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Angiotensin II Increases Vascular and Renal Endothelin-1 and Functional Endothelin Converting Enzyme Activity *in Vivo:* Role of ET_A Receptors for Endothelin Regulation

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Angiotensin II (Ang II)-stimulated expression of endothelin-1 (ET-1) mRNA is blocked by ET_A antagonists *in vitro.* We studied effects of Ang II (200 ng/kg/min) and ET_A antagonist LU135252 (50 mg/kg/d) in WKY rats *in vivo* investigating vascular and renal ET-1 protein expression, functional endothelin converting enzyme (ECE) activity, and clearance of ^{125I}ET-1. Infusion of Ang II for two weeks increased ET-1 protein content in aorta (4.7-fold) and femoral artery (1.6-fold) with and without endothelium and in kidneys (3-fold, $p<0.05$) and enhanced functional ECE activity ($p<0.05$). The Ang II-induced increase in tissue ET-1 content and functional ECE activity was completely prevented by LU135252 ($p<0.05$). Chronic treatment of control animals with LU135252 lowered basal vascular but not renal ET-1 content ($p<0.05$ vs. control). Clearance of ^{125I}ET-1 was unaffected by the treatments. It is concluded that Ang II increases ET-1 protein and functional ECE activity in vascular smooth muscle and kidney through ET_A-receptors *in vivo.*

Angiotensin II (Ang II), a vasoconstrictor and mediator of vascular growth, stimulates expression of endothelin-1 (ET-1) in vitro[1, 2]. In vascular smooth muscle cells and cardiac fibroblasts ET-1 acts in an autocrine fashion [3, 4]: autocrine stimulation of ET-1-induced proliferation of vascular smooth muscle cells is regulated by an ET_A receptor-coupled mechanism [5]; furthermore, in rat cardiac fibroblasts, ET-1 mRNA expression stimulated by ET-1 or Ang II is blocked by a selective ET_A-antagonist in vitro [4]. Endothelin synthesis depends on activity of endothelin converting enzymes (ECE) [6-8] which cleave ET-1 from its precursor big-endothelin-1 (bigET-1) [9, 10]. ET-1 is rapidly internalized by vascular smooth muscle cells through clathrin-mediated endocytosis [11, 12] and cleared from the circulation by receptor-operated mechanisms in lung and kidney [13, 14]

The effects of Ang II and the role of ET_A receptors on the endothelin system in vascular and renal tissue *in vivo* are unclear. Furthermore, it remains to be determined whether increased levels of circulating Ang II affect the endothelin system on the level of the endothelium and/or vascular smooth muscle and whether it affects target organs such as the kidney. Therefore, we investigated the effects of chronic treatment with Ang II with or without the selective ET A antagonist LU135252 on vascular ET-1 content and functional ECE activity in rat aorta and femoral artery, determined ET-1 content in the kidney and measured clearance of ^{125I}ET-1.

We here demonstrate by quantitative radioimmunoassay that Ang II increases non-endothelial vascular and renal ET-1 protein and enhances functional ECE activity *in vivo* without affecting clearance of ET-1. ET_A-antagonist LU135252 prevented Ang II-induced changes suggesting that Ang II modulates ET-1 protein tissue content and functional ECE activity through ET_A-receptor-coupled mechanisms.

MATERIALS AND METHODS

Animals and clearance studies. Ten week old male Wistar Kyoto rats (IFFA CREDO, L'Arbesle, France, 220-250 g) were divided into three groups and treated for two weeks with Ang II (200 ng/kg/min) or saline (osmotic minipump Model 2002 [15]) or Ang II plus ET_A-receptor antagonist LU 135252 (50 mg/kg/day, with chow). Rats were anesthetized (thiopental, 50 mg/kg, intraperitoneal) and blood pressure was recorded intra-arterially after canulation of the left femoral artery. In a subset of animals (n=3 per group), clearance studies were performed by 0.3 mL trace bolus injections of non-pressor doses of approximately 200.000 dpm (0.13 pmol/L) of radiolabelled (^{125I}ET-1) peptide through the left jugular vein. 50 μL of blood sampled from the carotid artery was collected over 2 minutes [14, 16]. Radioactivity was determined in a gamma counter (Canberra Packard) and counts/minute versus time were fitted to a mono-exponential decay curve [13, 14]

