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Vascular Endothelin and Angiotensin Receptors Regulation in Inflammatory Arterial Disorders

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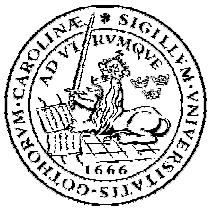

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DOCTORAL THESIS

The public defence of this thesis for the degree of Doctor of Philosophy in Medicine will, with due permission from Lund University, take place in

Segerfalkssalen, Wallenberg Neuroscience Centre, Lund University, Sweden on Friday, January 22, 2010 at 13:00

Faculty opponent: Assistant Professor Claes Nordborg
Department of Pathology, Gothenburg University, Gothenburg, Sweden

English summary

The present thesis is aimed to examine the hypothesis that the degree of vascular inflammation correlates with the expression of vascular endothelin and angiotensin receptors. The receptor changes were studied in subcutaneous resistance arteries in patients with different degrees of ischemic heart disease (IHD). In addition, patients with giant cell arteritis (GCA) were also investigated because of the massive inflammatory activity in affected vessels. For functional studies of the resistance arteries, sensitive myographs were used. Receptor protein levels were measured quantitatively using immunohistochemistry or western blot.

Main results: 1. Patients with suspected acute coronary syndrome (angina pectoris) have increased expression of AT_1 and ET_B receptors in vascular smooth muscle cells (VSMC) in subcutaneous resistance arteries. 2. The level of the up-regulation of contractile AT_1 and ET_B receptors depends on the degree of underlying IHD. The phenomenon is also correlated to the level of the systolic blood pressure. 3. Temporal arteries from patients with GCA have up-regulation of AT_1 and ET_B receptors in the VSMC in the medial layer of the arterial wall. The degrees of up-regulation of ET_B receptors are directly correlated to the degree of the systemic inflammatory response (CRP). 4. Accompanying lymphocytic infiltrates as well as giant cells in the lesions of the temporal arteries express high immunostaining of endothelin-1 and up-regulation of ET_B and AT_1 receptors as well. 5. De novo expression of tissue endothelin-1 was observed in the VSMC in the medial layer as well in the neointima in affected arteries. 6. ET_B receptors mediate contraction of subcutaneous resistance arteries in IHD.

The present thesis demonstrated that there is increased ET_B and AT_1 receptor expression in VSMC in IHD and in GCA which correlates with the degree of inflammation.

Ivan Dimitrijevic, MD
Lund, December 22, 2009

List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals. The papers are appended at the end of this thesis.

I. Increased expression of endothelin ET_B and angiotensin AT_1 receptors in peripheral resistance arteries of patients with suspected acute coronary syndrome
Ivan Dimitrijevic, Ulf Ekelund, Marie-Louise Edvinsson and Lars Edvinsson
Heart Vessels 2009 24:393–398

II. Increased vascular endothelin type B and angiotensin type 1 receptors in patients with ischemic heart disease
Ivan Dimitrijevic, Marie-Louise Edvinsson, Malin Malmsjö, Per-Ola Kimblad and Lars Edvinsson
BMC Cardiovascular Disorders 2009, 9:40

III. Increased angiotensin II type 1 receptor expression in temporal arteries from patients with giant cell arteritis
Ivan Dimitrijevic, Malin Malmsjö, Christina Andersson, Pehr Rissler, Lars Edvinsson.
Ophthalmology 2009, 116, Issue 5, 990-996

IV. Increased tissue endothelin-1 and endothelin-B receptor expression in temporal arteries from patients with giant cell arteritis
Ivan Dimitrijevic, Christina Andersson, Pehr Rissler, Lars Edvinsson
Ophthalmology in press

Contents

Abbreviations

ACE	angiotensin converting enzyme
Ang II	angiotensin type II
AP	angina pectoris
ARB	angiotensin receptor blockers
AT_1	angiotensin receptor type 1
AT_2	angiotensin receptor type 2
BMI	body mass index
CABG	coronary artery bypass graft
CRP	C - reactive protein
DAB	3-3-diaminobenzidine tetrahydrochloride
DBP	diastolic blood pressure
EC	endothelial cells
ESR	erythrocyte sedimentation rate
ET_A	endothelin receptor type A
ET_B	endothelin receptor type B
ET-1	endothelin-1
GCA	giant cell arteritis
GPCR	g-protein coupled receptors
HbA1c	glycated haemoglobin
HRP	horse radish peroxidase
IHD	ischemic heart disease
IL-1	interleukin-1
IL-6	interleukin-6
INF-γ	interferon gamma
LDL	low-density lipoprotein cholesterol
MTX	methotrexate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
RAAS	renin angiotensin aldosterone system
SBP	systolic blood pressure
SDS	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S6c	sarafotoxin 6c
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor alpha
VCAM	vascular cell adhesion molecule
VSMC	vascular smooth muscle cells

Introduction

In the vascular system, peptide hormones play a significant role in the regulation of normal vessel physiology as well as during pathophysiological conditions. The actions of these peptide hormones are mediated through specific receptors which belong to the family of seven transmembrane G-protein coupled receptors [1]. Today two commonly used pharmacological interventions are directed against these receptors in different cardiovascular disorders, e.g. angiotensin II (Ang II) AT_1 receptor blockers (losartan, candesartan, i.a.) and endothelin-1 (ET-1) receptor antagonists (bosentan). The renin-angiotensin-aldosteron system (RAAS) and endothelin system involve the most important vasoconstrictive peptides in man and their dysfunction inflicts hypertension and vascular remodelling, leading to substantial morbidity and mortality from conditions such as myocardial infarction, congestive heart failure and stroke.

Inflammation is gaining more attention in attempts to resolve the questions behind the development of cardiovascular disorders [2]. Evidence supports a pivotal role for inflammation in all phases of atherosclerosis and immunologic inflammation of arteries. This thesis was undertaken to study how different degrees of inflammatory vessel disorders correlate to the function, engagement and expression of the RAAS and the ET-1 systems and their receptors in arterial vessel walls.

Angiotensin II and angiotensin receptors

Ang II is an oligopeptide consisting of eight amino acids and is the main effector of the RAAS. Ang II is involved in the regulation of electrolyte balance, fluid volume, blood pressure and aldosterone production. RAAS is dependent on a cascade of enzymatic reactions; initially, angiotensinogen is released into the circulation by the liver in response to glomerular hypoperfusion. Renin, produced by the kidney, converts angiotensinogen to angiotensin I (Ang I) through its enzymatic capability. Ang I is in turn cleaved by angiotensin converting enzyme (ACE) into Ang II. Ang II has direct vascular effects. The ACE concentration is highest in the lungs; however, ACE is also produced in the vascular endothelium of many tissues, thus synthesizing Ang II at a variety of sites, including the brain, vascular endothelium and the kidney. Locally acting RAAS has been suggested to play a major part in vasoconstriction [3, 4] in hypertension and in tissue injury, and regulated independently of circulating Ang II [5] Synthesis of Ang II has been shown to be catalyzed by other enzymes beyond renin and the ACE system, such as

cathepsins and chymase [6]. In VSMC, tissue Ang II generation may be more important than the circulating Ang II in the regulation of regional blood flow and in vascular disorders.

Three important direct vascular effects by Ang II need to be underlined. Apart from vasoconstriction, Ang II may induce expression of the *proinflammatory* phenotype of human VSMC [7]. Cytokines such as TNF-α [8] or IL-6 are activated by Ang II, the later via NF-κB activation [9] which may result in the expression of vascular cell adhesion molecule (VCAM) [10] facilitating recruitment of inflammatory cells to the vessel. *Remodelling* is driven by increased expressions of autocrine growth factors in VSMC [11, 12] and altering the extracellular matrix composition [13] and mitogenesis [14].

Two Ang II receptors that mediates the biologic effects have been identified and cloned in man; the AT_1 and AT_2 receptors [15, 16]. AT_3 and AT_4 are two other less characterized receptors mainly expressed in cell lines or neuronal tissue, areas with limited involvement in vascular tissue. AT_1 and AT_2 receptors are distributed heterogeneously in human tissues and blood vessels [17]. AT_1 receptors are predominantly located on VSMC, while AT_2 receptors are located on endothelial cells (EC) [18]. Also, AT_1 receptor activation is may result in progression of atherosclerotic lesions, inflammation and plaque rupture [19, 20], where AT_1 receptor blockers have been shown to prevent the inflammatory effect of Ang II. In resistance arteries the ratio of the media width to lumen diameter was diminished (improved) in patients treated with AT_1 receptor blockers [21] and treatment with AT_1 receptor blockers reduced the risk of cardiovascular death [22].

