p38 and ERK1/2 MAPKs mediate the interplay of TNF-α and IL-10 in regulating oxidative stress and cardiac myocyte apoptosis

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Dhingra S, Sharma AK, Singla DK, Singal PK, p38 and ERK1/2 MAPKs mediate the interplay of TNF-α and IL-10 in regulating oxidative stress and cardiac myocyte apoptosis. Am J Physiol Heart Circ Physiol 293: H3524–H3531, 2007. First published September 28, 2007; doi:10.1152/ajpheart.00919.2007.—It is known that TNF-α increases the production of ROS and decreases antioxidant enzymes, resulting in an increase in oxidative stress. IL-10 appears to modulate increases the production of ROS and decreases antioxidant enzymes, IL-10/10 ratio 1 for 4 h. H2O2 (100 μM) as a positive control and the antioxidant Trolox (20 μmol/l) were used to confirm the involvement of oxidative stress. H2O2 treatment increased oxidative stress and apoptosis; TNF-α mimicked these effects. Exposure to TNF-α significantly increased ROS production, caused cell injury, and increased the number of apoptotic cells and Bax-to-Bcl-xl ratio. This change was associated with an increase in the phospho-p38 MAPK-to-total p38 MAPK ratio and a decrease in the phospho-ERK1/2-to-total ERK1/2 ratio. IL-10 treatment by itself had no effect on these parameters, but it prevented the above-listed changes caused by TNF-α. The antioxidant Trolox modulated TNF-α-induced changes in Bax/Bcl-xl, cell injury, and MAPKs. Preexposure of cells to the p38 MAPK inhibitor SB-203580 prevented TNF-α-induced changes. Inhibition of the ERK pathway with PD-98059 attenuated the protective role of IL-10 against TNF-α-induced apoptosis. This study provides evidence in support of the essential role of p38 and ERK1/2 MAPKs in the interactive role of TNF-α and IL-10 in cardiac myocyte apoptosis.

Both oxidative stress and inflammation are considered to be important factors in the pathogenesis of heart failure (14, 25). TNF-α, a proinflammatory cytokine, is involved in the pathogenesis of cardiovascular disease, including myocardial infarction (17). TNF-α released during ischemia triggers a cytokine cascade, which is further amplified during reperfusion and may result in extensive myocardial damage and apoptosis (10, 17). The anti-inflammatory cytokine IL-10 inhibits the production of various proinflammatory cytokines including TNF-α (5). However, IL-10 has also been shown to act as an antagonist to TNF-α by inhibiting TNF-α-induced oxidative stress (15).

Cellular responses to external stimuli are partly regulated by the activation of three major MAPK signaling pathways, including ERK1/2, p38 MAPK, and JNKs. It is also known that cellular stresses upregulate JNK and p38 MAPK, which are thought to be involved in cardiac myocyte apoptosis and cardiac pathologies (22, 30). On the other hand, growth factors and hypertrophic stimuli activate ERK1/2, which mediates cardiac myocyte growth and also offers cell protection (29, 34). TNF-α has been shown to have a variety of intracellular effects by initiating the activation of p38 MAPK, which triggers the activation of apoptotic pathways through the generation of oxidative stress, activation of Bax (a proapoptotic protein), cytochrome c release, and activation of caspases (3, 21). The molecular mechanisms of IL-10 anti-inflammatory effects involve activation of the ERK pathway as well as enhancing the expression of an antiapoptotic protein, Bcl2 (7, 23). The interaction of these two cytokines in downstream pathways is not well understood. These studies, however, suggest that the two cytokines, TNF-α and IL-10, may interact in a complex manner, such that the final physiological and pathophysiological effect is the sum total of the TNF-α and IL-10 interaction.

The present study investigated the role of p38 and ERK1/2 MAPKs in the interactive role of TNF-α and IL-10 in cardiac myocyte apoptosis. Since oxidative stress has been suggested to mediate TNF-α-induced cardiac myocyte apoptosis, the antioxidant Trolox was also used to examine the cause and effect relationship. Exposure to H2O2 was used as a positive oxidative stress stimulus for comparison.

