The expression of renin and the formation of angiotensin II in bovine aortic endothelial cells

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Abstract

One controversy in the field of vascular angiotensin generation has surrounded the nature and particularly the source of vascular renin. This study investigated the expression of renin protein and its mRNA in aortic endothelial cells using immunocytochemistry, Western blotting, *in situ* hybridization and reverse transcription PCR (RT-PCR). Using a monoclonal antibody against human renin, immunocytochemical analysis revealed positive immunoreactivity in the cytoplasm of cultured bovine aortic endothelial cells. Immunoblotting of solubilized proteins separated by SDS-PAGE from cultured aortic endothelial cells identified two immunoreactive species with molecular masses of approximately 37–40 kDa. *In situ* hybridization showed that renin mRNA was localized in the cytoplasm of these cells. Using RT-PCR of RNA extracted from bovine aortic endothelial cells with primers specific for human renin, a clear single band was detected, which had the predicted size of 142 bp for (pro)renin.

Angiotensin II (Ang II) was assayed in conditioned medium (CM) from cultured bovine aortic endothelial

cells, and in addition, the effects of Ang II and CM on the proliferation of aorta smooth muscle cells (ASMC) were also studied. The results showed that CM contained Ang II equivalent to 15.05 ± 4.67 pg/ 10^6 cells. Assay of smooth muscle cell proliferation by cell number, and by tritiated thymidine uptake, showed that proliferative responses in the presence of Ang II at a concentration of 10^{-6} M were evident within 1 day of subculture, and cell numbers were nearly twice those of controls after 2 days. Thymidine incorporation into ASMC was also increased by Ang II in a dose-dependent manner and by endothelial cell CM. In both cases, stimulated proliferation was inhibited by the Ang II type 1 (AT1) receptor selective antagonist, losartan. These findings suggest that these vascular endothelial cells are a source of locally synthesized renin that may thus be involved in vascular Ang II generation. They also suggest that Ang II produced by the endothelial cells may be secreted and stimulate ASMC proliferation via the AT1 receptor.

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Introduction

Renin is a glycoproteolytic enzyme that is responsible for the first step in the formation of angiotensin II (Ang II). The main source of circulating renin is thought to be the juxtaglomerular cells of the afferent arterioles of the kidney (Gomez *et al.* 1990). The behaviour of the circulating renin–angiotensin system is well known; however, the actions of renin and the generation of Ang II at the tissue level are less well understood. Although the presence of a renin-like enzyme in the vascular wall was noted very soon after a biochemical assay for renin activity became available, and activity like that of angiotensin-converting enzyme (ACE), which converts angiotensin I to Ang II, was localized to the vascular wall, the concept that angiotensin I is also primarily generated in the vascular wall, rather than in the circulation, has evolved more slowly (Campbell 1985). The main controversy in the field of vascular angiotensin generation has surrounded the

nature and particularly the source of vascular renin (Swales & Heagerty 1987). Although indirect evidence also suggests the presence of renin in human resistance vessels and in perfused rat hindlimb preparations, this has not been shown conclusively (Bund *et al.* 1989, Muller & Luft 1998). The question remains whether renin is generated locally in the vascular wall, or instead is taken up from the circulation to act locally (Muller & Luft 1998). In the present study, we have used primary cultures of pure endothelial cells to determine whether renin expression and Ang II formation occur in the endothelial cells of the rat bovine aorta.

