. The cross-reactivity of the $ET\text{-}1_{(1\text{-}21)}$ antiserum with $ET\text{-}1_{(1\text{-}31)}$ was found to be 0.04%. Basal arterial and venous plasma ET-1 concentrations were similar in patients and controls. In the patients, infusion of the low and high doses of big ET-1 increased venous plasma levels of ET-1 three-fold and four-fold, respectively (P<0.05; Fig. 10C). By contrast, infusion of the low dose of big ET-1 did not increase venous plasma ET-1 levels in the control group (Fig. 10C). The high dose of big ET-1 elevated venous plasma ET-1 also in controls. Administration of the high dose, but not the low dose, of big ET-1 resulted in a significant elevation of arterial ET-1 levels in the patients. Infusion of big ET-1 did not significantly alter the arterial levels of ET-1 in the controls. The net formation of ET-1, calculated as the veno-arterial plasma concentration difference multiplied by the forearm plasma flow, increased markedly in the patients but not in the controls during infusion of the low dose of big ET-1 (Fig. 10D). During the infusion of the high dose there was no significant difference in net formation of ET-1

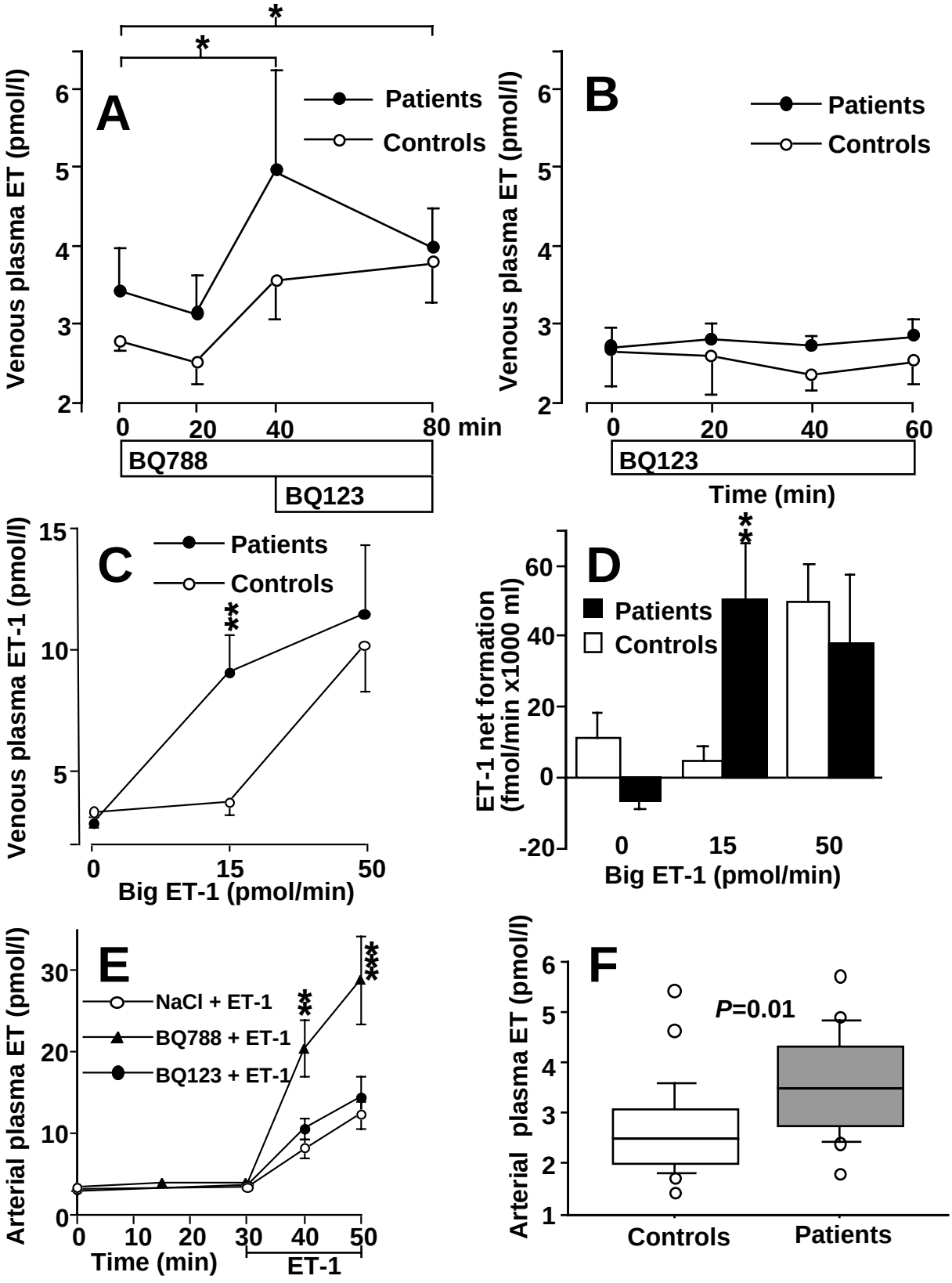

Figure 10. Plasma ET-like immunoreactivity. Study II: Deep venous plasma levels of ET before and during infusion of (A) BQ788 alone and in combination with BQ123 (controls n=6, patients n=9) and (B) BQ123 alone (controls n=8, patients n=10). Study IV: Plasma ET-1 in (C) a deep forearm vein in the basal state and at the end of each infusion period of big ET-1. (D) Net formation of ET-1 during infusion of big ET-1 in healthy controls and patients with atherosclerosis (n=6-8). Study V: (E) Systemic artery plasma ET-1 before and during intra-venous infusion of ET-1 in the presence of NaCl, in the presence of ET_B receptor blockade (BQ788) and in the presence of selective ET_A receptor blockade (BQ123; n=6). Mean values and SEM. Study I-IV: (F) Basal arterial ET in patients with atherosclerosis (n=17) and age-matched controls (n=25). The boxplot indicates the median value, the 25th to 75th percentile, 95 % confidence interval and outliers. *P<0.05; **P<0.01; ***P<0.001 compared to controls.

between the two groups. These findings suggest that the increased formation of ET-1 in response to big ET-1 administration in patients with atherosclerosis as compared to healthy controls may be due to increased activity of ECE in the patients, which may explain the enhanced vasoconstrictor response to big ET-1 in the atherosclerotic patients.

