Angiotensin II-induced hypertension is associated with a selective inhibition of EDHF-mediated responses in the rat mesenteric artery*

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List of nonstandard abbreviations: Cx, connexin; EDHF, endothelium-derived hyperpolarizing factor; NO, nitric oxide; Ang II, Angiotensin II; RWPs, red wine polyphenols; 18α-GA, 18α-glycyrrhetinic acid; MnTMPyP, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin; ODQ, 1H-(1,2,4)-oxadiazolo(4,2-a)quinoxalin-1-one.

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Abstract

Hypertension has been shown to be associated with impaired endothelium-derived hyperpolarizing factor (EDHF)-mediated arterial relaxation and hyperpolarization. Treatments of hypertensive rats with inhibitors of the renin-angiotensin system have been shown to restore both EDHF-mediated responses and the expression of connexins involved in the intercellular transfer of the hyperpolarization, in mesenteric arteries. The present study was designed to determine whether chronic treatment of rats with angiotensin II impairs EDHF-mediated responses and the expression of connexins in the mesenteric arterial wall. Male Wistar rats were treated with angiotensin II (0.4 mg/kg/day) during 21 days using osmotic mini-pumps. Arterial pressure was measured by tail-cuff plethysmography. Contractile responses and membrane potential were measured in isolated mesenteric arteries. The expression of the three connexins Cx37, Cx40 and Cx43 was quantified in segments of mesenteric arteries by immunohistochemistry and quantitative real time RT-PCR. Angiotensin II administration increased the mean systolic blood pressure. EDHF-mediated relaxation and hyperpolarization to acetylcholine and red wine polyphenols were significantly impaired in mesenteric arteries from angiotensin II-treated rats in comparison with control animals whereas NO-mediated relaxation was unaltered. The expression of connexins Cx37, Cx40 and Cx43 was significantly decreased in the mesenteric artery from angiotensin II-treated rats. These findings indicate that angiotensin II-induced hypertension is associated with a selective impairment of EDHF-mediated relaxation and hyperpolarization in the rat mesenteric artery. The inhibition of EDHF-mediated responses is due, at least partly, to a decreased expression of connexins Cx37, Cx40 and Cx43 in the arterial wall.
Introduction

Endothelial cells play a major role in the control of vascular homeostasis, in part by the production and release of vasorelaxing factors. Among these factors contributing to the protective effect of endothelial cells are nitric oxide (NO) (Furchgott and Zawadzki, 1980; Palmer et al., 1987), prostacyclin (Moncada and Vane, 1978) and the endothelium-derived hyperpolarizing factor (EDHF), which is associated with hyperpolarization of vascular smooth muscle cells (for review, see Busse et al., 2002; Feletou and Vanhoutte, 2006). In most cases, this hyperpolarization reflects the activation of endothelial small and intermediate conductance calcium-activated potassium channels (SKCa and IKCa, respectively).

Although EDHF plays only a minor role in the control of vascular tone in most large arteries in contrast to NO, its importance increases as the vessel size decreases (Shimokawa et al., 1996). In addition, the nature of EDHF has been the matter of much debate. It is now accepted that EDHF may vary according to the arterial bed and animal species investigated (for review, see Busse et al., 2002; Feletou and Vanhoutte, 2006). Besides epoxyeicosatrienoic acids (Fisslthaler et al., 1999), hydrogen peroxide (Matoba et al., 2000) and potassium ions (Edwards et al., 1998), gap junctions provide a satisfactory explanation in several vessels for the propagation of endothelial hyperpolarization to the underlying smooth muscle cells, which characterizes EDHF-mediated responses in arteries (Griffith et al., 2004). In many arteries, these pathways may operate in parallel and, for instance, in the rat mesenteric artery, during submaximal contraction to phenylephrine, myoendothelial gap junctions and endothelium-derived K+ ions support EDHF-mediated responses (Dora et al., 2003; Edwards et al., 1998). Hydrogen peroxide is a rather weak relaxing and hyperpolarizing factor; in addition, if epoxyeicosatrienoic acids are endothelium-derived...
hyperpolarizing factors, principally in porcine and bovine coronary arteries, they may also regulate endothelial calcium homeostasis as well as the activity of endothelial calcium-activated potassium channels and modulate gap junctional communication (for review, see Feletou and Vanhoutte, 2006).

The physiological role of gap junctional intercellular communication has been particularly well investigated in vascular smooth muscle in which cellular propagation of an electrical or a chemical signal through gap junctions plays a key role in the conduction of vasomotor responses, rapidly propagating vasodilatation or vasoconstriction (Christ et al., 1996). In blood vessels, gap junctions allow the direct coupling between adjacent endothelial cells or adjacent smooth muscle cells or even connect endothelial to smooth muscle cells in structures corresponding to myoendothelial gap junctions. At least three connexins (Cx) are involved in the formation of vascular gap junctions, Cx37, Cx40 and Cx43 (Christ et al., 1996; Hill et al., 2002). A close correlation between the distribution of myoendothelial gap junctions along the mesenteric arterial tree and EDHF-mediated responses has been suggested (Sandow and Hill, 2000). The functional role of gap junctions in EDHF-mediated responses has indeed been confirmed in the rat mesenteric artery (Edwards et al., 1999; Sandow et al., 2002; Mather et al., 2005) as well as in other arteries.