Materials and Methods

Cell culture

Bovine aortic endothelial cells (BAEC) were harvested enzymatically from bovine aorta following established

methods (Jaffe *et al.* 1973, Gunbrone 1974, Booyse *et al.* 1975) with minor modifications. Cells were grown in RPMI-1640 medium (ICN-Flow Ltd, High Wycombe, Bucks, UK) with 10% new-born bovine serum (Sigma Chemical Co., Poole, Dorset, UK) and 5% fetal bovine serum (FBS) (Sigma), 100 units/ml penicillin (Gibco, Paisley, Strathclyde, UK), 100 µg/ml streptomycin (Gibco), and 4 µmol/l L-glutamine (Gibco). The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. The medium was changed once every 2 days. After confluence within 2 weeks, the cells were rinsed with PBS, and subsequently trypsinized with 0.08% trypsin in PBS by incubating them in this solution for 1 min at 37 °C. The resulting suspension of cells was pipetted into a 75 cm² tissue culture flask containing 10 ml medium and incubated as mentioned previously. The second passaged cells were used for experiments.

Bovine and rat aortic smooth muscle cells (BASMC, RASMC) were isolated by the media explant method and cultured over several passages according to Ross (1971). Cells were grown in 5% CO₂ in air at 37 °C using RPMI-1640 culture supplemented with 100 units/ml of penicillin, 100 µg/ml streptomycin, 4 µmol/l L-glutamine and 20% FBS. Experiments were performed with cells from passages three to five.

Conditioned medium (CM)

BAEC at the stage of exponential cell proliferation were washed and 10 ml of the usual culture medium containing 2% serum replacement (Sigma) instead of 20% FBS were added. Forty-eight hours later, this medium (endothelial cell conditioned medium, ECCM) was removed and immediately filtered through a 0.22 µm filter. CM from exponentially growing BASMC (SMCCM) was collected in the same manner. Before adding it to cells, CM was mixed 2:1 with medium RPMI-1640 containing 2% serum replacement. Collected media were stored at -20 °C prior to use.

Cell count

A suspension of RASMC (10⁵ cells/ml) was prepared on the first day of the experiment using RPMI-1640 supplemented with 20% FBS. One millilitre of this cell suspension was distributed to each well of a 24-well multiwell dish. The medium was replaced 24 h after the subculture with 20% FBS/RPMI-1640 containing 10⁻⁶ M, 10⁻⁷ M or 10⁻⁸ M Ang II with or without losartan (10⁻⁵ M) with three wells per group. Experiments were terminated at various time points by washing cells with PBS and resuspending them in 0.3 ml PBS containing 0.125% trypsin and 0.02% EDTA. Digestion was stopped by addition of 0.7 ml 20% FBS/RPMI-1640 to each well. Cells were counted in a haemocytometer using light microscopy.

Tritiated thymidine uptake

RASMC suspensions (0.3 × 10⁵ cells/ml) were prepared as above. The medium was replaced with appropriate experimental media as above, 24 h after subculture, with six wells per group. After a further 24 h, 10 µl [³H]methylthymidine (Amersham International, Amersham, Bucks, UK), 0.1 mCi/ml were added to each well. Twenty-four hours after the addition of radioactive thymidine, media were aspirated and the cultured cells were rinsed three times with cold PBS (Oikawa *et al.* 1987). The cells were then dissolved in 0.5 ml 0.1 M NaOH and a 0.3 ml aliquot was mixed with 3.5 ml scintillation fluid (Packard Instruments BV Chemical Operations, Groningen, The Netherlands) and, after standing overnight at room temperature, tritium content was assayed in a Wallac 1410 liquid scintillation counter. BASMC suspensions were prepared in the same manner as RASMC. Twenty-four hours after subculture, the medium was replaced with ECCM and SMCCM separately. After a further 24 h, [³H]methylthymidine (10 µl, 0.1 mCi/ml) was added to each well. Twenty-four hours after the addition of radioactive thymidine, tritium content was assayed in the same method as for RASMC.

Ang II assay

ECCM was concentrated by freeze drying. The 10-fold concentrated medium was assayed for Ang II using an angiotensin RIA kit (Nichols Institute Diagnostics, Saffron-Waldon, UK). RPMI-1640 medium containing 2% serum replacement was used as control.

