Inhibition of Tumor Necrosis Factor-α Improves Postischemic Recovery of Hypertrophied Hearts

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Background—Tumor necrosis factor (TNF)-α has been implicated in the pathogenesis of heart failure and ischemia-reperfusion injury. Effects of TNF-α are initiated by membrane receptors coupled to sphingomyelinase signaling and include altered metabolism and calcium cycling, contractile dysfunction, and cell death. We postulate that pressure-overload hypertrophy results in increased myocardial TNF-α expression and that it contributes to decreased contractility in hypertrophied infant hearts subjected to ischemia-reperfusion.

Methods and Results—Neonatal rabbits underwent aortic banding to induce LV hypertrophy. Myocardial TNF-α protein expression increased progressively with LV hypertrophy. Serum TNF-α was detected only after the onset of heart failure. Before onset of ventricular dilatation and heart failure (determined by serial echocardiograms), hearts from aortic banded and age-matched control rabbits were perfused in the Langendorff mode and subjected to 45 minutes of ischemia and 30 minutes of reperfusion. Postischemic recovery was impaired in hypertrophied hearts, but addition of neutralizing anti-rabbit TNF-α antibody to cardioplegia and perfusate solutions restored postischemic function. This effect was mimicked by treatment with the ceramidase inhibitor N-oleoyl ethanolamine. TNF-α inhibition also was associated with faster postischemic recovery of phosphocreatine, ATP, and pH as assessed by 31P nuclear magnetic resonance spectroscopy. Intracellular calcium handling, measured by Rhod 2 spectrofluorometry, demonstrated lower diastolic calcium levels and higher systolic calcium transients in anti-TNF-α treated hearts.

Conclusions—TNF-α is expressed in myocardium during compensated pressure-overload hypertrophy and contributes to postischemic myocardial dysfunction. Inhibition of TNF-α signaling significantly improves postischemic contractile function, myocardial energetics, and intracellular calcium handling. (Circulation. 2001;104[suppl I]:I-350-I-355.)

Key Words: calcium ■ ischemia ■ hypertrophy ■ heart failure ■ reperfusion

Both clinical and experimental studies have shown that ventricular hypertrophy is associated with contractile dysfunction, impaired tolerance to ischemia, and increased risk during cardiac surgery. Molecular and biochemical modifications known to occur during pressure overload hypertrophy include alterations to contractile protein isoforms, increased dependence on glycolysis for energy metabolism, and changes in intracellular calcium regulation and excitation-contraction coupling.1 During myocardial ischemia, hypertrophied hearts exhibit accelerated loss of high-energy phosphates, greater accumulation of tissue lactate, earlier onset of ischemic contracture, and accelerated calcium overload during early reperfusion.2

Proinflammatory cytokine tumor necrosis factor (TNF)-α is released from circulating or resident immune cells in addition to several other cell types. TNF-α is synthesized as a larger precursor, cleaved to the active form at the cell membrane, and released to act in an endocrine, autocrine, or paracrine fashion. Typical stimuli for TNF production include bacterial products such as lipopolysaccharide and reactive oxygen species. TNF-α also has been implicated in the pathogenesis of heart failure, ischemia-reperfusion injury, and cardiac dysfunction seen during sepsis, shock, or myocarditis and after heart transplantation.3–6 Elevated levels of TNF-α have been detected in patients with and in experimental models of LV pressure overload.7,8 Effects of TNF-α are initiated by membrane receptors coupled to sphingomyelinase signaling; they include altered glycolytic and mitochondrial metabolism; stimulation of other signaling pathways, such as protein kinase C; induction of nitric oxide synthesis; abnormal calcium cycling; contractile dysfunction; and cell death.9–12

We hypothesized that in an infant rabbit model of pressure-overload hypertrophy, analogous to the clinical situation seen in patients with aortic arch obstruction, TNF-α is expressed in the heart before the onset of LV dilatation and heart failure and contributes to postischemic contractile dysfunction. Strategies that inhibit biological actions of TNF-α on the heart should improve tolerance to myocardial ischemia in hypertrophied hearts.
Methods

Animal Model
All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (publication No. 86–23, revised 1996). Protocol was reviewed and approved by the Animal Care Committee at Children’s Hospital Boston.

