Extracellular Human Thioredoxin-1 Inhibits Lipopolysaccharide-induced Interleukin-1β Expression in Human Monocyte-derived Macrophages*

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Ludivine Billiet†, Christophe Furman†, Guilhem Larigauderie‡, Corinne Copin‡, Korbinian Brand‡, Jean-Charles Fruchart†, and Mustapha Rouis†,‡

From the †U-545 INSERM, Institut Pasteur de Lille and Université Lille 2, 59019 Lille, France and the ‡Institute of Clinical Chemistry and Pathobiochemistry Klinikum rechts der Isar, Technische Universität München, Ismaninger Strasse 22, 81675 München, Germany

Oxidative stress plays an important role in atherosclerotic vascular disease, and several recent studies were focused on thioredoxin-1 (Trx-1) and its potential protective role against oxidative stress. Since human monocyte-derived macrophages (HMDM) are important cells in several inflammatory diseases including atherosclerosis, we conducted this study to evaluate the impact of extracellular recombinant human Trx-1 (rhTrx-1) on gene expression in lipopolysaccharide-activated HMDM. Our results showed that rhTrx-1 was capable of reducing interleukin (IL)-1 β mRNA and protein synthesis in a dose-dependent manner. This effect was partly mediated through a reduction of NF-κB activation as analyzed by transient transfection and gel shift assays. In addition, we showed that the attenuation of NF-κB activity was the result of the reduction of both p50 and p65 subunit mRNA and protein synthesis on one hand and of the induction of IκBα mRNA and protein expression on the other hand. Moreover, inhibition of endogenous Trx-1 mRNA was also observed, suggesting a contribution to the diminution of NF-κB activity since endogenous Trx-1, in contrast to the exogenous Trx-1, activates the NF-κB system. Finally, H2O2-oxidized rhTrx-1 reduced IL-1β mRNA synthesis in lipopolysaccharide-activated HMDM. This result highly suggested that the rhTrx-1 used in this study could be oxidized in the culture medium and, in turn, reduced IL-1β mRNA and protein synthesis. Taken together, these data indicated a potential new mechanism through which extracellular rhTrx-1 exerts an anti-inflammatory function in HMDM.

Increasing evidence indicates that oxidative stress plays an important role in atherosclerotic vascular disease (1). However, the protective mechanisms of antioxidants, including dietary antioxidants such as ascorbate (vitamin C) and α-tocopherol (vitamin E), have not been well defined (1). Nevertheless, recent attention was focused on thioredoxin-1 (Trx-1) and its potential protective role against oxidative stress (see review (2)). Indeed, Trx-1, a 12-kDa highly conserved protein in almost all species, is the major carrier of redox potential in cells (3). Trx-1 functions by the reversible oxidization of two Trx-specific redox-active cysteine residues (Cys-32 and Cys-35) to form a disulfide bond that, in turn, can be reduced by the action of Trx-1 reductase and NADPH.

Intracellular Trx-1 exerts most of its antioxidant properties through scavenging of reactive oxygen species (4). In addition, it acts as a cofactor for several enzymes such as nucleoside diphosphate reductase (5), T7 DNA polymerase (6), and 3-phosphoadenosylsulphate reductase (7). Moreover, mammalian Trx-1 plays an important role in the regulation of redox-sensitive transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) (8, 9) and in the regulation of glucocorticoid receptor-mediated signal transduction (9–11).