Effect of ET-1 and ET receptor blockade on pulmonary and systemic ET-levels in healthy controls (V)

During intra-venous infusion of ET-1 the increase in systemic arterial ET-1 levels was smaller than the increase in pulmonary arterial ET-1 levels. The systemic (Fig. 10E) and pulmonary arterial ET-1 levels rose significantly more in the presence of the ET_B receptor antagonist BQ788 than in the control protocol. This finding suggests that the ET_B receptor functions as a clearance receptor and may modulate vascular tone by altering the plasma concentration of ET-1. The ET_A receptor antagonist BQ123 did not affect the increase in ET-1 levels. The plasma half life of ET-1 was similar in all three protocols.

Basal difference in ET levels between patients and controls (I-IV)

When we pooled all subjects from study I-IV we found that the patients with atherosclerosis had significantly higher basal venous and arterial ET levels than the age-matched controls (3.5±0.2 vs. 2.7±0.2 pmol/l in arterial plasma; P=0.01; Fig. 10F).

Immunohistochemical analysis of ECE-1 (IV)

Immunohistochemistry revealed a prominent staining for ECE-1 in the endothelium as well as in intimal cells and smooth muscle cells in the media of normal radial arteries. ECE-1 immunoreactivity was also detected in cells in the fibrous cap of the atherosclerotic lesions of radial arteries.

Ultrasound examination (II-IV)

Brachial artery wall thickness

Ultrasound examination of the brachial artery revealed that the anterior wall thickness and the mean of anterior and posterior wall thickness in relation to the inner diameter was significantly larger in the patients than in the controls pooled in study II-IV. This finding may indicate that the patients had structural changes in the forearm vascular bed. There was no evidence of significant stenotic lesions or flow obstructions in any subject.

Flow-mediated dilatation of the brachial artery (II)

Flow-mediated dilatation tended to be smaller in the patients than in the controls, but this difference was not statistically significant. There was no significant difference between patients and controls in endothelium-independent vasodilatation of the brachial artery induced by sublingual nitroglycerin.

General Discussion

The role of ET-1 in atherosclerosis

Atherosclerosis is a major cause of morbidity and mortality. Coronary atherosclerosis and its complications, resulting in acute coronary syndromes, are the most common cause of death in the western world. Previous studies strongly support the role of ET-1 in the pathogenesis of atherosclerosis and this has implications for the therapeutic potential of ET receptor antagonists.[104] Several studies performed on experimental animals suggest that ET-1 contributes to the development and progression of atherosclerosis. In the hamster model of atherosclerosis, ET_A receptor blockade inhibited the formation of the fatty streak by reducing the number and the size of the macrophage-foam cells.[152] Furthermore, ET_A receptor blockade inhibits progression of atherosclerosis in the aorta of apolipoprotein E deficient mice.[192] In healthy humans the plasma levels of ET-1 are low.[209] However, the plasma levels of ET-1 are elevated in subjects with risk factors for atherosclerosis such as hypercholesterolemia[143] and type II diabetes mellitus[145] as well as in those with established atherosclerosis (Fig. 11).[142,164,210] There are also data demonstrating enhanced tissue expression of ET-1 and its receptors, in particular the ET_B receptor, in atherosclerotic arteries. A recent study demonstrated that the acute phase reactant CRP, which is an important prognostic marker in patients with acute coronary syndromes, induces inflammatory effects via release of ET-1.[162] The functional consequences of the altered ET system in atherosclerosis had not previously been investigated in patients with atherosclerosis. In study I we found that the vasoconstrictor response to ET-1 was not changed compared to age-matched controls, in agreement with findings in patients with cardiovascular risk factors such as diabetes mellitus.[211] In study II however, we found that co-administration the ET_A receptor antagonist BQ123 and the ET_B receptor antagonist BQ788 induced a marked (two-fold) increase in FBF in the patients, whereas the same administration procedure caused no significant increase in FBF in the controls. In the controls, on the other hand, the vasodilator response to combined ET_A and ET_B receptor blockade was blunted in comparison with selective ET_A receptor blockade, which is in agreement with previous observations.[199] These findings clearly demonstrate that the vasoconstrictor tone mediated by endogenous ET-1 is more pronounced in patients with atherosclerosis than in healthy controls (Fig. 11), extending the previous *in vitro* findings to functional consequences in patients with atherosclerosis.

Importance of the ET_B receptor in atherosclerosis

The effects of ET-1 are mediated via both ET_A and ET_B receptors and many potential therapeutic benefits of ET receptor antagonists, particularly in cardiovascular disease, have been investigated.[212] There has been much discussion about which receptors should be blocked to obtain the best perfusion and endothelial function of atherosclerotic vascular beds.[154] The ET_A receptor located on the smooth muscle cell mediates potent vasoconstriction,[15] promotes hypertrophy,[213] proliferation[105] and stimulates neutrophil adhesion to the vessel wall.[115] This receptor subtype contributes to detrimental effects in the development of atherosclerosis and should consequently be blocked in order to obtain therapeutic effects. The debate has concerned whether a selective ET_A receptor antagonist or a dual ET_A/ET_B receptor blocker should be used.[121] As mentioned in the introduction, the ET_A receptor seems to be the major subtype mediating vasoconstriction in healthy humans,[102,214] although the situation appears to be different in patients with atherosclerosis. Binding studies *in vitro* suggest that ET_B receptors are upregulated in the tunica media of athero-

