Tumor Necrosis Factor-α Confers Resistance to Hypoxic Injury in the Adult Mammalian Cardiac Myocyte

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Background — Previous studies in isolated cardiac myocytes have shown that tumor necrosis factor (TNF-α) provokes increased expression of 27- and 70-kD stress proteins as well as manganese superoxide dismutase, suggesting that TNF-α might play a role in mediating stress responses in the heart.

Methods and Results — To determine whether TNF-α stimulation would protect isolated cardiac myocytes against environmental stress, myocyte cultures were pretreated with TNF-α for 12 hours and then subjected to continuous hypoxic injury (O₂ content, 3 to 5 ppm) for 12 hours, followed by reoxygenation. Cell injury was assessed in terms of lactate dehydrogenase (LDH) release, ⁴⁵Ca²⁺ uptake, and MTT metabolism. Pretreatment with TNF-α concentrations ≥50 U/mL significantly attenuated LDH release by hypoxic cells compared with diluent-treated hypoxic cells. Similar findings were observed with respect to ⁴⁵Ca²⁺ uptake and MTT metabolism in TNF-α–pretreated cells that were subjected to prolonged hypoxia. To determine the mechanism for the TNF-α–induced protective effect, the cells were pretreated with heat shock protein (HSP) 72 antisense oligonucleotides. These studies showed that the protective effect of TNF-α was not inhibited by antisense oligonucleotides, despite use of a concentration of antisense that was sufficient to attenuate the TNF-α–induced increase in HSP 72 expression. Subsequent studies using mutated TNF ligands showed that activation of both types 1 and 2 TNF receptors was sufficient to confer a protective response in isolated cardiac myocytes through an as yet unknown pathway(s).

Conclusions — Taken together, the above observations demonstrate that TNF-α pretreatment confers resistance to hypoxic stress in the adult cardiac myocyte through a novel mechanism that appears to be different from but not necessarily exclusive of the protective response conferred by HSP 72 expression. (Circulation. 1998;97:1392-1400.)

Key Words: myocytes • hypoxia • proteins • genes

Tumor necrosis factor-α is a proinflammatory cytokine with pleiotropic biological effects. When TNF-α is elaborated in low concentrations, this cytokine acts primarily in an autocrine/paracrine/juxtacrine manner and is thought to play an important role in regional tissue homeostasis as well as in the regulation of local host defense responses.¹–³ When elaborated in higher concentrations, however, TNF-α can produce devastating endocrine effects that include metabolic wasting (cachexia), microvascular coagulation, and lethal hemodynamic collapse.⁴–⁷ Thus, TNF-α may produce either adaptive homeostatic responses or devastating maladaptive effects, depending on the duration and degree to which this cytokine is expressed.

Experimental studies from several laboratories have shown that the adult mammalian heart synthesizes TNF-α mRNA and protein de novo after certain forms of stress.⁸–¹⁰ Although the precise biological role for stress-induced TNF-α expression within the heart is unknown, two lines of evidence suggest that the expression of TNF-α may serve an important autocrine and/or paracrine homeostatic role in the heart. First, TNF-α gene expression is not constitutive within the heart but rather is temporally coupled to stressful environmental stimuli.⁸–¹⁰ TNF-α biosynthesis within the heart is not only extremely rapid in onset, occurring within 60 minutes after the onset of a stressful stimulus, but also rapid in offset, in that TNF-α mRNA levels return to baseline within 90 minutes after the removal of the inciting stress.⁸ Second, TNF-α stimulation has been shown to reduce LDH release threefold in Langendorff-perfused rat hearts that had been subjected to a 20-minute period of global ischemia.¹¹ Taken together, the above observations suggest that the coordinated and tightly regulated expression of TNF-α mRNA and protein within the heart may serve to protect the heart against environmental stress through as yet unknown mechanisms.

Relevant to the above discussion is the recent set of observations that TNF-α stimulation provokes increased expression of the 27- and 72-kD HSPs in fetal and adult cardiac myocytes, respectively.¹²,¹³ Given that the 70-kD family of HSPs are thought to protect the heart against environmental injury,¹⁴,¹⁵ we hypothesized that TNF-α stimulation might protect the isolated cardiac myocyte against environmental...
stress by upregulating HSP 72 expression. Accordingly, the purpose of this study was to determine whether TNF-\(\alpha\) stimulation would protect the isolated cardiac myocyte against hypoxic stress. This study demonstrates that TNF-\(\alpha\) pretreatment confers resistance to hypoxic stress in the adult cardiac myocyte through a novel mechanism that appears to be different from but not necessarily exclusive of the protective response conferred by increased HSP 72 expression.

**Methods**

**Cardiac Myocyte Isolation and Culture**

The methods for isolating adult feline cardiac myocytes, the characteristics of the cell culture system, and the serum-free cell culture conditions used in this study have all been described previously in considerable detail.\(^{13,16–18}\) Endotoxin-free 0.1\% human serum albumin was always used as the carrier protein for the recombinant human TNF-\(\alpha\) (Genzyme) that was used throughout these experiments; 0.1\% human serum albumin was always used as the control (diluent) solution.

