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Modulation of the Thioredoxin System During Inflammatory Responses and Its Effect on Heme Oxygenase-1 Expression

KUNIAKI EJIMA,[1,2,4] MATTHEW D. LAYNE,[1–3] IRVITH M. CARVAJAL,[1,2] HIROKI NANRI,[4] BONNA ITH,[1,2] SHAW-FANG YET,[1–3] and MARK A. PERRELLA[1–3]

ABSTRACT

Heme oxygenase (HO) enzymes catalyze the initial reaction in heme catabolism. HO-1 is an inducible isoform that is up-regulated by diverse stimuli, including inflammatory cytokines and factors that promote oxidative stress. HO-1 is a cytoprotective enzyme that degrades heme, a potent oxidant, to generate carbon monoxide, biliverdin (subsequently reduced to bilirubin), and iron. Recently, we found that thioredoxin (TRX), a disulfide reductase enzyme known to be important for the binding of transcription factors to DNA, contributes to the induction of HO-1 by inflammatory mediators. In the present study, we extended this observation and determined that, similar to HO-1, TRX and TRX reductase (TR) are induced by bacterial lipopolysaccharide in macrophages at the level of mRNA and protein. However, maximal induction of TRX and TR precedes that of HO-1. Increased expression of HO-1 in the cytoplasm of inflammatory cells corresponds to a translocation of TRX into the nucleus of these cells. Finally, transfection of TRX into macrophages promoted an increase in HO-1 protein. Taken together, these data support the concept that the TRX system contributes to the up-regulation of HO-1 under conditions associated with increased oxidative stress. *Antioxid. Redox Signal.* 4, 569–575.

INTRODUCTION

HEME OXYGENASE (HO) ENZYMES catalyze the initial reaction in heme catabolism (5, 14, 15). HO-1 is the 32-kDa isoform that is induced by diverse stimuli, including inflammatory cytokines and factors that promote oxidative stress *in vitro* (1, 12) and *in vivo* (3, 6, 13, 31, 33, 35, 36). HO-1 is a cytoprotective enzyme (20) that degrades heme, a potent oxidant, to generate carbon monoxide, biliverdin, and iron. Carbon monoxide is a vasodilatory gas that recently has been reported to have antiinflammatory properties (21, 22), biliverdin is reduced to bilirubin that has antioxidant properties (27), and iron is sequestered by ferritin. Taking into account the properties of HO-1 and the subsequent products of heme catabolism, HO-1 appears to play an important role in protecting cells and tissues in the setting of increased oxidative stress.

The extracellular environment and the surface of cells contain proteins that are rich in disulfide bonds. The presence of these disulfide bonds reflects an oxidative extracellular environment. In contrast, the inside of the cell is kept in a reduced state through the actions of disulfide reductases. One of the major disulfide reductases that helps to maintain this reduced intracellular environment is thioredoxin (TRX) (2, 10). TRX itself is kept in a reduced form by TRX reductase (TR) and NADPH, collectively known as the TRX system. In addition to its role in maintaining intracellular proteins in a reduced state, TRX has been shown to regulate the activation of transcription factors (8, 9, 26). In its reduced form, TRX can reactivate oxidatively inactivated transcription factors, including the activator protein-1 (AP-1) family members, Jun and Fos (19). TRX does not interact directly with AP-1 proteins, but TRX activates AP-1 by directly associating with intranuclear redox factor 1 (Ref-1) (8, 25). Ref-1 then reduces

[1]Pulmonary and Critical Care and [2]Cardiovascular Divisions, Brigham and Women's Hospital, Boston, MA 02115.
[3]Department of Medicine, Harvard Medical School, Boston, MA 02115.
[4]Department of Health Development, University of Occupational and Environmental Health, Kitakyushu 807-8555, Japan.

conserved cysteines in Fos and Jun, and enhances their DNA-binding activity (34). TRX is typically located in the cytosol (11), but it translocates into the nucleus and interacts with Ref-1 in response to various stimuli associated with oxidative stress.

