Original Contributions

Left Ventricular Insulin-like Growth Factor I Increases in Early Renal Hypertension

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Increasing interest has been directed toward the possible role of trophically acting molecules as modulators or initiators, or both, of myocardial hypertrophy. The aim of the present study was to investigate the possible role of one such molecule, namely, insulin-like growth factor I, in myocardial hypertrophy developed in response to renal artery stenosis. Two-kidney, one clip Goldblatt hypertension was induced in Wistar rats weighing 180 g, and sham-operated animals were used as controls. Blood pressure was increased as early as 2 days after clipping (133±4 versus 116±4 mm Hg, p<0.05), and the increase persisted 4 and 7 days after clipping (148±6 versus 129±3 mm Hg, p<0.01 and 171±5 versus 139±3 mm Hg, p<0.01, respectively). Left ventricular weight followed a similar pattern (373±7 versus 350±8 mg, NS, 415±11 versus 386±9 mg, p<0.01, and 466±11 versus 391±10 mg, p<0.01 at 2, 4, and 7 days after clipping, respectively), but no changes in body weight between the groups were observed. Insulin-like growth factor I messenger RNA (mRNA) was quantified using a solution hybridization assay. After 4 days of renal hypertension, there was a significant increase in left ventricular insulin-like growth factor I mRNA (2.0•10^{-18}±0.48•10^{-18} versus 0.4•10^{-18}±0.07•10^{-18} mol·µg DNA^{-1}), which was no longer detectable 7 days after clipping. When insulin-like growth factor I protein was visualized with immunohistochemistry, an enhanced abundance of the protein was demonstrated in the left ventricle 4 as well as 7 days after clipping, and the protein seemed mainly confined to the subendocardial layers where wall stress is highest. In the right ventricle, no changes in weight, insulin-like growth factor I mRNA, or protein could be detected. In conclusion, two-kidney, one clip Goldblatt hypertension induced an increase in insulin-like growth factor I mRNA as well as protein in the left but not in the right ventricle. The principal localization of the increase in insulin-like growth factor I to the subendocardial layers of the left ventricle suggests wall stress as the most probable initiating stimulus for the enhanced synthesis of insulin-like growth factor I. (Hypertension 1992;19:25-32)

When challenged by a chronically increased systemic pressure load, the heart responds with an adaptive left ventricular hypertrophy to normalize wall stress and myocardial oxygen consumption (see Friberg). However, the precise mechanism for transforming the mechanical stimuli of increased pressure or volume work into the hypertrophic response is not known. In addition to mechanical stimuli, different trophic factors such as catecholamines and angiotensin II may influence the hypertrophic process. The rapidly developing left ventricular hypertrophy after experimentally induced renal artery stenosis (two-kidney, one clip [2K1C] Goldblatt hypertension) has been suggested to constitute a local response to the increased pressure load. However, in addition to the intermittent blood pressure peaks that occur soon after renal artery constriction, the level of circulating angiotensin II is markedly elevated, which might contribute to the development of left ventricular hypertrophy as may also locally produced growth factors exercising autocrine or paracrine trophic effects. Moreover, interest has also been directed toward the role of proto-oncogenes as possible factors involved in regulation of myocardial hypertrophy. Insulin-like growth factor I (IGF-I), which is identical to somatomedin-C, is a 70-amino acid peptide that has a high degree of sequence homology to insulin. The IGFs have traditionally been considered to be synthesized in the liver under growth hormone regulation and released into the circulation...
to mediate the growth-promoting effects of growth hormone. However, there is now considerable evidence showing synthesis of IGF-I in multiple extrahepatic tissues, including heart and skeletal muscle, and paracrine/autocrine mechanisms of action have been proposed.

Studies using specific complementary DNA (cDNA) probes for IGF-I have demonstrated the presence of IGF-I messenger RNA (mRNA) in a variety of rat tissues including heart and skeletal muscle. Expression of IGF-I mRNA in rat heart was found to be partly regulated by growth hormone with decreased levels after hypophysectomy, and IGF-I mRNA levels were restored after growth hormone treatment. Moreover, increased expression of IGF-I mRNA has been demonstrated in tissues regenerating after injury, both in intact and hypophysectomized rats, suggesting that IGF-I mRNA expression may be independent of growth hormone under certain conditions.