**Cardiac Myocyte Evaluation**

**Model of Hypoxic Stress in Cardiac Myocytes**

Adult feline cardiac myocytes cultures were subjected to hypoxic stress in a commercially available acrylic plastic hypoxia chamber (Billups-Rothenberg) in which the ambient oxygen in the chamber was replaced by a gas mixture composed of 90\% nitrogen and 10\% CO\(_2\). The degree of hypoxia in the chamber was monitored with an oxygen probe (Lazar, Inc) that was mounted in the hypoxia chamber; the tip of the oxygen probe was submersed in deH\(_2\)O to continuously monitor the dissolved oxygen content (ppm) during hypoxic culture conditions. Under normoxic conditions, the oxygen content in the chamber was 18 to 20 ppm; under hypoxic conditions, the oxygen content declined to 3 to 5 ppm and remained stable at this level for up to 12 hours. To increase the degree of cell injury, the cardiac myocytes were incubated in modified DMEM culture medium without supplemental glucose or glutamine (“DMEM base,” Gibco BRL) during hypoxia to prevent the cells from utilizing the glycolytic pathway. At the end of the hypoxic period, the cells were returned to medium 199 and transferred to a water-jacketed incubator (95\% O\(_2/5\%\) CO\(_2\)) to begin reoxygenation.

In preliminary experiments, we determined that up to 8 hours of hypoxic injury produced only mild cell injury, as determined by the degree of LDH released into the culture medium, whereas 12 hours of continuous hypoxia followed by reoxygenation resulted in a significant twofold to threefold increase in LDH release compared with parallel cultures of cardiac myocytes that had been maintained under normoxic conditions. Moreover, we further determined that this amount of hypoxic injury was sufficient to release \(\approx 50\%\) of the total LDH content of the myocytes. Accordingly, for the experiments detailed below, we exposed the cardiac myocytes to 12 hours of continuous hypoxia followed by reoxygenation to produce significant (but not uniformly lethal) cell injury. Insofar as we planned to use LDH as a marker of cell injury, we confirmed that pre-treating the cells for 12 hours with TNF-\(\alpha\) did not significantly alter the ratio of cytosolic LDH to total cell protein in normoxic TNF-\(\alpha\)-treated (1.27\(\pm\)0.08 U/\(\mu\)g protein) or normoxic diluent-treated (1.23\(\pm\)0.05 U/\(\mu\)g protein) cardiac myocytes (\(P=68\)).

**Characterization of the In Vitro Hypoxia Model System**

Three interrelated studies were performed to characterize the hypoxia/reoxygenation model system in the diluent-treated and TNF-\(\alpha\)-treated cells. First, to determine whether the experimental conditions altered the total cell number, we examined the total DNA content in the diluent-treated and TNF-\(\alpha\) (200 U/mL)-treated myocytes studied under normoxic and hypoxic conditions. DNA content was determined fluorometrically by determining the amount of binding of Hoechst 33258 dye to cellular DNA, exactly according to the manufacturer’s suggestions (Hoefer Scientific Instruments). Calf thymus DNA was used as a standard. Second, to determine the myocyte protein content in the diluent- and TNF-\(\alpha\) (200 U/mL)-treated cultures, we measured total protein content per dish in the normoxic and hypoxic cultures (BCA assay, Pierce). Third, to determine whether hypoxic injury induced endogenous TNF-\(\alpha\) production, we examined TNF-\(\alpha\) release in the supernatants from normoxic and hypoxic myocyte cultures that had been pretreated with TNF-\(\alpha\) or diluent. The cell supernatants were then assayed for the presence or absence of TNF-\(\alpha\) with an ELISA (Bio-source Cytoscreen US Ultrasensitive) that recognizes both human and feline TNF-\(\alpha\). The culture conditions for the above three studies were identical to those described immediately below.

**Effect of TNF-\(\alpha\) on Hypoxic Cell Injury**

A 2-mL suspension of freshly isolated cardiac myocytes was plated at a final concentration of \(5\times10^5\) cells/mL onto laminin-coated (20 \(\mu\)g/mL) polystyrene Petri dishes. Medium was changed with medium 199 on day 1 of culture. On the basis of previous studies from this laboratory showing that stimulation with TNF-\(\alpha\) led to a maximal increase in HSP 72 expression by 12 hours,\(^{13}\) the cultures were pretreated continuously for 12 hours with a range of concentrations of TNF-\(\alpha\) (10 to 1000 U/mL); control cultures were treated with diluent alone. The culture medium was then changed to DMEM base, followed by 12 hours of continuous hypoxia as described above; control cultures were switched to DMEM base and maintained under normoxic conditions for 12 hours. Supplemental TNF-\(\alpha\) was not added to the normoxic or hypoxic cultures that were maintained in DMEM base. Three indices of cell injury were examined after the 12-hour period of exposure of the myocyte cultures to normoxic or hypoxic culture conditions: LDH release into the culture medium, \(^{45}\)Ca\(^{2+}\) uptake by the cardiac myocytes, and metabolism MTT.\(^{10,20}\) For the cells that were exposed to hypoxic culture conditions, all studies were performed immediately on reoxygenation (\(\approx 15\) to 30 minutes) of the cultures; cells exposed to normoxic conditions were studied in a parallel time fashion. The specificity of the TNF-\(\alpha\)-induced effects was determined by use of an anti-TNF-\(\alpha\) antibody to neutralize the effects of TNF-\(\alpha\) exactly as we have described previously.\(^{22}\)

