Effect of Tumor Necrosis Factor–Alpha on Endothelial and Inducible Nitric Oxide Synthase Messenger Ribonucleic Acid Expression and Nitric Oxide Synthesis in Ischemic and Nonischemic Isolated Rat Heart

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OBJECTIVES The present study aimed to investigate the influence of endogenous tumor necrosis factor–alpha (TNF-α) that was synthesized during ischemia and exogenous TNF-α on endothelial and inducible nitric oxide synthase (eNOS and iNOS) messenger ribonucleic acid (mRNA) expression and nitric oxide (NO) production in the isolated rat heart.

BACKGROUND Tumor necrosis factor–alpha is recognized as being a proinflammatory cytokine with a significant cardiodepressant effect. One of the proposed mechanisms for TNF-α-induced cardiac contractile dysfunction is increased NO production via iNOS mRNA upregulation, but the role of NO in TNF-α-induced myocardial dysfunction is highly controversial.

METHODS Isolated rat hearts studied by a modified Langendorff model were randomly divided into subgroups to investigate the effect of 1-h global cardioplegic ischemia or the effect of 1-h perfusion with exogenous TNF-α on the expression of eNOS mRNA and iNOS mRNA and on NO production.

RESULTS After 1 h of ischemia, there were significant increases in TNF levels in the effluent (from hearts), and eNOS mRNA expression had declined (from 0.91 ± 0.08 to 0.68 ± 0.19, p < 0.001); but there were no changes in iNOS mRNA expression, and NO was below detectable levels. Perfusion of isolated hearts with TNF-α had a cardiodepressant effect and decreased eNOS mRNA expression to 0.67 ± 0.04 (p < 0.002). Inducible nitric oxide synthase mRNA was unchanged, and NO was below detectable levels.

CONCLUSIONS We believe this is the first study to directly show that TNF-α does not increase NO synthesis and release but does downregulate eNOS mRNA in the ischemic and nonischemic isolated rat heart. (J Am Coll Cardiol 2003;42:1299–305) © 2003 by the American College of Cardiology Foundation

Tumor necrosis factor–alpha (TNF-α) is a trimeric 17-kDa polypeptide produced by monocytes and macrophages, which are known to be proinflammatory cytokines (1,2). Its classic trilogy action on the body is the induction of necrosis, shock, and cachexia. Tumor necrosis factor–alpha has a potent negative inotropic effect (3,4), and the hemodynamic influence of TNF-α is characterized by decreased myocardial contraction and reduced ejection fraction, hypotension, decreased systemic vascular resistance, and biventricular dilatation (5,6). Recent studies have demonstrated that TNF-α is produced during ischemia-reperfusion injury and activates nuclear factor kappa-β, which initiates the cytokine cascade and facilitates the expression of chemokines and adhesion molecules (7,8). Experiments with mice lacking TNF-α have demonstrated an improvement in myocardial ischemia-reperfusion injury (9).

Myocardial cells themselves can produce TNF-α in the isolated perfused heart under endotoxin treatment (10). Our group previously reported that TNF-α is released from the isolated heart undergoing ischemia and that this cytokine production is directly correlated with the degree of myocardial dysfunction (11,12). Moreover, the administration of monoclonal antibodies to TNF-α eliminates this cytokine in effluent and attenuates the postischemic myocardial injury (13).

One of the mechanisms suggested for TNF-α cardiac contractile dysfunction is increased nitric oxide (NO) production by the myocardium, which causes depression of cardiac function. In the heart, NO is synthesized from L-arginine by two types of nitric oxide synthases (NOS): endothelial (eNOS) and inducible (iNOS). A constitutive
isofrom of NOS, eNOS, which is calcium/calmodulin regulated and was originally described in endothelial cells, causes continuous NO production and plays an important role in vascular tone regulation (14). The second type of NOS described in cardiac myocytes is calcium-independent iNOS, which can be activated by cytokines, leading to NO overproduction and causing a negative inotropic effect, probably by activation of the enzyme guanylate cyclase (15,16). Some studies have found that cardiac myocytes express both eNOS and iNOS (16).