New Zealand White rabbits underwent banding of the descending thoracic aorta at 10 days of age. The band was placed snugly around the aorta, and animals developed progressive stenosis as they grew. Using serial echocardiography, we previously determined the time course of development of LV hypertrophy and its progression to failure. Six to seven weeks after banding, LV mass/volume ratio increased by >30%, which indicates the maximal degree of compensated hypertrophy before onset of ventricular dilatation and failure. In the present study, we confirmed development of LV hypertrophy echocardiographically, but hearts were excised after specific time intervals and degree of hypertrophy quantified by use of the LV wet weight/body weight ratio. All ischemia-reperfusion experiments were performed 6 weeks after banding. Age-matched nonhypertrophied hearts were used in control experiments.

Animals were euthanized by intravenous injection of ketamine (100 mg/kg), xylazine (2.5 mg/kg), and heparin (1000 U), and hearts were excised and perfused in the Langendorff mode with modified Krebs-Henseleit buffer exactly as previously described. A fluid-filled balloon was used to measure LV function and inflated to achieve a diastolic pressure of 7 mm Hg. The same balloon volume was used for postischemic function measurements. Temperature was maintained at 37°C throughout the experiment. In a separate set of experiments, hearts were excised at 2, 4, 6, and 8 weeks after banding and studied for myocardial expression of TNF-α (see below).

Experimental Protocol
After 30 minutes of stabilization, hearts were subjected to 45 minutes of ischemia at 37°C followed by 30 minutes of reperfusion. Cardiac arrest was achieved by infusing 50 mL of Krebs-Henseleit buffer (also at 37°C) containing 22.5 mmol/L of KCl. Role of TNF signaling was evaluated by use of 2 different methods, and the animals were randomized (nonblinded) into respective groups. Neutralizing goat anti-rabbit TNF-α antibody (3 μg/mL, no azide, low endotoxin; PharMingen Int'l) was added to perfusate for 10 minutes before and 5 minutes after ischemia and to the cardioplegic solution. This antibody was selected for its ability to neutralize biological activity of rabbit TNF-α. The 50% neutralization dose for this lot was estimated to be 1 μg/mL in, so that quantitative neutralization of tissue TNF-α could be expected. In untreated hearts, 3 μg/mL of nonspecific goat anti-mouse IgG was added. In separate groups, hearts were perfused with ceramide inhibitor N-oleoyl ethanolamine (NOE 5 μmol/L dissolved in DMSO) for 15 minutes before ischemia; NOE 5 μmol/L also was added to the cardioplegic solution.

Western Immunoblotting
Protein extracts were prepared, and samples of 30 μg total protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with polyclonal purified azide-containing goat anti-rabbit TNF-α antibody (PharMingen) followed by incubation with horseradish peroxidase–conjugated anti-goat IgG secondary antibody and detection by use of enhanced chemiluminescence. Heparinized blood samples were collected from the same animals and centrifuged and protein content was determined, undiluted serum samples that contained 40 μg of protein were separated in SDS gels and processed as described above. Laser densitometry was used to quantify intensity of the bands, and data are expressed as percentage density at time point “failure” (8 weeks).

Calcium Measurements
In separate experiments, hearts were loaded with the Ca2+-sensitive dye Rhod 2-AM after 15 minutes of posts ischemic reperfusion by perfusion with the cell-permeable acetoxy methyl ester (Rhod 2-AM), and Ca2+ measurements were performed after a 15-minute washout period. These methods have been previously described and validated in detail. At the end of each experiment, 2,′,2′-dithiobipyridine (100 μmol/L) was infused over a period of 2 minutes followed by bolus injection of calcium ionophore A23187 (calciumycin) in 10% calcium solution to measure maximum calcium-induced fluorescence (Fmax).

Systolic and diastolic calcium concentration were calculated by use of the following equation:

\[ [Ca^{2+}] = K_d \times (F_0 - F)/(A/A_{max})(F_{max} - F) - (F - F_0) \]

where \( K_d \) is the dissociation constant for Rhod 2 with calcium, \( F \) is fluorescence at a specific time point, \( F_0 \) is tissue autofluorescence, and \( A \) and \( A_{max} \) are tissue light absorbance at the respective time point and at the end of the experiment, respectively.

31P Nuclear Magnetic Resonance Spectroscopy
In other experiments, hearts were perfused with phosphate-free modified KH buffer by use of a customized perfusion system. Isolated heart in its perfusion chamber was positioned within a 20-mm solenoid radio frequency coil. Nuclear magnetic resonance (NMR) spectra were acquired in an 8.45-T vertical bore Bruker spectrometer (Bruker Instruments). Spectra were obtained by signal-averaging 120 scans with a 2-s delay, resulting in a time resolution of 4.5 minutes. Phosphocreatine (PCr) and β-ATP peak areas were quantified by integration of respective peak after baseline correction with software provided by Bruker. Data are expressed as percentage of preschematic values. Intracellular pH was calculated from shift of inorganic phosphate (P) peak by use of the following equation:

\[ pH = 6.9 + \log(\frac{x-3.28}{5.7-x}) \]

where \( x \) is the position of the P, peak with respect to the PCr peak in parts per million.

