



Thioredoxin reductase 1 upregulates MCP-1 release in human endothelial cells

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ABSTRACT

To know if thioredoxin reductase 1 (TrxR1) plays a role in antioxidant defense mechanisms against atherosclerosis, effect of TrxR1 on expression/release of monocyte chemoattractant protein (MCP-1) was investigated in activated human endothelial-like EAhy926 cells. The MCP-1 release and expression, cellular generation of reactive oxygen species (ROS), nuclear translocation and DNA-binding activity of NF- κ B subunit p65 were assayed in cells either overexpressing recombinant TrxR1 or having their endogenous TrxR1 knocked down. It was found that overexpression of TrxR1 enhanced, while knockdown of TrxR1 reduced MCP-1 release and expression. Upregulation of MCP-1 by TrxR1 was associated with increasing generation of intracellular ROS generation, enhanced nuclear translocation and DNA-binding activity of NF- κ B. Assay using NF- κ B reporter revealed that TrxR1 upregulated transcriptional activity of NF- κ B. This study suggests that TrxR1 enhances ROS generation, NF- κ B activity and subsequent MCP-1 expression in endothelial cells, and may promote rather than prevent vascular endothelium from forming atherosclerotic plaque.

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Introduction

The view that atherosclerosis is indeed a chronic inflammatory disease initiated by monocyte adhesion to activated endothelial cells (EC) is now widely accepted [1]. A bulk of evidences suggests that the inflammatory response in vascular injury involves recruitment and activation of monocytes through activation of monocyte chemoattractant protein-1 (MCP-1) [1]. As a potent CC chemokine for monocytes, MCP-1 plays an essential role in the recruitment of monocytes/macrophages to vascular lesions [2]. Expression of MCP-1 is tightly regulated and has been shown to occur at the transcriptional level by various stimuli such as oxidized low-density lipoprotein and TNF- α , lipopolysaccharide (LPS) [3]. In addition, many other factors were also reported to down modulate MCP-1 gene expression such as estradiol, troglitazone and TGF- β ₁ [3]. It has been reported that reactive oxygen species (ROS) derived from Rac1-activated NADPH oxidase upregulated TNF- α -induced MCP-1 expression, while the antioxidants, such as pyrrolidine dithiocarbamate (PDTTC) and *N*-acetylcysteine (NAC), significantly inhibited the IL-4-induced MCP-1 mRNA expression in human vascular endothelial cells [4]. Depletion of the endogenous Nox4 by transfection of siRNA for Nox4 into human aortic endothelial cells resulted in a failure to induce ROS

generation and subsequent expression of MCP-1 in response to LPS [5]. However, no investigation on the possible role of thioredoxin reductases, which play important role in regulation of cellular redox status, in regulating MCP-1 release and expression has been reported.

Thioredoxin reductases are members of the nucleotide-disulfide oxidoreductase family and ubiquitously found in mammalian tissues. All mammalian TrxRs are homodimeric selenocysteine-containing enzymes and the major selenoproteins expressed in human vascular endothelial cells [6]. They reduce and thereby activate thioredoxins which serves as reducing equivalent and catalyzes many redox reactions [7]. In human coronary atherosclerotic specimens, Trx expression is enhanced throughout the vessel wall and the greatest increases were observed in endothelial cells and infiltrating macrophages within the neointimal plaques [8,9]. An almost twofold increase in TrxR1 mRNA was recently found in the atherosclerotic plaques relative to surrounding healthy areas of the artery specimen taken from the same patients [10]. The high expression of Trx and TrxR in the atherosclerotic plaques suggests that Trx and TrxR may cooperate to work for antioxidant defense mechanisms in atherosclerosis. However, up to now, there is no evidence to show if TrxR really protects the vascular endothelium from forming atherosclerotic plaque. For this reason, we established two endothelial cell models, the human endothelial-like EAhy926 cells either overexpressing TrxR1 or having their endogenous TrxR1 knocked down, and investigated how TrxR1 modulate the expression of MCP-1 in the endothelial-like cells. It was surprisingly found that TrxR1 upregulated, rather than downregulated the MCP-1 release and expression.

