Angiotensin II Stimulates Gene Expression of Cardiac Insulin-Like Growth Factor I and Its Receptor Through Effects on Blood Pressure and Food Intake

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Abstract—Angiotensin II (Ang II) is known to act as a growth factor and may be involved in cardiac remodeling. We have shown that insulin-like growth factor-I (IGF-I) is an autocrine mediator of growth responses to Ang II in vascular smooth muscle cells in vitro, and we hypothesized that IGF-I also serves as an important modulator of cardiovascular growth in vivo. To study the effect of Ang II on cardiac IGF-I, we infused rats for 3, 7, or 14 days with Ang II through osmotic minipumps. After 7 days, left ventricular mass normalized for body weight was increased by 20% (P<0.01) in Ang II rats compared with pair-fed control rats that were given a restricted amount of food identical to that eaten by the anorexic, Ang II–infused rats. Ang II increased left ventricular IGF-I mRNA levels by 1.5- to 1.8-fold compared with ad libitum–fed or pair-fed control rats (P<0.05). Cardiac IGF-I protein was increased correspondingly and was localized on the cardiomyocytes. Treatment with hydralazine abolished the induction of IGF-I mRNA, which indicates that Ang II induces cardiac IGF-I mRNA expression through a pressor-mediated mechanism. IGF-I receptor (IGF-IR) mRNA was induced 2.1-fold in Ang II rats compared with ad libitum–fed rats (P<0.01). However, this increase was also observed in pair-fed controls and is thus due to the anorexigenic effect of Ang II. We have recently shown that circulating IGF-I levels are reduced in response to Ang II infusion. Elevation of IGF-I levels by coinfusion of IGF-I and Ang II significantly increased left ventricular index by 16% compared with rats infused with Ang II alone (P<0.05). In conclusion, autocrine upregulation of cardiac IGF-I and IGF-IR mRNA by Ang II occurs through hemodynamic and nonhemodynamic mechanisms, respectively, and may modulate cardiac structural changes that occur in hypertension. (Hypertension. 1999;34:1053-1059.)

Key Words: hypertrophy, cardiac heart failure growth substances angiotensin II immunohistochemistry

The heart adapts to increased systemic pressure by developing hypertrophy, a response that is thought to be mediated by the local induction of growth factors. Evidence exists that insulin-like growth factor-I (IGF-I) is one of the growth factors that play a role in pressure-induced hypertrophy. Several recent studies suggest that the upregulation of IGF-I may provide a compensatory mechanism by ameliorating myocyte growth and contractility in an attempt to sustain ventricular pump function. However, humoral factors can change cardiac mass independent of hemodynamics. A well-known example is acromegaly, in which elevated circulating levels of growth hormone (GH) lead to increased cardiac mass in large part through an increase in IGF-I. Thus, IGF-I may be involved in pressure-dependent as well as pressure-independent forms of cardiac hypertrophy.

Angiotensin (Ang) II is another important factor in this context; it causes hypertrophy not only through its blood pressure–raising action, but probably also through direct trophic effects on the fibroblast or cardiomyocyte. Whereas the initial hypertrophic response to overload seems to support contractility of the heart, continuous exposure to high blood pressure will result in pathological hypertrophy. The beneficial effects of angiotensin-converting enzyme inhibitors or Ang II receptor antagonists in animal models of cardiac hypertrophy as well as in human cardiac disease strongly support that Ang II is involved in this pathological hypertrophy.

Diseases such as congestive heart failure and chronic renal failure frequently go hand in hand with cachexia. In fact, cachexia was shown to be an independent risk factor leading to increased mortality in congestive heart failure. Moreover, cachexia can be associated with low circulating levels of IGF-I. Conditions of GH or IGF-I deficiency in humans are associated with cardiac atrophy and impaired cardiac function. If it is true that IGF-I supports cardiac function, low circulating IGF-I levels may be one of the direct reasons for increased mortality in patients with congestive heart failure.