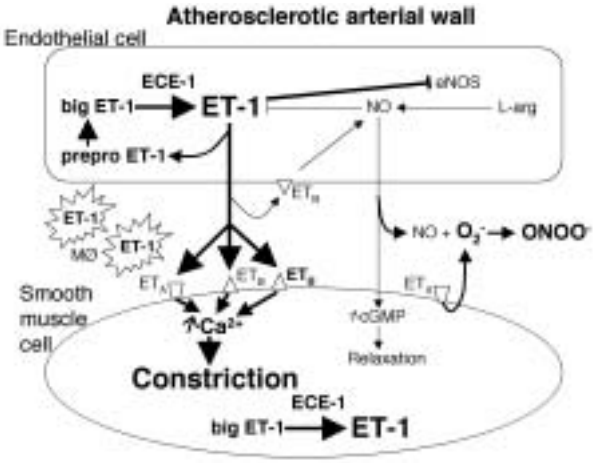

Figure 11. Schematic illustration of the biosynthesis of endothelin-1 (ET-1) and nitric oxide (NO) and their effects on the endothelial cells and vascular smooth muscle cells in the atherosclerotic arterial wall. In atherosclerosis the production and release of ET-1 is increased, whereas the biological activity of NO is decreased due to reduced production and/or enhanced inactivation by superoxide (O_2^-). ET-1 may inhibit endothelial NO synthase (eNOS) activity and increase superoxide production. The increased production of ET-1 may be due to reduced NO-mediated inhibition of ET-1 production, increased activity of ET converting enzyme-1 (ECE-1) and/or induction of ET-1 synthesis in vascular smooth muscle cells and macrophages (MØ). Reduced biological activity of NO and enhanced production of ET-1 results in enhanced vasoconstrictor tone and pro-inflammatory activity with activation of macrophages which infiltrate the vessel wall. The vasoconstrictor tone is further enhanced by upregulation of ET_B receptors localized on the smooth muscle cell. In atherosclerosis there is a predominant vasoconstrictor tone, inflammation and endothelial dysfunction. ET-1 seems to be an important factor for the development of these events in the atherosclerotic process. ET receptor antagonists may be of importance for the limitation of atherosclerotic progression and complications. Due to the upregulation of ET_B receptors in atherosclerosis, combined ET_A/ET_B receptor blockade have more pronounced vasodilator effects than selective ET_A receptor blockade.

sclerotic human coronary arteries (Fig. 11).[153,170] Therefore it was of importance to elucidate the functional consequences of this ET_B receptor upregulation. The main finding of study I is that the vasoconstrictor response to ET_B receptor stimulation by sarafotoxin 6c is enhanced in patients with atherosclerosis. The vasoconstrictor response to ET-1 was not changed, however. This indicates a selective enhancement of the sensitivity of contractile ET_B receptors in the forearm vasculature of atherosclerotic patients. Study I provides indications of a functional consequence of an altered ET_B receptor population in these patients (Fig. 11).

It may be argued that the enhanced forearm vasoconstrictor response to sarafotoxin 6c in the atherosclerotic patients is due to a reduction in the EDV evoked by endothelial ET_B receptor stimulation.[183,215] Several studies have demonstrated reduced EDV in atherosclerosis and hypercholesterolemia due to reduced bioavailability of NO.[216] Since the total response to sarafotoxin 6c is a net effect of the dilator and the constrictor components, impairment of the dilator component would result in an enhanced vasoconstrictor response to ET_B receptor stimulation. However, the initial increase in FBF by sarafotoxin 6c in the patients was not smaller than that observed in the controls. This clearly suggests that the subsequent more pronounced reduction in blood flow in atherosclerotic patients was related to enhancement of the vasoconstrictor component of ET_B receptor stimulation. Enhanced sensitivity to ET_B receptor stimulation does not seem to be limited to atherosclerosis, however. Love and coworkers found an increased forearm vasoconstrictor effect of sarafotoxin 6c but a blunted vasoconstrictor response to ET-1 in patients with congestive heart failure.[103] It was also recently demonstrated that ET_B receptor blockade resulted in forearm vasodilatation in hypertensive patients but vasoconstriction in normotensive controls,[136] suggesting enhanced ET_B-mediated vasoconstriction in hypertension. Thus, the findings in study I and previous studies indicate that there is an enhanced ET_B receptor-mediated vasoconstriction in atherosclerosis as well as in other cardiovascular diseases such as heart failure and hypertension.

The reports of *in vitro* upregulation of ET_B receptors together with the findings in study I indicate that dual ET_A/ET_B receptor blockade may be more efficient than selective ET_A receptor blockade in atherosclerosis. We thus formulated the hypothesis that combined ET_A and ET_B receptor blockade would result in a greater vasodilator response than selective ET_A receptor blockade in patients with atherosclerosis. On the other hand, the vasodilatation induced by ET_A receptor antagonism seems to be mediated by NO and is attenuated by simultaneous ET_B

receptor blockade in healthy men.[199] Therefore, it is of importance to clarify how dual ET_A/ET_B receptor blockade affects blood flow in patients with atherosclerosis.

The main finding of study II is that combined ET_A and ET_B receptor blockade does indeed evoke a significantly more pronounced vasodilator response in the forearm of patients with atherosclerosis as compared to selective ET_A receptor blockade and compared to healthy controls. Selective ET_B receptor blockade resulted in a slight but significant increase in FBF in patients as compared to a slight decrease in FBF in controls. Finally, there was no difference in the vasodilator response to selective ET_A receptor blockade between the two groups. These findings suggest that the ET_B receptor plays a more important role for the vasoconstrictor tone mediated by endogenous ET-1 in atherosclerotic patients, in agreement with previous suggestions of the ET_B receptor becoming more important as atherosclerosis progresses.[154] The observations in study II, together with the finding of increased vasoconstriction to ET_B receptor stimulation in patients with atherosclerosis in study I and the *in vitro* findings of ET_B receptor upregulation in the atherosclerotic human coronary artery[170] and aorta[153] suggest that there is a shift towards more ET_B-mediated vasoconstriction in atherosclerosis. These observations illustrate the importance of evaluating the hemodynamic effects of the receptor antagonists in patients with cardiovascular disease since the responses are very different from those found in healthy controls. The findings demonstrate that dual ET_A and ET_B receptor antagonism is more effective than selective ET_A receptor antagonism in increasing blood flow and might therefore be of greater therapeutic value in patients with atherosclerosis.

