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Ischemic heart disease down-regulates angiotensin type 1 receptor mRNA in human coronary arteries

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Abstract

Angiotensin II is important in the development of cardiovascular disease. In the present study, angiotensin II receptor mRNA levels were quantified by real-time polymerase chain reaction (real-time PCR) in human coronary arteries from patients with ischemic heart disease and controls. Furthermore, the suitability of artery culture for studying angiotensin receptor changes was evaluated by in vitro pharmacology and real-time PCR. The angiotensin type 1 (AT_1) receptor mRNA levels were down-regulated in human coronary arteries from patients with ischemic heart disease as compared to controls ($P<0.05$). Culture of coronary arteries for 48 h induced down-regulation of the angiotensin AT_1 and AT_2 receptor mRNA levels and also a less efficacious angiotensin II-induced vasoconstriction ($E_{max}=103\pm2\%$ before and $23\pm7\%$ after artery culture, $P<0.001$). Artery culture may thus be a suitable method for studying angiotensin receptor regulation.

Keywords: Cardiovascular disease; Coronary artery; Angiotensin II; Gene expression; Receptor; Vasoconstriction

1. Introduction

Angiotensin II is the principal effector molecule of the renin–angiotensin system and is essential for maintaining systemic hemodynamics and vascular tone. Angiotensin II is the product of a bioenzymatic cascade in which renin cleaves angiotensinogen to angiotensin I, and subsequently, angiotensin-converting enzyme converts angiotensin I to angiotensin II. The formation of angiotensin II has been demonstrated, not only in the systemic circulation, but also in a number of local tissues including the human vasculature (Naftilan, 1994). Angiotensin II is a potent vasoconstrictor and a growth factor that regulates cell growth and fibrosis (Baker and Aceto, 1990; Brilla et al., 1991; Levy et al., 1996; Weber, 2000) and has been implicated in the pathology of heart failure, hypertension and atherosclerosis (Goodfriend et al., 1996). Inhibition of the renin–angiotensin system by use of angiotensin-converting enzyme blockers or the angiotensin II type 1 (AT_1) receptor blockers is currently used in the clinic for the treatment of heart failure and hypertension (Theal et al., 2003).

Two angiotensin II receptors have been identified in man, angiotensin AT_1 and AT_2 receptors (Furuta et al., 1992; Tsuzuki et al., 1994), which are members of the G-protein coupled, seven-transmembrane domain receptor family. The vascular effects of angiotensin II are primarily mediated by smooth muscle cell angiotensin AT_1 receptors that induce vasoconstriction and mitogenesis (Allen et al., 2000; Unger et al., 1996). Conversely, angiotensin AT_2 receptors are located on endothelial cells and are known to induce vasodilatation by the release of nitric oxide and prostaglandins, inhibit cell growth and stimulate apoptosis (Carey et al., 2000; Horiuchi et al., 1997; Touyz et al., 1999). In

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human coronary arteries, angiotensin II induces vasoconstriction mainly by angiotensin AT_1 receptors (Maassen-VanDenBrink et al., 1999) but also via angiotensin AT_2 receptors on smooth muscle cells (Pantev et al., 2002).

The angiotensin II receptor expression varies in cardiovascular pathology such as vascular injury and cardiac remodelling (Akishita et al., 2000; Masaki et al., 1998). It has been shown by autoradiography that angiotensin AT_1 receptors are down-regulated during ischemic heart disease (Katugampola and Davenport, 2000). The mechanisms involved in inducing angiotensin II receptor changes during cardiovascular disease are not fully understood, although increased levels of circulating humoral factors including cytokines and growth factors may contribute (Harrison et al., 2003). Artery conservation in culture medium has been suggested as an experimental method for inducing changes in receptor expression to delineate the mechanisms involved. Inasmuch as artery culture has been shown to alter receptor expression that resembles that, in cardiovascular disease, a comparison between vascular disease and artery culture has been made before. Endothelin type B receptor mRNA is up-regulated in human coronary arteries after artery culture, which resembles the vascular changes in atherosclerosis and coronary artery diseases (Dagassan et al., 1996; Wackenfors et al., 2004; Wenzel et al., 1996). Likewise, serotonin type 1B receptors are up-regulated after artery culture (Hoel et al., 2001) and also after subarachnoidal hemorrhage (Hansen-Schwartz et al., 2003). No study has yet been performed to evaluate artery culture as a method for angiotensin II receptor regulation.

