Interaction of angiotensin II and the insulin-like growth factor system in vascular smooth muscle cells

THOMAS GUSTAFSSON, PETER ANDERSSON, YUN CHEN, JAN OLOF MAGNUSSON, AND HANS J. ARNQVIST
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Gustafsson, Thomas, Peter Andersson, Yun Chen, Jan Olof Magnusson, and Hans J. Arnqvist. Interaction of angiotensin II and the insulin-like growth factor system in vascular smooth muscle cells. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H499–H507, 1999.—We studied the effects and interactions of ANG II and the insulin-like growth factor (IGF) system in cultured rat aortic smooth muscle cells. ANG II (1 µM) and IGF-I (10 nM) stimulated both DNA and protein synthesis. The effects of the two peptides in combination were additive or more than additive. The AT1 receptor antagonist losartan (10 and 100 µM) blocked their synergistic effect on DNA synthesis. IGF binding protein (IGFBP)-1 inhibited the effect of IGF-I but not that of ANG II on DNA synthesis. IGF-I stimulated gene expression of IGFBP-2 and IGFBP-4. ANG II decreased IGFBP-1, IGFBP-2, and IGFBP-4 transcripts but increased the IGFBP-4 receptor transcript. IGF-I and ANG II in combination had similar effects on gene expression as IGF-I alone. The IGFBP-2 and IGFBP-4 peptides could be detected in the conditioned medium. Our results show that ANG II and IGF-I have synergistic effects on vascular smooth muscle cells and that they interact in several ways.

IGF-I receives its effect through activation of the type I IGF receptor (2). The gene expression of IGF-I as well as IGF-I peptide is induced in experimentally induced smooth muscle hypertrophy (1, 9, 16), and both IGF-I mRNA as well as IGF-I receptor mRNA levels are increased in balloon injury-induced proliferation of VSMC (5). In vitro, IGF-I is a weak mitogen for VSMC (2).

In circulation and in the tissues, the availability of IGF-I is regulated by six different but related IGF binding proteins (IGFBP). VSMC are known to secrete IGFBP-2 and IGFBP-4, which are able to modulate IGF action at the cellular level, mainly by regulating its availability to the IGF-I receptor (8, 10, 21). The gene expression of IGFBP-2 and -4 is regulated during the development of smooth muscle hypertrophy (9). Selected transgenic overexpression of IGF-I in mouse aortic smooth muscle has recently been shown to cause hyperplasia of aortic smooth muscle cells and to increase the wet weight of the aorta (33). Targeted overexpression of IGFBP-4 in mouse aortic smooth muscle resulted in hypoplasia and decreased wet weight of the aorta (34).

IGF-I interacts with other growth factors and enhances in vitro the effects of platelet-derived growth factor (PDGF)-BB, basic fibroblast growth factor (bFGF), and epidermal growth factor on DNA synthesis in VSMC (31, 36). ANG II, which influences peripheral vasoconstriction, aldosterone secretion, and electrolyte balance, is also involved in VSMC hypertrophy and growth (30). ANG II has been reported to interact with bFGF, transforming growth factor-β, and IGF-I (12, 20). Because both IGF-I and ANG II are involved in smooth muscle hypertrophy, we wanted to evaluate their possible interaction on trophic processes. The effects of IGF-I and ANG II and their interaction on DNA and protein synthesis in cultured rat aortic smooth muscle cells were studied. We also examined whether ANG II and IGF-I regulate gene expression of IGF-I, IGF-I receptor, IGFBP-2, and IGFBP-4.

