Increased vascular endothelin type B and angiotensin type 1 receptors in patients with ischemic heart disease

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Short title: ET\textsubscript{B} and AT\textsubscript{1} receptors in ischemic heart disease

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Abstract

Background
Endothelin-1 and angiotensin II are strong vasoconstrictors. Patients with ischemic heart disease have elevated plasma levels of endothelin-1 and angiotensin II and show increased vascular tone. The aim of the present study was to examine the endothelin and angiotensin II receptor expression in the subcutaneous arteries from patients with different degrees of ischemic heart disease.

Methods
Subcutaneous arteries were obtained, by biopsy from the abdomen, from patients undergoing coronary artery bypass graft (CABG) surgery because of ischemic heart disease (n=10), patients with angina pectoris without established myocardial infarction (n=15) and matched cardiovascular healthy controls (n=10). Endothelin type A (ET_A) and type B (ET_B) and angiotensin type 1 (AT_1) and type 2 (AT_2) receptors expression was examined using immunohistochemistry.

Results
ET_A and, to a lesser extent, ET_B receptors staining was observed in the vascular smooth muscle cells. The level of ET_B receptor expression was higher in the patients undergoing CABG surgery (250% ± 23%; p<0.05) and in the patients with angina pectoris (199% ± 6%; p<0.05), than in the healthy controls (100% ± 28%). No such difference was found for the ET_A receptors. In the endothelial cells, primarily ET_B receptor immunostaining was observed.
AT₁ and, to a lesser extent, AT₂ receptors immunostaining was seen in the vascular smooth muscle cells. The levels of AT₁ receptor expression were higher in both the angina pectoris (128% ± 25%; p<0.05) and in the CABG patients (203% ± 41%; p<0.05), than in the healthy controls (100% ± 25%). No such difference was found for the AT₂ receptors. In endothelial cells, primarily AT₂ receptor immunostaining was observed and did not change in heart disease.

Conclusions
These results demonstrate for the first time, upregulation of ET₉ and AT₁ receptors on vascular smooth muscle cell in ischemic heart disease. These receptors may play a role in the pathophysiology of ischemic heart disease and could provide important targets for pharmaceutical interventions.

Index terms: Vasculature • ischemic heart disease • endothelin • angiotensin •
CABG surgery • immunohistochemistry
Background

The renin-angiotensin and the endothelin system are essential in vascular homeostasis and have become maladaptive in cardiovascular diseases [1]. Angiotensin II and endothelin-1 are formed in the endothelium and induce potent vasoconstriction and proliferation of vascular smooth muscle cells [2, 3]. The continuous interaction between endothelin-1 and angiotensin II participate in the control of vessel tone and changes in the endothelin and renin-angiotensin-systems can give rise to dysfunctional vessels such as that seen in patients with cardiovascular risk factors [4]. Endothelin-1 and angiotensin II have therefore been suggested to play a role in the development if cardiovascular diseases, including hypertension[5], chronic heart failure [6] and atherosclerosis [7].

Endothelin-1 mediates its effect through two distinct G-protein coupled receptors; the endothelin type A (ET\textsubscript{A}) and type B (ET\textsubscript{B}) receptors. During physiologic conditions, the ET\textsubscript{A} receptor is the dominant receptor subtype expressed in vascular smooth muscle cells and mediates contraction, while the ET\textsubscript{B} receptor is primarily located on endothelial cells and mediates vasodilatation via the release of nitric oxide and prostaglandins [8]. However, ET\textsubscript{B} receptors on vascular smooth muscle cells have been observed to be upregulated during pathological conditions such as atherosclerosis [9], congestive heart failure [10], and ischemic heart disease [11]. Endothelin receptors on vascular smooth muscle cells are both mitogenic, leading to atherosclerosis, and can induce strong vasoconstriction resulting in elevated vascular tone which contribute to the development of cardiovascular disease.
Two angiotensin II receptors have been identified in man, AT\textsubscript{1} and AT\textsubscript{2} receptors, which are members of the G-protein coupled, seven-transmembrane domain receptor family. The vascular effects of angiotensin II are primarily mediated by AT\textsubscript{1} receptors located on smooth muscle cells which induce vasoconstriction and mitogenesis. Conversely, AT\textsubscript{2} receptors are located on endothelial cells and are known to induce vasodilatation, inhibit cell growth and stimulate apoptosis [12].

