Load-Dependent and -Independent Regulation of Proinflammatory Cytokine and Cytokine Receptor Gene Expression in the Adult Mammalian Heart

Georg Baumgarten, MD; Pascal Knuefermann, MD; Dinesh Kalra, MD; Feng Gao, MD; George E. Taffet, MD; Lloyd Michael, PhD; Perry J. Blackshear, MD, PhD; Ester Carballo, MD, PhD; Natarajan Sivasubramanian, PhD; Douglas L. Mann, MD

Background—Although previous studies have examined the effects of acute hemodynamic pressure overload on proinflammatory cytokine gene expression, the effects of sustained hemodynamic overloading have not been examined.

Methods and Results—Sustained hemodynamic pressure overloading was produced in mice by transverse constriction of the aorta. Proinflammatory cytokine and cytokine receptor gene expression were determined by ribonuclease protection assays (RPA) at 6 hours and at 3, 7, 14 and 35 days after banding. M-mode echocardiography was used to assess left ventricular structure and function at identical time points. RPA showed that tumor necrosis factor (TNF), interleukin (IL)-1β, and IL-6 mRNA levels were maximal at 6 hours and returned to baseline levels within 72 hours. There was a significant increase in IL-1RII and IL-6Rα receptor mRNA levels after overloading but no significant increase in TNFR1, TNFR2, IL-1RI, or gp130 mRNA levels. The transient increase in expression of proinflammatory cytokine gene expression was not explained by changes in left ventricular loading conditions, left ventricular wall stress, desensitization of proinflammatory genes, or decreased nuclear factor-κB activation. It is interesting that transverse constriction of the aorta provoked an increase in the expression of tristetraprolin, a homeostatic zinc finger protein that is known to destabilize TNF mRNA.

Conclusion—Sustained hemodynamic overloading provokes a transient increase in proinflammatory cytokine and cytokine receptor gene expression; however, the decrease in proinflammatory cytokine gene expression occurred in the absence of changes in loading conditions, suggesting that the expression of proinflammatory cytokines in the heart is regulated, at least in part, by load-dependent and load-independent mechanisms. (Circulation. 2002;105:2192-2197.)

Key Words: hypotrophy ■ gene expression ■ growth factors ■ cytokines ■ remodeling

Recent studies from this and other laboratories have shown that proinflammatory cytokines, including tumor necrosis factor (TNF), interleukin (IL)-1β, and IL-6 are expressed within the heart in response to either mechanical overload or ischemic injury (reviewed in Mann1). Although the precise role that these “stress-activated” cytokines play in the myocardium is not known, recent evidence suggests that loss of TNF- or gp130 receptor–mediated signaling is associated with accelerated cardiac myocyte apoptosis and augmented tissue injury after acute cardiac stress, suggesting that cytokine signaling may confer beneficial cytoprotective responses within the myocardium.2,3 Sustained overexpression of intramyocardial cytokines, however, is sufficient to provoke overt cardiac decompensation through increased myocyte loss, decreased myocyte contractility, and increased degradation and/or fibrosis of the extracellular matrix.4,5

Given that cytokine signaling may provoke both beneficial and detrimental effects in the heart, it is essential to understand how these molecules are regulated in response to acute environmental injury. Although several studies have examined the mechanisms that govern the expression of cytokines in the heart in response to acute cardiac injury, virtually nothing is known with regard to the mechanisms that are responsible for governing the sustained expression of proinflammatory cytokines in response to ongoing cardiac injury. Accordingly, to begin to address these issues, we have examined the effects of sustained hemodynamic overloading on proinflammatory cytokine and cytokine receptor gene
expression in relation to changes in left ventricle (LV) structure and function.

Methods

Hemodynamic Overload–Induced Cytokine and Cytokine Receptor Gene Expression

Murine Model of Hemodynamic Overloading

Hemodynamic pressure overloading was achieved by transverse aortic constriction (TAC), as described.6 Experiments were performed in C57×ICR hybrid mice (C57BL6, Jackson Labs, Maine; ICR, Herlan, Tex), of either sex, maintained on a diet of standard mouse chow and water (see Data Supplement). Additional studies were also performed on mice that were doubly deficient in the type 1 TNF receptor (TNFR1; p55) and the type 2 TNF receptor (TNFR2; p75).2