Immunocytochemistry

After fixation in 100% cold acetone for 15 min, cells were incubated in 0.3% hydrogen peroxide-methanol for 15 min to block endogenous peroxidase activity, and in 20% BSA in PBS for 20 min to block non-specific staining. Cells were then incubated at 37 °C for 90 min with anti-renin (human) antibody 2D12 (diluted in PBS 1:1000; generously given by Prof. P Corvol, INSERM, Paris, France). Slides were then treated for 30 min at room temperature with biotinylated anti-mouse IgG diluted 1:400 in 0.01 M PBS and then avidin-biotin-peroxidase (ABC kit; Sigma) for 45 min at room temperature. Peroxidase activity was visualized by the diaminobenzidine reaction according to the manufacturer's instructions. Primary antibody was omitted in negative control slides, which consistently remained unstained.

Western blotting

Cells were harvested by trypsinization, washed with PBS and centrifuged. The supernatant was removed and 200 µl 1% Triton X-100 solubilization buffer containing 50 mM

Tris-HCl (pH 7.4), 1 µg/ml each of protease inhibitors aprotinin and soybean trypsin inhibitor and 30 µg/ml phenylmethylsulphonyl fluoride (Sigma) were added to the cell pellet, mixed at 4 °C for 40 min, and the mixture centrifuged again. The solubilized fraction, equivalent to 400 µg protein, as estimated by the Lowry method (Lowry *et al.* 1951), was loaded into each well and subjected to SDS-PAGE using the methods of Laemmli (1970) with prestained SDS-PAGE molecular weight standards (Sigma) in an adjacent well. The gel (7.5% polyacrylamide) was run overnight at 30 V or for 3 h at 200 V. Proteins were electrotransferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham) overnight at 30 V using the following transfer buffer: 18.2 g trizma base and 71.3 g glycine in 1 litre methanol and 2 litres water. Non-specific binding sites on the gel were then blocked with 10% milk powder for 3 h. Membranes were washed four times for 10 min with 0.1% Triton X-100 in PBS and incubated with primary anti-renin antibody (2D12) diluted 1:1000 in PBS containing 0.1% Tween-20 (Sigma) (PBS-T) for 1 h. Membranes were then washed twice for 10 min in PBS-T, incubated for 1 h with horseradish peroxidase-linked sheep anti-mouse IgG (Amersham) diluted 1:5000 in PBS-T. Positive bands were visualized using ECL Western blotting detection reagent (Amersham).

In situ hybridization

Preparation of probe Human renin oligonucleotide antisense probe (45 bases; PE-Applied Biosystems, Warrington, Cheshire, UK) was used. Human renin oligonucleotide sense probe (45 bases) was used as negative control. The sequences of sense and antisense probe were 5'-CCTCAATCCGAGAAAGCCTGAAGGAACGAGGTGTGGACATG-3' and 5'-CATGTCCACACCTCGTTCCTTCAGGCTTCTCGGATTGAGG-3' respectively. The sense and antisense probe correspond to nucleotides 142–187. These oligonucleotides were labelled with digoxigenin (DIG) using a DIG Oligonucleotide Tailing Kit (Boehringer-Mannheim, Lewes, East Sussex, UK). The probes were stored at a final concentration of 50 ng/µl at –20 °C prior to use.

Preparation of cells Cells were washed with PBS at 37 °C, fixed with 4% (w/v) formaldehyde for 20 min, washed with PBS at room temperature, dehydrated in 70, 90 and 100% ethanol, and stored until required at –70 °C. Before hybridization, cells were rehydrated by incubating successively in 100, 90 and 70% ethanol, and treated for 30 min at 37 °C with 100 mM Tris-HCl, 50 mM EDTA, pH 8.0 containing proteinase K at 20 µg/ml, and finally prehybridized for 20 min at 37 °C with prehybridization buffer containing 50% formamide, 4 × SSC, 10 mM Tris-HCl (pH 7.5), 1 × Denhardt's solution (Sigma), 500 µg/ml salmon testis DNA (Sigma)

and 10% dextran sulphate (Sigma) according to the manufacturer's protocol.