In the present study, angiotensin AT_1 and AT_2 receptor mRNA levels in human coronary arteries from patients with ischemic heart disease and controls were quantified by real-time polymerase chain reaction (real-time PCR). Furthermore, the suitability of artery culture as a method for inducing changes in angiotensin II receptor mRNA expression and vasocontractile effects were analysed by real-time PCR and in vitro pharmacology in cultured and noncultured human coronary arteries.

2. Materials and methods

2.1. Tissue collection

For the real-time PCR experiments, a nonatherosclerotic section of the left anterior descending artery was obtained during postmortem autopsy from patients suffering from ischemic heart disease (six patients) and from patients without known cardiovascular disease (controls, six patients). There was no difference in delay between the time of death and the time for coronary artery obtention between the two groups. All the patients with ischemic heart disease had died from myocardial infarction. In the control group, the cause of death was pneumonia (two patients), stroke (one patient), acute respiratory distress syndrome (one patient), pulmonary embolus (one patient) and trauma (one patient). The control patients did not have ischemic heart disease diagnosed by the time of death. After removal of the endothelium, the vessels were snap frozen in liquid nitrogen and stored at −80 °C.

Human coronary arteries used for culture were obtained from 16 hearts that were explanted in the process of heart transplantation from patients with dilated cardiomyopathy. The arteries were collected from the epicardial of the left ventricle, immersed into cold bicarbonate buffer solution (for composition, see below), transported to the laboratory on ice and used for the in vitro experiments.

2.2. In vitro pharmacology and artery culture

The vessels were dissected free from adhering tissue, and the luminal side was gently rubbed with a metal wire to disrupt the endothelium. The vessels were cut into cylindrical segments (3–4-mm long). The segments from each patient were divided into two groups; one was conservated in culture medium for 48 h and then used for in vitro pharmacology experiments, and the other was used immediately for in vitro pharmacology experiments. The artery segments that were conservated in culture medium were placed in a plate containing 1 ml Dulbecco's mod eagle 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 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 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 segments were studied at the same time in separate tissue baths. The segments were allowed to stabilise 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 mM) buffer solution. Cumulative concentration–response curves were constructed for all experiments by the addition of increasing concentrations of angiotensin II (0.01 nM–10 μM). In the experiments using antagonists, candesartan (1 and 10 nM) or S−(+)-1-[(4-(Dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid ditrifluoroacetate (PD123319, 0.1 μM) were added 1 h prior to angiotensin II. Thereafter, the experiments were terminated by the addition of the endothelium-dependent vasodilator Adenosine 5′ -[*b*-thio]-diphosphate trilithium salt (ADPβS, 10 μM) to check that the endothelium was properly removed. Abolished dilatation indicated a properly removed endothelium. For method details, see Wackenfors et al. (2004).

2.3. Real-time PCR

Receptor mRNA expression levels were quantified in coronary arteries from patients with ischemic heart disease and controls as well as in cultured and noncultured coronary arteries (see above). TRIzol®LS was used for the RNA extraction according to the manufacturer's instructions (Life Technologies, Paisley, UK). Reverse transcription of total RNA to cDNA was performed with the GeneAmp RNA PCR kit in a DNA Thermal cycler (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). First strand cDNA was synthesised from 0.5–1 μg total RNA in a 100-μl reaction volume with random hexamers as primers. Real-time PCR was performed in a GeneAmp 5700 Sequence Detection System using the GeneAmp SYBR® Green kit (Perkin-Elmer), with the cDNA synthesised above as template in a 50-μl reaction. The GeneAmp 5700 Sequence Detection System monitors the amplification 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 angiotensin AT_1 and AT_2 receptors were designed as follows:

Angiotensin AT_1 receptor forward;	5′ - ACC TGG CTA TTG TTC ACC CAA -3′
reverse;	5′ - ACA AGC ATT GTG CGT CGA AG -3′
Angiotensin AT_2 receptor forward;	5′ - CCT CGC TGT GGC TGA TTT ACT C -3′
reverse;	5′ - CTT TGC ACA TCA CAG GTC CAA -3′

The genes for β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as references, inasmuch as they are continuously expressed in cells.