MATERIALS AND METHODS

Isolation and culture of VSMC. Rat aortic smooth muscle cells were isolated and cultured according to a modified method of Nilsson et al. (28). Male Sprague-Dawley rats (8–10 wk old; ALAB, Stockholm, Sweden) weighing ~200–250 g were used. The aorta was cut into small pieces, which were digested for 1 h at 37°C in 0.1% collagenase in Ham’s F-12 medium, containing 10% newborn calf serum, 0.05 mg/ml ascorbic acid, 2 µg/ml Fungizone, and 0.01 mg/ml gentamicin. The pieces of the aorta were then transferred into fresh collagenase/Ham’s F-12 medium and incubated for another 20 h. The cell suspension was filtered through a nylon filter (pore size, 48 µm; Schweizerische Seidengaze Fabrik, Zurich, Switzerland), washed in Ham’s F-12 medium, and then plated in culturing flasks in Ham’s F-12 medium, and then plated in culturing flasks in Ham’s F-12 medium.

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the VSMC were counted in a Bürker chamber and then seeded out in equal numbers in 24-well plates. The cells were grown to near-confluency and were then starved in serum-free F-12 medium for 24 h. The culture medium was then changed to fresh serum-free F-12 medium with the addition of [3H]thymidine (1 μCi/ml), and the cells were incubated for 24 h in the presence or absence of different additions as given in the text. Triplicate wells were used for the different conditions. The VSMC were washed with F-12 medium, and DNA was precipitated with ice-cold 5% TCA. The cells were treated with 0.1 M KOH, and 500 µl of the solution (1 ml) was used for dissolving the cells. The plate was swirled, and 500 µl of the solution (1 ml) was removed to scintillation vials for the subsequent measurement of radioactivity in a liquid scintillation counter.

[3H]leucine incorporation into protein. Protein synthesis was studied by measuring incorporation of [3H]leucine into protein. The cells were serum starved for 24 h and then incubated with or without peptides for 18 h. [3H]leucine was then pulsed in a final concentration of 1 µCi/ml, and the incubation was continued for another 90 min. The cells were then washed with ice-cold phosphate-buffered saline containing 0.9 M EDTA (pH 7.4) and then with 5% TCA. A solution containing 5% SDS, 20 mM Na2CO3, and 2 mM EDTA was used for dissolving the cells. The plate was swirled, and 500 µl of the solution (1 ml) was removed to scintillation vials.

Measurement of mRNA and DNA. Near-confluent cells, cultured in Petri dishes, were deprived of serum for 24 h and then incubated with or without polypeptides at indicated concentrations for 6 or 24 h. The cells were then harvested in SET buffer (1% SDS, 20 mM Tris, and 10 mM EDTA, pH 7.5) and homogenized with a Polytron. Proteinase K was added, and the samples were extracted with phenol and chloroform according to the method of Durnam and Palmiter (15). Total nucleic acids were precipitated in 95% ethanol. The DNA content was measured as described by Labarca and Paigen (24). The mRNA levels for IGF-I, IGF-I receptor, IGFBP-2, and IGFBP-4 were analyzed with a solution hybridization assay using 35S-UTP-labeled RNA probes. The probe for IGF-I was synthesized from 153 bp of cDNA from exon 3 in the mouse. This probe could detect all the IGF-I transcripts. Because the mouse sequence is 92% homologous to the corresponding rat sequence, it could be used for detection in the rat. The IGF-I receptor probe contained 265 bases, complementary to a part of the 5'-untranslated region, to a sequence coding for the signal peptide and 53 amino acids of the α-subunit. The IGFBP-2 probe consisted of 397 bases complementary to exons 2 and 3 and part of exon 4 of the IGFBP-2 gene. The probe for IGFBP-4 consisted of 444 bases of a rat cDNA clone. The probes were synthesized as described by Melton et al. (27). The samples were hybridized with each probe for 18 h at 70°C. The hybridization solution contained a total volume of 40 µl: 0.6 M NaCl, 20 mM Tris, 4 mM EDTA, 0.75 mM dithiothreitol, 25% formamide, and 0.1% SDS. At least 10,000 cpm 32P-UTP-labeled probe was used per hybridization. RNases were then added, and the double-stranded RNA was precipitated in 6 M TCA. The hybrids were collected on filter, and the radioactivity was counted in a liquid scintillation counter. In each assay, a standard curve was included. The curve was created by hybridizing known amounts of in vitro-synthesized standard (sense) RNA with the probe (antisense).