Hybridization The slides were incubated with hybridization solution containing 1 µg/ml DIG-tail labelled oligonucleotide probe in prehybridization buffer in a moist chamber at 42 °C overnight. The slides were washed twice for 15 min in 2 × SSC at room temperature, twice for 15 min in 1 × SSC and twice for 30 min in 0.1 × SSC.

Immunodetection of *in situ* hybridization signal The slides were washed briefly with DIG-buffer I (100 mM Tris-HCl, pH 7.5; 150 mM NaCl), blocked with buffer I containing 0.1% Triton X-100 and 2% normal sheep serum for 30 min, and further incubated for 2 h with buffer I containing 500 × diluted anti-DIG polyclonal antibody-conjugated alkaline phosphatase (Boehringer-Mannheim), 0.1% Triton X-100, 1% normal sheep serum at room temperature. The slides were washed twice for 15 min in buffer I and incubated once for 10 min in buffer II (100 mM Tris-HCl, pH 9.5; 100 mM NaCl, 50 mM MgCl₂) and incubated with colour-substrate solution containing nitroblue tetrazolium (450 µg/ml), 5-bromo-4-chloro-3-indolylphosphatase (175 µg/ml) and levamisole (1 mM) in buffer II. The colour reaction was developed in a moist chamber for 48 h in the dark. The reaction was stopped by adding buffer III (10 mM Tris-HCl, pH 8.1; 1 mM EDTA).

Isolation of total cellular RNA and reverse transcription-PCR (RT-PCR)

Isolation of total cellular RNA Total cellular RNA was extracted from cultured BAEC using the RNeasy Total Pure kit (Qiagen, Crawley, UK) according to the manufacturer's protocol, and quantified spectrophotometrically by measuring absorbance at 260 and 280 nm.

RT-PCR The RNA was reverse-transcribed as follows. A 20 µl reaction volume was used. The following components were added to an RNAase-free microcentrifuge tube: 5 µg total RNA, 4 µl 5 × first strand Buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl₂ pH 8.3 at room temperature), 2 µl 0.1 M dithiothreitol, 1 µl 10 mM dNTP (mix 10 mM each dATP, dGTP, dCTP and dTTP at neutral pH; Gibco), 1 µl Random Hexamers (3 µg/µl; Gibco), 1 µl reverse transcriptase (200 U/µl; Gibco), and water to make up the volume to 20 µl. The contents of the tube were mixed gently. After incubation at 23 °C for 20 min and 42 °C for 60 min, the samples were heated at 95 °C for 5 min to terminate the reactions, and were stored at –20 °C until use. The sequence of sense and antisense primers for human renin were designed according to published sequences. Oligonucleotide primers were custom made by Gibco. The sequences of sense and antisense primers for human renin were

Figure 1 The effect of Ang II on the proliferation of RASMC in the absence (a) and presence (b) of losartan (DuP), 10^{-5} M. Cells were distributed on a 24-well multiwell dish. At each time point, cells were harvested and counted. The increase in cell number was dependent on the concentration of Ang II, and was inhibited by losartan. Value are means $\pm$ S.E., $n=3$ throughout. Cell numbers in groups with Ang II 10^{-5} M were nearly twice those of controls after 2 days, $P<0.05$, significantly different from controls. (c) bFGF was used as a positive control.

5' GTGTCTGTGGGGTCATCC 3' and 5' ATCAAAC AGCCTCTTCTTGGC 3' respectively, corresponding to bases 2062–2079 and 2182–2202 (exon 7) of the cloned full-length sequence. The predicted sizes of the amplified renin cDNA products were 142 bp. After incubation for 3 min at 94 °C, 5 μ l of the single-stranded cDNA in the 20 μ l reaction mixture were amplified with 2 pmol each of sense and antisense primers and 2.5 units of Taq DNA polymerase in 50 μ l 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each dNTP. The reactions

were performed for 1 min at 95 °C and 1 min at 58 °C for 35 cycles. The RT-PCR products in 10 μ l aliquots were electrophoresed on a 1.4% agarose gel.