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Materials and methods

Plasmids construction. Human TrxR1 cDNA (GenBank Accession No. BG772375) was PCR amplified from original plasmid containing TrxR1 cDNA using the 5' primer, 5'-CGGAATTCGCCACCATGGACGCCCTGAAGATCTTC-3' containing an EcoRI site and a Kozak consensus sequence upstream of the start codon ATG, and the 3' primer, 5'-GCCGATCCGCCAAATGAGATGAGGACGTGA-3' including a BamHI site. The PCR product was subcloned into the mammalian bicistronic expression vector pIRESneo2, which permits the translation of two open reading frames from one mRNA, and makes all survived colonies to stably express TrxR1. This construct includes a 1.5 kb fragment downstream of the stop codon TAA containing a selenocysteine insertion sequence and the first three of the six AU-rich elements located in the 3'-UTR [11]. The constructed TrxR1-expression vector, pIRESneo2-TrxR1, were confirmed by DNA sequencing.

Cell culture and transfection. The EAhy926 cell line provided by Dr. C.J. Edgell, (University of North Carolina, USA) [12] were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C. EAhy926 cells were transfected with pIRESneo2-TrxR1 or pIRESneo2 by electroporation and selected with G418 (400 µg/ml) for 4 weeks, and then screened by immunoblot analysis for TrxR1 expression. The cells overexpressing TrxR1 are referred as EAhy-TrxR1 cells, while the cells expressing only neomycin-resistant gene is referred as EAhy-neo cells. In all experiments, in order to achieve sufficient synthesis of selenocysteine in cells the cells were pre-incubated in the medium supplemented with 40 nM sodium selenite for 48 before usage.

Assay of TrxR1 activity. Cells were lysed with lysis buffer (1% Triton X-100 and 1 mM PMSF in PE: 50 mM potassium phosphate, 2 mM EDTA and pH 7.5). After sonication on ice, the lysate was centrifuged at 15,000g, 4 °C for 30 min. The supernatant was heated at 55 °C for 5 min, and filtered in a centrifugal filter of 30-kDa cut-off. The retentate was washed three times with PE buffer and used as crude cell extract. TrxR activity was determined by a modified DTNB reduction assay [13].

Knockdown of TrxR1 by siRNA. The siRNA for TrxR1 was used as 5'-CAGGUGCUUGUGGACAUCAAdTdT-3' [14]. The nonspecific oligonucleotide 5'-UUCUCCGAACGUGUCACGUTT-3' was used as negative control (mock RNA). Cells were seeded and cultured overnight, transfected with 150 nM TrxR1 siRNA or mock RNA using VigoFect transfection reagent (Vigorous Biotechnology, Beijing). All Experiments were performed 72 h after transfection.

ELISA assay of MCP-1. 1×10^5 endothelial cells were seeded in each well of 24-well plate. After stimulation with 50 ng/ml LPS or 50 µg/ml oxLDL for 6 h, supernatant of each culture medium was collected. MCP-1 concentration in the supernatant was measured by an enzyme-linked immunosorbent assay (ELISA) according to the literature [15].

Immunoblotting. Cell lysates or nuclear protein extract obtained from the cell lysate were prepared using NE-PER Reagents (Pierce), and resolved by electrophoresis on 12% acrylamide gels as previously described [16]. The resolved protein bands were electrotransferred onto nitrocellulose membrane, then incubated with appropriate antibodies for at least 4 h. Specific protein bands were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Electrophoretic mobility-shift assay of NF-κB. Nuclear extracts were isolated using NE-PER Kit (Pierce) and the protein contents were determined with BCA protein assay Kit (Pierce). Biotinylated double-stranded oligonucleotide (5'-biotin-AGTTGAGGGGACTTTC CAGGC-3') was used as probe for specific binding of NF-κB. EMSA was performed according to the manual of LightShift Chemiluminescent EMSA Kit (Pierce).

