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Transcriptional Up-Regulation in Expression of 5-Hydroxytryptamine_{2A} and Transcriptional Down-Regulation of Angiotensin II type 1 Receptors during Organ Culture of Rat Mesenteric Artery

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Abstract: The purpose of this study was to investigate in rat mesenteric artery if there is up-regulation of 5-hydroxytryptamine (5-HT) receptors and angiotensin II receptors and the potential role of protein kinase C activation in the smooth muscle cells during organ culture. Angiotensin II, 5-HT and potassium induced contraction of ring segments without endothelium, monitored by a sensitive in vitro pharmacology method. After the culture of the arterial ring segments for 24 hr, the concentration-contraction curves induced by 5-HT slightly shifted towards to the left with pEC_{50} from 6.64±0.11 to 6.84±0.11 and a significant increase in E_{max} from 147±11% to 246±15% (P<0.05), compared with that obtained in fresh segments. In contrast, the angiotensin II concentration-contraction curve only showed a significant decrease in E_{max} from 99±10% to 37±8%. Specific antagonists for the 5-HT type 2A receptors (5-HT_{2A}) and angiotensin II type 1 receptors (AT_{1}) demonstrated that the contractions occurred via 5-HT_{2A} and AT_{1} receptors, respectively. Real-time PCR revealed that the 5-HT_{2A} receptor mRNA was up-regulated in parallel with the contractile response while there was a down-regulation of AT_{1} receptor mRNA. Transcriptional inhibitor actinomycin D and specific protein kinase C inhibitor Ro31-8220 demonstrated that it was a transcriptional mechanism with involvement of protein kinase C that regulated the enhanced expression of 5-HT_{2A} receptors in the mesenteric artery.

5-Hydroxytryptamine (5-HT) and angiotensin II (AngII) are signaling molecules that have important roles in cardiovascular regulation (Turla & Webb 1989; Chester et al. 1993; Yildiz et al. 1998; Mizuno et al. 1989). Both have been seen to be synthesized in vascular endothelial cells (Lincoln et al. 1990). Their dominating receptors in smooth muscle cells of rat mesenteric artery are of the 5-HT type 2A (5-HT_{2A}) and Ang II type 1 (AT_{1}) receptors (Zemin et al. 1999). They are considered to be involved both in regulation of tone in the smooth muscle cells and in remodeling via mitogenesis (Florian & Watts 1998; Watts 1998; Ytterberg & Edvinsson 2001; Fang & Marwick 2002). Elevated levels of Ang II and 5-HT are seen in stroke (Nishimura & Suzuki 1995; Walther et al. 2002), and in congestive heart failure (Gschwend et al. 2003). As a feedback mechanism, there is local release of nitric oxide and prostacyclin, which overweighs the vascular constrictors but these may be particularly important in vascular disease with altered receptor expression. In situations of elevated levels of 5-HT or Ang II their impact on the smooth muscle cells might be pronounced, particularly, when the agonist concentrations in the concentration-response curves are logarithmic. We have previously shown enhanced expression of the endothelin type B receptor (ET_{B}) in experimental subarachnoid haemorrhage (Alafaci et al. 1990; Hansen-Schwartz et al. 2002), in stroke (Stenman et al. 2002) and in ischaemic heart disease (Wackenfors et al. 2004) which results in markedly enhanced responses to the agonists. The ET_{B} receptor may not only change its phenotype from a relaxant to a contractile ET_{B} receptor phenotype but also in increased receptor number as a result of organ culture (Adner et al. 1996). The mechanisms behind this phenomenon involve mitogen activated-protein kinase (Uddman et al. 2003), protein kinase C (Uddman et al. 2002) and transcription factors (Möller et al. 1997). However, we do not know if this phenomenon is unique to ET_{B} receptors or if alterations in other G-protein coupled receptors may occur during organ culture. There are data supporting since there is increased efficacy to Ang II in stroke (Mizuno et al. 1999; Stenman & Edvinsson 2004) and to 5-HT in SAH (Saida et al. 1997; Miranda et al. 1996) which imply a change in receptor expression (Zhu et al. 2000), while the elevated circulating level of Ang II in heart failure may results in the down-regulation of Ang II receptors (De Gasparo et al. 1994; Regitz-Zagrosek et al. 1994; Haywood et al. 1997).

