Overexpression of Insulin-Like Growth Factor-1 Attenuates the Myocyte Renin-Angiotensin System in Transgenic Mice

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Abstract—Constitutive overexpression of insulin-like growth factor-1 (IGF-1) in myocytes protects them from apoptosis and interferes with myocyte hypertrophy in the normal and pathological heart. Conversely, angiotensin II (Ang II) triggers cell death and promotes myocyte hypertrophy. Moreover, activation of p53 upregulates the cellular renin-angiotensin system (RAS). Therefore, IGF-1 overexpression in FVB.Igf+/- mice may downregulate the local RAS through the attenuation of p53 and p53-inducible genes. On this basis, p53 DNA binding activity to angiotensinogen (Aogen), bax, and the AT1 receptor was determined in left ventricular myocytes from FVB.Igf-/- and FVB.Igf+/- mice. The quantity of Bax, Bcl-2, Aogen, and AT1 receptor in these cells was evaluated. The presence of Mdm2-p53 complexes was also established. Finally, Ang II levels in myocytes were measured. Upregulation of IGF-1 in myocytes was associated with a protein-to-protein interaction between Mdm2 and p53, which attenuated p53 transcriptional activity for bax, Aogen, and AT1 receptor. Similarly, the amount of Bax, Aogen, and AT1 receptor proteins in these cells decreased. In contrast, the expression of Bcl-2 remained constant. The downregulation of Aogen in myocytes from FVB.Igf+/- mice was characterized by a reduction in Ang II. In conclusion, IGF-1 negatively influences the myocyte RAS through the upregulation of Mdm2 and its binding to p53. This may represent the molecular mechanism responsible for the effects of IGF-1 on cell viability and myocyte hypertrophy in the nonpathological and pathological heart in vivo. (Circ Res. 1999;84:752-762.)

Key Words: insulin-like growth factor-1 ■ p53 ■ mdm2 ■ angiotensin II ■ transgenic mice

An upregulation of insulin-like growth factor-1 (IGF-1) in the myocardium occurs under a variety of pathological conditions characterized by an increase in pressure and/or volume loads on the heart.1-7 This response has been associated with myocyte hypertrophy.1,2,4,5 The reentry of this cell population into the cell cycle,3,6 myocyte proliferation (for a review, see Reference 7), and a change in the responsiveness of myofilaments to Ca2+8,9 Additionaly, this growth factor increases the rate of protein accumulation, enhancing anabolic and inhibiting catabolic pathways in the cells.10 Therapeutic interventions with IGF-1 attenuate myocyte necrosis and apoptosis after ischemia-reperfusion injury.11 Moreover, the administration of IGF-1 improves experimental myocardial recovery and function after infarction,4,5 although its mechanism remains to be defined. Similar results have been reported in humans affected by idiopathic dilated cardiomyopathy,12 but the events implicated in these beneficial effects of IGF-1 have not been clarified. Constitutive overexpression of IGF-1 prevents the activation of myocyte death in the surviving myocardium of the postinfarcted mouse heart, limiting ventricular dilation, wall stress, and reactive hypertrophy.13 Baseline levels of ongoing myocyte necrosis and apoptosis in control FVB.Igf+/- transgenic mice are significantly lower than those in FVB.Igf-/- nontransgenic littermates,13 further emphasizing the role of IGF-1 in cell survival. In this regard, IGF-1 protects from apoptosis cerebellar neurons,14 hemopoietic cells,15 fibroblasts,16 preovulatory follicles,17 neuroblastoma cells,18 and transplanted tumors.19 Although several factors may be involved in the preservation of cell viability by IGF-1, including the generation of nitric oxide20 and the suppression of interleukin-1β-converting enzyme,21 it is uncertain whether IGF-1 activates these mechanisms in the myocardium. Angiotensin II (Ang II) may represent a major cause of cell death in the heart. This hormone triggers apoptosis in neonatal22 and adult23 myocytes in vitro, and sarcomere stretching is coupled with the synthesis and release of Ang II24,25 and the transmission of a death signal to myocytes.23,25,26 The tumor suppressor p53 upregulates the local renin-angiotensin system (RAS), leading to the formation of Ang II.24,25 In addition, p53 decreases the expression of genes opposing cell death, such as Bcl-2,25-29 and enhances genes promoting apoptosis, such as Bax.25-27,29 In this report, the hypothesis was raised that IGF-1 interferes with the activity of p53, depressing the myocyte RAS and the induction of p53-dependent genes. Such an attenuation of the detrimental impact of these gene...
products on the heart by IGF-1 may be mediated by its ability to phosphorylate the amino terminus of p53, stimulating the transcription of mdm2.\textsuperscript{30,31} Uptregulation of mdm2 may downregulate p53 function by forming mdm2-p53 protein complexes.\textsuperscript{32} These possibilities were tested by evaluating p53 binding to the promoter of angiotensinogen (Aogen), AT\textsubscript{1}, receptor, and bax genes in left ventricular myocytes isolated from FVB.\textsuperscript{Igf}\textsubscript{−/−} and FVB.\textsuperscript{Igf}\textsubscript{+/−} mice at 3 months of age. Additionally, the expression of Aogen, AT\textsubscript{1}, receptor, p53, Bax, and Bcl-2 in these cells was established. The level of protein interaction between mdm2 and p53 was also documented. Finally, the percentage of myocytes containing Ang II was measured by confocal microscopy and complemented with immunocytological analysis of the amount of this peptide in the cells. Transgenic mice were generated by utilizing a construct in which the human IGF-1B cDNA was placed under the control of the rat α-myosin heavy-chain promoter.\textsuperscript{33}

