Interleukin 6 Mediates Myocardial Fibrosis, Concentric Hypertrophy, and Diastolic Dysfunction in Rats

Giselle C. Meléndez, Jennifer L. McLarty, Scott P. Levick, Yan Du, Joseph S. Janicki, Gregory L. Brower

Abstract—Although there is a correlation between hypertension and levels of interleukin (IL) 6, the exact role this cytokine plays in myocardial remodeling is unknown. This is complicated by the variable tissue and circulating levels of IL-6 reported in numerous experimental models of hypertension. Accordingly, we explored the hypothesis that elevated levels of IL-6 mediate adverse myocardial remodeling. To this end, adult male Sprague-Dawley rats were infused with IL-6 (2.5 μg · kg⁻¹ · h⁻¹, IP) for 7 days via osmotic minipump and compared with vehicle-infused, aged-matched controls. Left ventricular function was evaluated using a blood-perfused isolated heart preparation. Myocardial interstitial collagen volume fraction and isolated cardiomyocyte size were also assessed. Isolated adult cardiac fibroblast experiments were performed to determine the importance of the soluble IL-6 receptor in mediating cardiac fibrosis. IL-6 infusions in vivo resulted in concentric left ventricular hypertrophy, increased ventricular stiffness, a marked increase in collagen volume fraction (6.2% versus 1.7%; \( P < 0.001 \)), and proportional increases in cardiomyocyte width and length, all independent of blood pressure. The soluble IL-6 receptor in combination with IL-6 was found to be essential to producing increased collagen concentration by isolated cardiac fibroblasts and also played a role in mediating a phenotypic conversion to myofibroblasts. These novel observations demonstrate that IL-6 induces a myocardial phenotype almost identical to that of the hypertensive heart, identifying IL-6 as potentially important in this remodeling process. (Hypertension. 2010;56:225-231.)

Key Words: myocardial fibrosis ■ cardiomyocyte dimensions ■ ventricular compliance ■ ventricular function ■ cytokine

Inflammation is a key component in the myocardial remodeling process that takes place in response to hypertension.¹⁻⁵ However, the respective roles of specific cytokines in this process are not well defined. Interleukin (IL) 6 has attracted attention with regard to myocardial dysfunction because increased levels correlate with the severity of heart failure and are strongly prognostic of 1-year mortality.⁶,⁷ There is a growing body of evidence that a similar association exists in hypertensive patients.⁸⁻¹⁰ Lee et al¹¹ found that induction of IL-6 by angiotensin II contributes to elevations in blood pressure; however, the contribution of IL-6 to myocardial remodeling has not been firmly established. Hirota et al¹² demonstrated that concomitant overexpression of both IL-6 and the IL-6 receptor in mice induced concentric hypertrophy typical of that occurring in a hypertensive heart. Although these observations suggest that IL-6 may directly mediate hypertrophic remodeling associated with hypertension, no studies have directly investigated the role of IL-6 in mediating cardiac fibrosis or diastolic dysfunction, features that are also characteristic of the hypertensive heart. The effects of IL-6 on collagen synthesis by isolated cardiac fibroblasts have been inconsistent.¹³,¹⁴ Siwik et al¹⁴ found that IL-6 produced a modest decrease in collagen synthesis, together with increased matrix metalloproteinase activity in neonatal cardiac fibroblasts. However, these studies did not investigate the role of the soluble IL-6 receptor (sIL-6R). We hypothesized that elevations of IL-6 in vivo would produce cardiac fibrosis, in addition to inducing cardiac hypertrophy, and, furthermore, that the sIL-6R would be important in the regulation of collagen by adult cardiac fibroblasts.