2.4. Drugs and solutions

The bicarbonate buffer solution for the in vitro pharmacology experiments was of the following composition (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 (1000 mg l^{-1} D-glucose) contained sodium pyruvate (100 mg l^{-1}) and was supplemented with penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}; Gibco BRL, Praisley, UK). Angiotensin II (Sigma, USA) was dissolved in 0.1% bovine serum albumin. ADPβS (Sigma) was dissolved in 0.9% saline. Candesartan and PD123319 were generous gifts from Prof. Peter Morsing, AstraZeneca, Sweden. These substances were dissolved in 0.9% saline. Oligonucleotides and reagents for the real-time PCR assay were purchased from Perkin-Elmer.

2.5. Calculations and statistics

In vitro pharmacology: All calculations and statistics were performed using GraphPad 4.0 software. E_{max} refers to the maximum contraction calculated as percent of the contractile capacity of 63.5 mM 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. Statistical significance was accepted when $P<0.05$, using students' *t*-test when comparing two groups and ANOVA (analysis of variance) when comparing more than two groups. ANOVA was carried out with Dunnetts correction. Values are presented as means±S.E.M.

Real-time PCR: All experiments were performed in triplicate. The amount of angiotensin AT_1 and AT_2 receptor mRNA was calculated as relative to the level of the mRNA expression of the two housekeeping genes, GAPDH and β-actin, in the same sample. Thereafter the results were compared, and if similar, the findings were confirmed. The following formula was used for calculation of the amount of angiotensin AT_1 and AT_2 receptor mRNA: $X_0/R_0=2^{CtR-CtX}$, where X_0=original amount of angiotensin II receptor mRNA, $R_{0=}$original amount of GAPDH or β-actin mRNA, CtR=C_T-value for GAPDH or β-actin and CtX=C_T-value for the angiotensin II receptor. Statistical analyses were performed using student's *t*-test where $P<0.05$ was considered significant. Values are presented as means±S.E.M relative to GAPDH mRNA levels.

2.6. Ethics

The project was approved by the Ethics Committee of Lund University in Sweden and conforms to the principles outlined in the Declaration of Helsinki.

2.7. Limitation of the study

The supply of human material is limited. Whole arteries can be collected from the explanted hearts during heart transplantation, while only biopsies of arteries can be obtained during autopsy due to ethical reasons. The material from autopsy is therefore not sufficient for in vitro pharmacology experiments, and only real-time PCR experiments can be performed. For obvious reasons collection of human coronary arteries takes time, thereby the limited size of material.

3. Results

Angiotensin AT_1 receptor mRNA expression levels were lower in the endothelium-denuded human coronary arteries from patients that had died from ischemic heart disease as compared to patients that had died from other causes than ischemic heart disease, controls ($P<0.05$; Fig. 1A). There was no difference in angiotensin AT_2 receptor mRNA levels (P= n.s.; Fig. 1B). Angiotensin AT_1 and AT_2 mRNA expression levels were similar when using β-actin for reference gene as compared to GAPDH (data not shown).

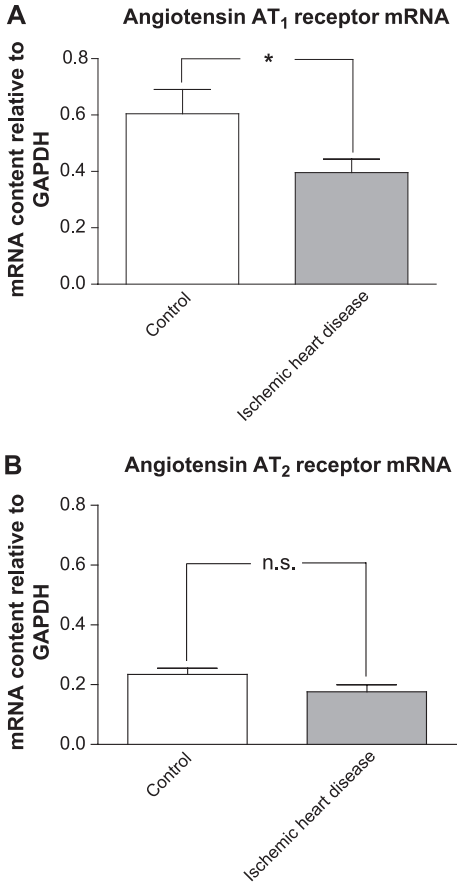

Fig. 1. Angiotensin AT_1 receptor (A) and angiotensin AT_2 receptor (B) mRNA levels assessed by real-time PCR in human coronary arteries from control patients and patients with ischemic heart disease. Values are presented as mean values±S.E.M relative to the GAPDH levels. Statistical significance was accepted when $P<0.05$(*), using students' t-test. (n.s.=non significant).