Immunodetection of IGFBP-2 and IGFBP-4 in conditioned medium. Near-confluent VSMC grown in Petri dishes were starved in serum-free F-12 medium for 24 h. After the cells were stimulated with IGF-I, ANG II, or IGF-I in combination with ANG II for 24 h, the conditioned medium (5 ml) was collected. EDTA in a final concentration of 5 mM was added to prevent protein degradation. Fifty microliters of 0.1% BSA and 5 ml of 10% TCA were added to precipitate the protein. The samples were incubated at 4°C overnight and were then centrifuged at 11,000 rpm at 4°C for 30 min. The pellet was washed twice with 95% ethanol and was then dissolved in 250 µl of electrode buffer. Forty microliters of the sample were mixed with 20 µl of sample buffer and then applied on 15% SDS-PAGE. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membrane for 1 h (200 µA) with transfer buffer (15 mM Tris, 120 mM glycine, and 5% methanol, pH 8.3). The sheets were saturated with 0.2% polyvinyl alcohol dissolved in Tris-buffered saline (TBS-Tween, 0.2%), pH 7.6 (overnight), and incubated for 1 h with polyclonal antibodies (1:3,000) raised against rat IGFBP-2 or IGFBP-4. After washing six times (5 min each) with TBS-Tween, a donkey anti-mouse IgG horseradish peroxidase-labeled antibody (1:3,000) was added for 1 h. After washing (6 times, 5 min), the detection was visualized by enhanced chemiluminescence (ECL) analysis with the Amersham ECL system (Amersham) using ECL Hyperfilms for exposure.

Chemicals. Proteinase K was from Merck (Darmstadt, Germany). 35S-UTP, [3H]thymidine, and [3H]leucine were from Amersham International, and the chemicals for anti-sense (probe) and sense (standard) synthesis were from Promega (Madison, WI). RNase A, RNase T1, and herring sperm DNA were obtained from Boehringer (Mannheim, Germany). Phenol was from Fischer Scientific (Fair Lawn, NJ), trypsin was from Difco Labs (Detroit, MI), and ANG II and collagenase type I were from Sigma (St. Louis, MO). IGFBP-1 was a gift from Dr. Mats Lake (KabiGen, Sweden). Scintillation liquid (Ultima Gold) was obtained from Packard Instruments (Meriden, CT). The monoclonal antibody against α-smooth muscle actin was from Sigma Immunchemicals (La Jolla, CA). Chemicals and solutions for cell culture were received from Gibco BRL Life Technologies (Täby, Sweden). Polyclonal antibodies to IGFBP-2 and IGFBP-4 were from Austral Biologicals, and the PVDF membrane was from Du Pont. Recombinant human IGF-1 was a kind gift from Dr. A. Söknner, KabiPharmacia Peptide Hormones (Stockholm, Sweden).

Statistics. Values are given as means ± SE. Statistical comparisons were made by Student’s t-test, and if more than two groups were compared, ANOVA and Scheffé’s method were used. A value of P < 0.05 was considered significant.

RESULTS

Effects on DNA and protein synthesis. Both IGF-I and ANG II dose-dependently stimulated [3H]thymidine incorporation into the VSMC up to concentrations of 10⁻⁸ and 10⁻⁶ M, respectively (Fig. 1, A and B). When added in combination, IGF-I (10⁻⁸ M) and ANG II (10⁻⁸ M) had additive or more than additive effects on DNA synthesis (Figs. 2A and 3, A and B), and they also had additive effects on protein synthesis (Fig. 2B).

Because ANG II is known to mediate the majority of its biological effects through activation of the ANG II type 1 (AT₁) receptor (26), which is the predominant ANG II receptor in VSMC (25), the AT₁ receptor antagonist losartan was tested. The effect of ANG II on DNA and protein synthesis was totally blocked by the addition of 10 or 100 µM losartan. In addition, 100 µM losartan inhibited the synergistic effect of IGF-I and ANG II on DNA synthesis and also inhibited the effect
on protein synthesis, although not significantly (Fig. 2, A and B). Losartan did not significantly alter the effect of IGF-I on DNA or protein synthesis (Fig. 2, A and B), and losartan alone did not have any effect on the parameters studied (data not shown).