**Materials and Methods**

**Myocyte Isolation**

Hearts were excised from male transgenic FVB.\textsuperscript{Igf}\textsubscript{−/−} and FVB.\textsuperscript{Igf}\textsubscript{+/−} nontransgenic littermates at 3 months of age.\textsuperscript{33} Myocytes from the left ventricle were enzymatically dissociated. After 9°C with HEPES-MEM gassed with 85% O\textsubscript{2}, 15% N\textsubscript{2}. (2) For Biochemical Corp) perfusion of the myocardium was carried out at 37°C with HEPES-MEM with the addition of 0.5 mmol/L EGTA. The washing solution was HEPES-MEM with the addition of 0.5 mmol/L EGTA.

**Western Blot of Bax, Bcl-2, Aogen, and AT\textsubscript{1}, Receptor**

For immunoblottting of Bax, Bcl-2, Aogen, and AT\textsubscript{1}, receptor, myocytes were lysed with 250 \textmu L of lysis buffer (50 mmol/L Tris-HCl pH 7.5; 5 mmol/L EDTA; 250 mmol/L NaCl; and 0.1% Triton X-100) containing the protease inhibitors 2 mmol/L PMSF, 1 \textmu mol/L aprotinin, 5 mmol/L EDTA, and 1 mmol/L Na\textsubscript{3}VO\textsubscript{4}; incubated in ice; and spun down at 14 000 rpm. Equivalents of 50 \mu g of protein were separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose filters, blocked with 6% powdered milk, and exposed to rabbit polyclonal anti-human Bcl-2 (AC21, Santa Cruz), anti-human Bax (P19, Santa Cruz), mouse anti-rat Aogen (Swant, Bellinzona, Switzerland), and rabbit polyclonal anti-rat AT\textsubscript{1} receptor (AB1525, Chemicon, Temecula, Calif) antibodies at a concentration of 1 \mu g/mL in Tris-buffered saline–TWEEN (TBST). Bound antibodies were detected by peroxidase-conjugated anti-mouse or anti-rabbit IgG. Bcl-2 was detected as a 27-kDa band, Bax as a 19-kDa band, Aogen as a 56- to 58-kDa band, and AT\textsubscript{1}, as a 41-kDa band. Mouse serum and the supernatant from the myocyte lysates immunoprecipitated with Aogen antibody were used as positive and negative controls, respectively. Purified AT\textsubscript{1} receptor protein from S9 cells (BioSignal, Montreal, Canada) and the supernatant from myocyte lysates immunoprecipitated with AT\textsubscript{1} antibody were used as positive and negative controls, respectively.