Hypertension is often associated with endothelial dysfunction (Mombouli and Vanhoutte, 1999). Several studies suggest that there is a close association between altered connexin expression in the arterial wall, changes in the renin-angiotensin system and hypertension. However, up to now, a clear demonstration of an effect of angiotensin II, the most active component of the renin-angiotensin system, on vascular connexin expression is still missing. Therefore, our study aimed to determine whether there is an
alteration of EDHF-mediated responses and a modification of connexin expression in the arterial wall in response to chronic angiotensin II administration to rats.

EDHF-mediated responses were induced, ex vivo in the rat mesenteric artery, by two independent stimuli, acetylcholine and red wine polyphenols (RWP). Indeed, acetylcholine is mainly dependent on an increase in intracellular free calcium concentration in endothelial cells. RWP has been shown to induce arterial relaxations mainly through the redox-sensitive activation of the PI3-kinase pathway (Ndiaye et al., 2004, 2005). Therefore, we sought to verify whether angiotensin II is able to discriminate the two distinct endothelial intracellular pathways in response to the two stimuli, acetylcholine and RWP.
Methods

In vivo treatment of rats with angiotensin II

This study has been approved by the local ethics committee of animal experimentation (CREMEAS).

Male Wistar rats (12 weeks-old) were anesthetized with intraperitoneal administration of ketamine (80 mg/kg) and xylamine (10 mg/kg). A 1 cm-long incision was made in the midscapular region and an osmotic minipump (Alzet, model 2004) was implanted. Angiotensin II, contained in the osmotic minipumps, was dissolved in 0.15 mol/L NaCl containing 0.01 N acetic acid as previously described (Sarr et al., 2006). The infusion rate was 0.4 mg/kg/day. Control rats were age-matched animals, which underwent an identical surgical procedure without pump implantation. After 21 days of angiotensin II administration, rats were euthanized and thereafter blood was taken into EDTA-containing tubes from the abdominal aorta. Blood was centrifuged at 4°C for 10 min at 2,000 g. Plasma samples were then frozen in liquid nitrogen and stored at -80°C for a later on determination of the plasma renin activity. The main superior mesenteric artery was excised and bathed in Krebs bicarbonate solution (in mmol/L: NaCl 119, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.18, CaCl₂ 1.25, NaHCO₃ 25 and D-glucose 11, pH 7.4, 37°C) for dissection.

Blood pressure measurement

Systolic blood pressure and heart rate were measured in both conscious control rats and angiotensin II-treated rats by tail-cuff plethysmography connected to a computerized system (LE 5002®, BIOSEB, Chaville, France). Rats were trained in the blood pressure device to accustom them to the procedure for 3 days before the osmotic minipump
implantation. On each day of blood pressure determination, 10 measurements were obtained and averaged for each rat.

**Determination of plasma renin activity**

Plasma renin activity was measured by determining the level of angiotensin I generated during a 30-min incubation of plasma at 37°C in the presence of 8-hydroxyquinoline (5 mmol/L). Angiotensin I was measured by radioimmunoassay.

**Vascular reactivity studies**

The main superior mesenteric artery was cleaned of connective tissue and cut into rings (2-3 mm in length). Rings were suspended in organ baths containing oxygenated (95% O₂, 5% CO₂) Krebs bicarbonate solution for the determination of changes in isometric tension. The rings were stretched step by step until an optimal resting tension of 1 g was reached and then allowed to equilibrate for at least 60 min. After the equilibration period, the rings were exposed to high K⁺-containing Krebs bicarbonate solution (80 mmol/L) until reproducible contractile responses were obtained. High K⁺ solution was prepared by equimolar substitution of NaCl with KCl. Thereafter, the rings were precontracted with phenylephrine (1 µmol/L) to about 80% of the maximal contraction to high K⁺ solution and the relaxation to acetylcholine (1 µmol/L) was determined. After washout and a further 30-min equilibration period, rings were again contracted with phenylephrine (1 µmol/L) before a concentration-relaxation curve to either acetylcholine (0.1 nmol/L to 10 µmol/L), RWPs (0.1 to 300 µg/mL), sodium nitroprusside (0.1 nmol/L to 10 µmol/L) or levcromakalim (0.1 nmol/L to 10 µmol/L) was constructed. Sodium nitroprusside- and levcromakalim-induced relaxations were
examined in endothelium-denuded rings of mesenteric artery. In some experiments, rings were exposed to an inhibitor for 45 to 120 min before contraction with phenylephrine. The NO-mediated component of relaxation was recorded in the presence of indomethacin (10 µmol/L) and charybdotoxin plus apamin (100 nmol/L each) to rule out the formation of vasoactive prostanoids and EDHF, respectively. The EDHF-mediated component of relaxation was studied in the presence of indomethacin (10 µmol/L) and Nω-nitro-L-arginine (100 µmol/L) to rule out the formation of vasoactive prostanoids and NO, respectively. In some experiments, the relaxation was analyzed in the presence of all inhibitors (indomethacin, Nω-nitro-L-arginine and charybdotoxin (100 nmol/L) plus apamin (100 nmol/L)); the effect of this combination in which charybdotoxin was replaced with TRAM-34 (1 µmol/L) was also analyzed. In control experiments, the effect of a combination of indomethacin, Nω-nitro-L-arginine (300 µmol/L), carboxy-PTIO (300 µmol/L) and ODQ (1 µmol/L) was also investigated on EDHF-mediated relaxation. Relaxations were expressed as a percentage of the contraction induced by phenylephrine.

Blood pressure measurement and vascular reactivity studies were also performed after only 14 days of angiotensin II administration (0.4 mg/kg/day).