Assay of NF-κB transcriptional activity using NF-κB-dependent luciferase reporter. The pNF-κB-luciferase reporter plasmid (Clontech) consisting of four sequential κB consensus sites upstream of the herpes simplex virus-TK TATA-like promoter and luciferase gene was used for assay of NF-κB activity under various conditions. In the assay, HEK293 cells were cotransfected with 1 µg of NF-κB-luciferase plasmid, 1 µg of pIRESneo2-TrxR1 vector and 100 ng of the internal control plasmid pCMV-β-galactosidase using VigoFect transfection reagent in DMEM for 48 h. Since HEK293 cells are lack of the expression of endogenous TLR4, CD14 and MD-2 which is necessary for cellular responses to LPS [17], the cells were also cotransfected with 0.2 µg of pDisplay-HA-hTLR4, pcDNA3-hCD14 and pFlag-CMV1-hMD2 vector provided by Dr. L. Hajjar (University of Washington, Seattle). The cells transfected with 1 µg of pIRESneo2 vector (Clontech) was used as control. After transfection, the cells were stimulated with 10 µg/ml LPS for 8 h. Then, the luciferase activity was assayed by a home-made luminometer and normalized against the β-galactosidase activity as the measure for transcriptional activity of the endogenous NF-κB in the cells.

Intracellular ROS detection. DHE was used to detect intracellular superoxide anions (O_2^-) generation by fluorescence microscopy. The reaction of DHE with forms a red fluorescent product (2-hydroxyethidium) which intercalates with DNA [18] 1×10^4 TrxR1-overexpressing cells and control cells were plated on glass-bottomed dishes and cultured for 48 h. Then, culture medium was replaced with 1 ml HBSS. The fluorescence images of the attached cells on the bottom glass were taken every 2 min for 1 s at Ex/Em = 535/605 nm on an Olympus IX-71 microscope equipped with AquaCosmos Microscopic Image Acquisition and Analysis System. After 8-min imaging, 20 µM of DHE was added in dish, and fluorescence from the red fluorescence product formed by DHE oxidation in attached cells were continuously imaged for 30 min. The fluorescence images of 20 randomly selected cells on each dish were used to calculate kinetic curves of the average fluorescence of the red fluorescence product over the cells. DHE solution was freshly prepared and kept at nitrogen atmosphere to reduce oxidation. Since the DHE-derived fluorescence in the cells, which were loaded with DHE and imaged later, appeared slightly lower than the fluorescence in the cells which loaded with DHE and imaged early due to unavoidable oxidation of DHE, the fluorescence images were taken in following order: EAhy-neo, EAhy-TrxR1, EAhy-neo and EAhy-TrxR1 cells.

Statistics. All data obtained in this study are presented as mean ± SE of three independent measurements, and were subjected to Student's *t*-test. Statistical significance of the data are shown as single asterisk ($p < 0.05$) and double asterisks ($p < 0.01$) in corresponding figures.

Results

Overexpression of TrxR1 upregulates MCP-1 secretion

To know if TrxR1 plays any role in regulation of MCP-1 in vascular endothelium, a stable TrxR1-overexpressing EAhy926 cell line was established by transfecting the Lab-constructed TrxR1-expression vector, pIRESneo2-TrxR1, into cells. The TrxR1 activity in the transfected cells was determined as DTNB reduction rate. As Fig. 1A shows, activity of TrxR1 in the TrxR1-overexpressing cells (EAhy-TrxR1 cells) was 34% higher than that in controls (EAhy-neo cells), which reflects the abundance of TrxR1 observed in human atherosclerotic lesions to a certain degree. The immunoblotting also showed about 30% higher expression of TrxR1 in the TrxR1-overexpressing cells (Fig. 1B). The almost equal increase in TrxR1 activity and protein expression in the TrxR1-overexpressing

