ANTIOXIDANTS & REDOX SIGNALING
Volume 8, Numbers 9 & 10, 2006

Forum Rapid Letter

Thioredoxin-1 Suppresses Systemic Inflammatory Responses Against Cigarette Smoking

ATSUYASU SATO,[1] TOMIJIRO HARA,[2] HAJIME NAKAMURA,[3] NORIKO KATO,[2,4] YUMA HOSHINO,[3] NORIHIKO KONDO,[2] MICHIAKI MISHIMA,[1] and JUNJI YODOI[2,3]

ABSTRACT

Thioredoxin-1 (TRX) is a small redox-active protein with antioxidative effects and redox-regulating functions. Cigarette smoking is a major etiological factor in the pathogenesis of a variety of diseases and recruits systemic immune and inflammatory responses. This report demonstrates that TRX attenuates the systemic inflammatory responses induced by cigarette smoking. The mRNA expressions of tumor necrosis factor alpha (TNF-alpha) and macrophage migration inhibitory factor (MIF) were suppressed in the spleen of TRX overexpressing transgenic mice (TRX-tg) exposed to cigarette smoking, compared with control C57BL/6 mice. In addition, protein carbonylation, a marker of cellular protein oxidation, was enhanced by cigarette smoking in the tissues of heart and liver in control mice more than in TRX-tg mice. These findings suggest that TRX may suppress the systemic inflammatory responses against cigarette smoking. *Antioxid. Redox Signal.* 8, 1891–1896.

INTRODUCTION

CIGARETTE SMOKING is a major etiological factor in lung diseases such as chronic obstructive pulmonary disease (COPD) and lung cancer (25). Moreover, cigarette smoking induces systemic inflammation by increasing oxidative stress and has been implicated as an aggravating factor in systemic diseases such as atherosclerosis, coronary artery disease, and metabolic syndrome (13, 21, 25). Cigarette smoking enhances the activation of redox-sensitive transcription factors such as nuclear factor-kappa B (NF-kappa B) and activator protein-1 (AP-1) which are critical to the induction of interleukin (IL)-8, IL-6, and tumor necrosis factor-alpha (TNF-alpha) (28, 29), leading to systemic inflammation as well as local inflammation (9, 10).

Thioredoxin-1 (TRX) is a small (12 kDa) redox-active protein, which has a conserved active site [-Cys–Gly–Pro–Cys-], and functions in reducing protein disulfide bonds or scavenging hydrogen peroxide together with peroxiredoxins. Human TRX was originally identified as a soluble cytokine-like factor named adult T-cell leukemia (ATL)-derived factor from the supernatants of human T cell leukemia virus type-I transformed ATL2 cells (31). Recently, it was reported that serum TRX concentrations were elevated higher in smoking patients than in nonsmoking patients (20). Furthermore, TRX overexpressing transgenic mice (TRX-tg) show marked resistance against diesel exhaust particle (DEP)-induced lung damage, influenza virus-induced pneumonia, adriamycin-induced cardiotoxicity, and thioacetamide-induced acute hepatitis (14, 22, 23, 30). The induction of endogenous TRX prevents ethanol-induced cytotoxicity and photooxidative retinal damage (11, 34). Moreover, the administration of recombinant human TRX also attenuates oxidative stress-associated disorders (7, 12, 19). Accumulating evidence suggests that augmentation of TRX by either administration of recombinant human TRX or TRX inducer is associated with increased tolerance against oxidative stress (7, 34).

Macrophage migration inhibitory factor (MIF), which was initially identified as a soluble factor in the culture medium of activated T cells (1), is a proinflammatory cytokine and

[1]Department of Respiratory Medicine, Kyoto University Graduate School of Medicine; [2]Department of Biological Responses, Institute for Virus Research, Kyoto University; [3]Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital; [4]Redox Bioscience Inc., Kyoto, Japan.
AS and TH contributed equally.

glucocorticoid-induced immunomodulatory, primarily produced by macrophages in response to a variety of inflammatory stimuli (4). MIF also contains redox-active cysteines and is considered a member of the TRX family. It is suggested that MIF-mediated immune processes might be regulated by the cysteine-mediated redox mechanism (16, 17). Recently, we found that acute local lung inflammation is significantly attenuated in TRX-tg compared with control mice (Sato *et al.*, in preparation). In this study, we investigated systemic inflammatory responses in TRX-tg and control mice.

MATERIALS AND METHODS

Mice

C57BL/6 mice as a control were purchased from Japan SLC Inc. (Hamamatsu, shizuoka-prefecture, Japan). TRX-tg were generated from C57BL/6 mice on a transgene composed of beta-actin promoter and the human TRX gene as described previously (33). All animals were matched for sex (male) and age (9–12 weeks). The animal research committee of Kyoto University approved this animal experiment for this article.