Results

Effects of Ang II and CM on the proliferation of ASMC

Increased proliferation of RASMC in the presence of Ang II was evident from day 1, though sharply reduced after

Figure 2 Incorporation of [^3H]thymidine into RASMC incubated with serum-free medium containing Ang II with or without losartan, 10^{-5} M for 48 h. Data are means $\pm$ S.E., $n=6$. *Not significantly different from controls. ** $P<0.01$.

day 2 similar to the effect of bFGF. The number of RASMC in groups treated with 10^{-6} M Ang II was nearly twice that of controls. The increase in cell number was dependent on the concentration of Ang II, and was inhibited by losartan (Fig. 1).

Tritiated thymidine incorporation into RASMC increased with Ang II concentration. The Ang II-induced increase in tritiated thymidine incorporation was inhibited by losartan (Fig. 2).

ECCM increased thymidine incorporation into BASMC, but SMCCM did not. This ECCM-induced increase in thymidine incorporation was inhibited by losartan (Fig. 3).

Ang II assay

Ang II generation was demonstrated in ECCM. The amounts of Ang II in ECCM is significantly ($P<0.05$) higher than that of controls (Table 1).

Figure 3 Incorporation of [^3H]thymidine into RASMC incubated with ECCM and SMCCM with or without losartan (DUP), 10^{-5} M for 48 h. Data are means $\pm$ S.E., $n=6$. *Not significantly different from controls. ** $P<0.01$.

Table 1 Ang II in CM from BAEC

	Concentration (pg/ 10^6 cells)	<i>n</i>	<i>P</i>
BAEC	15.05 ± 4.67	3	<0.05
Control	3.2 ± 1.04	3	—

Immunocytochemistry

Immunoreactive renin was found to be present in the cytoplasmic compartment of cultured bovine endothelial cells, though possibly more concentrated in the perinuclear region (Fig. 4a). Negative control slides remained unstained (Fig. 4b).

Immunoblotting

Evidence that the protein recognized in bovine endothelial cells by immunostaining was indeed renin was obtained by immunoblotting after SDS-PAGE. Using solubilized cell fractions from cultured endothelial cells, two immunoreactive bands were obtained with molecular masses of approximately 37–40 kDa, similar to the expected values for renin and prorenin (Fig. 5).

In situ hybridization

The specific signals of the antisense probe were located in the cytoplasm of cultured endothelial cells (Fig. 6a). No signal was detected in negative controls in which sections were hybridized with the sense probe (Fig. 6b).

RT-PCR

In RT-PCR, using bovine endothelial cell RNA and primers derived from the human renin sequence, a single band was detected with the size predicted of 142 bp (Fig. 7).

Discussion

In addition to its systemic origin, there is abundant evidence that Ang II can also be formed by localized tissue renin–angiotensin systems. Many of these, for example in the reproductive tract, have been shown to have functions that are distinct from those of the vascular and homeostatic roles of the systemic system.

Though elements of a tissue renin–angiotensin system have been identified in the vascular wall of the major vessels (Unger & Gohlke 1990, 1992, Lindpaintner & Ganten 1991, Johnston 1992, Lee & Lindpaintner 1993, Swales & Samani 1993, Von Lutterotti *et al.* 1994) there is less general agreement about their origin (Okamura *et al.* 1992, Unger & Gohlke 1992, Hilgers & Mann 1993). A

Figure 4 (a) Immunoreactive renin was found to be present in the cytoplasmic compartment of cultured bovine endothelial cells, though possibly more concentrated in the perinuclear region (arrow). (b) Negative control slides remained unstained.

key example is renin (Taddei & Salvetti 1992). Is the renin that has been located in endothelial cells indeed synthesized there, or is it taken up from the circulation (Dzau 1993)? If the former, why, it may be asked, should circulating renin evidently be so clearly associated with vascular function? If the latter is true, however, what is the function of renin uptake?

