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# Ischemic heart disease induce upregulation of endothelin receptor mRNA in human coronary arteries

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**Abstract**

Endothelin has been implicated in the pathogenesis of ischemic heart disease and congestive heart failure. The aims were to quantify endothelin type A ($ET_A$) and type B ($ET_B$) receptor mRNA levels in human coronary arteries from patients with ischemic heart disease, congestive heart failure and controls using real-time polymerase chain reaction (real-time PCR). In addition, the suitability of organ culture as a model mimicking endothelin receptor changes in cardiovascular disease was evaluated by in vitro pharmacology and real-time PCR. Endothelin $ET_A$ and $ET_B$ receptor mRNA levels were significantly higher in arteries from patients with ischemic heart disease ($0.23 \pm 0.04$ and $0.35 \pm 0.06$) as compared to congestive heart failure ($0.09 \pm 0.02$ and $0.07 \pm 0.01$) and controls ($0.08 \pm 0.02$ and $0.08 \pm 0.01$). After organ culture, the endothelin $ET_B$ receptor mRNA levels were elevated, and the sarafotoxin 6c-induced vasoconstriction was more efficacious. Increased endothelin receptor activity may contribute to the increased vascular tone and development of atherosclerotic disease in ischemic heart disease in man.


*Keywords:* Cardiovascular disease; Coronary artery; Endothelin; Gene expression; Endothelin receptor; Vasoconstriction

## 1. Introduction

Endothelin-1 is one of the most potent vasoconstrictors known (Yanagisawa et al., 1988). Circulating endothelin-1 level is elevated and is used as a prognostic marker in ischemic heart disease and congestive heart failure (Mundhenke et al., 1999; Neunteufl et al., 2002). Three isoforms of the endothelins have been identified in mammals, namely, endothelin-1, endothelin-2 and endothelin-3 (Yanagisawa et al., 1988). Endothelin-1 is the most abundant in the human cardiovascular system (Inoue et al., 1989). Endothelial cells are the major source of endothelin-1, although present in other vascular cell types such as smooth muscle cells and macrophages (Ehrenreich et al., 1990; Kanse et al., 1991). Endothelin plays an important role in the control of basal coronary artery tone. Besides its vasoactive properties, endothelin also acts as a mitogen of vascular smooth muscle cells, stimulates extracellular matrix synthesis and attracts monocytes in the process of atherosclerosis (Achmad and Rao, 1992; Alberts et al., 1994; Rizvi et al., 1996; Weissberg et al., 1990).

In humans, endothelin-1 mediates its action via two G-protein-coupled receptor subtypes; the endothelin type A ($ET_A$) and type B ($ET_B$) receptors (Arai et al., 1990; Sakurai et al., 1990). Endothelin $ET_A$ receptor is the dominant receptor subtype on vascular smooth muscle cells and mediates contraction, while the endothelin $ET_B$ receptor is located on endothelial cells and mediates vasodilatation via the release of nitric oxide (Maguire and Davenport, 1995). In experimental cerebral vasospasm, stroke and atherosclerosis, endothelin $ET_B$ receptors increase on smooth muscle cells and play an increasing role in inducing vasoconstriction (Dagassan et al., 1996; Roux et al., 1995; Stenman et al., 2002; Wenzel et al., 1996). Organ culture of human omental

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and rat mesenteric arteries for 1–2 days induce the same type of upregulation of contractile endothelin $ET_B$ receptors on smooth muscle cells (Adner et al., 1995, 1996; Möller et al., 1997) and has therefore been suggested as an experimental model for studying endothelin receptor plasticity in cardiovascular disease. No study has yet been performed to evaluate organ culture as a method for studying the changes that occur in coronary arteries in man during cardiovascular disease.

The present study was carried out with two aims: firstly, to examine the endothelin receptor expression in coronary arteries from patients with ischemic heart disease and congestive heart failure as compared to cardiovascular healthy controls; secondly, to evaluate if organ culture induce similar changes in endothelin receptor expression as occurs during the development of cardiovascular disease.