**Immunoprecipitation and Immunoblotting of mdm2 and p53**

Aliquots of myocyte lysates were obtained from transgenic and nontransgenic mice (see the previous paragraph). Two separate immunoprecipitation assays were performed: (1) Two hundred micrograms of soluble protein extracts was incubated with 3 \mu g of mouse monoclonal anti-human mdm2 antibody (Sm1p14, Santa Cruz) and 250 \mu L of HNTG buffer (20 mmol/L HEPES, pH 7.5; 150 mmol/L NaCl; 0.1% Triton X-100; and 10% glycerol) containing the protease inhibitors 0.2 mmol/L PMSF, 2 \mu g/\mu L aprotinin, and 0.2 mmol/L Na\textsubscript{3}VO\textsubscript{4}, overnight at 4°C. Subsequently, 50 \mu L of protein A–agarose (Pierce) was added to each sample. After these washings with a buffer containing 20 mmol/L Tris-HCl (pH 7.4), 300 mmol/L NaCl, 2 mmol/L EDTA, and 2 mmol/L EGTA, samples were spun at 14 000 rpm for 2 minutes. Loading buffer was added to each pellet, and immunoprecipitated proteins were separated by 10% SDS-PAGE. Proteins were transferred to nitrocellulose filters and exposed to rabbit polyclonal anti-human mdm2 antibodies (C-18 and 5’-CTGCCCAG-CATTATCTCAACTT-3’). As a noncompetitor, mouse monoclonal anti-human p53 antibody (AB-11, Calbiochem, Cambridge, Mass) was used to immunoprecipitate the myocyte lysates. p53 was detected as a 53-kDa band, and mdm2 as a 90-, 76-, or 57- to 58-kDa band.
Ang II Labeling

Frozen sections of myocardium were fixed in 3.7% formaldehyde and incubated with antiserum to Ang II (Peninsula Laboratories Inc, Belmont, Calif) diluted 1:20 in PBS and with FITC-labeled goat anti-rabbit IgG. Specificity was determined by preabsorption of 10 μL of antibody with 0.05 mg of antigen for 2 hours at 37°C. Nonimmune rabbit serum was used as an additional control. Tissue sections were stained with rabbit polyclonal laminin antibody (Sigma Chemical Co, St Louis, Mo) to identify the boundaries of myocytes. Myocyte cytoplasm was identified by α-sarcomeric actin antibody (clone 5C5, Sigma) labeling. Sections were examined at 3100 (numerical aperture 1.4) with an MRC-1000 confocal microscope (Bio-Rad Laboratories). Myocytes containing granules positive for Ang II and the number of granules per myocyte profile were evaluated for each animal.

Ang II Concentration

Myocytes were suspended in 1 mol/L acetic acid, homogenized, and centrifuged at 15 000 rpm for 30 minutes at 4°C. Supernatants were dried, reconstituted with 0.1% trifluoroacetic acid, and purified on a C18 Sep-Pak column (Waters Associates). This fraction was eluted from the column with 30% acetonitrile in 5 mL of 0.1% trifluoroacetic acid, dried, and dissolved in 0.25 mL TBST solution. Samples of 50 μL were analyzed in a microtiter plate by using an anti–Ang II antibody (Peninsula ELISA) and a tracer, biotinylated Ang II. The microtiter plate was washed 5 times with TBST and treated with streptavidin–horseradish peroxidase. The color reaction was developed with 100 μL of tetramethylbenzidine substrate and terminated by addition of 2N HCl. The absorbance was recorded at 450 nm, and the concentration was calculated from the standard curve generated each time for Ang II.

Data Analysis

Results are presented as mean±SD. Autoradiograms were analyzed by an image analyzer (Gel Doc 1000, Bio-Rad). Significance between 2 measurements, P<0.05, was determined by Student’s t test; n values are listed in the text or figure legends.

Results

Myocyte Isolation

A total of 28 FVB.Igf+/− and 28 FVB.Igf+/− mice were included in this part of the study. Body weight was 22±3 and 23±4 g in nontransgenic and transgenic mice, respectively. Corresponding heart weight values were 156±15 and 225±22 mg. The isolation procedure, which included the use of collagenase perfusion, did not allow separation of the 2 ventricles and the measurement of their respective weights. However, dissection was performed later to obtain myocytes from the left ventricle only. The yield of myocytes was 2.3±0.2×10^6 and 2.4±0.1×10^6 cells per left ventricle in nontransgenic and transgenic mice, respectively. Intact myocytes accounted for 85±6% and 86±7% in nontransgenics and transgenics, respectively. Contamination from nonmyocytes was 2±2% in both groups of mice. Figure 1A and 1B illustrates that myocytes were rod-shaped and that the level of contamination from interstitial cells was minimal. These myocytes exhibited integrity of the plasma membrane and were Ca^2+ tolerant. In summary, the myocyte isolation protocol provided a consistent preparation of intact, viable, mouse myocytes.

