Action of Angiotensin II on DNA Synthesis by Human Saphenous Vein in Organ Culture

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Abstract—Angiotensin II (Ang II), an effector peptide of the renin-angiotensin system, has been reported to stimulate growth of blood vessels in vivo and smooth muscle cells in culture. In this study, the effect of Ang II on DNA synthesis was examined in deendothelialized human saphenous vein in organ culture. After 7 days’ exposure to medium containing 0.4% fetal calf serum plus Ang II, there was a marked increase in DNA synthesis. The effect of Ang II was comparable to the response to platelet-derived growth factor. Responses to Ang II were partially inhibited by the AT1 receptor antagonist candesartan. An AT2 receptor antagonist, PD123319, had no effect on Ang II–induced DNA synthesis, either alone or in combination with candesartan. The Ang II peptide analogues [Sar1,Ile8]-Ang II (saralasin) and [Sar1,Thr3]-Ang II (sarthran) acted as agonists, increasing DNA synthesis. In the presence of saralasin, responses to Ang II were inhibited. Tyrphostin-23, a tyrosine kinase inhibitor, prevented Ang II–induced DNA synthesis and reduced DNA synthesis in tissues incubated in medium containing only 0.4% fetal calf serum. In conclusion, Ang II stimulates DNA synthesis in human saphenous vein in organ culture. The effect of Ang II was more marked than has been previously reported in isolated cultured saphenous vein smooth muscle cells, and this effect is mediated in part by an angiotensin type 1 receptor. It is possible that an undefined receptor for Ang II may also be involved in the stimulation of DNA synthesis in this preparation. (Hypertension. 2000;36:917-921.)

Key Words: human saphenous vein ■ muscle, smooth ■ DNA synthesis ■ angiotensin II ■ organ culture ■ angiotensin I

Angiotensin II (Ang II) is an important regulator of vascular smooth muscle cell growth: infusion of Ang II stimulates vascular hypertrophy in rats,1-3 independently of changes in blood pressure,1,3 and also enhances neointimal formation after vascular injury.4 Inhibition of Ang II formation by angiotensin-converting enzyme inhibitors (ACEI) reverses vascular hypertrophy associated with hypertension1 and inhibits neointimal growth after vascular injury in rats.6 However, such effects may be species dependent, because ACEI has been reported to have little or no effect on the response to vascular injury in rabbits,7 pigs,8 or baboons.9 In addition, an ACEI also failed to reduce the incidence of restenosis in a recent clinical trial.10

Angiotensin type 1 (AT1) receptors are considered to mediate most of the effects of Ang II on vascular growth,11 although angiotensin type 2 (AT2) receptors may also contribute.12-14 Rodent vascular injury models have been reported to overexpress AT1 receptors,15 and an AT1 antagonist inhibits neointimal hyperplasia in injured rat carotid arteries.16 In contrast, an AT2 antagonist had no effect on intimal hyperplasia in a porcine vascular injury model.17

Ang II has also been reported to promote the growth of cultured smooth muscle cells. Ang II stimulated [3H]-thymidine and [3H]-leucine incorporation in smooth muscle cells derived from human aorta and subcutaneous arteries.18 In contrast, in human coronary smooth muscle cells, Ang II stimulated protein synthesis but not DNA synthesis.19 In human saphenous vein–derived smooth muscle cells, Ang II induced some DNA synthesis in one study20 but failed to stimulate proliferation or protein synthesis in another.21 Although these data provide some evidence for trophic effects of Ang II, isolated cells may lack cell-cell and cell-matrix interactions that influence responses to growth factors. We therefore examined the effect of Ang II on DNA synthesis in human saphenous vein segments in an organ culture system in which such interactions are maintained. These studies also examined the involvements of receptor subtypes and the possible role of tyrosine kinase activation in Ang II–induced responses.