## 2. Materials and methods

### 2.1. Tissue collection

Coronary arteries were obtained during postmortem autopsy from patients suffering from ischemic heart disease ischemic heart disease (six patients), congestive heart failure (eight patients) and from patients without known cardiovascular disease (controls, nine patients). In these arteries, endothelin receptor mRNA levels were measured by real-time polymerase chain reaction (real-time PCR). All the patients with ischemic heart disease had died from myocardial infarction. In the group of patients without known cardiovascular disease (controls), the cause of death was pneumonia (nine patients), stroke (three patients), acute respiratory distress syndrome (one patient), sepsis (one patient) and trauma (one patient). The patients without cardiovascular disease did not have heart failure or any atherosclerotic disease diagnosed by the time of death. Coronary arteries used for organ culture were obtained from eight human hearts that were explanted in the process of heart transplantation from patients suffering from dilated cardiomyopathy. The arteries (inner diameter 2–3 mm and outer diameter 3–4 mm) were harvested from the epicardial of the left ventricle, immersed into cold buffer solution (for composition, see below), transported to the laboratory on ice and immediately used for further experiments.

### 2.2. Real-time PCR

After removal of the endothelium (see below), the vessels were snap frozen in liquid nitrogen and put in a −70 °C freezer. Total cellular RNA was extracted using the FastRNA kit-green (Bio 101, Carlsbad, CA, USA) following the suppliers' instructions. Reverse transcription of total RNA to cDNA was carried out using the GeneAmp RNA PCR kit (PE Applied Biosystems, Foster City, CA, USA) in a Perkin-Elmer DNA Thermal cycler. First-strand cDNA was synthesized from 0.5 to 1 μg total RNA in a 100-μl reaction volume using random hexamers as primers. Real-time PCR was performed in a GeneAmp 5700 Sequence Detection System (Perkin-Elmer, Applied Biosystems) using the GeneAmp SYBR® Green kit (Perkin-Elmer, Applied Biosystems) with the cDNA synthesized above as template in a 50-μl reaction volume. The GeneAmp 5700 Sequence Detection System monitors the growth of DNA in real-time using an optic imaging system, via the binding of a fluorescent dye to double-stranded DNA. Specific primers for the human endothelin $ET_A$ and endothelin $ET_B$ receptors were designed as follows:

| | | |
|---|---|---|
| Endothelin $ET_A$ receptor | forward | 5′ -ATTGCCCTCAGCGAACAC-3′ |
| | reverse | 5′ -CAACCAAGCAGAAAGACGGTC-3′ |
| Endothelin $ET_B$ receptor | forward | 5′ -GATACGACAACTTCCGCTCCA-3′ |
| | reverse | 5′ -GTCCACGATGAGGACAATGAG-3′ |

The genes for β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as references, since they are continuously expressed in cells. For method details, see Wackenfors et al. (2003).

### 2.3. In vitro pharmacology and organ culture procedure

Once in the laboratory, the vessels from operating room were dissected free from adhering tissue, and the luminal side was gently rubbed with a metal wire to disrupt the endothelium. The vessels were then cut into cylindrical segments (3–4 mm long). The segments from each patient were divided into two groups; one was put in organ culture for 48 h, and the other was immediately used for pharmacological experiments. The time point chosen has previously been shown to result in endothelin $ET_B$ receptor upregulation in arteries that reaches its maximum at 48 h (Möller et al., 2002). The artery segments for organ culture were placed in a 48-well plate (one segment in each well) containing 1 ml Dulbecco's Modified Eagle's Medium (DMEM) (for composition, see below) and incubated at 37 °C in humidified 5% $CO_2$ in air, as described previously (Adner et al., 1996). For the in vitro pharmacology experiments, the segments from both cultured and non-cultured arteries were mounted on two L-shaped metal prongs, one of which was connected to a force displacement transducer for continuous recording of the isometric tension (Högestätt et al., 1983). The mounted artery segments were immersed in temperature-controlled (37 °C) tissue baths containing a bicarbonate-based buffer solution, which was continuously gassed with 5% $CO_2$ in $O_2$ resulting in a pH of 7.4. Eight to sixteen ring segments were studied at the same time in separate tissue baths. The

