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Regulatory role of thioredoxin in homocysteine-induced monocyte chemoattractant protein-1 secretion in monocytes/macrophages

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Abstract **We have previously shown that homocysteine (Hcy) can induce monocyte chemoattractant protein-1 (MCP-1) secretion via reactive oxygen species (ROS) in human monocytes. Here, we show that Hcy upregulates expression of an important antioxidative protein, thioredoxin (Trx), via NADPH oxidase in human monocytes in vitro. The increase of Trx expression and activity inhibited Hcy-induced ROS production and MCP-1 secretion. Of note, 2-week hyperhomocysteinemia (HHcy) ApoE$^{-/-}$ mice showed accelerated lesion formation and parallel lower Trx expression in macrophages than ApoE$^{-/-}$ mice, suggesting that HHcy-induced sustained oxidative stress in vivo might account for impaired Trx and hence increased ROS production and MCP-1 secretion from macrophages, and subsequently accelerated atherogenesis.**

Keywords: Atherosclerosis; Chemokine; Antioxidation; Macrophage; Thioredoxin

1. Introduction

Atherosclerosis is the leading cause of cardiovascular morbidity and mortality in the world. Hyperhomocysteinemia (HHcy) has been recognized as an independent risk factor for this disease for more than 30 years [1]. Despite studies reporting that diet-induced mild to moderate HHcy accelerates atherosclerotic lesion development in ApoE$^{-/-}$ mice [2], the mechanisms are still not completely understood.

Recent studies have elucidated that the development of atherosclerosis involves reactive oxygen species (ROS)-induced oxidative stress and inflammation [3]. During the early stage of atherogenesis, monocytes and T cells are recruited to the subendothelium. The cell recruitment is mainly regulated by adhesion molecules and chemokines. One of the most important chemokines is monocyte chemoattractant protein-1 (MCP-1). Gu et al. [4] reported that the ablation of MCP-1 greatly decreased the lesion size in atherosclerotic LDLR$^{-/-}$ mice. We have previously demonstrated that homocysteine (Hcy) enhances vascular inflammation by promoting the expression and secretion of MCP-1 and IL-8 in human monocytes in vitro, and ROS may mediate the Hcy-induced MCP-1 expression [5].

Human thioredoxin (Trx) is a ubiquitously expressed multifunctional protein crucial for the regulation of transcription factors, modulation of immune response, and especially for antioxidative defense [6]. Trx reduces the oxidized form of Trx peroxidase, which then scavenges ROS, such as H_2O_2 [7], and acts as a redox regulator of signaling molecules and transcription factors. Trx could maintain a reduced intracellular microenvironment by reducing protein disulfides under oxidative stress, thereby mediating the cellular response to redox state [8].

We aimed to determine the role of Trx in Hcy-induced MCP-1 secretion from monocytes both in vitro and in vivo. Our data show that Hcy elevated Trx protein in a NADPH oxidase-dependent manner in human monocytes. Overexpression of Trx inhibited Hcy-induced ROS production as well as MCP-1 secretion which might delay atherogenesis. Interestingly, Trx expression was significantly decreased after long-term HHcy stimulation in early stage of ApoE$^{-/-}$ mice, which might hence accelerate atherogenesis.

2. Materials and methods

2.1. Cell culture

Primary human monocytes were isolated from blood of healthy donors as described previously [9]. Murine peritoneal macrophages were isolated by flushing the peritoneal cavity with ice-cold PBS containing 10% FBS and 1 mM EDTA. Human monocytes (THP-1) and type II alveolar epithelial cells (A549) were obtained from ATCC. For experiments, THP-1 cells were differentiated for 24 h with 10 nM PMA. THP-1 cells were grown in RMPI-1640 containing 10% FBS. A549 cells were cultured in DMEM (Hyclone, Logan, UT) supplemented with 10% FBS.

2.2. Preparation of cell extracts and Western blotting

Cells extracts were prepared and separated by 12% SDS–PAGE, and then transferred onto a nitrocellulose membrane as described previously [9]. Membranes were immunoblotted with anti-Trx antibody (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA) and then were incubated with IRDye™-conjugated secondary antibody for 1 h. The immunofluorescence band was detected by use of the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

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Abbreviations: Trx, thioredoxin; MCP-1, monocyte chemoattractant protein-1; ROS, reactive oxygen species; Hcy, homocysteine; HHcy, hyperhomocysteinemia

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2.3. Plasmid transfection

A549 cells were transfected with 1.5 μg pcDNA3.1-Trx expression plasmid or pcDNA3.1 plasmid for 24 h by use of cationic polymer transfection reagent (JetPEI, France) prior to measure ROS generation or for western blotting.