Statistical Analysis
Data are expressed as mean±SE, and statistical analysis was performed by use of the SPSS software package (version 9.0, SPSS Inc). Differences between groups were tested for significance by 1-way ANOVA with Tukey’s post hoc test for multiple comparisons. If normal distribution and equal variance testing was passed, Student’s t test was used to compare individual data sets. A 2-tailed \( p \) value of <0.05 was considered statistically significant throughout.

Results
TNF-α Expression in LV Hypertrophy
All animals included in the present study had developed significant LV hypertrophy at 6 weeks after banding, as demonstrated by LV wet weight/body weight ratio (Figure 1). Echocardiograms at this time demonstrated compensated LV hypertrophy (increased LV mass/volume ratio [30% increase] and normal shortening fraction [not shown]). Clinical signs of decompensation, such as pleural or pericardial effusions, ascites, tachypnea, or cyanosis, were not detected until 1 to 2 weeks later (ie, between 7 to 8 weeks after banding). Within 2 weeks after banding, low levels of TNF-α protein expression in LV protein extracts were detected in some but not all hearts (Figure 2). However, at 4 weeks after banding, TNF-α protein consistently was detected; it was further increased at 6 weeks. Maximum TNF-α protein levels were detected after LV decompensation at 8 weeks. Serum TNF-α did not increase during early stages of hypertrophy but was elevated after onset of LV failure (Figure 2).
**Preischemic LV Function**

No significant differences were seen in preischemic LV function between hypertrophied and nonhypertrophied hearts. Baseline LV developed pressure at a diastolic pressure of 7 mm Hg was 102±3 mm Hg in hypertrophied hearts and 110±4 mm Hg in nonhypertrophied hearts (P=0.3). Coronary flow was 45±4 mL/min in nonhypertrophied hearts and 51±3 mL/min in hypertrophied hearts (P=0.04), but myocardial oxygen consumption (MV\textsubscript{O}\textsubscript{2}) was not different (0.87±0.07 versus 0.85±0.06 mL·min\textsuperscript{-1}·g\textsuperscript{-1} dry wt; P=0.6).

**Postischemic LV Function**

In nonhypertrophied hearts, postischemic developed pressure recovered to 92±3% of preischemic level, diastolic pressure was 9.3±1.3 mm Hg (P=0.003 versus preischemia), coronary flow was 42±3 mL/min, and MV\textsubscript{O}\textsubscript{2} was 0.77±0.06 mL·min\textsuperscript{-1}·g\textsuperscript{-1}. Treatment with anti-TNF-\textalpha antibody had no effect on postischemic recovery of nonhypertrophied hearts (Figure 3).

In hypertrophied hearts treated with mouse anti-rabbit control antibody, LV developed pressure was significantly lower and diastolic pressure significantly higher compared with nonhypertrophied hearts (Figure 3). However, treatment with anti-rabbit TNF-\textalpha antibody resulted in significant improvement of postischemic developed and diastolic pressures without change in coronary flow (48±5 mL/min in hypertrophy plus control antibody versus 48±3 mL/min in hypertrophy plus anti-TNF-\textalpha; P=0.6). Despite the increase in contractility, MV\textsubscript{O}\textsubscript{2} was lower in anti-TNF-\textalpha treated hearts (0.79±0.07 versus 0.88±0.04 mL·min\textsuperscript{-1}·g\textsuperscript{-1}; P=0.04), consistent with improved contractile efficiency. The beneficial effect of anti-TNF-\textalpha antibody on postischemic LV function was mimicked by treatment with ceramidase inhibitor NOE (Figure 3).

**Intracellular Calcium Handling**

Diastolic [Ca\textsuperscript{2+}], was significantly higher in postischemic hypertrophied hearts than in nonhypertrophied hearts. In addition, amplitude of calcium transients was depressed. However, treatment with anti-TNF-\textalpha antibody was associated with significantly lower diastolic [Ca\textsuperscript{2+}], compared with sham antibody-treated hypertrophied hearts. Calcium transient amplitude was also higher in hypertrophied hearts treated with anti-TNF-\textalpha. Treatment with NOE also led to a significant reduction of postischemic diastolic [Ca\textsuperscript{2+}], associated with an increase in [Ca\textsuperscript{2+}], transient amplitude that did not reach statistical significance (Figure 4).

