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# Decreased serum levels of thioredoxin in patients with coronary artery disease plus hyperhomocysteinemia is strongly associated with the disease severity

Yunfei Wu[a,1], Lijuan Yang[b,1], Liangwei Zhong[a,*]

[a] College of Life Sciences, Graduate University of Chinese Academy of Sciences, Yuquan Road 19(A), 100049 Beijing, China
[b] Department of Endocrinology, Chinese PLA General Hospital, 100853 Beijing, China

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ABSTRACT

*Objective:* Elevation of homocysteine and thioredoxin (Trx) levels was found in some patients with coronary artery diseases (CAD). However, their correlations with CAD were not clear. Dysfunction of thioredoxin/thioredoxin reductase (TrxR) may cause oxidative stress that is common to CAD. We seek to determine the association among homocysteine, Trx/TrxR and CAD.
*Methods:* Serum samples were collected from 150 CAD patients under statin treatment and 122 non-CAD controls. Risk factors for atherosclerosis including homocysteine, lipids and glucose levels were analyzed. Trx/TrxR activities and protein levels were determined using super-insulin assay and Western blot, respectively. One-way ANOVA, Tukey's post hoc test and Spearman's rank correlation coefficient were used for statistical analysis. CAD severity was evaluated by angiographic Gensini score.
*Results:* Compared with non-CAD group, CAD group had significantly increased TrxR activity ($P < 0.05$) and homocysteine levels ($P < 0.01$), but not Trx activity. After further dividing CAD group using homocysteine below 15 μM as reference, Trx activity decreased significantly in CAD group with high homocysteine, and was inversely associated with homocysteine levels ($r = -0.199$, $P < 0.05$) that was, however, weakly positively associated with TrxR activity. Neither lipids nor glucose significantly affected Trx/TrxR activity. Association of CAD severity with low Trx plus high homocysteine was strong ($r = -0.458$, $P < 0.001$), but with high homocysteine alone was rather weak ($r = 0.125$, $P = 0.225$).
*Conclusion:* In CAD patients, high homocysteine levels may cause low Trx activity, which is closely correlated to the extent and severity of CAD.



## 1. Introduction

Elevation of plasma homocysteine was found to be weakly correlated with the extent of coronary artery disease (CAD), but strongly related with mortality of CAD patients [1]. In these observations the mechanism remains unclear. The toxic effects of homocysteine were frequently attributed to direct or indirect perturbation of redox homeostasis [2]. Studies on certain cell lines had associated homocysteine-induced oxidative stress with an increase in NADPH oxidase and a decrease in Trx [3]. Up-regulation of Trx expression may significantly reduce homocysteine-induced reactive oxygen species production [4]. However, clinical relationship between homocysteine and Trx levels in the human body remains unknown.

Human cytosolic Trx (hTrx1) is critical for cellular oxidation-reduction (redox) events. The reduced hTrx1 exerts antioxidant functions through Trx peroxidase [5], methionine sulfoxide reductase [6] or glutathione [7]. There are close association of cell growth or apoptosis with the availability of hTrx1 because reduced form of hTrx1 acts as an electron donor to ribonucleotide reductase [8] or as a negative regulator of apoptosis signal-regulating kinase 1 (ASK1) [9]. During reduction of substrates, hTrx1 is oxidized. In the human body, reduction of oxidized hTrx1 by NADPH is catalyzed by selenoprotein TrxR [10]. Human TrxR may catalyze the NADPH-dependent reduction of $H_2O_2$ [11], lipid hydroperoxides [12] and dehydroascorbate [13] as well. Trx, TrxR and NADPH, collectively called Trx system, play powerful roles in defence mechanism against oxidative stress, nitrosative stress [14] and in redox regulation of cell survival [15].

The presence of oxidative stress and/or inflammation often leads to up-regulation of Trx/TrxR [16], and release of the oxidized Trx1 into extracellular space [17]. Enhanced levels of Trx1 were observed in patients with CAD [18], acute myocardial infarction [19] or in coronary culprit lesions [20]. Although a few reports described the effect of homocysteine on Trx expression, their results appeared different from each other [3,4]. It is worthwhile to examine whether relation between Trx and homocysteine has clinical implications.