MATERIALS AND METHODS

Isolation of Adult Cardiac Myocytes

All animal study protocols were approved by the University of Manitoba Animal Care Committee following guidelines established by Canadian Council on Animal Care. Ventricular myocytes were isolated from the hearts of normal adult male Sprague-Dawley rats (250–300 g) using a previously described procedure (22). After excision, hearts were mounted on a modified Langendorff perfusion apparatus. The perfusate was modified Krebs buffer containing 110 mM NaCl, 2.6 mM KCl, 1.2 mM KH2PO4; 1.2 mM MgSO4, 25 mM NaHCO3, and 11 mM glucose (pH 7.4). The Ca2+-free perfusion was then switched to recirculating mode with the buffer containing 25 μM calcium, 0.1% (wt/vol) collagenase, and 0.25% (wt/vol) BSA for 20 min. Collagenase-digested ventricles were chopped into small pieces and gently passed through pipettes with progressively smaller tip diameters and with increasing concentrations of CaCl2. The suspension was filtered through a nylon mesh (200 μm) and was allowed to settle for 10 min. The supernatant was discarded, and the cell pellet was resuspended in medium 199 (M199) containing CaCl2 (1.8 mM). Myocytes (1 × 10^5 myocytes/dish) were plated on 4% serum-coated cell culture dishes. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
polystyrene tissue culture dishes. Plated myocytes were incubated in serum-free culture medium supplemented with antibiotics (100 μg/ml streptomycin-penicillin) at 37°C under a 5% CO₂-95% air atmosphere. Two hours after cells had been plated, the culture medium was changed to remove unattached dead cells, and viable myocytes were incubated overnight.

**Treatment of Cardiac Myocytes**

After an initial incubation for a period of 24 h, quiescent cardiac myocytes were treated with one of the following at a time: H₂O₂ (100 μM), TNF-α (10 ng/ml), IL-10 (10 ng/ml), and a combination of IL-10 + TNF-α (ratio 1) for 4 h. Both TNF-α and IL-10 were purchased from R&D Systems (Minneapolis, MN). This dose and treatment protocol is based on that of our previous study (15). For a study of the role of oxidative stress in TNF-α-induced changes in MAPK (p38 and ERK1/2) activation and in proapoptotic as well as anti-apoptotic proteins (Bax and Bcl-xl), cells were treated with 20 μM Trolox (a water-soluble antioxidant) for 30 min and then incubated with the combination of Trolox (20 μM) and TNF-α (10 ng/ml) for 4 h. To study the physiological role of p38 and ERK1/2 MAPKs in TNF-α-induced effects, cardiac myocytes were pretreated with 25 μM SB-203580 (a p38 MAPK inhibitor), 25 μM PD-98059 (an ERK1/2 inhibitor), or DMSO (vehicle solution) for 15 min.

**Western Blot Analysis for MAPKs**

Phosphorylated and total p38 and ERK1/2 MAPKs were examined by Western blot analysis using MAPK antibody kits (Cell Signaling Technology). For protein isolation, control and treated cardiac myocytes in different treatment groups were suspended in PBS containing protease inhibitor cocktail for mammalian tissues (Sigma-Aldrich, Oakville, ON, Canada). Protein samples (30 μg) were then subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Roche Diagnostics). Membrane-bound proteins were detected with a BM chemiluminescence blotting substrate (POD) kit (Roche Diagnostics). Bands were visualized with Fluor S-Multilager MAX system (Bio-Rad Laboratories) and quantified by image analysis software (Quantity One, Bio-Rad Laboratories).

**Endogenous Production of ROS**

Oxidative stress was monitored by the measurement of ROS generation using a previously described method (31). Treated cardiac myocytes in culture dishes were washed with PBS and incubated with 10 μM solution of the fluorescent probe 5-(6)-chloromethyl-2',7'-dihydrofluorescein diacetate (DCFDA; Molecular Probes, Eugene, OR) at 37°C in PBS for 30 min in a humidified chamber. Fluorescent images were taken with the Olympus BX 51 fluorescence microscope. Fluorescence intensity was measured using digital imaging processing software (Image Pro Plus).