Cytokine and Cytokine Receptor Gene Expression

The time course of cytokine and cytokine receptor gene expression was determined in sham-operated and banded animals at 6 hours and at 1, 3, 7, and 35 days after TAC. The level of expression of TNF, IL-1β, IL-6 mRNA, and their respective cognate receptors TNFR1, TNFR2, IL-1RI, IL-RII, IL-6Rα, and gp130 mRNA were determined using a custom-designed multiprobe riboquant ribonuclease protection assay (Pharmingen), as described (see Data Supplement for details).7

Cytokine Protein Expression

To determine the effects of TAC on proinflammatory cytokine protein production, we examined TNF, IL-1β, and IL-6 protein levels in sham-operated and banded animals at 6 and 72 hours. Protein levels were measured by ELISA (R&D Systems), as described.5 Data were expressed as picograms per milligram of protein.

Cellular Source for Proinflammatory Cytokine Gene Expression

To determine the cellular source for proinflammatory cytokine gene expression after TAC, in situ hybridization was performed according to the method of Yang et al.8 with minor modifications. The relevant cDNAs for TNF (1 to 708), IL-1β (172 to 565), and IL-6 (27 to 429) were obtained by reverse transcription–polymerase chain reaction cloning. Myocardial sections from banded and sham-operated hearts (6 hours) were fixed, sectioned, and processed using tyramide signal amplification and avidin alkaline phosphatase conjugation, as described (see Data Supplement).

Effect of Hemodynamic Overloading on LV Structure and Function

Two-dimensional targeted M-mode echocardiography and Doppler echocardiography were used to measure LV structure and hemodynamic parameters after hemodynamic overloading, as described.4 Images were obtained at days 1, 3, 7, 14, and 35 in the banded and sham-operated animals (see Data Supplement).

Mechanisms for Proinflammatory Cytokine and Cytokine Receptor Gene Expression After Hemodynamic Overloading

Assessment of LV Loading Conditions

To determine whether changes in cytokine gene expression were related to changes in LV loading conditions, we assessed peak flow velocity in the left carotid artery, distal to the aortic constriction, and the fractional shortening/velocity ratio, a noninvasive method for determining aortic constriction.10 A 20-MHz pulsed Doppler probe coupled to a fast-Fourier transform spectrum analyzer (model SP-25A, Medasonics) was used to record flow velocity in the right and left carotid arteries on days 1 and 3 after transverse aortic constriction, as described (see Data Supplement).11

Provocation With Lipopolysaccharide

To determine whether changes in cytokine gene expression after TAC were related to changes in responsiveness to external stimuli, we treated banded (day 3) and sham-operated animals (day 3) with lipopolysaccharide (LPS). LPS (10 mg/kg dissolved in 1000 mL of PBS, Escherichia coli, ST 206603, Sigma-Aldrich Inc) was administered as a single intraperitoneal bolus, and the hearts were rapidly excised under deep anesthesia 6 hours after LPS stimulation. Myocardial cytokine mRNA expression was determined by ribonuclease protection assay, as described above.

Nuclear Factor-κB Activation

Insofar as previous studies have shown that the nuclear factor-κB (NF-κB) is an important regulator of TNF, IL-1β, and IL-6 gene expression, we examined NF-κB activation at baseline and at 6 hours and 3 days after the onset of hemodynamic overloading by performing electrophoretic mobility shift assays in banded and naive hearts (see Data Supplement).

Tristetraprolin Expression

Recent studies have shown that tristetraprolin (TTP [Nup475, TIS 11, GOS 24]), a zinc finger protein that binds to mRNA species with adenylate and uridylate-rich elements in the 3’-untranslated region, is responsible for destabilizing TNF mRNA in different cell types.12 To determine whether TAC was a sufficient stimulus to increase TTP expression, we examined TTP mRNA levels (RPA) at 6, 24, and 72 hours, as well as TTP protein levels at 6 and 72 hours (Western blotting) in the banded and sham-operated animals (see Data Supplement).

Statistics

Data are presented as mean±SEM. One-way ANOVA was used to test for differences in cytokine, cytokine receptor, and TTP mRNA levels and for differences in LV structure and function. When appropriate, post-hoc multiple comparison testing was performed to test for differences between control (Dunnett’s test) and experimental groups (Newman-Keuls test). Nonpaired t tests were used to test for differences in the degree of aortic constriction, the ratio of LV wall thickness to LV radius (h/R ratio), and TTP protein levels. Significant differences were said to exist at P<0.05.