segments were allowed to stabilize at a resting tension of 4 mN for 1 h before the experiments were started. The contractile capacity of each vessel segment was examined by exposure to a $K^+$-rich (63.5 mmol/l) buffer solution. Cumulative concentration–response curves were constructed for endothelin-1 and sarafotoxin 6c. To check that the endothelium was properly removed, the experiments were terminated by the addition of 10 μM adenosine 5′ -*O*-thiodiphosphate (ADPβS). Abolished dilatation indicated a properly removed endothelium. For method details, see Malmsjö et al. (2000).

### 2.4. Drugs and solutions

The bicarbonate buffer solution for the in vitro pharmacology experiments was of the following composition in mM; 119, NaCl; 15, $NaHCO_3$; 4.6, KCl; 1.2, $MgCl_2$; 1.2, $NaH_2PO_4$; 1.5, $CaCl_2$; and 5.5, glucose. Serum-free DMEM for organ culture (1000 mg $l^{-1}$ D-glucose) contained sodium pyruvate (100 mg $l^{-1}$) (Gibco, Praisley, UK) and was supplemented with penicillin (100 U $ml^{-1}$) and streptomyocin (100 μg $ml^{-1}$) (Gibco). endothelin-1 and sarafotoxin 6c were purchased from Sigma (USA) and dissolved in 0.9% saline with 10% albumin. Oligonucleotides and reagents for the PCR assay were purchased from Perkin-Elmer, Applied Biosystems.

### 2.5. Calculations and statistics

#### 2.5.1. In vitro pharmacology

All calculations and statistics were performed using GraphPad Prism 3.02 software. $E_{max}$ refers to the maximum contraction calculated as percent of the contractile capacity of 63.5 mmol/l $K^+$. The negative logarithm of the drug concentration that elicited 50% contraction ($pEC_{50}$) was determined by linear regression analysis using the values immediately above and below half-maximum response. The pharmacological experiments were performed in arteries from six to eight patients for each substance, and statistical significance was accepted when $P<0.05$, using Student's *t*-test. All differences referred to in the text have been statistically verified. Values are presented as means ± S.E.M.

#### 2.5.2. Real-time PCR

A total of 20 experiments were performed on coronary arteries from six patients suffering form ischemic heart disease (expressed as "$n=6(20)$" in the text), 22 experiments were performed on 8 arteries from patients with congestive heart failure ($n=8(22)$), 22 experiments were performed on arteries from 9 patients with absence of cardiovascular disease ($n=9(22)$) and 15 experiments were performed on arteries from 5 patients with congestive heart failure after organ culture with subsequent real-time PCR. The amount of endothelin $ET_A$ and endothelin $ET_B$ receptor mRNA was calculated as relative to the amount of β-actin mRNA and GAPDH mRNA in the same sample by the formula: $X_0/R_0=2^{CtR-CtX}$, where $X_0$ = original amount of endothelin receptor mRNA, $R_0$ = original amount of β-actin mRNA, CtR = $C_T$-value for β-actin and CtX = $C_T$-value for the endothelin receptor. Statistical analyses performed using Student's *t*-test and one-way analysis of variance (ANOVA) with Newman–Keuls multiple comparison test, where $P<0.05$ was considered significant. All differences referred to in the text have been statistically verified. Values are presented as means ± S.E.M. relative to β-actin mRNA levels.