Materials and Methods

Adult male Sprague-Dawley rats weighing between 250 and 300 g were housed under standard environmental conditions and maintained on commercial rat chow and tap water ad libitum. This investigation conformed with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the experimental protocol had Institutional Animal Care and Use Committee approval. Anesthesia for the nonterminal surgical procedure was achieved by inhalation of isoflurane (2%). Analgesia was achieved by administration of buprenorphine HCL (0.025 mg · kg⁻¹, SQ) to the rats at the time of surgery. At the experimental end point, the rats were anesthetized with pentobarbital (70 mg · kg⁻¹, IP) and euthanized by removal of the heart.
Implantation of Osmotic Pumps and Experimental Protocol

IL-6 (Invitrogen) was administered at a rate of 2.5 μg · kg⁻¹ · h⁻¹ for 7 days using osmotic minipumps (ALZET model 2001, Durect Corporation) implanted in the peritoneal cavity. The lipopolysaccharide IL-6 was dissolved in a vehicle solution containing 10% rat albumin. At the experimental end point, blood pressure and body weights were measured and left ventricular (LV) function assessed.

Assessment of Ventricular Size and Function

LV volume and function were evaluated ex vivo using a blood-perfused isolated heart preparation, as described previously. Briefly, the descending thoracic aorta was cannulated for continuous retrograde perfusion of the heart. The heart was then extirpated and attached to a pressurized reservoir (95 to 105 mm Hg) containing arterial blood from a support rat. The pulmonary artery was then transected to allow unrestricted drainage of coronary venous flow, which was collected in a reservoir and returned to the support rat, where it was reoxygenated. The left atrium was removed, and a compliant latex balloon was inserted through the mitral valve orifice into the LV to obtain ventricular pressure-volume relationships. Once the heart developed stable isovolumetric contractions, the balloon volume, which produced an LV end-diastolic pressure (EDP) of 0 mm Hg (V0) was determined. The balloon volume was then increased in 10-μL increments until an LV EDP of 25 mm Hg was attained. After each increase in balloon volume, the end-diastolic and peak isovolumetric pressures were recorded. After completion of these functional studies, the atria and great vessels were removed and the right ventricle (RV) and LV plus septum were separated and weighed. A midventricular transmural LV section was placed in 4% paraformaldehyde and the remaining tissue snap-frozen in liquid nitrogen and stored at −80°C for further analysis.

Morphological and Histological Evaluation

LV tissue from vehicle (n=6) and IL-6–infused (n=8) rats was processed for routine histopathology, and 5-μm-thick paraffin-embedded sections were stained with hematoxylin-eosin for the evaluation of myocardial morphology. A serial section was stained with picrosirius red, and 20 randomly chosen fields per section were analyzed by light microscopy (×400) to determine the average collagen volume fraction, as described previously. Perivascular areas were excluded from the collagen volume fraction analysis.

Determination of Cardiomyocyte Size

Cardiomyocytes from the LV of vehicle (n=6) and IL-6–infused (n=8) rats were isolated using a modified KOH procedure initially described by Gerdes et al18 to assess the extent of cellular remodeling. Briefly, formalin-fixed tissue from a midlevel transmural section of the LV was trimmed into 1-mm³ pieces and placed in a 0.1-mol/L KOH solution at room temperature for 24 hours. The tissue was then transferred to a 0.1-mol/L PBS solution and continuously agitated for 10 minutes. Cardiac myocytes thus obtained were purified using a 10% Ficoll density gradient. Finally, the cells were resuspended in 10% buffered formalin and transferred to a glass microscope slide, using a cytopsin apparatus, and stained with 1.5% galloycyanin and 5.0% chromium potassium sulfate dodecahydrate. The length and width of 50 rod-shaped cardiomyocytes were measured for each individual heart using ImagePro Plus software (Media Cybernetics, LP).

Measurement of IL-6 and Tumor Necrosis Factor-α

Myocardial levels of IL-6 and tumor necrosis factor (TNF)-α were determined using commercially available kits (Quantikine, R&D Systems) in vehicle (n=6) and IL-6–infused (n=8) rats. Approximately 100 mg of LV tissue were homogenized for the assays. All of the samples were run in duplicate with the average of the 2 replicates reported.