p53 DNA Binding Activity

To determine whether IGF-1 overexpression in myocytes affected the transcriptional activity of p53, binding of p53 to the promoter of bax, Aogen, and AT1 receptor was measured by gel retardation assay. The bax promoter contains 1 perfect and 3 imperfect consensus sequences for p53. An oligonucleotide of 46 bp, including the binding sites for p53, was used as a probe in a mobility shift analysis, and 1 p53 shifted complex was noted (Figure 2A). In comparison with myocyte nuclear extracts from FVB.Igf+/− mice, the optical density (OD) of the p53 band from FVB.Igf+/− was reduced (nontransgenics, OD=5.3±0.9, n=6; transgenics, OD=2.5±0.5, n=6; P<0.001). The specificity of the assay was confirmed by documenting that exposure to excess unlabeled self-oligonucleotide or preincubation with a p53 antibody opposed the appearance of a p53 shifted complex. Conversely, the addition of an irrelevant antibody or an unlabeled, mutated form of bax did not interfere with p53 DNA binding.
The promoter of Aogen contains 7 of 10 matches with the consensus sequence of p53. An oligonucleotide of 25 bp was used as a probe in a gel retardation assay. Figure 2B illustrates that 1 complex with shifted gel mobility was detected in myocyte nuclear extracts from nontransgenic and transgenic mice. The intensity of the p53 band was more apparent in FVB.Igf^+/+ than in FVB.Igf^-/- animals (nontransgensics, OD = 3.6 ± 0.6, n = 6; transgensics, OD = 1.4 ± 0.3, n = 6; P < 0.001). The specificity of the reaction was established by inhibiting the formation of a shifted complex with the exposure of nuclear extracts to an excess of unlabeled self-oligonucleotides (C) and with a monoclonal p53 antibody (Ab). Addition of an irrelevant antibody (Irr) or preincubation with an unlabeled mutated form of bax (Bax mut) did not interfere with p53 binding. Bax indicates bax probe in the absence of nuclear extracts; Ao, Aogen probe in the absence of nuclear extracts; AT1, AT1 probe in the absence of nuclear extracts; and SV-T2, nuclear extracts obtained from SV-T2 cells used as a positive control. D, Pattern of proteins corresponding to nuclear preparations for mobility shift assays; Coomassie blue staining.

The promoter of AT1 receptor shares 7 of 10 matches with the p53 binding motif. A probe of 25 bp including this imperfect p53 consensus site was end-labeled and used in a band shift assay. A p53 shifted complex was visualized (Figure 2C). p53 binding was decreased slightly in myocytes from FVB.Igf^-/- mice (nontransgensics, OD = 1.54 ± 0.47, n = 10; transgensics, OD = 1.06 ± 0.46, n = 10; P < 0.035). The specificity of the assay was determined as described above for Aogen. Finally, consistency in protein loading, lack of protein degradation, and uniformity in the relative purity of the nuclear extracts are shown in FVB.Igf^-/- and FVB.Igf^+/+ mice (Figure 2D). The OD of the actin band was reduced but was consistent throughout. In summary, constitutive overexpression of IGF-1 in myocytes was associated with decreased p53 binding to the promoter of bax, Aogen, and AT1 receptor.
Expression of Bax, Bcl-2, Aogen, and AT1 Receptor in Myocytes

p53 upregulates transcription of bax, whereas a negative regulatory element is present in the promoter of bcl-2. Because differences in p53 function were documented between control myocytes and myocytes overexpressing IGF-1, the amount of Bax and Bcl-2 was measured by Western blotting in these 2 groups of cells (Figure 3A and 3B). In comparison with FVB. Igf2/2 (NT) and FVB. Igf1/2 (T) mice, the expression of Bax, a 19-kDa protein, was decreased 55% in FVB. Igf1/2 (nontransgenics, OD = 2.2 ± 0.8, n = 6; transgenics, OD = 1.0 ± 0.3, n = 6; P < 0.001). However, Bcl-2, a 27-kDa protein, did not vary with IGF-1 in myocytes (nontransgenics, OD = 5.2 ± 0.8, n = 11; transgenics, OD = 4.8 ± 0.6, n = 11; P = 0.2), resulting in an increase in the Bcl-2-to-Bax protein ratio in the cell. Figure 4A illustrates the amount of Aogen in myocytes from FVB. Igf2/2 and FVB. Igf1/2 mice, which appeared as a double band at 56 to 58 kDa. Aogen quantity was 53% lower in cells overexpressing IGF-1, and this difference was significant (nontransgenics, OD = 1.1 ± 0.24, n = 11; transgenics, OD = 0.52 ± 0.26, n = 11; P < 0.001). Similarly, AT1 receptor, a 41-kDa protein (Figure 4B), decreased 45% in FVB. Igf1/2 (nontransgenics, OD = 0.49 ± 0.12, n = 6; transgenics, OD = 0.27 ± 0.16, n = 6; P < 0.03). In summary, constitutive overexpression of IGF-1 in myocytes was characterized by an attenuation of Bax, Aogen, and AT1 receptor proteins, whereas the quantity of Bcl-2 remained constant.