2.4. Measurement of ROS generation

The generation of ROS in A549 cells was detected by horseradish peroxidase-catalysed chemiluminescence in a light-tight box with a BPCL Ultra-weak luminescence analyzer (Beijing, China) at 37 °C as described in Ref. [10]. Transfection of A549 cells was performed as previously described, with 10 μg/ml horseradish peroxidase, 0.5 mM luminol, and 100 μM Hcy in a total volume of 1 ml.

2.5. Measurement of MCP-1 protein secretion

Cultured human monocytes were treated with Hcy and some were pretreated with 1 μM sodium selenite for 24 h, then the supernatant was harvested 3 days later. MCP-1 concentration was determined on ELISA (R&D Systems, Minneapolis, MN) [9].

2.6. Animals and tissue sample preparations

Female ApoE$^{-/-}$ mice, 6 weeks old, were fed normal mouse chow and water with or without 1.8 g/L DL-Hcy added ($n = 8$ mice) for 2 weeks. Mice were anesthetized, then blood was drawn and serum harvested for determination of plasma Hcy and lipids. Subsequently, peritoneal macrophages were isolated for quantitative real-time PCR analysis. After being removed, the heart was cut transversely. Cryostat

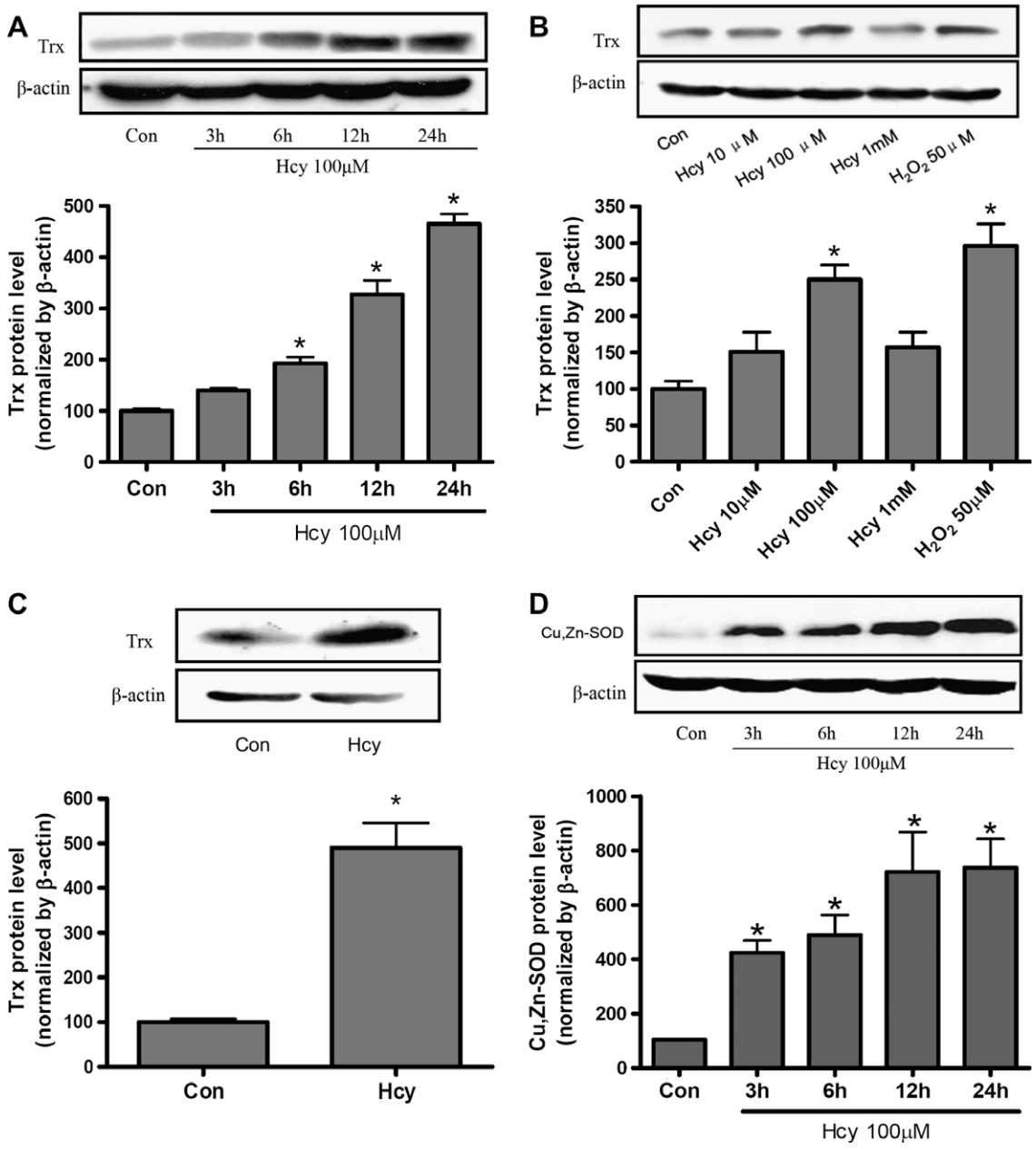

Fig. 1. Effect of Hcy on Trx and Cu, Zn-SOD protein levels in monocytes. Western blot of Trx protein expression in differentiated THP-1 cells incubated with Hcy for various times, (A) and various concentrations or H_2O_2 as positive control, (B); primary cultured human monocytes incubated in the absence or presence of Hcy (100 μM) for 12 h, (C); and Cu, Zn-SOD protein expression in differentiated THP-1 cells incubated with Hcy for various times (D). $^*P < 0.05$ vs. control (Con), $n = 3$.

aortic root sections (7 μm) were then collected for oil red O staining. All treatment of laboratory animals and experimental procedures were approved by the Institutional Authority for Laboratory Animals Care of Peking University Health Science Center.

2.7. RNA extraction and quantitative real-time PCR analysis