**Apoptosis**

Pro- and anti-apoptotic proteins (Bax and Bcl-xl) were examined by Western blot analysis using rat antibodies (Cell Signaling Technology) as described above. These experiments were complemented by Hoescht 33258 staining of nuclei. After treatment, cardiac myocytes in culture dishes were washed three times with PBS and incubated with Hoescht 33258 (1 μg/ml) for 10 min in a humidified chamber, protected from light, at 37°C. After cells had been stained, plates were examined using a fluorescent microscope (Olympus BX 51). Myocytes were observed for apoptosis and quantified by counting the percentage of cells containing fragmented nuclei.

**Evaluation of Cellular Damage**

Cellular damage was evaluated by measurement of the release of creatine kinase (CK) in the culture medium after treatment. A spectrophotometric assay was performed for CK with a UV-Rate assay kit (Stanbio Laboratory, Boerne, TX).

**Protein Estimation**

Total protein concentration was determined using BSA as the standard (6).

**Statistical Analysis**

Data are expressed as means ± SE. For statistical analysis of the data, groups were compared by one-way ANOVA, and Bonferroni’s test was performed to identify differences between groups. A value of P < 0.05 was considered significant.

**RESULTS**

**Activation of p38 and ERK1/2 MAPKs**

Phosphorylation of p38 and ERK1/2 MAPKs was examined in cardiac myocytes exposed to TNF-α (10 ng/ml), IL-10 (10 ng/ml), and IL-10 + TNF-α (ratio 1). TNF-α exposure led to a significant increase (P < 0.001) in p38 MAPK phosphorylation compared with the control group. IL-10 treatment alone did not show any change, whereas TNF-α-induced increases in p38 MAPK phosphorylation were prevented by IL-10 (Fig. 1A).

ERK1/2 phosphorylation was decreased by 61% in TNF-α-exposed cardiac myocytes compared with the control group. There were no changes observed in ERK1/2 phosphorylation by IL-10 treatment alone, whereas TNF-α-induced decreases in ERK1/2 were completely prevented by IL-10 treatment (Fig. 1B).

**Effect of Trolox on TNF-α induced changes.** The antioxidant Trolox was used to study the role of TNF-α-induced oxidative stress in the activation of MAPKs (p38 and ERK1/2) in cardiac myocytes. Trolox completely prevented TNF-α-induced changes in p38 and ERK1/2 phosphorylation (Fig. 1, A and B). Trolox by itself had no effect on these two MAPKs.

**Oxidative Stress Experiments**

Oxidative stress was monitored in terms of production of ROS. The production of intracellular ROS was increased by 150% in TNF-α-exposed cardiac myocytes. ROS levels were not different between control and IL-10-treated cells. However, TNF-α-induced increases in ROS production were significantly prevented by IL-10. H₂O₂, used as a positive control, significantly increased (+150%) ROS production in cardiac myocytes as measured by green fluorescence intensity (Fig. 2).

**TNF-α-Induced Apoptosis and Cell Injury and Modulation by IL-10**

The pro- to anti-apoptotic protein (Bax to Bcl-xl) ratio was significantly increased (P < 0.001) in cardiac myocytes exposed to TNF-α. IL-10 alone did not show any effect on the Bax-to-Bcl-xl ratio, whereas TNF-α induced an almost 400% increase in the Bax-to-Bcl-xl ratio, which was significantly (P < 0.001) prevented by IL-10 (Fig. 3A). Antioxidant Trolox treatment also completely prevented TNF-α-induced increases in the Bax-to-Bcl-xl ratio. Trolox by itself had no effect on this ratio in untreated cardiac myocytes (Fig. 3A).

Cell injury was evaluated by the measurement of CK release by cells in the culture medium after treatment with TNF-α, IL-10, and Trolox. TNF-α exposure led to an increase in
cellular damage in cardiac myocytes, as it significantly increased (P < 0.001) CK activity. IL-10 alone did not show any significant change, whereas TNF-α-induced increases in CK release by cardiac myocytes were significantly (P < 0.001) prevented by IL-10. Similarly, H₂O₂ treatment significantly increased numbers of apoptotic cells (Fig. 4, A and B).

**Effects of p38 and ERK1/2 MAPK Inhibition on TNF-α-Induced Apoptosis**

Cardiac myocytes were pretreated with 25 μM SB-203580 (a p38 MAPK inhibitor) or 25 μM PD-98059 (an ERK1/2 inhibitor) for 15 min (Fig. 5, A and B). In the presence of the p38 MAPK inhibitor, TNF-α exposure did not cause any significant change in ROS generation (Fig. 5A). Furthermore, there were no significant increases observed in the Bax-to-Bcl-xl ratio (Fig. 6A) as well as in numbers of apoptotic nuclei (Fig. 6B). IL-10 treatment alone as well as along with TNF-α did not show any changes in ROS generation and apoptosis.