Antibodies

The expressions of mouse MIF and TNF-alpha proteins were determined using anti-mouse MIF polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-mouse TNF-alpha polyclonal antibody (Santa Cruz Biotechnology).

Cigarette and short-term cigarette smoke exposure

The purchased cigarettes were University of Kentucky research grade (code 2R4F) (8, 35). Mice were divided into two groups, TRX-tg (n = 3) and control mice (n = 3), placed in polycarbonate chambers, and exposed to cigarette smoke. Mice were exposed to cigarette smoke generated from 40 filter-cut standard cigarettes per day for 3 days, using SG-200 smoke generator equipment (Shibata Scientific Technology Ltd., Tokyo, Japan). After the smoke exposure, mice were followed up for a day and then sacrificed on day 4.

RNA isolation and real-time RT–PCR analysis

Splenic total RNA was isolated with a RNeasy Mini Kit (Qiagen GmbH Hilden, Germany) according to the protocol of the manufacturer. One microgram of splenic total RNA was subsequently used for the synthesis of first-stand cDNA with Super Script III RNase H minus reverse transcriptase (Invitrogen Inc., Breda, Netherlands) in 20 µl final volume containing 250 ng of random hexamers and 40 units of RNase OUT inhibitor. After RT reaction, all cDNA was used for quantitative PCR analysis.

Quantitative RT–PCR analysis was performed using the ABI Prism 7000 sequence detection system (Applied Biosystems, Branchburg, NJ). The following gene expression assays (Assay-on-Demand, Applied Biosystems) suitable for this system were used. They are based on 5′ nuclease chemistry and consist of two unlabeled PCR primers for amplification and a 6-FAM dye-labeled TaqMan MGB probe for detecting the sequence of interest TNF and MIF. Template cDNA, 25 µl (equivalent to 100 ng of total RNA), were added to 25 µl of PCR reaction mix containing TaqMan Universal PCR Master Mix (Applied Biosystems) and 2.5 µl assay-on-demand specific for the indicated transcripts. Samples were incubated for 5 min at 50°C, and for 10 min at 95°C, after which target amplification was carried out with 40 two-staged cycles of 15 sec at 95°C and 1 min at 60°C. Fold induction was calculated using ribosomal RNA as a control in the individual experiments.

TNF-alpha and MIF production measured by Western blot analysis

The spleen was washed with ice-cold PBS and lysed with lysis buffer (20 m*M* Tris-HCl; pH 7.4, 150 m*M* NaCl, 1% Nonidet P-40, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* phenylmethylsulfonyl fluoride, 1% Triton X-100, 2.5 m*M*), and the extracts were cleaned by centrifugation. Equal amounts of protein (50 µg protein per lane), estimated by the Bradford method using a protein assay (Bio-Rad Laboratories, Hercules, CA), were electrophoresed on 15% SDS-polyacrylamide gel, and then the protein was electrophoretically transferred to a poly membrane (Millipore Corporation, Billerica, MA). After blocking with 10% skim milk for immunoassay (Nacalai Tesque, Inc., Kyoto, Japan) in Tris-buffered saline containing 0.05% Tween20 at 4°C overnight, the membrane was incubated with the first antibody. Afterward, in the case of MIF, it was incubated with anti-rabbit IgG, horseradish peroxidase linked antibody (GE Healthcare Bio-Sciences, Little Chalfont, UK), and in the case of TNF-alpha, it was incubated with anti-goat IgG, horse radish peroxidase-linked antibody (DAKO, Glostrup, Denmark). Chemiluminescence was detected by Chemi-Lumi One (Nacalai Tesque, Inc.) according to the protocol of the manufacturer. The densitometry analysis was performed by NIH image (the Research Services Branch of the National Institute of Mental Health, Washington DC).

Oxidized protein measured by OxyBlot analysis

Protein carbonylation was detected by using OxiBlot protein oxidation detection kit (Intergen, Purchase, NY). This kit provides reagents for sensitive immunodetection of carbonyl groups. The 2,4-dinitrophenylhydrazone (DNP)-derivatized protein samples (5 µg of protein/lane) were separated on a 12% SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis. The membrane was incubated with primary antibody, specific to the DNP moiety of proteins, and then with the peroxidase-linked secondary antibody, as provided from the manufacturer. Chemiluminescence was detected by Chemi-Lumi One (Nacalai Tesque, Inc.) according to the protocol of the manufacturer.