Total RNA from murine peritoneal macrophages was isolated by use of Trizol reagent (Promega, Madison, WI). Total RNA was reverse transcribed with use of a reverse transcription system (Promega, Madison, WI). All amplification reactions involved use of the Mx3000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA) on the basis of SYBR Green I fluorescence. The primer pairs used were as follows: for mouse Trx1, upstream: 5′-TCT GCT ACG TGG TGT GGA CCT-3′ and downstream: 5′-TCC TTG TTA GCA CCG GAG AAC-3′; for mouse β-actin, upstream: 5′-ATC TGG CAC CAC ACC TTC-3′ and downstream: 5′-AGC CAG GTC CAG ACG CA-3′.

2.8. Plasma Hcy and lipids

Plasma total Hcy level was quantified by gas chromatography–mass spectrometry. Plasma total cholesterol (TC) and total triglyceride (TG) levels were assayed with use of kits from Zhong Sheng Bio-technology (Beijing, China).

2.9. Statistical analysis

Data are presented as means ± SEM and analyzed by one-way ANOVA and then the Student–Newman–Keuls test for multiple comparisons; $n = 3$–8 refers to data from 3 to 8 individual mice or 3 to 8 independent cell isolations. Unpaired Student's t-test was used for comparisons between two groups. A $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Hcy upregulated Trx protein expression in human monocytes in vitro

ROS is an important mediator for Hcy-induced MCP-1 secretion from human monocytes [5]. To examine whether Hcy directly affects expression of the redox regulator Trx in human monocytes in vitro, differentiated THP-1 cells were challenged with Hcy. The results showed that Hcy induced a time- and concentration-dependent upregulation of Trx protein expression (Fig. 1A and B). As a positive control, H_2O_2 (50 μM) also caused Trx upregulation in THP-1 cells (Fig. 1B). As well, Hcy also increased Trx expression in primary human monocytes (Fig. 1C). In addition, the effect of Hcy (100 μM) on the expression of other antioxidants were studied and a time-dependent upregulation of Cu, Zn-SOD