Results

Hemodynamic Overload–Induced Cytokine and Cytokine Receptor Gene Expression

Figure 1 shows that relative to sham-operated controls, cytokine gene expression was maximal 6 hours after TAC, decreased significantly by 24 hours, and had returned to baseline levels by 72 hours (P<0.01, ANOVA). Figure 1 further shows that relative to sham-operated controls, there was a significant increase in IL-RII mRNA and IL6Rα mRNA levels after TAC, whereas there was no significant (P>0.05, ANOVA) increase in the mRNA levels for TNFR1, TNFR2, IL-RI, or gp130. The Table shows that there was a significant (P<0.03) increase in TNF protein at 6 hours after hemodynamic overloading relative to that of the sham-operated animals, whereas the changes in IL1-β and IL-6 protein were not significantly different from that of control and thus did not increase in parallel with the changes in IL1-β and IL-6 mRNA expression. TNF, IL-1β, and IL-6 protein levels at 72 hours were similar in the sham-operated and banded animals.

To determine the relative contribution of TNF signaling to TAC-induced expression of IL-1β and IL-6, the above experiments were repeated in TNFR1/TNFR2-deficient mice.2 Figure 2 shows that IL-1β mRNA levels were signif-
Significantly (P<0.05) elevated in the TNFR1/TNFR2 knockout animals, whereas IL-6 mRNA levels were not significantly different relative to those of sham-operated animals. It is interesting that IL-1β and IL-6 mRNA levels were significantly less than those observed in banded mice, with intact TNF signaling depicted in Figure 1 (P<0.09 and P<0.001, respectively).

Cellular Source for Proinflammatory Cytokine Gene Expression

Figure 3 summarizes the results of the in situ hybridization studies using antisense probes for TNF, IL-1β, and IL-6. As shown, there was cytoplasmic labeling of endothelial cells in blood vessels with the TNF antisense probe (Figure 3A), and cytoplasmic labeling of the cardiac myocytes (Figure 3D).

Effect of Hemodynamic Overloading on Proinflammatory Cytokine Levels

<table>
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<th>Sham at 6 h</th>
<th>Band at 6 h</th>
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<td>(n=6)</td>
<td>(n=3)</td>
<td>(n=6)</td>
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<td>TNF, pg/mg</td>
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<td>0.6±0.2*</td>
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<tr>
<td>IL-1β, pg/mg</td>
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<td>5.59±0.6</td>
<td>4.4±1.0</td>
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<tr>
<td>IL-6, pg/mg</td>
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<td>12.5±1.8</td>
<td>4.3±1.8</td>
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</tbody>
</table>

Data are mean±SEM. Protein levels for TNF, IL-1β, and IL-6 were determined in whole heart extracts from hemodynamically overloaded (banded) and sham-operated animals at 6 and 72 hours after hemodynamic overloading (*P<0.03 compared with sham-operated animals).

However, there was no labeling of the cardiac myocytes (Figure 3G) or endothelial cells with the sense TNF probe. Similarly, Figure 3B and 3E showed that there was labeling of endothelial cells within blood vessels (Figure 3B), and labeling of cardiac myocytes (3E) using the IL-1β antisense probes. Figure 3C and 3F showed that smooth muscle and endothelial cells in blood vessels (Figure 3C) and cardiac myocytes (Figure 3F) were labeled with the IL-6 antisense probes. Figure 3H and 3I show, respectively, that the sense probes for IL-1β and IL-6 did not hybridize to any cell types in the banded hearts. Similar findings were obtained in 2 additional banded hearts.
Effect of Hemodynamic Overloading on LV Structure and Function

TAC resulted in a significant (P < 0.05) increase in LV wall thickness by days 7 and 35 (Figure 4A). In addition, there was a small but significant (P < 0.05) increase in LV end-diastolic dimension at 35 days (Figure 4B). There was no significant change (P > 0.05), however, in LV fractional shortening compared with that of sham-operated animals (Figure 4C).