LDH levels were measured after 12 hours of hypoxia in DMEM base with a colorimetric assay (Sigma Chemical Co) that measures the conversion of pyruvic acid to lactic acid by LDH. To account for variations in LDH release secondary to differences in cell number from culture preparation to culture preparation, the extent of LDH release was normalized by the micromgramgs of myocyte protein (BCA assay) per dish. Final values were expressed as U/\(\mu\)g cell protein. Previously, we have shown that LDH release in hypoxic/reoxygenated adult feline myocytes correlates inversely with cell viability (\(r=−0.78\); \(P<0.0001\) ), as determined by the ratio of live and dead cells that take up calcein acetoxyethylster and ethidium homodimer-1, respectively.\(^{22}\)

\(^{45}\)Ca\(^{2+}\) uptake, which was used as a second parameter to assess sarcolemmal integrity, was determined in the following manner. Immediately on reoxygenation, the cells were incubated for 60 minutes in a low-sodium (25 mmol/L NaCl) \(^{45}\)Ca\(^{2+}\)-free Krebs-Henseleit buffer supplemented with verapamil (10 \(\times\) mol/L) to block both the Na\(^+\)–Ca\(^{2+}\) exchanger and the L-type Ca\(^{2+}\) channel, respectively. Next, 5 \(\mu\)Ci/mL of \(^{45}\)Ca\(^{2+}\) was added to the culture medium, and the extent of \(^{45}\)Ca\(^{2+}\) uptake of cardiac myocytes was examined at 0, 0.5, 1, 2, and 5 minutes. The extent of \(^{45}\)Ca\(^{2+}\) uptake was
normalized by micrograms of protein (BCA assay, Pierce) per dish; final values were expressed as cpm/μg protein.

**MTT Metabolism**

MTT is a tetrazolium salt that turns blue when reduced to formazan by the respiratory enzymes present in functioning mitochondria. MTT metabolism can be quantified simply in a variety of cell types, including cardiac myocytes, by determination of the extent to which cells reduce MTT to formazan; the amount of formazan that is produced by the cells can then be quantified spectrophotometrically. To confirm that the degree of MTT metabolism reflected the total number of viable adult cardiac myocytes, in preliminary control experiments we plated increasing concentrations of cardiac myocytes (from 300 to 10,000 cells/well) in 96-well microtiter plates (Falcon, Becton Dickinson) previously coated with 0.2% laminin. The cells were allowed to remain in culture for 24 hours, at which point 20 μL/well of MTT stock (12 mmol/L [5 mg/mL] in PBS) was added; the cells were then returned to the incubator for an additional 4 hours, after which the reaction was stopped by the addition of 10% SDS (pH 7.2). The degree of MTT metabolism was determined spectrophotometrically at 600 nm (Molecular Devices) after an overnight incubation at 37°C.

To examine the effect of 12 hours of hypoxia on MTT metabolism by cardiac myocytes, 104 cells/well were plated onto laminin-coated 96-well microtiter plates and cultured in medium 199 for 24 hours. The culture medium was then switched to DMEM base, and the cells were subjected to hypoxia for 12 hours. On reoxygenation after hypoxia, 100 μL of the culture medium was removed and 20 μL of MTT added directly to the well, and the degree of MTT metabolism was determined as described above. MTT metabolism was expressed as a percentage of control values obtained for cardiac myocytes maintained under normoxic conditions, which were arbitrarily assigned a value of 100%.

**Effect of TNF-α-Induced HSP 72 Expression on Hypoxic Cell Injury**

To assess the role of TNF-α-induced HSP 72 expression in hypoxic injury, a 14-base phosphorothioate antisense oligonucleotide (5′-CAGGTCGATGCCGA-3′) was used to block HSP 72 synthesis. The antisense oligonucleotide, which was synthesized by the Baylor Nucleic Acid Core Facility, was designed on the basis of a highly conserved region of HSP 72 (5′-TGGCAATCGGCCTG-3′) that corresponds to bases 508 to 521 in the human gene. Previously, we have shown that the uptake of the phosphorothioate antisense oligonucleotides by cardiac myocytes is maximal by 12 hours. Moreover, we have shown that pre-treating the cardiac myocytes with 2.5 μmol/L of antisense oligonucleotide for 12 hours was sufficient to significantly block the endogenous increase in HSP 72 expression after hypoxic stress; concentrations of antisense oligonucleotide >2.5 μmol/L did not blunt the HSP 72 response further. The specificity of the antisense strategy used here was demonstrated in previous studies that showed that HSP 72 antisense oligonucleotides had no effect on HSP 60 levels in feline cardiac myocytes, as well as by studies that showed that an irrelevant antisense oligonucleotide to the MHC (major histocompatibility complex) class I gene had no effect on the level of HSP 72 expression.