Angiotensin II induced a less efficacious vasoconstriction after 48 h of artery culture in the endothelium-denuded human coronary arteries (E_{max}=103±2% before and 23±7% after artery culture, $P<0.001$; Fig. 2), while there was no difference in potency (pEC_{50}=7.5±0.1 before and 7.5±0.1 after artery culture, P=n.s.; Fig. 2). Likewise, the

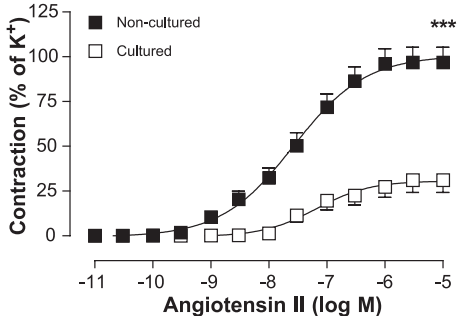

Fig. 2. Concentrationresponse curves to angiotensin II in noncultured and cultured human coronary arteries. Vasoconstriction is expressed as a percentage of the maximal contraction induced by 63.5 mM K^+ and presented as mean values±S.E.M. Statistical significance was accepted when $P<0.05$(*), using students' t-test ($P<0.001$=***).

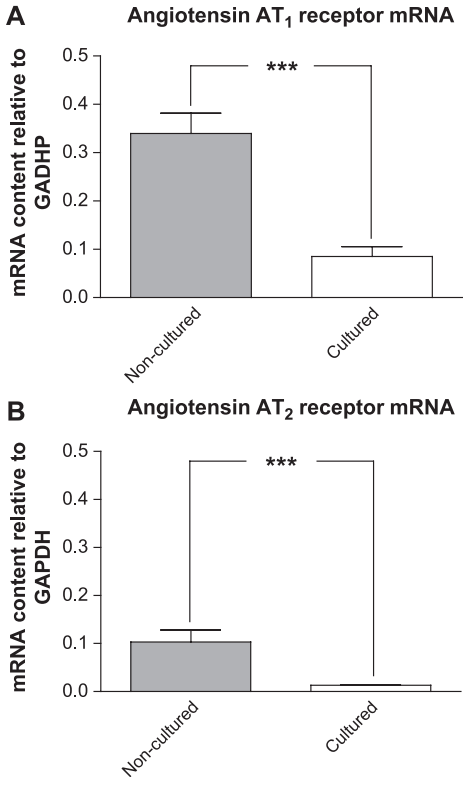

Fig. 3. Angiotensin AT_1 receptor (A) and angiotensin AT_2 receptor (B) mRNA levels, assessed by real-time PCR, in noncultured and cultured human coronary arteries. Values are presented as mean values±S.E.M relative to the GAPDH levels. Statistical significance was accepted when $P<0.05$(*), using students' t-test ($P<0.01$=**).

angiotensin AT_1 and AT_2 receptor mRNA expression levels were lower after artery culture ($P<0.001$; Fig. 3A,B).

The selective angiotensin AT_1 receptor antagonist candesartan (1 nM) inhibited the angiotensin II-induced vasoconstriction in noncultured (E_{max}=51±3%, $P<0.05$; Fig. 4A) and in cultured coronary arteries (E_{max}=2±1%, $P<0.01$; Fig. 4C). The selective angiotensin AT_2 receptor antagonist PD123319 (0.1 μM) also inhibited the angiotensin II contraction both before (E_{max}=63±1%, $P<0.05$; Fig. 4B) and after artery culture (E_{max}=10±1%, $P<0.01$; Fig. 4D).

The K^+ contractions were not affected by artery culture (9.0±1.1 mN before and 11.6±1.3 mN after artery culture, P=n.s.), indicating that the smooth muscle cell function was intact. After endothelium-denudation, vascular relaxations to 10 μM ADPβS were abolished, indicating that the endothelium was properly removed.