The present study was designed to examine if organ culture of arterial segments change vascular smooth muscle cells receptor expression. The rat mesenteric artery has been used as a representative of a peripheral resistance vessel (Ralevic et al. 1991), and presently we have examined if there are alterations in the expression of Ang II and 5-HT.
receptors in rat mesenteric artery with functional and molecular methods. Actinomycin D (Strohm et al. 2002) and the specific protein kinase C inhibitor Ro 31-8220 (Trachsel & Keller 1995) were used to study transcription and involvement of protein kinase C, respectively.

Materials and Methods

Tissue preparation and organ culture procedure. Sprague-Dawley rats (body weight 250–300 g) were anesthetized with CO2 and exsanguinated. The superior mesenteric artery was removed gently, immersed into cold buffer solution (for composition, see below) and dissected free of adhering tissue under a microscope. The endothelium was denuded by perfusion of the vessel for 10 sec. with 0.1% Triton X-100 followed by another 10 sec. with a physiologic buffer solution (Hamel et al. 1987). The vessels were then cut into 1 mm long cylindrical segments, used directly (fresh group) or incubated for 6 hr–2 days at 37°C in humified 5% CO2 in O2 (organ culture group). The segments for organ culture were placed in a 96-well plate, one segment in each well, containing 300 μl Dulbecco’s modified Eagle’s medium (DMEM) containing L-glutamine (584 mg/l) and supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). The medium was changed to fresh daily. The experimental protocol was approved by Lund University Animal Ethic Committee (M217-03).

In vitro pharmacology. Fresh or incubated segments were immersed in temperature-controlled (37°C) tissue baths containing a bicarbonate buffer solution. The solution was continuously aerated with 5% CO2 in O2 resulting in a pH of 7.4. Each segment was mounted on a Grass FT-03 transducer (Grass Instr., Quincy, USA) connected to a PowerLab (ADInstruments, Hastings, UK) unit for continuous recording of isometric tension. A resting tone of about 2.5 mN was applied to each segment and the segments were allowed to stabilize at this tension for at least one hour before being exposed to a potassium-rich (60 mM) buffer solution with the same composition as the standard solution except that NaCl was replaced by an equimolar concentration of KCl. The potassium-induced contraction was used as a reference for the contractile capacity, and the segments was used only if potassium elicited reproducible responses over 1.0 mN. Concentration-response curves for vasoconstrictors were obtained by cumulative administration of the agents.

Buffer solutions and drugs. Standard buffer solution (mM): NaCl 119; NaHCO3 15; KCl 4.6; MgCl2 1.2; NaH2PO4 1.2; CaCl2 1.5; glucose 5.5. Analytical grade chemicals and double distilled water were used for preparing all solutions. Dulbecco’s modified Eagle’s medium, penicillin and streptomycin were purchased from Gibco BRL, Paisley, Scotland, UK. Ang II, 5-HT, Ro 31-8220, ketanserin, candesartan, cyclohexamide and actinomycin D (Sigma, St. Louis, USA) were dissolved in sterile water with bovine serum albumin (0.1% w/v).

Real time RT-PCR. Smooth muscle isolated from fresh or the cultured segments was homogenated in 1 ml of the RNAproTM solution (Q-BIOgene, CA, USA) by using a FastPrep® instrument (Q-BIOgene, CA, USA). The total RNA was extracted following a protocol from the FastRNA® Pro kit supplier. Reverse transcription of total RNA to cDNA was carried out using the Gene Amp RT kit (PE Applied Biosystems) in a Perkin-Elmer 2400 PCR machine at 42°C for 30 min. The real-time quantitative PCR was performed with the GeneAmp SYBR Green PCR kit (PE Applied Biosystems) in a Perkin-Elmer real-time PCR machine (PE, GeneAmp 5700 sequence detection system). The system automatically monitors the binding of a fluorescent dye to double-strand DNA by real-time detection of the fluorescence during each cycle of PCR amplification. Specific primers for rat AT1A and 5-HT2A receptors were designed based on gene bank data by using primer expression 2 software (PE Applied Biosystems).