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Tissue preparations and organ chamber studies. After sacrifice, aorta and femoral artery were removed and dissected in cold (4°C) Krebs ringer bicarbonate solution (composition in mmol/L: NaCl 118.6, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.1, edetate calcium disodium 0.026, glucose 11.1). Arterial rings (with and without endothelium) were suspended in organ chambers, connected to force transducers (UTC-2, Gould Statham, CA, U.S.A.) and equillibrated with potassium chloride as described [15]. Presence or absence of endothelium was tested in preconstricted arteries (norepinephrine, 0.1-0.5 μmol/L) by relaxation to acetylcholine, [15]. Contractions to big-ET-1 (0.1 -100 nmol/L) and ET-1 (0.1 -100 nmol/L) were obtained and normalized to potassium chloride. Functional ECE activity in intact vascular rings was calculated as described [17]: briefly, in rings of the same animal, contractions to the ECE substrate (big-ET-1, 10, 30, and 100 nmol/L) were obtained and divided by contractions to the ECE product (ET-1, 10, 30, and 100 nmol/L), ratios of contractions (normalized to potassium chloride 100 mmol/L) were expressed as arbitrary units [17]. Tissue from whole kidneys, aorta and femoral artery (with and without endothelium, which was removed mechanically [18]) was snap frozen in liquid nitrogen and kept at −80°C until determination of endothelin-1.

Quantitative measurement of tissue endothelin-1 protein content. ET-1 protein was extracted from vascular and renal tissue using a slightly modified protocol of that described by Hisaki [10]. Briefly, vessels were homogenized using a polytron for 60 seconds in 2 mL of ice cold chloroform:methanol 2:1 containing 1 mmol/L N-ethylmaleimide and 0.1% trifluoroacetic acid. Homogenates were left overnight at 4°C, then 0.8 mL of distilled water was added. The mixture was vortexed and centrifuged at 4000 rpm for 15 minutes and the supernatant was removed. 1 mL aliquots of the extract were diluted with 9 mL of 4% acetic acid and then extracted as described[15]. Eluates were dried in a speed-vac and reconstituted in working assay buffer for the radioimmunoassay. Overall recovery for ET-1 added to chloroform/methanol tissue homogenates and taken through all extraction steps was 65±3% with inter and intra-assay coefficients of 5.6% and 10%, respectively. ET-1 protein was measured by a quantitative radioimmunoassay [15]; ET-1 peptide was identified by reverse-phase HPLC [15] and related to wet weight (pg/g tissue).

Materials. Acetylcholine chloride, big endothelin 1-38 (human), norepinephrine bitartrate salt and potassium chloride were from Sigma Chemicals Co. (Buchs, Switzerland), endothelin-1 was from Novabiochem AG (Läufelfingen, Switzerland) and (3-[^{125}I]-iodotyrosyl)-ET-1 (2000 Ci/mmol/L, purity: >90%) was from Amersham (Amersham, U.K.).

Statistical analysis. Data are means ±SEM, n refers to the number of animals. Contractions to bigET-1 and ET-1 were normalized to KCl 100mmol/L. The area under the concentration-response curve (AUC) was calculated as described ([17], expressed in arbitrary units), sensitivity (EC_{50}) was calculated as described [15] and expressed as its negative logarithm (pD_2). Statistical significance was analyzed by two-way ANOVA followed by Bonferroni correction. ET-1 clearance data was analyzed using the non-parametric Mann-Whitney U-test, a $P < 0.05$ was considered significant.

RESULTS

Blood Pressure and Contractions in Aorta and Femoral Artery in Vitro

Compared to control rats (135±5 vs. 136±4 mmHg, n.s.), angiotensin II treatment increased systolic blood pressure (from 136±4 to 162±3 mmHg, $p<0.05$), which was partly reduced by LU135252 (131±3 vs. 146±3 mmHg, $p<0.05$ vs. Ang II). Treatment of control WKY rats with LU135252 had no significant effect on blood pressure (136±3 vs. 132±6 mmHg, n.s.). Contractions to KCl in aorta and femoral artery were not significantly affected (*Table 1*). Sensitivity (pD_2 values) and maximal responses of contractions to bigET-1 and ET-1 were higher in aorta ($p<0.05$, *Table 1*). Ang II-treatment increased the sensitivity, AUC and maximal responses of contractions to bigET-1 in aorta and femoral artery (n=7-10, $p<0.05$, *Table 1*). Changes in vascular reactivity in Ang II-treated animals were largely normalized by LU135252 ($p<0.05$, *Table 1*).