We have recently reported that infusion of rats with Ang II through osmotic minipumps results in significant body
weight loss as well as a strong reduction in circulating levels of IGF-I independent of increased blood pressure.\textsuperscript{19} The present study uses the same model to investigate whether circulating IGF-I, cardiac IGF-I, and cardiac IGF–II receptor (IGF-IR) are involved in the hypertrophic response to Ang II infusion. First, we determined whether the IGF-I and IGF-IR mRNAs were upregulated in the heart, despite the downregulation of circulating IGF-I. Next, we analyzed whether the observed changes in IGF-I mRNA were direct effects of Ang II at the level of the cardiomyocyte or whether they were caused by the blood pressure–raising potential of Ang II using the vasodilator hydralazine. Finally, we investigated the effect of augmenting circulating IGF-I levels on cardiac mass by coinfusing rats with Ang II and IGF-I.

Methods

Animals
All experimental procedures were performed in accordance with institutional guidelines. All experiments involved the implantation of osmotic minipumps (Alzet model 2001 or 2002, Alza Corp). Male Sprague-Dawley rats (weight, 175 to 325 g) were housed in individual metabolic cages under standard conditions. Rats were anesthetized with 80 mg/kg ketamine and 12 mg/kg xylazine, and the pumps were placed subcutaneously in the subcapular region. Ang II was dissolved in saline acidified with 0.01 mol/L acetic acid to maintain its stability and was infused at a rate of 500 ng·kg\textsuperscript{-1}·min\textsuperscript{-1}. Vehicle-infused control animals were given a restricted amount of food identical to that eaten by the Ang II–infused rats and are thus referred to as pair-fed controls. In some experiments, vehicle-infused control animals were fed ad libitum; these are referred to as ad libitum–fed controls. In confusion experiments, a second minipump was implanted that contained recombinant IGF-I (kindly provided by Genentech Inc) for infusion at a rate of 1.4 μg·kg\textsuperscript{-1}·d\textsuperscript{-1}.

To determine the effect of blood pressure, control- or Ang II–infused rats received drinking water with or without hydralazine (10 mg·kg\textsuperscript{-1}·d\textsuperscript{-1}) or losartan (25 mg·kg\textsuperscript{-1}·d\textsuperscript{-1}). Losartan was kindly provided by Dr R. Smith, DuPont Merck, Wilmington, Del.

Both antihypertensive treatments were started 2 days before implantation of the minipumps.

Three, 7, or 14 days after implantation of the pumps, rats were anesthetized and euthanatized after aortic blood was taken, mixed in saline acidified with 100 U/mL heparin, and stored at −20°C until analysis by radioimmunoassay as described previously.\textsuperscript{19} The heart was excised and the left ventricle was dissected, weighed, and snap-frozen in liquid nitrogen.

IGF-I and IGF-IR mRNA Levels

Total RNA was prepared from frozen left ventricular muscle with an acid phenol–guanidinium reagent (Tri-Reagent, Molecular Research Center) according to the manufacturer’s instructions. RNA was quantified and purity was assessed by measuring absorptions at 260 and 280 nm. Solution hybridization and RNase protection assays were performed as previously described.\textsuperscript{2} Briefly, 20 μg of total RNA was hybridized overnight in hybridization buffer (Ambion, Inc) with 5×\textsuperscript{10} cpm riboprobe (see below). After RNase digestion with 40 μg/mL ribonuclease A and 100 U/mL ribonuclease T\textsubscript{1}, the samples underwent treatment with proteinase K, phenol extraction, ethanol precipitation, and analysis on a 6% denaturing polyacrylamide urea sequencing gel.