It was recently shown by Engelmann et al. that ventricular RNA from neonatal normotensive and spontaneously hypertensive rats contained IGF-I mRNA in low abundance with a relative higher abundance in the hypertensive rats. In addition, in situ hybridization revealed IGF-I mRNA expression localized in myocytes and occasionally in endothelial cells.

To elucidate a possible functional role of IGF-I as a possible “second messenger” or permissive factor during developing cardiac hypertrophy, 2K1C hypertension was induced in adult male rats. Abundance of IGF-I mRNA was quantified using a solution hybridization assay, and the distribution of the IGF-I protein across the myocardial wall was visualized by immunohistochemistry.

**Methods**

**General Procedures**

Goldblatt or 2K1C hypertension was induced in male normotensive Wistar rats (Mollegaard Breeding Center Ltd., Ejby, Denmark), weighing about 180 g, by placing a silver clip (diameter, 0.18 mm) around the left renal artery leaving the right kidney intact. A sham operation merely exposing the left renal artery was performed in control rats. Anesthesia was induced by the short-lasting barbiturate methohexital sodium (Brevital; Eli Lilly and Co., Indianapolis, Ind.) (75 mg · kg⁻¹ body wt i.p.). Ninety-seven percent (60 of 62) of the rats survived surgery, and no deaths occurred during the course of the study. Experiments were then performed on groups of 10 2K1C and 10 sham-operated rats 2, 4, or 7 days postoperatively.

On the day of experimentation, rats were weighed, and their systolic blood pressure and heart rate were measured by tail plethysmography (Narco BioSystems, Houston, Tex.). The animals were then anesthetized by methohexital sodium, and the hearts were excised. The atria and great vessels were trimmed away, and the ventricles were separated, blotted, and weighed before being frozen in liquid nitrogen (N₂) and then stored at −20°C until further analysis could be performed. The time from excision to freezing never exceeded 5 minutes.

**Preparation of Total Nucleic Acid**

The tissue was homogenized and digested with proteinase-K added to a sodium dodecyl sulfate-containing buffer, as previously described. A subsequent extraction with phenolchloroform was then performed.

**Solution Hybridization Assay**

Quantitation of IGF-I mRNA was achieved using an RNA-probe labeled with [³²S]uridine-triphosphate, as described in detail previously. The radioactive probe and the unlabeled synthetic IGF-I mRNA strand were prepared according to the method of Melton et al. In brief, the DNA clone used is a 153-base pair genomic subclone (In pSP 64 in both orientations) of mouse IGF-I corresponding to exon 3 (by analogy to human IGF-I). Studies by Bell et al. and Shimatsu and Rotwein suggest that two forms of IGF-I mRNA exist in both mouse and rat: IGF-IA mRNA and IGF-IB mRNA. The structure of the probe used in the present study corresponds to a part of the C-peptide, the whole A- and D-peptide, and the part of the E-peptide that is identical in both the 1A and 1B prepro-IGF-I molecules. Therefore, the probe permitted the detection of both the 1A and 1B forms of IGF-I mRNA. The mouse IGF-I complementary RNA (cRNA) probe was hybridized at 70°C to total nuclear acid (TNA) samples essentially as described. Incubations were performed in microcentrifuge vials (Eppendorf) in a volume of 40 μl containing 0.6 mol NaCl/l, 30 mmol Tris-HCl/l (pH 7.5), 4 mmol EDTA/l, 0.1% sodium dodecyl sulfate, 10 mmol dithiothreitol/l, and 25% (vol/vol) formamide. After overnight incubation, the samples were treated with RNase for 45 minutes at 37°C by adding 1.0 ml of a solution containing 40 μg RNase A and 2 μg RNase T1 (Boehringer-Mannheim, Mannheim, FRG) and 100 μg of herring sperm DNA (Sigma Chemical Co., St. Louis, Mo.) to each sample. The radioactivity protected from RNase digestion by hybridization to mRNA was precipitated by the addition of 100 μl trichloroacetic acid (6 mol/l) and collected on glass fiber filter (Whatman GF/C; Whatman Ltd, Maidstone, UK). The hybridization signal of each sample was compared with a standard curve constructed from incubations with known amounts of IGF-I mRNA in a standard tissue TNA preparation. This standard TNA preparation was originally compared with the synthetic nucleotide mRNA strand. Each TNA sample was analyzed in triplicate. The DNA content was assayed according to Labarca and Paigen, and results are expressed as IGF-I mRNA/DNA (10⁻¹⁸ mol/μg).