Expression of p53 and Mdm2 in Myocytes

To identify the mechanism by which IGF-1 may decrease p53 function in myocytes, the expression of Mdm2 and its interaction with p53 were determined. Mdm2-p53 protein complexes are coupled with attenuated p53 binding activity. Additionally, Mdm2 reduces the stability of p53, further affecting its function. To prevent dissociation of protein complexes, cell lysates were prepared in the absence of SDS and were immunoprecipitated with p53 antibody. The coimmunoprecipitated proteins were run on SDS-PAGE and then exposed to p53 antibody. The specificity of the p53 band was confirmed by the absence of an identical band in the presence of irrelevant antibody. Under these conditions, the IgG heavy-chain band...
Figure 5. A, Immunoprecipitation (IP) with p53 antibody (Ab) and Western blot (WB) with p53 antibody of myocyte lysates obtained from FVB.Igf−/− (NT) and FVB.Igf+/− (T) mice. Irr indicates IP with irrelevant antibody (Irr) was used as a negative control; (−), supernatant of myocyte lysates immunoprecipitated with p53 Ab was used as a negative control for p53. B, IP with p53 Ab and WB with Mdm2 Ab of myocyte lysates obtained from NT and T mice. Irr indicates IP with Irr Ab was used as a negative control; (−), supernatant of myocyte lysates immunoprecipitated with Mdm2 Ab was used as a negative control for Mdm2; and (−), supernatant of myocyte lysates immunoprecipitated with p53 Ab was used as a positive control for Mdm2. C, IP with Mdm2 Ab and WB with Mdm2 Ab of myocyte lysates obtained from NT and T mice. Irr indicates IP with Irr Ab was used as a negative control; (−), supernatant of myocyte lysates immunoprecipitated with Mdm2 Ab was used as a negative control for Mdm2; and (−), supernatant of myocyte lysates immunoprecipitated with p53 Ab was used as a negative control for p53. D, IP with Mdm2 Ab and WB with p53 Ab of myocyte lysates obtained from NT and T mice. Irr indicates IP with Irr Ab was used as a negative control; (−), supernatant of myocyte lysates immunoprecipitated with p53 Ab was used as a negative control for p53. E, Sequential IP with Mdm2 Ab of supernatant after IP with Mdm2 Ab of myocyte lysates obtained from NT and T mice. F, Sequential IP with Mdm2 Ab of supernatant after IP with Mdm2 Ab of myocyte lysates obtained from NT and T mice.

was not detected in this blot. The aggregate amount of p53 was 93% higher in FVB.Igf−/− mice than in FVB.Igf+/− animals (nontransgensics, OD = 5.8 ± 0.8, n = 6; transgensics, OD = 3.0 ± 0.5, n = 6; P < 0.001). To identify the fraction of Mdm2 bound to p53, the same blots employed for the measurement of p53 in myocytes were exposed to Mdm2 antibody. Two bands, corresponding to Mdm2 p90 and p57 to 58, were recognized in transgenic mice only (Figure 5B; nontransgensics, OD = not detectable, n = 6; transgensics, p90: OD = 0.9 ± 0.3, n = 6; p57 to 58: OD = 1.4 ± 0.4, n = 6). These 2 isoforms of Mdm2 can bind to p53 because they possess an amino-terminal hydrophobic cleft. Conversely, the other isoforms of Mdm2, p85 and p76, lack this region. The band corresponding to the IgG heavy chain is shown as an example in Figure 5B in the samples precipitated with both p53 antibody and irrelevant antibody. The total amount of Mdm2 in myocyte lysates was identified in an identical manner (Figure 5C). Mdm2 p90, p76, and p57 to 58 were detected; Mdm2 p90 increased 6-fold (nontransgensics, OD = 0.4 ± 0.4, n = 6; transgensics, OD = 2.4 ± 0.7, n = 6; P < 0.001), and Mdm2 p57 to 58 increased 50% (nontransgensics, OD = 3.6 ± 0.9, n = 6; transgensics, OD = 5.4 ± 1.9, n = 6; P = 0.07) in myocytes from FVB.Igf+/− mice. Additionally, Mdm2 p76 was barely detectable in nontransgenic and transgenic mice, and Mdm2 p85 was not apparent. The IgG heavy-chain band was present below the band corresponding to Mdm2 p57 to 58 in samples precipitated with both Mdm2 antibody and irrelevant antibody (not shown in this photomicrograph; see Figure 5B). When the blots probed with Mdm2 antibody were exposed to p53 antibody, p53 bound to Mdm2 was visible exclusively in myocytes overexpressing IGF-1 (Figure 5D; nontransgensics, OD = not detectable, n = 6; transgensics, OD = 0.8 ± 0.4, n = 6). The supernatant remaining after immunoprecipitation with Mdm2 antibody was sequentially immunoprecipitated with p53 antibody to identify the fraction of p53 free from Mdm2-p53 complexes (Figure 5E). The portion of free p53 was higher in FVB.Igf−/− mice (nontransgensics, OD = 1.9 ± 0.6, n = 6; transgensics, OD = 0.6 ± 0.4, n = 6; P < 0.002). Conversely, Mdm2 p90 and p57 to 58, which were not included in p53-Mdm2 complexes (Figure 5F), were greater in FVB.Igf+/− (Mdm2 p90: nontransgensics, OD = not detectable, n = 6; transgensics, OD = 0.41 ± 0.15, n = 6; Mdm2 p57 to 58: nontransgensics, OD = 0.6 ± 0.4, n = 6; transgensics, OD = 1.5 ± 0.7, n = 6; P < 0.02). Mdm2 p76 was seen as a faint band. In summary, IGF-1 overexpression was
associated with the induction of Mdm2, which resulted in the formation of Mdm2-p53 complexes in myocytes.