In addition to its intracellular role, intact Trx-1 and its truncated form (Trx-80) can be released by cells and exert both cytokine-like and chemokine-like activities (12–16). Indeed, Trx-1 secretion has been reported to occur in conditions associated with oxidative stress and inflammation, such as human immunodeficiency virus infection (17), hepatitis C infection (18), and rheumatoid arthritis (19). In addition, data reported in a recent review indicated that serum Trx-1 levels were found to be significantly increased in patients with acute coronary syndromes and dilated cardiomyopathy (2). Similarly, serum Trx-1 concentrations were higher in patients with several cardiovascular risk factors than in normal subjects (20). Moreover, results suggesting a possible association between Trx-1 secretion and the severity of heart failure were reported (2). In contrast, beneficial effects of elevated plasma Trx-1 levels induced by injection of recombinant human Trx-1 have been described in ischemic reperfusion injury (21, 22) and in lipopolysaccharide-induced neutrophil chemotaxis in the mouse air pouch model (23, 24). In addition, overexpression of human Trx-1 in transgenic mice attenuated focal ischemic brain damage (25). These data suggest that Trx-1 plays a number of biological roles in both intracellular and extracellular compartments.

Since human monocyte-derived macrophages (HMDM) are important cells involved in several inflammatory diseases including atherosclerosis, we conducted this study to evaluate the impact of extracellular human Trx-1 on gene expression using a DNA array approach. We found an inhibitory effect of Trx-1 on the expression of several important inflammatory genes such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8) in LPS-activated HMDM. Given the fact that monocytes, macrophages, and macrophage-derived foam cells are the main sources of IL-1β (26–31) on the one hand and the wide range of biological activities that IL-1β can exert in immunological responses and in other numerous acute and chronic inflammatory disorders including diabetes mellitus, rheuma-
toid arthritis, and atherosclerosis (32–36) on the other hand, we decided to focus our study on this cytokine to elucidate the mechanism by which recombinant human Trx-1 (rhTrx-1) inhibits its synthesis.

**EXPERIMENTAL PROCEDURES**

**Isolation and Culture of Human Monocytes**—Mononuclear cells were isolated from buffy coats of healthy normolipidemic donors using Ficoll gradient centrifugation and were subsequently cultured in RPMI 1640 medium containing gentamicin (40 μg/ml), glutamine (0.05%), and 10% pooled human serum (PromoCell, Heidelberg, Germany) at a density of 6 × 10⁶ cells/well in 60-mm well Primaria plastic culture dishes (Polylabo, Strasbourg, France). Differentiation of monocytes into macrophages was allowed to occur spontaneously by adhesion of cells to the culture dish and continued maturation by subsequent 12 days of culture. For analysis, these cells were washed three times with phosphate-buffered saline and incubated 2 h with or without LPS (Sigma) at 1 μg/ml. The cells were then incubated in the presence or absence of various concentrations of rhTrx-1 (American Diagnostica) for 6 or 24 h in the presence of RPMI medium supplemented with gentamicin (40 μg/ml), glutamine (0.05%), and 1% Nutridoma-HU.

**FIGURE 1.** Effect of rhTrx-1 on LPS-induced IL-1β mRNA and protein expression in HMDM. HMDM cells were activated with LPS (1 μg/ml) for 2 h and then treated with or without rhTrx-1 (1–5 μg/ml) for 6 (A) and 24 (B and C) h. IL-1β and cyclophilin mRNA (for normalization) were evaluated by Q-PCR. The results are the means ± S.D. of three separate experiments, each performed in triplicate (*, p < 0.05; **, p < 0.01). Secreted IL-1β protein levels were determined by ELISA (C). The results are the means ± S.D. of five separate experiments, each performed in triplicate (*, p < 0.05).

**FIGURE 2.** Effect of rhTrx-1 on LPS-induced IL-1β promoter activity in HMDM. Cells were first transiently transfected with pGL3(NF-κB)-luciferase expression vector and pCH110, a β-galactosidase vector (for normalization) for 20 h and then activated for 2 h with LPS (1 μg/ml). The cells were then untreated or treated with rhTrx-1 (5 μg/ml) for an additional 24 h and lysed, and both luciferase and β-galactosidase activities were determined according to “Experimental Procedures”. The results are the means ± S.D. of five separate experiments, each performed in triplicate (*, p < 0.01).
(Roche Diagnostics). The viability of the cells was assessed by measuring lactate dehydrogenase release (lactate dehydrogenase kit, Roche Diagnostics). No statistical difference was detected between the level of cytotoxicity in control and treated cells (viability ± S.D. of three separate experiments, each performed in triplicate (**, p < 0.01)). In parallel, the I-kBα protein was evaluated, and the results are the means ± S.D. of three independent Western blots (C). One representative Western blot was inserted.