**Membrane potential measurement**

Segments of the main superior mesenteric artery were slit open longitudinally and pinned to the sylgard base of a heated (37°C) organ chamber with the luminal side (endothelial layer) upward and superfused (5 mL/min) with heated (37°C) Krebs bicarbonate solution containing indomethacin (10 µmol/L) and Nω-nitro-L-arginine (100 µmol/L) to rule out the formation of vasoactive prostanoids and NO, respectively. The membrane potential of smooth muscle cells was recorded with glass capillary
microelectrodes (tip resistance of 40 to 80 MΩ) filled with KCl (3 mol/L) and connected to the headstage of a high impedance amplifier (intra 767, World Precision Instruments, Sarasota, Florida, USA) as described previously (Chataigneau et al., 1998); an Ag/AgCl pellet, in contact with the bathing solution and directly connected to the amplifier, served as the reference electrode. Impalement of smooth muscle cells was performed from the intimal side and successful impalements were signaled by a sudden negative drop in potential from the baseline (zero potential reference) to a stable negative potential for at least two minutes. The preparations were superfused for at least 60 min prior to any recording. All drugs were applied by continuous superfusion.

**Immunohistochemical determination of connexin distribution in the mesenteric arterial wall**

The main mesenteric artery was removed, embedded in OCT compound and snap-frozen. Frozen arteries were cryosectionned at 7 µm. Sections were air-dried for 1 hour and stored at -80°C until use. The slides were first treated with 5% goat serum in PBS containing 0.1% Triton X100 for 60 minutes to block any non-specific binding. After rinsing, the sections were then incubated overnight at 4°C with the antibodies directed against connexins Cx37, Cx40 and Cx43 (dilutions from 1:100 to 1:200). Sections were then washed with PBS, incubated with the secondary antibody (Alexa 488-conjugated goat anti-rabbit IgG, dilution 1:200) diluted in the same buffer for 2 hours at room temperature in the dark, and washed before being mounted and cover slipped. For negative controls, primary antibodies were omitted.

The samples were observed using a confocal laser-scanning microscope (Bio-Rad MRC-1024) equipped with a krypton/argon laser and fitted with the appropriate filters.
for the detection of Alexa 488 fluorescence. Quantification of connexin expression was performed using a Sharp 2000 software.

**Quantitative real-time RT-PCR analysis**

Total RNA was extracted from rat main mesenteric arteries using the Rneasy® Micro KIT (Qiagen; Courtaboeuf, France). One (1) µg of total RNA was reverse transcribed by a modified Moloney murine leukemia virus-derived reverse transcriptase using the iScript™ cDNA synthesis kit according to the manufacture's instructions (Bio-Rad; Hercules, CA, USA). Real-time PCR was performed using the protocol recommended for the iQ™ SYBR Green Supermix from Bio-Rad in a MyIQ™ thermocycler (Bio-Rad). Primers (Sigma Proligo) were designed with the help of the sequences of rat connexins deposited in Genbank (Accession numbers M76532, AF021806, M19317 and BC087743 for Cx37, Cx40, Cx43 and GAPDH, respectively). The sequences of the primers were: rat Cx37, forward 5'- TCGAGTGTAACACAGCCCAG -3', reverse 5'- CCGCCGAGACAGGTAATGA -3'; rat Cx40 forward 5'- TGCAGGAAAAGCAGAAGCTG-3', reverse 5'- GAGGACAATCTTCCCGTTCA-3', rat Cx43 forward 5'- GACTGCTTCCTCTCACGTC-3', reverse 5' -TTCACGCGATCCTTAACGCC-3', GAPDH, forward 5'- CCATCACCATCTTCCAGGAG -3', reverse 5'- CGGAGATGATGACCCCTTGT -3'.

The cycle conditions comprised a 3 min period of polymerase activation at 95°C, and 42 cycles at 95°C for 15 s, 15 s at 59°C and 10 s at 72°C. Analysis of DNA melting curves demonstrated a single peak for the whole set of primers. Amplification products were size controlled on a 2% gel agarose. Quantitative data were normalized relative to the internal house-keeping control GAPDH gene. Results are expressed as percentage of mRNA of connexins in angiotensin II-treated rats compared with untreated rats.
Preparation of Red Wine Polyphenols (RWPs)

RWPs dry powder, obtained from French red wine (Corbières A.O.C., France), was provided by Dr. M. Moutounet (Institut National de la Recherche Agronomique, Montpellier, France) and analyzed by Dr. P.-L. Teissedre (Département d’Oenologie, Université de Montpellier, France). For the preparation of RWPs dry powder, as previously described (Andriambeloson et al., 1998), phenolic compounds were adsorbed on a preparative column and alcohol was desorbed. The alcoholic-eluent was gently evaporated; the concentrated residue was lyophilized and finely sprayed to obtain RWPs dry powder. One liter of red wine produced 2.9 g of RWPs which contained 471 mg/g of total phenolic compounds expressed as gallic acid. The extract contained 8.6 mg/g catechin, 8.7 mg/g epicatechin, dimers (B1: 6.9 mg/g, B2: 8.0 mg/g, B3: 20.7 mg/g and B4: 0.7 mg/g), anthocyanins (malvidin-3-glucoside: 11.7 mg/g, peonidin-3-glucoside: 0.66 mg/g and cyanidin-3-glucoside: 0.06 mg/g) and phenolic acids (gallic acid: 5.0 mg/g, caffeic acid: 2.5 mg/g and caftaric acid: 12.5 mg/g).