**Ang II Labeling of Myocytes**

To analyze the possibility that IGF-1 may decrease the formation of Ang II, 2 approaches were used: (1) quantitative evaluation of the percentage of myocytes labeled by Ang II antibody by confocal microscopy and (2) measurement of Ang II in myocytes by immunochemical assay. Figure 6A illustrates by green fluorescence the discrete sites of Ang II labeling, and Figure 6B depicts by red fluorescence the myocytes stained by α-sarcomeric actin. These 2 images are shown together in Figure 6C; the fluorescent dots correspond to the localization of Ang II in the myocardium. Myocyte profiles, defined by laminin staining, contained a minimum of 1 to a maximum of 20 stained sites per cell. Preabsorption of the primary Ang II antibody with Ang II resulted in the absence of immunostaining (Figure 6D). Similarly, substitution of the Ang II antibody with nonimmune rabbit serum was characterized by the lack of staining in the myocardium (not shown).

One hundred to 200 left ventricular myocytes were examined at random in each of 5 nontransgenic and 5 transgenic mice, for a total of 700 cells per group. This type of determination showed that 53 ± 3% and 37 ± 8% of myocytes were labeled in FVB.\textit{Igf}^{+/−} and FVB.\textit{Igf}^{+/+} mice, respectively. The 30% lower magnitude of Ang II labeling in transgenics was statistically significant (\(P<0.001\)). Additionally, the number of Ang II–positive sites per square millimeter of all myocytes was 14,619 ± 4254 in FVB.\textit{Igf}^{+/−} and 7315 ± 3630 in FVB.\textit{Igf}^{+/+} mice. The 2-fold greater value in nontransgenics was significant (\(P<0.02\)). Finally, the distribution of Ang II–positive dots in labeled cells and the fraction of negative myocytes are shown in Figure 7. In comparison with nontransgenic mice, this type of analysis documented that Ang II was consistently reduced in all cell categories of transgenic animals. In summary, constitutive overexpression of IGF-1 led to a reduction in the formation of Ang II in myocytes.
Ang II Quantity in Myocytes

To assess whether IGF-1 interfered with the accumulation of Ang II, this peptide was measured by ELISA in left ventricular myocytes isolated from nontransgenic and transgenic animals. This and the previous analysis were not influenced by the volume of myocytes, which could have affected the number of labeled sites per cell profile as well as the total amount of Ang II per cell. The volumes of mononucleated, binucleated, trinucleated, and tetranucleated myocytes were essentially identical in FVB.IGF-1/− and FVB.IGF/+ mice up to 7 months of age. Figure 8 illustrates that the quantity of Ang II per 10⁶ myocytes was 2.1-fold higher in nontransgenics (n = 12) than in transgenics (n = 12) and that this difference was statistically significant (P < 0.001). The results with the immunochemical assay confirmed and strengthened the measurements made by confocal microscopy. In summary, constitutive overexpression of IGF-1 was coupled with a reduction in Ang II concentration in myocytes.