5-HT2A receptor primers;
Forward: 5'-CTCAGTCACCATCGACAG-3'
Reverse: 5'-TTGTGCGCAGGCTCAG-3'

AT1A receptor primers;
Forward: 5'-GGA TGG TTC GCA GAG AGA GTA CAT
Reverse: 5'-CCT GGC CTC TGT TAC TGT TGG

The house keeping gene, GAPDH mRNA continuously expressed to a constant amount in the cells, was compared with the house keeping gene EF-1 in a pilot study by real-time PCR (Stenman et al. 2002). GAPDH was used as a reference in this study, but both were equally well constant in the tests.

GAPDH primers
Forward: 5'-GGCCTTCGCTGTTCTAC-3'
Reverse: 5'-CGGCATGTCAGACCAAC-3'

The PCR reaction was performed in a 50 μl volume and started at a temperature of 50°C for 2 min., 95°C for 10 min. and the following 40 PCR cycles with 95°C for 15 sec. and 60°C for 1 min. Dissociation curves were run after the real time PCR to identify the specific PCR products. All primers were designed using the Primer Express 2.0 software (PE Applied Biosystems) and synthesized by TAG Copenhagen A/S (Denmark).

Data were analyzed with the comparative cycle threshold (CT) method. To evaluate the amount of 5-HT2A and AT1A mRNA in a sample, GAPDH mRNA was assessed in the same sample simultaneously. The CT values of GAPDH mRNA were used as a reference to quantify the relative amount of 5-HT2A and AT1A mRNA. The relative amount of mRNA was calculated with the CT values of 5-HT2A and AT1A receptor mRNA in relation to the CT values of GAPDH mRNA in the sample.

Calculation and statistics. Data are expressed as mean values ±S.E.M. Contractile responses in each segment are expressed as a percentage of the potassium-induced contraction. Emax represents the maximal contraction induced by an agonist. The pEC50 value was calculated from the line between the concentrations above and below the midpoint of the concentration-response curve. Statistical analysis was performed with unpaired Student’s t-test or one-way ANOVA with Dunnett’s post test. Differences were considered significant at P<0.05.

Results

5-Hydroxytryptamine receptors.

Contractile response. 5-HT, a general 5-HT receptor agonist, resulted in a concentration-dependent contraction. In fresh arterial segments the Emax and pEC50 values were 147±11% and 6.64±0.11, respectively. After 24 hr of incubation, the curves slightly shifted to the left with the pEC50 values from 6.64±0.11 to 6.84±0.11 and the maximal contraction increased significantly from 147±11% to 246±15% (P<0.05) (fig. 1A, table 1). The selective 5-HT2A receptor antagonist ketanserin (10−9–10−8 M) shifted the concentration-response in a concentration-dependent manner to the right without any change in maximum contraction (fig. 1B, table 1), indicating that the contraction induced by 5-HT was via a 5-HT2A receptor subtype (Watts 2002). The pEC50 values of 5-HT with ketanserin 10−9 M and 10−8 M decreased.
from 6.74±0.09 (without ketanserin) to 6.55±0.06 and
5.22±0.07, respectively. A Schild plot of the data yielded a
pA2 value of 9.43 (slope=1) (Watts 2002). 5-CT, a selective
5-HT1 agonist, gave rise to a concentration-dependent con-
traction in the fresh arterial segments. The concentration-
contraction curves of 5-CT did not shift obviously following
organ culture for 24 or 48 hr and there was no significantly
difference in the maximum contraction or the pEC50 values
(data not shown).

Transcription and translation inhibition experiments. In or-
der to examine the mechanisms of up-regulation of 5-HT2A
receptors, we used the transcription inhibitor actinomycin
D (Strohm et al. 2002) and the translation inhibitor cyclo-
hexamide (Möller et al. 1997). The results revealed that
both inhibitors significantly decreased the enhanced 5-HT2A
receptor-mediated contraction. The enhanced contraction
had returned to that seen in fresh arterial segments in ac-
tinomycin D (5×10^-6 M) treated segments while cyclohexa-
mide (5×10^-5 M) only partially reduced the up-regulation
(fig. 3, table 1). Furthermore, the involvement of protein
kinase C was addressed by using its specific inhibitor Ro
31-8220 (10^-6 M). The presence of Ro31-8220 during organ
culture resulted in an attenuated 5-HT concentration-con-
traction curve with an Emax of 158±12% compared to that
seen after organ culture in the absence of Ro31-8220
(246±15%, P<0.01) (fig. 4, table 1).