Materials

Antibodies were purchased as indicated: rabbit anti-rat connexin-37 polyclonal antibody (Alpha Diagnostic, San Antonio, TX, USA), rabbit anti-mouse connexin-40 polyclonal antibody (Chemicon, Temecula, CA, USA), rabbit anti-rat connexin-43 polyclonal antibody (Zymed, San Francisco, CA, USA) and Alexa fluor-488 labelled to goat anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands).

Chemicals: apamin and charybdotoxin were obtained from Latoxan (Valence, France). ODQ (1H-(1,2,4)-oxadiazolo(4,2-a)quinoxalin-1-one) was from Tocris Bioscience. Nω-nitro-L-arginine, indomethacin, acetylcholine, sodium nitroprusside, levromakalim,
wortmannin, 18α-glycyrrhetinic acid, carbenoxolone, angiotensin II acetate Human, phenylephrine and carboxy-PTIO were all obtained from Sigma-Aldrich. The superoxide dismutase mimetic, MnTMPyP (Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin) was provided by Alexis Biochemicals Corporation (San Diego, CA, USA). TRAM-34 (1-[(2-chlorophenyl)(diphenyl)methyl]-1H-pyrazole) was a generous gift from Dr Heike Wulff (University of California, Davis).

RWPs were dissolved in a solution of ethanol and deionized water (50 % v/v). A stock solution of indomethacin was prepared in a sodium bicarbonate (10 mmol/L) solution. A stock solution of 18α-glycyrrhetinic acid and ODQ was prepared in absolute dimethyl sulfoxide. The other compounds were dissolved in deionized water. Concentrations are expressed as final concentrations (molar or µg/ml) in the bath solution.

Alzet osmotic minipumps were purchased from Charles River Laboratories International (Witmington, MA, USA).

**Statistical analysis**

Values are expressed as means ± SEM. n indicates the number of animals. Statistical analysis was performed with Student’s t-test for paired data or ANOVA followed by Fischer’s protected least significant difference test to compare two treatments where appropriate. Values of p<0.05 were considered to be statistically significant.
Results

Characterization of acetylcholine- and RWP-induced EDHF-mediated relaxation in mesenteric arteries from control rats

The cumulative addition of acetylcholine caused concentration-dependent relaxations in isolated mesenteric arteries from control rats, in the presence of indomethacin (10 µmol/L) and Nω-nitro-L-arginine (100 µmol/L) (Fig. 1). These relaxations were abolished by the combination of apamin (100 nmol/L) plus charybdotoxin (100 nmol/L) and also by the combination of apamin (100 nmol/L) plus TRAM-34 (1 µmol/L), a more selective blocker of IKCa (data not shown). Furthermore, acetylcholine-induced relaxations were unaltered by the combination of indomethacin (10 µmol/L), Nω-nitro-L-arginine (300 µmol/L), carboxy-PTIO (300 µmol/L, a NO scavenger) and ODQ (1 µmol/L, a selective inhibitor of guanylyl cyclase) (data not shown). Altogether, these results indicate that relaxations obtained in the presence of indomethacin and Nω-nitro-L-arginine are mediated by EDHF.

The two different stimuli of EDHF-mediated responses, acetylcholine and RWPs, were then compared in mesenteric arteries from control rats, in the presence of indomethacin and Nω-nitro-L-arginine (Fig. 1). Acetylcholine-induced EDHF-mediated relaxation was not modified by MnTMPyP, a superoxide dismutase mimetic (Fig. 1A), whereas this later compound abolished EDHF-mediated relaxation to RWPs (Fig. 1B). Similarly, wortmannin, a PI3-kinase inhibitor, abolished EDHF-mediated relaxation induced by RWPs (Fig. 1D) without altering that to acetylcholine (Fig. 1C).

Gap junctions, which are formed of connexins, have been shown to play a crucial role in electrical coupling in the arterial wall and also in EDHF-mediated responses, at least in some vascular beds (Griffith et al., 2004; Mather et al., 2005). In isolated mesenteric...
arteries from control rats, 18α-glycyrrhetinic acid (18 α–GA), a well recognized inhibitor of gap junctional communication (Griffith et al., 2004), induced an almost complete inhibition of both EDHF-mediated relaxation to acetylcholine (Fig. 2A) and RWPs (Fig. 2B) without altering relaxations evoked by either sodium nitroprusside (Fig. 2C) or levromakalim (Fig. 2D). Similar results were obtained with carbenoxolone (100 µmol/L, Fig. 3), a water-soluble hemisuccinate derivative of 18β-glycyrrhetinic acid. Altogether, these results suggest that gap junctions are involved in EDHF signaling in mesenteric arteries.

**Chronic administration of angiotensin II induces hypertension**

Chronic administration of angiotensin II (0.4 mg/kg/day during 21 days) induced a significant increase in systolic blood pressure to a maximal value of 178.2 ± 7.8 mm Hg (n=11) after 20 days in comparison to control rats (124.0 ± 3.4 mm Hg, n=14) (Fig. 4A). In contrast, heart rate was unaffected by angiotensin II treatment (Fig. 4A). The angiotensin II-induced increased systolic blood pressure was associated with a pronounced reduction of the plasma renin activity (39.1 ± 4.1 ng/AngI/mL/h, n=14 and 7.2 ± 1.7 ng/AngI/mL/h, n=11 in control and angiotensin II-treated rats, respectively) (Fig. 4B).