Finkel et al. (17) demonstrated that NOS inhibition prevents the myocardial-depressant effect of TNF-α and concluded that the negative inotropic effect is mediated by NO. Wang and Zweier (18) observed increased NO and peroxinitrite release from the isolated rat heart after 30 min of global ischemia. Pretreatment with the NOS inhibitors resulted in a fourfold increase in postischemic functional recovery. The role of NO in TNF-α-induced myocardial dysfunction, however, is highly controversial. Avontuur et al. (19) showed that inhibition of NO synthesis causes myocardial ischemia in endotoxemic rats. Yocoyama et al. (4) reported that increased levels of NO did not mediate the negative inotropic effect of TNF-α-induced myocardial contractile abnormalities. In another study, TNF-α showed no significant effect on NO production in cultured cardiac myocytes (20). Lastly, NOS inhibitors did not prevent a decrease of either intracellular calcium or the amplitude of cell shortening caused by TNF-α in isolated ventricular myocytes (21). Most of these above-cited studies incorporated evidence provided indirectly by NOS inhibitors.

The present study investigated whether endogenous (paracrine) TNF-α that was synthesized during global cardioplegic ischemia and exogenous TNF-α given in perfusion solution have a direct influence on eNOS and iNOS messenger ribonucleic acid (mRNA) expression and NO release in the isolated rat heart.

**METHODS**

Male Wistar rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg). Their hearts were rapidly excised, immersed in cold saline (4°C), mounted on the stainless-steel cannula of a modified Langendorff apparatus, and perfused with Krebs-Henseleit (KH) solution exactly as detailed in four of our previously reported studies (11–13).

Left ventricular (LV) hemodynamic parameters (peak systolic developed pressure) and coronary flow (CF) were continuously measured at 10-min intervals, and the first derivative of the rise in LV pressure (dP/dt max) and time-pressure integral were calculated.

**Ethics.** All animals received humane care as described in “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 (NIH Publication No. 80-23, revised 1985).

**Experimental protocol.** The rats were randomly divided into two subgroups to assess the effect of 1-h global cardioplegic ischemia on TNF-α and NO synthesis and production. Control measurements were recorded after a 15-min period of stabilization (baseline). All hearts were perfused therefor for a 30-min period. Left ventricular hemodynamic parameters were measured at 10-min intervals. Effluent for TNF-α and NO levels was withdrawn at the end of the 30-min period (n = 8). Warm cardioplegia was then administered for 2 min (37°C, perfusion pressure 73 mm Hg, KCl 16 mEq/L in KH solution) followed by a 60-min period of global ischemia at 31°C to the arrested hearts (group A, n = 8).

Group B was similar to group A, but these rats received monoclonal rabbit anti-rat TNF-α antibodies (anti-TNF-α Ab, n = 8). Concentration of anti-TNF-α Ab was selected according to specific activity: approximately 1 μg of these antibodies completely neutralized 25 pg of rat TNF-α. We found that the TNF-α level in effluent was 805 ± 230 pg/ml after a 60-min period of global ischemia. We used 1.5 μg/ml of anti-TNF-α antibodies (total dose 45 μg) for total neutralization of TNF-α in the heart tissue, after which TNF-α was not found in the effluent from the coronary sinus of ischemic hearts.

The first milliliter of perfusion effluent was withdrawn at the end of ischemia to determine TNF-α and NO levels in both groups. Additional hearts were assayed at baseline (n = 7) and immediately at the end of ischemia (n = 7 for each group) to determine LV TNF-α, eNOS, and iNOS mRNA expression.

Two additional subgroups (groups C and D) formed by other rats were used to investigate the direct effect of exogenous TNF-α on nonischemic perfused hearts. Control measurements were recorded after a 15-min period of stabilization. Thereafter, the hearts were perfused for a 60-min period with KH solution to which either saline (group C, n = 10, “controls”) or 16 ng/ml (pathophysiologically relevant concentration) TNF-α (group D, n = 7) were added to KH solution using an infusion pump (0.5 ml/min). The dose of TNF-α was selected according the results of group A’s postischemic TNF-α level measurements. Hemodynamic measurements were recorded every
10 min, and effluent was withdrawn to determine TNF-α and NO levels. Five hearts in each group were removed for LV iNOS and eNOS mRNA assays immediately at the end of the 60-min perfusion period.

**TNF-α determination.** Tumor necrosis factor-α levels were detected in the effluent from the coronary sinus of the isolated rat hearts. Tumor necrosis factor-α activity was measured using the commercially available enzyme-linked immunoassay kit Cytoscreen TM rat kit TNF-α, Immunoassay kit (Biosource, Camarillo, California). The limit of detection was 4 pg/ml.

**NO measurement.** Effluent nitrite (NO3)- and nitrate (NO2)-stable metabolites of NO representing NO production were detected in the heart perfusate samples and cardiac myocyte culture supernatant as described previously (22). The limit of detection was 2 μmol/l.