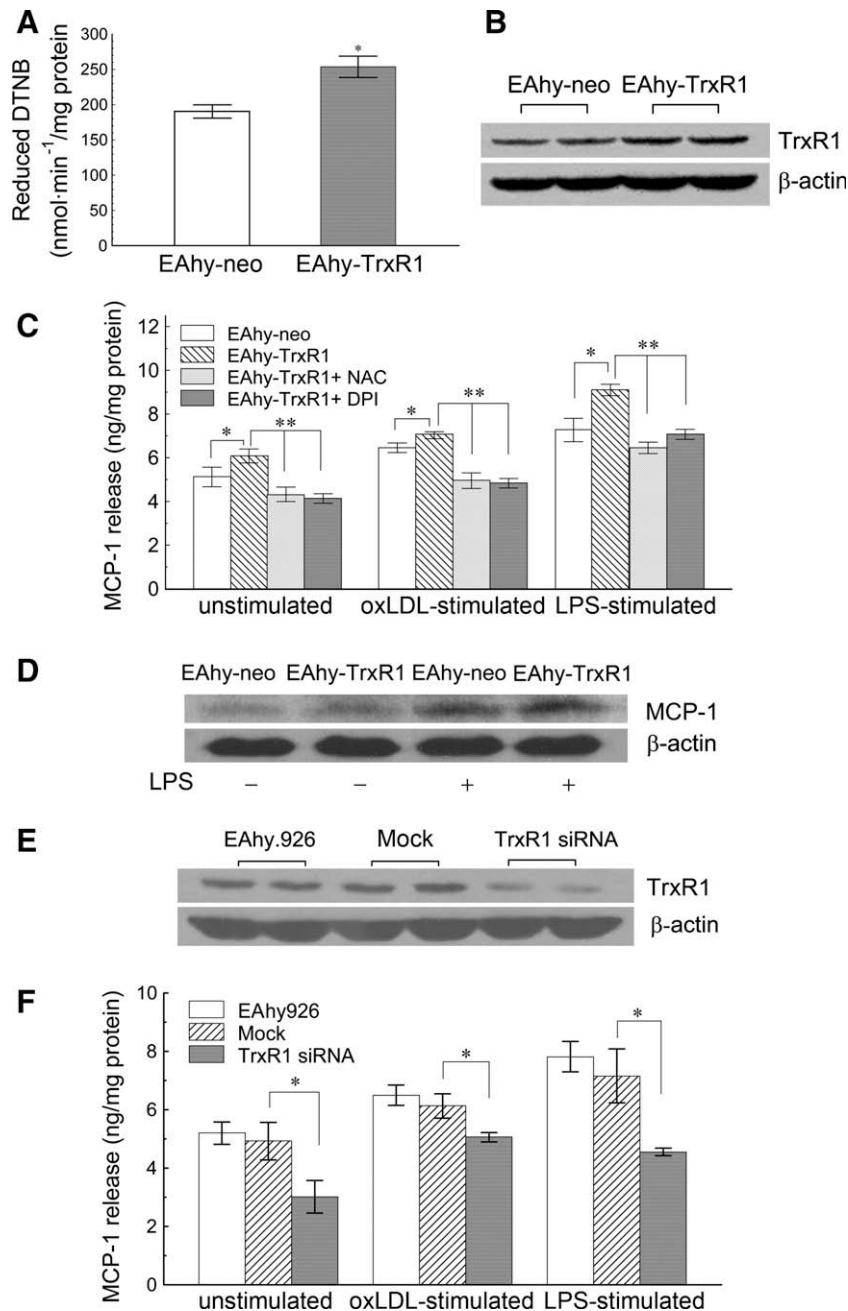


Fig. 1. TrxR1 activity/expression and MCP-1 release in the TrxR1-overexpressing and TrxR1-knockdown EAhy926 cells. (A) TrxR1 activity in TrxR1-overexpressing cells (EAhy-TrxR1) and their controls (EAhy-neo) assayed by DTNB reduction. (B) A typical immunoblot of the TrxR1 in the two cell lines. (C) MCP-1 release in the TrxR1-overexpressing cells. The cells were pre-incubated with or without 10 mM NAC for 4 h or 10 μ M DPI for 30 min, and then stimulated by 50 μ g/ml oxLDL or 50 ng/ml LPS for 6 h. (D) MCP-1 expression in TrxR1-overexpressing cells and their controls (EAhy-neo cells) which were left untreated or stimulated with 50 ng/ml LPS for 6 h. (E) Immunoblot of TrxR1 in the wild-type cells, the cells transfected with 150 nM siRNA for TrxR1 and scrambled oligonucleotide (mock RNA), respectively. The immunoblot analysis was performed 72 h after transfection. (F) MCP-1 release in the TrxR1-knockdown cells and their controls (mock) after stimulation with 50 μ g/ml oxLDL or 50 ng/ml LPS for 6 h. All above measurement on MCP-1 release and expression were performed at least three times.