In a separate experiment, β-actin mRNA was analyzed in the left and right ventricles at 4 days...
after induction of renal hypertension or performance of sham operation using the same protocol as described above. The probe used was a 1.9 kb pair fragment of rat \(\beta\)-actin cloned into a pGEM vector (Promega, Madison, Wis.).

**Immunohistochemistry**

Hearts were rapidly removed and frozen in liquid nitrogen. Transverse cryostat sections, 6-\(\mu\)m thick, were prepared from the right and left ventricle of each heart. The sections were fixed in methanol at \(-20^\circ\)C and were then transferred to phosphate-buffered saline. After preincubation with 5% fat-free milk in phosphate-buffered saline, the sections were incubated at 4\(^\circ\)C overnight with a polyclonal antiserum raised in rabbits against human IGF-I (K1792; kindly supplied by KabiGen AB, Stockholm, Sweden; described by Nilsson et al\(^{22}\)) and then with donkey anti-rabbit immunoglobulin, peroxidase-linked F(ab')2 fragment (Amersham, Little Chalfont, UK) for 1 hour at room temperature. The reaction was visualized by the diaminobenzidine reaction.

Control sections incubated without the primary antiserum showed unspecific staining of scattered erythrocytes but were otherwise negative. In sections incubated with the primary antiserum preadsorbed with purified IGF-I, a significant reduction in staining intensity was seen (Figure 1).

**Statistical Methods**

Statistical methods used were two-way analysis of variance, followed by Student-Newman-Keuls multiple test between individual groups.

**Results**

To elucidate a possible role of IGF-I during development of cardiac hypertrophy, 2K1C hypertension was produced in male Wistar rats. Postoperative body weight developed in a similar manner in 2K1C and sham-operated animals (180±2.2 g versus 183±1.9 g, 199±3.0 g versus 209±2.0 g, and 217±1.0 g versus 213±1.5 g at 2, 4, and 7 days, respectively). Two days after surgery blood pressure was significantly elevated in clipped rats compared with sham-operated controls (Figure 2). The elevated blood pressure persisted throughout the 7-day period of study. Left ventricular weight remained largely unchanged 2 days after clipping but increased on the fourth and seventh day after clipping, whereas no change in right ventricular weight was evident at any time point (Figures 3 and 4).

Quantitation of IGF-I mRNA, achieved using a solution hybridization assay, revealed a fivefold increase of left ventricular levels of IGF-I mRNA 4 days after clipping compared with sham-operated controls (Figure 3). This difference was, however, not evident after 7 days of renal artery constriction. In addition, as shown in Figure 4, the right ventricular IGF-I mRNA content was unaltered in renal hypertensive rats when compared with normotensive control rats.

To investigate whether the increased levels of IGF-I mRNA were due to a specific activation of the IGF-I gene or part of a generalized increase in RNA, levels of \(\beta\)-actin mRNA were determined in the left

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**FIGURE 1.** Photomicrographs show serial sections from the left ventricle 4 days after induction of renal hypertension with a clip stenosing the left renal artery. Panel a: Incubation with antiserum. Panel b: Incubation with antiserum preadsorbed with synthetic insulin-like growth factor I. Bar, 50 \(\mu\)m.