* Corresponding author. Tel.: +86 10 88256266; fax: +86 10 88256266.
*E-mail address:* liazho@gucas.ac.cn (L. Zhong).
[1] These authors contributed equally to this work.

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We here demonstrate that homocysteine critically affects Trx levels in human serum. The molecular link between them may provide a new insight into the mechanism by which Trx mediates homocysteine-induced cardiovascular events.

## 2. Materials and methods

### 2.1. Materials

Calf liver TrxR and recombinant hTrx1 were prepared in this Lab. Monoclonal antibodies against hTrx1 and human TrxR were purchased from Santa Cruz Biotechnology, Inc. Peroxidase-labeled human anti-mouse IgG antibodies and chemiluminescence ECL kit were purchased from Beyotime, China. PVDF membrane (Hybond-C Extra) was from Amersham Biosciences. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), NADPH and insulin were purchased from Sigma Corporation, USA. Abbott kit, CHOL kit, HDL-C plus 3rd generation kit, LDL-C plus 2nd generation kit, Total Protein Gen.2 and Gluco-quant glucose/HK kit were purchased from Roche Pharmaceuticals, USA.

### 2.2. Clinical analysis for serum levels of homocysteine, lipids and glucose

All analyses were performed with Hitachi 7600 automatic chemistry analyzer (Beijing Strong Biotechnologies, Inc., Beijing, China). The levels of serum total homocysteine were determined using an enzymatic recycling method [21]. Abbott kit, CHOL kit, HDL-C plus 3rd generation kit, LDL-C plus 2nd generation kit, Total Protein Gen.2 and Gluco-quant glucose/HK kit were used to determine serum levels of total homocysteine, total cholesterol/triglyceride, HDL-C, LDL-C, total protein and blood glucose, respectively.

Using following criteria, study groups were further divided into subgroups: hyperhomocysteinemia (total serum homocysteine concentrations $\geq$15 μM), dyslipidemia [total cholesterol concentrations (TC) $\geq$5.7 mM, HDL cholesterol (HDL-C) concentrations $\leq$0.9 mM, LDL cholesterol (LDL-C) concentrations $\geq$3.4 mM, total triglyceride concentrations (TG) $\geq$1.7 mM] and hyperglycemia (fasting blood glucose concentrations $\geq$6.1 mM).

### 2.3. Study groups

#### 2.3.1. CAD patient group

A total of 150 patients with CAD, which was confirmed by clinical history, standard diagnostic techniques and coronary angiography, were selected from the hospitalized patients in Chinese PLA General Hospital, Beijing. Patients with abnormal hepatic/renal function and cancer were excluded. All of these patients had started statin therapy (with simvastatin, atorvastatin calcium or pravastatin sodium 20 mg daily) long before this study. Using serum levels of total homocysteine (Hcy) below 15 μM as a reference, patients were further divided into “CAD (normal Hcy) group” and “CAD (high Hcy) group”.

#### 2.3.2. Non-CAD control group

Control individuals were selected among the persons who underwent an annual regular physical checkup. 122 individuals were included. These individuals had no history or symptoms of CAD. Similarly, they were further divided into two subgroups: “non-CAD (normal Hcy) group” and “non-CAD (high Hcy) group” as well.

The investigation conforms to the principles outlined in the Declaration of Helsinki, and was approved by the medical ethics committee of Chinese PLA General Hospital. Informed consent was obtained.

### 2.4. Measurement of serum Trx/TrxR activity

Selected serum samples were frozen at −80 °C until testing for Trx/TrxR. The activity was measured in a 96-well plate using the enzyme mark instrument (Multiskan MK3, Thermo, USA).