### 2.6. Ethics

The project was approved by the Ethics Committee of Lund University in Sweden and Szeged University in

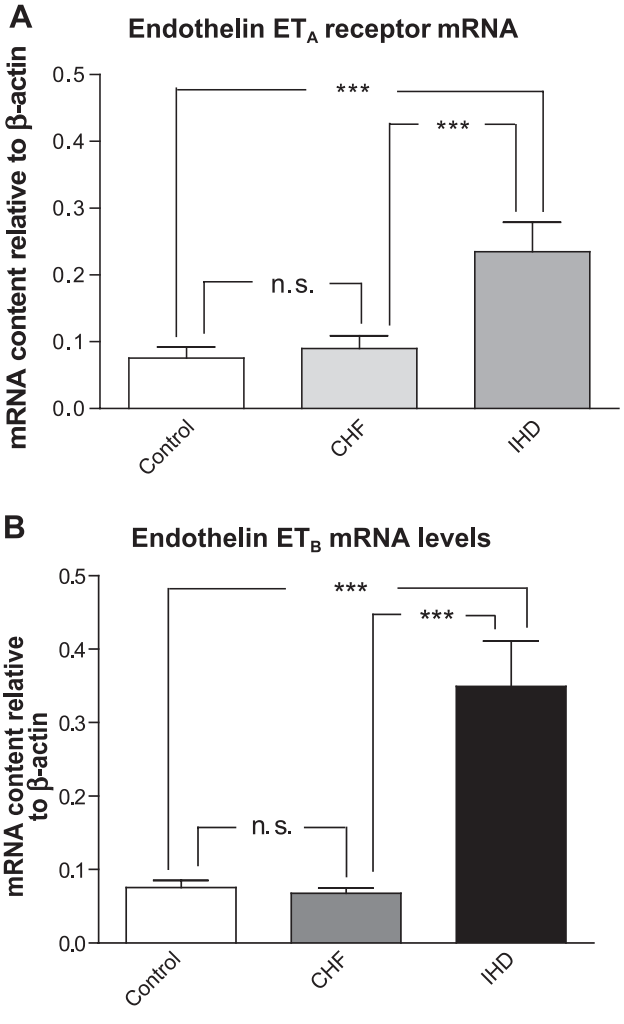


Fig. 1. Endothelin $ET_A$ receptor mRNA levels (A) and endothelin $ET_B$ receptor mRNA levels (B), assessed by real-time PCR, in the human coronary arteries from patients without known cardiovascular disease (control, $n=9(22)$), from patients suffering from congestive heart failure (congestive heart failure, $n=8(22)$) and ischemic heart disease (ischemic heart disease, $n=6(20)$). Values are presented as mean values ± S.E.M. relative to the β-actin levels. Statistical analyses were performed using one-way ANOVA with Newman–Keuls multiple comparison test, where $P<0.05$ was considered significant.