For determination of IGF-I mRNA levels, 2 riboprobes were used; initial studies were performed with a probe transcribed from a plasmid provided by Dr P. Rotwein, Washington University School of Medicine, St Louis, Mo, that included exon 4 and noncoding sequence as described previously.\textsuperscript{2} The 956-base riboprobe gives a 182-bp protected band after hybridization and RNase digestion. For later studies, a new plasmid was created by subcloning the full-length rat IGF-I cDNA sequence\textsuperscript{20} (provided by Dr G.I. Bell, Howard Hughes Medical Institute, Chicago, Ill) into the EcoRI site of pGEM-9Zf(−). Transcription with SP6 of the Hinf I linearized plasmid results in a full-length probe of 299 bases, and after hybridization and RNase digestion, the protected fragment is 263 bp long. The 2 probes gave identical results in terms of changes in IGF-I mRNA levels in response to Ang II infusion.

For determination of IGF-IR mRNA levels, a 203-bp EcoRI and KpnI rat IGF-IR cDNA fragment in pGEM-ZZ was used to generate a radiolabeled antisense probe, as previously described.\textsuperscript{21} The full-length probe is 251 bp, and the protected fragment is 195 bp in length. As an additional control for RNA loading and to determine the specificity of observed changes in IGF-IR mRNA levels, a GAPDH riboprobe was included in the hybridization mixture. This probe gives a 133-bp protected band after RNase digestion. Autoradiographic signals for IGF-I and IGF-IR were quantified in arbitrary units by use of a phosphomager (Molecular Dynamics), and signals were normalized by use of the corresponding GAPDH values to correct for variations in RNA loading.

Tissue Extraction and Radioimmunoassay for IGF-I

Tissue concentrations of IGF-I were determined as described by D’Ercole et al.\textsuperscript{22} Briefly, left ventricles were pulverized under liquid nitrogen, further disrupted using a Potter homogenizer, and extracted in 1 mol/L acetic acid for 2 hours at 4°C. After centrifugation (10 minutes at 1000g), the supernatants were neutralized with NaOH. Protein concentration of the tissue extract was determined by use of the BioRad protein assay. Radioimmunoassays were performed as described previously\textsuperscript{19} with 40 μg of protein extracted from each left ventricle. IGF-I polyclonal antibody UB 286 (raised by Drs L.E. Underwood and J.J. van Wyk, University of North Carolina, Chapel Hill) was donated by the US National Hormone and Pituatory Program.

Immunohistochemistry

For immunocytochemistry, left ventricles were fixed with 4% formaldehyde and embedded in paraffin with routine procedures. Deparaffinized sections 6 μm thick were preincubated for 1 hour with 5% normal goat serum (Vector Laboratories, Inc) in PBS, incubated overnight with the polyclonal anti-IGF-I antibody described above (dilution, 1:1000), washed 4× for 15 minutes with PBS, and then incubated with a biotin-conjugated goat anti-rabbit antibody (Vector Laboratories) for 1 hour. The sections were incubated for 30 minutes with an alkaline phosphatase–conjugated biotin-streptavidin complex (Vector Laboratories), following the supplier’s instructions. The antibody binding sites were visualized with fast red (Vector Laboratories). The sections were counterstained with Harris’ hematoxylin and mounted with GVA mounting solution (Zymed).

Statistical Analysis

All data represent means of 4 to 10 rats per group per time point, and values are expressed as mean±SE. Results were analyzed by unpaired Student’s \textit{t} test when results from 2 experimental groups were compared or by ANOVA when data from ≥3 groups were studied. For data analyzed by ANOVA, pairwise comparisons were made by Tukey’s test.

Results

Effect of Ang II Infusion on Left Ventricular IGF-I mRNA

Recently, we showed that Ang II infusion causes anorexia.\textsuperscript{19} Thus, 2 types of control rats were included in the present study: vehicle-infused animals that were fed ad libitum and vehicle-infused rats that were pair-fed in conjunction with the Ang II–infused rats. A significant increase (20%) in left...
ventricular mass was observed after 3 days of Ang II infusion versus pair-fed controls ($P<0.01$). After 7 days, the difference between Ang II–treated and pair-fed controls was still significant (12%; $P<0.05$). Our prior results showed that blood pressure was higher at both 3 and 7 days. Figure 1 shows that at 7 days of infusion in the Ang II–infused hypertensive rats, left ventricular IGF-I mRNA levels were increased. This response was not due to reduced food intake, because no increase in IGF-I mRNA was observed in pair-fed animals (Figure 1A). Compared with pair-fed animals, a 1.5±0.2-fold (n=4) increase in IGF-I mRNA was detectable at 3 days of Ang II infusion and a 1.8±0.3-fold (n=5; $P<0.05$) increase was reached by day 7 (Figure 1B) that persisted for up to 2 weeks of infusion (data not shown).