4. Discussion

In the present study, the angiotensin AT_1 receptor mRNA expression level was decreased in coronary arteries from patients that had died from ischemic heart disease as compared with patients that had died from other causes. The angiotensin AT_2 receptor mRNA levels were

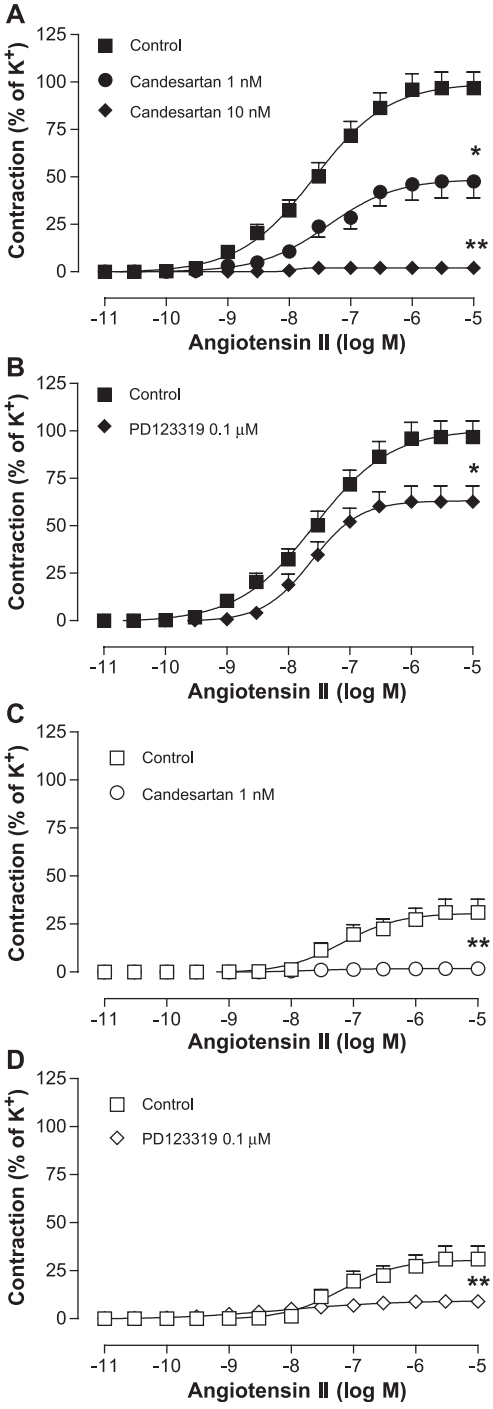

Fig. 4. Concentration–response curves to angiotensin II in noncultured (A and B) and in cultured (C and D) human coronary arteries in the presence of 1 and 10 nM candesartan or 0.1 μM PD123319. Vasoconstriction is expressed as a percentage of the maximal contraction induced by 63.5 mM K^+ and presented as mean values±S.E.M. Statistical significance was accepted when $P<0.05$, using students' *t*-test when comparing two groups and ANOVA (analysis of variance) when comparing more than two groups. ANOVA was carried out with Dunnetts correction.

unchanged. The angiotensin II-induced vasoconstriction was inhibited by candesartan and PD123319, indicating the presence of both angiotensin AT_1 and AT_2 receptors in smooth muscle cells. Artery culture was evaluated as an experimental method for angiotensin II receptor regulation. A similar decrease in angiotensin AT_1 receptor mRNA levels was seen in coronary arteries after culture as in ischemic heart disease. Artery culture may therefore provide a suitable method for studying angiotensin II receptor regulation.

Before the experiments were started, the endothelium was removed mechanically to minimise the influence that a varying endothelium function would imply in theses arteries from patients with ischemic heart disease. Real-time PCR experiments showed that both angiotensin AT_1 and AT_2 receptor mRNA were present in the coronary artery smooth muscle cells. Furthermore, the angiotensin II vasoconstriction was inhibited by candesartan and PD123319, indicating the presence of functional angiotensin AT_1 and AT_2 receptors. Angiotensin AT_1 receptors have been suggested to be of major importance in stimulating vasoconstriction in human arteries (Garcha et al., 1999; Pantev et al., 2002), while angiotensin AT_2 receptors are mainly believed to mediate endothelium-dependent dilatation (Carey et al., 2000). In a previous study, angiotensin AT_2 receptors were visualised in the smooth muscle cells of arteries by immunohistochemistry (Zhuo et al., 1998). The present study demonstrates the presence of angiotensin AT_2 receptor mRNA in human coronary arteries and an angiotensin AT_2 receptor mediated vasoconstriction. In this study, the mRNA expression levels were lower for the angiotensin AT_2 receptor as compared with the angiotensin AT_1 receptor, supporting the assumption that angiotensin AT_1 receptors are of major importance (Katugampola and Davenport, 2000).

