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DIRECT EVIDENCE FOR LOCAL GENERATION AND RELEASE OF ANGIOTENSIN II IN HUMAN VASCULAR TISSUE

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Summary: A direct measurement of both angiotensins I and II immunoreactive substances was made in the perfusate from isolated human umbilical vein perfused with Krebs-Ringer solution which was free of any component of the renin-angiotensin system. The identity of the immunoreactive peptides was confirmed as angiotensin I and angiotensin II by high-performance liquid chromatography in reference to standard compounds. The rate of release of angiotensins was 41.9±7.4 and 63.4±12.0 pg for angiotensins I and II, respectively, during the first perfusion period of 30 min, and it remained stable at least for 3 hours. Angiotensin-converting enzyme inhibitor captopril, added to the perfusion medium (10^{-9} to $5x10^{-6}$ M), suppressed immunoreactive angiotensin II release in a dose-dependent fashion; the maximal percent inhibition of angiotensin II release evoked by captopril ($5x10^{-6}$ M) was approximately 56%. These results taken together with the previous observations of presence of essential components of the renin-angiotensin system in vascular tissue provide direct evidence for local generation and subsequent release of angiotensin II in vascular beds of human beings.

The presence of essential components of the renin-angiotensin system, such as renin enzyme, angiotensinogen, angiotensin converting enzyme, and angiotensin receptors, has been shown in vascular tissues (1-6). Although earlier reports of renin in homogenates of vascular tissues (7-9) may be explained largely by adherence of plasma renin of renal origin to vascular

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Abbreviations: Ang I, angiotensin I; Ang II, angiotensin II; Ang III, angiotensin III; BSA, bovine serum albumin; TFA, trifluoroacetic acid; PMSF, phenylmethylsulfonyl fluoride; HPLC, high-performance liquid chromatography.

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beds, rigorous studies in recent years have revealed the presence of specific immunoreactive renin in arterial tissue (10,11), cultured smooth muscle cells (12), and endothelial cells (13). In addition, angiotensinogen messenger RNA (mRNA) could be detected in vascular tissues (3), and angiotensin converting enzyme and angiotensin II (Ang II) receptors are present in the vasculature. Thus, Ang II probably is formed locally in the vascular tissues, is released from it, and binds to Ang II receptors in vascular smooth muscle. Indeed, we previously demonstrated that sizable amounts of immunoreactive angiotensin I (Ang I) and Ang II were released continuously for several hours from diverse vascular beds such as mesenteric arteries (14) and hind legs (15,16) of rats when these vascular tissues were isolated and then perfused with angiotensinogen-free medium. It was also shown in these studies that such release of Ang II is not due to mere leakage from the vascular beds but due to regulated mechanism (14-16), finding providing experimental evidence for production and subsequent secretion of Ang II from vascular tissue.

To further continue the investigation of the mechanism by which Ang II is generated in vascular tissues, we have studied the release of Ang I and Ang II from isolated human umbilical vein perfused with a medium which was free of any component of the renin-angiotensin system.

Materials and Methods

Perfusion of umbilical vein: Whole human umbilical cords were kept in ice-cold saline immediately after they were obtained at delivery. A cannula was inserted into the umbilical vein, which was then perfused with Krebs-Ringer solution (112 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11.2 mM D(+)-glucose), containing 0.4% (w/V) bovine serum albumin (BSA), in a water-jacketed container maintained at 37°C. The solution was aerated with a mixture of 5% CO_2 and 95% O_2 to obtain a pH of 7.4. The tissues were perfused at a constant flow rate of 6 ml/min with a roller pump (Polystaltic Pump, Buchler, Fort Lee, NJ). The perfusion pressure was recorded with a pressure transducer (Model CP-01, Century Technology Co, Inglewood, CA) connected to a polygraph (Model S-0411, Nihon Koden Co., Tokyo). During perfusion of the umbilical cord the perfusion pressure was approximately 25 to 30 mmHg. To avoid contamination of plasma angiotensin, the reparations were thoroughly washed with Krebs-Ringer solution for 30 min before the sample collections were started.

Determinations of angiotensins: Angiotensins that were released from the perfused umbilical cord were isolated by solid-phase extraction with an octadecasilyl-silica cartridge (Sep-Pak C_{18}, Waters Associates, Milford, MA) as described previously (14-16). In brief, the cartridge was connected to the water-jacketed container to allow the perfusate to pass through the cartridge.