Mechanisms for Proinflammatory Cytokine and Cytokine Receptor Gene Regulation After Hemodynamic Overloading

Assessment of LV Loading Conditions

Figure 5A shows that the ratio of peak aortic blood flow velocity in the left carotid artery/right carotid artery was ≈5.4 ± 0.8 on day 1 and 9.1 ± 2.4 on day 3, suggesting that the degree of aortic constriction did not decrease between days 1 and 3. Figure 5B shows that the fractional shortening/velocity ratio (an indirect measure of the degree of aortic constriction10) was not significantly (P = 0.28) different between days 1 and 3 in the banded animals, again suggesting there was no change in the degree of aortic constriction between days 1 and 3. Figure 5C, shows that there was no significant difference (P = 0.11) in the h/R ratio in the banded animals, suggesting that LV wall stress did not change between days 1 and 3 after hemodynamic overloading.

Provocation With LPS

To determine whether the decrease in cytokine gene expression was secondary to decreased responsiveness to external stimuli, we treated banded and sham-operated animals with LPS on day 3, when cytokine gene expression had returned to baseline levels. Figure 6 shows that LPS administration resulted in a significant increase in TNF (P < 0.05), IL-1β (P < 0.05), and IL-6 gene expression (P < 0.05) relative to levels in LPS-treated sham-operated animals, suggesting that cytokine gene expression was hyperresponsive to external stimuli in the banded hearts.

NF-κB Activation

There was a persistent increase in NF-κB–DNA-binding complexes detectable at 6 and 72 hours after aortic banding, whereas these complexes were not detected in the hearts of naive animals (see Data Supplement).

TTP Expression

Insofar as the decrease in proinflammatory cytokine gene expression did not appear to be secondary to decreased

Figure 4. Effect of hemodynamic overloading on LV structure and function. A 2D directed M-mode echocardiogram was used to assess changes in LV posterior wall thickness (A), LV end-diastolic dimension (EDD) (B), and the percent LV fractional shortening (FS) (C) on days 1, 3, 7, 14, and 35 after aortic banding (n = 4 animals/group per time point). Data are expressed as the ratio of each parameter in banded/sham-operated animals at the corresponding time points. *P < 0.05 vs control values.

Figure 5. Assessment of hemodynamic overloading. A, Doppler echocardiography was used to assess the ratio of peak flow velocity in the right carotid artery (RCA) to left carotid artery (LCA) on days 1 and 3 (n = 4 animals/time point) in banded animals (Figure 4A) as a measure of the degree of aortic constriction. B, The fractional shortening velocity ratio (an indirect measure of the fractional shortening velocity ratio) was determined using the ratio of LV fractional shortening (% FS) to peak right carotid artery flow velocity on days 1 and 3 (n = 5 animals/time point) in the banded animals (Figure 4B). C, To determine whether there were changes in LV wall stress as a measure of time, we measured the h/R ratio on days 1 and 3 in the sham-operated and banded animals (n = 4 animals/time point).

Figure 6. LPS provocation in banded and sham-operated animals. Banded (day 3) and sham-operated animals (day 3) were treated with LPS. LPS was administered as a single intraperitoneal bolus, and the hearts rapidly excised under deep anesthesia 6 hours after LPS stimulation. Myocardial cytokine mRNA expression was determined by ribonuclease protection assays (A), and the results of group data (n = 5 animals/group) were expressed as the ratio of the mRNA level in banded/sham animals 6 hours after LPS provocation (B).
NF-κB activation, we analyzed gene expression for tristetraprolin. As shown in Figure 7A, there was a significant 5-fold increase in TTP mRNA levels at 6 hours, followed by a return in TTP levels toward baseline values by 1 and 3 days after aortic banding (P<0.001). Western blot analysis showed that there was a significant (P=0.015) increase in TTP protein levels at 3 days in the banded animals, whereas there was no change in TTP protein levels in the sham-operated animals (Figure 7B). Moreover, when we determined the relative ratio of TTP/TNF mRNA at 6 hours, followed by a return in TTP protein levels in the sham-operated animals (n=4 animals/group) by Western blot analysis. C, The ratio of the level of TNF mRNA to TTP mRNA was examined at 6 hours and at days 1 and 3 in banded animals. *P<0.001 vs values on days 1 and 3.