The present study is the first to quantify angiotensin II receptor mRNA expression by real-time PCR in human coronary arteries from patients with ischemic heart disease. The aim was to compare the levels of angiotensin AT_1 and AT_2 receptor expression in the vascular wall from patients that had died from myocardial infarction with patients that had died from other causes than ischemic heart disease. The angiotensin AT_1 receptor density has been studied before by immunohistochemistry and has been shown to correlate with the degree of atherosclerosis, being higher in the atherosclerotic plaque where inflammatory cells and myofibroblasts are present (Gross et al., 2002). On the other hand, in a previous study by Katugampola and Davenport (2000), a down-regulation of angiotensin AT_1 receptors in human coronary arteries in patients with ischemic heart disease were illustrated with autoradiography. The aim of the present study was to examine the changes in angiotensin II receptor expression levels in the nonatherosclerotic vascular wall from coronary arteries from patients with ischemic heart disease and not in the atherosclerotic plaque itself. The angiotensin AT_1 receptor mRNA levels were down-regulated in coronary arteries from patients with ischemic heart disease as compared to patients that had died from other causes than ischemic heart disease.

Inasmuch as nonatherosclerotic sections of the arteries were studied, the change in angiotensin AT_1 receptor mRNA expression levels may be due to the presence of ischemia and not the degree of atherosclerosis. The angiotensin AT_2 receptor mRNA levels were unaffected. In the present study, we show the effect of ischemic heart disease on angiotensin II receptor expression, although an effect of other background factors, e.g., medication, cannot be excluded. Due to the obvious limited supply of human coronary arteries, and thereby the small groups, any within group comparisons could not be performed.

The cause of angiotensin II receptor mRNA down-regulation during ischemic heart disease is indefinite, although elevated angiotensin II levels in the plasma and increased renin–angiotensin system activity in the atherosclerotic lesion may down-regulate the angiotensin AT_1 receptors by a negative feedback system, causing receptor internalisation (Anderson et al., 1993; Diet et al., 1996; Schieffer et al., 2000; Zimmerman and Davisson, 2004). Angiotensin AT_1 receptors induce vasoconstriction, mitogenesis, cell migration, production of extra cellular matrix components and inflammation (Allen et al., 2000; Kim and Iwao, 2000). Renin–angiotensin system activity has been shown to be increased in ischemic heart disease, and the subsequent stimulation of angiotensin AT_1 receptors results in progression of atherosclerotic lesions, inflammation and plaque rupture (Diet et al., 1996; Schieffer et al., 2000). Decreasing the number of angiotensin AT_1 receptors during ischemic heart disease may be a counter-regulatory system aimed to minimise these negative effects. In contrast to the angiotensin AT_1 receptor, the angiotensin AT_2 receptor does not internalise in response to agonist binding and therefore remains available on the plasma membrane without desensitisation for long biological responses (Csikos et al., 1998). This may provide an explanation to why angiotensin AT_1 but not angiotensin AT_2 receptor mRNA levels were down-regulated in the present study. Furthermore, the angiotensin AT_2 receptor has been suggested to down-regulate the expression of angiotensin AT_1 receptors (Su et al., 2002).

Culture of human coronary arteries for 48 h induced down-regulation of angiotensin AT_1 and AT_2 receptor mRNA levels and a decreased angiotensin II-induced vasoconstriction. This angiotensin II contraction was mediated by angiotensin AT_1 and AT_2 receptors, inasmuch as candesartan (an angiotensin AT_1 receptor inhibitor) and PD123319 (an angiotensin AT_2 receptor inhibitor) had inhibitory effects both before and after artery culture. The decreased angiotensin AT_1 receptor mRNA expression levels after artery culture resemble the results from the patients with ischemic heart disease. A comparison between vascular disease and the artery culture has been made before. Contractile endothelin type B receptors in human coronary arteries are up-regulated after 48 h of culture, thereby mimicking the changes that occur in atherosclerosis and coronary artery diseases (Dagassan et al., 1996; Wackenfors et al., 2004; Wenzel et al., 1996). Likewise, serotonin type 1B receptors are up-regulated after artery culture (Hoel et al., 2001) and also after subarachnoidal hemorrhage (Hansen-Schwartz et al., 2003). Artery culture may therefore provide an experimental method in which the development of receptor changes on smooth muscle cells can be studied in detail to further delineate the mechanisms of action. Although it cannot be certain that the mechanisms responsible for the angiotensin II receptor changes are the same in culture as in ischemic heart disease, artery culture is an easily accessible method for inducing and studying receptor changes. Culture in the presence of the different humoral factors or intracellular messenger inhibitors may reveal important pathways that are involved in the receptor regulation.

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