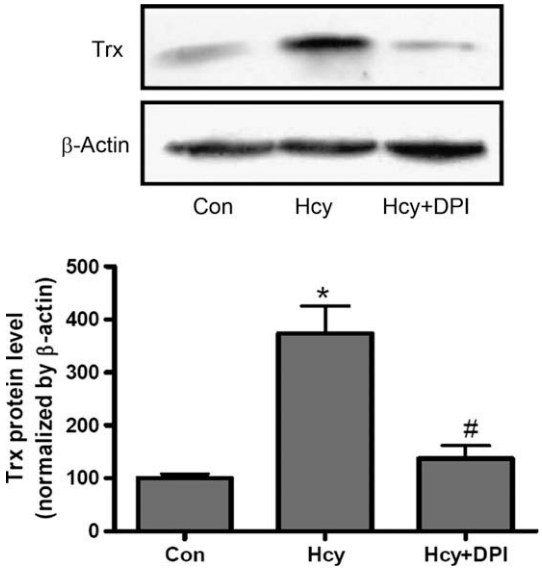

Fig. 2. Role of NADPH oxidase in Hcy-induced Trx protein expression. Western blot of Trx protein expression in differentiated THP-1 cells pretreated with the NADPH oxidase inhibitor DPI (10 μM) for 30 min and stimulated with Hcy (100 μM) for 12 h. Data represent results from three independent experiments. *: $P < 0.05$ vs. control (Con), $n = 3$; #: $P < 0.05$ vs. Hcy, $n = 3$.

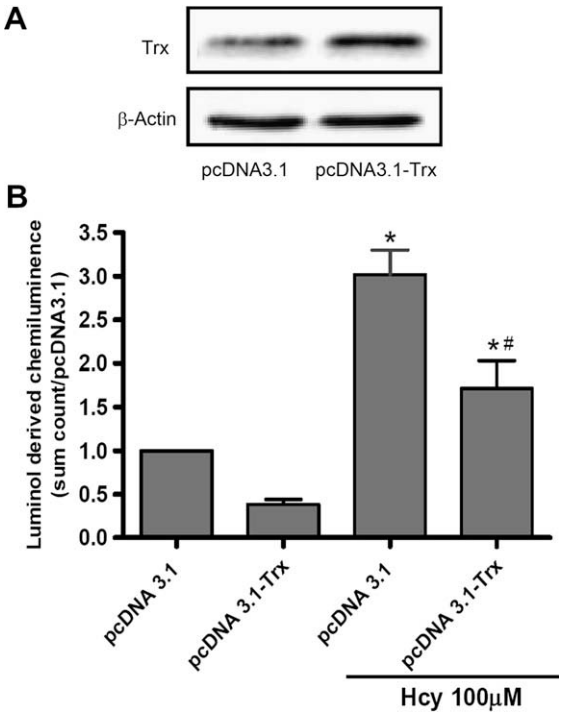

Fig. 3. Effect of Trx overexpression on Hcy-induced ROS production in A549 cells. Western blot of Trx protein expression in A549 cells transiently transfected with pcDNA3.1 and pcDNA3.1-Trx plasmid, for 24 h (A). Transfected cells were stimulated with Hcy (100 μM) for 20 min; ROS production was initiated by NADPH and measured by luminol-derived chemiluminescence (B). The sum count represents the amount of ROS production. *$P < 0.05$ vs. pcDNA3.1 alone, #$P < 0.05$ vs. Hcy + pcDNA 3.1, $n = 3$.

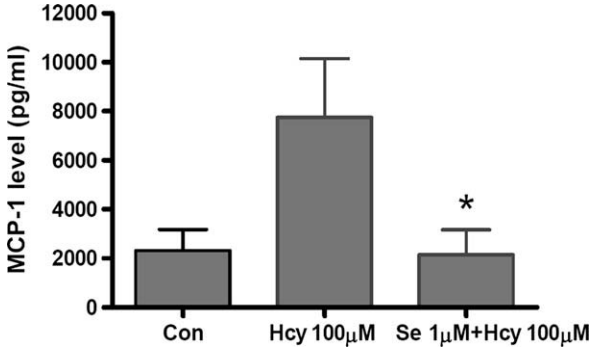

Fig. 4. Role of Trx activity in Hcy-induced MCP-1 secretion in primary human monocytes. ELISA results of MCP-1 secretion in primary human monocytes pretreated with selenium for 24 h and then stimulated with Hcy (100 μM) for 3 days. *$P < 0.05$ vs. Hcy alone, $n = 6$.