**Myocardial tissue TNF-α, eNOS, and iNOS mRNA determination.** Immediately after stabilization (baseline) or after 1 h of global cardioplegic ischemia, the LV myocardium was excised and placed in cold Hank’s balanced solution. Total RNA was extracted from myocardial samples using the guanidinium thiocyanate method (23). Ribonucleic acid pellets were maintained at -20°C with 75% ethanol until assay. Dried sediments were dissolved in sterile RNase free water and quantitated spectrophotometrically at λ = 260 nm.

Reverse-transcription polymerase chain reaction (PCR) amplification and quantitative analysis were prepared as previously described (12). The primer sequences, annealing temperature, number of cycles, and PCR product size are listed in Table 1. All TNF-α, eNOS, and iNOS band intensities were normalized by respective for glyceraldehyde-phosphate dehydrogenase values. Each PCR reaction was performed at least twice, and four to five hearts were used for each experimental group.

**Drugs.** Polyclonal rabbit anti-rat TNF-α Ab was purchased from R&D Systems (Minneapolis, Minnesota). Specific activity was as follows: approximately 1 μg of these antibodies completely neutralized 25 pg of rat TNF-α. Recombinant human TNF-α was purchased from Reprogen Ltd. (Rehovot, Israel).

**Statistics.** The results are presented as mean ± SD. All hemodynamic measurements were subjected to two-way analysis of variance with repeated measures. This design includes one between-subject factor (the experimental group) and one within-subject factor (the time of measurement). Whenever a significant time trend was demonstrated, we used contrast analysis to compare each measurement to its successive one (SIMPLE; SPSS Inc., Chicago, Illinois). Only one contrast was used to eliminate the problem of multiple comparisons. Student t tests were used to assess significant differences between relative optical densities of the mRNA bands in groups and between groups. Significance was established at p < 0.05. All statistical analyses were performed by the Statistical Department at our Medical Center.

**RESULTS**

The baseline values (groups A and B) for different LV hemodynamic parameters are given in Table 2. No significant differences were found between the experimental groups. A two-way analysis of variance (comparing group means and group-time interaction) was conducted to reveal possible differences in LV hemodynamic parameters for the experimental groups before cardioplegic ischemia (baseline values, 10, 20, and 30 min perfusion); none of the p values attained statistical significance.

**Table 1. Specification of the Primer Sets Used to Analyze Messenger Ribonucleic Acid Expression**

<table>
<thead>
<tr>
<th>Size</th>
<th>Primer Set</th>
<th>Number of Cycles</th>
<th>Annealing Temperature (°C)</th>
<th>PCR Product (bp)</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>Sense CACGCTTCTTGCTTACTGA</td>
<td>30</td>
<td>57</td>
<td>546</td>
</tr>
<tr>
<td></td>
<td>Antisense GGACTCCGTATGCTCAATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>Sense CCGGAATTCGAATCCGCCGACAAG</td>
<td>45</td>
<td>65</td>
<td>614</td>
</tr>
<tr>
<td></td>
<td>Antisense GCCGGATCCCAGGGGATTGTCCTGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>Sense GTGTTCCACAGGAGATGTTG</td>
<td>35</td>
<td>60</td>
<td>576</td>
</tr>
<tr>
<td></td>
<td>Antisense CTCCGCCCCACTGAGGTCGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense AATGCATCCTGCACCACCAA</td>
<td>30</td>
<td>60</td>
<td>515</td>
</tr>
<tr>
<td></td>
<td>Antisense GTAGGATATGTTGTCAT</td>
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</tr>
</tbody>
</table>

eNOS = endothelial nitric oxide synthase; GAPDH = glyceraldehyde-phosphate dehydrogenase; iNOS = inducible nitric oxide synthase; PCR = polymerase chain reaction; TNF-α = tumor necrosis factor-alpha.