Cartridges, which were exchanged at a 30-min intervals, had been moistened with 5 ml of methanol and prewashed with 10 ml of Krebs-Ringer solution just before use. After washing with 10 ml of 0.1% trifluoroacetic acid (TFA), angiotensins were eluted with the mixture of methanol : distilled water : TFA (80:19.9:0.1, vol ratio). The eluate was evaporated to dryness in a vacuum centrifuge (Model MC-90, Taiyo Scientific Instruments Co., Tokyo) and resuspended in 0.1 M Tris-acetate buffer containing 2.6 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1% BSA, pH 7.4, for radioimmunoassay. Under these conditions, the lowest detectable amount of immunoreactive Ang II was 1 pg/tube, and the blank values were always less than 1 pg/tube. The Ang II antiserum showed less than 0.01% cross-reactivity with Ang I but 100% cross-reactivity with angiotensin III (Ang III; Ang-(2-8) heptapeptides), Ang-(3-8) hexapeptide, and Ang-(4-8) pentapeptide.

Identification of angiotensins by high-performance liquid chromatography (HPLC): The peptides were fractionated following elution from the Sep-Pak on a Nova Pak C_{18} column (0.39x15 cm, Waters Associates). Elution was effected using a 20 min exponential gradient following curve 7 of a Waters Associates gradient controller. The starting solvent was 89% buffer A and 11% buffer B and the final solvent was 68% buffer A and 32% buffer B. The flow rate was 1.5 ml/min. Buffer A consisted of 25 mM phosphate buffer, pH 7.6, containing 5% acetonitrile, and buffer B consisted of 95% acetonitrile. Fractions were collected at 0.2 min intervals and evaporated to dryness in a vacuum centrifuge as above and subjected to radioimmunoassay of angiotensins.

Statistical analysis: Data are given as means $\pm$ SEM. Statistical analysis was performed by one-way analysis of variance and Student's t-test where appropriate.

Results and Discussion

The release of angiotensins from isolated perfused umbilical veins was examined up to 3 hours. To equilibrate the preparations and to eliminate the possible contamination of plasma angiotensins, the tissues were thoroughly washed with Krebs-Ringer solution (about 200 ml) over a period of 30 min before sample collection was started. Under these conditions, the rate of release of angiotensins was 41.9$\pm$7.4 and 63.4$\pm$12.0 pg for Ang I and Ang II, respectively, during the first perfusion period of 30 min. Although the variability in the rate of the release of angiotensins between different umbilical cords was considerable ranging from 15.8 to 108.5 pg/30 min for Ang I; 28.1 to 128.2 pg/30 min for Ang II for unknown reasons, individual cords examined showed a stable rate of the peptides release at least up to 3 hours, which gave a constant mean value for both Ang I and Ang II release, as shown in Fig. 1.

To determine whether these immunoreactive angiotensin peptides were indeed Ang I and Ang II, the sample was examined by HPLC. As shown in Fig. 2, the Ang

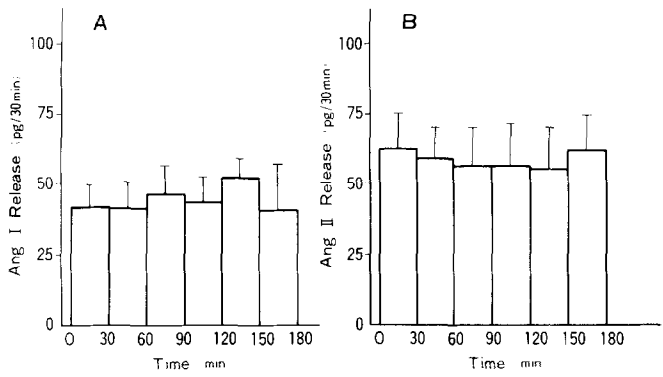

Fig. 1. Time course of angiotensin I (A) and angiotensin II (B) release from isolated perfused human umbilical veins (n=5). Each column shows mean ± SEM value of release of angiotensin I (Ang I) and II (Ang II) for 30 min.

II immunoreactive substance was eluted in a sharp, single peak at the position where standard Ang II emerged. Ang I immunoreactivity also emerged as a single peak at a position where authentic Ang I emerged. Ang III was detected on the same HPLC, but the peak of the Ang III was very small as compared with those for Ang I and Ang II. These results provide evidence for production of Ang I and Ang II in the human vascular tissues as the two angiotensins were generated continuously in the absence of external supply of angiotensinogen in the perfusion medium.

To further address the mechanism of the local production of angiotensins, we examined the effect of a specific angiotensin-converting enzyme inhibitor, captopril, on the release of Ang I as well as Ang II (Fig. 3). Captopril, added to the perfusion medium (10^{-9} to 5×10^{-6} M), caused a significant decrease of Ang II release in a dose-dependent manner ($F=10.92$, $p<0.001$), with a significant, reciprocal increase of Ang I release ($F=3.65$, $p<0.05$). At the highest concentration of captopril (5×10^{-6} M), it induced an approximately 56% decrease in the basal rate of Ang II release. The finding clearly indicates that Ang I is converted to Ang II either intracellularly or extracellularly, since angiotensin converting enzyme is localized not only in the luminal surface of the vascular endothelium (17) but also in the subcellular particles of various tissues (18,19) including blood vessel walls (5).