**FIGURE 2.** Line plot shows effect of two-kidney, one clip (2K1C) procedure on blood pressure. Male normotensive Wistar rats weighing approximately 180 g were equipped with a silver clip around the left renal artery leaving the contralateral kidney intact. In control rats, a sham operation was performed merely exposing the left renal artery. On the day of experimentation 2, 4, or 7 days after operation, systolic blood pressure was measured by tail plethysmography. Blood pressure levels are expressed as millimeters of mercury. Values are mean±SEM. *\(p<0.05\); **\(p<0.01\).
Figure 3. Bar graphs show effect of two-kidney, one clip (2K1C) hypertension on left ventricular weight (upper panel) and insulin-like growth factor I (IGF-I) messenger RNA (mRNA) content (lower panel). Male normotensive Wistar rats weighing approximately 180 g were equipped with a silver clip around the left renal artery leaving the contralateral kidney intact. In control rats, a sham operation was performed merely exposing the left renal artery. Experiments were then performed 2, 4, or 7 days after operation when the rats were weighed, and hearts were excised and processed (see "Methods"). Left ventricular weight is expressed as grams and levels of IGF-I mRNA are expressed as $10^{-18}$ moles per microgram DNA. Values are mean±SEM. **p<0.01.

Figure 4. Bar graphs show effect of two-kidney, one clip (2K1C) hypertension on right ventricular weight (upper panel) and insulin-like growth factor I (IGF-I) messenger RNA (mRNA) content (lower panel). Male normotensive Wistar rats weighing approximately 180 g were equipped with a silver clip around the left renal artery leaving the contralateral kidney intact. In control rats, a sham operation was performed merely exposing the left renal artery. Experiments were then performed 2, 4, or 7 days after operation when the rats were weighed, and hearts were excised and processed (see "Methods"). Right ventricular weight is expressed as grams and levels of IGF-I mRNA are expressed as $10^{-18}$ moles per microgram DNA. Values are mean±SEM.

TABLE 1. Effect of Two-Kidney, One Clip Hypertension on Left and Right Ventricular β-Actin mRNA Content 4 Days After Operation

<table>
<thead>
<tr>
<th></th>
<th>Control values (%)</th>
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<tbody>
<tr>
<td><strong>Left ventricle</strong></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>100</td>
</tr>
<tr>
<td>2K1C</td>
<td>102</td>
</tr>
<tr>
<td><strong>Right ventricle</strong></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>100</td>
</tr>
<tr>
<td>2K1C</td>
<td>101</td>
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Quantities of β-actin mRNA are expressed as a percentage of the control values (sham-operated rats=100%). 2K1C, two-kidney, one clip.
FIGURE 5. Photomicrographs show cryostat sections from rat hearts processed to demonstrate insulin-like growth factor I (IGF-I) immunoreactivity by indirect immunohistochemistry. Panel A: Section from the left ventricle of a sham-operated animal. No staining is seen in myocytes. Connective tissue shows unspecific staining. Panels B and C: Section from the left ventricle 4 days after induction of renal hypertension showing myocytes in cross (panel B) and longitudinal (panel C) sections. There is a focal expression of IGF-I immunoreactivity showing distinctly IGF-I positive cells (arrow) mingled with negative cells (arrow heads). Panel D: Section from the right ventricle of the same animal as in panel B. There is no staining of myocytes. Panel E: Section from the left ventricle 7 days after the operation. Low magnification showing IGF-I positive myocytes mainly in the subendocardial region (left, arrows). Panel F: Higher magnification of panel E shows sharp demarcation between unstained and IGF-I positive myocytes (arrows). Peroxidase/DAB. Bars, 100 μm.
stained cells were observed in the subepicardial region (Figure 5E).

Discussion

The main findings in this study are that early renal hypertension in Wistar rats results in increased levels of IGF-I mRNA and IGF-I protein in the left but not in the right ventricle. An elevated resting blood pressure was found as soon as the second day after renal artery constriction, whereas expression of IGF-I mRNA was still unchanged from control rats at this time. The increase in IGF-I mRNA peaked after 4 days and then declined, and the pattern of IGF-I protein was similar at 4 and 7 days after renal artery constriction.