To determine whether pre-treating the cardiac myocytes with antisense oligonucleotide would block the TNF-α-induced increase in HSP 72 expression, we first examined HSP 72 expression in diluent- and TNF-α (200 U/mL)-stimulated cardiac myocytes that had been treated previously with 2.5 μmol/L antisense oligonucleotides; the appropriate negative controls for these experiments consisted of cardiac myocytes treated with 2.5 μmol/L “sense” phosphorothioate oligonucleotides (5′-TGGCAATCGGCCTG-3′). For each of these experiments, the cells were first treated with sense or antisense oligonucleotides for 12 hours, followed by treatment with diluent or TNF-α for 12 hours in the presence of sense or antisense oligonucleotides. HSP 72 expression was determined by ELISA exactly as we have described previously. To account for variations in HSP 72 expression arising from cell number alone, the amount of HSP 72 per culture was normalized by the amount of protein per culture.

To determine whether pretreatment with HSP 72 antisense oligonucleotides would attenuate any potential protective effects conferred by TNF-α, cardiac myocytes were first cultured continuously for 12 hours in the presence of 2.5 μmol/L antisense or 2.5 μmol/L sense oligonucleotides. The myocyte cultures that had been pretreated with antisense and sense oligonucleotides were then stimulated with TNF-α for an additional 12 hours before the cells were subjected to hypoxic stress. Cell injury was performed exactly as described above, with the exception that the DMEM base culture medium was supplemented with either 2.5 μmol/L sense or 2.5 μmol/L antisense oligonucleotides. At the end of the 12-hour period of hypoxic stress, cell injury was assessed in terms of LDH release, 45Ca uptake, and MTT metabolism exactly as described above.

**Effect of Mutated TNF Ligands on Hypoxic Cell Injury**

To determine whether the protective effects of TNF-α were mediated through activation of the type 1 TNF receptor (TNFR1), the type 2 TNF receptor (TNFR2), or both TNF receptors, we pretreated the cells with mutated TNF ligands that bind selectively to human TNFR1 (corresponding mutant, TNFM1) and TNFR2 (corresponding mutant, TNFM2; both provided by W. Lesslauer, F. Hoffman-LaRoche, Basel, Switzerland) or a combination of TNFM1 and TNFM2; cells pretreated with wild-type TNF-α (200 U/mL) served as the appropriate positive controls. The specificity of the mutated TNF ligands for binding to feline TNFR1 and TNFR2 has been validated previously. The myocyte cultures were pretreated for 12 hours with TNFM1, TNFM2, or a combination of TNFM1 and TNFM2 and were then subjected to normoxic and hypoxic culture conditions exactly as described above for wild-type TNF-α. Cell injury was assessed in terms of LDH release, 45Ca uptake (5 minutes), and MTT metabolism.

**Statistical Analysis**

Each value is expressed as a mean ± SEM. One-way ANOVA was used to test for mean differences in LDH release, 45Ca uptake, and MTT metabolism. If an overall significant difference in LDH release, 45Ca uptake, and MTT metabolism was observed by ANOVA, then post hoc ANOVA testing was performed between diluent-treated normoxic controls and experimental groups (Dunnett’s test) or between experimental groups (Newman-Keuls). Two-way ANOVA was used to test for mean differences in 45Ca uptake in normoxic and hypoxic/reoxygenated myocytes as a function of time. Significant differences were said to exist at P < .05.

**Results**

**Cardiac Myocyte Evaluation**

**Characterization of the In Vitro Hypoxia Model System**

Three interrelated studies were performed to characterize the hypoxia model system used to produce cell injury. Fig 1A shows that the DNA content per culture dish was not significantly different (P = .99 by ANOVA) in the diluent and TNF-α-treated cells that were studied under normoxic and hypoxic conditions, suggesting that total cell number was not different in the four groups. Although there were statistically significant differences in the overall protein levels per dish within the different groups (P < .02 by ANOVA), post hoc ANOVA testing indicated that the differences in protein content within the individual groups were not significantly greater from control values (P > .05 by Dunnett’s test). The small increases in protein content in the TNF-α-treated cells are consistent with our previous observation that TNF-α provokes a modest increase in the rate of protein synthesis in adult cardiac myocytes. To determine whether hypoxic...
injury provoked endogenous TNF-α production, we examined TNF-α release in the supernatants from normoxic and hypoxic myocyte cultures that had been pretreated with TNF-α or diluent. As shown in Fig 1C, the level of TNF-α production in the supernatants in the normoxic and hypoxic diluent-treated cells was negligible and was not significantly different between groups (P > 0.05 by Dunnett’s test). In contrast, the levels of TNF-α were significantly greater (P < 0.01 for both by Dunnett’s test) in the normoxic and hypoxic cells that had been pretreated with TNF-α. Given that the levels of TNF-α that were recovered from the cytokine-pretreated cells represents ~1% to 2% of the total amount of TNF-α that the cells were pretreated with, it is likely that the TNF-α levels observed in the supernatants from these cultures represents TNF-α that was released from TNF receptors that were occupied during the 12-hour period of TNF-α pretreatment. Importantly, the TNF-α levels in the supernatants from the hypoxic TNF-α-pretreated cells were not significantly greater (P > 0.05 by Dunnett’s test) than in the normoxic TNF-α-pretreated cells, suggesting that there was no increase in the endogenous TNF-α secretion in the cytokine-pretreated hypoxic cells.