Expression of angiotensin receptors is tightly regulated by negative feedback from Ang II. Typically, exposure of VSMC to Ang II makes the receptors desensitized [23] and AT_1 receptors endocytosized within 10 minutes after Ang II activation, leaving 60% of the receptors to be degraded within the lysosomes; the rest are recycled back to the plasma membrane [24]. This agonist-antagonist system is not the only way Ang II receptor expression can be manipulated. Alternative pathways regulating AT_1 receptor expression exist in VSMC referred as *heterologous* AT_1 receptor regulation that can alter the receptor expression at the cell surface. For instance, different growth factors, cytokines and hormones either up or down regulate the AT_1 receptor expression in VSMC (Table 1). AT_1 receptor expression is elevated in subjects with increased cholesterol blood levels [25] as well in VSMC in hyperinsulinemia [26]. Also proinflammatory factors such as IL-1α or C reactive protein (CRP) have been shown to increase the expression of AT_1 receptors in vascular tissue [27, 28] exaggerating the pathological effects of the AT_1 receptor,

indicating that different clinical situations can induce alteration of the AT_1 receptor expression in the vessels.

Table 1 Examples of agonists that have the capability of shifting the levels of Ang II type 1 (AT_1) receptor expressed in VSMC [29].

Agonists that down regulate	Agonists that up regulate
Angiotensin II	Interleukin-1
ATP	Insulin
Nitric oxide	Interleukin-6
Estrogen	Cholesterol
Statins	C-reactive protein
Forskolin	Glucocorticoids

The AT_2 receptors are mainly known to induce vasodilatation, inhibit cell growth and stimulate apoptosis. The expression is greater in fetal tissues and decline in the expression after birth [30].

Endothelin-1 and endothelin receptors

ET-1 is a 21-amino-acid highly potent vasoconstricting peptide, initially isolated from cultured porcine aortic EC [31]. The endothelin (ET) family consists of three different 21-amino acid peptides, ET-1, ET-2 and ET-3 [32] with two important disulfide bonds between the thiol groups of cystine residues, offering stability and correct configuration of the peptide [33]. ET-1 is formed through a stepwise cascade starting out with preproET undergoing cleavage by furin-like peptidases forming bigET. BigET is then converted to the mature 21-amino-acid peptide ET by endothelin converting enzymes, that belongs to the metalloprotease family located both on the inside and outside of cells [34, 35]. Peptides from the Sarafotoxin family, originally isolated from the venom of the Atractaspis engaddensis snake, are very similar by sequence and biological activity to the members of the ET-family [36].

The most abundant isoform of ET in the vasculature is ET-1, produced by the EC [32]. Other cell types and organs also produce ET-1, the myocardium [37], macrophages [38], VSMC [39], central nervous system and kidney, but at very low concentrations. The plasma concentration of ET-1 in healthy persons ranges from 1-10 pmol/l [40]. This could reflect a rapid elimination of ET-1 from the bloodstream [41] but rather is a consequence of ET-1 acting in a local autocrine fashion [35] with concentrations within the vascular wall ≥100-fold that of plasma level. This is due to the fact that 80% of ET-1 is secreted on the basal side of EC towards the underlying VSMC [42]. A number of different factors as well as several pathologic conditions can manipulate the production of ET-1 in the blood vessel wall. Cardiovascular stress such as increased blood pressure, vasoactive substances [43] such as Ang II or different mediators of inflammation [44], TNF-α [35], all have the ability to increase the ET-1 production in the vessel wall. Increased ET-1 expression significantly contributes to the development and maintenance of cardiovascular disorders [45].

ET-1 mediates its biological effects through two distinct G-protein coupled receptors; the endothelin type A (ET_A) and type B (ET_B) receptors [46, 47]. During physiologic conditions, the ET_A receptor is the dominant receptor subtype expressed in VSMC and mediates vasoconstriction.

The ET_B receptor is primarily located on EC and principally mediates vasodilatation via the release of nitric oxide and prostaglandins [48] but can also induce vasoconstriction in vessels as well [49]. It is important to know that these studies were primarily conducted on large conductance and muscular arteries rather than on small resistance arterioles important in the regulation of the peripheral

resistance. Importantly ET-1 is more potent as a mitogen on the VSMC expressing ET_B receptors as compared to those mainly expressing ET_A[50].

A number of factors modify the expression of endothelin receptors. Corticosteroids down regulate both ET_A and ET_B receptors [51] whereas insulin and nitric oxide increase the ET_A receptor expression in VSMC [41]. Elevated blood pressure [52] or IL-1β and TNF-α increase ET_B reactivity and mRNA expression [53]. Several detailed reviews have appeared carefully describing the endothelin system and factors that regulate ET-1 and ET_A/ET_B [54, 55].

Subcutaneous arteries as a model for generalized microvascular function

Atherosclerotic vascular disease has often deleterious effects on target organs such as the brain and heart and compromises concurrent functional alterations of the small resistance vessels [56]. However, it is difficult to examine them in vivo and to follow disease progression; hence subcutaneous vasculature might serve as a surrogate.

An early marker of vascular disease is reduction in endothelial function in small vessels [56]. This predisposes to vasoconstriction, enhanced leukocyte adhesion, platelet activation, and development of atherosclerosis [57]. Subcutaneous arteries/arterioles (figure 1) might be an attractive and a representative model to examine the generalized function of the microvasculature ex vivo [58]. There are sometimes similarities in vasomotor behaviour between small resistance vessels and larger arteries with similar functional alterations, e.g. with impaired vasodilatation [59]. Thus, dysfunction of arteries is a generalized process that occurs in a similar fashion in several vascular beds [60] Understanding the mechanisms behind the impairment of vessel relaxation in the coronary microvasculature is of particular interest in atherosclerosis and specifically what happens in VSMC that are the target of therapies in different cardiovascular disorders. The VSMC layer gradually decreases with the size of the artery; as blood pressure control mainly occurs in small resistance arteries, the location where Ang II has its main regulatory effect makes them of particular importance as well.

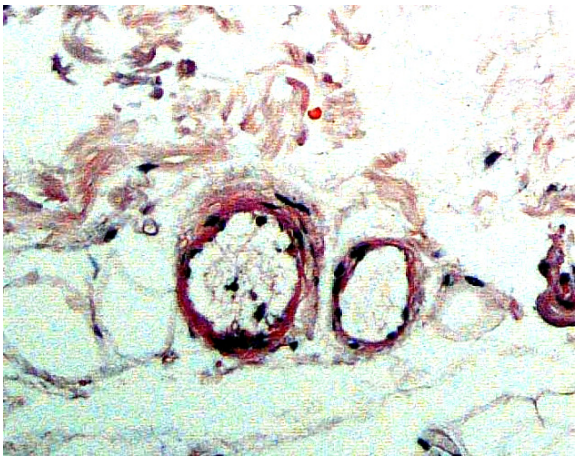
In IHD, 25% to 50% of the patients are presented as asymtomatc silent ischemia [61]. It is unclear what kind of changes take place early on in IHD, making the

understanding of the mechanisms behind the impairment of vessel relaxation/contraction in the coronary microvasculature of particular interest.

Non-invasive functional studies of subcutaneous arteries have emerged as a model to assess the vascular function in different diseases such as hypertension [62] and atherosclerotic coronary disease [63]. As vascular dysfunction in coronary artery disease includes increased vasoconstriction, available non-invasive techniques used to assess the vascular function in IHD have reported similarities in the responsiveness of the subcutaneous circulation that highly correlates to brachial artery flow mediated dilation [63] and also endothelial dilatory responses are similar in large and small subcutaneous resistance arteries in hypertensive patients [64].

ET-1 exaggerates the vasoconstrictor response in highly atherosclerotic coronary arteries [65] as Ang II does. In such situations ET receptor inhibitors may significantly reduce the hemodynamic significance of coronary stenoses and thereby reduce myocardial ischemia. To understand which of the ET-1 and Ang II receptors is important to modulate in IHD, methods for precise quantification are needed, since the non-invasive techniques do not allow for this kind of analysis. Hence the assessment of subcutaneous arteries may prove useful.

Figure 1. Typical subcutaneous resistance artery biopsy from patients undergoing CABG surgery, Hematoxylin-Eosin stained.



Ang II and endothelins in inflammatory vascular disorders

Inflammation may be triggered by several mechanisms and functions as a defensive process in removing injurious stimulus in tissue damage. Active inflammation consists of two simultaneously ongoing processes, tissue destruction and attempts at repair. In the arterial wall, chronic inflammation usually starts as asymptomatic response compared to acute inflammation that typically involves a long list of symptoms.