AT\textsubscript{2} receptors have been shown, although in smaller amounts, on vascular smooth muscle cells, to induce vasoconstriction. Angiotensin II is a potent vasoconstrictor and a growth factor that regulates cell growth and fibrosis, and has been implicated in the pathology of heart failure, hypertension and atherosclerosis [12].

Previous studies on the effects of endothelin-1 and angiotensin II in the human peripheral vasculature have mainly been performed using a forearm blood flow model. To our knowledge, this is the first detailed \textit{in vitro} assessment of tissue samples from the peripheral vasculature in patients. The peripheral vasculature is important for regulating total peripheral resistance. Therefore, small peripheral arteries and arterioles, obtained from the subcutaneous tissue in patients, were used for the present study. Fifteen patients with angina pectoris without established myocardial infarction, 10 patients undergoing coronary artery bypass graft (CABG) surgery because of ischemic heart disease and 10 cardiovascular healthy controls, were included in the study. Endothelin ET\textsubscript{A} and ET\textsubscript{B} and angiotensin AT\textsubscript{1} and AT\textsubscript{2} receptor expression on vascular smooth muscle cells were examined using immunohistochemistry staining.
Methods

Study groups

Three different groups of patients were included in the study.

1. Cardiovascular healthy controls (controls). This group consisted of ten healthy volunteers without previous history of chest pain, cardiovascular disease or prior cardiac medications.

2. Patients with angina pectoris without established myocardial infarction (patients with angina pectoris). These fifteen patients were admitted to the medical emergency unit with angina pectoris without signs of myocardial infarction as measured by electrocardiogram and biomarkers for myocardial injury.

3. Patients undergoing coronary artery bypass graft surgery (CABG). These ten patients were undergoing CABG because of ischemic heart disease confirmed by coronary angiography showing three vessel disease.

The patient background characteristics are defined in Table 1.

Tissue collection

A biopsy was taken from the subcutaneous tissue of the abdomen. For the control group and the patient with angina pectoris, this was done during local anaesthesia (2 ml, 1% Xylocain; Astra Zeneca, Sweden) and for the patients undergoing CABG surgery the biopsy was taken under general anesthesia during the initiation of surgery. A 1 cm³ of subcutaneous tissue, containing small arteries, was removed from the abdomen. The tissue was frozen in ice-cold isopentane and stored at -80°C.
**Ethics**

The project was approved by the Ethics Committee of Lund University in Sweden (LU Dnr:308/2004) and conforms to the principles outlined in the Declaration of Helsinki. Each individual provided written consent to the procedure.

**Immunohistochemistry**

The biopsies were stored at -80°C prior to sectioning into 8-µm-thick slices in a calibrated Microm HM500M cryostat (Microm). The sections were fixed and rehydrated in phosphate-buffered saline (PBS). The sections were incubated overnight with rabbit anti-human ET\textsubscript{B} (16207, IBL) diluted 1:400, goat anti-human ET\textsubscript{A} (sc-21194, Santa Cruz Biotechnologies) diluted 1:50, rabbit anti-human AT\textsubscript{1} (sc-1173, Santa Cruz Biotechnologies) diluted 1:50, rabbit anti-human AT\textsubscript{2} (sc-9040, Santa Cruz Biotechnologies) diluted 1:50, mouse anti-human CD-31 (M0823, DAKO) diluted 1:100, mouse anti-human alpha-actin (M0851, DAKO) diluted 1:1000. All dilutions were done in PBS with 10% fetal calf serum. Antibodies to alpha-actin was used to localize smooth muscle cells and antibodies to CD-31 was used to localize endothelial cells. The secondary antibodies used were donkey anti-rabbit Cy\textsuperscript{TM3} conjugated (711-165-152, Jackson ImmunoResearch) 1:100, donkey anti-goat Cy\textsuperscript{TM3} conjugated (705-165-003, Jackson ImmunoResearch) 1:100 and donkey anti-mouse Texas Red conjugated (715-076-150, Jackson ImmunoResearch) 1:200 in PBS. As control, only secondary antibodies were used. The antibodies were directed against a part of the respective receptor protein; this amino acid sequence was used as control...
to block the antigenic site. The data from both immunocytochemistry and western blot are given at the respective company home page.