cells may suggest that the overexpressed TrxR1 contains activity-required selenocysteine.

The basal and stimulated MCP-1 secretion from TrxR1-overexpressing cells was then compared with those in wild-type and neomycin-resistant gene-transfected cells using ELISA. As Fig. 1C shows, basal, the oxidized LDL-stimulated and LPS-stimulated MCP-1 release were all enhanced by overexpression of TrxR1. It was also found that pretreatment of the cells with either 10 mM antioxidant NAC for 4 h or 10 μ M DPI, a specific NADPH oxidase inhibitor [19], for 30 min, significantly reduced the release of MCP-1 from the TrxR1-overexpressing cells, indicating that ROS

is involved in the upregulation of MCP-1 secretion by TrxR1. Protein expression of MCP-1 was also assayed by Western blotting to see if gene expression of MCP-1 is upregulated by overexpression of TrxR1. As Fig. 1D shows, the results clearly indicate that TrxR1 upregulates MCP-1 release through upregulation of gene expression of this chemoattractant protein.

Knockdown of the endogenous TrxR1 reduces MCP-1 release

To avoid any possible side-effect caused by overexpression of an exogenous gene in cells, the effect of TrxR1 on MCP-1 release

was also looked at in the cells having their endogenous TrxR1 knocked down. As immunoblot analysis showed, expression of the endogenous TrxR1 was reduced to approximately 35% of its original level by transfection of 150 nM TrxR1 siRNA (Fig. 1E) into the cells. The ELISA showed that 65% depletion of the endogenous TrxR1 reduced the basal, the oxLDL- and LPS-stimulated release of MCP-1 by about 40%, 17% and 36%, respectively, in comparison with that in the cells transfected with scramble RNA (mock) (Fig. 1F). Viability of the TrxR1-knockdown cells was checked by MTT test and found almost not decreased (see Supplemental data). The observed reduction of MCP-1 secretion by silencing TrxR1 is well consistent with the observation that overexpression of TrxR1-enhanced MCP-1 secretion from the endothelial cells.

TrxR1 promotes translocation of NF- κ B subunit p65 into nucleus

Since NF- κ B is a major nuclear factor for the transcription of MCP-1 [20], and nuclear translocation of NF- κ B subunit p65 is an essential step for initiating transcription of MCP-1, the effect of TrxR1 on the nuclear translocation of p65 was investigated by either overexpression of TrxR1 or knockdown of endogenous TrxR1 in EAhy926 cells. The immunoblot analysis showed that knockdown of TrxR1 significantly reduced the nuclear p65 in unstimulated and the LPS-stimulated cells by about 40% and 22%, respectively, in comparison with that in the wild-type cells or the scramble RNA-transfected cells (Fig. 2A and B).

Immunoblotting was also performed to probe the nuclear contents of p65 in the TrxR1-overexpressing cells (Fig. 2C and D). Three independent determinations showed that nuclear content of p65 was raised in the TrxR1-overexpressing cells whatever the cells were stimulated by LPS or not. Both the elevation of nuclear p65 in the TrxR1-overexpressing cells and reduction of nuclear p65 in the TrxR1-knockdown cells suggest that TrxR1 may act as a promoter for the translocation of NF- κ B subunit p65 into nucleus.