Recently, we showed that TRX is capable of increasing the promoter activity of HO-1 (31). These data suggested that beyond its ability to regulate the function of proteins through thiol-disulfide exchange reactions, TRX may also have beneficial effects during oxidative stress by transcriptionally upregulating HO-1, a cytoprotective enzyme. Thus, the present study was designed to extend our previous observations and determine whether: (a) TRX and TR (cytoplasmic form of TR) are regulated in a manner comparable to HO-1 in inflammatory cells exposed to lipopolysaccharide (LPS), an important pathophysiologic oxidative stimulus; (b) TRX is expressed and translocated from the cytoplasm to the nucleus, in a temporal pattern consistent with HO-1 expression, in inflammatory cells *in vitro* and *in vivo;* and (c) TRX can regulate the expression of HO-1 protein.

MATERIALS AND METHODS

Materials

LPS from *Escherichia coli* (serotype 026:B6) was obtained from Sigma (St. Louis, MO, U.S.A.). Recombinant human TRX and TRX antibody for immunostaining (goat anti-human) were purchased from American Diagnostica (Greenwich, CT, U.S.A.). The TRX and TR antibodies used for western blot analysis were generated in the laboratory of Dr. Hiroki Nanri (7). HO-1 antibody was purchased from StressGen Biotechnology Corp (Victoria, BC, Canada). Bio-PORTER protein delivery reagent was obtained from Gene Therapy Systems, Inc. (San Diego, CA, U.S.A.).

Cell culture

RAW 264.7 (RAW) cells, a murine macrophage cell line, were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.) and maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Western blot analyses

RAW cells were plated on 10-cm culture dishes (4×10^6 cells/dish) and allowed to grow for ~12–16 h. To obtain whole-cell protein extracts, cells were washed twice with cold phosphate-buffered saline (PBS) and collected in lysis buffer [25 m*M* Tris-HCl (pH 7.4), 50 m*M* NaCl, 0.5% sodium deoxycholate, 2% NP-40, 0.2% sodium dodecyl sulfate] containing complete protease inhibitor (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.). LPS (doses ranging from 10 to 500 ng/ml) was added to the culture medium, and cells were harvested at different time points (0, 6, 12, and 24 h). An equal amount of soluble protein was analyzed by western blotting as described (36) using either a polyclonal HO-1 antiserum diluted 1:1,000, a polyclonal TRX antiserum diluted 1:1,000, or a polyclonal anti-TR antiserum diluted 1:200. Specific proteins were detected with an enhanced chemiluminescence reagent (Pierce Chemical Co., Rockford, IL, U.S.A.) and evaluated by densitometric analysis with the NIH Image software (National Institutes of Health). For each western blot performed, an identical protein gel was run and stained with Coomassie Brilliant Blue R 250 staining solution to verify protein loading.

Northern blot analyses

LPS (doses ranging from 10 to 500 ng/ml) was added to the culture medium, and cells were harvested at different time points (0, 6, 12, and 24 h) after LPS administration. Total RNA was obtained from cultured RAW cells by silica gel-membrane spin technology (RNeasy Mini kit, Qiagen, Valencia, CA, U.S.A.). RNA was fractionated on 1.3% formaldehyde-agarose gels and transferred to nitrocellulose filters. The filters were hybridized at 68°C for 1 hour with ^{32}P-labeled rat HO-1 cDNA, human TRX cDNA, or mouse TR cDNA in QuickHyb solution (Stratagene, La Jolla, CA, U.S.A.). Hybridization with the HO-1, TRX, and TR probes revealed single bands (HO-1, ~1.3 kb; TRX, ~0.5 kb; and TR, ~3.3 kb). The hybridized filters were then washed in 0.2× saline–sodium citrate buffer, 0.1% sodium dodecyl sulfate at 55°C and autoradiographed with Kodak XAR film at −80°C for 2–12 h or phosphor screens for 1–2 h. To correct for differences in RNA loading, the filters were hybridized with a ^{32}P-labeled oligonucleotide probe complementary to 18S ribosomal RNA. Radioactivity was measured on a PhosphorImager running the Image Quant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Mouse model of ischemia and reperfusion in the heart

Mice were subjected to 1 h of myocardial ischemia and 24 h reperfusion as described previously (37). All ischemia and reperfusion experiments in mice were performed in accordance with National Institutes of Health guidelines and approved by the Harvard Medical Area Standing Committee on animals.