The increase in FBF induced by combined ET_A and ET_B blockade in the patients was larger than what could be expected from the increase in blood flow induced by the ET_A and ET_B receptor antagonists separately. Thus, the combined effect was more than additive. The reason for this is unclear. One possibility is that ET_B receptor blockade displaces ET-1 from ET_B clearance receptors,[95] as demonstrated by the increase in venous plasma levels of ET during administration of BQ788, and that this will result in increased stimulation of ET_A receptors. However, if this was an important mechanism, the selective ET_B antagonist would most likely reduce blood flow, which was not the case in the patients. Another possibility is that crosstalk exists between the two receptors such that if only one receptor is blocked, the other receptor can compensate for the loss of activity.[80-82]

Since ET_B receptors are present both on endothelial cells where they cause dilatation, and on smooth muscle cells where they cause vasoconstriction, the overall effect mediated by ET-1 on the ET_B receptor depends on a balance between these two actions. In healthy blood vessels it seems as if the balance of effects of ET-1 favors basal vasodilatation via the endothelial ET_B receptor.[199] Our results demonstrating vasoconstriction in response to selective ET_B receptor blockade with BQ788 in the somewhat older healthy controls supports this view. However, the vasodilator response evoked by BQ788 in the atherosclerotic patients suggests that this balance is shifted towards vasoconstriction via the smooth muscle cell ET_B receptors in these patients. The finding that the vasodilator response to sarafotoxin 6c was not impaired in atherosclerotic patients in study I suggests that the vasodilator response to selective ET_B blockade in the atherosclerotic patients in study II is due to an upregulation of ET_B receptors on smooth muscle cells, rather than to an impairment of ET_B-mediated vasodilatation.

Greater vasodilatation in response to non-selective ET_A and ET_B blockade than to selective ET_A blockade has also been reported in patients with hypertension.[136] The results in study II differ from those found in hypercholesterolemic subjects in whom combined ET receptor blockade evoked less vasodilatation than selective ET_A receptor blockade.[217] In study II, LDL cholesterol did not differ between the two study groups. Upregulation of vasoconstrictive ET_B receptors has also been described in experimental prehepatic portal hypertension,[134] pulmonary hypertension[218] and in patients with renal disease and high-grade proteinuria.[219] Thus, upregulation of ET_B receptors on smooth muscle cells may occur in several cardiovascular disorders, including atherosclerosis.

Conversion of big ET-1 to ET-1

The increased production of ET-1 in atherosclerosis may in part be due to enhanced expression and activity of ECE-1 in the vascular wall.[69,71,72] Therefore it was of interest to elucidate whether the activity of ECE-1 is enhanced in patients with atherosclerosis. The main finding of study IV is that big ET-1 evokes a significantly more pronounced vasoconstrictor response in the forearm of patients with atherosclerosis than in healthy controls. This was accompanied by an increased formation of ET-1 during the infusion of the low dose of big ET-1 in the patients. These findings suggest an enhanced conversion of big ET-1 to ET-1 *in vivo* which may explain the more prominent vasoconstrictor response in the patients (Fig. 11).

The biological activity of big ET-1 is critically dependent on conversion to mature ET-1[200,220] which exerts the biological effect via stimulation of the ET_A and ET_B receptors. Thus, the difference in vasoconstrictor response to big ET-1 between the patients and the controls could be due either to an augmented activity of ECE-1 which results in higher levels of ET-1 or to an increase in the vasoconstriction induced by ET-1. In study I we found no difference in the forearm vasoconstrictor response to administration of ET-1 between patients with atherosclerosis and controls. The patients and the controls included in that study are comparable to those of study IV in terms of age and atherosclerotic disease. In fact two patients and two controls participated in both studies. Therefore, it seems unlikely that the vascular bed is more sensitive to vasoconstriction induced by ET-1. Instead, a more likely mechanism behind the presently demonstrated enhanced vasoconstrictor effect of big ET-1 in the patients is enhanced conversion of big ET-1 to ET-1.

The venous plasma levels of ET-1 were higher and the net formation of ET-1 was larger in the patients only during administration of the low dose of big ET-1, while the high dose resulted in similar venous plasma levels and net formation of ET-1 in both groups. It is possible that the high dose evoked maximum conversion of big ET-1 in the forearm. Whether this may be due to saturation of the local ECE-1 system in its catalytic capacity, feedback regulation of enzyme activity or any other mechanism remains to be determined. Moreover, since the blood flow was more profoundly reduced in the patients, leading to longer circulation time, the veno-arterial concentration differences may in fact be underestimated in the patients. The finding that arterial plasma levels of ET-1 increased two-fold during infusion of the high dose of big ET-1 in patients but did not increase in controls supports the view that conversion to ET-1 was higher in the patient group. The differences in plasma ET-1 levels between patients and controls observed during administration of big ET-1 does not seem to be related to differences in local clearance of ET-1 since administration of ET-1 results in similar local venous plasma levels of ET-1 in atherosclerotic patients and in controls in study I.

The immunohistochemical analysis of the radial artery in study IV revealed presence of ECE-1 not only in the endothelium, but also in the underlying intima as well as in smooth muscle cells of the media. In addition, ECE-1 immunoreactivity was detected in the atherosclerotic plaque itself. This novel histological finding of atherosclerotic lesions expressing ECE-1 immunoreactivity in arteries of the forearm extends previous observations of ECE-1 in macrophage-rich regions of atherosclerotic human coronary arteries[69] as well as in the atherosclerotic rabbit aorta.[72] The additional expression of ECE-1 in the atherosclerotic plaque may contribute to the conversion of big ET-1 to ET-1.

Big ET-1 may be converted to either $ET\text{-}1_{(1\text{-}21)}$ by ECE-1 or to $ET\text{-}1_{(1\text{-}31)}$ by chymase (Fig. 2).[62] Chymase is produced and released by mast cells, which are known to be present in atherosclerotic arteries.[221,222] Therefore, the increased levels of ET-1-like immunoreactivity in atherosclerotic patients could partly be due to an enhanced conversion of big ET-1 to $ET\text{-}1_{(1\text{-}31)}$ by chymase. This may be of functional importance since the fragment $ET\text{-}1_{(1\text{-}31)}$ induces vasoconstriction via an ET_A receptor-mediated effect.[18] It was therefore of importance to elucidate whether the observed enhanced production of ET-1 was due to formation of $ET\text{-}1_{(1\text{-}21)}$ or $ET\text{-}1_{(1\text{-}31)}$. The ET-1 antiserum used was found to have negligible cross-reactivity with the $ET\text{-}1_{(1\text{-}31)}$ fragment (0.04%). This suggests that the increased level of ET-1 reflects the $ET\text{-}1_{(1\text{-}21)}$ fragment and is due

to enhanced activity of ECE-1 rather than being an effect of conversion by chymase (Fig. 11).