(Fig. 1D) but neither HO-1 nor Gpx was observed in differentiated THP-1 cells (data not shown).

3.2. Hcy induced Trx expression via NADPH oxidase in human monocytes

Trx expression can be induced via the antioxidant responsive element in its gene promoter upon oxidative stress [11]. Since we previously reported, Hcy could induce ROS production primarily via NADPH oxidase in human monocytes [5], we investigated whether Hcy-induced Trx expression depended on NADPH oxidase. Hcy-induced upregulation of Trx in THP-1 cells could be attenuated by DPI (10 μM), a specific inhibitor of NADPH oxidase (Fig. 2), suggesting that Hcy-induced upregulation of Trx depends on NADPH oxidase activity.

3.3. Trx overexpression suppressed Hcy-induced ROS production

A general and important function of Trx in cell signaling and defense against oxidative stress is to donate an electron donor to the ubiquitous family of Trx peroxidases, which catalyzes the reduction of H_2O_2 [7]. To evaluate the role of Trx in Hcy-induced production of ROS, we transfected Trx plasmids into A549 cells. Trx expression was significantly increased in transfected cells (Fig. 3A). Horseradish peroxidase-catalysed luminol chemiluminescence was used to monitor production of ROS, especially H_2O_2, in cells. Hcy treatment (100 μM) significantly enhanced ROS production, which was suppressed by Trx overexpression (Fig. 3B). These data imply that overexpression of Trx can suppress Hcy-induced ROS production. Therefore, Hcy-induced upregulation of Trx might represent a compensation to inhibit oxidative stress within a short time.