ET-1 Protein Content in Kidney, Aorta, and Femoral Artery

In control animals, ET-1 protein was 19±2 (kidney, n=5), 44±8 (aorta, n=7), and 127±11 pg/gram tissue (femoral artery, n=6). Ang II treatment increased ET-1 protein by 3-fold in the kidney (n=6, $p<0.05$), by 4.7-fold in the aorta (n=7, $p<0.05$), and 1.6-fold in the femoral artery (n=6, $p<0.05$) (*Figure 1*). Concomitant treatment with LU135252 reduced tissue ET-1 protein content to control values in kidneys and in aorta and femoral artery independent from endothelium removal (*Figure 1* and *Table 1*). In separate experiments, treatment of control animals with LU135252 for two weeks reduced ET-1 protein in aorta (28±4 pg/g, n=7, $p<0.05$ vs. control) and femoral artery (79±8 pg/g, n=6, $p<0.05$ vs. control) but not in the kidney (18±7 pg/g, n=5, n.s. vs. control).

Functional ECE Activity in Aorta and Femoral Artery

Ang II treatment increased functional ECE-activity (ratio of response to bigET-1/ET-1). The increase was greater in the aorta (10-fold at 30 nmol/L) than in the femoral artery (2-fold at 30 nmol/L, *Figure 2*). Increased functional ECE activity was reduced by LU135252 to control (aorta) or below control levels (femoral artery). At concentrations of 100 nmol/L of bigET-1 and ET-1, LU135252 had no effect on ECE activity (*Figure 2*). In control animals, LU135252 lowered functional ECE activity in femoral artery (0.32 vs. 0.05 units at 30 nmol/L, $p<0.05$) but not in aorta (0.05 vs. 0.06 units at 30 nmol/L, n.s.).

Clearance of Radiolabelled ^{125I}Endothelin-1

Compared to control animals (n=3), chronic treatment with Ang II with (n=3) or without LU135252 (n=3) had no effect on clearance of ^{125I}ET-1 (*Figure 3*).

DISCUSSION

This study demonstrates that chronic infusion of Ang II increases ET-1 protein content in vascular and renal tissue of WKY rats *in vivo* by an endothelium-independent mechanism and enhances functional vascular ECE activity in isolated rat aorta and femoral artery.

TABLE 1

Effects of Angiotensin II and ET_A-Antagonist LU135252 on Contractions in Response to Potassium Chloride (KCl), Vascular ET-1 Protein Content (ET-1 Protein), Sensitivity (pD_2), Area under the Curve (AUC) and Maximum Responses (Maximum, Percent of KCl 100 mmol/L) of Contractions in Response to Big Endothelin-1 (Big ET-1) and Endothelin-1 (ET-1) in Aorta and Femoral Artery with or without Endothelium (±/−Endo)

	Aorta			Femoral Artery		
Group	Control	Ang II	AngII + LU	Control	Ang II	AngII + LU
KCl (g)	2.83 ± 0.17	2.57 ± 0.15	3.16 ± 0.12	2.50 ± 0.1	2.11 ± 0.17	2.39 ± 0.17
ET-1 protein (pg/mg, −Endo)	44 ± 10	137 ± 20*	53 ± 3†	34 ± 12	159 ± 49*	60 ± 20†
big ET-1 (pD2)	8.30 ± 0.01	8.41 ± 0.08*	8.25 ± 0.01†	7.37 ± 0.04‡	7.7 ± 0.1*‡	7.26 ± 0.01†‡
big ET-1 (AUC)	25 ± 2	49 ± 9*	23 ± 2†	33 ± 6	83 ± 13*	34 ± 1†
big ET-1 (Maximum, +Endo)	75 ± 5	108 ± 5*	83 ± 6†	78 ± 8	109 ± 8*	111 ± 4
big ET-1 (Maximum, −Endo)	84 ± 6	109 ± 8*	78 ± 10†	73 ± 8	114 ± 3*	103 ± 9
ET-1 (pD2)	9.13 ± 0.03	9.05 ± 0.05	9.2 ± 0.04†	7.98 ± 0.03‡	8.15 ± 0.03*‡	7.78 ± 0.05†‡
ET-1 (AUC)	159 ± 8	132 ± 12	167 ± 8†	115 ± 5	128 ± 11	103 ± 8
ET-1 (Maximum)	137 ± 6	119 ± 10	138 ± 4	110 ± 4	108 ± 8	121 ± 5

Data are means ± SEM. *$p < 0.05$ vs. control, †$p < 0.05$ vs. salt, ‡$p < 0.05$ femoral artery vs aorta; ANOVA with Bonferroni.