**High-Energy Phosphates and pH**

No significant differences between nonhypertrophied hearts, hypertrophied hearts, and hypertrophied hearts treated with anti-TNF-\textalpha antibody regarding the time course of PCr loss during ischemia. However, PCr recovered faster during reperfusion in anti-TNF-\textalpha-treated hearts than in sham-antibody–
treated hypertrophied hearts (Figure 5). ATP declined more rapidly during ischemia in hypertrophied hearts and recovered significantly faster after treatment in those treated with anti-TNF-α antibody. In contrast, at the end of the 30-minute reperfusion period, ATP in control-antibody treated hypertrophied hearts still had not recovered to preischemic levels. During ischemia, intracellular pH dropped faster in hypertrophied hearts but recovered more rapidly after treatment with anti-TNF-α antibody.

**Discussion**

Results of our present study demonstrate that TNF-α is expressed in the heart during compensated pressure overload hypertrophy and contributes to reduced myocardial function during ischemia-reperfusion. In a surgically relevant model of 45-minute global ischemia with cardioplegic arrest and 30 minutes of reperfusion, Nonhypertrophied hearts were treated with control antibody (control+sham AB) or anti-rabbit TNF-α antibody (Hyper+antiTNF-α). Hypertrophied hearts were treated with control antibody (Hyper+sham AB), anti-rabbit TNF-α antibody (Hyper+antiTNF-α), or NOE (Hyper+NOE). Data are mean±SE, n=6 per group.

![Figure 3. LV developed (A) and diastolic (B) pressure after 45 minutes of ischemia with cardioplegic arrest and 30 minutes of reperfusion. Nonhypertrophied hearts were treated with control antibody (control+sham AB) or anti-rabbit TNF-α antibody (Hyper+antiTNF-α). Hypertrophied hearts were treated with control antibody (Hyper+sham AB), anti-rabbit TNF-α antibody (Hyper+antiTNF-α), or NOE (Hyper+NOE). Data are mean±SE, n=6 per group.](image)

![Figure 4. Measurements of myocardial calcium transients. A, Representative (Ca^{2+}) transients in a nonischemic hypertrophied heart (Control), postischemic hypertrophied heart (Postischemia), and postischemic anti-TNF-α-treated heart (Anti-TNF-α). B, Diastolic intracellular calcium (Ca^{2+}) and amplitude of the calcium transient (C) measured in whole heart after 45 minutes of ischemia and 30 minutes of reperfusion. Nonhypertrophied hearts were treated with control antibody (control+sham AB) or anti-rabbit TNF-α antibody (Hyper+antiTNF-α). Hypertrophied hearts were treated with control antibody (Hyper+sham AB), anti-rabbit TNF-α antibody (Hyper+antiTNF-α), or NOE (Hyper+NOE). Data are mean±SE, n=5 per group.](image)

**TNF-α and Hypertrophy**

Expression of TNF-α mRNA and protein has been demonstrated in cardiomyocytes, which are thought to account for at least half of the amount of TNF-α protein expressed in heart during sepsis. TNF-α is produced by both myocytes and nonmyocytes during the course of heart failure and ischemia-reperfusion injury. Kapadia et al detected TNF-α mRNA and protein synthesis in isolated feline hearts within 30 to 60 minutes after onset of LV pressure overload to a magnitude sufficient to depress cardiomyocyte contractility. TNF-α has been shown to be elevated in patients with LV overload, and, to lesser degree, in volume overload. In addition to its potential cytotoxic effects, TNF-α also can stimulate hypertrophic growth response in cardiac myocytes, which suggests that TNF-α expressed in hearts subjected to pressure overload contributes to development of myocyte hypertrophy. In our chronic model, myocardial TNF-α increased 2 to 4 weeks...
after the aortic band was placed, probably because it takes ≥1 week for animals to have grown sufficiently for aortic constriction to develop and for LV pressure load to increase. TNF-α levels in serum increased significantly only after onset of ventricular dilatation and failure. Whether this was due to increasing release from the heart or release from other tissue beds in response to developing heart failure is uncertain.