Real-time PCR. To confirm the transcriptional mechanisms
and the involvement of protein kinase C, changes in mRNA
levels for 5-HT2A receptors were analyzed before and after
organ culture by real-time PCR. The mRNA level of 5-
HT2A receptors was significantly increased (P<0.05) follow-
ing organ culture. The presence of the transcription inhibi-
tor actinomycin D or the specific protein kinase C inhibitor
Ro31-8220, but not the translation inhibitor cyclohexa-
de, significantly decreased the up-regulation of mRNA for
the 5-HT2A receptors (fig. 5A).

<table>
<thead>
<tr>
<th>Table 1.</th>
<th>Overview of E_max and pEC50 values of 5-HT and Ang II induced contraction on rat mesenteric arteries.</th>
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</thead>
<tbody>
<tr>
<td>Agonist</td>
<td>N</td>
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<tr>
<td>5-HT</td>
<td></td>
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<tr>
<td>Fresh</td>
<td>16</td>
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<td>Organ culture 24 hr</td>
<td>16</td>
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<tr>
<td>Antagonists</td>
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<td>Control</td>
<td>10</td>
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<tr>
<td>Ketanserin 10^-9 M</td>
<td>10</td>
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<tr>
<td>Ketanserin 10^-8 M</td>
<td>10</td>
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<tr>
<td>Inhibitors</td>
<td></td>
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<tr>
<td>Control</td>
<td>16</td>
</tr>
<tr>
<td>Actinomycin D 5×10^-6 M</td>
<td>11</td>
</tr>
<tr>
<td>Cyclohexamide 5×10^-5 M</td>
<td>11</td>
</tr>
<tr>
<td>Ro 31-8220 10^-6 M</td>
<td>16</td>
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<tr>
<td>Angiotensin II</td>
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<tr>
<td>Fresh</td>
<td>11</td>
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<td>Organ culture 24 hr</td>
<td>8</td>
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<tr>
<td>Organ culture 48 hr</td>
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<td>Antagonists</td>
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<td>Candesartan 10^-12 M</td>
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<td>15</td>
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<tr>
<td>Candesartan 10^-8 M</td>
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<td>Inhibitors</td>
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<td>Actinomycin D 5×10^-6 M</td>
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<tr>
<td>Cyclohexamide 5×10^-5 M</td>
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<tr>
<td>Ro 31-8220 10^-6 M</td>
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</tbody>
</table>

Maximum contractile effect E_max are expressed as percent of 60 mM
K^+-induced contraction. pEC50 values mean negative logarithm of
the molar concentration that produced half maximum contraction.
Data are shown as mean±S.E.M. N denotes the number of vessel
segments. *P<0.01 and **P<0.05 versus fresh or control segments,
respectively.

Fig. 1. Concentration response curves for 5-HT in fresh vessels and
following 24 hr organ culture (A) or in presence of selective 5-HT2A
receptor antagonist ketanserin (10^-8 M, 10^-9 M) on rat mesenteric
arteries after incubation 24 hr (B). Data are derived from 10–16
identical experiments and shown as mean±S.E.M.
Angiotensin II receptors.

Contractile response. Ang II caused a concentration-dependent contractile response in fresh mesenteric artery segments. The maximum contraction was $99 \pm 10\%$ and the $pEC_{50}$ value $8.93 \pm 0.17$. The contraction response curves were depressed markedly after organ culture for 24 hr or 48 hr (fig. 2A, table 1). The AT$_1$ receptor antagonist, candesartan, shifted the concentration-contractile curve to right and also reduced the maximum contraction (fig. 2B, table 1). The antagonism was similar to that previously seen in rabbit aorta (Morsing et al. 1999).

Real-time PCR. The presences of mRNA for the AT$_{1A}$ receptors was analyzed before and after organ culture by real-time PCR. In concert with the functional data above, the mRNA level of AT$_{1A}$ was markedly decreased after organ culture of the mesenteric artery for 24 hr ($P<0.05$) (fig. 5B).
Fig. 5. The comparison of mRNA level on rat mesenteric arteries of fresh, organ culture for 24 hr and co-cultured with different inhibitors: translation inhibitor cyclohexamide, transcriptional inhibitor actinomycin and protein kinase C inhibitor Ro 31-8220 (A). The mRNA expression level of AT1 receptor on fresh rat mesenteric segments or after organ culture for 24 hr (B). Data are derived from 3–5 identical experiments and shown as mean±S.E.M. Fresh versus organ culture *P<0.01, **P<0.001. Organ culture versus inhibitor *P<0.05.