Calculations and statistics

The experiments were performed using arteries from 15 patients with angina pectoris, 10 patients undergoing CABG surgery and 10 controls. The samples were examined in a fluorescence microscope (Olympus optical Co, LTD, Bx60F5) absolute fluorescence intensity was measured with ImageJ (http://rsb.info.nih.gov/ij/). Mean fluorescence values in the area selected were obtained. Each sample was investigated blinded to the observer, in three sections and at four different regions, and the mean values were calculated. Statistical analysis was performed using ANOVA with Bonferroni’s or Dunnet’s post-test test for multiple comparisons. Significance was defined as P<0.05 (*). The data are given in the text as percentage difference relative to the mean fluorescence seen in the control. The results are presented as mean values ± standard deviation (SD) and N equals number of patients.
Results

Endothelin receptor expression

In healthy controls, the vascular smooth muscle cells stained for ET_A and, to a lesser extent, ET_B receptors. The immunostaining intensities for ET_B receptors were higher in arteries from the patients undergoing CABG surgery (250% ± 23%; p<0.05) and from the patients with angina pectoris (199% ± 6%; p<0.05), than from the healthy controls (Figure 1 and 3). Furthermore, the levels of ET_B receptor expression were higher in the smooth muscle cell layer in the arteries from the patients undergoing CABG surgery that in the arteries from the patient with angina pectoris (Figure 1B). The levels of ET_A receptor expression, in the smooth muscle cells, were similar in all three groups (p = n.s.). In endothelial cells, it was primarily ET_B receptor immunostaining that was observed.

Angiotensin II receptor expression

The vascular smooth muscle cells stained for AT_1 and, to a lesser extent, AT_2 receptors. The levels of AT_1 receptor expression were higher in both the angina pectoris (128% ± 25%; p<0.05) and in the CABG patients (203% ± 41%; p<0.05), than from the healthy controls (Figure 2 and 3). In addition, the levels of AT_1 receptor expression were higher in the smooth muscle cell layer in arteries from patients undergoing CABG surgery that in arteries from patient with angina pectoris (p<0.05). AT_2 receptor expression was similar in all three groups. In endothelial cells, only AT_2 receptor immunostaining was observed. Since the number of cells in the endothelial cell layer is limited, no comparison was made between the groups.
**Actin and CD-31 immunostaining**

Dubble immunostaining showed co-localization between on one hand the AT₁ and ET₂ receptors and on the other hand smooth muscle cell alpha-actin in resistance arteries; as shown in the illustration (Figure 4) there was a clear co-localization between the two receptor subtypes and alpha-actin both in control and in CABG subjects. CD-31 was used to stain endothelial cells; we observed weak co-localization between the receptors and CD31 (Figure 4).

**Demographics**

The patients’ background characteristics are described in Table 1. Taken together, the systolic blood pressure and the plasma levels of N-terminal pro B-type natriuretic peptide and C reactive protein were higher in the patient with ischemic heart disease (both CABG and angina pectoris) than in the cardiovascular healthy controls.
Discussion

*Endothelin receptor expression*

Our results clearly show that the levels of $\text{ET}_B$ receptor expression are higher in the vascular smooth muscle cell layer of arteries from patients with angina pectoris than from healthy controls. The levels of expression are even higher in arteries from patients undergoing CABG surgery.

Upregulated $\text{ET}_B$ receptors on vascular smooth muscle cells have previously been shown in diabetes, hypertension and in human atherosclerotic coronary arteries and in atherosclerotic plaques [9, 14]. Plasma levels of endothelin-1 are elevated in patients with ischemic heart disease and heart failure and has been suggested as a prognostic marker [15]. The circulating endothelin-1 levels are further increased in patients undergoing CABG surgery [16]. This increased activity in the endothelin system may contribute to the smooth muscle cell proliferation, vasoconstriction and decreased perfusion pressure in atherosclerotic disease [17-19].

*Mechanisms underlying the upregulation of $\text{ET}_B$ receptors*

The mechanism underlying the increased $\text{ET}_B$ receptor expression is not known, but could depend on increased transcription of $\text{ET}_B$ receptor mRNA triggered by some of the many humoral factors that are increased in ischemic heart disease. Upregulation of $\text{ET}_B$ receptors is known to rely on increased transcription and subsequent translation of receptor mRNA [20]. In the human genome, the 5´-flanking region of the genes encoding the endothelin receptors contain several regulatory elements, like GATA-motifs and E-boxes [21, 22]. This indicates that the genes might be activated by for example inflammatory components. Indeed, the presence of
interleukin-1β and TNF-α stimulates results in upregulation of vascular ET<sub>B</sub> receptors [23]. In the present study, the patients with ischemic heart disease had higher plasma levels of C reactive protein. Atherosclerosis is known to induce a proinflammatory response [24] and this may regulate endothelin receptor expression.