**FIGURE 4.** Effect of rhTrx-1 on I-kBα mRNA and protein expression in LPS-activated HMDM. HMDM cells were activated or not with LPS (1 μg/ml) for 2 h, and then the LPS-activated cells were treated with or without rhTrx-1 (5 μg/ml) for 6 (A) or 24 (B and C) h. I-kBα mRNA was measured using Q-PCR. The results are the means ± S.D. of three separate experiments, each performed in triplicate (**, p < 0.01). In parallel, the I-kBα protein was evaluated, and the results are the means ± S.D. of three independent Western blots (C). One representative Western blot was inserted.

RNA and Protein Extraction—To obtain both total cellular protein and RNA using the PARIS kit (Ambion), whole cells were first homogenized in ice-cold cell disruption buffer. A part of the cell lysate was used for protein analysis, and the other part was immediately mixed with an equal volume of lysis/binding solution containing a high concentration of guanidinium thiocyanate for RNA purification using an RNA binding glass fiber filter. After three rapid washing steps, RNA was eluted and quantitated.

Affymetrix Oligonucleotide Arrays—First strand cDNA was synthesized by incubating 3 μg of total RNA with SuperScript II (Invitrogen) and T7-oligo(dT). Second strand cDNA was synthesized using DNA polymerase I (New England Biolabs Inc. Beverly, MA), DNA ligase (New England Biolabs), RNase H (Invitrogen), and T4 DNA polymerase (Invitrogen) as described by Park et al. (37). Double-stranded DNA was purified using phenol:chloroform:isoamyl alcohol on a Phase Lock Gel tube (Eppendorf) followed by ethanol precipitation. The DNA was dissolved in nuclease-free water. cRNA was synthesized from double-stranded cDNA using T7 RNA polymerase and purified using RNaseasy Mini kit (Qiagen, Courtaboeuf, France). cRNA was fragmented for 35 min at 94 °C in a solution of 40 mM Tris acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate. Fragmented cRNA samples were mixed with eukaryotic hybridization controls (Affymetrix), salmon sperm DNA, and acetylated bovine serum albumin and hybridized to microarrays for 16 h, rotating at 60 rpm, at 45 °C. After hybridization and washing, scanning of microarrays was performed according to the standard protocols (Affymetrix). The Affymetrix GeneChip information was extracted, and data were compared according to Park et al. (37).