We used IGFBP-1 as a blocking agent of IGF-I. IGFBP-1 (500 ng/ml) inhibited the IGF-I-induced thymidine incorporation (Fig. 2C). To see whether ANG II needs endogenous IGF-I for inducing DNA synthesis, as previously proposed (12), IGFBP-1 was added together with ANG II. IGFBP-1 (500 ng/ml) did not alter the effect by ANG II (Fig. 2C) and did not alone have any effect on thymidine incorporation (data not shown).

To further investigate the interaction of IGF-I and ANG II on DNA synthesis, the peptides were added in combination with one peptide’s concentration fixed and the other concentration varied. IGF-I was fixed at $10^{-8}$ M and ANG II at $10^{-6}$ M. Dose-response curves for IGF-I and ANG II alone were included in the figures for comparison. As shown in Fig. 3, A and B, the synergistic action of IGF-I and ANG II appeared at an IGF-I concentration of $10^{-11}$ to $10^{-10}$ M, when ANG II was kept constant and at an ANG II concentration of $10^{-8}$ to $10^{-7}$ M, when IGF-I was kept constant, i.e., at about the same concentrations at which the peptides added alone induced DNA synthesis.

Gene expression of IGF-I, IGF-I receptor, IGFBP-2, and IGFBP-4. IGF-I did not have any effect on IGF-I mRNA levels in VSMC. ANG II tended to induce a decrease of IGF-I mRNA after 6 h of incubation, and this effect was significant after 24 h. As shown in Fig. 4A, IGF-I and ANG II in combination tended to have similar effects on the IGF-I mRNA level as ANG II alone, i.e., inhibitory actions.

IGF-I receptor mRNA levels in the VSMC were not affected by IGF-I stimulation. ANG II as well as IGF-I and ANG II added in combination caused an increase of the IGF-I receptor mRNA already after 6 h. This effect was sustained for up to 24 h (Fig. 4B).

The basal levels of IGFBP-2 mRNA were increased by IGF-I stimulation after 6 h of incubation, and this effect was sustained for up to 24 h. ANG II decreased IGFBP-2 mRNA when incubated for 24 h, and there was a tendency of a decrease already after 6 h of incubation. The combination of IGF-I and ANG II stimulated IGFBP-2 mRNA after 6 h, an effect which was turned into a significant inhibition after 24 h (Fig. 4C).

As for IGFBP-2 mRNA, IGF-I also stimulated IGFBP-4 mRNA levels after 6 and 24 h of incubation, whereas the effect of ANG II was inhibitory already after 6 h and sustained in the 24-h incubation. IGF-I and ANG II in combination had similar inhibitory actions on IGFBP-4 mRNA as ANG II alone (Fig. 4D).

Analysis of endogenous IGFBP-2 and -4 in the conditioned medium by SDS-PAGE and immunoblot. The IGFBP-2 band was determined to be $\sim 35$ kDa, and an immunoreactive band, probably a proteolytic fragment of IGFBP-2, was detected at 25 kDa after 24-h incubation (Fig. 5A). Neither IGF-I, ANG II, nor the peptides...
added in combination significantly altered the IGFBP-2 band or the 25-kDa band as determined by optical density measurements (Table 1).

IGFBP-4 was detected at ~26 kDa, and an immuno-reactive band was detected at 16 kDa (probably a proteolytic fragment) after 24 h of incubation (Fig. 5B).

Neither of the peptides nor the peptides in combination caused any significant changes of the IGFBP-4 band intensity compared with control. IGF-I increased the amounts of the 16-kDa immunoreactive band, whereas ANG II or IGF-I and ANG II in combination had no effect (Table 1).
DISCUSSION

The present investigation shows additive effects of ANG II and IGF-I on DNA and protein synthesis in VSMC. ANG II increased the gene expression of IGF-I receptor and decreased that of IGF-I, IGFBP-2, and IGFBP-4, whereas IGF-I increased the gene expression of IGFBP-2 and -4. This indicates different actions of ANG II and IGF-I in VSMC as well as a complex interaction of ANG II and the IGF system.