**Angiotensin II receptor expression**

In the present study, the levels of AT<sub>1</sub> receptor expression were higher in subcutaneous arteries from patients with ischemic heart disease than in the healthy controls. The levels of expression were even higher in the arteries from patients undergoing CABG surgery. The plasma levels of angiotensin II are known to be elevated in different heart conditions, such as heart failure [11], hypertension [25], hypoxia [26], hypercholesterolemia [27] and hyperglycemia [28], and associated with altered expression of vascular angiotensin II receptors. Angiotensin II is also a potent vasoconstrictor and mitogen of coronary artery smooth muscle cells. The stimulation of AT<sub>1</sub> receptors results in progression of atherosclerotic lesions, inflammation and plaque rupture. Increased expression of AT<sub>1</sub> receptors might make the vasculature prone to develop spasm and atherosclerotic plaques and thus further increase peripheral vascular resistance.

**Mechanisms underlying the upregulation of AT<sub>1</sub> receptors**

The mechanisms underlying the upregulation of AT<sub>1</sub> receptor expression in the patients with ischemic heart disease cannot be deduced from the present study. Inflammatory mediators, such as IL-1 and IL-6, may up-regulate AT<sub>1</sub> receptors and enhance angiotensin II stimulated vessel hypertrophy [29]. In the present study, the plasma levels of C reactive protein was observed elevated in the patients with
ischemic heart disease. It has previously been shown that high levels of C reactive protein correlates with high levels of AT₁ receptor expression [30]. Increased AT₁ receptor expression has therefore been associated with vascular inflammation. C reactive protein is known to independently predict risk for myocardial infarction, stroke, peripheral artery disease and sudden cardiac death even among apparently healthy individuals [31].

**Clinical perspective**

Both endothelin-1 and angiotensin II have strong impact in cardiovascular disease as discussed above; here we will mention another situation where they are involved. CABG surgery is hampered by the deleterious vasospasm in arterial grafts. It has been suggested that impaired vasomotor function may contribute to this vasospasm [32]. Plasma levels of endothelin and angiotensin II are elevated during CABG surgery [16]. Both endothelin and angiotensin II receptors have been shown to mediate strong vasoconstrictor effects in bypass grafts [11]. Many measures have been undertaken to control the vessel tone during surgery, including dilating the vessels with potent vasodilators such as papaverine, sodium nitroprusside and nifedipine and distending the vessel with NaCl. It might be important to consider endothelin and angiotensin II receptor antagonists for this indication. Especially since the present results show increased levels of endothelin and angiotensin II receptor expression in patients undergoing CABG surgery.

Angiotensin converting enzyme (ACE) and angiotensin AT₁ receptor antagonists are frequently being used to prevent the development of target organ damage in atherosclerotic disease and in hypertension[33]. Whether endothelin receptor
antagonists will become part of the therapeutic armamentarium in ischemic heart disease remains unclear, and none of these agents is currently being developed for this indication. However, the mixed endothelin blocker Bosentan is used in treatment of pulmonary hypertension [34]. New endothelin antagonists devoid of side effects and perhaps specific for either of the receptor subtypes are discussed in treatment of vasospasm after subarachnoid haemorrhage [35], or alternative inhibitors of the endothelin converting enzymes that generate endothelin-1 may in the future become available to block the endothelin system [5].

Limitations
The present study has demonstrated increased expression of ET₉ and AT₁ receptors in patients with ischemic heart disease. However, the patients have confounding factors. The systolic blood pressure was higher in the patients with ischemic heart disease than in the healthy controls. Furthermore, the prevalence of medication with statins, beta blockers, ACE, and ARB was common in patients with ischemic heart disease while non-existing in healthy controls. The effects of these confounding factors on the endothelin and angiotensin II receptor expression cannot be concluded from the present study.