Protein kinase C inhibitor experiments. In order to study if protein kinase C activity was involved in the down-regulation of AT1 receptors, the specific protein kinase C inhibitor Ro 31-8220 (10⁻⁶ M) was present during organ culture. Furthermore, inhibitors for transcription and translation, actinomycin D and cyclohexamide, were also studied. The presence of either of the three above inhibitors for 24 hr resulted in the total loss of the Ang II induced contractile responses; it decreased to zero in the organ cultured vessel segments (fig. 6).

Discussion

The present study has for the first time shown that organ culture of rat mesenteric artery results in altered expression of 5-HT2A and AT1 receptors in the smooth muscle cells. Alteration in the expression of these G-protein-coupled receptors may prove to be a key event in vascular disease resulting in vascular smooth muscle spasm, proliferation and vascular remodeling. We have previously demonstrated that the ETB receptor shows plasticity by changing from a relaxant to a contractile phenotype during organ culture of cerebral and peripheral arteries, occurred not only in vitro but also in vivo e.g. in ischaemic stroke (Stenman et al. 2002) and subarachnoid haemorrhage (Hansen-Schwartz et al. 2003). The present study has revealed that there are alterations in the expression of 5-HT2A and AT1 receptors during organ culture of rat mesenteric arteries evident both at mRNA and functional levels. For ETB receptors in cerebral arteries, this has been shown also at the protein level (Henriksson et al. 2004). The up-regulation of the 5-HT2A receptor occurs via a transcriptional mechanism that involves protein kinase C. The results are well in line with our previous studies that have demonstrated that similar mechanisms are involved in the up-regulation of ETB receptors in vivo and in vitro (Uddman et al. 2002). Protein kinase C activity seems to be a key factor in the transcription of G-protein-coupled receptors and thus blockage of protein kinase C activity during organ culture regulates this. We do not think that smooth muscle cell phenotype changes occurred in the present organ culture of the artery ring segments, since it is known that the contractile response to potassium did not change in the segments cultured for 5 days (Adner et al. 1996). In the present study, the segments only cultured for 1–2 days and the culture medium did not contain any serum and growth factors. Smooth muscle cell phenotype changes in primary culture requires longer than 6 days of culture period and also growth factors (Chamley-Campbell et al. 1981).

The postsynaptic 5-HT receptors in the peripheral circulation, e.g. the mesenteric artery of the rat, consist mainly
of the 5-HT\textsubscript{2A} type as based on responses to 5-HT and antagonism by ketanserin with a pA\textsubscript{2} 9.43; this is in concert with published data for 5-HT\textsubscript{2} receptors, pA\textsubscript{2}=9.3 for 5-HT\textsubscript{2A} and 6.5 for 5-HT\textsubscript{2C} (Hoyer et al. 1994; Watts 2002), and the presence of mRNA of 5-HT\textsubscript{2A} receptors. In fresh rat mesenteric arteries, 5-HT produced a concentration-dependent contraction with an E\textsubscript{max} of 147±11% which increased after organ culture for 24 hr. The selective 5-HT\textsubscript{2A} receptor antagonist, ketanserin shifted the concentration-contraction curves of 5-HT to the right in a parallel manner without any change of E\textsubscript{max} which shows that it was a specific up-regulation of 5-HT\textsubscript{2A} receptors. The up-regulated receptor has the same pA\textsubscript{2} for ketanserin as that seen in fresh arteries. Real-time PCR is a method that allows the quantitative demonstration of receptor mRNA and here we have showed that the up-regulation is evident also at the mRNA level. This suggests a transcriptional mechanism responsible for the up-regulation. The transcription inhibitor actinomycin and the translation inhibitor cyclohexamide confirmed that the event occurs via a transcriptional mechanism. In agreement with the present study, Adner et al. (2002) have shown up-regulation of 5-HT\textsubscript{2A} receptors in smooth muscle cells during organ culture of mice tracheal segments.