Thus, Ang II infusion increases cardiac IGF-I mRNA expression, in contrast to its known suppressor effect on circulating IGF-I levels. To determine whether Ang II downregulation of circulating IGF-I resulted in autocrine induction of cardiac IGF-I through a feedback mechanism, we designed an additional experiment. We reversed the suppressive effect of Ang II on circulating IGF-I levels by confusing recombinant IGF-I with Ang II. Under this condition, cardiac IGF-I mRNA expression was significantly ($P<0.05$) increased compared with pair-fed rats (Figure 1B) at both time points. The difference in IGF-I mRNA levels between Ang II–infused and Ang II and IGF-I–infused rats at day 7 did not reach statistical significance. These data indicate that Ang II induces left ventricular IGF-I mRNA through a mechanism independent of food intake or changes in circulating IGF-I.

**Effect of Ang II Infusion on IGF-I Protein Expression**

To determine whether the Ang II–induced increase in cardiac IGF-I mRNA correlates with an increase in IGF-I protein, we prepared protein extracts from the left ventricle of 5 Ang II–infused rats and 6 control rats and measured IGF-I using a radioimmunoassay. Left ventricles from Ang II–infused rats contained significantly more IGF-I than ventricles from control rats (23% increase; $P<0.001$; Figure 1C). To localize IGF-I expression in the heart, we performed immunohistochemistry using paraffin sections from control and Ang II–infused rats. As shown in Figure 2, IGF-I immunoreactivity was predominantly located on the cardiomyocytes. Control sections using nonimmune serum showed no staining (data not shown).

**Effect of Antihypertensive Treatment on Ang II–Induced Changes in Left Ventricular IGF-I mRNA**

Pharmacological treatment of rats with either losartan or hydralazine beginning 2 days before implantation of the minipumps reduced blood pressure to control levels, as published previously. Cardiac IGF-I mRNA expression in these animals is shown in Figure 3 (representative RNase protection assay); quantification of results from 4 to 6 animals in each experimental group demonstrated that Ang II raised IGF-I mRNA levels (normalized with GAPDH) 1.7±0.1-fold at day 7 compared with ad libitum-fed controls.

**Figure 1.** Effect of Ang II infusion on left ventricular IGF-I mRNA and protein levels. A and B, RNA was prepared from left ventricle and hybridized with $^{32}$P-labeled antisense IGF-I and GAPDH riboprobes. RNase protection assay was used as described in Methods. A, Representative autoradiogram of the results obtained with Ang II–infused compared with ad libitum–fed and pair-fed control animals, studied at 1 week of infusion. B, Mean±SE of determinations from 4 to 6 animals per condition at 3 and 7 days of infusion. C, IGF-I protein levels determined by radioimmunoassay after acid extraction of the left ventricles. *$P<0.05$, **$P<0.01$ vs pair-fed control.
This Ang II–induced increase was blocked by administration of losartan (Figure 3B). Losartan alone did not affect IGF-I mRNA levels versus results from control rats. Normalization of blood pressure by hydralazine also prevented the increase in IGF-I mRNA levels in Ang II–infused rats, because rats treated with Ang II and hydralazine had IGF-I mRNA levels that were indistinguishable from control rats receiving hydralazine or from control rats not given pharmacological treatment. In conclusion, both losartan and hydralazine normalized blood pressure and prevented the induction of left ventricular IGF-I mRNA expression. Thus, the increase in left ventricular IGF-I mRNA is a response to the hemodynamic action of Ang II, an AT1 receptor–mediated effect.