Real-time PCR Conditions—For real-time PCR (Q-PCR) analysis, 5 μg of total RNA were reverse-transcribed using a random hexamer primer (Clontech). To avoid DNA contamination, a DNase I (Invitrogen) treatment was performed before each reverse transcription. All Q-PCR analyses were carried out using the Mx4000 multiplex quantitative PCR system (Stratagene). PCR cycling conditions were as follows: initial denaturation at 95 °C for 5–10 min followed by 40 cycles of 95 °C for 30 s, 1 min of annealing (annealing temperature adapted for the specific primer set used), and 1 min of extension at 72 °C. Fluorescence data were collected during the annealing stage of amplification. The presence of specific amplicon was established when fluorescence signal intensity for each molecular probe exceeded the instrument-defined calculated background noise threshold level, based on the fluorescence parameters for no-sample controls. cDNA of cyclophilin was used as an endogenous control to standardize the amount of cDNA in each sample. However, β-actin and 28S gene expressions, in contrast to glyceraldehyde-3-phosphate dehydrogenase, were not affected by rhTrx-1 treatment and therefore can be used, like cyclophilin, for normalization. The following primer pairs were used: for IL-1β, forward, 5′-CAAGCTTCTCAGGAGAATG-3′, and reverse, 5′-GCAGTTCACTGATACGTCAACAG-3′; for I-kBα, forward, 5′-TTGGGATGTAGTGCAATGCT-3′, and reverse, 5′-CTGACGTATCCACATCTAC-3′; for p50,
forward, 5′-GCAATCACCACCTTCATTC-3′, and reverse, 5′-CGTGACAGATCATTTC-3′; for endogenous Trx-1, forward, 5′-GACGCTGCAGGTGATAA-3′, and reverse, 5′-CTGACAGTCATCCACATCTAC-3′; for 28 S gene, forward, 5′-AAACTCTGGTGGAGGTCCGT-3′, and reverse, 5′-CTTACAAGTGGCCCACTA-3′; and for cyclophilin, forward, 5′-GCATACGGGTCCTGGCATCTTGTCC-3′, and reverse, 5′-ATGGTGATCTTCTTGCTGGTCTTGC-3′. cDNA standard sample was replaced with water in all no-sample controls.

Quantification of Secreted IL-1β by the ELISA Procedure—Extracellular IL-1β in HMDM culture media was quantitated by a sandwich ELISA kit (R&D Systems, Lille, France) containing two monoclonal antibodies specific to human IL-1β antigen.

Western Blot Analysis—For Western analyses, the cells were stimulated with LPS for 2 h and then with rhTrx-1 as indicated above, washed, and lysed, and the protein concentrations were measured by Peterson’s method with bovine serum albumin as the standard. Samples (25 µg) were denatured with SDS loading buffer at 95 °C for 5 min and subjected to SDS-PAGE (10% gel). The samples were transferred to a nitrocellulose membrane and probed for rhTrx-1 using anti-Trx-1 primary antibody (Abcam) (1:1000 dilution) and incubated with peroxidase-conjugated anti-mouse IgG (Bio-Rad) (1:500 dilution). Oxidized and reduced human apolipoprotein A-I (hApoA-I) were used as a negative control.

Carboxymethylation of rhTrx-1—rhTrx-1 was reduced with 4 mM DTT for 10 min at room temperature or oxidized with 1 mM H2O2 for 10 min at room temperature. Separation of the redox forms of Trx-1 was based upon the procedures of Watson et al. (38). rhTrx-1 was carboxymethylated in guanidine-Tris solution (6 M guanidine-HCl, 50 mM, Tris, pH 8.3, 3 mM EDTA, 0.5% (v/v) Triton X-100) containing 50 mM iodoacetic acid. After incubation at 37 °C for 30 min, excess iodoacetic acid was removed by Sephadex chromatography (MicroSpin G-25 columns, Amersham Biosciences). Eluates were diluted in 5× sample buffer (0.1 M Tris, pH 6.8, 10% (v/v) glycerol, 0.05% (w/v) bromphenol blue) and separated on a discontinuous native polyacrylamide gel (5% stacking gel, 15% resolving gel). Gels were electroblotted to nitrocellulose membrane and probed for rhTrx-1 using anti-Trx-1 primary antibody (Abcam) (1:1000 dilution) and incubated with peroxidase-conjugated anti-mouse IgG (Bio-Rad) (1:500 dilution). Oxidized and reduced human apolipoprotein A-I (hApoA-I) were used as a negative control.