Conclusions
The present study clearly shows that patients with ischemic heart disease have upregulated ET₉ and AT₁ receptors in the smooth muscle cells of peripheral resistance arteries. The level of ET₉ and AT₁ expression correlates with the degree of
ischemic heart disease, being highest in the patients undergoing CABG surgery. There was no difference in the expression of ET\textsubscript{A} or AT\textsubscript{2} receptors between the study groups. The changes in ET\textsubscript{B} and AT\textsubscript{1} receptor expression may be signs of remodeling of the vasculature, which is characteristic of ongoing ischemic heart disease.

**Competing interests**

The authors have no competing interest.

**Authors’ contributions**

Ivan Dimitrijevic planned and executed the immunofluorescence experiments, analyzed the data, preformed the surgical procedure and wrote the manuscript. Marie-Louise Edvinsson acquired the patient data, the biopsies and reviewed the manuscript. Malin Malmsjö helped in the writing of the manuscript. Per-Ola Kimblad performed the surgical procedure and reviewed the manuscript. Lars Edvinsson conceived the study, assisted in planning of the project and the writing of the manuscript.

**Acknowledgment**

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References


Figure legends

Table 1

Background characteristics for patients undergoing coronary artery bypass graft (CABG) surgery because of ischemic heart disease, patients with angina pectoris without established myocardial infarction and cardiovascular healthy controls. Values are expressed as mean ± SD. Statistical analysis was performed using ANOVA with Dunnet’s post-test for multiple comparisons. Significance was defined as P<0.05 (*). Abbreviations are: SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; NT-proBNP, N-terminal pro B-type natriuretic peptide; ARB, angiotensin receptor blockers; ACE, angiotensin converting enzyme and BMI, body mass index.

Figure 1

ET\textsubscript{A} and ET\textsubscript{B} receptor protein expression, assessed by immunohistochemistry, in human subcutaneous arteries from patients undergoing coronary artery bypass graft (CABG) surgery because of ischemic heart disease (n=10), patients with angina pectoris without established myocardial infarction (n=15) and cardiovascular healthy controls (n=10). Values are expressed as mean ± SD. Statistical analysis was performed using ANOVA with Dunnet’s post-test for multiple comparisons. Significance was defined as P<0.05 (*).

Figure 2
AT₁ and AT₂ receptor protein expression, assessed by immunohistochemistry, in human subcutaneous arteries from patients undergoing coronary artery bypass graft (CABG) surgery because of ischemic heart disease (n=10), patients with angina pectoris without established myocardial infarction (n=15) and cardiovascular healthy controls (n=10). Values are expressed as mean ± SD. Statistical analysis was performed using ANOVA with Dunnet’s post-test for multiple comparisons. Significance was defined as P<0.05 (*).

**Figure 3**

Representative examples showing immunofluorescence staining experiments for ET₉ and AT₁ receptors in subcutaneous arteries from patients undergoing coronary artery bypass graft (CABG) surgery because of ischemic heart disease (n=10), patients with angina pectoris without established myocardial infarction (n=15) and cardiovascular healthy controls (n=10). Note that the immunostaining intensity for both ET₉ and AT₁ receptors is higher in the arteries from patients with ischemic heart disease then from healthy controls. Significance defined as * p<0.05. Bar = 40µm.

**Figure 4**

Immunostaining and co localization of the AT₁ and ET₉ receptors to alpha-SMC and endothelin in resistance arteries of control patients. Antibodies to AT₁ (green, A1, arrow) and smooth muscle actin (red, A2 arrow), A3 (merged A1 and A2). Antibodies to ET₉ (green, B1, arrow) and smooth muscle actin (red, B2, arrow), B3 (merged B1 and B2). Antibodies to AT₁ (green, C1, arrow) and CD-31 (red, C2, arrow), C3
(merged C1+C2). Antibodies to ET$_B$ (green, D1, arrow) and CD-31 (red, D2, arrow), D3 (merged D1 and D2). Bar: (A–D) = 40\,\mu m.
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*Significant difference compared to Control
Figure 2

Bar graphs showing the ET receptor staining intensity for different groups:

- **A** shows the staining intensity for ETα receptors.
  - Control, Angina, and CABG groups are compared.

- **B** shows the staining intensity for ETβ receptors.
  - Control, Angina, and CABG groups are compared.

Significance markers (*) are used to indicate statistical differences between groups.
Figure 3

A

AT$_1$ receptor staining intensity (arbitrary units)

Control  Angina  CABG

B

AT$_2$ receptor staining intensity (arbitrary units)

Control  Angina  CABG