Inflammation is actively involved in the development of atherosclerosis [66] involving both humoral and cellular pathways [67] which also is true for giant cell arteritis that shares many common pathways in the development of vessel injury in these two distinctively different disorders. The pathology of arterial disease centres on the inflammation process on the vessel walls. It is remarkable that many of the underlying inflammatory mediators and processes are affected by the angiotensin and endothelin system. Ang II and ET-1 have been shown to stimulate cells to produce the inflammatory cytokines [68-71] indicating their importance in inflammation.

ET-1 has been implicated in progression of atherosclerotic plaques [72]. In arteries, ET-1 may induce vessel inflammation by recruiting leukocytes, stimulating the production of adhesion molecules, and inducing cytokine expression (e.g. IL-1, IL-6, TNF-α). It may induce vessel fibrosis by activating fibrotic transforming growth factor-β (TGF-β [44], increasing collagen synthesis [73], and stimulating fibroblast proliferation [74]. Moreover, ET-1 exerts trophic effects in VSMC leading to remodelling of the artery [75]. Increased circulating levels of ET-1 have been observed in GCA, even though ET-1 has local autocrine and paracrine actions; this is usually regarded as a "spill-over" of locally formed ET-1.

The Ang II receptors possess a plethora of response actions such as inducing cell growth [76, 77] , proliferation [78] and control of extracellular matrix formation [79]; these are typical activities seen in the vessel wall during an inflammatory process.

Ang II affects human leukocyte function via AT_1 receptor activation which results in increased production of proinflammatory cytokines such as TNF-α [8] [80]. Ang II modulates the immune response via the production of nuclear factor kappa light-chain-enhancer (NF-κB) in the cytoplasm of mature phagocytes resulting in transcription of inflammatory chemokines and cytokines [81]. Once activated, this inflammatory cascade can end in a kind of "cytokine storm" leading to further

immune activation. Dysfunction of the angiotensin axis leads to activation of AT receptors with alteration of the vessel function, leading to hypertension, which involves cardiac, renal and vascular remodelling. This may end with enhanced morbidity and mortality from conditions such as myocardial infarction, congestive heart failure and stroke.

Ang II and ET-1 have been shown to stimulate immune cells to produce inflammatory cytokines IL-6 and TNF-α [8, 80, 82]; these cytokines are highly active in vessel lesions in GCA and atherosclerosis.

Inflammatory vessel disorders

Arterial lesions originating from inflammation leads to characteristic morphological changes, one of which is intimal hyperplasia with infiltration of VSMC. However, the pathophysiological alterations that take place leads to more dangerous features involving increased vasoconstriction. The precise mechanisms behind these changes are not clear but inflammation orchestrating the complex signals targeting the vessel wall and the VSMC has a major role. Under normal circumstances VSMC principally have contractile abilities, however inflammation can alter the phenotype of VSMC inducing a highly active cytokine and extracellular matrix producing VSMC that can proliferative and migrate [83]. Also the immune cells participate by infiltrating the vessel wall. There macrophages produce matrix metalloproteinases, oxygen free radicals and secrete IL-1β, IL-6, and TGF-β and T cells also secrete IFN-α and IL-2 contributing to the change in the microenvironment of the vessel wall.

Despite tremendous improvements in the therapy of IHD over the last decades, our understanding of the disease and its underlying vascular pathophysiology is still incomplete. As an example, there seems to be an increased risk of future ischemic cardiac events and premature death even in chest pain patients where current ongoing IHD is ruled out [84]. But early on in the atherosclerotic disease, EC and VSMC represent the main components of the vessel wall that undergoes changes in vascular disorders. Typical changes of the arteries are that they are prone to have increased contraction. Angiotensin and endothelin, which are the main regulators of vasomotor dynamics in vessels, are deranged in arterial disorders [85, 86].

GCA is a vascular disease characterized by granulomatous panarteritis of large and medium-sized arteries [87]. The inflammation is often clinically limited to cranial branches of the aorta with involvement of the temporal arteries. Clinical presentation varies and involves fever of unknown origin to scalp necrosis and jaw

claudication. Systemic manifestation is related to the inflammatory process while organ involvement is mainly related to vascular occlusion [88]. The erythrocyte sedimentation rate (ESR) is elevated while a normal ESR value indicates less likelihood of disease. High serum IL-6 concentration shows close relation to the disease activity in GCA [89]. The endothelial dysfunction seen in GCA patients during active disease, is normalized by corticosteroids [90]. Still prolonged therapy does not always induce complete remission and side effects are common. Several corticosteroid sparing medications have been investigated, but so far alternative immunosuppressive agents have been amazingly useless in treating GCA. Anyhow the results of two randomized controlled trials using methotrexate in GCA have led to somewhat conflicting conclusions.

CRP is a biochemical marker of systemic inflammation and is associated with cardiovascular disease as confirmed by numerous epidemiological studies [91]. Production of CRP by VSMC and macrophages, both as the protein and the mRNA, has been observed in atherosclerotic lesions [92, 93]. This supports the de novo synthesis in the vessel wall and the idea of CRP secretion by cells in the vessel lesion by paracrine/autocrine loops that result in elevated local concentrations of CRP. CRP may up-regulate AT_1 receptor mRNA and protein, revealed as an increase in the number of AT_1 receptor binding sites in VSMC [28]. In addition, Ang II–induced VSMC migration and proliferation are furthermore enhanced by CRP, and stimulates the production of extracellular matrix, effects attenuated by the angiotensin AT_1 receptor blocker losartan [94]. Available evidence thus suggests that CRP exerts a direct effect at the level of VSMC. One might assume that the angiotensin I converting enzyme (ACE) levels, responsible for generating Ang II, are elevated in GCA which is seen in other disorders with granulomas such as sarcoidosis. In a limited number of GCA case reports, the ACE serum levels were normal [95-97]. ACE is primarily produced by EC in the lungs and kidneys; hence it is surprising that I could not find any investigation of ACE expression in temporal artery samples.

Accumulating evidence supports the function of ET-1, in addition to its direct vasoconstrictor properties, as a pro-inflammatory molecule. For instance monocytes are stimulated to produce different cytokines by ET-1 [98]. ET-1 has an important role in atherosclerosis [72], inflammatory airway diseases [99] and cutaneous inflammation [100]. Furthermore mitogenic and proliferative effects on cells are mediated by ET-1 [45] hence participating in vascular remodelling. Also fibroblasts can be stimulated by ET-1, and hence increasing the production of collagen [101] . Thus in cardiovascular disorders such as heart failure [102] or myocardial ischemia [103] inflammation is partly driven by ET-1.

Aims of the thesis

- To examine if there is a change in the expression of endothelin and angiotensin receptors in subcutaneous arteries in patients with suspected but ruled out acute coronary syndrome.

- To correlate the altered expression of ET_B and AT_1 receptors in subcutaneous arteries in patients with different degrees of ischemic heart disease and the functional responses of the altered receptors.

- To measure and localize the AT_1 and AT_2 receptor expression in temporal arteries in patients with giant cell arteritis.

- To measure and localize the ET-1, ET_B and ET_A receptor expression in temporal arteries in patients with giant cell arteritis and to correlate the receptor expression to the degree of systemic inflammation using CRP as a marker.

Methods

Ethics

The ethics committee at Lund University Hospital (papers I-IV) approved the studies. Where applicable; information about the study was given by a nurse or a physician and informed consent was given by all study subjects. All participants were assigned to individual study codes used in experiments for integrity purposes.

Subject inclusion, tissue collection and human surgery procedures

Patients with ischemic heart disease (I-II)

Subcutaneous resistance arteries were removed from the abdominal wall in patients with different degrees of IHD. All patients had normal and non-ischemic electrocardiograms with normal blood markers of myocardial injury and no prior history of myocardial infarction (ACS), while the coronary artery bypass graft (CABG) patients had three vessel heart disease verified by angiography. By using the CABG group, comparison of the impact of different degrees of IHD on the receptor expression was enabled.

Obtained arterial tissue was placed in cold buffer on ice and transported to the laboratory, either frozen in ice-cold isopentane and stored at -80°C for immunohistochemistry or Western blot analyzed for their contractile properties in a sensitive myograph.

Patients with giant cell arteritis (III-IV)

The retrospective immunohistochemical study of temporal arteries included archival formalin fixed, paraffin-embedded tissue from the department of Pathology, Lund University. Positive biopsies of temporal arteritis from patients with GCA were included in the studies. The original pathology reports were reviewed to verify the diagnosis GCA. Then a pathologist specialized in the subject participated in selecting the cases for the study in conjunction with criteria based on the American College of Rheumatology 1990 for the classification of GCA (that have a sensitivity of 94% and a specificity of 91%) [104]. Also, we were cautious not to include cases with underlying cardiovascular disorders or atherosclerosis lesions, which was possible by careful chart review.