Human IL-1β Promoter Analyses—The pGL3(NF-κB)-luciferase expression vector was made according to Gray et al. (39). Briefly, a fragment of IL-1β promoter (position −3690 to −3267) was linked

**FIGURE 5.** Effect of rhTrx-1 on p50 mRNA and protein expression in LPS-activated HMDM. HMDM cells were activated or not with LPS (1 µg/ml) for 2 h, and then the LPS-activated cells were treated with or without rh-Trx-1 (5 µg/ml) for 6 (A) and 24 (B and C) h. p50 mRNA was measured using Q-PCR. The results are the means ± S.D. of three separate experiments, each performed in triplicate (**p < 0.01). In parallel, the p50 protein level was evaluated, and the results are the means ± S.D. of three independent Western blots (C). One representative Western blot was inserted.
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upstream of a luciferase gene (KpnI-XhoI). This construct was obtained with the forward primer 5′-AGAAACTGGGACATACCAGGCGACAA-3′ and the backward primer 5′-CTCTCTCGAGGGCAGGCAATCC-3′. The pGL3(NF-κB)-luciferase plasmid was transiently transfected into the primary HMDM cells using jetPEI-Man transfection reagent (Qbiogene, Illkirch, France). A β-galactosidase expression vector (Amersham Biosciences) was co-transfected as a control for transfection efficiency.

Twenty hours following transfection, a fresh RPMI 1640 medium containing 1% Nutridoma-HU was added. Thereafter, the cells were incubated with 1 μg/ml LPS for 2 h and then with 5 μg/ml rhTrx-1. After 24 h, the cells were washed with phosphate-buffered saline, lysed in 100 μl of passive lysis buffer (Promega) at room temperature for 30 min, and subjected to luciferase (Promega) and β-galactosidase assays (Roche Diagnostics).

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assays—
Nuclear proteins were extracted from 1.2 × 10⁶ human macrophages grown in 100-mm² culture dishes that were scraped into phosphate-buffered saline and centrifuged for 10 min at 1800 × g as described previously (40). For electrophoretic mobility shift assays, the probe used was as follows. For κB, 5′-CAGAGGGCTTTCCGAGA-3′, briefly, double-stranded oligonucleotides were generated by annealing equimo-

FIGURE 6. Effect of rhTrx-1 on p65 mRNA and protein expression in LPS-activated HMDM.

HMDM cells were activated or not with LPS (1 μg/ml) for 2 h, and then the LPS-activated cells were treated with or without rhTrx-1 (5 μg/ml) for 6 (A) and 24 (B) h. p65 mRNA was measured using Q-PCR. The results are the means ± S.D. of three separate experiments, each performed in triplicate (*, p < 0.05, **, p < 0.01). In parallel, three different Western blots were performed to evaluate the p50 protein levels. The results are the means ± S.D. One representative Western blot was inserted.

FIGURE 7. Effect of exogenous rhTrx-1 on endogenous Trx-1 mRNA synthesis in HMDM.

Cells were activated or not with LPS (1 μg/ml) for 2 h, and the LPS-activated cells were treated with or without rhTrx-1 for 6 (A) and 24 (B) h. Endogenous Trx-1 mRNA levels were determined by Q-PCR. The results are the means ± S.D. of three separate experiments, each performed in triplicate (**, p < 0.01).
lar complementary oligonucleotides, either labeled with the Klenow fragment of DNA polymerase I and [α-32P]dCTP (K8) or end-labeled with [γ-32P]ATP (3000 mCi/mmol, PerkinElmer Life Sciences) using T4 polynucleotide kinase and purified by Chroma-spin 30 (Clontech) as described in Furman et al. (40).

RESULTS

To evaluate the impact of rhTrx-1 on the expression of inflammatory genes in HMDM, we stimulated the cells with LPS at 1 μg/ml for 2 h followed by the addition of rhTrx-1 at different concentrations ranging from 1 to 5 μg/ml for 6 and 24 h. The effect of rhTrx-1 on gene expression was determined using DNA array approach. The results showed a strong down-regulation of the expression of several known potent inflammatory genes such as IL-1β, IL-6, IL-8, and TNF-α in the presence of rhTrx-1 (data not shown). These results were confirmed by using the real-time PCR approach (not shown for IL-6, IL-8, and TNF-α).