Figure 1. Characterization of hypoxia model system. Myocyte cultures were pretreated continuously for 12 hours with 200 U/mL TNF-α (solid bars); control cultures were treated with diluent alone (open bars). Cardiac myocytes were then cultured under normoxic or hypoxic conditions, and total DNA content (A) and protein content (B) were determined as described in “Methods” (n=8 culture dishes/group from two separate myocyte isolations). To determine whether hypoxic injury induced endogenous TNF-α production, we also examined TNF-α secretion by measuring cytokine levels in supernatants from normoxic and hypoxic myocyte cultures (n=8 culture dishes/group from two separate myocyte isolations) pretreated with TNF-α or diluent (C). To facilitate comparison between myocyte isolations, data are depicted as fold increase in μg DNA/dish, μg cell protein/dish, and TNF-α release (pg/mL) vs values obtained in normoxic diluent-treated myocyte cultures. Respective values (mean±SEM) for DNA content, protein content, and TNF-α release in diluent-treated normoxic cardiac myocytes were 12.0±1.9 μg DNA, 258±14.3 μg protein, and 1.32±0.2 pg/mL TNF-α. *P < 0.05 vs normoxic diluent controls.

Figure 2. Effect of TNF-α pretreatment on LDH release after hypoxic injury stress. Myocyte cultures were pretreated continuously for 12 hours with 10 to 1000 U/mL TNF-α (solid bars); control cultures were treated with diluent alone (open bars). Cardiac myocytes were cultured under normoxic or hypoxic conditions as described in “Methods”; LDH release was assessed spectrophotometrically at end of 12-hour period of normoxia (n=6 cultures/group) or hypoxia (n=8 cultures/group). Specificity of TNF-α-induced effects was determined with anti-TNF-α antibody to neutralize effects of TNF-α (hatched bar). To facilitate comparison between myocyte isolations, data are depicted as fold increase in LDH release vs diluent-treated normoxic myocytes. One-way ANOVA indicated significant overall differences (P < 0.001) between groups. Extent (mean±SEM) of LDH release for diluent-treated normoxic cells was 1.0±0.2 U/μg cell protein. *P < 0.05 vs hypoxic diluent-treated cells.

Effect of TNF-α on Hypoxic Cell Injury

LDH Release

Fig 2 shows three important findings with respect to the effect of TNF-α pretreatment on LDH release by normoxic and hypoxic/reoxygenated cardiac myocytes. First, pretreatment of normoxic cardiac myocytes with TNF-α (1000 U/mL) had no significant effect (P > 0.05) on LDH release compared with diluent-treated normoxic cardiac myocytes. Second, 12 hours of continuous hypoxic stress resulted in a significant 2.3-fold increase (P < 0.05) in LDH release in diluent-treated cardiac myocytes compared with diluent-treated normoxic controls. Third, pretreatment with TNF-α concentrations ≥50 U/mL significantly attenuated (P < 0.05) LDH release compared with hypoxic/reoxygenated myocyte cultures that had been treated with diluent alone. The specificity of the TNF-α-induced effects was demonstrated by the finding that the protective resistance conferred by TNF-α could be completely abrogated by a polyclonal anti-TNF-α antibody. Although TNF-α pretreatment significantly reduced the extent of LDH release in hypoxic/reoxygenated cells, the degree of LDH release in the hypoxic/reoxygenated cytokine-treated cells was still significantly greater (P < 0.05) than was observed in diluent-treated normoxic cells, indicating that the protective effect of TNF-α was incomplete.

"Ca" Uptake

Fig 3A shows that hypoxic stress resulted in an overall increase in "Ca" uptake in diluent-treated hypoxic cells compared with diluent-treated normoxic control cells. As shown, the difference in "Ca" uptake was most apparent at 5 minutes. Two-way ANOVA indicated that there was a significant overall increase in "Ca" uptake both within
Figure 3. Effect of TNF-α pretreatment on 45Ca2+ uptake after hypoxic stress. A, Time course of 45Ca2+ uptake at 0, 0.5, 1, 2, and 5 minutes in isolated cardiac myocytes (see “Methods” for details) after 12 hours of culture under normoxic or hypoxic/reoxygenation culture conditions. Data are cpm/μg cell protein. B, Effect of 12 hours of pretreatment with diluent (open bars) or 200 U/mL of TNF-α (solid bars) on 5-minute 45Ca2+ uptake in cardiac myocytes cultured under normoxic (n=8 cultures/group) and hypoxic (n=10 cultures/group) conditions. Data in B are fold increase in 45Ca2+ uptake vs diluent-treated normoxic cells, to facilitate comparison between myocyte isolations. One-way ANOVA indicated significant overall differences (P<.001) between groups. Extent (mean±SEM) of 5-minute 45Ca2+ uptake in diluent-treated normoxic control cells in these experiments was 88.2±8.7 cpm/μg cell protein. *P<.05 vs diluent-treated hypoxic cells.