Immunostaining procedures

For immunocytochemistry, RAW cells were plated on coverslips (Lab-Tek II, Naperville, IL, U.S.A.) and grown in medium (2.5×10^4 cells/well) for 12 h. Cells were then stimulated in the absence (vehicle) or presence of LPS (10 ng/ml) for 24 h. Cells were then washed twice with PBS and fixed in 4% paraformaldehyde for 15 min. After washing twice with Cadenza buffer (Shandon, Pittsburgh, PA, U.S.A.), endogenous peroxidase activity was inactivated with 3% hydrogen peroxide for 20 min. After two more washes with Cadenza buffer, blocking solutions were made in Cadenza buffer containing 10% normal goat serum for the analysis of HO-1, or 10% normal rabbit serum for the analysis of TRX. After 20 min, the cells were incubated with rabbit anti-human HO-1 (1:2,000 in Cadenza) or goat anti-human TRX (1:2,000 in Cadenza) for 1 h at room temperature. The secondary antibodies used were biotinylated goat anti-rabbit IgG for the

analysis of HO-1 or rabbit anti-goat IgG for the analysis of TRX, diluted 1:200 in Cadenza buffer. The incubation was carried out at room temperature for 1 h. After washing twice with Cadenza buffer, slides were incubated for 1 h at room temperature in avidin and then developed with peroxidase diaminobenzidine kit (Vector Laboratories Inc., Burlingame, CA, U.S.A.). For immunohistochemistry, ventricles of mice subjected to myocardial ischemia and reperfusion were fixed in methyl Carnoys solution and embedded in paraffin. To detect the expression of HO-1 and TRX proteins, we also immunostained the heart sections with HO-1 and TRX antibodies as described previously (31, 35, 37). Counterstaining with methyl green was performed for immunohistochemistry to localize nuclei of the cells.

Transfections

RAW cells were transfected using the BioPORTER protein delivery reagent. Cells were plated on six-well tissue culture dishes (2.5×10^5 cells/well) and allowed to grow for 12–16 h. After recombinant human TRX protein was diluted in PBS, TRX was added to the tubes containing dry film of BioPORTER reagent and incubated for 5 min at room temperature. Cells were washed with serum-free medium, and then incubated with serum-free medium containing TRX protein for 3 h at 37°C. After the cells were washed three times with PBS, the cell extracts were prepared. The expression of HO-1 protein and the transfection efficiency of TRX were examined using western blot analyses and visual inspection after immunostaining, respectively.

RESULTS

Analysis of TR, TRX, and HO-1 proteins in macrophages exposed to LPS

To assess the regulation of TRX and TR proteins by an oxidative stimulus in comparison with HO-1 protein, we exposed RAW cells to LPS and performed western blot analyses. Both TRX and TR proteins increased in a dose- and time-dependent manner (Fig. 1A and B, respectively), similar to HO-1 protein. Peak induction of TRX (threefold) and TR (threefold) occurred with the LPS dose of 500 ng/ml (Fig. 1A), and protein levels for TRX and TR were increased as early as 6 h, preceding the most robust HO-1 protein induction (sixfold at 24 h) (Fig. 1B).

Analysis of TR, TRX, and HO-1 mRNA in macrophages exposed to LPS

The levels of TRX, TR, and HO-1 mRNA in RAW cells exposed to LPS were also examined (Fig. 2). The mRNA levels of TRX and TR were maximally induced by LPS after 6 h, similar to protein levels as shown in Fig. 1B. This induction preceded the most robust induction of HO-1 (12 and 24 h) by LPS in the macrophages (Fig. 2). Taken together with Fig. 1, these data suggest TRX and TR are induced by an oxidative stimulus such as LPS in macrophages, and the maximal induction of TRX and TR appears to precede that of HO-1.