TrxR1 enhances the activity of NF- κ B

To know if TrxR1 regulates transcriptional activity of NF- κ B, the DNA-binding activity of the NF- κ B in nuclear extract of either TrxR1-overexpressing or TrxR1-knockdown cells was determined by electrophoretic mobility-shift assay (EMSA) under LPS-stimulation. The band for NF- κ B–probe complex was identified by the significant reduction in the presence of anti-p65 antibody in the nuclear extract from the LPS-stimulated TrxR1-overexpressing cells (see band 10 in Fig. 4A). The EMSA results showed that knockdown of endogenous TrxR1 reduced, while overexpression of TrxR1 enhanced the ability of NF- κ B in binding to the double-stranded oligonucleotide containing a κ B site in LPS-stimulated cells. It suggests that TrxR1 upregulates the transcriptional (or DNA-binding) activity of NF- κ B.

To confirm the upregulation of NF- κ B activity by TrxR1 at transcription level, the NF- κ B-dependent luciferase reporter consisting of a four sequential κ B consensus sites-containing promoter was cotransfected with of TrxR1-expression vector into HEK293 cells. As Fig. 3B shows, transcriptional activity of NF- κ B was significantly enhanced by transient overexpression of TrxR1 in either basal or LPS-stimulated cells, but substantially reduced when the cells were pretreated with either 10 mM antioxidant NAC for 4 h or 10 μ M DPI for 30 min.

TrxR1 promotes intracellular ROS

Since the foregoing results showed that either the antioxidant NAC or the NADPH oxidase inhibitor DPI significantly re-

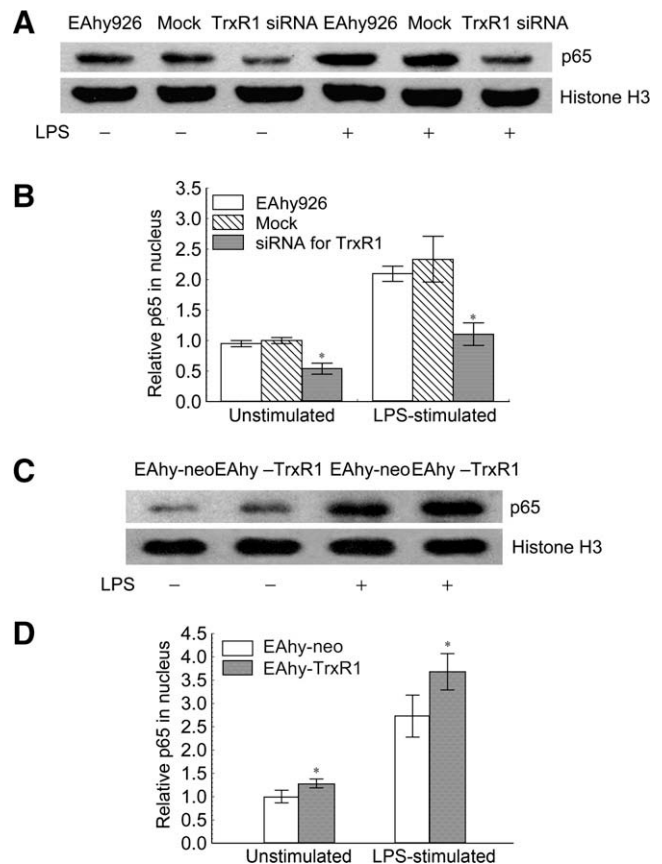


Fig. 2. Effect of TrxR1 on the nuclear content of NF- κ B subunit p65 in EAhy926 endothelial cells. (A) Immunoblot analysis of p65 in the nuclear extracts of wild-type cells, the cells transfected with mock RNA and the TrxR1-knockdown cells which were left untreated or stimulated with 50 ng/ml LPS for 60 min. (B) Relative nuclear content of p65 in unstimulated and the LPS-stimulated cells. (C) Immunoblot analysis of p65 in the nuclear extracts of the TrxR1-overexpressing cells and their controls after stimulation with or without 50 ng/ml LPS for 60 min. (D) Relative nuclear content of p65 in unstimulated and the LPS-stimulated TrxR1-overexpressing cells. All data in (B) and (D) are mean \pm SE based on three independent determinations.