IGF-I stimulated DNA synthesis in VSMC with a dose-response curve similar to previous reports (7, 10, 19). ANG II also stimulated DNA synthesis in a dose-dependent manner, in accordance with some reports (12, 14), whereas others have found that ANG II fails to elicit any stimulation on DNA synthesis (3, 23). This variation in the growth response to ANG II could be due to different culture conditions; cell density, aging, and many other factors. IGF-I and ANG II were also found to stimulate leucine incorporation. IGF-I in nanomolar concentrations has been reported to stimulate protein synthesis in intact bovine arteries (4), and ANG II has been found to cause dose-dependent stimulation of protein synthesis in cultured rat VSMC (3).

There is evidence that both IGF-I and ANG II are involved in the development of smooth muscle hypertrophy/hyperplasia (2, 30, 33), but to our knowledge, their interaction on these processes has not been studied. We found that IGF-I (10^{-8} M) and ANG II (10^{-8} M) added together had synergistic effects on DNA as well as on protein synthesis. This suggests that they stimulate smooth muscle cell growth largely by separate mechanisms. It has been reported that insulin can enhance the effect of ANG II (100 nM) on DNA synthesis in rat VSMC at high insulin concentrations (10^{-7} to 10^{-5} M) (22). Insulin receptors are few or absent in VSMC, and at high concentrations, insulin may act through IGF-I receptors (2). The dose-response curve obtained for the IGF-I effects in the present study suggests that its effect was elicited through the IGF-I receptor. By the addition of the AT1 receptor antagonist losartan, we were able to block the ANG II effect as well as the synergistic effect of IGF-I and ANG II in combination on DNA synthesis, whereas losartan did not alter the effect of IGF-I. Losartan also tended to block the synergistic effects on protein synthesis, although there was no significance. These results indicate that the growth-promoting effects are mediated through IGF-I and AT1 receptors.

To further study the interaction and the synergistic effects of IGF-I and ANG II, we created dose-response curves where one peptide’s concentration was fixed at a high concentration while the concentration of the other peptide was varied. Our results indicate that the synergistic effect appears at doses at which the peptides alone induce DNA synthesis.

We have previously shown that exogenous IGFBP-1, -2, and -4 have inhibitory effects on IGF-I action (18). In that study, IGFBP-1 was shown to be the most potent inhibitor of IGF-I-induced DNA synthesis in VSMC. Because it has been reported that endogenous IGF-I is critical for ANG II-induced DNA synthesis in VSMC (12), we designed an experiment in which we used IGFBP-1 as a blocking agent of secreted IGF-I. IGFBP-1 (500 ng/ml) significantly decreased the exogenous IGF-I effect on DNA synthesis, whereas it did not alter the effect of ANG II. These results suggest that autocrine IGF-I is not needed for ANG II-stimulated growth,
since IGFBP-1 showed no inhibitory effect on ANG II action. The results are in line with the concept that IGF-I and ANG II act through activation of different signal mechanisms.

We further investigated how IGF-I (10^-8 M) and ANG II (10^-6 M) regulate gene expression of IGF-I, IGF-I receptor, IGFBP-2, and IGFBP-4 in VSMC. Treating the cells with ANG II or ANG II and IGF-I in combination did rapidly cause an increase in the IGF-I receptor mRNA level. In agreement with these findings, Veres et al. (35) have reported an increase of the IGF-I receptor number in the plasma membrane of rat VSMC, when treated with ANG II. An increase of the IGF-I receptor level may therefore be of importance for inducing a potentiation of the effects of IGF-I by ANG II. ANG II also induced a pronounced downregulation of IGF-I mRNA after 24 h of stimulation, in contrast to the increase reported by Delafontaine and Lou (12). A decrease in IGF-I mRNA has also been shown to be caused by bFGF and PDGF-BB in VSMC (6, 17).