Statistical analysis

The mean ± SD was determined for each group in the individual experiments. Statistical analysis was performed by Student's t test.

RESULTS

Proinflammatory cytokines induced by short-term cigarette smoking were suppressed in TRX-tg mice

To elucidate the relationship between TRX and cigarette smoking-induced inflammation, we performed real-time RT–PCR and Western blot analysis. The splenic mRNA level of MIF after cigarette smoking was lower in TRX-tg mice than in control mice (0.727 ± 0.039 vs. 1.457 ± 0.486, $p = 0.05$, Fig. 1a). In addition, the splenic mRNA level of TNF-alpha after cigarette smoking was also lower in TRX-tg than in control mice (0.269 ± 0.137 vs. 0.880 ± 0.104, $p = 0.025$, Fig. 1b). On the other hand, the level of TNF-alpha protein expression was decreased in TRX-tg mice and control mice after cigarette smoking exposure. The levels of MIF protein expression was clearly decreased in TRX-tg mice after cigarette smoking exposure and was slightly decreased in control mice after cigarette smoking exposure (Fig. 2).

Protein oxidation by short-term cigarette smoking were suppressed in TRX-tg mice

To assess the systemic oxidative stress in tissues other than the spleen, the protein carbonylation, a marker of oxidized protein, was analyzed in the heart and the liver. By the OxiBlot analysis, the oxidized protein was enhanced in the heart and in the liver by cigarette smoking in control mice more than in TRX-tg mice (Fig. 3).

DISCUSSION

The association between TNF-alpha and smoking has been reported previously (2, 24, 36), suggesting that TNF-alpha has a crucial role in systemic inflammation (3, 6, 26, 32). In this study, we have shown that the overexpression of TRX in TRX-tg suppressed the mRNA induction of TNF-alpha in the spleen by cigarette smoking.

MIF represents an essential proinflammatory mediator of innate immunity in antimicrobial defense and stress responses (5), and may play a role in antigen presentation (27). Moreover, MIF also has a redox-active catalytic center of TRX-type [-Cys-Gly-Rho-Cys-] and is considered a member of the TRX families. The disulfide-mediated enzymatic reaction may be important for the immunological effects of this cytokine (16). Interestingly, we demonstrated that the mRNA production of MIF by cigarette smoking is suppressed in TRX-tg, compared with control mice. It is suggested that the downregulation of MIF in TRX-tg might be due to the reciprocal interaction of TRX family members between TRX and MIF (17). It is also reported that TNF-alpha upregulates local MIF expression by both infiltrating macrophages in rat crescentic glomerulonephritis. In addition, TNF-alpha regulates systemic MIF production (18). Hence, the downregulation of MIF in the current study might be related to the downregulation of TNF-alpha in TRX-tg. Collectively, systemic inflammation caused by cigarette smoking may be attenuated in TRX-tg by the downregulation of proinflammatory cytokines of TNF-alpha and MIF.

The present study showed that the systemic oxidative stress was increased by cigarette smoking as determined by protein carbonylation in the heart and liver. Moreover, TRX overexpression suppressed systemic oxidative stress caused by cigarette smoking. It is suggested that systemic hypoxia may be involved in the systemic oxidative stress caused by cigarette smoking. As previously reported (15), TRX overexpression introduces the resistance against hypoxia in the tissues. In addition, TRX attenuates the cigarette smoking-in-