**TNF-α and Myocardial Ischemia**

Using a blood-free, crystalloid-perfused model, Gurevitch et al\(^7\) detected increased TNF-α mRNA and TNF-α protein synthesis in addition to TNF-receptor upregulation in myocardium early after coronary artery ligation and concluded that TNF-α is part of the myocardial stress response to injury. We did not specifically study TNF-α expression during ischemia in our model, because the abovementioned groups have demonstrated conclusively that myocardial TNF-α synthesis occurs in response to ischemic stimulus. However, on the basis of our finding that treatment with anti-TNF-α antibody did not improve functional recovery of nonhypertrophied hearts, we conclude that, at least in rabbit heart, myocardial TNF-α synthesis during 45 minutes of ischemia with cardioplegic arrest does not reach levels sufficient to impair postischemic recovery. Nonetheless, it is possible that TNF production during ischemia was sufficient or even accelerated in hypertrophied hearts to contribute in a meaningful fashion to that already present in the myocardium in association with the development of hypertrophy. In either event, we conclude that TNF-α expression during compensated hypertrophy can be, to a large extent, responsible for the increased myocardial dysfunction that occurs in hypertrophied hearts after ischemia-reperfusion.

**TNF-α and Contractility**

TNF-α activates intracellular signaling by means of ≥2 specific receptors (TNF-R1 and TNF-R2). Providing anti-TNF-α antibody was effective because it binds TNF in the extracellular space, thereby preventing receptor binding and interrupting autocrine or paracrine signaling. Potential mechanisms by which TNF-α can impair myocardial function are multiple. TNF-α can rapidly impair calcium handling resulting in negative inotropic effects.\(^{10,20,21}\) TNF-α binding to sarcolemmal TNF receptors triggers breakdown of membrane sphingolipids, and the second messenger sphingosine is then cleaved from sphingomyelin by the enzyme ceramidase. Sphingosine has been shown to directly inhibit calcium release from the sarcoplasmic Ca\(^{2+}\) release channel (ryanodine receptor) independent of activation of other mechanisms. Furthermore, sarcoplasmic reticulum calcium release is closely coupled to sarcolemmal calcium influx through L-type calcium channels. Inhibition of the L-type calcium channel also will result in reduced amplitude of the calcium transient, and both TNF-α and sphingosine reduce L-type channel calcium current in isolated cells.\(^{22}\) In our model, ceramidase inhibition with NOE significantly improved contractility in postischemic hypertrophied hearts in association with increased amplitude of the calcium transient, which supports the idea that TNF receptor signaling is involved. Additional cellular effects of TNF-α signaling include altered substrate metabolism, mitochondrial dysfunction, oxyradical production, production of other proinflammatory mediators and signals (eg, cyclooxygenase products), and induction of nitric oxide synthesis. We observed improved recovery of high-energy phosphates and pH during reperfusion after TNF-α inhibition and speculate that improved energy metabolism in treated hearts led to improved contractile protein function and enhanced calcium removal through sarcoplasm-
mic reticulum calcium ATPase. Alternatively, ischemia-reperfusion is known to be accompanied by cytosolic calcium overload (as well as contractile protein calcium insensitivity), due in part to activation of multiple protein kinases and oxyradical effects on calcium regulatory proteins. Another possible explanation for lower diastolic calcium concentrations in hearts treated with anti-TNF-α strategies is that the primary abnormality was abnormal calcium cycling during reperfusion that was further exacerbated by TNF-α, with ATP depletion occurring secondary to increased calcium overload and futile calcium cycling in untreated hearts.

Although our present study focused on the detrimental effects of TNF-α, evidence also exists in favor of protective effects of TNF-α. For example, recent evidence in a genetically engineered mouse model indicated that TNF-α reduced myocardial infarct size by reducing apoptotic cell death. TFN-α and other proinflammatory cytokines can confer protection in the setting of ischemia-reperfusion by inducing production of antioxidant enzymes and other stress-related proteins. With regard to the present experiments, TNF-α may induce depression of cytosolic calcium levels, which results in negative inotropy in the immediate postischemic period but may, at the same time, serve to limit calcium overload and its consequences, including ATP consumption and apoptotic or necrotic cell death. Similarly, these effects could confer overall protection and improvement in function (particularly in the case of longer periods of ischemia and potential infarction), which would not have been detected by the acute experiments conducted in the present study.

We conclude that TNF-α is expressed in hypertrophied myocardium before onset of ventricular dilatation and heart failure and contributes significantly to increased susceptibility to ischemia observed in hypertrophied hearts. Strategies to neutralize biological activity of TNF-α currently are in development and may prove useful to decrease risk of postischemic contractile dysfunction in patients with LV hypertrophy.

Acknowledgments
The present work was supported in part by NIH grants HL-52589 and HL-66186 (Dr McGowan) and HL-46207 (Dr Nido). Dr Stamm was supported by a grant from the German Research Foundation (STA 497/2-1). Dr Moran was the Tommy Kaplan Fellow in the Department of Cardiology, Children’s Hospital Boston.

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doi: 10.1161/hc37t1.094851

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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