**Table 2. Baseline Measurements**

<table>
<thead>
<tr>
<th>Group</th>
<th>Peak systolic pressure (mm Hg)</th>
<th>dP/dt max (mm Hg/s)</th>
<th>Time-pressure integral (mm Hg x s)</th>
<th>Coronary flow (ml/min)</th>
</tr>
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<tr>
<td>A</td>
<td>121 ± 4</td>
<td>4015 ± 381</td>
<td>8.3 ± 0.5</td>
<td>17.6 ± 0.7</td>
</tr>
<tr>
<td>B</td>
<td>123 ± 5</td>
<td>4176 ± 174</td>
<td>8.5 ± 0.7</td>
<td>18.6 ± 1.2</td>
</tr>
</tbody>
</table>

Data presented as mean values ± SE. All variables were identical for all groups of hearts (p = nonsignificant).

dP/dt max = first derivative of the rise of left ventricular pressure.
Effect of global cardioplegic ischemia on TNF-α, eNOS, and iNOS mRNA expression. The baseline TNF-α mRNA expression that had been detected in LV specimens after a period of stabilization and that did not change after a 90-min period of nonischemic perfusion served as an additional control (n = 7) to the postischemic TNF-α, iNOS, and eNOS mRNA measurements. Intensities of the bands at baseline and at the end of perfusion were 0.55 ± 0.2 and 0.53 ± 0.1, respectively (p = NS, Fig. 1A). One hour of global cardioplegic ischemia led to a 1.6-fold increase of TNF-α mRNA expression, which was significantly higher than the levels detected in nonischemic, normally perfused hearts (p < 0.003, Fig. 1A).

Baseline eNOS and iNOS mRNA expression was detected in LV samples after a period of stabilization and did not change after a 90-min period of nonischemic perfusion (Fig. 1B and 1C). No upregulation of iNOS mRNA expression was registered in postischemic LV myocardial tissue compared with baseline values or after a 90-min period of nonischemic perfusion. There was, however, a significant downregulation of eNOS mRNA expression (p < 0.01, Fig. 1C) after 1 h of global cardioplegic ischemia.

Effect of global cardioplegic ischemia on TNF-α and NO release. Tumor necrosis factor-α was not detected in the myocardial effluent after 15 min of stabilization, before ischemia (after 30 min of perfusion), or after 90 min of normal nonischemic perfusion. Significant amounts of TNF-α were detected in the effluent after 1 h of global cardioplegic ischemia upon the first minute of reperfusion (805 ± 230 pg/ml). The NO2 and NO3 values in the effluent from isolated perfused hearts in all groups were below detectable levels.

Effect of TNF-α depletion on myocardial TNF-α, eNOS, and iNOS mRNA expression and release. Anti-TNF-α Ab was added to the cardioplegic solution to neutralize TNF-α in the ischemic heart. In hearts treated with anti-TNF-α Ab, TNF-α mRNA expression was at basal levels after 1 h of global ischemia (Fig. 1A), which was significantly lower than the levels detected in untreated ischemic hearts.

There was an increase of eNOS mRNA in the ischemic heart pretreated with anti-TNF-α Ab compared with untreated ischemic hearts (1.07 ± 0.1 and 0.67 ± 0.2, respectively, p < 0.01). There were no significant differences in iNOS mRNA band intensities between anti-TNF-α Ab-treated and control hearts (Fig. 1B and 1C). Tumor necrosis factor-α protein production and NO2 and NO3 in the effluent from the ischemic hearts treated with anti-TNF-α Ab were below detectable levels.

Effect of exogenous TNF-α on LV hemodynamic performances. No significant differences in baseline values for LV hemodynamic parameters were found between groups C and D (Fig. 2). Hearts treated with TNF-α demonstrated a significant deterioration in all hemodynamic measurements after only 10 min of perfusion (Fig. 2) and, after 60 min of TNF-α treatment, peak-systolic pressure fell to 45.1...
± 3.7%; dP/dt max to 51.8 ± 5.5%, time-pressure integral to 73.5 ± 4.8%, and CF to 54.7 ± 2.8% (p < 0.001 for all measurements compared with baseline). The decrease of LV function parameters in TNF-α-treated hearts was significant compared with untreated hearts (Fig. 2, p < 0.0001 for all measurements).

Effect of exogenous TNF-α on eNOS and iNOS mRNA expression. Endothelial nitric oxide synthase mRNA expression in LV specimens after a period of stabilization did not change after a 60-min period of nonischemic perfusion, but 60 min of TNF-α treatment led to significant downregulation of eNOS mRNA expression (Fig. 3A; p < 0.01). There were no significant differences in iNOS mRNA band intensities between TNF-α-treated and control hearts (Fig. 3B).

DISCUSSION

In the present study, we have demonstrated that endogenous (i.e., released during ischemia) and exogenous TNF-α cause depression of LV function associated with undetectable NO levels in the effluent from the heart, unchanged iNOS mRNA expression, and eNOS mRNA downregulation.