(P<.001) and between (P<.01) groups when the diluent-treated normoxic and hypoxic cells were compared. Fig 3B shows that there was no significant difference (P>.05) in 45Ca2+ uptake (5 minutes) in the normoxic cardiac myocytes treated with diluent and TNF-α (200 U/mL). After hypoxic stress, however, there was a significant 2.5-fold increase (P<.05) in 45Ca2+ uptake (5 minutes) in the diluent-treated hypoxic cardiac myocytes compared with the diluent-treated normoxic control cells. As shown, TNF-α (200 U/mL) pretreatment significantly attenuated (P<.05) 45Ca2+ uptake (5 minutes) in the hypoxic/reoxygenated cardiac myocytes compared with diluent-treated hypoxic cells. Nonetheless, the degree of 45Ca2+ uptake was still significantly greater (P<.05) in the TNF-α-pretreated hypoxic/myocytes than in the diluent-treated normoxic cells, indicating that the protective effect of TNF-α was incomplete.

MTT Metabolism

Fig 4A shows that there was a direct linear relationship between the number of cardiac myocytes that were plated and the degree of MTT metabolism (r=.98, P=.0001; slope, P<.001). Fig 4B depicts the degree of MTT metabolism in normoxic and hypoxic cells in the presence and absence of TNF-α pretreatment. As shown in Fig 4B, there was no significant difference (P>.05) in the degree of MTT metabolism in the normoxic myocytes pretreated either with diluent or TNF-α (200 U/mL). In contrast, the degree of MTT metabolism was significantly reduced (P<.05) in the diluent-treated hypoxic cardiac myocytes compared with the diluent-treated normoxic controls. However, the salient finding shown by Fig 4B is that MTT metabolism was significantly greater (P>.05) in the TNF-α-pretreated hypoxic myocytes than in the diluent-treated hypoxic/reoxygenated myocytes. Finally, the degree of MTT metabolism was still significantly greater (P<.05) in the diluent-treated normoxic cells compared with TNF-α-pretreated hypoxic/reoxygenated myocytes, indicating that the protective effect of TNF-α was incomplete.

Effect of TNF-α-Induced HSP 72 Expression on Hypoxic Cell Injury

We have shown previously that stimulation with TNF-α increases the expression of HSP 72 in cardiac myocytes. Because increased expression of HSP 72 has been associated with enhanced resistance to ischemic injury, we sought to determine whether the mechanism for the protective effect of wild-type TNF-α was mediated, at least in part, through increased expression of HSP 72. Accordingly, we used specific antisense oligonucleotides to interfere with cytokine-induced HSP 72 expression; myocytes treated with sense oligonucleotides served as the appropriate controls. The control experiments illustrated in Fig 5 show that pretreatment with 2.5 μmol/L sense oligonucleotide or 2.5 μmol/L antisense oligonucleotide had no effect on myocyte HSP 72 levels compared with baseline levels of HSP 72 expression in diluent-treated cardiac myocytes. As shown, TNF-α stimulation resulted in a significant (P<.005) threefold increase in HSP 72 expression, as we have reported previously. However, the important finding shown in Fig 5 is that pretreating the myocytes with HSP 72 antisense oligonucleotides signif-
Effect of Mutated TNF Ligands on Hypoxic Cell Injury

Insofar as the above studies suggested that TNF-α-induced increase in HSP 72 expression was unlikely to explain the observed protective effect against hypoxic injury, we sought to determine whether the protective effects of TNF-α were mediated through activation of TNFR1, TNFR2, or both TNF receptors. Fig 6A through 6C illustrates three salient characteristics of the studies in which hypoxic/reoxygenated cardiac myocytes were pretreated with the TNFM1 ligand (0.1 nmol/L), the TNFM2 ligand (0.1 nmol/L), TNFM1 (0.1 nmol/L)+TNFM2 (0.1 nmol/L), and wild-type TNF-α (200 U/mL). First, pretreatment of the cardiac myocytes with TNFM1 or TNFM2 resulted in a significant overall decrease in the amount of LDH release (Fig 6A), 5-minute $^{45}$Ca$^{2+}$ uptake (Fig 6B), and a significant overall increase in MTT metabolism (Fig 6C) compared with hypoxic/reoxygenated myocytes treated with diluent alone ($P<.001$ by ANOVA for each). Second, the protective resistance conferred by the TNFM1 and TNFM2 ligands was not significantly different ($P>.05$) from that observed with wild-type TNF-α alone. Third, the protective resistance obtained with the combination of the TNFM1 and TNFM2 ligands was not significantly different ($P>.05$) from that observed after stimulation with TNF-α, TNFM1, or TNFM2 alone. Taken together, these studies suggest that the protective effects of wild-type TNF-α can be mimicked by activating either TNFR1 or TNFR2 separately, which in turn implies (but does not prove) that TNFR1 and TNFR2 have overlapping functions with respect to protection against hypoxic injury.