Control patients

Study (I-II)

Healthy volunteers matched for age and gender were included. All stated that they had no underlying cardiovascular disorder and were perfectly healthy without any ongoing pharmacological treatment.

Study (III-IV)

Using the original pathology reports as well as the clinical charts of the patients in conjunction with histopathology slides allowed for the verification, no presence of signs of inflammation were observed. The absence of inflammatory lesions in the temporal arteries led to the categorization of the sample as negative, age and gender matched controls were included.

Blood sampling

Study I-II

Blood samples for laboratory analysis were obtained from patients at the telemetry unit after the chest pain had resolved. For patients undergoing CABG surgery, blood samples were collected the day before the surgical intervention whereas for healthy controls the blood samples were withdrawn prior to the biopsy.

Study III-IV

Laboratory data were obtained by retrospective chart review.

Molecular techniques

Immunohistochemistry

Cell staining allowed for cellular location and measurement of angiotensin and endothelin receptors expressions in the arteries. Fluorescence (study I-II) and DAB immunostaining (study III-IV) were used.

Subcutaneous resistance artery biopsies were stored at -80 °C, preserving the antigens against decreased immunoreactivity prior to sectioning into 8-μm-thick slices. The sections were fixed, rehydrated and incubated overnight with antibodies directed against a part of the respective receptor protein.

Formalin fixed paraffin embedded blocks were cut into 4 µm sections and dried in 60 °C for 1h. After dewaxing and rehydration, the sections were treated with 10 mM citrate buffer pH 6.0 in a microwave oven for antigen retrieval, reversing conformational alterations of the receptors that might have occurred during the heating and the dehydration process during paraffin embedding. By using this procedure, formaldehyde cross links masking the epitopes for the antibodies (leading to decreased detection) is avoided. The automated stainer allowed for improved reproducibility and good reliability of the immunostaining.

Mayer´s Hematoxylin stain was used for counterstaining and primary antibodies were omitted for negative control, leading to no reaction due to inappropriate binding of the secondary antibody. Also, specificity was demonstrated by preadsorbing the antibodies with the desired antigen. The samples were examined using a microscope (Olympus optical Co, LTD, Bx60F5) and each section was photographed. The imaging was performed in a blinded approach, although it was possible to assume which patients had GCA due to the significant inflammatory responses. The absolute staining intensity was measured using the software ImageJ (http://rsb.info.nih.gov/ij/) which allows for computer controlled image processing. DAB stained digitalized images were processed in ImageJ that accurately delineate the stained tissue and permitted for measuring the mean staining intensity (in arbitrary units), see figure 2.

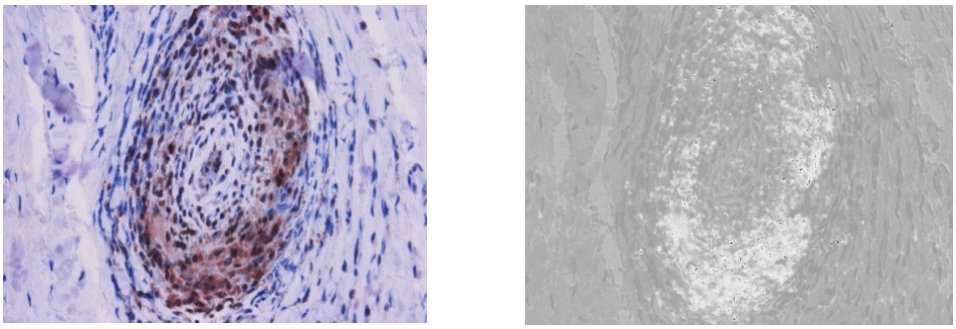


Figure 2. DAB stained image (left) digitally processed image for calculation of immunostaining intensities (right).

Western blot

For quantification of ET_B and AT_1 receptor protein expression, Western blots (Study II) were used. Subcutaneous resistance arteries were processed and proteins were denatured before separation with SDS-PAGE and blotting onto PVDF membranes followed by incubation with antibodies directed against AT_1 or ET_B receptors. For qualitative analysis ECL Plus Western blotting reagents were used.

In vitro pharmacology

Resistance arteries were cut into 1-mm-long cylindrical segments and mounted on two L-shaped metal prongs (Figure 3), unilaterally a force displacement transducer continuously recorded the isometric tension [105]. Mounted vessel segments were immersed in vessel baths at 37 °C containing bicarbonate based buffer solution. The buffer was continuously aerated with oxygen enriched with 5% CO_2 resulting in pH 7.4. Initially, vessels were stretched to a resting tone of 2 mN and was then allowed to stabilize at this tension for 1 h. Contractile capacity of each arterial vessel segment was controlled by exposure to a potassium rich (63.5 mM) buffer solution. Concentration-response curves were obtained by cumulative application of the ET_B receptor agonist, sarafotoxin 6c (S6c) at increasing concentrations and Ang II. Before the application of Ang II, the arteries were pretreated with the AT_2 receptor antagonist PD-123319 ($10^{-5.5}$ M) for 30 min. After washout, the vessels returned to baseline and ET-1 was then added at increasing concentrations (10^{-11}-10^{-6} mM). When ET_B receptors were desensitized [106] this facilitated ET-1 to act solely on the ET_A receptors. For details see Nilsson *et al.* [107].

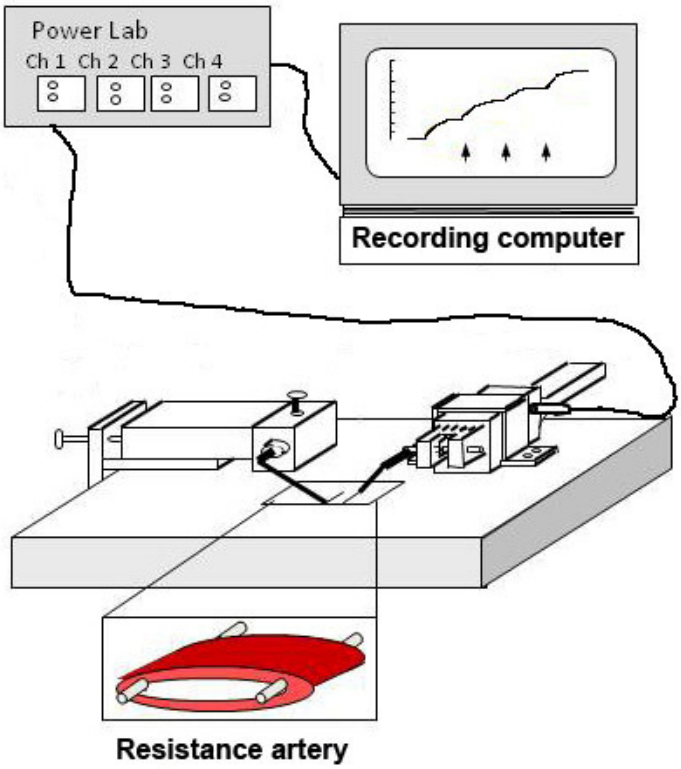


Figure 3. An in vitro myograph tissue bath system.

Calculations and statistical analysis

In paper I, Mann-Whitney test was the optimal way of comparing the two relatively small groups to obtain a more accurate P value as the two distributions were identical. In paper II, analyses were expanded involving three groups and ANOVA allowed for the comparison of the three groups. In addition Bonferroni's or Dunnet's post-test (multiple comparisons) either comparing the IHD patients to the control patients or IHD in between each other. For the in vitro pharmacology and Western blot analysis Student's t-test was used. In paper III-IV, Student's t-test was employed for the DAB immunohistochemistry staining comparisons. Statistical significance was defined as $p < 0.05$. Pearson correlation analyses were run to calculate the strength and direction of the relationship between receptor expression and laboratory variables, r, quantifies the direction and magnitude of correlation.