Treatment with the selective ET_A-receptor antagonist LU135252 completely prevented changes in ET-1 protein levels and functional ECE activity in angiotensin II-treated animals and reduced vascular but not renal ET-1 levels in control animals.

Interactions between the renin-angiotensin system and the endothelin system in vivo are still unclear. Acute administration of Ang II stimulates ET-1 mRNA and protein expression in cultured endothelial and vascular smooth muscle cells and cardiac fibroblasts in vitro [2-4, 19]. Furthermore, in vitro-studies have suggested a role for ET-receptors in regulating the action and expression of ET-1 and ET receptors through autocrine mechanisms [4, 5, 20, 21] as well as by modulating clearance of ET-1 [14]. We determined whether Ang II and ET_A receptors affect the endothelin system *in vivo* and investigated the effects on tissue ET-1 protein, functional ECE activity (using a bioassay of isolated vascular rings), and clearance of radiolabelled ET-1. This study is the first demonstration that Ang II treatment increases ET-1 protein in vascular and in renal tissue *in vivo*. Basal ET-1 protein levels were different between the tissues studied: vascular ET-1 levels were 2 to 8-fold higher than in renal tissue, suggesting that basal expression of ET-1 may play a important role in vascular homeostasis under normal conditions. Also, the response to stimulation by Ang II was greater in the aorta than in the femoral artery. As endothelium removal had little effect on ET-1 tissue content, these data indicate that Ang II increases ET-1 protein mainly at the level of vascular smooth muscle cells.

The ET_A receptor antagonist LU135252 prevented the increase of ET-1 protein induced by Ang II indicating that ET_A receptors are involved in the regulation of ET-1 tissue levels. Although the underlying mecha-

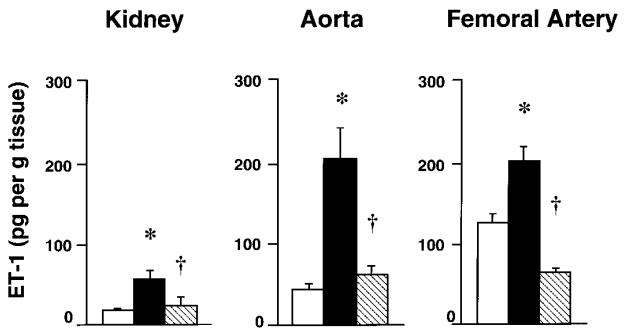

FIG. 1. Vascular and renal endothelin-1 protein concentrations (pg ET-1/g tissue wet weight) in control □, angiotensin II ■ and angiotensin II+LU135252 ▨-treated animals: Angiotensin II increased ET-1 protein in kidneys and arteries. These increases were completely prevented by ET_A-antagonist LU135252. *$p<0.05$ vs. control, †$p<0.05$ vs. Ang II.

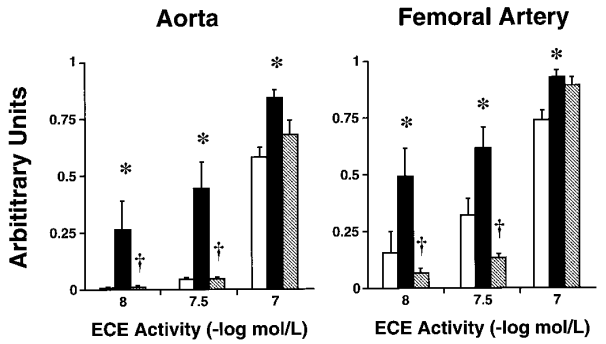

FIG. 2. Functional endothelin converting enzyme activity (ratio of responses to bigET-1/ ET-1, normalized to KCl) in aorta and femoral arteries control □, angiotensin II ■ and angiotensin II+LU135252 ▨-treated animals: Angiotensin II enhanced functional ECE activity in both arteries. ET_A-antagonist LU135252 normalized functional ECE activity. *$p<0.05$ vs. control, †$p<0.05$ vs. Ang II.