To measure Trx/TrxR activity, an assay mixture was prepared, which contained 0.25 M potassium phosphate, pH 7.5, 10 mM EDTA, 2 mM NADPH, 1.07 mM bovine insulin, and 40 μl of TrxR (for measuring Trx) or Trx (for measuring TrxR). The working solution of Trx or TrxR had been standardized through diluting its stock solution with 50 mM potassium phosphate buffer, pH 7.5 containing 1 mM EDTA (PE buffer), to give a final TrxR activity of Δ150 mA412 nm/min or a final Trx activity of Δ10 mA340 nm/min in 100 μl final reaction volume. The reaction was started by mixing 20 μl of serum, 30 μl of the assay mixture and 50 μl of distilled water, and performed at room temperature for 30 min. The reaction was terminated by adding 100 μl of 0.1 mM DTNB/8 M guanidine hydrochloride in 0.2 M potassium phosphate, pH 7.5. Trx/TrxR activity was determined by following an increase in absorbance at 412 nm due to Trx/TrxR-dependent reduction of DTNB to $TNB^{-}$. Activities (units) of Trx/TrxR are expressed as changes in A412 nm/mg protein·min.

A value of serum blank sample, in which 20 μl of PE buffer was used instead of serum, was determined to correct for non-Trx/TrxR-dependent reduction of DTNB.

Standard curves were obtained using the purified human cytosolic Trx and human placenta TrxR. The standard curve is linear over the range 0–100 ng/ml of Trx, or over the range 0–90 ng/ml of TrxR. The limit of detection is ∼20 ng/ml of Trx, or ∼15 ng/ml of TrxR, respectively.

### 2.5. Western blot analysis of serum Trx/TrxR protein levels

According to homocysteine concentrations and Trx/TrxR activity, serum samples were selected and diluted with phosphate buffered saline (PBS) to give a final protein concentration of 5 mg/ml. The diluted samples were loaded onto a 15% gel for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were electro-blotted onto a PVDF membrane, which was then treated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 1 h at room temperature. The Trx or TrxR was detected by incubating the membrane with mouse monoclonal IgG antibody raised against full-length hTrx1 or human TrxR, respectively, at room temperature for 2 h with shaking. Both antibodies were diluted 1:500 in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween 20). After wash three times in TBST, the membrane was incubated with a secondary antibody, i.e. anti-mouse IgG conjugated to horseradish peroxidase that was diluted 1:1000 in TBST. After 2 h, the membrane was again washed three times in TBST. The bound antibodies were visualized by the enhanced chemiluminescence (ECL) system with exposure to Amersham Hyperfilm MP at 25 °C.

### 2.6. Statistical analyses

Statistical data were expressed as means ± SD. One-way ANOVA was used to compare the mean values of selected groups. Tukey’s HSD post hoc test was used to determine which of the means for the four subgroups were significantly different from the others. Spearman’s rank correlation coefficient was used to assess the correlations between serum homocysteine levels and Trx/TrxR activity or the correlation between Trx activity/homocysteine levels and CAD severity. Statistical analyses were performed using SPSS software, version 13.0. *P*-values of less than 0.05 were considered as statistical significance.

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**Table 1**
Comparison of main clinical and biochemical features between CAD group and non-CAD group.

| | Non-CAD ($n$ = 122) | CAD ($n$ = 150) | $P$ |
|---|---|---|---|
| Mean age (years) | 56.5 ± 9.3[a] | 65.0 ± 11.4 | – |
| Men, $n$ (%) | 80 (66.7%) | 108 (72%) | – |
| Statin treatment, $n$ (%) | 0 | 150 (100%) | – |
| Homocysteine (μM) | 12.5 ± 5.33 | 17.0 ± 8.07 | <0.001 |
| Trx (mA412/mg·min) | 0.666 ± 0.410 | 0.723 ± 0.567 | 0.404 |
| TrxR (mA412/mg·min) | 0.983 ± 0.765 | 1.230 ± 1.061 | 0.040 |

[a] Data are expressed as mean ± SD. Three independent experiments were performed for measuring Trx/TrxR activity.

### 2.7. Measurement of CAD severity

Coronary angiograms of the CAD patients who had low Trx activity plus high homocysteine levels in serum were selected for evaluating CAD severity by Gensini score system [22]. The severity score was 0 for 0% stenosis of the coronary artery lumen, 1 for 1–25% stenosis, 2 for 26–50% stenosis, 4 for 51–75% stenosis, 8 for 76–90% stenosis, 16 for 91–99% stenosis, and 32 for total occlusion. Then this primary score is multiplied by a factor that takes into account the functional importance of the lesion's position in the coronary arterial tree, according to the method described by Gensini [22].