Discussion
There are 2 important findings in this study. First, hemodynamic pressure overloading provoked a robust, albeit transient, increase in proinflammatory cytokine and cytokine receptor gene expression in the adult mammalian heart. In this model of compensated hemodynamic overloading, the expression of TNF, IL-1β, and IL-6 mRNA was maximal at 6 hours and returned to baseline levels within 72 hours (Figure 1). Similarly TNF protein levels were significantly elevated 6 hours after hemodynamic overloading and returned to baseline levels within 72 hours. There was also a significant increase in IL-1RII and IL-6Rα receptor mRNA levels after overloading, whereas there was no significant increase in mRNA levels for TNFR1, TNFR2, IL-1RI, or gp130 mRNA (Figure 1). Consistent with previous reports, a variety of cells resident in the myocardium—including cardiac myocytes, endothelial cells, and smooth muscle cells—expressed proinflammatory cytokines after aortic banding (Figure 3). Furthermore, this study suggests that in the redundant cascade of proinflammatory cytokines, TNF plays an important but not obligatory role in the hemodynamic overload–induced expression of IL-1β and IL-6. That is, the increase in expression of IL-1β and IL-6 mRNA was significantly less in the banded TNFR1/TNFR2-deficient mice compared with banded wild-type mice. It is interesting that the transient increase in the expression of proinflammatory cytokines did not have obvious deleterious effects on LV function when assessed from day 1 to 35 after hemodynamic overloading; however, we cannot exclude the possibility that there may have been a decrease in LV function at earlier time points, when proinflammatory cytokine expression was highest.

The second, and perhaps more interesting, finding in this study was that the increased levels of proinflammatory cytokine and cytokine receptor mRNA returned to baseline values in the absence of measurable changes in LV loading conditions and/or LV wall stress (Figure 5). This observation suggested the interesting possibility that proinflammatory cytokine expression is modulated through a load-independent mechanism(s). The observed decrease in proinflammatory cytokine and cytokine receptor gene expression was not secondary to decreased activation of NF-κB, an important transcription factor for TNF, IL-1β, and IL-6,14 because we observed the presence of NF-κB–DNA-binding complexes in nuclear extracts from hearts that had been subjected to hemodynamic overloading for either 6 or 72 hours (Data Supplement). The decrease in proinflammatory cytokine gene expression did not appear to be secondary to desensitization of these genes, insofar as there was a significant hyperresponsive increase in proinflammatory cytokine gene expression in the LPS-treated banded animals relative to LPS-treated sham-operated animals (Figure 6). Although the mechanism for the finding is not known, it may relate to alterations in transcriptional and/or translational control of proinflammatory cytokine gene expression in the banded animals. Indeed, the observation that NF-κB activation was persistent in the banded animals 72 hours after hemodynamic overloading (Data Supplement) argues for alterations in transcriptional control. Finally, noting previous reports that suggested that a zinc finger protein termed TTP (Nup 475, TIS 11, GOS 24) was capable of deadenylating and destabilizing TNF mRNA,12 we asked whether hemodynamic overloading was a sufficient stimulus to provoke increased TTP mRNA and protein expression. In the present study, we show that TTP mRNA levels increased within 6 hours after the onset of hemodynamic overloading and decreased in concert with TNF mRNA levels at 24 and 72 hours (Figure 7A), suggesting that TNF and TTP were coordinately regulated in the heart after hemodynamic overloading. Importantly, the level of expression of TTP protein was significantly increased 72 hours after the onset of hemodynamic overloading (Figure 7B), thus providing a potential mechanistic basis for the decrease in TNF mRNA levels observed at this time point. Taken together, these studies suggest that the expression of TTP may represent a novel mechanism for modulating the
level of expression of proinflammatory cytokines in the heart after acute cardiac injury.

**Conclusion**

Given that sustained expression of proinflammatory cytokines can have deleterious effects in the heart, it is perhaps not surprising that nature has evolved a number of homeostatic mechanisms for delimiting the sustained expression of these molecules following environmental injury. What was surprising, however, was that the decrease in the expression of proinflammatory cytokines and cytokine receptor genes occurred in the absence of an obvious decrease in LV wall stress, which has traditionally been regarded as one of the major mechanisms for modulating gene expression after hemodynamic overloading. Although this study was not designed to delineate the complete ensemble of mechanisms that are responsible for downmodulating proinflammatory cytokines after cardiac injury, the results of this study do suggest that TTP may play an important role in modulating the expression of proinflammatory cytokines in the heart after acute environmental injury. Thus, these studies raise the interesting possibility that gain or loss of function of myocardial homeostatic regulatory proteins may determine whether the myocardial response to environmental injury is adaptive or maladaptive.

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**References**

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