**Chronic treatment with angiotensin II during 21 days is associated with a selective inhibition of EDHF-mediated relaxation in rat mesenteric arteries ex vivo**

The cumulative addition of acetylcholine caused concentration-dependent relaxations in isolated mesenteric arteries from both control and angiotensin II-treated rats (Fig. 5A, C). The NO-mediated component of relaxation (Fig. 5A) was not significantly different in the vessels from the two groups of rats. In contrast, the EDHF-mediated component
of relaxation (Fig 5C) was abolished in mesenteric artery rings from angiotensin II-treated rats. RWPs caused also concentration-dependent relaxations in isolated mesenteric arteries from the two groups of rats (Fig. 5B, D). Angiotensin II treatment was associated with an abolition of EDHF-mediated relaxation (Fig. 5D) without significant alteration of NO-mediated relaxation (Fig. 5B).

In the presence of indomethacin, Nω-nitro-L-arginine and charybdotoxin plus apamin, acetylcholine- and RWPs-induced relaxations were abolished in both control rats and angiotensin II-treated rats (data not shown).

The angiotensin II treatment did not affect the concentration-dependent relaxation evoked by levocromakalim (0.1 nmol/L to 10 µmol/L), an ATP-sensitive K⁺ channel opener (pD2: 6.20 ± 0.12 and 6.43 ± 0.27 in mesenteric arteries from control and angiotensin II-treated rats, respectively; relaxant effect at 10 µmol/L: 87.5 ± 4.0 % and 90.2 ± 7.2 %, in mesenteric arteries from control and angiotensin II-treated rats, respectively).

Altogether, these results indicate that the EDHF-mediated component of relaxation is selectively abolished by a chronic treatment with angiotensin II for 21 days.

It is noteworthy that a chronic treatment with angiotensin II for 14 days (0.4 mg/kg/day) induced a significant increase in systolic blood pressure (170.7 ± 6.8 mmHg versus 135.8 ± 4.7 mmHg, n=6, in angiotensin II-treated and control rats at day 14, respectively) without significant alteration in EDHF-mediated relaxations (data not shown).

**Chronic treatment with angiotensin II during 21 days is associated with an inhibition of EDHF-mediated hyperpolarization in rat mesenteric arteries ex vivo**
The membrane potential of rat mesenteric smooth muscle cells was recorded with the intracellular microelectrode technique in the presence of indomethacin and Nω-nitro-L-arginine (Fig. 6). Smooth muscle cells were significantly depolarized in arteries from rats treated with angiotensin II (resting membrane potential of -52.3 ± 1.6 mV, n=7 and -46.8 ± 1.6 mV, n=5, in mesenteric arteries from control and angiotensin II-treated rats, respectively). Acetylcholine-induced EDHF-mediated hyperpolarization which averaged -18.3 ± 2.5 mV (n=4) in mesenteric arteries from control rats was significantly reduced in mesenteric arteries from angiotensin II-treated rats (-10.3 ± 1.2 mV, n=3). As illustrated, RWPs elicited a slow developing and long lasting but transient hyperpolarization in mesenteric arteries from control rats (-15.0 ± 1.7 mV, n=4, Fig. 6A). This hyperpolarization was significantly and strongly reduced in mesenteric arteries from angiotensin II-treated rats (-5.0 ± 1.0 mV, n=3, Fig. 6A, B).

**Chronic treatment with angiotensin II is associated with a reduced expression of Cx37, Cx40 and Cx43 in the rat mesenteric arterial wall**

Connexins Cx37, Cx40 and Cx43 represent the three major isoforms, which have been described in the vascular system (Hill et al., 2002). We have determined whether the expression of these three connexins is altered in the mesenteric arteries from angiotensin II-treated rats.

As shown in Fig. 7, Cx37 and Cx43 were observed throughout the arterial wall in mesenteric arteries from control rats (Fig. 7A, C) whereas Cx40 was mainly detected in the endothelial layer (Fig. 7B). The expression of the connexins was significantly reduced to 37.6 ± 11.5 %, 35.2 ± 8.8 % and 42.6 ± 7.2 % of control value (100 %) for Cx37, Cx40 and Cx43, respectively, in the mesenteric artery from angiotensin II-treated rats (Fig. 7A, B, C).
The expression of the three connexins has also been assessed by mRNA analysis using quantitative real time RT-PCR. As shown in Fig. 8, the levels of mRNAs for Cx37, Cx40 and Cx43 were also significantly decreased in the mesenteric artery from angiotensin II-treated rats (reduction to 54.0 ± 15.1 %, 44.1 ± 9.4 % and 44.3 ± 13.6 % of control value for Cx37, Cx40 and Cx43, respectively).
Discussion

The present findings demonstrate that chronic administration of angiotensin II induces hypertension in rats in association with a selective impairment of EDHF-mediated responses but without alteration of NO-mediated relaxation. This effect correlates with a decrease in Cx37, Cx40 and Cx43 expression, the three connexins which compose gap junctions in the mesenteric arterial wall.

As expected, chronic administration of angiotensin II induced hypertension in rats and a pronounced reduction of the plasma renin activity. This latter effect is indicative of a high impregnation of rats with angiotensin II and is probably due to a negative feedback control of angiotensin II on renin secretion (Tanabe et al., 1999). Elevated plasma levels of angiotensin II are found in patients with severe forms of hypertension (Boyd et al., 1972; Sim and Qui, 2003). The experimental model of hypertension induced by angiotensin II is therefore of potential clinical importance.