Furthermore, analysis of the mechanisms triggering the up-regulation revealed that protein kinase C has a central role for ET\textsubscript{B} receptors (Uddman et al. 2002). In our study, the protein kinase C inhibitor Ro 31-8220 abolished both the increase in 5-HT\textsubscript{2A} receptor mRNA and reduced the contractile effects of 5-HT to that seen in fresh arteries. Thus, protein kinase C activity is involved in the up-regulation of 5-HT\textsubscript{2A} receptors. Protein kinase C is known to play an important role as an intracellular signal for activation of transcriptional factor nuclear factor-\kappaB (NF-\kappaB) and activation protein-1 (AP-1) (Sliva et al. 2002; Chuang et al. 2003). Since the protein kinase C inhibitor significantly inhibited the up-regulation of 5-HT\textsubscript{2A} receptors at both functional and mRNA levels, we have demonstrated that protein kinase C activity is required for the up-regulation. A similar phenomenon with protein kinase C has been demonstrated for ET\textsubscript{B} receptors and in addition the downstream extracellular signal-regulated kinase 1/2 (ERK1/2) is involved in the up-regulation of 5-HT\textsubscript{2A} receptors. Protein kinase C is known to play an important role as an intracellular signal for activation of transcriptional factor nuclear factor-\kappaB (NF-\kappaB) and activation protein-1 (AP-1) (Sliva et al. 2002; Chuang et al. 2003). Since the protein kinase C inhibitor significantly inhibited the up-regulation of 5-HT\textsubscript{2A} receptors at both functional and mRNA levels, we have demonstrated that protein kinase C activity is required for the up-regulation. A similar phenomenon with protein kinase C has been demonstrated for ET\textsubscript{B} receptors and in addition the downstream extracellular signal-regulated kinase 1/2 (ERK1/2) is involved in the up-regulation. Thus, blockade of protein kinase C activity might be a novel strategy of use in the regulation of the up-regulated G-protein-coupled receptor expression in vascular disease.

Ang II, the major effector molecule in the renin-angiotensin system, plays a central role in the control of sodium excretion and fluid volume as well as of vascular tone. The main actions of Ang II are mediated via a specific membrane-bound G-protein-coupled receptors AT\textsubscript{1} receptor, which plays an important role in the development and progression of many cardiovascular diseases. Available AT receptor blockers have been analysed in details and behave more or less as insurmountable antagonists (Ytterberg & Edvinsson 2001; Pantev et al. 2002). In heart failure there is down-regulation of AT receptors (Haywood et al. 1997; Touyz & Schiffrin 2000). This has been suggested as a feedback loop due to the elevated Ang II level in the circulation of subjects with heart failure (Makita et al. 1992; Lassegue et al. 1995). The intracellular mechanism behind the regulation of Ang II receptors is unclear, but has sometimes been considered due to enhanced receptor internalization. The contractile response to Ang II was in our study decreased in parallel with down-regulation of AT\textsubscript{1A} receptor mRNA during organ culture of rat mesenteric arteries for 24 hr. This demonstrates a transcriptional mechanism for the down-regulation of AT\textsubscript{1A} receptors. We do not know further intracellular mechanisms responsible for the down-regulation of AT\textsubscript{1} receptors, particularly since the experiments with actinomycin D or the protein kinase C inhibitor did not provide any clues. Production of Ang II, nitric oxide (Bucher et al. 2001), prostacyclin (Dusting et al. 1981; Oriji 1999) and protein kinase A activity (Xu & Murphy 2000) are putatively involved in the down-regulation since they have been shown to suppress G-protein-coupled receptor expression. Down-regulation of AT\textsubscript{1} receptors occur in human heart failure, failing human ventricular myocardium, hypertrophied human myocardium and atrial fibrillation (De Gasparo et al. 1994; Regitz-Zagrosek et al. 1994; Nozawa et al. 1996; Haywood et al. 1997; Tsutsumi et al. 1998) possibly via a mechanism similar to that seen during organ culture (Doan et al. 2004).

In conclusion, the present study demonstrated that 5-HT\textsubscript{2A} receptors were transcriptionally up-regulated whereas the AT\textsubscript{1} receptors were down-regulated at the transcriptional level in rat mesenteric artery following organ culture. Protein kinase C activity was involved in the up-regulation of 5-HT\textsubscript{2A} receptors during organ culture. Understanding of the G-protein-coupled receptor alterations that occur in organ culture may provide clues to their functions in cardiovascular disease.

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