In recent years, an increasing interest has been focused on trophic factors or molecules acting as modulators of cardiovascular structural adaptation in various forms of hemodynamic overload. There are a vast number of potential endogenous molecules in the myocardium that could exercise a hypertrophic influence. Hammond et al. found that protein synthesis in Langendorff-perfused hearts was stimulated when homogenate from hearts of dogs with aortic constriction was added to the perfusate. A similar stimulating effect in isolated neonatal cardiomyocytes has been shown for hearts of adult spontaneously hypertensive rats. This group also purified the stimulatory factor and found it to be a protein with a molecular weight of 8,500 daltons. It is tempting to speculate that this protein in fact might be IGF-I, which has been shown to have a molecular weight of 7,650 daltons. Speaking in favor of this hypothesis is the finding that IGF-I mRNA is more abundant in hearts from 3-day-old spontaneously hypertensive rats than in normotensive Wistar-Kyoto controls. Moreover, it has previously been demonstrated with immunohistochemistry that expression of IGF-I is transiently increased in activated satellite cells during regeneration after toxic or ischemic injury. Furthermore, extracted IGF-I mRNA levels measured with solution hybridization showed a 20-fold increase during the regeneration process. High levels of IGF-I mRNA have also been seen in hypophysectomized rats after injury, and similar results were obtained in aortic smooth muscle cells after balloon denudation. These latter findings indicate that IGF-I expression, under some conditions, is independent of growth hormone and under such conditions autocrine/paracrine mechanisms for IGF-I regulation and IGF-I action have been proposed.

The stimulus for the increase in left ventricular IGF-I observed in the present study is not clear. However, the fact that the increase of both IGF-I mRNA and protein was seen solely in the left and not in the right ventricle directs attention toward a stimulus that would affect the left ventricle only. This implies that mechanical factors would be likely to be involved. Increased coronary perfusion pressure stimulates protein synthesis in isolated perfused hearts as well as external load in the absence of other trophic factors in isolated feline cardiomyocytes and the mere presence of contractions in cultured neonatal cardiomyocytes. However, coronary perfusion pressure and flow as well as contractions per se will also affect both ventricles to the same extent and are thus less probable as stimuli to IGF-I synthesis. The increased left ventricular work load would be a more likely candidate to explain the raised IGF-I levels in the left ventricle, and this assumption is also strengthened by the present observation that IGF-I protein seems to be more abundant in the inner layers of the myocardium (Figure 5E). In these layers tension, as well as wall stress, is high and declines toward the epicardial surface. Wall stress may thus play an important role as stimulus for IGF-I synthesis under the present conditions. This may also explain why no elevation of right ventricular IGF-I levels was found; renal hypertension is not associated with an increased pressure in the pulmonary circulation, and consequently a normal wall tension is expected in the right ventricle. This hypothesis is further substantiated by the recent finding that hydrostatic pressure can increase secretion of IGF-I from cultured endothelial cells. Moreover, increasing tension (active as well as passive) has been shown to increase protein synthesis in isolated papillary muscle through an increase in Na" influx, probably through fast stretch-activated cation channels, and IGFs can act synergistically with monovalent ion influx to increase DNA synthesis. Thus, from the present in vivo results, one can suggest a possible link between increased tension/wall stress, increased myocardial IGF-I synthesis, and a hypertrophic response in the myocardium, at least in this early phase of hypertension. However, direct experiments to establish the existence of such a link still remain to be performed.