Discussion

Findings in the present study indicate that the upregulation of IGF-1 in myocytes of FVB.IGF/+ mice was characterized by the formation of Mdm2-p53 protein complexes that attenuated p53 transcriptional activity with respect to bax, Aogen, and AT1 receptor. These modulations at the level of the genes resulted in a decreased quantity of Bax, Aogen, and AT1 receptor proteins in myocytes. Because the amount of Bcl-2 protein was not altered, the Bcl-2-to-Bax protein ratio increased in myocytes overexpressing IGF-1. Conversely, the downregulation of Aogen was coupled with a reduction in the accumulation of Ang II in the cells, suggesting that IGF-1 negatively influenced the local RAS through the inhibition of p53 function. These observations may provide the molecular basis for the protective effect of IGF-1 against apoptosis in myocytes in vivo. Additionally, by opposing the synthesis of Ang II, this growth factor may limit myocyte cellular hypertrophy in FVB.IGF/+ mice during postnatal maturation and aging.

IGF-1, p53 Function, and Mdm2

In recent years, several studies have documented that p53 functional activity is not dependent on p53 quantity. The tumor suppressor is present in the cell in 2 distinct forms, transcriptionally active and latent. Changes in the relative proportion of these tetrmeric proteins may occur without alterations in the absolute amounts found by Western blotting. The localization of p53 in the nucleus reflects the induction of p53 activity, whereas the cytoplasmic distribution of this protein corresponds to loss of p53 function. Additionally, interaction of p53 with cellular proteins may increase its stability or enhance its degradation, further affecting the steady state of this transcription factor in the cell. How these various regulatory characteristics influence the expression of p53 in ventricular myocytes of transgenic mice is difficult to ascertain. These multiple levels of modulation of p53 quantity complicate interpretation of the decrease in p53 protein in myocytes overexpressing IGF-1. Similarly complex is the understanding of the differential impact of p53 on the protein levels of Bax, Aogen, AT1 receptor, and Bcl-2 in these cells.

Phosphorylation of the p53 molecule can occur at several sites clustered at the carboxy and amino termini of this protein. This posttranslational modification may result in the generation of subspecies of p53 that differ in the extent and/or site of phosphorylation. At least 9 kinases have been implicated in the phosphorylation of p53 in vitro and in vivo, and these specific enzymatic reactions have been linked to selective activation or repression of p53 function. Phosphorylation of the carboxy terminal by protein kinase C (PKC) activates DNA binding of a latent, inactive form of p53 that enhances transcription. Conversely, phosphorylation of the amino terminal by Raf-1 kinase and mitogen-activated protein kinase inhibits p53 binding activity, thus downregulating transcription. Distal events coupled with ligand binding to surface IGF-1 receptors on myocytes may lead to phosphorylation of the amino terminal of p53, decreasing its impact on p53-inducible genes, such as bax, Aogen, and AT1 receptor. Attenuation in the formation of Ang II in myocytes overexpressing IGF-1 may affect the stimulation of PKC and PKC-mediated phosphorylation of the carboxy terminal of p53. This may result in the lack of change in Bcl-2, which has a p53 negative regulatory element in its promoter region.

A relevant aspect concerning the inhibition of p53 binding activity by IGF-1 involves the expression of the protooncogene mdm2, which possesses in its promoter 2 perfect consensus sequences for p53. mdm2 decreases the stability of p53 by enhancing the degradation of this protein. This phenomenon is modulated by a protein-to-protein interaction between Mdm2 and p53 and stimulation of the ubiquitin-proteasome pathway. Additionally, the downregulation of p53 function by Mdm2 leads to inactivation of transcription of p53-inducible genes. Mdm2-p53 complexes maintain the ability to link to DNA, but this form of binding is not...
specific. However, it has the capacity to interfere with several basal transcription factors, such as TATA-binding protein and transcription factor IID, impairing constitutional levels of transcription. The observations in the present study of Mdm2-p53 complexes in myocytes overexpressing IGF-1 suggest that similar mechanisms may be operative under these conditions.