Isolated Cardiac Fibroblast Studies

Primary cultures of adult cardiac fibroblasts were obtained from male Sprague-Dawley rats (n=4). Briefly, hearts were homogenized and digested with Liberase 3 (Roche) with fibroblasts purified by selective attachment to the plastic culture ware. These cells were maintained in DMEM containing 10% neonatal bovine serum and 5% FCS, with medium replacement every other day and used before passage number 3. One million fibroblasts were allowed to adhere in DMEM with 10% neonatal bovine serum and 5% FCS for 24 hours before rinsing with Mesosona salt solution and serum starvation for 24 hours in DMEM-F12. Medium was then replaced with DMEM (1.5% FBS) containing 10 or 50 ng/mL of IL-6 for 24 hours. Additional experiments were performed where IL-6 incubation occurred in the presence of varying concentrations of the sIL-6R (ie, 0.5, 5.0, and 50.0 ng/mL). Cardiac fibroblasts were incubated in IL-6 (50 ng/mL in 1.5% FBS DME) for 1 hour before the sIL-6R treatment. The sIL-6R (Peprotech) was diluted in DMEM and added to the pretreated medium to achieve final concentrations of 0.5, 5.0, and 50.0 ng/mL for 24 hours. The concentrations of IL-6 and sIL-6R were published previously by Yamaguchi et al. They reported that IL-6 signaling in the presence of the soluble form of IL-6 receptor leads to the phosphorylation of IL-6 signal transducer gp130 in human gingival fibroblasts. Although we used adult rat cardiac fibroblasts, we postulated that the same concentrations of IL-6 and sIL-6R would produce the same signaling events. The concentrations of IL-6 used were shown previously to cause a response in skin and lung fibroblasts. Collagen synthesis was determined by hydroxyproline analysis of collected medium, as described by Edwards and O’Brien.

Western Blot Analysis

Western blot analysis was used to determine the relative amounts of α-smooth muscle actin (SMA). Male adult cardiac fibroblasts were treated as stated previously. After the 24-hour treatment, fibroblasts were rinsed with PBS and extracted in PBS containing protease inhibitor mixture (Sigma-Aldrich). The protein concentrations of the fibroblast homogenate were determined with a standardized colorimetric assay (Bio-Rad Protein Assay). Thirty micrograms of extracted protein were loaded onto 10% SDS-polyacrylamide gels and underwent electrophoresis at 150 V for 1 hour. The separated proteins were then transferred to 0.45-μm nitrocellulose membranes (ThermoScientific) at 30 V overnight. After transfer, nitrocellulose membranes were stained with Poncseu solution (Sigma-Aldrich) to confirm the transference of proteins to the membrane. The membrane was then rinsed with PBS and incubated with mouse monoclonal antibody against α-SMA (Santa Cruz Biotechnology) at a dilution of 1:500 in 5% milk/TBS-T for 2 hours. Membranes were then rinsed several times with TBS-T and incubated with horseradish peroxidase-conjugated goat antiantibody mouse (1:2000 dilution, Santa Cruz Biotechnology) for 2 hours. Membranes were then rinsed several times in TBS-T and developed using the enhanced chemiluminescence detection kit (ThermoScientific). The luminescent signal was detected by exposure to x-ray film (Phenix Research Products) for 5 minutes. Band density was measured using a GS 800 Calibrated Densitometer and Quality One Software by Bio-Rad. The membrane was later reprobed for GAPDH as an internal control. Results are presented as the ratio of α-SMA/GAPDH.

Statistical Analysis

Statistical analysis was performed using SPSS 11.5 software (SPSS). Results are presented as mean±SD or SEM, as appropriate. Unpaired t test was used to make group comparisons, except for the sIL-6R comparisons in Figure 4, where a 1-way ANOVA with Bonferroni posthoc test was performed. The end-diastolic pressure-volume curves for each heart were fit to a third-order nonlinear regression (Graph Pad), and the volumes corresponding with 2.5 mm Hg pressure increments were determined. The volumes for each pressure increment were then averaged to obtain the pressure-volume.
relationship for each group. Statistical significance was taken to be $P<0.05$.