As shown in the present study, acetylcholine and RWPs induced both NO- and EDHF-mediated relaxation in the rat mesenteric artery. The NO-mediated component of relaxation was unaltered in mesenteric arteries from angiotensin II-treated rats. In contrast, in large elastic arteries such as aorta in which NO is the main endothelium-derived relaxing factor, chronic administration of angiotensin II induced a significant inhibition of NO-mediated relaxation (Sarr et al., 2006). The decrease has been attributable, in part, to an increased vascular superoxide anion production, which inactivates NO. However, in agreement with our results, it has been observed that NO-mediated relaxation is not altered whereas EDHF-mediated relaxation is significantly impaired in isolated mesenteric arteries from SHR in comparison to control rats (Onaka et al., 1998; Kahonen et al., 1999; Goto et al., 2000). In summary, in hypertension, it
seems that NO-mediated responses are either decreased or unaltered (Feletou and Vanhoutte, 2004).

Our study is the first to demonstrate that chronic in vivo administration of angiotensin II to rats is associated with a selective abolition of EDHF-mediated relaxation and a decreased EDHF-mediated hyperpolarization as assessed in the rat mesenteric artery ex vivo. Both abolition of EDHF-mediated relaxation and reduction of EDHF-mediated hyperpolarization have been observed in mesenteric arteries from SHR in comparison to age-matched normotensive WKY (Fujii et al., 1992; Goto et al., 2000). The restoration of these responses by either an angiotensin-converting enzyme inhibitor, an angiotensin type 1 (AT₁) receptor antagonist or their combination was already an indication of the negative influence of the renin-angiotensin system on EDHF-mediated responses (Onaka et al., 1998; Kahonen et al., 1999, Goto et al., 2000). However, none of these studies has demonstrated a relationship between administration of angiotensin II and EDHF. In our study, the absence of modification of NO-mediated relaxation and levocromakalim-induced relaxation indicates that the treatment with angiotensin II does not alter the capacity of smooth muscle cells to relax or to hyperpolarize but is rather selective of EDHF signaling.

The present findings provide evidence that RWP-induced EDHF-mediated relaxation is sensitive to both MnTMPyP, a superoxide dismutase mimetic, and wortmannin, a PI3-kinase inhibitor, whereas acetylcholine-induced EDHF-mediated relaxation is unaffected by these two inhibitors in isolated mesenteric arteries from normotensive animals. These results strongly suggest that RWP induces a redox-sensitive activation of the PI3-kinase/Akt pathway leading to EDHF-mediated relaxation in the rat mesenteric artery as previously demonstrated in the porcine coronary artery (Ndaiye et al., 2004). The inhibition of EDHF-mediated relaxation by chronic administration of
angiotensin II could be observed independently of the nature of the agonist (acetylcholine or RWPs) indicating that angiotensin II is not able to discriminate the two associated endothelial intracellular pathways. A possible explanation is, therefore, that chronic treatment with angiotensin II induces an alteration of a downstream endothelial mechanism leading to EDHF mediated relaxation of vascular smooth muscle such as an alteration of the intercellular coupling by gap junctions.

Support for the hypothesis of a functional participation of gap junctions to EDHF-mediated responses has come from studies in which gap junctions were pharmacologically blocked, either with connexin-mimetic peptides or with glycyrrhetinic acid derivatives; 18α-glycyrrhetinic acid is a well recognized inhibitor of gap junctional communication (Goldberg et al., 1996; Griffith et al., 2004). In the present study, 18α-glycyrrhetinic acid induced an almost complete blockade of acetylcholine- and RWPs-induced EDHF-mediated relaxation without affecting sodium nitroprusside- and levcromakalim-induced relaxations. Our results are therefore in agreement with previous studies showing that 18α-glycyrrhetinic acid induces a significant inhibition of EDHF-mediated responses in the isolated, perfused or not, rat mesenteric artery (Harris et al., 2000; Goto et al., 2002) and confirm that gap junctions contribute to EDHF-mediated relaxation in this artery. The role of gap junctions is further supported by the data with carbenoxolone. In addition, our study provides evidence of a participation of gap junctions to RWPs-induced EDHF-mediated relaxation.

Our immunohistochemical data show that Cx37 and Cx43 are found both in the media and the intima whereas Cx40 is mostly localized to the intima of mesenteric arteries from normotensive control rats (Kansui et al., 2004; Rummery and Hill, 2004; present study). The present findings also demonstrate for the first time that chronic
administration of angiotensin II down-regulates by about 50% the expression of Cx37, Cx40 and Cx43 as assessed by quantitative real-time RT-PCR and by immunohistochemistry in cross-sections from the main superior mesenteric arterial wall. Reduced EDHF-mediated responses are accompanied by an altered vascular expression of Cx37, Cx40 and Cx43 in arteries from hypertensive animals (Rummery and Hill, 2004). The endothelial expression of Cx37, Cx40 and Cx43 is decreased in the caudal artery from SHR compared with WKY (Rummery et al., 2002). However, it has also been previously observed that the endothelial expression of Cx37 and Cx40 is reduced whereas that of Cx43 is increased in mesenteric arteries from SHR in comparison to normotensive animals (Kansui et al., 2004). A chronic treatment with candesartan, an AT₁ receptor antagonist, significantly increased the endothelial expression of Cx37 and Cx40 and decreased that of Cx43 (Kansui et al., 2004). This apparent discrepancy with our study is probably related to the animal model or the cell types examined. Altogether, these data and the present findings strongly suggest that there is a close relationship between the renin-angiotensin system and vascular gap junctions and underline the major role of angiotensin II and/or hypertension in the control of connexin expression in vascular cells. There was a higher level of inhibition of EDHF-mediated relaxation in comparison to EDHF-mediated hyperpolarization, in response to chronic angiotensin II treatment. This difference could be explained by the fact that gap junctions seem to play a greater role in transmitting endothelial cell hyperpolarization to smooth muscle cells following depolarization with phenylephrine than under basal conditions (i.e. in the absence of stimulation with phenylephrine, a condition that was used in electrophysiological experiments in the present study), in mesenteric arteries (Edwards et al., 1999).
It is not known which connexins are precisely involved in EDHF-mediated responses. However, using a strategy to selectively load inhibitory connexin antibodies and peptides directed against Cx40 into endothelial cells, it has been recently shown that Cx40 is the critical connexin isoform involved in heterocellular signaling in rat mesenteric arteries (Mather et al, 2005). Indeed, Cx40 is probably the key connexin involved in EDHF-mediated responses and the conduction of vasodilatation in murine and rat arteries (De Wit et al., 2000; Figueroa et al., 2003; Mather et al, 2005).