Since ET-1 is suggested to contribute to vasoconstriction, thrombosis, vascular growth, inflammation, endothelial dysfunction and the progression of atherosclerosis, enhanced ECE-1 activity may be of pathophysiological importance for patients with atherosclerosis. However, ECE-1 inhibition may be too unspecific a pharmacological tool to be of use in a clinical setting since ECE-1 also hydrolyzes peptides such as bradykinin, substance P and insulin.[64] Moreover, big ET-1 is cleaved also by other enzymes such as non-ECE metalloproteases (Fig. 2).

ET-1 and endothelial dysfunction

Endothelial dysfunction characterized by reduced bioavailability of NO in the coronary arteries[190] and the forearm vascular bed[191] is associated with increased risk for cardiovascular events. Moreover, in patients with atherosclerosis, circulating levels of ET-1 are elevated[142] and immunoreactive staining for ET-1 is increased in the vasculature.[164] However, little is known about the consequences of elevated ET-1 levels in atherosclerosis on NO bioavailability. ET receptor antagonism has been reported to improve EDV in isolated arteries from apolipoprotein E-deficient mice[192] and in isolated internal mammary arteries from patients with coronary artery disease.[193] However, the involvement of ET-1 in endothelial dysfunction in patients with atherosclerosis is unknown.

The main findings of study III are that ET-1 significantly decreases EDV induced by acetylcholine in the forearm of healthy humans, and that selective ET_A receptor blockade significantly improves EDV in patients with atherosclerosis. These findings suggest that elevated levels of ET-1 impair endothelial function in the human forearm and that it can be improved by ET_A receptor blockade in patients with atherosclerosis.

The vasodilator response to acetylcholine was inhibited by L-NMMA indicating that its effect to a large part is mediated by NO, as described in previous studies.[223-225] During the infusion of ET-1, FBF was reduced by approximately 50%, which may have affected the bioactive concentration of acetylcholine since it is known to be rapidly inactivated and its biological activity is altered by changes in blood flow.[226,227] However, noradrenaline, infused at a dose which reduced FBF by a similar degree, did not attenuate the response to acetylcholine. This finding clearly demonstrates that the decreased vasodilator response to acetylcholine during ET-1 administration is not due to reduced FBF *per se*. The per cent increase in FBF induced by the endothelium-independent vasodilator SNP was enhanced during infusion of ET-1, suggesting that the ability of the smooth muscle cells to relax was not impaired by ET-1. Therefore, a likely mechanism behind the decreased vasodilator response to acetylcholine during ET-1 is reduced bioavailability of endogenous NO. However, it cannot be excluded that ET-1 induced a small inhibition of the relaxing effect of NO on the smooth muscle cells since the SNP-induced increase in FBF expressed in absolute numbers was decreased during infusion of ET-1. In a previous study, Krum and co-workers[228] found no effect of ET-1 infusion on acetylcholine-induced vasodilatation in the human forearm. The reason for these differences in results is unclear. One important difference between the protocols is that the response to acetylcholine was tested after only 10 min of ET-1 infusion in the previous study, whereas ET-1 was administered for 60 min in the present study. Thus, the 10 min infusion of ET-1 may have been too short to induce endothelial dysfunction.

The second part of study III (Protocol 2) aimed at investigating the involvement of endogenous ET-1 in the impaired EDV in patients with atherosclerosis. Selective ET_A receptor blockade increased FBF to a similar extent in both patients with atherosclerosis and in healthy age-matched controls. Acetylcholine evoked similar degrees of vasodilatation before ET_A receptor blockade in both patients and controls, although there was a marginally larger mean response in the controls. Following 60 min administration of the ET_A receptor antagonist, EDV was significantly enhanced in the patients, but not in the controls. This observation suggests that in the patients, endogenous ET-1 acting through the ET_A receptor has a greater inhibitory effect on NO bioavailability than in the controls. This may be in

accordance with previous findings of enhanced expression of ET-1 in atherosclerotic arteries.[142] The general capacity of the forearm resistance vessels to dilate was not changed by ET_A receptor blockade since the vasodilator response to SNP was similar in both patients and controls before and during infusion of BQ123. Thus, the enhanced vasodilator response to acetylcholine during ET_A receptor blockade in the patients appears to be due to increased bioavailability of endogenous NO, in accordance with findings that 95% of the vasodilatation induced by ET_A receptor blockade can be inhibited by the NO blocker L-NMMA.[199]

Endothelial dysfunction is an early sign in atherosclerosis.[229,230] Several traditional risk factors for atherosclerosis have been implicated in the abnormal endothelial function. The findings in study III suggest that ET-1 contributes to this impaired EDV. A recent study extends our findings by demonstrating vasodilatation and improved EDV during ET_A receptor blockade in epicardial coronary arteries of patients with atherosclerosis.[231] It was further demonstrated that a low dose of the ET_A receptor antagonist LU 135252 improved the brachial artery EDV in patients with chronic heart failure.[232] However, the effect was not reproduced with a higher dose, which complicates the interpretation. Moreover, ET receptor blockade was shown to improve EDV in hypertensive patients.[233] These findings all support a role for ET-1 in the development of endothelial dysfunction.

The ET_B receptor located on the vascular endothelium is supposedly beneficial in healthy arteries by stimulating the release of NO. It may therefore be possible that blockade of the ET_B receptor in addition to ET_A receptor blockade does not result in improved EDV. However, preliminary data from an ongoing study in which EDV was examined before and following a 60 min intra-arterial infusion of BQ123 and BQ788 (both 10 nmol/min) in patients with atherosclerosis indicate that EDV is significantly enhanced also following dual ET_A/ET_B receptor blockade. This observation suggest that dual ET_A/ET_B receptor blockade is at least as efficient in improving EDV as selective ET_A receptor blockade in patients with atherosclerosis. In agreement, a recently published study demonstrated improved EDV in response to dual ET_A/ET_B receptor blockade in patients with hypertension.[233] This observation together with the finding in study II that the response to dual ET_A/ET_B receptor blockade evoked greater vasodilatation than selective ET_A receptor blockade in patients with atherosclerosis indicates that dual ET_A/ET_B receptor blockade may be preferable to selective ET_A receptor blockade in these patients.