**Effect of Ang II and IGF-I Infusion on Left Ventricular Weight**

The upregulation of cardiac IGF-I mRNA and protein by Ang II suggests that autocrine IGF-I may be involved in the hypertrophic response of the left ventricle to increased hemodynamic load. Circulating IGF-I produced by the liver may also be important for cardiac growth, but we have recently shown that circulating IGF-I levels are dramatically reduced in this model of hypertension. This response could negatively affect cardiac growth processes. IGF-I has been shown to be a potent survival factor for myocytes. To investigate whether increasing circulating IGF-I levels will enhance the hypertrophic response, we coinflused Ang II and IGF-I. This resulted in a significant, 2.2-fold increase in circulating IGF-I levels as measured by radioimmunoassay and caused an increase in left ventricular mass: after 7 days, cardiac mass of IGF-I and Ang II–coinflused rats was 14% higher than in rats infused with Ang II alone (P<0.05). The increase in left ventricular weight in rats coinflused with Ang II and IGF-I was 26% more than pair-fed controls (P<0.01).

**Effect of Ang II Infusion on IGF-IR mRNA**

We measured left ventricular IGF-IR mRNA by RNase protection assay. Ang II caused an increase in IGF-IR mRNA both at 1 (Figure 5A) and 2 weeks of infusion compared with vehicle-infused control animals that were fed ad libitum. At 7 days, the hypertrophic index (left ventricular weight in mg divided by body weight in g) was increased by 43±7%, 20±4%, and 26±7% in Ang II–infused rats, respectively, compared with pair-fed controls (P<0.01, P<0.01, and P<0.05, respectively). No difference in cardiac index between pair-fed and ad libitum–fed rats was measured (data not shown).
days, a $2.1 \pm 0.05$-fold increase ($P<0.01$) occurred in left ventricular IGF-IR mRNA (Figure 5B). A similar increase in IGF-IR mRNA occurred in hearts of pair-fed control rats; ie, no difference was detectable between Ang II and pair-fed control animals, which indicates that the induction of receptor mRNA is due to the anorexia induced by Ang II treatment. Thus, low food intake results in increased IGF-IR mRNA expression in the left ventricle. The increase in IGF-IR mRNA levels at 7 days is not due to low circulating IGF-I levels, because increasing the circulating IGF-I protein levels by coinfecting IGF-I and Ang II still resulted in elevated levels of IGF-IR mRNA (Figure 5B).

Discussion

Our main finding is that Ang II infusion causes an upregulation of cardiac IGF-I mRNA levels in the rat. Our prior data showed that Ang II stimulates IGF-I transcription in extrahypertrophic tissues, including vascular smooth muscle. However, Ang II is not the prime mediator of the increase in IGF-I mRNA in vivo, because the increase was inhibited when blood pressure was normalized with the vasodilator hydralazine. This apparent hemodynamic response corresponds well with data obtained with the use of other models of pressure and volume overload in which an induction of IGF-I mRNA was related to increased blood pressure. Those studies included models of high as well as low renin-hypertension. However, none provided evidence that the induction was hemodynamic rather than Ang II–mediated, because hemodynamic overload could induce Ang II expression locally, which in turn could induce IGF-I in an autocrine fashion. The present study shows that increased Ang II levels in the absence of an increase in hemodynamic load do not result in upregulation of IGF-I mRNA. These results strongly support the conclusion that the higher IGF-I mRNA level is due to elevated blood pressure. Whether Ang II has direct trophic effects on the heart independent of its hemodynamic action is still a matter of debate; if it does, the present results suggest that an induction of IGF-I mRNA is not part of this direct response.

Notably, circulating IGF-I levels are dramatically downregulated in the Ang II–infusion model. Despite this, Ang II induced an increase in cardiac mass, consistent with an active role for the local pressure-induced IGF-I mRNA. Direct evidence that circulating IGF-I also contributes to the hypertrophic response comes from the experiment in which coinfusion of Ang II and IGF-I resulted in an increase in left ventricular mass that was significantly greater than in rats infused with Ang II alone. This strongly suggests that both circulating as well as local IGF-I are important in the response to hemodynamic load.