The role of TNF-α in cardiac dysfunction. Tumor necrosis factor-α is an essential mediator of the profound cardiovascular changes observed during the shocked state associated with bacterial sepsis and its experimental counterpart, endotoxic shock (24,25). Recent investigations have confirmed that the TNF-α levels were increased in patients with congestive heart failure and demonstrated a direct relationship between circulating levels of TNF-α and clinical features of the disease (26). Tumor necrosis factor-α also plays an important role in reperfusion injury after myocardial revascularization (27). Tumor necrosis factor-α is synthesized and released from the isolated heart undergoing ischemia (11,12). The results of current experiments support this previous observation. Immunohistochemical studies localized TNF-α in ischemic hearts to cardiac myocytes and endothelial cells (13). The myocardium has been shown to be a major source of TNF-α in patients undergoing cardiopulmonary bypass (in vivo acute global
TNF-α-mediated cardiac depression (29,31). Tumor necrosis factor-α induces apoptosis (programmed cell death) in various cell types, including cardiac myocytes (2,31). Other possible mechanisms are direct cytotoxicity and oxidative stress (30).

Current hypothesis. Some previous studies using NO synthesis inhibitors confirm that NO is responsible for TNF-α-induced negative inotropism (17,18). It is proposed that TNF-α stimulates iNOS which, in turn, controls the conversion of L-arginine to NO. Excessive production of NO by cardiomyocytes causes contractile dysfunction and depression of cardiac function. Recent investigations, however, have shown that the NOS inhibitors do not ameliorate the decreased contractility of isolated perfused hearts observed with TNF-α (29). Tumor necrosis factor-α had no significant effect on NO and cGMP production in cultured cardiac myocytes (20). In a model of isolated cardiac myocytes, NOS inhibitors do not attenuate the negative inotropic effect of TNF-α, and incubation with TNF-α did not increase NO and cGMP production (4). NOS inhibitors did not prevent a decrease in either intracellular calcium or the amplitude of cell shortening caused by TNF in isolated ventricular myocytes (21). In addition, it has been shown that the massive cardiospecific overexpression of iNOS in transgenic mice is not associated with deleterious effects on cardiac hemodynamics and energetics (32). The participation of eNOS mRNA expression in cardiodepressant action of TNF-α has not been investigated in depth.

New insights. The present study clearly has demonstrated that endogenous (paracrine) TNF-α synthesized during 1 h of global cardioplegic ischemia or exogenous TNF-α added for 1 h to perfusion solution does not increase iNOS mRNA expression and NO release in the isolated rat heart. In the present study, we have demonstrated that the endogenous and exogenous TNF-α caused a decrease in eNOS mRNA expression. Moreover, depletion of TNF-α with anti-TNF-α Ab resulted in an increase in eNOS mRNA in the ischemic heart compared with untreated ischemic hearts, recovering eNOS mRNA expression to baseline levels.

We have shown that CF significantly decreased after 1 h of perfusion with a TNF-α-containing solution. Previous experiments demonstrated that TNF-α caused coronary constriction in the rat heart, which might accentuate direct negative inotropic action of TNF-α (29). In the current study, we found that eNOS mRNA downregulation in hearts undergoing ischemia or TNF-α perfusion may lead to a decrease in endothelial NO production, coronary vasoconstriction, and a decrease of CF. This might be one of the possible mechanisms of TNF-α-induced cardiodepressant action. It is theoretically possible that a more prolonged (3 to 6 h) TNF-α influence could lead to a change in iNOS mRNA expression, but this duration would be considered excessive in a model such as ours.

Study limitations. Our study had several limitations. First, it was performed in vitro. Second, nonblood perfusion...
excluded an influence of blood immunological and proinflammatory factors and also excluded the influence of several types of blood cells that are an important source of TNF-α and NO. Caution must be exercised when drawing direct clinical conclusions from the use of anti-TNF-α Ab in a clinical setting because our study was performed on an isolated rat heart model.

**Conclusions.** This study demonstrated that endogenous (i.e., released during ischemia) and exogenous TNF-α do not influence iNOS mRNA expression and that they do not enhance NO release. This study did reveal eNOS mRNA downregulation in the isolated rat heart. Depletion of endogenous TNF-α caused by anti-TNF-α Ab led to the decrease of TNF-α mRNA expression, the elimination of TNF-α protein from effluent solution, and an increase in eNOS mRNA expression to baseline levels in ischemic hearts. These findings suggest that eNOS downregulation occurs as a result of endogenous TNF-α synthesis. We hypothesize that the TNF-α-related eNOS mRNA downregulation that is associated with CF decreases and that LV dysfunction may be one of the mechanisms of TNF-α-mediated myocardial depression.

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