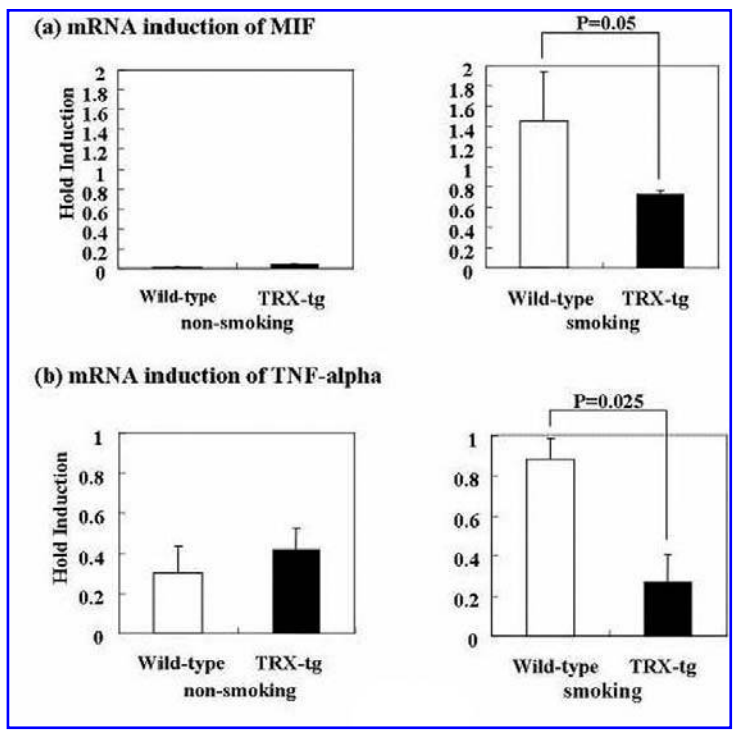

FIG. 1. TRX suppresses the splenic mRNA induction of proinflammatory cytokines induced by cigarette smoking. (**A**) The splenic mRNA transcription activity of MIF was enhanced by cigarette smoking in control mice ($n = 3$, *white bar*) more than in TRX-Tg ($n = 3$, *black bar*). (**B**) The splenic mRNA transcription activity of TNF-alpha was enhanced by cigarette smoking in control mice ($n = 3$, *white bar*) more than in TRX-tg mice ($n = 3$, *black bar*).

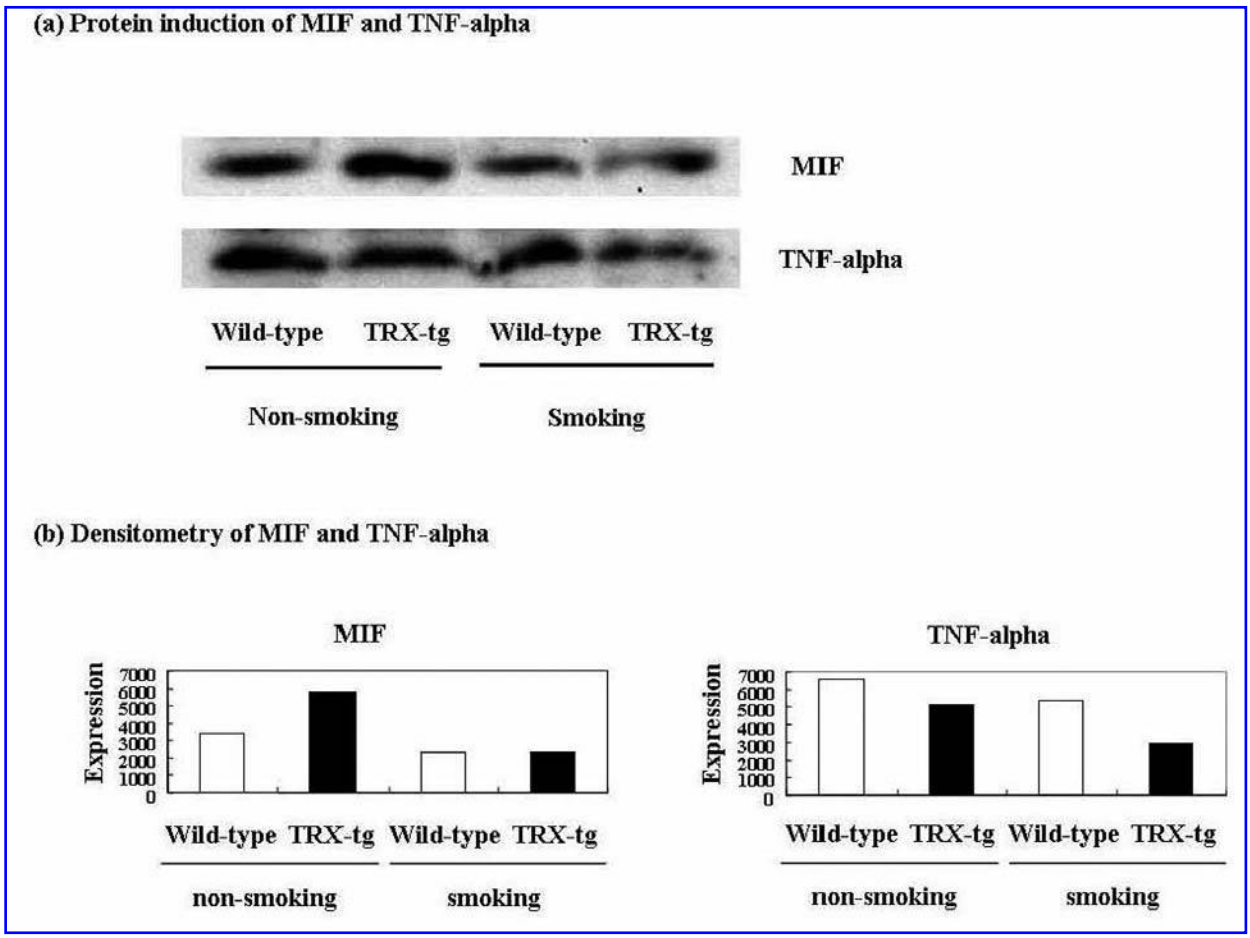

FIG. 2. Western blot analysis for the splenic protein expression of MIF and TNF-alpha by cigarette smoking exposure. (**A**) By Western blot analysis (TRX-tg mice (n = 1) and control mice (n = 1)). (**B**) By densitometry analysis [TRX-tg mice (n = 1) and control mice (n = 1)].