Discussion

The major conclusion to be drawn from this study is that TNF-α pretreatment confers resistance to hypoxic stress in the adult mammalian cardiac myocyte. This statement is supported by the following set of experimental observations. Fig 2 shows that pretreating the cardiac myocytes with $\geq$50 U/mL TNF-α significantly reduced the extent of LDH release compared with LDH release observed in diluent-treated hypoxic/reoxygenated myocytes. The observation that a neutralizing anti–TNF-α antibody completely abrogated the protective effect of TNF-α confirms the specificity of these findings. It is worth emphasizing that the concentration of TNF-α that was necessary and sufficient to confer resistance to hypoxic stress in the present study has not been shown to produce deleterious negative effects that were necessary and sufficient to confer resistance to hypoxic stress in the present study has not been shown to produce deleterious negative effects.

Effect of HSP 72 Sense and Antisense Oligonucleotides on TNF-α–Induced Resistance to Hypoxic Cell Injury

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<th>Diluent</th>
<th>TNF-α</th>
<th>TNF-α + Sense</th>
<th>TNF-α + Antisense</th>
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<td>LDH release, fold increase</td>
<td>2.4±0.2 (n=13)</td>
<td>1.6±0.2 (n=16)</td>
<td>1.6±0.1 (n=10)</td>
<td>1.7±0.1* (n=15)</td>
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<td>$^{45}$Ca$^{2+}$ uptake, fold increase</td>
<td>2.7±0.4 (n=13)</td>
<td>1.5±0.1 (n=16)</td>
<td>1.6±0* (n=6)</td>
<td>1.9±0.2* (n=15)</td>
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<td>MTT metabolism, % of control</td>
<td>62.2±3 (n=30)</td>
<td>83.6±2.7* (n=33)</td>
<td>85.3±3.4* (n=26)</td>
<td>79.7±2.6* (n=25)</td>
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Data are mean±SEM of the fold increase in LDH release and $^{45}$Ca$^{2+}$ uptake vs normoxic control cells or the mean±SEM of the percent of MTT metabolism vs normoxic control cells. Isolated cardiac myocytes were pretreated with TNF-α (200 U/mL) in the presence or absence of sense (2.5 μmol/L) or antisense (2.5 μmol/L) HSP 72 oligonucleotides. The number of experiments performed is shown below each group. One-way ANOVA indicated that there were significant overall differences in LDH release, $^{45}$Ca$^{2+}$ uptake, and MTT metabolism for each of the groups tested ($P<.01$). Post hoc ANOVA testing indicated that LDH release, $^{45}$Ca$^{2+}$ uptake significantly lower ($P<.05$), and the degree of MTT metabolism significantly higher ($P<.05$) in the hypoxic myocytes that had been pretreated with TNF-α in the presence and absence of sense and antisense oligonucleotides vs hypoxic myocytes treated with diluent alone. Importantly, the antisense oligonucleotides did not abrogate the protective effect conferred by TNF-α; i.e., there was no significant difference ($P=.05$) in the LDH release, $^{45}$Ca$^{2+}$ uptake, and MTT metabolism in the cells treated with TNF-α vs the cells treated with antisense oligonucleotides and TNF-α. LDH release and $^{45}$Ca$^{2+}$ uptake for diluent-treated normoxic cells was 1.1±0.2 U/μg cell protein and 72.5±5.4 cpm/μg cell protein, respectively. *$P<.05$ vs diluent-treated hypoxic cells.
improvement in MTT metabolism in hypoxic cells compared above for LDH release, Fig 3 shows that pretreating the cardiac hypoxic/reoxygenated cells treated with diluent alone. As a third measure of cellular injury, we also examined uptake in the hypoxic/reoxygenated myocyte cultures compared inotropic effects either in the intact ventricle or in the isolated contracting cardiac myocyte. Similar to the findings presented above for LDH release, Fig 3 shows that pretreating the cardiac myocytes with TNF-α led to a significant reduction in $^{45}$Ca$^{2+}$ uptake in the hypoxic/reoxygenated myocyte cultures compared with hypoxic myocyte cultures that had been treated with diluent alone. As a third measure of cellular injury, we also examined the effect of TNF-α pretreatment on mitochondrial MTT metabolism in cardiac myocytes. The important finding shown by Fig 4 is that TNF-α pretreatment resulted in a significant improvement in MTT metabolism in hypoxic cells compared with diluent-treated hypoxic cells. Thus, when assessed by three separate indices of cellular injury, TNF-α pretreatment was shown to confer resistance to hypoxic stress in adult cardiac myocytes.