The clinical relevance of our findings is related to the pathophysiology of hyperreninemic states such as congestive heart failure or chronic renal failure. These diseases are commonly complicated by cachexia, and it has been proposed...
that low IGF-I levels contribute to the increased mortality seen in heart failure patients. In this context, the other main finding of the present study, namely that IGF-IR mRNA is induced by anorexia, is important because the higher level of the receptor could facilitate trophic effects of IGF-I in the cardiomyocytes. The observation corresponds well with other studies that show that IGF-IR gene expression is highly regulated under many physiological and pathological conditions. In vivo, fasting increased IGF-I–specific binding in several tissues, and these changes were accompanied by increases in IGF-IR mRNA abundance. Often an increase in IGF-IR expression seems secondary to a decrease in the local tissue IGF-I concentration, because in some of the tissues, the local levels of IGF-I are decreased after a reduction in caloric intake. Our results clearly show that this is not the case in the heart, because local levels of IGF-I are increased, and, moreover, increased circulating levels of IGF-I by coinfusion of Ang II and IGF-I does not abolish the upregulation of IGF-IR mRNA.

Our results that Ang II causes hypertrophy even at lower doses are consistent with previous reports. Data published by Kim et al., who have analyzed left ventricular dry weight due to actual growth. Others have reported increased cardiac protein synthesis, increased fibroblast proliferation, and an induction of fibronectin expression associated with fibrosis. It has been suggested that in experimental arterial hypertension, myocyte and nonmyocyte compartments are under separate controls: myocyte hypertrophy is most closely related to ventricular loading, whereas circulating Ang II regulates interstitial fibrosis. Our new finding that IGF-I is located on the cardiomyocytes suggests strongly that IGF-I is involved in the myocyte response to increased afterload.

Although the mechanism whereby IGF-I and its receptor increase left ventricular mass in the model of Ang II infusion remains to be elucidated, studies in animals or cultured cells suggest that it may involve the prevention of apoptosis, or the stimulation of cells to reenter the cell cycle, or the stimulation of anabolic pathways, such as the enhancement of myofibril development or the reversion of hypertension-induced changes in cardiac protein expression. Regardless, in vivo evidence suggests that IGF-I may ameliorate myocyte growth and contractility and thereby sustain ventricular pump function. GH treatment improved cardiac function by increasing myocardial contractility in rats with postinfarction left ventricular dysfunction. IGF treatment resulted in improved hemodynamic parameters in rats with cardiac failure. In mice, treatment with GH and IGF-I induced hypertrophy and produced a positive inotropic effect without significant changes in expression of fetal and other selected myocardial genes, which suggests that treatment with GH and IGF-I induces a physiological type of hypertrophy. In humans, a preliminary study of GH in the treatment of dilated cardiomyopathy demonstrated that myocardial mass was increased and hemodynamics and myocardial energy metabolism were improved. Recently, a study demonstrated that in patients with dilated cardiomyopathy given recombinant human GH, a significant increase occurs in left ventricular mass; however, no improved function was seen.

In summary, our data demonstrate that Ang II infusion increases cardiac IGF-I mRNA expression by a hemodynamic mechanism and increases cardiac IGF-IR mRNA levels by a nonhemodynamic mechanism related to reduced food intake. The cardiac hypertrophic response to Ang II occurs despite a strong reduction in circulating IGF-I caused by Ang II. Coinfusion of IGF-I and Ang II produces an additional hypertrophic response, which suggests strongly that the reduction in circulating IGF-I induced by Ang II may partially blunt the cardiac hypertrophic response that accompanies induction of the autocrine cardiac IGF-I system by Ang II. The interaction between cardiac and systemic IGF-I regulation likely plays an important role in cardiac remodeling in response to Ang II.

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References

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