## 3. Results

### 3.1. Serum Trx/TrxR activity

One-way ANOVA was used to compare the mean values of selected groups. Main clinical and biochemical features of CAD group and non-CAD group are compared in Table 1. TrxR activity and homocysteine levels were significantly higher in CAD group than those in non-CAD group. There was no significant difference of Trx activity between the two groups.

However, interesting findings were observed when each group was further divided into two subgroups using serum levels of homocysteine below 15 μM as reference group. As shown in Table 2, the subgroup with high homocysteine had significantly lower activity of Trx, but not TrxR, than reference group had ($P$ = 0.004).

As dyslipidemia and hyperglycemia are common in CAD patients, we next examined the effects of lipids and glucose on Trx/TrxR activity. Neither lipids nor glucose were significantly correlated to the changes in TrxR/Trx activity (Table 2).

### 3.2. Serum Trx/TrxR protein levels

To see whether changes in Trx/TrxR activity were due to changes of their protein levels, Western blot analyses were performed. Upon reduction of serum samples with DTT, both Trx and TrxR appeared as a single band, corresponding to monomer of Trx (12 kDa) (Fig. 1A) or subunit of TrxR (55 kDa) (Fig. 1C). Compared with non-CAD individuals, Trx protein level was 1.5-fold higher in CAD patients with homocysteine below 15 μM, but was decreased by 34% in CAD patients with high homocysteine levels (Fig. 1A). TrxR protein level was 2.2-fold or 2.1-fold higher in CAD patients with homocysteine levels below or above 15 μM than that in non-CAD individuals (Fig. 1C). Under non-reducing conditions, Trx occurred as three major bands, corresponding to monomer, dimer or polymer/complex (Fig. 1B), and TrxR protein was mainly presented as the subunit dimer (Fig. 1D). The homocysteine-related changes in amounts of Trx/TrxR protein