Because IL-1β has a wide spectrum of inflammatory disorders including atherosclerosis, we selected this cytokine to determine the mechanism used by rhTrx-1 to inhibit its expression on HMDM.

Effect of rhTrx-1 on LPS-induced Human IL-1β Expression in HMDM—Since LPS is a well known potent inducer of IL-1β expression on macrophages (41–43), we first stimulated HMDM with LPS at 1 μg/ml for 2 h and thereafter evaluated the effect of rhTrx-1 treatment on IL-1β gene expression. IL-1β mRNA abundance (Fig. 1, A and B) as well as protein levels (Fig. 1C) were induced by LPS. The addition of rhTrx-1 for 6 or 24 h significantly reduced the mRNA levels of IL-1β in a dose-dependent manner (Fig. 1, A and B). The maximum inhibitory effect was observed at 5 μg/ml rhTrx-1 treatment. Higher concentrations up to 15 μg/ml did not enhance the inhibitory effect (not shown). At 5 μg/ml rhTrx-1, 97% of mRNA inhibition was observed after 6 h of treatment (p < 0.01) and 60% inhibition after treatment for 24 h (p < 0.05). Moreover, although IL-1β protein secretion was undetectable in cultured media after 6 h of treatment of LPS-activated HMDM with rhTrx-1, its secretion was reduced by 30% after 24 h of treatment with 5 μg/ml rhTrx-1 (p < 0.05) (Fig. 1C). In addition, treatment of HMDM with rhTrx-1 at 5 μg/ml for 6 h after their activation for 2 h with TNF-α at 10 ng/ml showed, like LPS, a decrease in p50 and p65 mRNA and an increase in 1-kbα mRNA synthesis (data not shown).

Effect of rhTrx-1 Treatment on LPS-induced Human IL-1β Promoter Activity in HMDM—A body of evidence indicates that LPS exerts its induction effect through a functional NF-κB site located between –3296 and –3287 bp in the human IL-1β promoter (39, 42, 44). Therefore, a plasmid construct designated pGL-3(NF-κB)-luciferase was made according to Gray et al. (39), and HMDM were transiently transfected and treated or untreated with rhTrx-1 (5 μg/ml). The result indicated a significant reduction (~26%, p < 0.01) in the luciferase activity in treated macrophages (Fig. 2). DNA mobility shift (Fig. 3, A and B) and supershift (not shown) assays revealed a decreased binding of the NF-κB dimer to the NF-κB motif when nuclear proteins were isolated from either 6-h or 24-h rhTrx-1-treated LPS-activated HMDM when compared with untreated LPS-activated macrophages.

Effect of rhTrx-1 on p50, p65, and 1-kbα mRNA and Protein Expressions in LPS-activated HMDM—To elucidate the mechanism by which rhTrx-1 reduced NF-κB activation, we investigated the impact of rhTrx-1 on p50, p65, and 1-kbα mRNA and protein expression in LPS-activated HMDM. Our result showed a significant induction of both 1-kbα mRNA (≥2-fold versus control, p < 0.01 for mRNA at 5 μg/ml rhTrx-1) (Fig. 4, A and B) and protein (≥2-fold versus control at 5 μg/ml rhTrx-1) (Fig. 4C). In contrast, inhibition of both p50 (Fig. 5, A and B) and p65 (Fig. 6, A and B) mRNA (30–50% inhibition versus control, p < 0.01) and p50 protein (Fig. 5C) and p65 protein (Fig. 6C) was found.

Effect of Exogenous rhTrx-1 on Endogenous Trx-1 mRNA Synthesis in HMDM—Since Trx-1, an important intracellular redox enzyme known to be induced in response to inflammation, was translocated into the nucleus to activate several transcription factors including NF-κB (8, 45), we evaluated the level of endogenous Trx-1 mRNA on LPS-activated macrophages treated with exogenous rhTrx-1 for 6 and 24 h, and the result showed a significant dose-dependent reduction of the intracellular Trx-1 mRNA abundance (Fig. 7, A and B). The maximal inhibition (~85% versus control, p < 0.01) was observed at 5 μg/ml rhTrx-1.