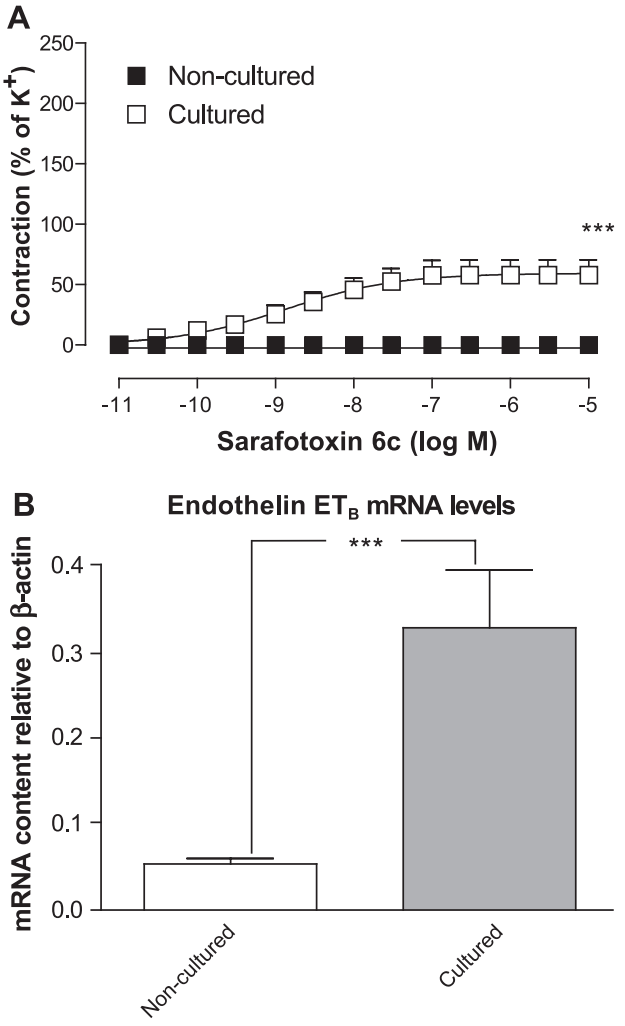


Fig. 2. (A) Concentration–response curves to sarafotoxin 6c in noncultured and cultured human coronary arteries. Vasoconstriction is expressed as a percentage of the maximal contraction induced by 63.5 mmol/l $K^+$ and presented as mean values ± S.E.M. (B) Endothelin $ET_B$ receptor mRNA levels, assessed by real-time PCR, in noncultured ($n = 5(15)$) and cultured ($n = 5(15)$) human coronary arteries. Values are presented as mean values ± S.E.M. relative to the β-actin levels. Statistical analyses were performed using Students' *t*-test, where $P < 0.05$ was considered significant.

Hungary and conforms to the principles outlined in the Declaration of Helsinki.

## 3. Results

The endothelin $ET_A$ and endothelin $ET_B$ receptor mRNA levels were significantly higher in coronary arteries from patients suffering from ischemic heart disease, as compared to arteries from patients with congestive heart failure and controls (endothelin $ET_A$ receptor mRNA = 0.23 ± 0.04 in ischemic heart disease, 0.09 ± 0.02 in congestive heart failure and 0.08 ± 0.02 in controls, $P < 0.001$, Fig. 1A; endothelin $ET_B$ receptor mRNA = 0.35 ± 0.06 in ischemic heart disease, 0.07 ± 0.01 in congestive heart failure and 0.08 ± 0.01 in controls, $P < 0.001$, Fig. 1B). There was no significant difference in the endothelin $ET_A$ or endothelin $ET_B$ receptor mRNA levels between the patients with congestive heart failure and controls ($P$ = n.s., Fig. 1A and B). Similar patterns of endothelin $ET_A$ and endothelin $ET_B$ receptor mRNA expression could be shown when using GAPDH for reference gene as compared to β-actin (data not shown), indicating that these genes were trustworthy as references in this material.

The endothelin $ET_B$ receptor agonist, sarafotoxin 6c, did not induce vasoconstriction in the endothelium-denuded human coronary arteries before organ culture. After 48 h of organ culture, the sarafotoxin 6c effect was more efficacious ($E_{max} = 59 \pm 1\%$, $pEC_{50} = 8.9 \pm 0.1$ after organ culture, Fig. 2A), indicating upregulation of endothelin $ET_B$ recep-