Mechanisms of ET-1 and NO interactions in atherosclerosis

The mechanism by which ET-1 reduces the bioavailability of NO remains to be clarified. One interesting possibility is that ET-1 increases superoxide production in the vascular wall[184,185,234] via an effect that may be coupled to the ET_A receptor[186] (Fig. 11). Superoxide will react with NO released from the endothelium and thereby reduce its bioavailability. However, the major producer of superoxide is the NAD(P)H oxidase which consists of two subunits ($gp91^{phox}$ and $p22^{phox}$).[235] In human endothelial cells, $gp91^{phox}$ has been identified as the rate-limiting subunit of the superoxide-forming NAD(P)H oxidase, and ET-1 has been shown to increase $gp91^{phox}$ expression and augment superoxide production in endothelial cells via the ET_B receptor.[236] Thus, also the ET_B receptor may contribute to the increased oxidative stress seen in conditions with elevated levels of ET-1. Another mechanism may be that ET-1 reduces NOS activity,[187] and a recent report describes that iNOS activity can be restored in dogs by combined ET_A/ET_B receptor blockade.[188] Interestingly, combined ET_A/ET_B receptor blockade increased eNOS activity more than selective ET_A receptor blockade in hypercholesterolemic pigs.[189] These data suggest that ET-1 increases oxidative stress in the vessel wall, leading to endothelial dysfunction and enhanced susceptibility to atherosclerosis.

The forearm as an experimental model

The major part of our studies were performed in the forearm vasculature. It may be argued that the forearm circulation does not properly reflect changes in other vascular beds. However, venous occlusion plethysmography has been used ex-

tensively to study human vascular physiology and pathophysiology *in vivo*.[194] Indeed, forearm venous occlusion plethysmography with local brachial artery infusion is an accurate, reproducible and convenient method and has therefore become one of the ”gold-standards” in the assessment of vascular function in health and disease.[237,238] The major advantage of this approach is that drugs can be administered at low doses, devoid of systemic hemodynamic effects. Recordings of blood flow are made during occlusion of venous return by a pneumatic cuff (Fig. 3). Venous occlusion plethysmography is considered to primarily reflect endothelial vasodilator function in the resistance vessels of the forearm. Since endothelium-derived markers have relatively poor specificity, endothelial function is therefore most commonly measured as the vasodilating response to physical or pharmacological stimuli, such as shear stress, acetylcholine, bradykinin, substance P or serotonin.[239] Each agonist acts through a cellular membrane receptor, with signal transduction through G proteins. Acetylcholine is most frequently used as endothelial vasodilator agonist. Evaluation of changes in blood flow in response to serial intra-arterial infusions of increasing doses of acetylcholine provides a measure of endothelial function. NO-donors such as SNP are used to stimulate the endothelium-independent vasodilatation via a direct action on the vascular smooth muscle. Endothelial function as determined using venous occlusion plethysmography with local acetylcholine infusion correlates with prognosis in hypertensive patients.[191] Furthermore, atheromatous lesions in arteries of the forearm correlate with atherosclerotic lesions of arteries in other vascular beds such as coronary arteries and carotid arteries.[240] EDV can be measured not only in the forearm circulation, but also directly in the coronary arteries. Endothelial dysfunction appears to be a systemic phenomenon, affecting resistance and conduit vessels in the forearm as well as the coronary circulation. Recent studies have shown that coronary endothelial dysfunction is a strong and independent predictor of long-term atherosclerotic disease progression and cardiovascular event rates.[190,241] Interestingly, there is a correlation between forearm vasomotor responses and those in the coronary arteries[225,242] and, therefore, the forearm vascular bed can probably be used as a surrogate for assessing endothelial function in the coronary arteries, minimizing the invasive nature of such investigations.

EDV was in study III determined by infusion of acetylcholine, and was characterized to be NO-dependent to a large part, in agreement with previous findings.[223,224] In study III the EDV was greater in the young healthy controls of protocol 1 than in the older controls included in protocol 2, although blood pressure and smoking habits were similar in the two groups. These observations are in agreement with previous observations suggesting that endothelial function is reduced at an older age,[243] although other uncontrolled factors, such as cholesterol levels, may have contributed to this difference. It was also noted that there was a trend towards an inverse dose-response relationship for acetylcholine in the atherosclerotic patients. This may be related to acetylcholine causing constriction via activation of muscarinic receptors on the vascular smooth muscle cell. In patients with severe endothelial dysfunction, acetylcholine may therefore evoke vasoconstriction at high doses.[244]

The change in vessel diameter and blood flow of conduit areries, usually in the brachial artery, can be examined using an external high-resolution ultrasound imaging,[245] as we did in study II. Flow-mediated vasodilatation, obtained in the form of hyperemia after a short period of arterial occlusion, stimulates the endothelium to release NO, which results in vasodilatation. Sublingual nitroglycerin is used to assess endothelium-independent vasodilatation. Close correlations have been demonstrated both between endothelium-dependent responses measured non-invasively in the brachial artery and invasively in the coronary arteries[246] and between these responses and the severity of coronary artery disease.[247]

The histological demonstration of atherosclerotic plaques in radial arteries in study IV supports the notion that forearm artery atheromatosis correlates with atherosclerotic lesions in the coronary and carotid arteries[240] and suggests that the forearm vascular bed is a suitable model to be used for studies in atherosclerotic patients. In study I, on a similar group of patients, we found no difference in the vasoconstrictor response to exogenous ET-1 between

patients and controls. It therefore seems unlikely that the difference in vascular response between patients and controls observed in study II and IV is due to morphological differences in brachial artery wall between the two groups.