Methods

Clinical Details

Segments of veins surplus to clinical requirement were obtained from 55 patients (43 men and 12 women, age range 50 to 78 years) who were undergoing coronary artery bypass surgery. Studies were in accordance with local ethics committee guidelines. Patients taking ACEI or AT, antagonists before surgery were excluded from the study.
Tissue Preparation and Culture

Segments of vein were transferred to the tissue culture laboratory in cold Hanks’ balanced salt solution buffered with 25 mmol/L HEPES and prepared for culture by a procedure based on that described by Soyombo et al. Briefly, the adventitia and extraneous adhering fat were removed, and the vein was cut open longitudinally. The luminal surface was gently rubbed with a sterile cotton bud to remove the endothelium, and the tissue was then cut into 5-mm segments. Segments were then pinned, luminal surface uppermost, onto culture dishes precoated with a layer of silicone elastomer. Culture dishes were filled with medium consisting of Dulbecco’s modified Eagle medium (DMEM) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and gentamicin (25 μg/mL). Veins were cultured in a humidified atmosphere of 5% (vol/vol) carbon dioxide in air at 37°C for 7 days in DMEM containing 0.4% serum (SFM), 30% serum, or SFM plus drugs. Antagonists or peptide analogues were incubated with tissues for at least 10 minutes before the addition of Ang II. Drugs and peptides were replenished daily throughout the culture period.

Measurement of DNA Synthesis

DNA synthesis was determined by measuring the incorporation of 3H-methylthymidine. 3H-methylthymidine (1 μCi/mL) was added to the culture dishes for 24 hours, and the experiment was then terminated by washing the vein segments twice with ice-cold PBS. All studies were performed in triplicate. Tissue wet weight was recorded, and veins were then lysed by exposure to a tissue solubilizer (NCS-II; Amersham) for 48 hours. The acid insoluble fraction was then extracted on ice with 10% (wt/vol) trichloroacetic acid. Radioactivity was determined by means of a scintillation counter, and the counts per minute (cpm) were normalized to micrograms of wet tissue weight. Initial experiments were performed to determine a time course for 3H-thymidine incorporation into human saphenous vein in organ culture. Veins were incubated in culture medium containing 0.4%, 1%, or 30% serum for 3 time points: 1 day, 3 days, and 7 days. At the end of these time points, 3H-thymidine incorporation was maximal in response to 30% serum after 7 days (~10-fold increase), and therefore this time point was chosen for all subsequent experiments.

Morphometric Analysis

After culture, vein segments from some treatment groups were fixed in 10% formalin, embedded in paraffin, and stained with Miller’s elastic and van Giessen stains. Randomly selected vein sections were also stained with hematoxylin-eosin or immunostained with a monoclonal Ki67 (Novacstra) antibody (counterstained with hematoxylin) to identify the nuclear antigen (Ki67) associated with cell proliferation. For quantification of Ki67 labeling, total cell number was determined by morphometric analysis of images generated by a Zeiss Axioscope equipped with a CCD camera and appropriate software (NIH Image). Intimal/neointimal thickness was defined by tissue on the luminal side of the internal elastic lamina (a trace of acellular thickening was present in this layer of all vein segments before culture). Medial thickness was defined by tissue enclosed by the internal and external elastic laminae.

Drugs

Candesartan and EXP3174 were gifts from Takeda Inc and DuPont, respectively. Other sources of drugs were as follows: Ang II (human), Novabiochem; PD123319, Research Biochemicals; and [Sar1,Ile8]-Ang II, [Sar1,Thr8]-Ang II, and tyrphostin-23, Sigma. Ang II is rapidly broken down by tissue proteases and therefore is able to achieve stable levels in culture. Ang II (1 to 100 nmol/L) was delivered via osmotic minipumps (Charles River) at an infusion rate of 0.5 μL/h. This method of administration resulted in concentrations of Ang II in culture medium of 8.5±0.7×10−9 mol/L as measured by radiimmunoassay on day 7 using Ang II (100 nmol/L). Candesartan stock was made up in dimethyl sulfoxide (DMSO); the final concentration of DMSO did not exceed 0.0001%. Other antagonists and peptides were made up in PBS.

Statistical Analysis

Treatment groups were compared by Student’s paired and unpaired t test as appropriate or repeated-measures ANOVA for comparison of 3 or more groups, followed by Dunnett’s test with Instat 3.02 (GraphPad Software Inc). Statistical significance was accepted at P<0.05. All values are mean±SEM of n observations as indicated below the legend in parentheses. Data were compared by ANOVA for repeated measures followed by Dunnett’s test. *P<0.05; **P<0.01.

Results

Ang II (1 to 100 nmol/L) caused a marked, concentration-dependent increase in DNA synthesis over 7 days (Figure 1A). Tyrophostin-23, a selective inhibitor of tyrosine kinases, reduced 3H-thymidine incorporation in SFM and also abolished the increase induced by Ang II (10 nmol/L). Responses to Ang II varied between individuals, but overall Ang II (10 nmol/L) significantly increased DNA synthesis compared with veins exposed to SFM (Figure 1B). Responses to Ang II were comparable in magnitude to those to platelet-derived growth factor-AB (PDGF-AB; 1 ng/mL), although less than those to 30% serum (Figure 1B).