Cardiac hypertrophy and possibly also myocardial IGF-I production may be modified by endocrine or neural factors with trophic properties. Growth hormone is obviously important for regulation of IGF-I synthesis, but as pointed out above, growth hormone--independent IGF-I synthesis has been described under various conditions. In the present study, a change in growth hormone levels would affect both the left and the right ventricle equally, and it is therefore not likely that growth hormone is the sole mediator of the increased IGF-I synthesis. Nevertheless, a normal growth hormone level may well be required to elicit the present IGF-I response since it seems to be a permissive factor for the development of left ventricular hypertrophy after aortic constriction and renal artery stenosis. Cat echolamines such as epinephrine or norepinephrine have been shown to induce hypertrophy of cultured neonatal cardiomyocytes via an α-receptor--dependent increase in protein synthesis. Similar findings have been reported in both isolated perfused working hearts and cultured myocytes from adult rats. Yet, in another study, cardiac sympathetic denervation
did not influence right ventricular hypertrophy after banding of the pulmonary artery.\(^3^7\) Thus, the discrepancy between in vitro and in vivo results regarding the effects of catecholamines makes their role in the development of cardiac hypertrophy unresolved. In the present model, sympathetic outflow to the heart may increase as a consequence of elevated levels of circulating angiotensin II, due to either enhanced presynaptic release of norepinephrine or facilitation of the effects of norepinephrine postjunctionally. This would most likely affect both ventricles in a similar manner, and thus, catecholamines would not by themselves explain the effect on left ventricular IGF-I synthesis. Angiotensin II represents yet another hormone that may be linked to development of myocardial hypertrophy. The circulating levels of angiotensin II increase in early renal hypertension, and in addition, angiotensin II has been shown to increase cardiac contractility and contractile frequency in cultured myocytes.\(^3^8\) Moreover, angiotensin II possesses hypertrophic properties in cultured neonatal chick heart cells,\(^3^9\) but in myocytes from adult rats, no stimulatory effect on protein synthesis could be detected in response to angiotensin II.\(^3^6\) This discrepancy may be attributed to the fact that angiotensin II receptors in adult rat hearts are mainly confined to the conduction system and are virtually absent in ventricular myocytes.\(^3^9\) Thus, the role of circulating angiotensin II in the development of cardiac hypertrophy is still unclear, and as pointed out above, it does not seem to be the most probable candidate as stimulus for the increased left ventricular IGF-I synthesis observed in the present study. Angiotensin II may also be produced locally in the myocardium, and it has recently been shown that angiotensin II is required for the development of left ventricular hypertrophy after aortic constriction, mimicking the situation for growth hormone.\(^4^0,4^1\) Furthermore, an increase in myocardial angiotensin converting enzyme has been demonstrated in rats with aortic stenosis,\(^4^2\) possibly generating higher tissue levels of angiotensin II than circulating concentrations would imply. Thus, although humoral or nervous stimuli by themselves do not seem to be able to induce the increased levels of left ventricular IGF-I, any such factor may be important through interactions with mechanical or hemodynamic stimuli.

In recent years an increasing number of investigations have emphasized the possible role of locally produced growth factors in the early stages of cardiovascular hypertrophy, and IGF-I is, definitely, not the only trophic molecule produced by the myocardium. The proto-oncogenes c-myc, c-fos, and c-Ha-ras increased in the myocardium in response to mechanical stimuli as well as vasoactive agents such as angiotensin II, vasopressin, and phenylephrine.\(^4^3-4^5\) Furthermore c-sis, which codes for the B chain of platelet-derived growth factor, is present in the myocardium and can also induce expression of c-myc and c-fos. Any of these factors could theoretically have influenced or modulated the production of IGF-I in the 2K1C model of hypertension. However, none of these studies have been able to show a definite functional role of the proto-oncogenes in the hypertrophic process. Furthermore, in most cases only the genetic precursor and not the actual peptide was measured, and the possibility exists that the genes were transcribed without translation into the peptide end product.

In conclusion, expression of IGF-I mRNA and synthesis of IGF-I protein were enhanced in the left but not in the right ventricle 4 days after renal artery constriction. The enhancement was detected after an established increase in blood pressure could be demonstrated. The augmentation of IGF-I synthesis seems to be coupled to the increased left ventricular workload and not to circulating humoral factors such as growth hormone or angiotensin II, thus suggesting an autocrine/paracrine regulation of IGF-I under these conditions. Because of the finding of more IGF-I protein in the endocardial than in the epicardial layers, wall stress seems to be the most likely candidate as mechanical stimulus for IGF-I synthesis. Thus, the present study demonstrates the ability of the myocardium itself to produce a molecule with possible trophic properties in direct response to a mechanical stimulus.

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