Effect of Oxidized or Reduced rhTrx-1 on LPS-induced IL-1β mRNA Synthesis in HMDM—Since Trx-1 functions by the reversible oxidation of two Trx-specific redox-active cysteine residues, we conducted
Experiments to identify the redox status involved in the diminution of IL-1β production in LPS-activated HMDM. Fig. 8A clearly shows a difference of the migration in a polyacrylamide gel between H2O2-treated and DTT-treated rhTrx-1, suggesting the efficiency of the oxidative modification of the protein by the H2O2 treatment. This result is in agreement with the result obtained by Watson et al. (38). Thereafter, LPS-activated HMDM were treated with the oxidized form of the rhTrx-1 or with its reduced form for 6 h. Fig. 8B indicated a significant down-regulation of IL-1β mRNA production in the presence of 1 and 5 μg/ml H2O2-treated macrophage (28% reduction, p < 0.05 and 63% reduction p < 0.01 versus control, respectively). DTT-reduced rhTrx-1 did not affect the expression of IL-1β (Fig. 8C). As a negative control, similar experiments were conducted in parallel with H2O2-oxidized or DTT-reduced hApoA-I, a major apolipoprotein involved in reverse cholesterol transport from macrophage foam cells to the liver. The results did not show any effect on IL-1β expression (Fig. 8). Finally, Fig. 9 shows a summary of the effect of LPS (Fig. 9A) and after the addition of extracellular rhTrx-1 (Fig. 9B) on LPS-activated HMDM.

Discussion

Accumulating evidence suggests that extensive inflammation occurs in arterial tissue (46, 47). Studies in animals and humans highlighted the role of inflammatory cells, mainly monocytes/macrophages, in coronary atheroma plaque formation (48). Excessive oxidant stress can regulate the activity of the redox-sensitive transcription factors, leading to the release of inflammatory cytokines and chemokines.

Trx-1, a small ubiquitous protein, is known to be an important intracellular redox regulator (49, 50). Within the cytoplasm, Trx-1 plays a role as a signaling molecule (51–53). In addition, it can be translocated to the nucleus, where it activates various intracellular proteins including transcription factors such as NF-κB, activator protein-1, and redox factor-1 (Ref-1) (45, 50). Trx-1 can also be secreted from cells and can exert important extracellular functions (17, 54–56). Nevertheless, the mechanism that mediates the extracellular Trx-1 effect, as well as its precise role in HMDM, is not well clarified.

In this study, the analysis of the mRNA expression pattern of LPS-activated HMDM treated or untreated with rhTrx-1 using the DNA array approach allowed the identification of IL-1β, IL-6, IL-8, and TNF-α, among others, as down-regulated inflammatory genes in response to rhTrx-1 (data not shown). This finding was confirmed by real-time PCR quantification (Fig. 1, A and B, for IL-1β) and the ELISA approach (Fig. 1C for IL-1β). Moreover, transfection and gel shift assays showed that extracellular rhTrx-1 was capable of partially reducing IL-1β promoter activity (Fig. 2) through a reduction of NF-κB dimer.
interaction with the specific DNA motif (Fig. 3, A and B). In addition, I-κBα mRNA and protein synthesis were enhanced (Fig. 4, A–C). In contrast, rhTrx-1 significantly reduced the expression of both p50 (Fig. 5, A–C) and p65 (Fig. 6, A–C) subunits and suppressed endogenous Trx-1 mRNA expression (Fig. 7, A and B). A similar finding was also observed when HMDM were activated with TNFα and treated with rhTrx-1 (data not shown), suggesting that LPS and TNFα might activate the NF-κB system through a same mechanism.