We examined the role of AT1 receptors in responses to Ang II by incubating veins with Ang II and the nonpeptide AT1 antagonist candesartan. Candesartan (1 nmol/L) had no significant effect on DNA synthesis in SFM or 30% serum (data not shown), but it significantly attenuated responses to Ang II (10 nmol/L) (Figure 2A). Higher concentrations of candesartan...
Figure 2. Effects of angiotensin receptor antagonists on Ang II–induced DNA synthesis by human saphenous vein. Vein was cultured in medium containing 0.4% fetal calf serum plus additional agents for 7 days. A, Selective nonpeptide antagonists. Effect of Ang II (All; 10 nmol/L), Ang II in the presence of 1 nmol/L candesartan (All+C), Ang II in the presence of 100 nmol/L PD123319 (All+P), or Ang II in the presence of both 1 nmol/L candesartan and 100 nmol/L PD123319 (All+C+P). Data are expressed as percent response to Ang II for each individual and are mean±SEM of n observations as indicated below the legend in parentheses. Data were compared by Student’s t test for paired data. *P<0.05. B, Nonselective peptide antagonists. Effect of Ang II (All; 10 nmol/L), 1 μmol/L [Sar1,Ile8]-Ang II (Sarile), 100 nmol/L [Sar1,Thr8]-Ang II (Sarthran), and Ang II (10 nmol/L) in the presence of 1 μmol/L sarile (All+Sarile). Data are expressed as percent response to Ang II for each individual and are mean±SEM of n observations as indicated below the legend in parentheses. Data were compared by Student’s t test for paired data. *P<0.05.

In addition to stimulation of DNA synthesis, Ang II caused a small and statistically insignificant increase in thickness of the vein media by 28±17% (n=7). There was no significant change in intimal thickness. Endothelial cell regeneration did not occur over the course of the study, as indicated by the lack of immunohistochemical staining for the endothelial cell antigen CD31 (data not shown). Ang II caused a significant increase in the percentage of Ki67-positive cells (taken as an index of proliferating cells) in the media of vein sections (SFM=2.2±1%; Ang II=6.2±0.7%; n=3; P<0.01). After hematoxylin-eosin staining of vein sections, medial nuclear density appeared to be increased by 30% serum and Ang II. Ang II and 30% serum did not induce detectable differences in histological appearance after Miller’s elastin or van Gieson staining.

Discussion

Organ culture studies may have advantages over those that use isolated cells, because cell-cell and cell-extracellular matrix interactions are preserved, and these may influence growth factor responses. In our studies, Ang II caused a marked concentration-dependent increase in DNA synthesis in veins cultured for 7 days in medium containing minimal (0.4%) serum. The responses to Ang II in this model were of comparable magnitude to those of a classic mitogen, PDGF. This finding contrasts with the modest stimulation of 3H-thymidine incorporation seen in an earlier study of isolated smooth muscle cells cultured from human saphenous vein.

Further studies indicated that exposure to Ang II was also accompanied by ~3-fold increase in proliferating cell nuclear antigen Ki67-positive cells in vein media, providing further evidence for hyperplasia in response to Ang II. The failure to observe a significant increase in neointima in response to Ang II in these preparations is likely to be due to the removal of the endothelium, because this has been reported to inhibit spontaneous neointima formation in this preparation. The extent of DNA synthesis seen in response to Ang II varied between individuals. The reasons for this are not known, but it perhaps should not be unexpected given the diversity of patient ages and conditions. Interindividual differences in the rate of growth and sensitivity to growth inhibitors have also been observed in vascular smooth muscle cells cultured from saphenous vein taken from different individuals.