duced the TrxR1-enhanced cellular release of MCP-1 (Fig. 1C), it suggests that ROS might be involved in the regulation of MCP-1 release by TrxR1. For this reason, the intracellular O_2^- generation was measured in TrxR1-overexpressing cells and their controls (EAhy-neo cells). Considering a possible decline of DHE fluorescence in the cells loading with DHE and being imaged later due to unavoidable oxidation of DHE, the control cells were loaded and imaged early and compared with that in TrxR1-overexpressing cells loaded with DHE and imaged immediately later. As Fig. 4 shows, the DHE-derived fluorescence and its rising slope in TrxR1-overexpressing cells, which are represented by kinetic curves 2 and 4, are obviously higher than that in control cells measured as curves 1 and 3, though the former were loaded with DHE and imaged later. The very precisely performed DHE fluorescence imaging of the cellular O_2^- generation demonstrates that overexpression of TrxR1-enhanced intracellular ROS generation.

Discussion

Since regulatory roles of cytosolic thioredoxin and many other antioxidants are dependent on the activity of cytosolic thioredoxin reductase, TrxR1 together with Trx1 has been recognized as an essential component for cellular redox control and antioxidant de-

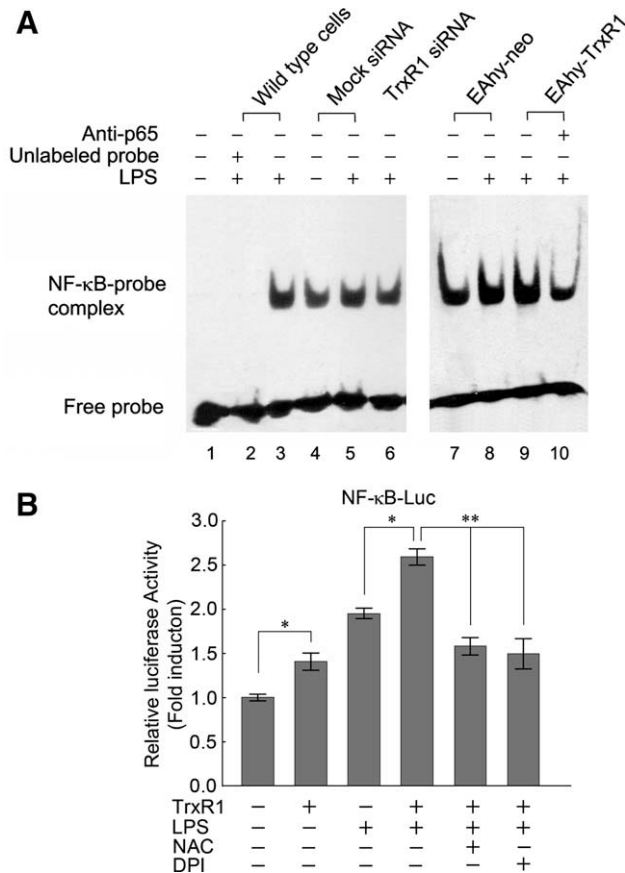


Fig. 3. Effect of TrxR1 on DNA-binding and transcriptional activity of NF- κ B in EAhy926 cells. (A) EMSA of the DNA-binding activity of NF- κ B in indicated cells. EMSA was performed using biotin-labeled DNA probe containing the NF- κ B consensus sequence and nuclear extracts from the wild-type cells (lanes 2 and 3), the cells transfected with scrambled oligonucleotide (mock RNA, lanes 4 and 5), the TrxR1-knockdown cells (lane 6), the cells expressing neomycin-resistant gene (EAhy-neo, lanes 7 and 8) and the TrxR1-overexpressing cells (EAhy-TrxR1, lanes 9 and 10) after stimulation with or without 50 ng/ml LPS for 60 min. Anti-p65 antibody was used to shift p65-probe complex (lane 10). (B) Transcription of the NF- κ B-Luc reporter in the HEK293 cells under various combined treatments of transfection of TrxR1, presence or absence of 10 mM NAC or 10 μ M DPI and 10 μ g/ml LPS-stimulation. All measurements on luciferase activity were triplicate.