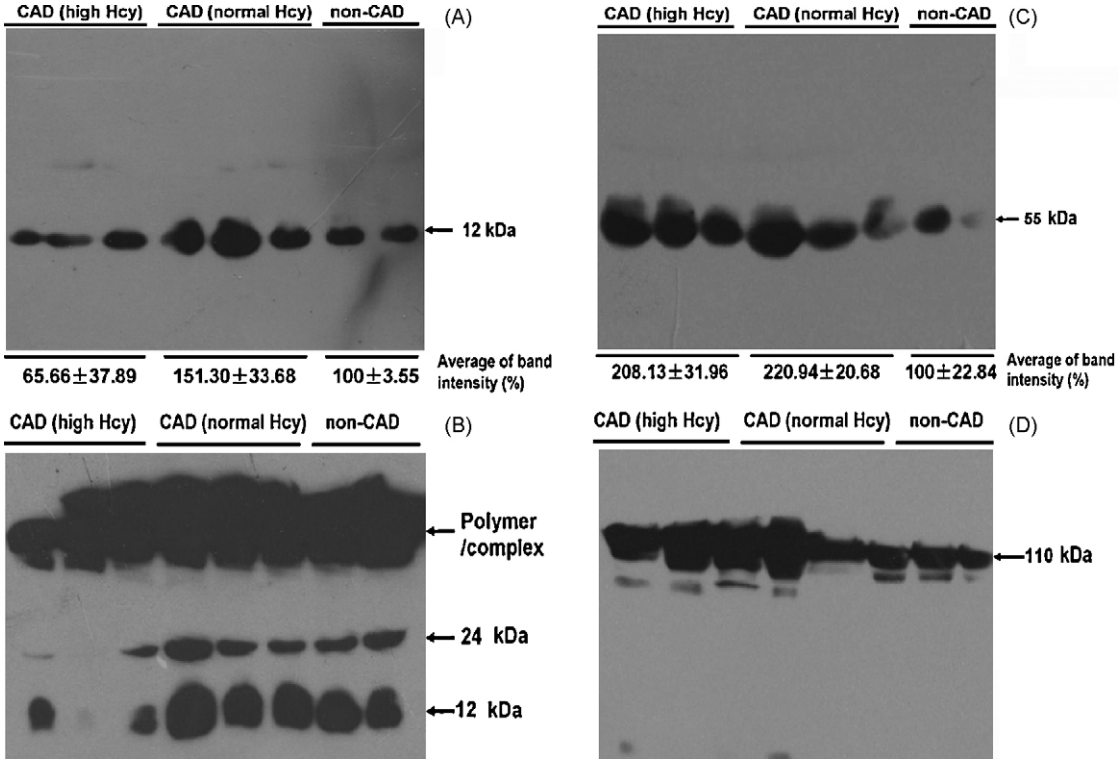


**Fig. 1.** Western blot analysis of Trx/TrxR in human serum. (A) DTT-reduced sera. Under these conditions, hTrx exists as a monomer. Its protein levels were higher in CAD patients with homocysteine below 15 μM [CAD (normal Hcy)] than that in CAD patients with homocysteine above 15 μM [CAD (high Hcy)], or that in non-CAD controls with homocysteine below 15 μM [non-CAD]. (B) Non-reduced sera. Under these conditions, hTrx exists mainly as monomer (12 kDa), dimer (24 kDa) and polymer/complex. (C) DTT-reduced sera. Under reducing conditions, there is no disulfide bond between two TrxR subunits, showing a band position at 55 kDa correlated with one subunit molecular weight. CAD patients had higher levels of TrxR than non-CAD controls had. Homocysteine showed positive but weak effect on TrxR. (D) Non-reduced sera. Under non-reducing conditions, two subunits of TrxR are covalently linked, showing a major band at 110 kDa correlated with 2× subunit molecular weight.

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**Table 2**
Effects of lipids/glucose/homocysteine on Trx/TrxR activity in CAD patients.

| | | TrxR (mA412 nm/mg) | | | Trx (mA412 nm/mg·min) | | |
|---|---|---|---|---|---|---|---|
| | | (*n*) | | *P* | (*n*) | | *P* |
| tHcy | <15 μM | 75 | 1.183 ± 0.919[a] | 0.626 | 53 | 0.889 ± 0.711 | 0.004 |
| | ≥15 μM | 65 | 1.270 ± 1.182 | | 67 | 0.592 ± 0.384 | |
| HDL-C | >0.9 mM | 40 | 1.450 ± 1.35 | 0.122 | 36 | 0.718 ± 0.635 | 0.951 |
| | ≤0.9 mM | 100 | 1.142 ± 0.917 | | 84 | 0.725 ± 0.544 | |
| LDL-C | <3.4 mM | 120 | 1.272 ± 0.988 | 0.259 | 104 | 0.726 ± 0.567 | 0.900 |
| | ≥3.4 mM | 20 | 0.980 ± 0.594 | | 15 | 0.707 ± 0.605 | |
| TG | <1.7 mM | 82 | 1.312 ± 1.149 | 0.269 | 67 | 0.729 ± 0.530 | 0.902 |
| | ≥1.7 mM | 57 | 1.108 ± 0.938 | | 53 | 0.716 ± 0.622 | |
| TC | <5.7 mM | 125 | 1.266 ± 1.105 | 0.173 | 108 | 0.721 ± 0.565 | 0.780 |
| | ≥5.7 mM | 13 | 0.919 ± 0.601 | | 11 | 0.756 ± 0.662 | |
| Glucose | <6.1 mM | 105 | 1.131 ± 0.880 | 0.057 | 88 | 0.700 ± 0.561 | 0.433 |
| | ≥6.1 mM | 35 | 1.525 ± 1.465 | | 32 | 0.791 ± 0.597 | |

[a] Data are expressed as mean ± SD. tHcy: total homocysteine; TG: triglyceride; TC: total cholesterol.

under reducing conditions appeared similar to that under non-reducing conditions.