Pretreatment for 15 min with the ERK1/2 inhibitor significantly (P < 0.001) increased ROS generation in TNF-α-exposed cardiac myocytes (Fig. 5B). Modulation of TNF-α-induced oxidative stress by IL-10 treatment was not apparent after inhibition of ERK1/2 (Fig. 5B). Furthermore, TNF-α-induced increases in the Bax-to-Bcl-xl were not tempered by IL-10 in ERK1/2-inhibited cardiac myocytes (Fig. 7A), and

![Fig. 1. A and B, top: Western blot analysis of the effects of treatment with TNF-α (10 ng/ml), IL-10 (10 ng/ml), IL-10 + TNF-α (ratio 1), Trolox (20 μmol/l), and Trolox + TNF-α for 4 h on MAPK [p38 (A) and ERK1/2 (B)] phosphorylation in adult rat cardiac myocytes. β-Actin was used as an internal control. Bottom, histogram showing densitometric analysis. Values are ratios of phosphorylated to total p38 or ERK1/2 expression. Relative levels of protein expression were normalized to β-actin. Data are expressed as means ± SE from 4 experiments. **P < 0.01 and ***P < 0.001, significantly different from its control; ###P < 0.01 and ####P < 0.001 vs. TNF-α.

Cardiac myocyte apoptosis was also studied after cells had been stained with Hoescht 33258. In control culture dishes, cells were rod shaped and binucleated, and nuclei had a normal appearance. TNF-α exposure resulted in nuclear fragmentation, and numbers of apoptotic cells significantly (P < 0.001) increased compared with the control group. IL-10 treatment alone did not show any significant change, whereas TNF-α-induced increases in apoptotic cells were significantly (P < 0.001) prevented by IL-10. Similarly, H₂O₂ treatment significantly increased numbers of apoptotic cells (Fig. 4, A and B).
modulation of TNF-α-induced cardiac myocyte apoptosis by IL-10 was completely prevented by ERK1/2 inhibition (Fig. 7B).

**DISCUSSION**

Previously, we have shown that TNF-α increases oxidative stress and downregulates antioxidant enzymes in isolated cardiac myocytes (15). IL-10 was shown to modulate this effect of TNF-α (15). The present study demonstrates that TNF-α-induced oxidative stress is capable of upregulating p38 MAPK phosphorylation and downregulates ERK1/2 phosphorylation with the net result of an activation of the apoptotic signaling cascade and apoptosis. IL-10, on the other hand, had no effect on baseline values, but it prevented TNF-α-induced changes.

The present data showed that direct exposure of cardiac myocytes to TNF-α resulted in an increase in oxidative stress, as indicated by an increase in ROS signals. In this regard, it has been shown that oxidative stress mediates TNF-α-induced mitochondrial DNA damage and dysfunction in cardiac myocytes (28). TNF-α has been reported to stimulate oxidative stress in the failing heart in patients with dilated cardiomyopathy (32) and with heart failure (9, 12). Exogenous administration of IL-10 has been shown to protect against TNF-α-induced oxidative stress. Since in nonchallenged cardiac myocytes IL-10 caused no changes in levels of intracellular ROS generation, IL-10 may be acting as an internal antagonist to TNF-α-induced oxidative stress and the cascade of changes (15). It has been reported that IL-10, at a dose of 10 ng/ml, inhibited TNF-α release from human peripheral blood mononuclear cells (5). Furthermore, IL-10 has been shown to suppress LPS-induced, inflammatory stimulus-mediated increases in TNF-α release from LPS-stimulated human peripheral blood mononuclear cells (5).
in ROS (13). IL-10 treatment reduced renal ischemia-reperfusion-induced lipid peroxidation and improved the redox ratio (16). The present study, however, reports for the first time that IL-10 modulation of TNF-α-induced oxidative stress may be at a site downstream to its receptor activation.