duced lung damage as shown elsewhere (Sato A, *et al*. in preparation). Therefore, the mechanisms whereby TRX suppresses the systemic oxidative stress may be explained partly by the direct protection of other tissues from hypoxia and partly by the secondary effect via the prevention of lung damage. Interestingly, TNF-alpha is also involved in the systemic inflammation associated with chronic hypoxia in patients with COPD (32).

In conclusion, the present study provides the evidence that TRX may have a potential to suppress the systemic inflammatory responses against cigarette smoking. This is the first report about the relationship between TRX and systemic inflammation caused by cigarette smoking, but further studies are needed to clarify the underlying mechanism (s) by using either flow-cytometry analysis or histological assessment.

ACKNOWLEDGMENTS

The authors would like to thank Mr. Masahiro Takenaka from Redox Bioscience Inc. and also would like to thank Takeda Pharmaceutical Co. LTD. for their technical contribution to this work. We acknowledge Dr. Yasuya Inomata and Dr. Kazuo Murata for the critical reading of this manuscript. This work was supported by Grant-in-Aid for the Creation of Innovations through Business-Academic-Public Sector Cooperation and Open Competition for the Development of Innovative Technology, from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

ABBREVIATIONS

AP-1, activator protein-1; ATL, adult T-cell leukemia; COPD, chronic obstructive pulmonary disease; DEP, diesel exhaust particle; DNP, 2,4-dinitrophenylhydrazone; IL, interleukin; MIF, macrophage migration inhibitory factor; NF-kappa B, nuclear factor-kappa B; TNF, tumor necrosis factor; TRX, thioredoxin-1; TRX-tg, thioredoxin-1 overexpressing transgenic mice.

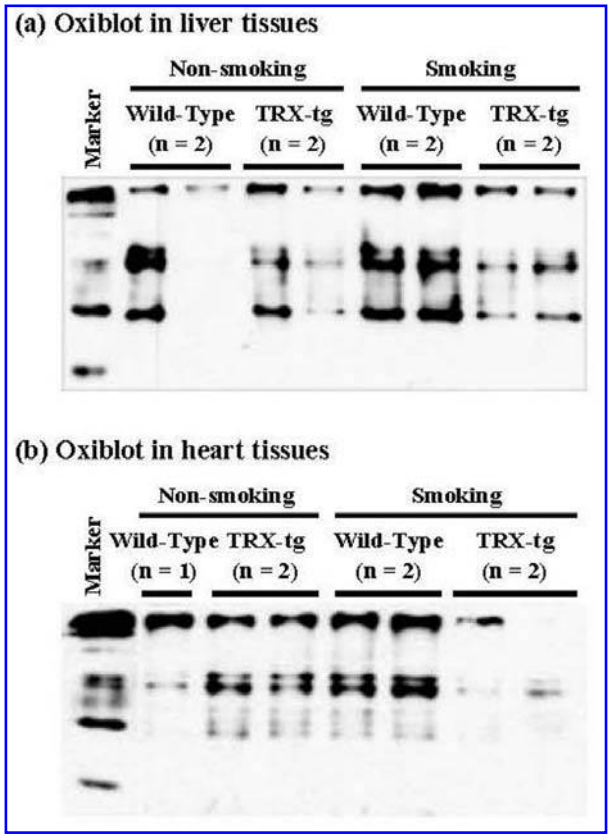

FIG. 3. TRX suppressed tissue protein oxidation induced by cigarette smoking. Tissue protein oxidation was enhanced by cigarette smoking (**A**) in the heart and (**B**) in the liver in control mice more than in TRX-tg mice.

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Address reprint requests to:
Dr. Junji Yodoi
Department of Biological Responses
Institute for Virus Research
Kyoto University
53 Kawaharacho, Shogoin
Sakyo-ku, 606-8507
Kyoto, Japan

E-mail: yodoi@virus.kyoto-u.ac.jp

Date of first submission to ARS Central, April 4, 2006; date of acceptance, April 14, 2006.

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