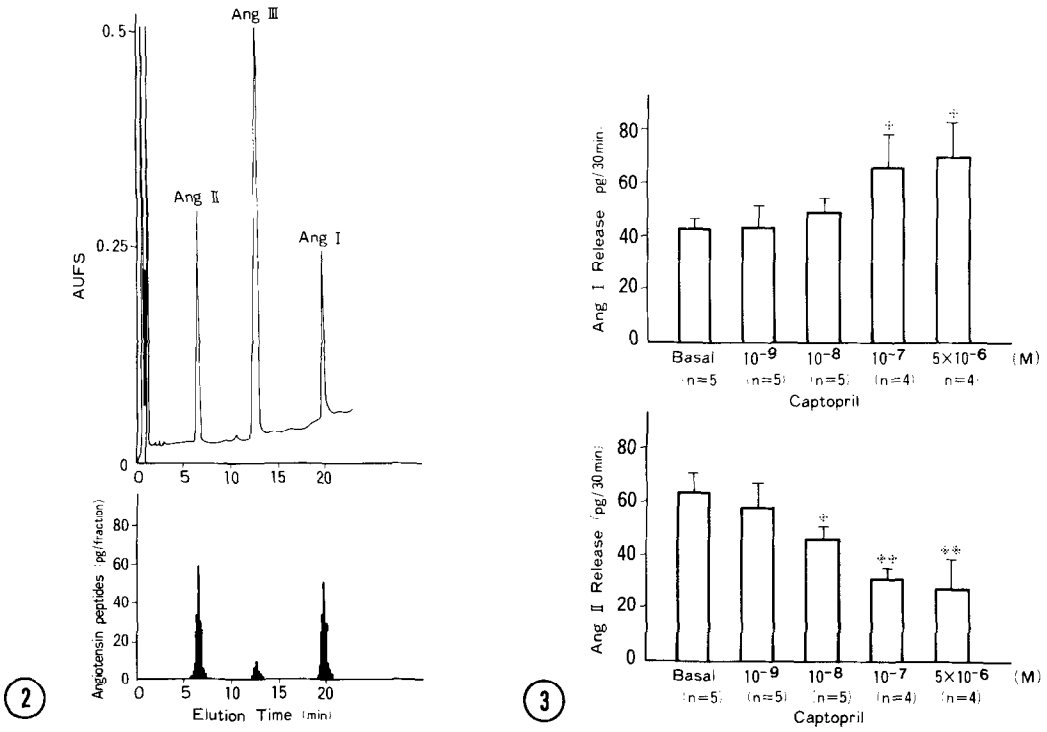

Fig. 2. Chromatographic separation as a function of time (min) of a mixture of synthetic angiotensin peptides (Ang I, Ang II and Ang III) detected by absorbance at 214 nm by reverse phase high-performance liquid chromatography (HPLC) (upper panel). Reverse phase column (Nova Pak C_{18}, 0.39 x15 cm) with a particle size of four microns using a nonlinear phosphate-acetonitrile gradient elution system. Standard injection volumes were between 5 and 50 µl, flow rate was 1.5 ml/min. Elution time of Ang I, Ang II and Ang III was 19.7, 6.3 and 12.6 min, respectively. A typical chromatographic separation of a sample extracted from perfusate of human umbilical veins (lower panel). Fractions were subjected to radioimmunoassay of Ang I, Ang II and Ang III.

Fig. 3. Effect of captopril on angiotensin I (Ang I, upper panel) and II (Ang II, lower panel) release from isolated perfused human umbilical veins. $^{*}p<0.05$, $^{**}p<0.01$ compared with basal values.

Previously, we demonstrated that sizable amounts of Ang I and Ang II were released continuously for several hours from diverse vascular beds such as mesenteric arteries (14) and hindquarters (15,16) of rats when these vascular tissues were isolated and then perfused with angiotensinogen-free medium. It was also clearly shown in these studies that release of Ang II was suppressed by either angiotensin converting enzyme inhibitors (captopril and SA 446) (15) or prostaglandin synthesis inhibitors (indomethacin and meclofenamate) (16), but was markedly stimulated by adrenergic agonist isoproterenolol (14). These

findings suggest that such release of Ang II is not due to mere leakage from the vascular beds but due to regulated mechanism. Therefore, although the results of the present study are preliminary, these past and present findings strongly support the hypothesis that human vascular tissue has the potential to produce Ang II and secretes it in a regulated mechanism. It is thus possible that secreted Ang II binds to receptors on the same or neighboring cells in an autocrine or paracrine fashion to exert its biological effects, such as stimulation of prostaglandin biosynthesis (20), activation of vascular smooth muscle tone, and potentiation of sympathetic activity (21-23).

Finally, to our best knowledge, this is the first demonstration of release of Ang II from human vascular tissue. However, since the umbilical cord vasculature used in the present study was not arteries but veins, further efforts remain to be made to see if and how angiotensins are released from human arterial tissues, in addition to its physiological relevance to regulation of vascular tone.

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