The earliest manifestation of endothelial function could be expected to be an alteration in the small vessel tone before the development of structural alterations in large and small arteries.[248] The wave forms of these small arteries can be assessed noninvasively by pulse contour analysis, most conveniently from a transducer placed over the radial artery.[249] Pulse wave analysis can detect oscillations and reflected waves generated at branch points in the small arteries, providing an oscillatory elasticity index for microvasculature function.[250] There is a striking reduction in this elasticity index in subjects with hypertension, diabetes or hyperlipidemia, in smokers and in the presence of atherosclerotic disease.[251] The availability of this method may be an opportunity to screen individuals for early evidence of endothelial dysfunction.

ET-1 in the renal and splanchnic circulation

We observed no effects of BQ123 on basal splanchnic or renal hemodynamics. This indicates that activation of the ET_A receptor by endogenous ET-1 does not contribute significantly to basal splanchnic or renal vascular tone in healthy humans. This finding contrasts with the observation in the human forearm that ET_A receptor blockade evokes vasodilatation.[199] Thus, there seem to be regional variations in the contribution of ET-1 to basal vascular tone via the ET_A receptor. The vasoconstrictor effect induced by exogenous ET-1 in the splanchnic vascular bed was completely blocked by BQ123. In addition, BQ123 greatly attenuated the vasoconstriction induced by ET-1 in the renal vasculature. These findings suggest that the constrictor effects of ET-1 in the splanchnic and renal vascular beds of healthy humans are mainly ET_A receptor-mediated, which is similar to the situation in the forearm. The situation may be different in atherosclerosis (study I and II), renal failure[219] and portal hypertension[126] when ET_B receptors seem to be up-regulated and thereby may contribute to a greater extent to the vascular tone induced by ET-1.

Administration of the ET_B receptor antagonist BQ788 reduced splanchnic and renal blood flow, with corresponding increases in vascular resistance. This may be due to a basal vasodilator tone mediated via endothelial ET_B receptors. An alternative explanation is that ET_B receptors act as clearance receptors.[95] Accordingly, binding of BQ788 to ET_B clearance receptors resulted in elevated plasma concentration of ET-1 which may then cause vasoconstriction via the ET_A receptor. The observation that the splanchnic and renal vasoconstriction induced by ET-1 was enhanced in the presence of BQ788 may also be due to higher plasma concentrations of ET-1 in the presence of BQ788 than under control conditions. The larger vasoconstrictor response could thus be explained by increased stimulation of the ET_A receptor. Taken together, these observations suggest that the ET_B receptor is important for clearance of circulating ET-1 whereas the ET_A receptor is the most important receptor for mediating vasoconstriction in the renal and splanchnic vascular beds.

There was an increase in MAP and SVR in response to ET-1 both under control conditions and in the presence of BQ788, but not in the presence of BQ123. These observations suggest that these effects of ET-1 are mainly ET_A receptor-mediated in agreement with previous observations.[252]

ET receptor blockers – potential targets in cardiovascular disease

There are many potential therapeutic targets for ET receptor blockers in cardiovascular disease. In patients with essential hypertension, bosentan – a dual ET_A/ET_B receptor blocker - shows an antihypertensive efficacy similar to that of the ACE inhibitor enalapril.[253] In experimental renal disease, chronic ET receptor blockade reduces hypertension-associated[254,255] and other forms of renal and vascular injury[256,257] and also prolongs survival.[258,259] Two of the major factors determining the severity of stroke and its sequelae, ischemic brain injury and vasospasm, are effectively reduced by ET receptor blockade.[259,260] In myocardial ischemia and reperfusion injury,

ET receptor blockade limits infarct size and reduces the inflammatory response, a mechanism related to increased NO bioavailability.[168,169] Restenosis is a major problem following balloon angioplasty and ET receptor blockade is effective in reducing neointima formation after balloon angioplasty in both rodents and pigs.[261,262] Moreover, after heart transplantation in rats, ET receptor blockade inhibits transplant-associated arteriosclerosis and reduces reperfusion injury.[263]

Patients with primary pulmonary hypertension have enhanced expression of ET-1,[130] and increased concentration[131] of ET-1 in the circulation , and ET-1 plasma levels correlate with the severity of primary pulmonary hypertension.[132] A recently completed randomized, placebo controlled trial of bosentan, an oral dual ET_A/ET_B receptor antagonist, showed significant improvement in exercise capacity, functional class and pulmonary hemodynamics in patients with pulmonary arterial hypertension.[264] Bosentan (Tracleer®) is the first, and so far only ET receptor blocker approved for clinical use, for the indication pulmonary hypertension.

Congestive heart failure is associated with elevated plasma levels of ET-1 and these levels correlate inversely with prognosis.[265] Administration of ET receptor antagonists increases cardiac index and reduces pulmonary arterial pressure in patients with congestive heart failure. There are also ongoning clinical trials in patients with congestive heart failure, although the first trials, REACH and ENABLE, did not show promising results.[266]

ET receptor blockade may have an important role in the prevention of progression of atherosclerotic plaques. Long-term ET_A receptor blockade reduced the extent of atherosclerosis in apolipoprotein E-deficient mice, without affecting blood pressure or plasma cholesterol. It also restored EDV and prevented increased vascular ET-1 content.[192] Also in hypercholesterolemic pigs, impaired EDV is improved after ET receptor blockade.[267] These data are in agreement with our findings of improved EDV in response to ET_A receptor blockade in patients with atherosclerosis in study III. In patients with coronary artery disease, the combined ET_A/ET_B receptor antagonist, bosentan, causes vasodilatation.[268] Interestingly, ET_A receptor blockade has been shown to effectively dilate epicardial coronary arteries in patients with atherosclerosis, especially at areas of stenosis, whereas there were no changes in patients without signs of coronary atherosclerosis.[269] Furthermore, distal coronary vasoconstriction after coronary angioplasty is attenuated by ET_A receptor antagonism.[270] These findings, together with the fact that the tissue distribution of ET-1 is highly upregulated at areas of atherosclerotic plaques,[148] and our findings in study II of enhanced vasodilatation in response to dual ET_A/ET_B receptor blockade in patients with atherosclerosis but not in age-matched controls, suggest that the vasodilator effects of ET receptor antagonists are more pronounced in atherosclerotic arteries, especially at regions of stenosis, than in non-stenotic arteries. This may indicate that the problem with steal phenomenon (more vasodilatation in normal than in stenotic arteries) may be less pronounced with ET receptor blockers in comparison with other vasodilators such as calcium antagonists.[271] Potential complications with ET receptor blockers are hepatic side effects like those seen in the REACH trial. Because of their teratogenic effects,[21] ET receptor antagonists are contraindicated in women of child-bearing age. In clinical trials, the antagonists were occasionally associated with an increase in heart rate, facial flush, and headache.[272] Additional hypotensive effects may occur if ET antagonists are combined with ACE-inhibitors.[273,274]