fense [21,22]. Besides that, as increased expression of TrxR was found in human atherosclerotic plaques and foam cells [10], it seems logical that TrxR would protect the vascular endothelium from forming atherosclerotic plaque. However, no solid evidence has been obtained to verify this plausible expectation. This study provided good evidence arguing to the common speculation that increased TrxR in human atherosclerotic plaques may work for antioxidant defense mechanisms in atherosclerosis. We showed that TrxR1 upregulated intracellular ROS generation and release of MCP-1, a critical chemotactic factor for initiation of atherosclerosis, by assessing the effect of TrxR1 in the cells either overexpressing recombinant TrxR1 or knocking down the endogenous TrxR1. The observed upregulation of ROS generation in the cells by TrxR1 may be an even more novel finding for revisiting this enzyme.

With respect to the mechanisms involved in the upregulation of MCP-1 by TrxR1, we showed that overexpression of TrxR1 enhanced, while knockdown of endogenous TrxR1 reduced LPS-induced nuclear translocation of NF- κ B and its DNA-binding activity. Since overexpression of TrxR1-enhanced TNF- α -induced DNA-binding activity of NF- κ B and expression of the NF- κ B-targeted E-selectin and cyclooxygenase-2 genes was reported by

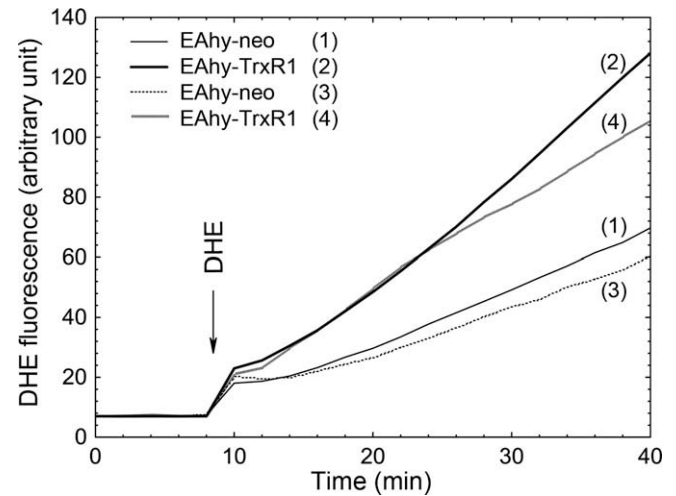


Fig. 4. Kinetic curves of the DHE-derived fluorescence in TrxR1-overexpressing and control cells attached on glass-bottomed dishes. DHE (20 μ M) was added between the images taken at 8 and 10 min. The number of curve indicates the temporal order of microscopic measurement. Each curve was calculated based on the fluorescence images of 20 randomly selected cells.

other investigators [23], this study provides new evidence for upregulation of another NF- κ B-targeted gene by TrxR1. Furthermore, the reporter experiments in this study revealed that ROS is involved in the upregulation of NF- κ B activity by TrxR1, since both NAC and DPI suppressed the TrxR1-enhanced transcriptional activity of NF- κ B (see Fig. 3B).

As monocyte chemoattractant protein (MCP)-1 has a potent chemoattractant activity for monocytes/macrophages and the accumulation of monocytes/macrophages in the intima of artery vessel is an important early event in atherosclerosis, MCP-1 has been considered as a critical chemokine involved in the pathogenesis atherosclerosis. Despite this study may imply a negative role played by TrxR1 in the pathogenesis atherosclerosis, the antioxidant activity of thioredoxin reductase, such as reducing and thereby activating thioredoxin, catalyzing many redox reactions is still well recognized. This study probably revealed the duality of this enzyme. In one hand, TrxR1 catalyzes the reduction of the disulfide in oxidized thioredoxin with NADPH as hydrogen donor. On the other hand, TrxR1 promotes ROS generation through upregulating the activity of NADPH oxidase. Further investigation is needed for understanding the dualistic nature of thioredoxin reductase.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2009.06.100](https://doi.org/10.1016/j.bbrc.2009.06.100).

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