In this study, we found that IGF-I stimulation increases IGFBP-2 and IGFBP-4 mRNA levels, whereas ANG II decreases their mRNA levels in VSMC. According to Cohick et al. (10), IGF-I treatment affected neither IGFBP-2 nor IGFBP-4 mRNA levels in porcine VSMC. Giannella-Neto et al. (17) reported that PDGF-BB increased gene expression of IGFBP-4 in rat VSMC. The increase in IGFBP-2 and IGFBP-4 mRNA when treating the cells with high IGF-I concentrations could indicate an induction of these IGFBP to protect the cells from IGF-I overstimulation.

Both IGFBP-2 and IGFBP-4 could be detected in the conditioned medium. In addition, we further detected one immunoreactive band each for the IGFBP. We were not able to detect any significant changes in intensity of the main band when treating the cells with IGF-I or ANG II, as determined by optical density measurements (Table 1). Cohick et al. (10) showed that IGF-I did not regulate IGFBP-2 secretion but decreased the IGFBP-4 amount present in the conditioned medium from porcine VSMC. Giannella-Neto et al. (17) showed that PDGF isoforms did not significantly alter release of the IGFBP-2 but evoked a five- to sixfold increase in the IGFBP-4 amounts in the conditioned medium from rat VSMC. In the present study, IGF-I stimulated the formation of the IGFBP-4 fragment without altering the intensity of the main band, indicating an increase in the metabolic processing of IGFBP-4. IGFBP-4-dependent degradation of endogenous IGFBP-4 has previously been reported in VSMC (10, 21). An active metabolic processing of the IGFBP can be significant for its regulatory role. Taken together, IGF-I and ANG II markedly regulate the mRNA for IGFBP-2 and IGFBP-4 in VSMC, and both these peptides can be detected in conditioned medium. At least for IGFBP-4, an increase in a proteolytic fragment was detected.

In conclusion, our study shows that there is a close interaction of ANG II and the IGF system in vascular smooth muscle cells. As it appears, IGF-I and ANG II act largely through separate signaling mechanisms.

Fig. 5. IGFBP-2 (A) and IGFBP-4 (B) levels in conditioned medium. Near-confluent rat vascular smooth muscle cells were stimulated with IGF-I (10^-8 M), ANG II (10^-6 M), or IGF-I and ANG II in combination. After 24 h, conditioned medium was collected, concentrated, and subjected to Western immunoblot analysis. Representative data are shown (n = 3). Intensity measurements (optical densitometric units) for respective blots are shown in Table 1. Human recombinant IGFBP-2 (hrIGFBP-2) and IGFBP-4 (hrIGFBP-4) were used as controls.

Table 1. Summary of the Western immunoblot data of IGFBP-2 and IGFBP-4, evaluated by optical density measurements

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<th>IGFBP-2</th>
<th>IGFBP-4</th>
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<tr>
<td></td>
<td>Main</td>
<td>Fragment</td>
</tr>
<tr>
<td>Control</td>
<td>1.35 ± 0.30</td>
<td>0.29 ± 0.15</td>
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<tr>
<td>IGF-I</td>
<td>1.30 ± 0.32</td>
<td>0.40 ± 0.20</td>
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<tr>
<td>ANG II</td>
<td>1.04 ± 0.26</td>
<td>0.30 ± 0.16</td>
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<tr>
<td>IGF-I + ANG II</td>
<td>0.78 ± 0.21</td>
<td>0.25 ± 0.15</td>
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Values are means ± SE of optical densitometry units of 3 experiments. IGF, insulin-like growth factor; IGFBP, IGF binding protein. Statistical comparison was made according to Scheffe’s method. *P < 0.05 compared with control.
and have synergistic trophic effects. Endogenous IGF-I is not critical for eliciting the effect of ANG II. However, our data suggest that ANG II may enhance the IGF-I effect by upregulating the IGF-I receptor mRNA level.

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