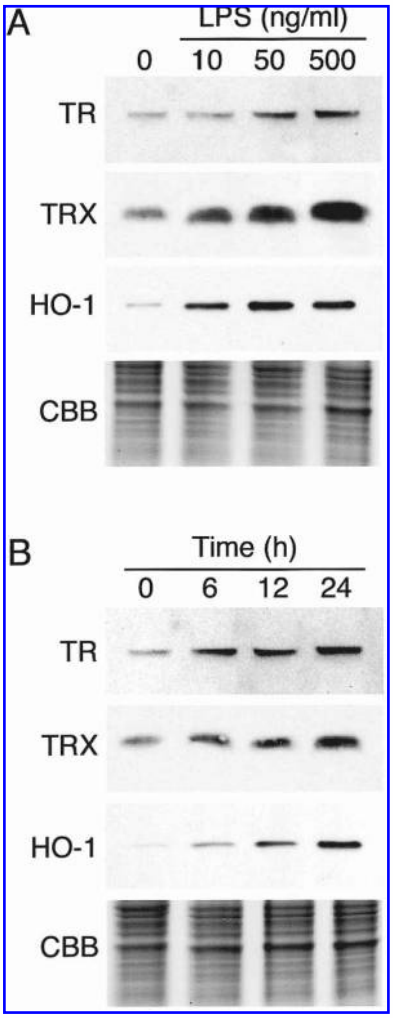

FIG. 1. Effect of LPS on TR, TRX, and HO-1 protein levels in macrophages. (A) Total protein extract (30 μg/lane) from RAW cells after 24 h of LPS stimulation (at the indicated concentrations) was subjected to western blotting using antibodies directed against TR, TRX, and HO-1. **(B)** RAW cells were stimulated with LPS (500 ng/ml), and total protein was harvested at various time points (0, 6, 12, and 24 h). Western blot analysis was performed as described in A. For A and B, identical gels were run and stained with Coomassie Brilliant Blue (CBB) to assess the equality of protein loading. Data in A and B show a representative experiment that was performed three separate times.

Translocation of TRX and induction of HO-1 in macrophages exposed to LPS in vitro

For TRX to have its effect on gene transcription, it must translocate into the nucleus of cells. Thus, to determine whether the translocation of TRX into the nucleus of macrophages occurs after LPS stimulation, and to determine whether this translocation occurs in conjunction with an increase in HO-1 expression, we immunostained RAW cells exposed to vehicle (Control) or LPS. In the absence of LPS, faint staining for TRX (Fig. 3A, upper left panel) and HO-1 (Fig. 3A, lower left panel) was present in the cytoplasm of

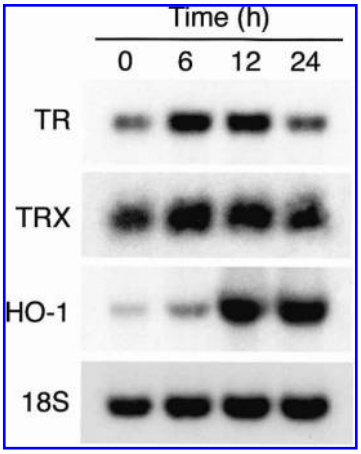

FIG. 2. Effect of LPS on TR, TRX, and HO-1 mRNA levels in macrophages. RAW cells were stimulated with LPS (500 ng/ml) and harvested at various time points (0, 6, 12, and 24 h). Total RNA (10 μg/lane) harvested from the RAW cells was subjected to northern blotting and hybridized with ^{32}P-labeled mouse TR cDNA, human TRX cDNA, and rat HO-1 cDNA. RNA loading was assessed by hybridization with 18S. Data in this figure show a representative experiment that was performed three separate times.

RAW cells. However, after the addition of LPS (10 ng/ml) for 24 h, intense staining for TRX was present in the nuclei of macrophages (Fig. 3A, upper right panel), and this staining corresponded to an intense cytoplasmic and perinuclear staining for HO-1 (Fig. 3A, lower right panel). Time-course experiments were also performed and demonstrated that TRX translocation and HO-1 induction occurred as early as 6 h after LPS stimulation (data not shown). These data demonstrate that, beyond an increase in the expression of TRX, LPS promotes a translocation of TRX into the nucleus that corresponds to increased HO-1 protein expression.

Induction of TRX and HO-1 in inflammatory cells after ischemia and reperfusion in the heart in vivo

To examine the expression of TRX and HO-1 proteins under inflammatory conditions *in vivo,* hearts from mice subjected to 1 hour of ischemia and 24 h of reperfusion injury were analyzed immunohistochemically. Inflammatory cells (predominantly neutrophils; data not shown) infiltrating the hearts after ischemia and reperfusion injury were strongly stained for both TRX and HO-1 (Fig. 3B, left and right panels, respectively, arrows).

Expression of HO-1 in macrophages transfected with TRX

To determine whether TRX may contribute to the up-regulation of HO-1, we examined the expression of HO-1 protein in RAW cells transfected with TRX protein. Western blot analysis revealed that HO-1 protein levels increased in RAW cells after transfection (3 h) with TRX protein, and peak induction of HO-1 (threefold) occurred at a TRX dose of 1 μg/ml (Fig. 4). Western blot analysis confirmed that the level of TRX increased in the cells (Fig. 4), and this increase was consistent with the increase in amount of TRX transfected. The threefold induction of HO-1 protein by TRX is certainly an underestimation of the peak HO-1 response as transfection efficiency of TRX protein into RAW cells was determined to be ~40%. Nevertheless, these data demonstrate that increased amounts of TRX protein lead to an increase in HO-1 protein expression in macrophages.