IGF-1 and Bcl-2 Family of Proteins

The members of the Bcl-2 family consist of agonists and antagonists of apoptosis. The ability of cells to sustain death signals is dependent on the level of expression of antiapoptotic gene products, such as Bcl-2, and proapoptotic gene products, such as Bax. Bcl-2 induces cell survival by forming heterodimers with Bax, which suppress the death-promoting effect of this protein. The quantity of Bax decreased in myocytes of transgenic mice, thus reflecting the attenuation in p53 binding to the bax promoter in these cells. Although the amount of Bcl-2 remained constant, the Bcl-2–to-Bax protein ratio increased in FVB.Igf1/−/− mouse myocytes, possibly enhancing their capacity to counteract death stimuli generated by humoral and mechanical factors. Moreover, IGF-1 can activate the phosphatidylinositol-3'-OH kinase that may stimulate the serine-threonine kinase Akt, a potent suppressor of cell death. Through this effector pathway, IGF-1 may induce phosphorylation of Bad, a proapoptotic member of the Bcl-2 family, neutralizing its action on cell survival. Constitutive overexpression of IGF-1 prevents the initiation of cell death in the surviving myocardium after infarction, and this phenomenon positively interferes with the development of postinfarction cardiac myopathy. Moreover, the administration of IGF-1 reduces cell death in ischemia/reperfusion injury, improves myocardial function chronically after infarction, and ameliorates ventricular performance in patients with idiopathic dilated cardiomyopathy. This beneficial influence of IGF-1 on the pathological heart may not be restricted to its consequences on gene products implicated in myocyte viability. Myocytes overexpressing IGF-1 exhibit a higher velocity of shortening and greater compliance characteristics. These physiological modifications may be critical in heart failure, in which an increased response to diastolic overload may enhance cardiac pump function via a more effective Frank-Starling relation.

IGF-1 and Myocyte RAS

Ventricular myocytes possess a cellular RAS and synthesize and secrete Ang II. Heart failure is characterized by an upregulation of this system and an enhanced formation of Ang II that has been implicated in the hypertrophic response of the myocardium. This growth-promoting effect of Ang II on adult myocytes has been confirmed in vitro. Attenuation of reactive hypertrophy, through interference with the systemic and local RAS in vivo, has improved morbidity and mortality in patients with ischemic and nonischemic cardiomyopathy. Recently, Ang II has been linked to the initiation of apoptosis in myocytes. IGF-1 decreased the quantity of Ang II in myocytes, and this effect may have inhibited activation of cell death and reduced myocyte hypertrophy in the viable myocardium after infarction in FVB.Igf1/−/− mice. Although the limiting factor in the generation of Ang II in various cell types remains to be defined, Aogen is critical for the stimulation of the entire system. The amount of Aogen protein was downregulated in myocytes overexpressing IGF-1, and the change in this precursor of Ang II was accompanied by a diminished hormone content per cell. Additionally, AT1 receptor protein was decreased, further attenuating the response of myocytes to Ang II. As suggested above, the alteration in p53 transcriptional activation of Aogen may be responsible for the lower concentration of Ang II in myocytes of transgenic mice. However, it cannot be excluded that defects in other components of the local RAS may be involved in the restriction of the synthesis of Ang II in the hearts of FVB.Igf1/−/− mice.

Limitations of the Study and Conclusions

There are several limitations in the present investigation that have to be acknowledged. The observation that p53 DNA binding to the promoter of bax, Aogen, and AT1 receptor was reduced in myocytes from FVB.Igf1/−/− mice does not exclude the possibility that the changes in gene expression produced by IGF-1 were mediated by p53-independent mechanisms. Although it has been shown that the sequences of the oligonucleotides used were critical for the binding of p53 to bax, Aogen, and AT1 receptor genes, other transcription factors could be implicated in the attenuation of the myocyte RAS in FVB.Igf1/−/− animals. This difficulty in the interpretation of the results cannot be overcome in in vivo experiments. Additionally, the weights of the hearts of transgenic mice were greater than those of nontransgensics, and cardiac hypertrophy may have affected gene expression. However, this seems unlikely, because myocyte volume was essentially identical in FVB.Igf1/−/− and FVB.Igf1/−/− mice and the difference in cardiac weight was the result of a larger number of cells in transgensics.

In conclusion, constitutive overexpression of IGF-1 in myocytes downregulates the formation of Ang II and the accumulation of Bax in the cells by attenuating the transcriptional activity of p53 and the induction of p53-dependent genes. This inhibition of p53 function appears to be mediated by the expression of Mdm2 and its ability to increase the degradation of p53 and decrease specific p53 DNA binding. Because Ang II promotes myocyte hypertrophy and apoptosis, IGF-1 may counteract these potential detrimental effects, thereby ameliorating the ability of the heart to sustain pathological loads acutely and chronically. Whether myocyte proliferation constitutes a third component of the beneficial consequences of IGF-1 on the heart, by substituting for cellular hypertrophy, remains an important unanswered question.

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