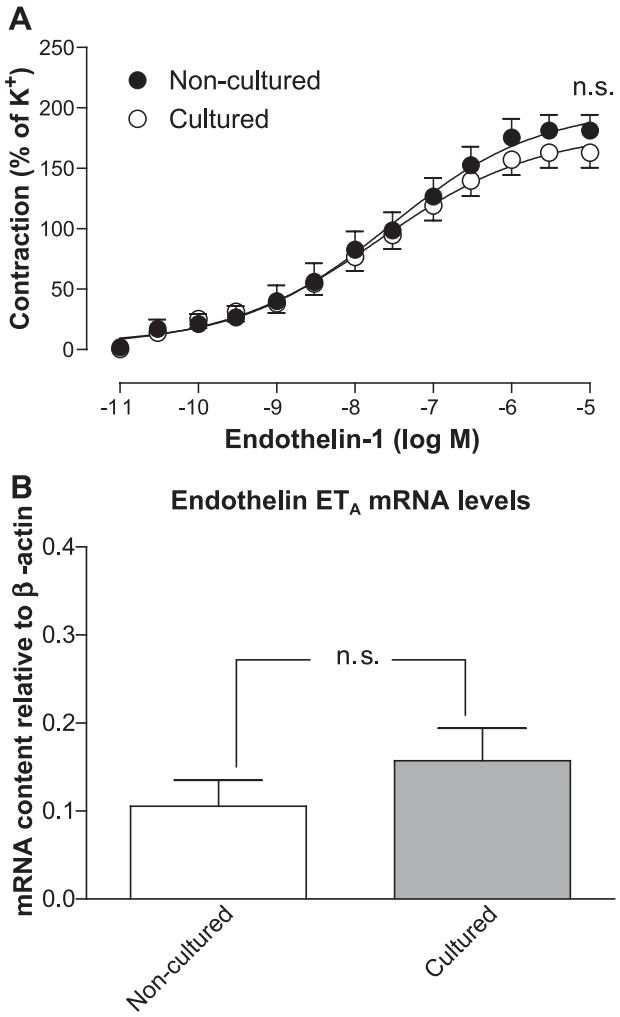


Fig. 3. (A) Concentration–response curves to endothelin-1 in noncultured and cultured human coronary arteries. Vasoconstriction is expressed as a percentage of the maximal contraction induced by 63.5 mmol/l $K^+$ and presented as mean values ± S.E.M. (B) Endothelin $ET_A$ receptor mRNA levels, assessed by real-time PCR, in noncultured ($n = 5(15)$) and cultured ($n = 5(15)$) human coronary arteries. Values are presented as mean values ± S.E.M. relative to the β-actin levels. Statistical analyses were performed using Students' *t*-test, where $P < 0.05$ was considered significant.

tors. Likewise, the endothelin $ET_B$ receptor mRNA levels, measured by real-time PCR, were elevated after organ culture ($0.05 \pm 0.01$ before organ culture; $0.29 \pm 0.06$ after organ culture, $P < 0.001$, Fig. 2B). The endothelin-1-induced vasoconstriction was not significantly changed by organ culture ($E_{max} = 200 \pm 8\%$, $pEC_{50} = 7.6 \pm 0.1$ before and $E_{max} = 180 \pm 8\%$, $pEC_{50} = 7.7 \pm 0.1$ after organ culture, $P$ = n.s., Fig. 3A). Endothelin $ET_A$ receptor mRNA levels showed a tendency towards being increased in cultured coronary arteries, although not significant ($P$ = n.s., Fig. 3B). The $K^+$ contraction was not significantly affected by organ culture ($P$ = n.s.), indicating unchanged smooth muscle cell function.

## 4. Discussion

This is the first study to show that endothelin $ET_A$ and endothelin $ET_B$ receptor mRNA levels from patients with ischemic heart disease and congestive heart failure can be quantified by real-time PCR in human coronary arteries. When compared to those of patients without known cardiovascular disease (controls), the study revealed a significant upregulation of endothelin $ET_A$ and endothelin $ET_B$ receptor mRNA contents in the coronary arteries from patients with ischemic heart disease, which may be due to atherosclerosis. Furthermore, organ culture was evaluated as an experimental model for endothelin receptor plasticity, which has never been done before in human coronary arteries. A similar pattern of upregulation of endothelin $ET_B$ receptor mRNA was seen after culturing the human coronary arteries for 48 h, as in coronary arteries from patients with ischemic heart disease.