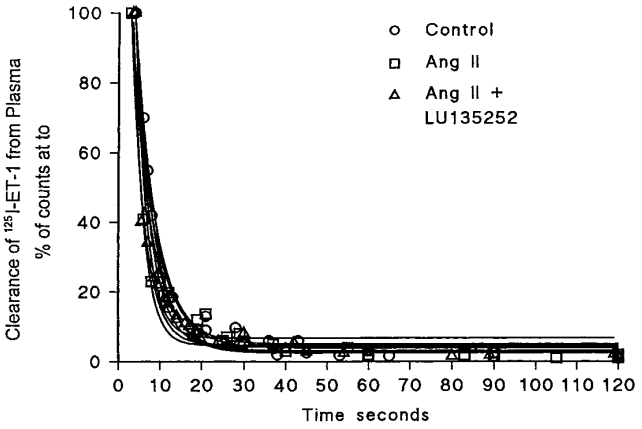

FIG. 3. Clearance of radiolabelled ^{125I}ET-1 in WKY rats: Treatments with Ang II with or without LU135252 had no effect on clearance of ET-1 (n.s.).

nisms are currently unclear, these findings could reflect an autocrine regulatory mechanism modulating ET-1 levels in vascular smooth muscle and renal tissue through ET_A receptors similar to those described in vitro [4, 5, 20] or an interference between LU135252 and ET-1 bound to the ET_A receptor [22]. Endothelial cells are unlikely to be the target for the effects of LU135252, since autocrine expression of ET-1 in these cells is regulated through ET_B receptors [21] and endothelial cell essentially do not express ET_A receptors. Our data further suggest that ET_A receptors regulate ET-1 content in vascular smooth muscle also under normal conditions, at least in certain vascular beds. In line with this interpretation, LU135252 reduced vascular but not renal ET-1 protein levels in control WKY rats. ET-1 protein content in Ang II-hypertensive animals was completely normalized, although LU135252 only in part lowered blood pressure. Furthermore, in control animals LU135252 lowered vascular ET-1 protein without affecting blood pressure. It was recently reported that overexpression of the ET-1 gene in transgenic mice is not associated with hypertension despite vascular hypertrophy [23] and that ET-1 overexpression using the preproendothelin-1 promotor increases vascular and tissue ET-1 levels without altering blood pressure [24, 25]. These results support the hypothesis that ET_A receptor-mediated regulation of tissue ET-1 protein as shown in this study may be pressure-independent. Indeed, preliminary studies from our laboratory demonstrate that the calcium antagonist verapamil lowers blood pressure to the same extent as ET_A blockade with LU135252 but that it does not affect Ang II-induced increases in tissue ET-1 levels in the aorta of these animals [26].

Synthesis of ET-1 is regulated by endothelin converting enzymes (ECE) [6-8] which utilize the precursor bigET-1 to form the active peptide ET-1 [9, 10]. Since ECE activity is difficult to assess in intact vascular rings, we applied an ECE bioassay incorporating responses to bigET-1 and ET-1 which give an functional estimate of ECE activity in intact rings [17]. First, we observed that functional ECE activity under basal conditions was different between aorta and femoral artery indicating an anatomical heterogeneity in line with a recent observation in aging rats [17]. Ang II treatment enhanced functional ECE activity to a greater extent in aorta than in femoral artery indicating that Ang II may not only affect the endothelin system by direct activation of ET-1 expression [2, 3] but also by stimulating its conversion which may differ between between certain vascular beds. Whether the different susceptibility to stimulation by Ang II is related to the difference in basal expression of ET-1 (being 3-fold higher in the femoral artery) and whether increased functional ECE activity contributes to increased tissue ET-1 levels, remains to be determined. As with ET-1 tissue levels, Ang II-induced activation of functional vascular ECE activity was inhibited by ET_A receptor blockade. LU135252 also lowered functional ECE activity in the femoral artery but not in the aorta of control animals. This indicates a potential role for ET_A receptors regulating conversion of bigET-1 under basal and stimulated conditions *in vivo*.

Synthesis and clearance are major determinants of ET-1 production [14, 16]. ET-1 is released into the circulation and abluminally towards the vessel wall [27] where it is rapidly taken up by vascular smooth muscle [11, 12]. We have demonstrated that clearance of ET-1 is unaffected by chronic treatment with Ang II alone or in combination with LU135252. This suggests that the increase in tissue ET-1 protein content is a local process in the vasculature and kidney and is likely not to be related to impaired clearance of circulating ET-1. Our previous observation showing that Ang II increases circulating ET-1 levels in Ang II-induced hypertension [15] is in line with this interpretation.

In conclusion, chronic Ang II infusion increased vascular and renal ET-1 protein and functional vascular ECE activity in WKY rats *in vivo*, these increases were independent of the endothelium. Clearance of ET-1 was not affected. ET_A-blockade with LU135252 reduced Ang II-induced ET-1 protein to basal levels in all tissues studied, normalized functional ECE activity and also reduced ET-1 protein in the vasculature of normotensive control animals. These results suggest that Ang II and ET_A receptors play an important role in the regulation of the vascular and renal the endothelin system*in vivo*.

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