### 3.3. Correlation between serum levels of Trx/homocysteine and severity of CAD

One-way ANOVA revealed a significant decrease of Trx activity in CAD group with high homocysteine levels ($P = 0.004$). Tukey's HSD post hoc test further indicated that CAD group with high homocysteine had much lower Trx activity than CAD group with homocysteine below 15 μM ($P = 0.007$), but Trx activity in non-CAD groups with normal or high homocysteine levels was not significantly different from one another (Fig. 2A). On the other hand, CAD subgroups had higher TrxR activity than non-CAD subgroups, but there was no statistical difference of TrxR activity among the four subgroups (Fig. 2B).

Spearman's rank correlation coefficients among homocysteine, Trx, TrxR and CAD severity were shown in Table 3. In CAD group, association of homocysteine with Trx activity was significantly negative ($r = -0.199$, $P = 0.030$), whereas association of homocystein with TrxR activity was slightly positive, but the difference was not statistically significant ($r = 0.001$, $P = 0.235$).

Of all CAD patients studied, coronary angiography was selected from 63 patients who had decreased Trx activity, accompanied by high homocysteine levels. The coronary angiography demonstrated that 11 patients (17.5%) had non-vessel lesion, 11 patients (17.5%) had single-vessel lesion, 21 patients (33.3%) had double-vessel lesion, 11 patients (17.5%) had triple-vessel lesion, and 9 patients (14.3%) had quadruple-vessel lesion. The Gensini score ranged from 0 to 400. High homocysteine plus decreased Trx activity was strongly, inversely associated with CAD severity ($r = -0.456$, $P < 0.001$). By contrast, high homocysteine alone was weakly associated with CAD severity ($r = 0.125$, $P = 0.255$), but the difference was not statistically significant (Table 3).

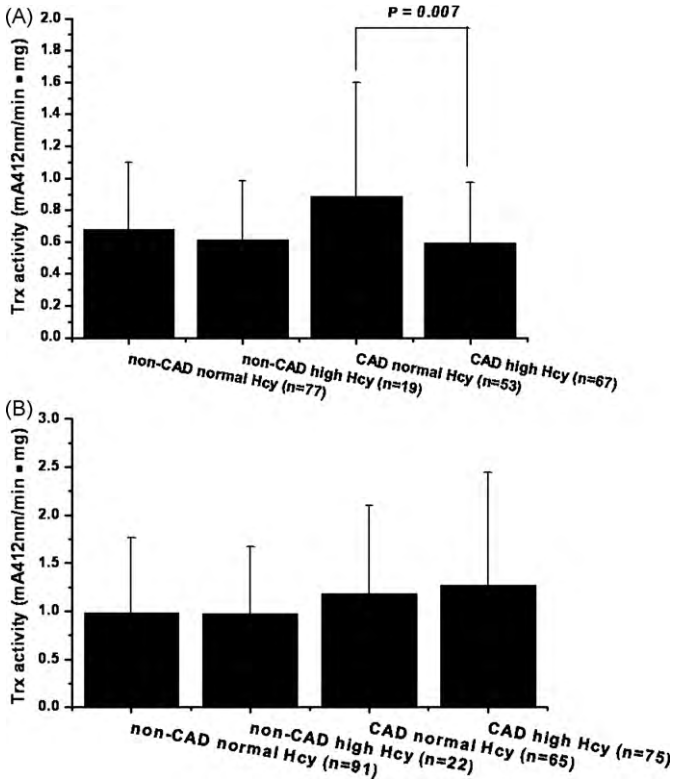


**Fig. 2.** Relation between homocysteine and Trx/TrxR. Levels of serum homocysteine were determined as described in Section 2. Activities of serum Trx/TrxR were analyzed with super-insulin assay. (A) Trx. Using homocysteine below 15 μM as reference, significant difference in Trx activity of the CAD groups was observed ($P = 0.004$, one-way ANOVA). Tukey's HSD test indicated that CAD group with high homocysteine had much lower Trx activity than CAD group with homocysteine below 15 μM ($P = 0.007$), but the Trx activity of non-CAD groups with normal or high homocysteine levels was not significantly different from one another. (B) TrxR. There was slight increase of TrxR activity in CAD groups, but TrxR activity was not significantly different from one another.