The exact cellular mechanisms by which connexin expression is decreased remains to be investigated. The role of reactive oxygen species on connexin expression should be examined as it is known that gap junctional communication is impaired by oxidative stress (Griffith et al., 2005) and that angiotensin II administration is associated with an increase in oxidative stress in arteries (Sarr et al., 2006).

It has been observed that despite a reduced functional activity and expression of SK3 channels, the EDHF-mediated responses were not altered in small mesenteric arteries from rats treated with 60 ng/kg/min angiotensin II (Hilgers and Webb, 2007). The absence of alteration of EDHF-mediated responses could be explained by the short duration of angiotensin II administration (14 days). Indeed, we have also observed that a chronic treatment with angiotensin II for 14 days, even with a higher dose (0.4 mg/kg/day), induced a significant increase in systolic blood pressure without significant alteration in EDHF-mediated relaxations. Our results suggest that the inhibition of EDHF-mediated responses is a process which takes place in the long run following chronic exposure to angiotensin II or angiotensin II-induced hypertension (for at least 21 days).

In conclusion, our study highlights a close relationship between chronic exposure to angiotensin II, the abolition of EDHF-mediated relaxations and the decrease in connexin
expression in mesenteric arteries. As EDHF is the predominant endothelial factor in small arteries, it is proposed that these latter modifications could well contribute to maintain and/or worsen hypertension.
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References


Footnotes

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Legends for Figures

**Figure 1:** Characterization of acetylcholine- and red wine polyphenols (RWP)-induced EDHF-mediated relaxation in isolated rat mesenteric arteries

(A-B) Effect of MnTMPyP (100 µmol/L) on EDHF-mediated relaxation induced by either acetylcholine (A) or RWPs (B) in isolated mesenteric arteries from normotensive rats (control group).

(C-D) Effect of wortmannin (30 nmol/L) on EDHF-mediated relaxation induced by either acetylcholine (C) or RWPs (D) in isolated mesenteric arteries from age-matched normotensive rats.

All experiments were performed in the presence of indomethacin (10 µmol/L) and Nω-nitro-L-arginine (100 µmol/L). Results are shown as mean ± SEM of 8 different rats. *p < 0.05.

**Figure 2:** Involvement of gap junctions in EDHF-mediated relaxation in isolated rat mesenteric arteries: 18α-glycyrrhetinic acid

Effect of 18α-glycyrrhetinic acid (18α-GA, 100 µmol/L) on EDHF-mediated relaxation induced by either acetylcholine (A) or red wine polyphenols (RWP, B), on sodium nitroprusside (SNP)-induced relaxation (C) and on levcromakalim (LEV)-induced relaxation (D), in isolated mesenteric arteries from normotensive rats (control group).

All experiments were performed in the presence of indomethacin (10 µmol/L) and Nω-nitro-L-arginine (100 µmol/L). Results are shown as mean ± SEM of 3-8 different rats. *p < 0.05.

**Figure 3:** Involvement of gap junctions in EDHF-mediated relaxation in isolated rat mesenteric arteries: carbenoxolone

Effect of carbenoxolone (100 µmol/L) on EDHF-mediated relaxation induced by either acetylcholine (A) or red wine polyphenols (R WP, B), on sodium nitroprusside (SNP)-(C) and levcromakalim (LEV)-(D) induced relaxations, in isolated mesenteric arteries from normotensive rats (control group).

All experiments were performed in the presence of indomethacin (10 µmol/L) and Nω-nitro-L-arginine (100 µmol/L). Results are shown as mean ± SEM of 5 different rats. *p < 0.05.
Figure 4: Chronic (21 days) treatment with angiotensin II (Ang II) causes a significant increase in systolic blood pressure in rats

Effects of a chronic administration of Ang II (0.4 mg/kg/day during 21 days with subcutaneous osmotic minipumps) on rat hemodynamic parameters (A) and on the plasma renin activity determined at day 21 (B). Results are shown as mean ± SEM of 11-14 different rats. *p <0.05. The arrows in A indicate the initiation of Ang II administration (day 0).