Results and Comments

Angiotensin and endothelin receptor up-regulation in ischemic heart disease (paper I-II)

Study I was conducted as a pilot study to develop a model for study of the alteration in receptor expression taking place in resistance arteries. There is an unexplained increased risk of future ischemic cardiac events and premature death, in chest pain patients where current IHD is ruled out [84]. Earlier data have pointed out increased Ang II and ET-1 serum levels in patients with IHD and atherosclerosis. Specifically AT_1 and ET_B receptors have been shown to be up regulated in different cardiovascular disorders such as atherosclerosis [49, 108-110]. In this study we used semi-quantitative immunohistochemistry, with antibodies previously used in our laboratory, directed against ET_A, ET_B, AT_1 and AT_2 receptor proteins, to measure the expression in subcutaneous resistance arteries from patients with chest pain, suspicious of unstable angina pectoris or myocardial infarction (i.e. acute coronary syndrome, ACS). Healthy subjects recruited by the research nurse from the local senior housing or relatives to the lab group, matched for age and gender, with no previous cardiac illness or medication were used as controls. Thus, the intention of the study was to evaluate if there exists early signs of receptor alteration in patients with suspected IHD. The subcutaneous resistance arteries served as a surrogate model for coronary microvasculature, as these are unfeasible to obtain.

The physiological parameters did not differ between the groups (Table in paper I) with the exception that the suspected ACS patients had increased systolic blood pressure (SBP) and slightly elevated $HbA1_c$ levels (Figure 4).

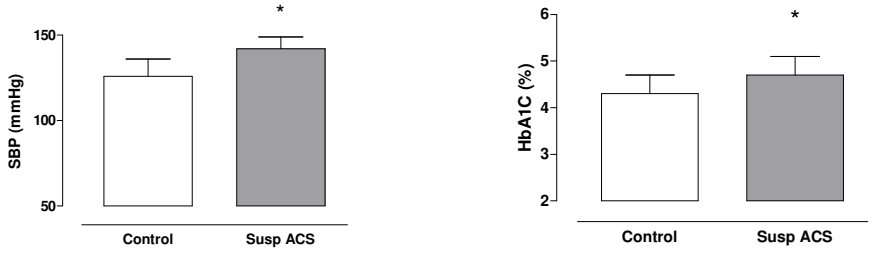


Figure 4. Systolic blood pressure (left) and HbA_{1C} (right) in patients with suspected ACS without established myocardial infarction and cardiovascular healthy controls. SBP = systolic blood pressure.

Interestingly, quantification of the immunofluorescence intensities revealed that the ET_B and AT_1 receptor expressions were higher in the VSMC in arteries from the patients with suspected ACS as compared to arteries from the control group (Figure 5). Similar up-regulation of ET_B and AT_1 receptors has been reported in atherosclerotic arteries [108, 110]. These findings together with the present results suggest that the activation of the endothelin and angiotensin systems is not only limited to muscular arteries of large diameter [108, 110] but also involves small resistance arteries in patients with early signs of IHD (present data), even prior to myocardial events. However, levels of ET_A and AT_2 receptor expression in VSMC in patients with suspected IHD showed no difference when compared to the healthy controls, which provides (i) good control of specificity of the disease process, and (ii) indicating possibly a less importance of these receptors in IHD.

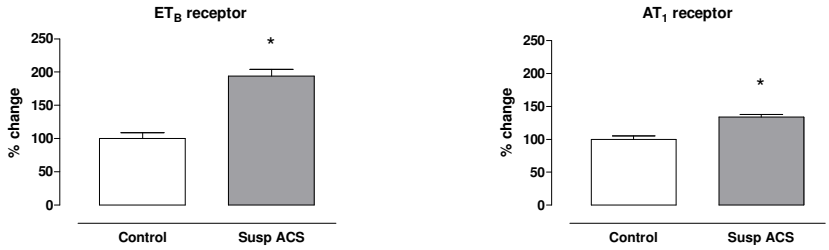


Figure 5. The ET_B and AT_1 receptor expressions were higher in the smooth muscle cells in the arteries/arterioles from the patients with susp ACS than in the arteries from the control group ($p<0.05$)

In situations where vasoconstriction and proinflammatory activities are unfavourable, such as in myocardial ischemia, AT_1 and ET_B receptors may be potential targets for pharmacological manipulations. The question whether the up-regulation of ET_B and AT_1 receptors in the resistance arteries in suspected ACS contributes to increased contractile responses, inflammation and/or proliferation in IHD cannot be concluded from the present study. Our laboratory has in a recent study reported that increased intraluminal perfusion pressure enhances the ET_B receptor and Ang II receptor expressions [111]. Thus, the increase in SBP noted may have confounded the result. Even if the glucose level was only slightly higher, still this might still have influenced the increase in AT_1 receptor expression. Up-regulation of AT_1 or ET_B receptors on VSMC have been associated with high glucose levels [112, 113]. Therefore to obtain a conceivable relation between the

clinical parameters expressed in the patients and the ET_B and AT_1 receptor expression, study II was staged in part to answer these questions. By specific inclusion of patients with two different degrees of IHD, patients with angina pectoris (AP) and patients undergoing coronary artery bypass grafting (CABG), were included.

The physiological parameters differed between the groups (Table in paper II). The differences are summarized in Table 2. The SBP and CRP were higher in the patients with IHD (both CABG and AP). CRP is an important and independent predictive risk factor for myocardial infarction, stroke, peripheral artery disease and sudden cardiac death even among apparently healthy individuals [114]. Also the plasma levels of N-terminal pro B-type natriuretic peptide (NT- proBNP) were higher in the CABG group than in the cardiovascular healthy controls and in AP indicating a slight ischemic heart failure.

Table 2

Clinical parameters	Control (n=10)	AP (n=15)	CABG (n=10)
NT-proBNP (ng/L)	106 ± 112	218 ± 243	1626 ±2157*
C-reactive protein (mg/dl)	2.1 ± 1.9	6.2 ± 15.3*	6.9 ± 6.1*
SBP (mmHg)	125 ± 9	142 ± 15*	140 ± 14*

Western blot and densitometry analysis showed that patients undergoing CABG surgery had a mean increase of ET_B receptor expression (133 ± 8%; $P<0.05$) as well as an increase of AT_1 receptor expression (137 ± 9%; $P<0.05$) that was significantly higher than in healthy controls.

The location of the receptor up-regulation was localized by immunohistochemistry to the VSMC as in study I and not in the endothelium or the adventitia. Quantification of the receptor expressions by measurement of the staining intensities revealed that both ET_B and AT_1 receptor expressions were increased in

VSMC and interestingly shown to be related to the burden of the atherosclerotic disease patients suffered from (figure 6). ET_B was found to a slight degree, as it should, in the EC but the levels of expression did not differ between the groups. Also we observed intact endothelium in all three groups.

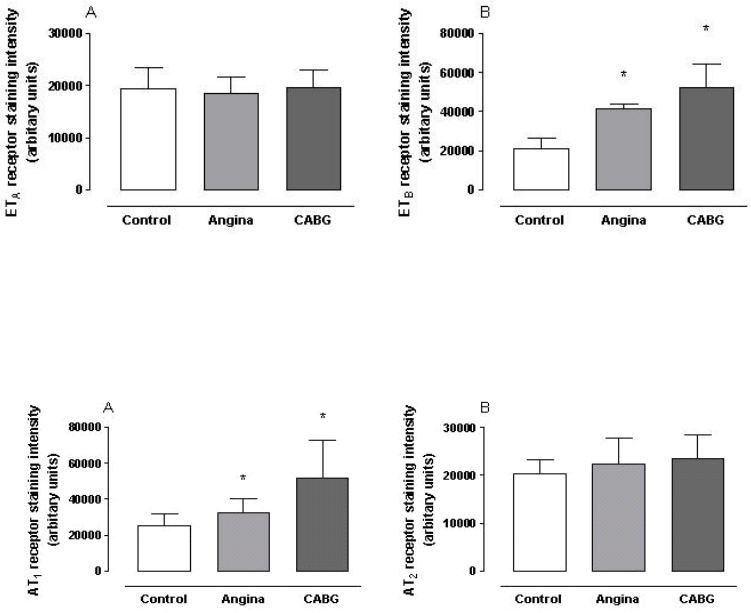


Figure 6. ET_A, ET_B, AT_1 and AT_2 receptor protein expression, assessed by immunohistochemistry, in human subcutaneous arteries from patients undergoing coronary artery bypass graft (CABG) surgery because of IHD (n=10), patients with AP without established myocardial infarction (n=15) and cardiovascular healthy controls (n=10). Values are expressed as mean ± SD. * $p<0.05$

In order to elucidate the impact of observed receptor up-regulation on the vasoconstriction of the resistance arteries, functional pharmacology experiments were performed. The selective ET_B receptor agonist S6c was used to study ET_B receptor-mediated contraction. Subsequently ET-1 induced vasoconstriction was studied when ET_B receptors were desensitized by the previous application of S6c leaving only ET_A receptors available for a contractile response [115]. S6c induced no vasoconstriction in healthy control vessels. On the other hand patients undergoing CABG showed a fairly strong and significant contractile response to the cumulative application of S6c ($P<0.05$, $n=5$) suggesting up-regulation of ET_B receptors at the functional level as well. Because the vessel size was very small we had to have new subcutaneous biopsies from the abdomen from reconstructive surgery of healthy subjects. The thoracic surgery could in some cases obtain slightly larger vessels than suitable for in vitro pharmacology. As ET-1 induced a potent vasoconstriction in both healthy controls and in patients undergoing CABG surgery, the conclusion was that there were no obvious changes in the ET_A receptor responses between the two groups.