**Results**

**Morphometric Parameters**

Average LV, RV, and lung weights, as well as systolic and diastolic blood pressures for vehicle and IL-6–infused groups, are presented in Table 1. LV weights of IL-6–infused hearts were significantly increased relative to vehicle-infused hearts. No significant differences in RV and lung weights were observed between the groups, indicating that the animals were still in compensated hypertrophy. IL-6 infusion did not significantly alter systolic or diastolic blood pressures.

**Histological Analysis**

The effect of IL-6 infusion on LV collagen volume fraction is presented in Figure 1. A $>3$-fold increase in interstitial myocardial collagen was observed in the IL-6–infused group compared with control ($6.2\pm0.4\%$ versus $1.7\pm0.2\%$, respectively; $P<0.001$). No evidence of inflammatory cell infiltration or necrosis was observed.

**LV Diastolic Function**

As can be seen in Figure 2, IL-6 infusion produced a leftward shift of the EDP-end-diatolic volume relationship together with an increased slope, indicative of a smaller and stiffer ventricular chamber. These changes were quantified as the difference in unstressed LV volume ($V_0$) and the volume required to increase EDP from 0 to 25 mm Hg ($\Delta V_{0.25}$). As detailed in Table 2, $V_0$ and $\Delta V_{0.25}$ were significantly decreased in the IL-6–infused group when compared with control.

**LV Cardiomyocyte Size**

Average LV cardiomyocyte length and width for IL-6 and vehicle-infused groups are presented in Table 3. Both length and width were significantly increased after 7 days of IL-6 infusion compared with cardiomyocytes from control hearts. However, the average cardiomyocyte length:width ratio remained similar for both groups.

**LV Levels of IL-6 and TNF-$\alpha$**

Levels of myocardial IL-6 and TNF-$\alpha$ from IL-6 and vehicle-infused rats are shown in Figure 3A and 3B, respectively. IL-6 levels were significantly increased in the IL-6–infused group compared with controls ($6.5\pm0.8$ versus $2.3\pm0.1$ pg/mL, respectively; $P<0.001$). TNF-$\alpha$ was not significantly increased in the IL-6–infused group compared with controls ($1.7\pm0.2$ versus $1.3\pm0.1$ pg/mL).

**Cardiac Fibroblast Collagen Synthesis and Myofibroblast Formation**

As can be seen in Figure 4A, incubation of isolated adult cardiac fibroblasts with IL-6 at a concentration of 10 ng/mL...
had no effect on hydroxyproline concentration, either alone or in the presence of varying concentrations of the sIL-6 receptor. However, IL-6 at a concentration of 50 ng/mL produced a significant concentration-dependent increase in hydroxyproline content for all 3 concentrations of the sIL-6R tested (Figure 4B). Similarly, IL-6 alone had no effect on fibroblast phenotype as assessed by α-SMA (Figure 4C). However, IL-6 administered in conjunction with sIL-6R at 50 ng/mL resulted in a dramatic increase in α-SMA in fibroblasts, indicative of phenotypic conversion to myofibroblasts (Figure 4D).

Discussion

Both experimental studies and epidemiology point to an emerging recognition of inflammatory cytokines as biomarkers of cardiovascular disease. However, the relative importance of specific cytokines in the regulation of arterial pressure and in the pathogenesis of hypertension has yet to be fully elucidated. Increased levels of circulating IL-6 in patients correlate with the severity of heart failure and are predictive of mortality. A similar relationship has also been identified between IL-6 and hypertension, with increases in mean arterial pressure in normal healthy men being significantly associated with elevated levels of circulating IL-6. However, the exact contribution of IL-6 to the pathogenesis of hypertension is unclear because of the variable levels of IL-6 reported in experimental models of hypertension.