Figure 5: Chronic (21 days) treatment with angiotensin II (Ang II) is associated with a selective abolition of acetylcholine (A, C)- and red wine polyphenols (RWPs, B, D)-induced EDHF-mediated relaxation in isolated rat mesenteric arteries

Acetylcholine- and RWPs-induced relaxations were recorded in isolated mesenteric arteries from age-matched normotensive rats (control) and Ang II-treated rats (Ang II) as follows:

(A, B) in the presence of indomethacin (10 µmol/L) and apamin (APA, 100 nmol/L) plus charybdotoxin (ChTx, 100 nmol/L) to rule out the formation of vasoactive prostanoids and EDHF, respectively (NO-mediated relaxation),

(C, D) in the presence of indomethacin (10 µmol/L) and Nω-nitro-L-arginine (L-NA, 100 µmol/L) to rule out the formation of vasoactive prostanoids and NO, respectively (EDHF-mediated relaxation).

Results are shown as mean ± SEM of 6-14 different rats. *p <0.05.

Figure 6: Chronic (21 days) treatment with angiotensin II (Ang II) is associated with a significant inhibition of EDHF-mediated hyperpolarization in isolated rat mesenteric arteries

(A) Original recordings illustrating red wine polyphenols (RWPs)-induced EDHF-mediated hyperpolarization of mesenteric artery smooth muscle cells from age-matched normotensive rats (control group) and Ang II-treated rats.

(B) Corresponding bargraph.

All experiments were performed in the presence of indomethacin (10 µmol/L) and Nω-nitro-L-arginine (100 µmol/L). Results are shown as mean ± SEM of 3-4 different rats. *p <0.05.
Figure 7: Effects of a chronic treatment with angiotensin II (Ang II) on the distribution of connexins Cx37, Cx40 and Cx43 in mesenteric arteries

The distribution of connexins Cx37 (A), Cx40 (B) and Cx43 (C) was determined in mesenteric artery sections from age-matched normotensive rats (control) and Ang II-treated rats (Ang II) using selective polyclonal antibodies and a fluorescence-tagged secondary antibody by confocal microscopy. Upper panel shows representative immunofluorescent staining and corresponding phase contrast; lower panel represents corresponding cumulative data. Similar observations have been done in 4 additional experiments. I: intima, M: media and A: adventitia. *p <0.05.

Figure 8: Chronic treatment with angiotensin II (Ang II) significantly decreases the expression of connexins Cx37, Cx40 and Cx43 in mesenteric arteries as assessed by quantitative real time RT-PCR

Cumulative data illustrating the differential expression of the three connexins Cx37 (A), Cx40 (B) and Cx43 (C) as assessed by mRNA analysis using quantitative real time RT-PCR in mesenteric arteries from age-matched normotensive rats (control) and Ang II-treated rats (Ang II). Quantitative data were normalized relative to the internal house-keeping control GAPDH gene. Results are expressed as percentage of mRNA of connexins in angiotensin II-treated rats compared with untreated rats (control). Results are shown as mean ± SEM of 5 different rats. *p <0.05.
Figure 1

A. B.

○ without inhibitor (n=8) ○ without inhibitor (n=8)
◆ MnTMPyP (n=8) ◆ MnTMPyP (n=8)

- Log [Acetylcholine], (mol/L)

0 25 50 75 100

10 9 8 7 6 5

0.1 1 10 100

[RWPs], (µg/ml)

C. D.

○ without inhibitor (n=8) ○ without inhibitor (n=8)
■ Wortmannin (n=8) ■ Wortmannin (n=8)

- Log [Acetylcholine], (mol/L)

0 25 50 75 100

10 9 8 7 6 5

0.1 1 10 100

[RWPs], (µg/ml)
Figure 3

A. Relaxation (%) vs. Log [Acetylcholine], (mol/L)
- Without inhibitor (n=5)
- Carbenoxolone (n=5)

B. Relaxation (%) vs. RWPs, (µg/ml)
- Without inhibitor (n=5)
- Carbenoxolone (n=5)

C. Relaxation (%) vs. Log [SNP], (mol/L)
- Without inhibitor (n=5)
- Carbenoxolone (n=5)

D. Relaxation (%) vs. Log [LEV], (mol/L)
- Without inhibitor (n=5)
- Carbenoxolone (n=5)
Figure 4

A

Systolic blood pressure
(mm Hg)

V

Ang II

Control group

Ang II-treated group

(n=14)

(n=11)

* 

Heart rate
(bpm)

B

Plasma renin activity
(ng Ang I/mL/h)

Control group

Ang II-treated group

(n=14)

(n=11)

*
Figure 5

A. NO-mediated relaxation

B. NO-mediated relaxation

C. EDHF-mediated relaxation

D. EDHF-mediated relaxation

-Log [Acetylcholine], (mol/L)

Relaxation (%)

Control (n=7)

Ang II (n=6)

Control (n=6)

Ang II (n=7)

[RWPs], (µg/ml)

Control (n=14)

Ang II (n=9)

Control (n=14)

Ang II (n=11)
**Figure 6**

A.  

[Graph showing membrane potential (mV) for Control group and Ang II-treated group. RWPs (100 µg/mL) result in hyperpolarization.]

B.  

[Bar chart showing RWP-induced hyperpolarization (mV) for Control group and Ang II-treated group. (n=4) and (n=3) indicated.]
Figure 7

A. Cx37

Control

Ang II

B. Cx40

Control

Ang II

C. Cx43

Control

Ang II

Fluorescence (% vs Control)

Control

Ang II

*
Figure 8

A. Cx37  B. Cx40  C. Cx43

Connexin expression corrected for GAPDH (% of control)

Control  Ang II  Control  Ang II  Control  Ang II

* * *