These data are in concert with a previous study that shows an up-regulation of ET_B receptors in human atherosclerotic lesions [109]. The elevated ET_B receptor-mediated contraction and the immunohistochemistry data were verified by protein analysis with Western blot. The increased levels of contractile ET_B receptors on the VSMC could play an important role in IHD since ET_B receptor activation in healthy controls did not induce any contractions at all. This amplified activity in the endothelin system and in resistance arteries could contribute to VSMC proliferation, vasoconstriction and decreased perfusion of the microvasculature in atherosclerotic disease [116, 117]. Anyhow, changes were only noted in IHD. Since endothelial dysfunction was not measured, the role of the endothelium and alteration of its vasodilator components cannot be concluded herein apart from the up-regulation of contractile ET_B receptors on VSMC.

However, the Ang II induced concentration-dependent vasoconstriction response did not differ between healthy controls and patients undergoing CABG surgery. It should be noted that we used an AT_2 receptor antagonist to exclude the possible influence of a relaxant AT_2 receptor. A reasonable explanation could be that the observed increased AT_1 receptor expression in resistance arteries has other capabilities apart from vasoconstriction or that the increased use of ARB could have influenced the obtained results.

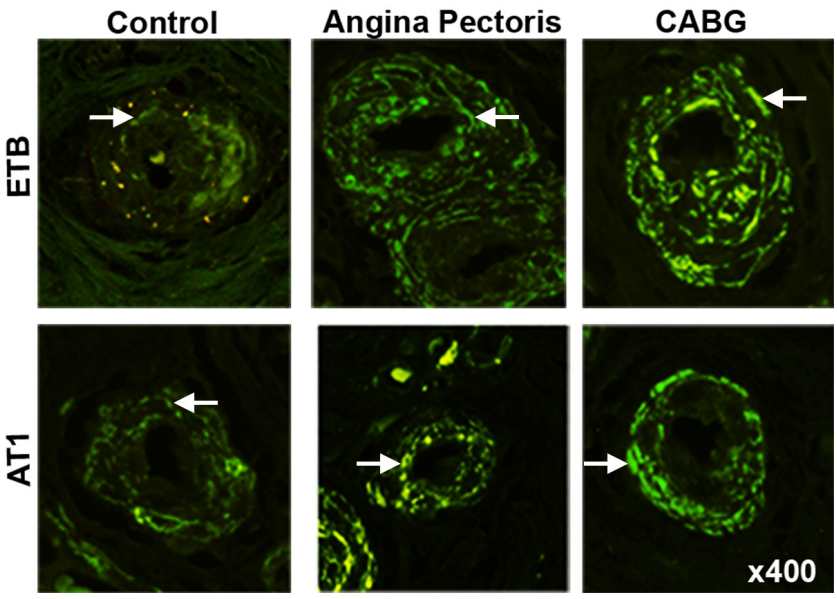


Figure 7. Representative examples showing immunofluorescence staining experiments for ET_B and AT_1 receptors in human subcutaneous arteries. Note that the immunostaining intensity for both ET_B and AT_1 receptors, indicated with arrows, is higher in the arteries from patients with IHD than from healthy controls.

Taken in to account that hypertension has been shown to contribute to increased endothelin receptor expression [107] it was important to consider this when explaining ET_B or AT_1 receptor up-regulation (figure 7). The blood pressure was elevated in AP and CABG patients but without significant difference between these two groups. Still there was a significantly higher expression of both ET_B and AT_1 receptor expression in the VSMC in the CABG groups as compared to the AP. Thus, the blood pressure alone could only partly explain the difference observed in the receptor expressions and other factors have to be taken into consideration (e.g. local inflammation).

Receptor expression and correlation to clinical parameter

The quantitative immunohistochemical data (figure 6) allowed for the analysis of a putative correlation to CRP, SBP or NT-proBNP levels with AT_1 and ET_B receptors immunoreactivity in the VSMC. One of these factors could have been heart failure secondary to ischemia, based on significantly increased NT-proBNP levels in the CABG patients, indicating some degree of heart failure, a condition associated with increased ET_B receptor-mediated systemic vasoconstriction [118]. Our laboratory has previously shown that mRNA levels of endothelin receptors are elevated in coronary arteries in IHD [119] as well as that Ang II and ET-1 receptor levels are increased in arteries in hypertension [111]. Anyhow contrary data concerning the AT_1 mRNA levels in coronary arteries in heart failure exists [120]. Still NT-proBNP levels showed no correlation to the expression of ET_B or AT_1 receptor in the VSMC in resistance arteries in patients with IHD. However, the systolic blood pressure showed a positive correlation to the ET_B receptor expression in patients with AP ($r=0.54$, $p<0.05$). Interestingly there was a difference in expression of ET_B and AT_1 receptors between the AP and CABG group even if they had similar SBP indicating that some other factor apart from SBP could have influenced the expression. Calculation of the receptor expression, in relation to CRP did not expose any positive correlation. In conclusion, the data demonstrates that hypertension positively correlates to the up-regulation of ET_B receptor in VSMC in subcutaneous resistance arteries from patients with AP but not in CABG patients. Systemic inflammation and heart failure did not influence the receptor expression. It is important to recognize that the study population was limited (low in number of individuals) which could have given a false negative result. It needs to be taken into account that the observed correlations between on one hand CRP, proBNP and blood pressure and on the other the Ang II and ET-1 receptor expression, is based on a small patient group and needs further exploration. Low levels of systemic CRP in the circulation tells little about the local concentrations of CRP in tissue that could have been much higher. Also SBP is elevated in situations with pain; however this was taken into account since the blood pressure measurements were obtained after chest pain was relieved.

In study I and II the level of ET_B receptor expression was higher in the VSMC layer of arteries from patients with IHD. The ET_B receptor expression was highest in arteries from patients undergoing CABG surgery. Up-regulated ET_B receptor expression on VSMC have been shown in patients with diabetes, hypertension as well as in human atherosclerotic coronary arteries and in atherosclerotic plaques [108, 111, 113, 121]. Plasma levels of ET-1 are elevated in patients with IHD or

heart failure; it has been suggested as a prognostic marker. In patients with ischemic or non-ischemic cardiomyopathy, ET-1 levels were an important predictive indicator for increased mortality during the first year in patients with decreased left ventricular function [122] as in patients with acute myocardial infarction [123]. The circulating ET-1 levels are further increased in patients undergoing CABG surgery [124]. This increased activity in the endothelin system may contribute to VSMC proliferation, vasoconstriction and decreased perfusion as well as attract monocytes in atherosclerotic disease [116, 117, 125]. Furthermore, CABG patients had significantly increased proBNP levels indicating some degree of heart failure, a situation associated with increased ET_B receptor-mediated systemic vasoconstriction [118]. Elevated ET_B contraction and increased receptor expression (verified by Western blot) in the present study, indicates that the increased levels of contractile ET_B receptors on the VSMC could play an important role in IHD. ET_B receptor activation in healthy controls did not induce any contractions at all.

Angiotensin and endothelin receptor expression in giant cell arteritis (paper III-IV)

Angiotensin receptors in GCA

Immunostaining intensities of Ang II receptors in sections of temporal arteries from patients with GCA were compared to sections of temporal arteries negative for GCA. Ideally, one should have compared to healthy control temporal arteries; such are however difficult to obtain.

Clinical parameters (see paper III) showed increased systemic inflammatory mediators in patients with giant cell arteritis, which is a common phenomenon in this disease. Immunostaining of AT_1 receptors were localized to the VSMC and to a lesser extent to the EC. In temporal arteries from patients with GCA, AT_1 receptor positive staining was observed in lymphocytes, histocytes, multinucleated giant cells and VSMC as well. AT_1 receptor staining was more intense in the temporal arteries from patients with GCA than in the controls. On the other hand, AT_2 receptor immunostaining was only faint and primarily localized in the EC and to a lesser extent on the VSMC. Interestingly there was no difference in AT_2 receptor expression observed between temporal arteries from the patients with GCA and controls. However, this was presumed to be due to AT_2 receptors not being involved in inflammation as much as to induce vasodilatation, inhibit cell growth and stimulate apoptosis [30].