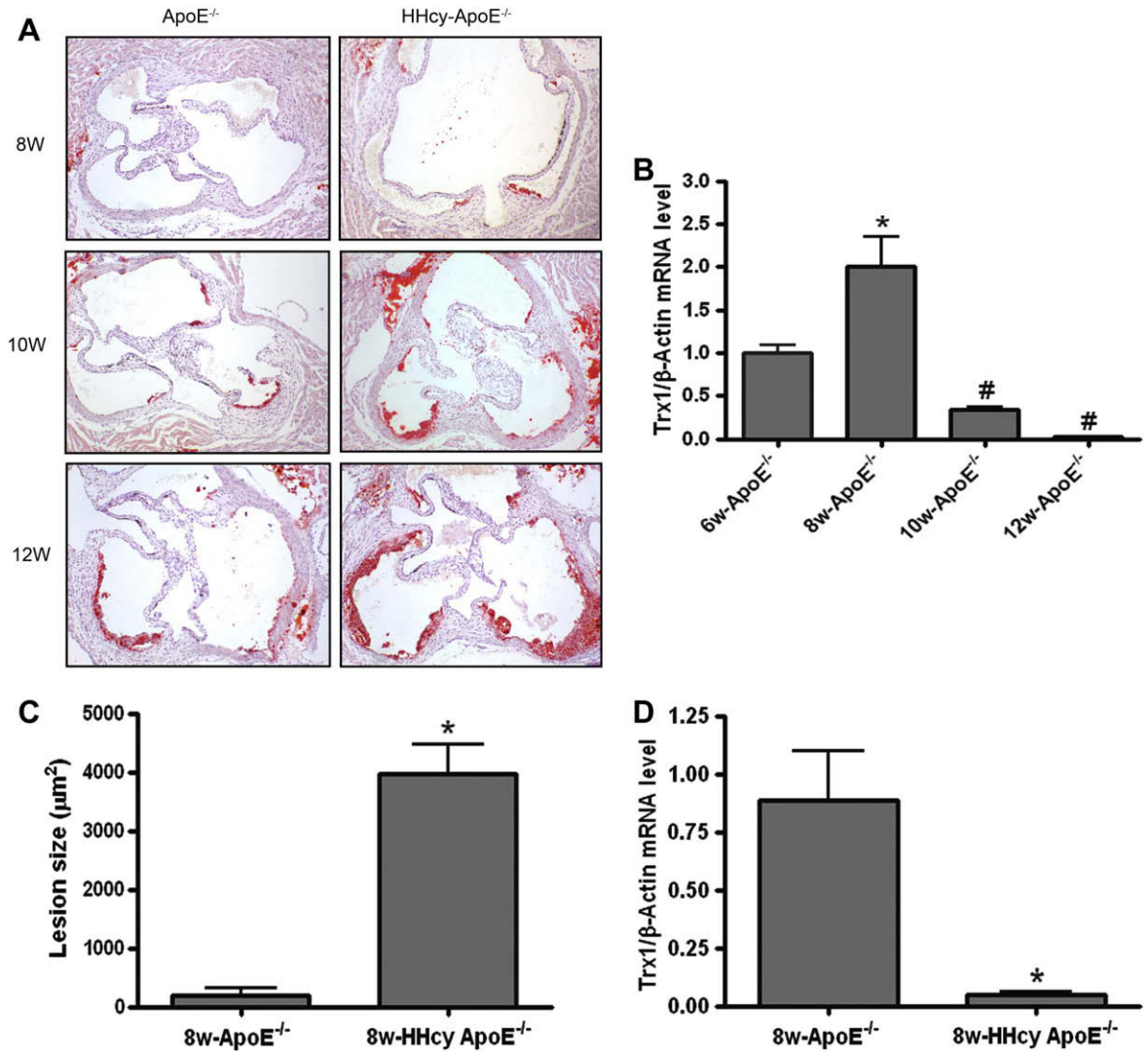

Fig. 5. HHcy accelerated the decrease of Trx expression in ApoE$^{-/-}$ mice. HHcy-accelerated early atherosclerotic lesions in 6-week-old ApoE$^{-/-}$ mice fed water with high Hcy content for 2, 4 and 6 weeks (8, 10, 12 w-HHcy ApoE$^{-/-}$). Representative serial sections of aortic roots stained with oil-red O (A). Trx mRNA level in peritoneal macrophages from 6, 8, 10 and 12-week-old ApoE$^{-/-}$ mice quantified by qRT-PCR. mRNA levels are relative to the expression of 6-week-old animals (6w-ApoE$^{-/-}$). $^{*}P < 0.05$ vs. 6-week-old ApoE$^{-/-}$, $n = 3$; $^{\#}P < 0.05$ vs. 8-week-old ApoE$^{-/-}$, $n = 3$ (B). Mean aortic root lesion areas in 8-week-old ApoE$^{-/-}$ mice (8w-ApoE$^{-/-}$) and 6-week-old ApoE$^{-/-}$ mice fed Hcy water for 2 weeks (8w-HHcy-ApoE$^{-/-}$) were quantified. $^{*}P < 0.05$, 8w-ApoE$^{-/-}$ vs. 8w-HHcy-ApoE$^{-/-}$, $n = 8$ (C). Trx mRNA level in peritoneal macrophages of 8-week-old ApoE$^{-/-}$ mice (8w-ApoE$^{-/-}$) and 6-week-old ApoE$^{-/-}$ mice fed Hcy water for 2 weeks (8w-HHcy-ApoE$^{-/-}$) were quantified by qRT-PCR. $^{*}P < 0.05$ 8w-ApoE$^{-/-}$ vs. 8w-HHcy-ApoE$^{-/-}$, $n = 6$ (D).

3.4. Increased Trx activity abolished Hcy-induced MCP-1 secretion in human monocytes

Our previous study showed pretreating cells with 1 μM sodium selenite for 24 h is an efficient method to enhance Trx reductase and Trx activity. We also ruled out any possible toxic effects of 1 μM sodium selenite on cells [12]. To evaluate the role of Trx in Hcy-induced MCP-1 secretion, we pretreated primary human monocytes with 1 μM sodium selenite for 24 h. Hcy (100 μM) significantly enhanced MCP-1 secretion which was abolished with 1 μM selenium pretreatment (Fig. 4), indicating that increased Trx activity can abolish Hcy-induced increase in MCP-1 secretion in human monocytes. The antioxidant Trx might protect against Hcy-induced inflammation in monocytes and further protect against Hcy-accelerated atherosclerosis by the compensation within a short time.