This increase in oxidative stress is also associated with an increase in cardiac myocyte injury, as has been shown in isolated cells as well as in vivo hearts (4, 8, 33). In the present study, TNF-α induced an increase in CK release, which was prevented by IL-10 treatment as well as by Trolox, suggesting that IL-10 acts downstream to prevent this effect of TNF-α. In a recent study (15) using H₂O₂ as an exogenous source of oxidative stress, a significant increase in CK release was reported.

In cardiac myocytes, apoptosis has been implicated in the pathogenesis of heart failure of numerous etiologies, including myocarditis, ischemia-reperfusion injury, chronic pressure overload, and congestive heart failure. The balance between proapoptotic (Bax) and antiapoptotic (Bcl-xl) proteins determines the ability of cells to either survive or undergo apoptosis after a certain stimulus or injury. In the present study, TNF-α caused a significant increase in apoptosis, as confirmed by increases in the Bax-to-Bcl-xl ratio and increases in numbers of apoptotic nuclei. TNF-α-induced cardiac myocyte apoptosis has also been suggested to be mediated by a sphingosine-dependent mechanism (17), induction of matrix metalloproteinase-2 activity (27), and the calpain/caspase-12 apoptotic pathway (2). In the present study, TNF-α-induced cardiac myocyte apoptosis was significantly decreased by IL-10. In this regard, it has been demonstrated that IL-10 regulates apoptosis by upregulating the expression of Bcl-xl in lymphocytes (20). Recombinant human IL-10 has also been reported...
to increase the expression of antiapoptotic protein Bcl2 and cell survival in primary human CD34+ hematopoietic progenitor cells (35). The fact that TNF-α-induced oxidative stress causes apoptosis was further confirmed by the decrease in TNF-α-induced apoptosis by the antioxidant Trolox. Trolox, a water-soluble antioxidant, has been found to scavenge lipid peroxyl radicals, thus preventing the lipid peroxidation cascade from damaging cellular and mitochondrial membranes. In this regard, Trolox protects different cells from ROS damage and reduces the oxidative stress induced by ischemia-reperfusion injury (24).

Oxidative stress is known to induce apoptosis in a variety of cell types, apparently by modifying intracellular signaling pathways. MAPKs are important transducers of cell signaling that coordinate the cellular response to various types of stimuli including TNF-α (19). p38 MAPK and JNK MAPK pathways are activated weakly in response to growth factors but are strongly activated in response to stresses such as UV radiation, ROS, osmotic changes, and TNF-α (18). In the present study, we have shown that TNF-α exposure led to a significant increase in p38 MAPK phosphorylation and downregulated pathways.
ERK1/2 phosphorylation. In this regard, it has been reported that TNF-α-induced p38 MAPK activation increases protein phosphatase 2A activity, which further downregulates the ERK pathway (11). TNF-α is also responsible for the activation of p38 MAPK through the activation of TNF-α receptor-associated factor (3).

Exposure of cells to SB-203580, a specific p38 MAPK inhibitor, prevented TNF-α-induced increases in oxidative stress and apoptosis, which further implies that p38 MAPK is a downstream target of TNF-α-induced oxidative stress in cardiac myocytes that activates the apoptotic signaling cascade. TNF-α-induced changes in MAPKs in the present study were also modulated by IL-10. It has been reported that IL-10 activates ERK1/2 by inducing tyrosine phosphorylation, therefore supporting cell survival and cell protection (23). Activation of ERK MAPK protects cardiac myocytes from oxidative stress-induced cell death (1). A growing number of reports have pointed out the role of ERK1/2 in counteregulating the proapoptotic effects elicited by p38 MAPK and JNK MAPK activation (26, 36, 37). In our study, TNF-α-induced decreases in ERK1/2 were also prevented by IL-10, thereby preventing TNF-α-induced apoptosis. Conversely, inhibition of the ERK MAPK pathway with PD-98059 attenuated the protective role of IL-10 against TNF-α-induced apoptosis.

In conclusion, this study provides evidence in support of the essential role of p38 and ERK1/2 MAPKs in the interactive pathway of TNF-α-induced oxidative stress, activation of MAPKs and IL-10 in regulating cardiac myocyte apoptosis. Stimulation of IL-10 or its downstream signaling pathways holds a good therapeutic potential against TNF-α-mediated cardiomyocyte dysfunction.

GRANTS

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