GCA and atherosclerosis have similar injury pathways, both of which involve vessel inflammation. Ang II modulates the immune response via the production of NF-κB in the cytoplasm of mature phagocytes, resulting in the transcription of inflammatory cytokines and chemokines, including IL-1β, IL-6, IL-8, IFNγ, TNF-α, monocyte chemotactic protein-1 and cell adhesion molecule 1 [81]. Angiotensin-converting enzyme inhibitors (ACEi) reduce the expression of monocyte chemo-attractant protein-1 and concomitant macrophage plaque infiltration in animal models of atherosclerosis [126]. In patients with carotid artery disease, the angiotensin receptor blocker irbersartan inhibits metalloproteinase activity, as well as T-cell and macrophage infiltration in the vascular wall [127].

Based on this, inhibitors of the RAAS might be an attractive alternative approach for the treatment of GCA with increased AT_1 receptor activity. Since corticosteroids are the only proven treatment for GCA, interest lies in identifying corticosteroid-sparing alternatives since numerous complications are associated with long-term corticosteroid treatment [128]. The complications appear to be dose related and corticosteroid-sparing medications may therefore be beneficial. Trials have examined methotrexate (MTX) [129, 130] and anti-TNF-α agents [131]. None of these therapies can, to date, be recommended for GCA. However the two randomized trials using MTX present divergent results. Hoffman et al. did not find any significant improvement by addition of MTX contrary to Jover et.al. who found 10 mg MTX in addition to corticosteroids reduced relapse. Interestingly TNF-α inhibition with infliximab in conjunction with corticosteroids had no effect on the proportion of relapses compared to placebo even if the involvement of TNF-α expression has been shown to be increased in GCA lesions. From these findings, an elegant approach reducing the corticosteroid use in GCA would be targeting the AT_1 receptors with inhibitors of the RAAS.

Given that the AT_1 receptor seems to be a good sensor of the inflammatory activity in the vessel wall in GCA it is fair to question if it is purely up-regulated secondary to ongoing inflammation.

Endothelin-1 and ET receptors in GCA

Elevated serum levels of ET-1 have been reported in GCA in a limited group of four study subjects [132], as well as in temporal arteries in GCA in a contemporary study by Lozano et.al. [133]. A general up-regulation of the components of the endothelin system was also noted in temporal arteries in GCA by Lozano et.al. as well. The specific role of ET-1, endothelin A (ET_A) and B (ET_B) receptors in GCA

is unknown but White et al. have shown that the increased vasoconstriction of temporal arteries upon inflammatory stimulation (exogenous cytokine application) is mediated by ET_B receptors [134]. The ET-1 and its receptors were investigated by immunohistochemistry in paper IV.

Increased ET-1 and ET_B receptor immunoreactivity was found in the medial layer of the temporal arteries (VSMC) and in EC, in patients with GCA. No alteration in the ET_A receptor expression in the VSMC or the EC was detected compared to Lozano et al. Even if the neointima had ET_A staining this was too little to be detected in our experiments. Interestingly, VSMC seems to undergo a phenotypic change seen in inflammation into a prosecretory state with ET-1 production. Compared to the controls there was a clear increase in tissue ET-1 and ET_B receptor immunoreactivity in the VSMC as quantified and compared to the healthy controls (for details see figure in paper IV). Elevated tissue ET-1 concentrations have also been found in atherosclerotic lesions; therefore cases with that kind of morphology were excluded in the study. However, it is unclear how and if atherosclerosis influenced the results by Lozano et al.

As expected, immunostaining of ET_B receptors was observed in the endothelial cell layer of tissue sections taken from control subjects. In the GCA patients there was an additional positive ET_B receptor expression in the VSMC layer of the temporal arteries (see figure paper IV). In patients with GCA, the ET_B receptor immunostaining was increased in VSMC (+75% ± 18%; $p < 0.05$) and in the endothelium (+49 ± 8%; $p < 0.05$) compared with controls. Multinucleated giant cells exhibited distinct ET_B receptor immunostaining in GCA.

The quantitative immunohistochemical data allowed for the analysis of a putative correlation to CRP or ESR levels. CRP levels were found to positively correlate to the degree of ET-1 ($r=0.75$,$p <0.05$) and ET_B ($r=0.65$, $p < 0.05$) receptor expression in temporal arteries from patients with GCA (Figure 8), whereas no significant correlation with ESR was found. This finding suggests that the ET-1 system via ET_B receptors is activated in GCA. However the included patients are only representative for cases with a positive temporal artery biopsy and it is difficult to make a general assumption on this. No changes were observed in ET_A receptor expression in VSMC or EC compared to controls.

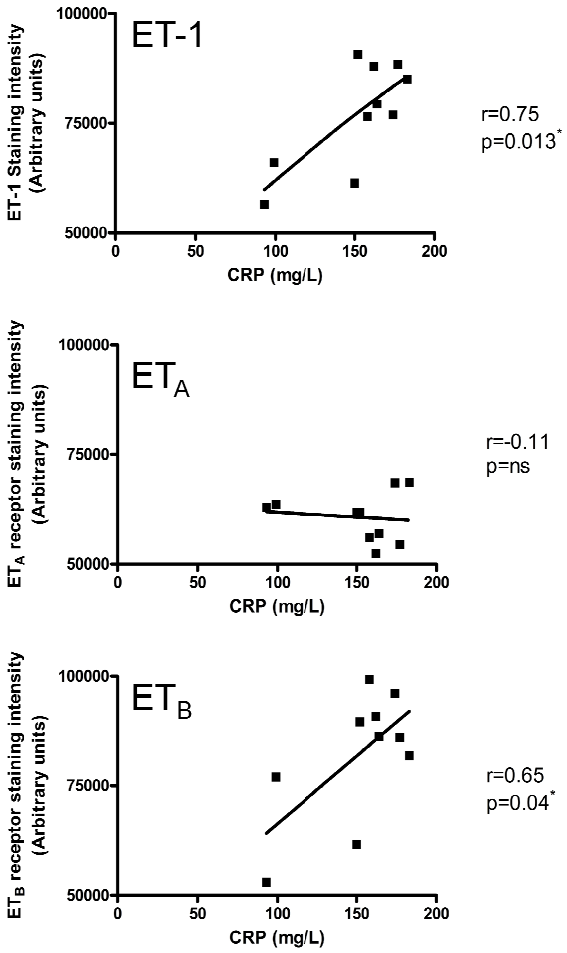


Figure 8. Demonstration of a possible association between the ET-1, ET_A and ET_B receptors immunostaining and levels of CRP. The linear regression line (solid) equation is for non-zero slope. Statistical analysis was performed using Pearson's coefficient of correlation. r = correlation coefficient, ns = not significant, p represent p-value and * significantly lower than $P < 0.05$. Note that the levels of ET-1 and ET_B receptors correlated significantly to the levels of CRP.

Major conclusions

The main hypothesis of the work was that there is a correlation between inflammation and receptors of the Ang II and ET-1 systems. One feature of dysfunctional arteries is increased vasoconstriction by activation of the Ang II or the ET-1 systems. Patients with different degrees of vessel inflammation were investigated concerning the receptor expression of Ang II and ET-1 in the VSMC. Subcutaneous resistance arteries from patients with IHD or temporal arteries from patients with GCA were investigated, because they have different degrees of inflammation. Focus was on the specific receptor alteration in inflammatory arterial disorders. Measured by immunohistochemistry (IHC) or western blot, quantification of the receptor expression was possible. In addition in vitro pharmacology allowed for the study of the functional consequences of the receptor alterations.

AT_1 receptor expression was increased in the VSMC in resistance arteries from patients with IHD and the degree depended on how much IHD there was. No alteration of the AT_2 receptor expression was observed. Interestingly, this phenomenon was similar in temporal arteries from patients with GCA, which is a completely different vascular inflammatory disorder but with very severe inflammation. However, the up regulation of AT_1 receptors in resistance arteries did not involve increased vasomotor response compared to healthy controls; it could involve other items such as vessel remodelling with increased VSMC proliferation, cell growth or extracellular matrix formation.

ET_B receptor expression was increased in the VSMC layer in resistance arteries from patients with IHD. No alteration of the ET_A receptor was observed. The level of up regulation of ET_B receptors was related to the degree of IHD. The IHC data was quantified and western blot also confirmed the observation. Interestingly, we observed a similar phenomenon was comparable in temporal arteries from patients with GCA. In contrast, however in GCA there is a strong positive correlation between the systemic inflammatory response and both the level of ET_B receptor expression in the VSMC and the level of ET-1 peptide respectively. In IHD there was no such correlation. However, vessels from patients with IHD show an increased vasoconstriction response upon ET_B receptor activation compared to healthy controls. ET_A receptor induced vasoconstriction did not vary depending on IHD.