Nevertheless, the data presented herein clearly demonstrate the ability of IL-6 to induce a pattern of myocardial remodeling consistent with that occurring in the hypertensive heart, including concentric hypertrophy, fibrosis, and diastolic dysfunction. In addition, we have identified a critical role for the sIL-6R in collagen synthesis induced by IL-6.

Our findings are the first to establish that pathological elevations in circulating IL-6 result in extensive cardiac fibrosis. The IL-6 family of cytokines has the capacity to regulate cell function through a cell surface receptor composed of 2 transmembrane proteins, a ligand-binding subunit designated as the IL-6 receptor and a signal transducing glycoprotein (gp130). The presence of the IL-6 receptor has been demonstrated previously in adult cardiac fibroblasts, where it was reported to be essential for fibroblast growth.

Table 2. Isolated Heart: LV Volume and Chamber Stiffness

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>V0, μL</th>
<th>∆V0.25, μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle infused</td>
<td>6</td>
<td>292±8</td>
<td>114±5</td>
</tr>
<tr>
<td>IL-6 infused</td>
<td>8</td>
<td>259±2*</td>
<td>79±1*</td>
</tr>
</tbody>
</table>

∗P<0.001 vs vehicle infused.

Values are mean±SEM; V0 indicates LV end-diastolic volume at an LV EDP of 0 mm Hg; ∆V0.25, LV end-diastolic volume at LVEDP of 25 mm Hg – V0.

Table 3. Cardiomyocyte Size

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Cell Width, μM</th>
<th>Cell Length, μM</th>
<th>CL:CW Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle infused</td>
<td>6</td>
<td>46.5±2.4</td>
<td>108.9±7.8</td>
<td>2.3:0.2</td>
</tr>
<tr>
<td>IL-6 infused</td>
<td>8</td>
<td>61.9±2.5*</td>
<td>138.2±15*</td>
<td>2.2:0.2</td>
</tr>
</tbody>
</table>

∗P<0.001 vs vehicle infused.

Values are mean±SEM; CL indicates cell length; CW, cell width.

Figure 3. Myocardial levels of IL-6 (A) and TNF-α (B) after vehicle and IL-6 infusions. All of the values are mean±SEM. ∗P<0.001 vs vehicle-infused group.

Furthermore, Siwik et al found that incubation of adult rat cardiac fibroblasts with IL-6 reduced collagen content in the medium by 11%. In addition, they also reported that matrix metalloproteinase activity was significantly increased after incubation of neonatal cardiac fibroblasts with IL-6. In contrast with their observation, we found no effect of IL-6 alone on collagen content in the medium of isolated adult cardiac fibroblasts. However, addition of the sIL-6R, which was not evaluated in the studies by Siwik et al, elicited concentration-dependent increases in collagen content. Incubation of fibroblasts with IL-6 in the presence of the sIL-6R produced a 4-fold increase in collagen content at the highest concentration of the sIL-6R. Moreover, this combined stimulus also induced a conversion in fibroblast phenotype to that of a myofibroblast, indicating the ability of the sIL-6R to regulate fibroblast function by several mechanisms. The sIL-6R is naturally occurring in the body and is the result of proteolysis of the membrane receptor or alternative mRNA splicing. Although most soluble receptors act as antagonists in the sense that they compete with the corresponding membrane-bound receptor for the specific ligand, this is not the case with the sIL-6R, which instead acts as an agonist to activate signal transduction on cells that are not stimulated by IL-6 alone. The IL-6/sIL-6R does this by interacting with membrane-bound gp130, which in turn leads to phosphorylation of downstream second messengers, such as Janus kinase, to induce signal transduction and activators of transcription, resulting in stimulation of various cellular events.