The effect of Ang II on DNA synthesis in our vascular model is in contrast with 2 recent studies in which Ang II did not influence DNA synthesis over 3 days of incubation with rat aorta and renal artery segments in organ culture. In those studies, protein synthesis and medial hypertrophy were induced. Aside from interspecies differences, several possibilities may explain the difference between the present and former studies. First, in the previous investigations, daily replenishment of Ang II was used. The biological half-life of Ang II in culture systems is extremely short, because it is broken down by tissue proteases. It is possible that the continuous exposure achieved in the present study with osmotic minipump infusions may better stimulate DNA synthesis. Second, the longer duration of stimulation with Ang II in the present study (7 days versus 3 days) may also be a contributory factor. Although Ang II can stimulate phospholipase C and generation of diacylglycerol and inositol 1,4,5-trisphosphate within seconds or minutes, tissue exposure to Ang II for >48 hours can activate a delayed stimulation of DNA synthesis through the autocrine or paracrine production of secondary growth factors.
Activation of tyrosine kinases is believed to participate in multiple steps of signal transduction initiated by a variety of growth factors, including PDGF and, more recently, Ang II. The effect of Ang II may involve direct effects on tyrosine kinases or transactivation of growth factor receptors. In the present study, Ang II–induced DNA synthesis was totally abolished by the tyrosine kinase inhibitor tyrphostin-23, implying a role for tyrosine kinases in the response to Ang II. Interestingly, 3H-methylthymidine incorporation in 0.4% serum was also inhibited by tyrphostin-23, which suggests that some DNA synthesis may occur even in the absence of any growth factor, possibly as a result of injury during vein preparation.

We are unaware of any previous reports concerning the identity of the angiotensin receptor subtype involved in Ang II–mediated growth in intact human vascular tissue in organ culture, although the trophic actions of Ang II on isolated smooth muscle cells derived from human and animal tissue are mediated by the AT1 receptor subtype. In our studies, the AT1 receptor antagonists candesartan and EXP3174 only partially inhibited Ang II–induced DNA synthesis. The concentration of candesartan was chosen on the basis of previous functional studies showing that the estimated affinity (Kd) of candesartan for the AT1 receptor in human arteries was ≈10 pmol/L. In previous studies, similar (and lower) concentrations of these antagonists have been shown to completely inhibit DNA synthesis in rat aortic and human aortic vascular smooth muscle cells in culture. The inability of candesartan and losartan to completely inhibit responses to Ang II implies involvement of another receptor subtype. However, the AT1 receptor antagonist PD123319, either alone or in combination with candesartan, had no effect on responses to Ang II. It is possible, therefore, that the effect of Ang II was mediated in part by a non-AT1/non-AT2 receptor. Consequently, we examined the effects of the nonselective Ang II peptide antagonists saralasin and sarthran. In many preparations, these agents act as antagonists of angiotensin receptors and the receptor for angiotensin 1–7. In our study, both saralasin and sarthran acted as agonists and increased DNA synthesis to levels comparable to Ang II itself. In the presence of saralasin, Ang II–induced DNA synthesis was abolished, which indicates that Ang II and the peptide antagonists act at common receptors. This effect of the peptide antagonists could involve the internalization or desensitization of receptors for Ang II. These observations appear consistent with an AT receptor of undefined subtype contributing to DNA synthesis in response to Ang II; however, additional studies, such as ligand binding studies with radiolabeled Ang II and AT1 antagonists, would be valuable in confirming this possibility.

Non-AT1/non-AT2 receptors have previously been described in tissues of avian species, and a unique Ang II binding site in human endometrium has also been described. Interestingly, saralasin has been reported to be a partial agonist at the atypical AT receptor found in the nasal salt gland of Anas platyrhynchos. A role for non-AT1/non-AT2 receptors in cellular growth in humans has also been proposed. Recently, non-AT1/non-AT2 receptors have been reported to mediate Ang II–induced growth in human cardiac fibroblasts and human keratinocytes in culture. Degradation products of the octapeptide Ang II (or its precursor molecules) may also exert actions on cells via non-AT1/non-AT2 receptors. Thus, angiotensin (1–7) and angiotensin IV (3–8) have been reported to promote or inhibit DNA synthesis in a variety of cell types. It would be important to examine the effects of these analogues and their antagonists in this preparation in future studies.

In summary, the present study demonstrates that Ang II induces marked DNA synthesis in saphenous vein organ culture model via a mechanism that involves activation of tyrosine kinases. Ang II–induced DNA synthesis is mediated in part by AT1 receptors, but it is likely that an atypical receptor (for which sarthran and sarile are agonists) may also contribute. Enhanced vascular smooth muscle cell growth is considered to have an important role in various pathophysiological processes, and consequently, defining the angiotensin receptor subtypes involved in this response may have important clinical implications.

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References


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