Reverse transcriptase–polymerase chain reaction and ligand binding studies have proven that both endothelin $ET_A$ and endothelin $ET_B$ receptor mRNA are present in human coronary artery smooth muscle cells (Maguire and Davenport, 1995). In the present study, the presence of endothelin $ET_A$ and endothelin $ET_B$ receptor mRNA in smooth muscle cells from human coronary arteries was verified and quantified by real-time PCR experiments. In the coronary arteries from patients with congestive heart failure, the endothelin $ET_A$ and endothelin $ET_B$ receptor mRNA levels were the same as in the patients without known cardiovascular disease (controls). Plasma levels of endothelin-1 have been observed elevated and suggested as a prognostic marker in patients with congestive heart failure (Mundhenke et al., 1999). Therefore, although the receptor quantity in these arteries was unchanged, high levels of endothelin-1 may contribute to the increased vascular tone seen in patients with congestive heart failure.

In human coronary arteries from the heart-explanted subjects, which have congestive heart failure, endothelin $ET_A$ alone mediated vasoconstriction, since the selective endothelin $ET_B$ agonist sarafotoxin 6c had no contractile effect. Previous results from our group have shown that endothelin $ET_B$ receptors do not mediate contraction in human coronary arteries since an endothelin $ET_B$ receptor antagonist had no effect (Saetrum Opgaard et al., 1996). This agrees well with the basis of ligand-binding studies; endothelin $ET_A$ receptors have been shown to be the dominant endothelin receptor subtype present in the human coronary arteries and accounting for more than 85% of the endothelin receptors (Davenport and Maguire, 1994). The endothelin-1 response could be blocked in a competitive manner by the endothelin $ET_A$ receptor agonist IRL1620, as has been shown before (Saetrum Opgaard et al., 1996). Despite the absence of a sarafotoxin 6c effect in the coronary arteries, a small amount of endothelin $ET_B$ receptor mRNA could be found by real-time PCR. The reason for this discrepancy is not known, although mRNA expression for a receptor does not necessarily mean that this translates into an active receptor protein on the cell surface. It has been suggested that endothelin $ET_B$ receptors may act as a sink for endothelin-1 (Miyauchi and Masaki, 1999).

We could, in the present study, quantify endothelin receptor mRNA levels by real-time PCR in human coronary arteries. Endothelin $ET_A$ and endothelin $ET_B$ receptor mRNA levels were increased in arteries from patients with ischemic heart disease, while unaffected by congestive heart failure as compared to controls. By Doppler analysis of blood flow in patients with coronary artery disease, it was demonstrated that endogenous endothelin contributes to the regulation of vascular tone in the skin microcirculation not only through endothelin $ET_A$ receptors but also possibly through endothelin $ET_B$ receptors (Wenzel et al., 1996). In addition, competition binding experiments using $^{125}I$ endothelin-1 and different selective and nonselective endothelin $ET_A$ and endothelin $ET_B$ receptor agonists or antagonists in humans demonstrate upregulation of endothelin $ET_B$ receptors on smooth muscle cells in atherosclerotic human coronary arteries (Dagassan et al., 1996). Furthermore, organ culture and immunohistochemical staining studies have, in arteries from dogs, shown that the endothelin $ET_B$ receptor activity is upregulated after chronic increases in blood flow (Barber et al., 1996). It is therefore believed that the development of atherosclerosis involves upregulation of endothelin receptors. Endothelin has been shown to act as a mitogen on vascular smooth muscle cells and stimulate extracellular matrix synthesis and chemoattractant on monocytes in the process of atherosclerosis (Achmad and Rao, 1992; Alberts et al., 1994; Rizvi et al., 1996; Weissberg et al., 1990). Besides increased levels of endothelin receptors in atherosclerotic disease, plasma levels of endothelin-1 are elevated and have been demonstrated to be a prognostic marker in ischemic heart disease (Neunteufl et al., 2002). Atherosclerotic coronary arteries are prone to inappropriate vasoconstriction that exacerbates the severity of coronary artery stenosis and thereby contribute to the pathogenesis of myocardial ischemia. Endogenous endothelin-1 tonically constricts human coronary arteries, especially those with atherosclerosis, and

accounts for nearly all the resting tone at coronary artery stenoses (Kinlay et al., 2001).