3.5. HHcy accelerated the decrease of Trx expression in $ApoE^{-/-}$ mice

We previously developed an animal model with HHcy by feeding 6-week-old $ApoE^{-/-}$ mice with water supplemented with Hcy for addition 4 weeks. Total plasma Hcy level was positively correlated with mean aortic root lesion area in the HHcy $ApoE^{-/-}$ mice [9]. To further evaluate the correlation between atherosclerotic lesion formation and Trx expression in $ApoE^{-/-}$ and HHcy $ApoE^{-/-}$ mice, we fed 6-week-old $ApoE^{-/-}$ mice with regular water or water containing high Hcy content for 2, 4 and 6 weeks (8–12-week-old HHcy $ApoE^{-/-}$). $ApoE^{-/-}$ mice fed with regular water developed spontaneous atherosclerotic plaques during 10–12-week-old, and HHcy accelerated atherosclerotic lesion formation in 8–12-week-old HHcy $ApoE^{-/-}$ compared with 8–12-week-old $ApoE^{-/-}$ mice alone (Fig. 5A). Hoen et al. [13] have reported that the expression of many antioxidant enzymes such as catalase-1, SOD, Gpx and glutathione S-transferase were significantly increased in the aortic arch of $ApoE^{-/-}$ mice during early stage of lesion formation, and then declined thereafter. In our current study, we found similar pattern of Trx expression in peritoneal macrophages from $ApoE^{-/-}$ mice. The relative mRNA level of Trx was higher, by 2-fold, in macrophages of 8-week-old $ApoE^{-/-}$ mice than in those of 6-week-old $ApoE^{-/-}$ mice, and then declined markedly with lesion development in 10–12-week-old $ApoE^{-/-}$ mice (Fig. 5B). Interestingly, although $ApoE^{-/-}$ mice fed with and without Hcy did not differ in plasma concentration of TC and TG (Table 1), 6-week-old $ApoE^{-/-}$ mice fed with Hcy water, even only for 2 weeks (8-week-old HHcy $ApoE^{-/-}$), showed accelerated lesion formation (Fig. 5C), and in accordance, decrease of Trx expression was earlier in these macrophages of 8-week-old HHcy $ApoE^{-/-}$ as compared with those of 8-week-old $ApoE^{-/-}$-alone significantly (Fig. 5D). These data suggest that HHcy-induced sustained oxidative stress might reduce the antioxidant capacity of the macrophage, which may, at least in part, account for macrophage inflammation and accelerate atherosclerotic formation in vivo.

Table 1
Plasma levels of Hcy, TC, and TG.

Groups	*n*	Hcy (μM)	TC (mM)	TG (mM)
8w-$ApoE^{-/-}$	8	5.7 ± 0.72	7.84 ± 0.54	1.07 ± 0.05
8w-HHcy $ApoE^{-/-}$[a]	8	16.81 ± 1.35[a]	8.02 ± 0.69	0.99 ± 0.06

TC, total cholesterol; TG, triglycerides.
[a]Six-week-old $ApoE^{-/-}$ mice were fed regular water or water with high Hcy content for 2 weeks. Values are mean ± S.E.M.; $P < 0.05$ as compared with 8w-$ApoE^{-/-}$ mice.

We also found that Hcy could induce a time-dependent upregulation of Cu, Zn-SOD in human monocytes. Nishio and Watanabe [14] showed with rat aorta smooth muscle cells that Hcy decreased Gpx activity and increased SOD activity but had no effect on catalase activity. Wilcken et al. [15] reported a positive correlation between extracellular SOD and total Hcy level in HHcy patients. Hcy-induced upregulation of SOD and Trx in monocytes might represent a protective antioxidant response to Hcy-induced oxidative damage and MCP-1 secretion and might contribute to reducing cardiovascular risk in patients with early HHcy. SOD expression following Hcy treatment of $ApoE^{-/-}$ mice may have the similar expression pattern with Trx in HHcy $ApoE^{-/-}$ mice. Additional studies are ongoing in our laboratory to further investigate Hcy effect on antioxidant, such as Trx and SOD expression in HHcy patients.

In conclusions, our studies demonstrate that the antioxidant Trx might play an important role in HHcy-accelerated atherosclerosis. In human monocytes in vitro, Hcy rapidly operates through NADPH oxidase to upregulate the expression of Trx to inhibit oxidative stress and MCP-1 secretion within a short time. However, this compensation is not strong enough to prevent HHcy-induced sustained oxidative stress, which finally inhibits the Trx expression and causes macrophage inflammation, and further accelerates the development of atherosclerosis. Thus, our findings provide new insight into understanding HHcy-mediated atherogenesis, suggesting a cue to prevent the progress of this process by modulating Trx activity.

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