In addition to cardiac fibrosis, we also found that IL-6 induced significant concentric LV hypertrophy. This finding is consistent with the previous reports by Hirota et al, who...
described a pattern of concentric hypertrophy in the hearts of mice overexpressing both IL-6 and the IL-6 receptor. However, our study extends these findings to demonstrate that individual cardiomyocytes underwent both elongation and thickening in response to IL-6 infusion. The fact that the cell length:width ratio was similar to normal cardiomyocytes indicates that this hypertrophy consisted of proportional growth. This is in agreement with the previous report by Korecky and Rakusan,34 who determined that cell length and width increase proportionally in concentric cardiac hypertrophy. Several studies have implicated cardiotrophin 1/gp130–induced phosphorylation of signal transducers and activators of transcription 3 as mediating myocardial hypertrophy,35–37 and Wollert et al,38 reported that cardiotrophin 1 stimulation in isolated neonatal cardiomyocytes induces hypertrophy consisting of in-series addition of sarcomeric units. Although this may reflect intrinsic differences between adult and neonatal cardiomyocytes or a differential response of cells to cardiotrophin 1 and IL-6, it seems likely that differential regulation of hypertrophy is more complex, involving induction of other in vivo pathways like the renin-angiotensin system. This is reflected in the findings of Lopez et al,37 who reported recently that in-series sarcomeric addition was induced by cardiotrophin 1 in adult cardiomyocytes isolated from normotensive Wistar rats, whereas a concentric hypertrophic response was observed in cardiomyocytes isolated from spontaneously hypertensive rats. Interestingly, cardiac fibroblasts stimulated with angiotensin II have been shown to secrete members of the IL-6 family, including IL-6 itself, which induced cardiomyocyte hypertrophy via activation of the gp130 receptor.39 In view of the possibility that increased LV stiffness could lead to higher left atrial and pulmonary pressures, it is interesting to note that RV hypertrophy was absent after IL-6 infusion. Although LV stiffness was increased in the IL-6–infused animals, we do not know the actual LV end diastolic pressures. Given that this was a 1-week infusion of IL-6, it may be that the pressures were not elevated long enough to result in RV hypertrophy within that time period. Furthermore, there is evidence from exercise studies in heart failure patients that pulmonary wedge pressure (a marker of LV pressure) is not coupled to right atrial pressure until a point of pericardial constraint is reached.40 Thus, in the IL-6–infused animals, a sufficiently large enough degree of LV hypertrophy and/or ventricular stiffness may not have been attained to influence the RV workload.

The IL-6–induced changes in myocardial structure were manifested functionally as a stiffer (decreased \(\frac{V_{0}}{H9004}\)) smaller ventricle (decreased \(V_{0}\)). The increased stiffness was most likely the result of the marked myocardial fibrosis, because it has been shown that myocyte hypertrophy does not intrinsically alter LV stiffness.41 Together these events replicate the concentric cardiac remodeling typical of pressure overload, where there is an increase in LV mass concomitant with decreased LV chamber size and significant fibrosis.
Consequently, these findings provide additional evidence suggesting that IL-6 may contribute to the development of diastolic dysfunction in hypertensive patients, resulting in the eventual transition to heart failure.

The increased levels of myocardial IL-6 confirm the effectiveness of the IL-6 infusion. However, IL-6 did not induce a significant increase in blood pressure, nor was there an inflammatory cell response in the myocardium, indicating that IL-6 release likely occurs downstream of inflammatory cell infiltration, and fibrosis and hypertrophy can be independent of blood pressure. Although increases in TNF-α are known to induce subsequent increases in IL-6, the sustained increase in IL-6 did not produce a corresponding induction of myocardial TNF-α levels.

**Perspectives**

This is the first report demonstrating the ability of IL-6 to induce a pattern of myocardial remodeling that includes cardiac fibrosis and concentric hypertrophy. Overall, the remodeling induced by infusion of IL-6 was remarkably similar to that seen in hypertension and suggests that IL-6 may be responsible for inducing adverse remodeling in the hypertensive heart, thereby contributing to LV diastolic dysfunction. Furthermore, this study establishes the necessity of the sIL-6R for IL-6 to increase collagen content by cardiac fibroblasts. Accordingly, future investigation is warranted to evaluate IL-6 as a potential therapeutic target.

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**Disclosures**

None.

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