This problem is addressed in this paper by using primary cultures of pure bovine vascular endothelial cells. The results presented here clearly show that renin in proliferated, second generation endothelial cells maintained in culture for 48 h, contains prorenin and renin, as judged by immunocytochemistry (Fig. 4) and the presence of immunoreactive components with appropriate molecular masses in immunoblotting gels (Fig. 5). These data alone

suggest that the (pro)renin detected is indeed synthesized within these cells – there can be no other source in such cultures. Final proof comes from *in situ* hybridization evidence (Fig. 6) and RT-PCR (Fig. 7), which demonstrate the presence of (pro)renin mRNA.

The concept that Ang II is also primarily generated in the vascular wall rather than in the circulation has evolved slowly (Campbell 1985). However, it is known that vascular endothelial cells may play an important role in the vascular structural remodelling that occurs, for example in chronic vascular disease (Xiao *et al.* 1993), and it is thought that Ang II may be involved (Berk *et al.* 1989, Daemen *et al.* 1991, Scott-Burden *et al.* 1991). In the present studies, to further confirm the existence of local renin–angiotensin system components, we also examined

Figure 5 Immunoblotting of SDS gel of solubilized proteins from cultured BAEC, using anti-renin (human) antibody 2D12 to the renin protein. Two immunoreactive bands with molecular masses of approximately 37–40 kDa are recognized.

Figure 6 *In situ* hybridization for the detection of renin mRNA with DIG-labelled antisense and sense probes. There is positive staining in the cytoplasm of cultured endothelial cells (a) and no positive cells are detected using the sense probe (b).

whether Ang II is secreted by these cells and in sufficient quantities to affect the proliferation of vascular smooth muscle cells (ASMC). The CM from exponentially proliferating BAEC contained Ang II in assayable amounts (Table 1), and in addition tritiated thymidine incorporation into ASMC was increased by ECCM, as well as by Ang II (Figs 2 and 3). Both of these stimulated increases, by ECCM as well as by Ang II, were inhibited by losartan. These data suggest that the vascular wall is not only a target for Ang II, but is also a site of its production. The results also suggest that Ang II may regulate ASMC proliferation in an autocrine manner. Although tritiated thymidine uptake was not significantly enhanced by SMCCM, it was significantly decreased in the presence of losartan (Fig. 3). This is also consistent with the data in Fig. 1, in which

RASMC growth in the presence of Ang II is inhibited by losartan.

The problem remaining then is: how does the function of systemically generated renin and Ang II differ from that in the tissue? One possibility that has been suggested is that the tissue and systemic systems have somewhat different roles, and whereas the systemic renin–angiotensin system may more directly control acute functions, such as changes in vascular tone, and blood pressure, it is the tissue-located renin–angiotensin system that is responsible for tissue maintenance and repair (Brilla *et al.* 1992, Johnston *et al.* 1992, Lee *et al.* 1992, Lee & Lindpaintner 1993). In one sense this may be consistent with the present results which, at least from these *in vitro* data, suggest that formation of renin within the tissue is constitutive, and does not require hormonal support from the blood, and hence is not subject to momentary changes in stimulation that result from acute responses to physiological demand. However, the therapeutic benefit deriving from ACE inhibition, or from Ang II type 1 receptor antagonists may well derive in part from inhibition of the tissue renin–angiotensin system in the blood vessel endothelium (Lee *et al.* 1992, Von Lutterotti *et al.* 1994).

The question of the regulation of endothelial renin synthesis remains to be resolved, however, in view of the relationship between renin and Ang II generation. The use of primary cultures of pure endothelial cells presents appropriate experimental approaches.

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Figure 7 Using RT-PCR of RNA extracted from bovine aorta endothelial cells with primers specific for renin, a clear single band was detected, which had the predicted size of 142 bp.

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