NF-κB, composed of homo- and heterodimeric complexes of members of the Rel family of proteins, is involved in the inducible expression of a wide variety of cellular genes (57). Within the cytoplasm, NF-κB is associated with the inhibitory I-κB proteins and therefore is present in an inactive form. Treatment of cells with various inducers results in the phosphorylation and degradation of I-κB. Therefore, NF-κB can be released and translocated into the nucleus, where it binds to a specific DNA motif. This binding has been reported to be stimulated by Trx-1, which can also be translocated into the nucleus (45). In this study, suppression of the IL-1β expression gene by extracellular rhTrx-1 treatment (Fig. 1) could be the result of the inhibition of p50, p65 (Figs. 5 and 6), and endogenous Trx-1 (Fig. 7) and of the enhancement of I-κBα synthesis (Fig. 4), leading to attenuation of NF-κB activity (Figs. 2 and 3).

Indeed, under oxidative stress or the inflammatory state, NF-κB activation in the nucleus is dependent on the Trx-1 up-regulation in the cytoplasm and its translocation into the nucleus (58). In this study, we showed, for the first time, an inhibition of cellular Trx-1 expression by extracellular human recombinant Trx-1 in HMDM treated with an inflammatory mediator. Taken together, these data indicated a potential new mechanism through which extracellular rhTrx-1 exerts an anti-inflammatory function in HMDM.

These data were consistent with previous reports indicating that extracellular Trx-1 exerts various beneficial effects. For example, it has been reported that circulating Trx-1 prevents LPS- and chemokine-induced neutrophil chemotaxis in mice (24), suppresses autoimmune myocarditis via its antioxidative damage and anti-inflammatory functions (56), decreases reperfusion-induced arrhythmias (59), decreases adriamycin-induced cytoxic injury (60), and prevents ischemic reperfusion injury in animal models (21, 22). In addition, overexpression of human Trx-1 in transgenic mice attenuated focal ischemic brain damage (25). Nevertheless, the rhTrx-1 concentrations (1 and 5 μg/ml) used in this in vitro study are higher than those measured in the plasma (~40 ng/ml) of different subjects with various cardiovascular risk factors (20). Therefore, our results, although suggestive, do not demonstrate that the phenomenon can occur in vivo. However, the rhTrx-1 concentration used in this study is in agreement with several reported in vitro studies such as the study of Kondo et al. (55), who used 10 μg/ml rhTrx.

Thioredoxin-1 functions by reversible oxidation. However, it is unknown whether the reduced form or the oxidized form of rhTrx-1 is involved in the various reported biological effects. Therefore, we addressed this question in this study, and we clearly showed that oxidized rhTrx-1, but not the DTT-reduced form or other oxidized unrelated proteins such as hApoA-I or hApoA-II (not shown), was specifically responsible for the reduction of IL-1β secretion by human macrophages. It is important to note that, in our study, the rhTrx-1 was under the reduced form when added to the LPS-activated cells, as stated by the supplier. Therefore, it is more likely that an oxidative modification of the protein occurs in the culture medium. This hypothesis is reinforced by the fact that the extracellular environment has been described to be predominantly oxidizing (61). The effect of rhTrx-1 in our study is probably mediated by a cell surface molecule and not following its infiltration to the cytoplasm (55), where it is less likely to exist under the oxidized form since the interior of the cell is highly reducing (61).

Excessive oxidative stress on the arterial wall, resulting, for example, from high levels of oxidized low density lipoprotein in hypercholesterolemic patients and/or from a contamination with a potent microbial mediator of inflammation, such as the lipopolysaccharide, is believed to be involved in the progression of atherosclerosis. Indeed, both substances activate macrophages to produce cytokines such as TNFα, IL-1, and IL-6 (62, 63). Since extracellular recombinant Trx-1, probably following its oxidative modification, suppressed all these cytokines, therefore, it appears to be a promising target for cardiovascular disease treatment.

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