A second, albeit unexpected, finding of the present study was that the protective effects of TNF-α were independent of cytokine-induced increased expression of HSP 72. As shown in Fig 5, pretreatment with antisense oligonucleotides that were sufficient to blunt TNF-α-induced increase in HSP 72 expression by $\approx 40\%$ did not blunt the protective effects of TNF-α. Moreover, the protective effects of TNF-α were mimicked by selective stimulation of TNFR2, which we have shown does not increase HSP 72 expression in adult feline cardiac myocytes. However, it should be emphasized that these studies do not necessarily vitiate an important protective role for HSP 72.

Although this study did not identify the mechanism(s) for the protective effect of TNF-α, the present report has provided a potentially important insight into the mechanism for this effect. That is, although the vast majority of TNF-α-induced effects in cells, including increased expression of manganese superoxide dismutase, are mediated by binding and oligomerization of TNFR1,26-27 the data from the present study show clearly that activation of either TNFR1 or TNFR2 confers a protective response that is not different from that obtained with wild-type TNF-α alone. The simplest interpretation for these findings is that TNFR1 and TNFR2 share overlapping functions with respect to the induction of protective stress proteins and/or signals in the cardiac myocyte. This point of view is further supported by the finding that the protective effects conferred by simultaneous activation of TNFR1 and TNFR2 together were not different from those obtained with either TNFR1 or TNFR2 alone, suggesting that activation of TNFR1 or TNFR2 alone is sufficient to supply the same protective response. If this interpretation is correct, it will be important in future studies to identify the potential biochemical signaling pathways that are redundant to both TNF receptors. One recognized limitation of the present study was that it was not possible to assess the degree of receptor shedding in the diluent-treated and hypoxic feline myocytes. Therefore, the absolute degree of TNF-α stimulation that is necessary to confer a protective response in myocytes cannot be determined precisely from these studies.

Conclusions

The thesis that the cell types residing within the mammalian myocardium both produce and respond to stress by synthesizing a variety of soluble protein factors is certainly not new28,29 and has long been accepted by the cardiovascular community. Indeed, it is becoming increasingly clear that peptide growth factors produced locally within the myocardium, such as acidic and basic fibroblast growth factors, platelet-derived growth factor, non–myocyte-derived growth factor, and angiotensin II, play an important role in modulating myocardial growth during tissue injury and repair.30-37 Nonetheless, despite the widespread and enthusiastic acceptance for the role of peptide growth factors in the heart, with the exception of endothelin and possibly transforming growth factor-$\beta$,38-42 very little is known with respect to the importance of other cytokines and, in particular, the role that the family of so-called proinflammatory cytokines may play in the heart. One obvious reason for this dearth of knowledge is that the canonical role that has been assigned to proinflammatory cytokines has been that of mediating infectious and/or immunological processes in the heart. That is to say, cyto-
kines have traditionally been thought of as secretory products generated by the immune system in the setting of classic infectious and/or cardiac inflammatory conditions, such as systemic sepsis and/or viral myocarditis.

The recent identification of rapid TNF-α biosynthesis by cardiac myocytes and nonmyocardial cell types within the myocardium in response to a variety of forms of stress not ordinarily associated with activation of the immune system has provided an important clue with respect to a significant homeostatic role for TNF-α expression in the heart. Indeed, the repeated observation that TNF-α is expressed in virtually all forms of cardiac injury in both large and small mammals, including but not limited to myocardial infarction, unstable angina, hemodynamic overloading, myocardial reperfusion injury, hypertrophic cardiomyopathy, and end-stage congestive heart failure, suggests but does not prove that TNF-α may act as a phyleogenetically conserved “innate stress response gene” in the heart. In this regard, the observation that TNF-α confers resistance to hypoxic stress in the adult cardiac myocyte expands on this concept and suggests that TNF-α production by the injured and/or stressed cardiac myocyte may serve as a local autocrine/paracrine/juxtacrine mechanism for protecting neighboring myocytes within the myocardium. In addition to the direct protective effects demonstrated for TNF-α in the present study, the short-term expression of myocardial TNF-α and quite possibly other cytokines such as interleukin-6 and cardiotoxin-1 may provide the heart with a panoply of additional homeostatic responses to environmental stress, including hypertrophic growth, increased regional myocardial blood flow, and increased resistance to ischemia-induced arrhythmias through the generation of nitric oxide, increased free radical scavenging through increased expression of manganese superoxide dismutase, and increased expression of 27- and 72-kD stress proteins.

The above statements notwithstanding, it is likely that the short-term beneficial effects of TNF-α may be lost if myocardial TNF-α expression becomes either sustained and/or excessive, in which case the salutary effects of TNF-α may be contravened by the known deleterious endocrine effects of TNF-α, including left ventricular dysfunction, pulmonary remodeling, and pulmonary edema. Therefore, to maximize the potential portfolio of beneficial homeostatic responses conferred by TNF-α in the heart, it will become increasingly important in future studies not only to define the mechanisms that are responsible for the protective effects of TNF-α but also to delineate the mechanisms that foster TNF-α overexpression in the heart.

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