DISCUSSION

HO-1 is a cytoprotective enzyme that plays an important role in the host defense against oxidative stress (20). This concept has been confirmed by studies assessing HO-1-deficient or HO-1-overexpressing mice exposed to different pathophysiologic states (4, 18, 23, 24, 32, 33, 36, 37, 38). Mice deficient in HO-1 are at increased risk for the consequences of oxidative stress in conditions such as chronic hypoxia (36), endotoxemia (24, 32), and ischemia in the kidney (33) and the heart (38). Targeted overexpression of HO-1 in cardiomyocytes (37) or neurons (4, 23) protects against the detrimental consequences of ischemia and/or reperfusion injury in the heart or brain, respectively. Moreover, overexpression of HO-1 in type II pneumocytes prevents the pulmonary inflammatory and vascular responses to chronic hypoxia in the lung (18).

The TRX system, similar to HO-1, has cytoprotective properties against oxidative stress and cell death (2, 30). This concept has also been confirmed *in vivo* by using transgenic mice overexpressing TRX (28). For example, Takagi and colleagues demonstrated that overexpression of TRX attenuated focal ischemic brain damage in mice (28). As discussed previously, beyond its cytoprotective role and its ability to maintain intracellular proteins in a reduced state, TRX has been shown to be important for the binding of transcription factors to DNA (8, 9, 26). Recently, we demonstrated that TRX contributes to the AP-1-driven, transcriptional up-regulation of HO-1 by inflammatory cytokines and LPS (31). Our study provided the first evidence that these two cytoprotective factors may be linked during exposure to pathophysiologic stimuli.

Both TRX and TR are induced by LPS in macrophages at the level of protein (Fig. 1) and mRNA (Fig. 2), and maximal induction of TRX and TR precedes that of HO-1. At the point in time in which HO-1 protein is induced by LPS in the cytoplasm of macrophages *in vitro* (Fig. 3A, lower right panel), TRX has translocated into the nucleus of these cells (Fig. 3A, upper right panel). A similar response occurs *in vivo,* as TRX is expressed in inflammatory cells of the heart after ischemia and reperfusion injury at the same time as HO-1 is expressed (Fig. 3B). Previously, we showed that TRX translocated into the nucleus of vascular smooth muscle cells, *in vitro* and *in vivo,* exposed to inflammatory cytokines and LPS (31). This response has also been shown *in vivo* in placental tissue after exposure to LPS (7), in the proximal tubules of kidneys exposed to ferric nitrilotriacetate (29), and *in vitro* in HeLa cells exposed to ultraviolet irradiation (17), phorbol 12-myristate 13-acetate (8), and hydrogen peroxide (16).

To determine more definitively whether TRX contributes to the regulation of HO-1, particularly at the protein level, we