## 4. Discussion

In CAD patients, serum Trx activity would go down if serum homocysteine levels go up. Moreover, high homocysteine plus decreased Trx is strongly related to CAD severity, but high homocysteine alone is only weakly related to CAD severity (Table 3). So, serum Trx seems related not only to the presence of CAD but also to the severity of CAD under conditions of hyperhomocysteinemia. As hTrx1 is able to quench singlet oxygen or hydroxyl radical [23], and inhibit p38 MAP-mediated endothelial apoptosis [24], our results suggest that hTrx1 may be involved in the mechanism underlying high homocysteine-induced oxidative stress and apoptosis.

A negative correlation between homocysteine levels and Trx protein levels indicates that homocysteine might have effects on Trx degradation or synthesis. From Fig. 1, we learn that redox status of human serum affects the interaction of Trx/TrxR with itself or other serum proteins, but does not have obvious effects on levels of Trx/TrxR protein. Through comparing the Trx pattern in serum after one day storage with that in serum after 2-month storage, we

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**Table 3**
Correlation coefficients among homocysteine, Trx, TrxR and CAD severity in CAD patients.

| | Patients ($n$ = 120) Trx | Patients ($n$ = 140) TrxR | Patients ($n$ = 63) Severity of CAD |
|---|---|---|---|
| Homocysteine↑[a] | $r$ = −0.199 $P$ = 0.030 | $r$ = 0.001 $P$ = 0.235 | $r$ = 0.125 $P$ = 0.255 |
| Homocysteine↑ + Trx↓ | – | – | $r$ = −0.458 $P$ < 0.001 |

[a] Symbol ↑ stands for an increase, and ↓ stands for a decrease.

observed a similar Trx pattern in both the fresh and stored human serum (data not shown), which ruled out the experimental artifact that serum storage caused the formation of dimeric and polymeric Trx. In fact, formation of Trx/TrxR dimer or polymer may protect the essential surface Cys residue, such as hTrx1-Cys73, from oxidative impairment [25]; serum Trx/TrxR remains active after stored for months under aerobic conditions, and the oxidized form of both Trx and TrxR exhibits high resistance against proteinases (Zhong's observation). Moreover, Trx in human serum appeared active after an initial lag phase (data not shown). The delay period might reflect that the monomeric Trx is gradually released from the high molecular weight complexes. Thus, a high homocysteine level does not seem to enhance degradation of Trx protein in serum. On the other hand, there are few reports about the effect of homocysteine on Trx expression, although their results appeared controversial [3,4]. The possibility that the decrease of serum Trx is the result of homocysteine-induced reduction in Trx expression cannot be ruled out. Our investigation is in progress.

Remarkably, high homocysteine levels inhibit selenoprotein glutathione peroxidase-1 [26], but slightly enhance human TrxR levels (Fig. 1), the underlying mechanism remains unclear. Perhaps, increased oxidative stress, caused by homocysteine-related decrease of hTrx1, could be one of the major factors for up-regulation of TrxR [27,28]. Elevation of TrxR may be in line for its role in catalyzing the reduction of hydroperoxides and lipid peroxides, such as 15(S)HPETE that is associated with atherosclerosis [12]. With the apparent mismatch between Trx (significantly decreased) and TrxR (slightly increased), it seems unlikely that selenium supplement is effective at increasing antioxidant capacity of Trx system under conditions of hyperhomocysteinemia, although serum selenium state is deeply involved in TrxR activity [29].

In addition, CAD patients under statins treatment had basically normal levels of serum lipids, but remained significantly higher homocyteine levels than non-CAD group had, which was also observed by other researchers [30]. These findings suggest that new treatments aiming at increasing Trx activity might increase cardiovascular benefit by statin therapy in CAD patients.

In conclusion, serum Trx activity seems to be mainly dependent on homocysteine levels, and is not significantly affected by serum lipids or glucose levels in CAD patients. In particular, high homocysteine plus low Trx is strongly related to CAD severity, further studies with large patient numbers may document whether serum Trx levels might be useful biomarker in homocysteine-induced atherosclerosis or CAD.

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