Endothelin receptor expression and function in inflammatory arterial disorders

ET_B receptors are up-regulated on VSMC in diabetes, hypertension and in atherosclerosis [108, 111, 113, 121]. In addition plasma levels of ET-1 are elevated in heart failure, IHD or GCA and has been suggested as a predictive marker [135] and of great importance in the development of cardiovascular disorder. During CABG surgery circulating ET-1 levels are additionally increased [124]. ET_B receptor expression was undoubtedly increased in the VSMC layer of resistance arteries from patients with IHD as well as in temporal arteries from patients with GCA and appeared to correlate with the degree of inflammation. VSMC might as a result of the increased ET-1 activity proliferate, induce vasoconstriction and decrease perfusion in atherosclerotic disease [116, 117]. Enhanced ET_B receptor mediated contraction in IHD could contribute and play an important role in IHD and inhibition of ET_B receptors serves as a potential and attractive approach modulating vasoconstriction.

Angiotensin II receptor expression

Different pathological situations in man have been shown to alter Ang II receptor expression in arteries; such as heart failure [120], hypertension [136], hypoxia [137], hypercholesterolemia [110] and hyperglycemia [138]. Stimulation of AT_1 receptors results in progression of atherosclerotic lesions, inflammation and plaque rupture apart from being a potent vasoconstrictor and mitogen for VSMC. Increased expression of AT_1 receptors might make the vasculature prone to develop spasm and atherosclerotic plaques, and thus further increase peripheral vascular resistance by reducing vessel lumen, situations that are important in threatening ischemia. However, no enhanced contraction of Ang II is mediated via AT_1 receptors. The reason for this could not be concluded from the experiments but could depend on increased use of AT_1 receptor antagonists in patients undergoing CABG surgery, thus blocking the AT_1 receptors. It is important, however, not to forget that AT_1 receptors apart from their contractile properties also possess other abilities such as regulating cell growth, differentiation and fibrosis which are important in the pathology of heart failure, hypertension and atherosclerosis ability especially important in the phenotypic switch and activation of VSMC.

Future perspective

The mechanisms behind up-regulation of ET_B and AT_1 receptors in IHD and GCA are not known. Today several ET receptor antagonists have been developed, principally to target cardiovascular disease states which also are true for AT_1 receptor antagonists. Currently high doses of corticosteroids are used in the treatment of GCA and no effective corticosteroid-sparing drugs are available. Inflammation in GCA may probably be modulated by inhibitors of the ET-1 or Ang II systems. Thus, inhibition of these systems may provide a corticosteroid-sparing alternative for the treatment of GCA especially upon threatening blindness with hampered circulation on behalf of vascular occlusion, a condition that today has no treatment. Moreover acute coronary disease is today treated by angioplasty adjusting the conductance arteries leaving the myocardial microcirculation without modification.

Investigation of which intracellular signaling pathways are activated by the Ang II or ET-1 is gaining more importance. This lies in interest of my future investigations.

Summary in Swedish

Kärlsjukdomar; som t.ex. åderförfettning och temporalis arterit (TA) kan leda till syrebrist (ischemi) i ändorgan med åtföljande infarcering. Skador på hjärtmuskeln kan resultera i hjärtinfarkt och vid TA kan man få synnedsättning eller blindhet. Båda tillstånden har allvarliga konsekvenser för patienterna och tycks bero på sjuklig kärlsammandragning eller förträngning. Trots utveckling av nya behandlingar och mediciner de senaste 40 åren för akuta ischemiska hjärtsjukdomar är dessa alltjämt sammankopplade med den högsta dödligheten i västvärlden och med svåra följdsjukdomar. Beträffande TA-behandling saknas det idag alternativ till kortison; vilken också är förknippat med mängder av biverkningar.

Kroppens artärer (blodkärl som leder blod från hjärtat till olika organ) ansvarar för att transportera syre och näringsämnen till organen i kroppen. De tar samtidigt upp skadliga nedbrytningsprodukter och metaboliter, och avlägsnar dessa från cirkulationen. Kärlen utsätts för kroppsegna och icke kroppsegna skadliga faktorer som tobaksrök, kolesterol, högt blodtryck och inflammatoriska produkter. Långvarig exponering av blodkärlen för dessa faktorer leder till uppkomsten av olika kärlsjukdomar. Vanligaste formen av kärlsjukdom är åderförfettning (arterioskleros) med en betydande inflammatorisk komponent. Åderförfettning drabbar alla artärer i kroppen, vanligast är kärlkramp eller hjärtinfarkt då förträngningarna i kranskärlen orsakar syrebrist (ischemi). Ischemisk hjärtsjukdom (IHD) är den största folksjukdomen i västvärlden och dominerar ekonomiskt dagens sjukvård.

En annan sjukdom med kärlinflammation är TA, som är begränsad och bl.a. angriper kärl i tinningregionen, vilket kan påverka den viktiga blodförsörjningen av ögonen med blindhet som konsekvens.

Inflammation är kroppens skydd mot de skadevållande faktorerna och central i uppkomsten av båda dessa kärlsjukdomar. Inflammationsprocessen är dock förknippad med bieffekter. En viktig bieffekt gemensam för inflammatoriska kärlsjukdomar är en försämring av blodkärlens väggar med sjuklig kärl-sammandragning. Avgörande komponenter för sjuklig kärlsammandragning är förhöjd halt av två av de signalsubstanser som ansvarar för kontraktion av kärlet, endothelin-1 (ET-1) och angiotensin II (Ang II). ET-1 och Ang II verkar genom inbindning till sina specifika budbärarstrukturer (receptorer), proteinmolekyler på cellytan som fungerar som molekylära strömbrytare och ansvarar för överförandet av signaler till cellen. Vid olika kärlsjukdomar har man sett att ET-1 och Ang II

receptorers uttryck kan uppregleras och ge upphov till förstärkt signalering, med åtföljande abnorm kärlsammandragning.

Syfte

Ändamålet med avhandlingen var att utforska hur ET-1 och Ang II receptorers uttryck ändras vid inflammatoriska hjärt- och kärlsjukdomar. Delstudie I-II karlägger förändringarna i uttryck av Ang II och ET-1 receptorer vid ischemisk hjärtsjukdom. I studie III-IV studerades artärer från patienter med TA för att fastställa huruvida modifikationerna av Ang II och ET-1 receptorerna är ett globalt fenomen vid kärlinflammation. Studie IV kartlägger även sambandet mellan graden av kärlinflammation och endotelinreceptoruttryck.

Metoder

Artärerna kommer från patienter med olika grader av ischemisk hjärtsjukdom, uttagna kirurgiskt från underhudsfett (Studie I-II) samt från lagrade biopsier av TA (Studie III-IV). Artärerna analyserades för graden av uttryck av signalmolekylerna Ang II och ET-1, samt deras receptorer i artärer med specifika antikroppar. Immunohistologiska analyser utfördes i samtliga arbeten för att kartlägga var i blodkärlen receptorförändringarna förekom. Metoden möjliggjorde även att mäta mängden av receptoruttryck indirekt. I delarbete II användes även sk. myografer; en metod som mäter graden av sammandragande eller vidgande effekter i artärer. För att styrka observationerna av receptor- uppreglering nyttjades även western blot, en metod som fastställer proteinuttryck.

Resultat

Oberoende av typen av underliggande inflammatorisk kärlsjukdom föreligger ett ökat uttryck av endotelin B receptorer (ET_B) samt Ang II typ 1 receptorer (AT_1) hos patienter med TA och IHD. Patienter med IHD uppvisade förstärkt kärlsammandragande svar på ET-1 vilket förmedlas via ET_B receptorerna. Graden av systemisk inflammation i artärer är direkt relaterad till graden av ET-1 och ET_B receptoruttryck vid TA.

Slutsats

Ökat uttryck av angiotensin och endotelin receptorer (AT_1 och ET_B) i artärerna är ett generellt fenomen som är oberoende av underliggande genes till kärlinflammation. Patienter med IHD har sjuklig sammandragning av artärerna som kan bero på en uppreglad ET_B receptorsignalering. Våra fynd har gett ny kunskap om effekten av angiotensin och endotelin på blodkärl och hur detta förändras vid inflammatorisk kärlsjukdom. Detta kan frambringa nya farmakologiska angreppspunkter och en mer riktad läkemedelsbehandling i framtiden.

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My Co authors

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