Organ culture was evaluated as an experimental tool to study endothelin receptor plasticity, which has never been done before in human coronary arteries. After 48 h of culture, three was an upregulation of endothelin $ET_B$ receptor mRNA. Furthermore, in cultured arteries, sarafotoxin 6c now induced a potent vasoconstriction, indicating the appearance of a contractile endothelin $ET_B$ receptor after organ culture. Endothelin $ET_A$ receptor mRNA showed a tendency towards being upregulated, although nonsignificant, while endothelin-1 was equally effective at inducing vasoconstriction in the cultured and noncultured arteries.

The pattern of endothelin $ET_B$ receptor upregulation during culture was comparable to that seen in coronary arteries from patients with ischemic heart disease patients. Similar comparisons between vascular disease and the organ culture model for studying endothelin receptor plasticity have been made before. In experimental stroke, endothelin $ET_B$ receptors are upregulated in the ischemic rat middle cerebral artery (Stenman et al., 2002) as well as after organ culture of cerebral and temporal arteries (Hansen-Schwartz et al., 2002; White et al., 1999). One day of organ culture induces an upregulation of contractile endothelin $ET_B$ receptors on smooth muscle cells on human omental and rat mesenteric arteries (Adner et al., 1996; Möller et al., 1997), thereby mimicking the changes that occur in cardiovascular disease (Dagassan et al., 1996; Wenzel et al., 1996). Organ culture may therefore provide an experimental model in which the development of receptor changes on smooth muscle cells can be studied in detail to further delineate the molecular mechanisms involved. Culture in the presence of different humoral factors or intracellular messenger inhibitors may reveal important pathways that lead to the development of cardiovascular disease. The method thereby combines the advantage of cell culturing techniques with the advantage of functional evaluation of intact blood vessels.

The cause of endothelin $ET_B$ receptor upregulation is unknown, although a study by Möller et al. (2002) concluded that the upregulation of endothelin $ET_B$ receptors following organ culture of endothelium-denuded rat mesenteric arteries was mediated via increased transcription and subsequent translation of endothelin $ET_B$ receptor mRNA. The mRNA level reaches its maximum at 24 h, while the contractile response has its maximum after 48 h (Möller et al., 2002). In the human genome, the 5′-flanking region of the genes encoding the endothelin receptors contains several regulatory elements, like GATA-motifs and E-boxes (Arai et al., 1993; Hosoda et al., 1992). This indicates that the genes might be activated by, for example, inflammatory components after infarction. In conjunction with an ischemic event, the pro-inflammatory cytokines interleukin-1β and tumour necrosis factor-α are released (Seekamp et al., 1993). An enhanced endothelin $ET_B$ receptor mediated contraction of the rat basilar and the human temporal artery have been reported to occur after incubation (Leseth et al., 1999; White et al., 1999), which supports the hypothesis that inflammatory components might be involved.

In conclusion, the present study has shown an upregulation of endothelin $ET_A$ and endothelin $ET_B$ receptors in coronary arteries from patients with ischemic heart disease. Such an increase in endothelin receptor activity may contribute to the smooth muscle cell proliferation, vasoconstriction and the decreased blood perfusion in atherosclerotic disease. Although these results from large vessels might not be reflective of arteriolar responses, an endothelin receptor antagonist could be of use in preventing the development of atherosclerotic disease and subsequent increase in vascular tone. Since not only endothelin $ET_A$ receptors but also endothelin $ET_B$ receptors mediate strong vasoconstriction in the coronary arteries from patients with ischemic heart disease, an endothelin $ET_A$ and endothelin $ET_B$ balanced antagonist might have beneficial effects. Similar endothelin receptor upregulation can be observed after organ culture as in ischemic heart disease. Organ culture may therefore provide a useful experimental model in which the development of changes in receptor expression on smooth muscle cells can be studied in detail to further delineate the extracellular hormones, inflammatory mediators and intracellular transduction pathways involved.

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