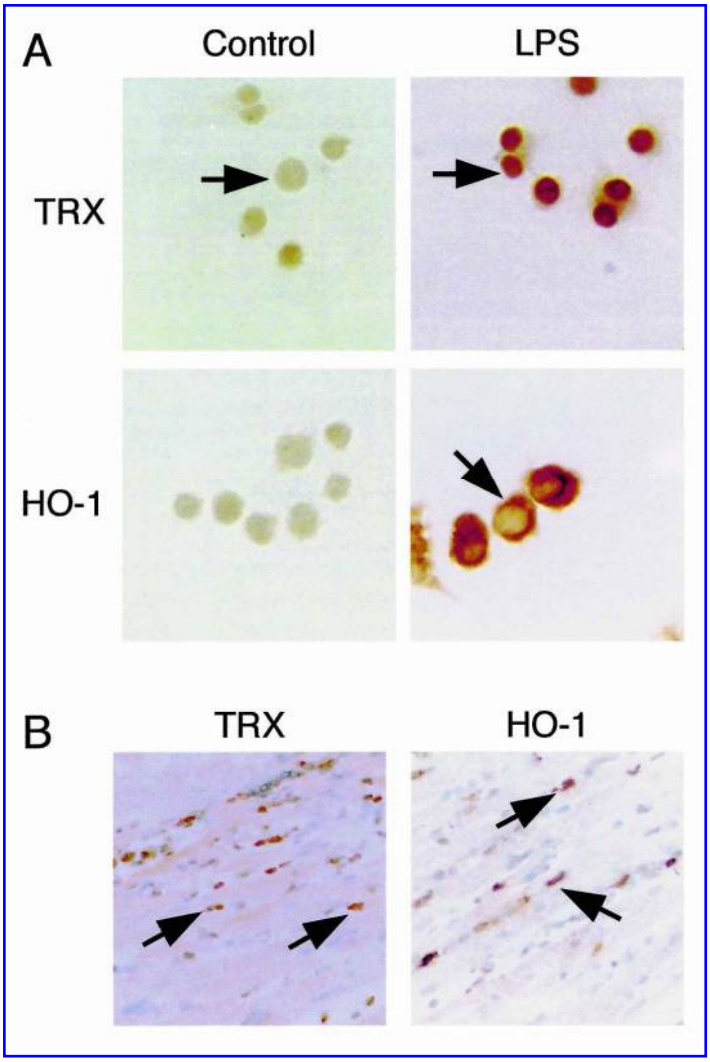

FIG. 3. Response of TRX and HO-1 to oxidative stimuli *in vitro* and *in vivo*. **(A)** Effect of LPS on localization and expression of TRX and HO-1 in cultured macrophages. RAW cells were treated with vehicle control (upper left and lower left panels) or LPS (10 ng/ml, upper right and lower right panels) for 24 h. Immunocytochemical analysis was performed as described under Materials and Methods. Arrows indicate the localization of TRX (upper left and upper right panels) or HO-1 (lower left and lower right panels) protein. Original magnification: ×400. Data in this figure show a representative experiment that was performed three separate times. **(B)** Effect of myocardial ischemia and reperfusion on the expression of TRX and HO-1 in inflammatory cells. Mouse hearts were subjected to 1 h of ischemia and 24 hours of reperfusion. Immunohistochemical analysis was performed as described under Materials and Methods. Arrows indicate the expression of TRX (left panel) or HO-1 (right panel) protein in inflammatory cells of the hearts. Original magnification: ×200. Data in this figure show a representative experiment that was performed three separate times.

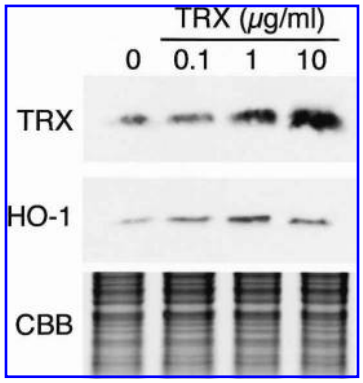

FIG. 4. Effect of TRX on HO-1 protein expression in macrophages. RAW cells were transfected with TRX protein at the indicated concentrations. Three hours after transfection, total protein extracts were prepared and 30 μg/lane was subjected to western blotting using antibodies directed against TRX and HO-1. An identical gel was run and stained with Coomassie Brilliant Blue (CBB) to assess the equality of protein loading. Data in this figure show a representative experiment that was performed two separate times.

transfected macrophages with reduced, recombinant TRX and assessed HO-1 protein levels. As demonstrated in Fig. 4, HO-1 protein levels increased in RAW cells after transfection with increasing amounts of TRX protein, and peak induction of HO-1 protein occurred at a TRX dose of 1 μg/ml. These data confirmed and extended our previous observation that TRX produced an increase in HO-1 promoter activity (31). Moreover, we have also previously demonstrated that an inhibitor of TR can decrease the induction of HO-1 by inflammatory cytokines and LPS (31). Taken together, these data support the concept that the TRX system contributes to the up-regulation of HO-1 under conditions associated with inflammation and increased oxidative stress.

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ABBREVIATIONS

AP-1, activator protein-1; HO-1, heme oxygenase-1; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; RAW, RAW 264.7; Ref-1, redox factor 1; TR, thioredoxin reductase; TRX, thioredoxin.

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Address reprint requests to:
Dr. Mark A. Perrella
Brigham and Women's Hospital
Pulmonary and Critical Care Division
75 Francis Street
Boston, MA 02115

E-mail: mperrella@rics.bwh.harvard.edu

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