Value of ET plasma levels

The release of arterial ET into the vascular compartment is small, since *in vitro* endothelial cells have been shown to secrete 80% of this peptide on the basolateral side towards the smooth muscle,[97] suggesting that plasma ET levels may, in fact, underestimate the degree of ET synthesis and release in atherosclerotic arteries. Nevertheless, several studies have demonstrated that plasma ET concentrations are elevated in patients with hypercholesterolemia ,[143] type II diabetes mellitus,[145] ischemic heart disease,[210] myocardial infarction,[275] pulmonary hypertension,[130] heart failure,[130,276] renal failure[277] and atherosclerosis.[69,142,164] Our finding of elevated venous and arterial ET levels in patients with atherosclerosis compared to age-matched controls is

in agreement with these observations. Furthermore, infusion of big ET-1 resulted in a significant reduction in blood flow, but only a modest increase in venous ET-1 concentration, in fact similar to ET-1 levels reported in patients with myocardial infarction.[275] This indicates that even a small elevation of plasma ET-1 levels may reflect a marked increase in ET-1 release and induce an increased vasoconstrictor tone in patients with cardiovascular disease. The expression of ET-1 is enhanced by ischemia[278,279] and mechanical injury.[166,280] Depending on the condition, increased ET levels may reflect increased production and/or reduced clearance of ET-1.[281,282] ET-1 levels also increase following treatment with ET_B receptor antagonists,[268,283] as seen in study II and V. The reason for this is most likely that ET_B receptors exert a clearance effect on ET-1.[95] This clearance function is present both in the pulmonary vascular bed (study V) and in the forearm (study II). On the other hand, the elimination of circulating ET-1 was very similar in the absence and presence of ET_B receptor blockade in study V. This suggests that clearance of circulating ET-1 via binding to the ET_B receptor only contributes to a small proportion of the total ET-1 clearance in humans in accordance with observations in the rat.[284] Since ET-levels correlate well with the seriousness of the pathophysiological condition,[142] ET-1 is a good neurohormonal marker in predicting clinical outcome in cardiovascular disease. ET-1 levels at 72 h post myocardial infarction and in renal failure predict long-term outcome, and in patients with congestive heart failure plasma levels of big ET-1 have been demonstrated to be a better predictor of 1-year outcome than plasma atrial natriuretic peptide and norepinephrine, NYHA class, age, and echocardiographic left ventricular parameters.[265]

Since endothelial dysfunction is an early and prognostic sign in the atherosclerotic process, markers of endothelial abnormalities have been sought. Among these are measurement of endothelium-derived substances.[285] ET-1 is one of the circulating markers that have been associated with conditions of endothelial dysfunction, such as atherosclerosis, hypercholesterolemia and cigarette smoking.[142,143] Other circulating markers of endothelial dysfunction include von Willebrand factor,[286] tissue plasminogen activator and plasminogen activator inhibitor-1.[287] Moreover, circulating adhesion molecules may serve as markers of endothelial damage of atherosclerosis.[287] The main problem is the relatively poor specificity of these markers, which may hamper their use in detection or monitoring of endothelial dysfunction.

SUMMARY AND CONCLUSIONS

In the present thesis we made the following findings:

The forearm vasoconstrictor response to dual ET_A/ET_B receptor stimulation by ET-1 is unchanged, whereas the vasoconstriction induced by ET_B receptor stimulation is enhanced in patients with atherosclerosis. This finding suggests that the vasoconstrictor ET_B receptor becomes functionally more important in atherosclerotic disease.

The vasodilator response to dual ET_A/ET_B receptor blockade in patients with atherosclerosis is greater than that to selective ET_A receptor blockade and compared to the response in healthy controls, indicating an enhanced ET_B-mediated vascular tone in these patients. These findings may have important therapeutic implications since dual ET_A/ET_B receptor antagonism seems to be more efficient than selective ET_A receptor antagonism in reducing vascular tone in patients with atherosclerosis.

Elevated levels of ET-1 impair EDV in the human forearm. Furthermore, ET_A receptor blockade improves EDV in patients with atherosclerosis. Since endothelial function is a prognostic factor in the progression of atherosclerotic disease, ET receptor blockade may have important therapeutic effects by improving endothelial function and increasing blood flow in patients with atherosclerosis.

The vasoconstrictor response to big ET-1 is enhanced in patients with atherosclerosis. The formation of $ET\text{-}1_{(1\text{-}21)}$ was significantly higher in atherosclerotic patients than in controls. These observations suggest an enhanced conversion of big ET-1 to ET-1 which may be due to enhanced activity of ECE-1.

ET-1-mediated splanchnic and renal vasoconstriction is blocked by selective ET_A receptor antagonism in healthy humans. Furthermore, ET_B receptor blockade reduces splanchnic and renal blood flow *per se* and enhances ET-1-mediated vasoconstriction. These findings demonstrate that the ET_A receptor plays a dominant role in mediating vasoconstriction in the splanchnic and renal vasculature. The results also suggest that ET_B receptors may play an important role in regulating plasma ET-1 levels as indicated by the increase after ET_B receptor blockade.

Atherosclerosis is associated with changes in synthesis, release and vascular actions of endothelial factors such as ET-1. Taken together, these findings suggest that ET-1 may play an important role in the enhanced vasoconstriction and endothelial dysfunction seen in patients with atherosclerosis. ET receptor blockade may be of therapeutic value by improving blood flow and endothelial function in these patients.

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