

Elevated serum levels of thioredoxin in patients with acute exacerbation of asthma

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Abstract

The pathogenesis of bronchial asthma is chronic airway inflammation caused by immune cells such as T lymphocytes and eosinophils. Eosinophils release cytotoxic products including reactive oxygen species at the site of inflammation, leading to epithelial damage. Human thioredoxin (TRX), a redox-regulating protein with antioxidant activity, is induced and secreted from cells by oxidative stress. This study was undertaken to investigate the clinical significance of TRX in the pathogenesis of asthma. We collected blood samples from 48 patients with bronchial asthma with or without attack, and measured serum ECP and pulmonary function as well as serum TRX. The serum TRX levels in patients with asthma were significantly increased in patients with mild (34.63 [28.40–42.73] ng/ml, medians with 25 and 75% interquartiles, $P = 0.0064$) and moderate (38.83 [35.14–50.80] ng/ml, $P = 0.0017$) asthma attacks compared with those during the asymptomatic period. The serum TRX levels were inversely correlated with FEV_{1.0}% ($r = -0.44$, $P = 0.039$) and %PEF ($r = -0.49$, $P = 0.020$) during attack. There was a significant correlation between the serum TRX and the serum eosinophil cationic protein ($r_s = 0.32$, $P = 0.016$). These findings suggest that serum TRX is related to the state of asthma exacerbation and allergic inflammation.

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1. Introduction

The pathogenesis of bronchial asthma is chronic airway inflammation caused by immune cells, involving T lymphocytes and eosinophils [1,2]. The activation of eosinophils by cytokines and chemoattractants is a critical event in the allergic inflammatory response. Eosinophils are recruited from the circulation into inflammatory foci by chemoattractants such as C5a, platelet-activating factor, and C-C chemokines. IL-5 prolongs eosinophil survival and primes other functions in the tissue. At the site of inflammation, eosinophils release cytotoxic products including eosinophil granule proteins and reactive oxygen species (ROS) causing epithelial damage [1,2]. We and others have shown that

eosinophil-active cytokines and chemoattractants elicit the production of ROS from eosinophils [3–5]. Consistent with the aforementioned findings, eosinophils in bronchoalveolar lavage fluid (BALF) from subjects who underwent allergen challenge spontaneously produce prominent amounts of superoxide anions [6]. The quantity of oxidants appears to be increased at the inflammatory sites of asthma, leading to the formation of antioxidant defenses that neutralize the effect of oxidants [7]. One example of this phenomenon is the enhanced glutathione level in BALF obtained from patients with asthma [8].

Thioredoxin (TRX) containing a redox-active disulfide (-Cys-Gly-Pro-Cys-) was originally discovered in *Escherichia coli* as a hydrogen donor for ribonucleotide reductase [9]. Human TRX produced by human T-cell leukemia virus-1 (HTLV-1) transformed T-cells was cloned as adult T-cell leukemia-derived factor (ADF) [10]. By thorough investigations accomplished during

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Table 1
Clinical characteristics of patients with asthma

Number	48
Age (years)	48.9 ± 16.5
Male/female	26/22
Attack	Remission: 30, mild: 19 (7) ^a , moderate: 7 (1) ^a
Severity	Intermittent: 21, mild persistent: 20, moderate persistent: 7 ^b
Atopy	Atopy: 33, non-atopy: 15
Treatment during remission ^c	
Inhaled steroid	28 (58.3%)
Maintenance oral predonisolone	5 (10.4%)
Alternative treatment	Methylxanthine: 35 (72.9%), seratroduct: 9 (18.8%), pranlukast: 2 (4.2%), oxitropium bromide: 2 (4.2%), azelastine: 2 (4.2%), oral β_2 agonist: 2 (4.2%), tazanolast: 1 (2.1%), suplastat tosilate: 1 (2.1%)

Age is expressed as mean ± S.D. Other data are expressed as number with percent in parentheses.

^a The number of patients evaluated during both attack and asymptomatic periods is presented in parentheses.

^b Disease severity of asthmatic patients with attack was evaluated in their asymptomatic period.

^c All patients were treated with inhaled β_2 agonist, when required.

more than a decade, TRX is currently known to possess both intracellular and extracellular activities. The former includes antioxidant activity which regulates redox-sensitive molecules such as NF- κ B, AP-1, and glucocorticoid receptors. In contrast, TRX exhibits extracellular cytokine-like activity similar to IL-2R α chain induction [11]. Recent studies have shown that TRX is a potent chemoattractant for neutrophils, monocytes, T-cells, and eosinophils [12,13]. Clinically, TRX appears to play a crucial role in the control of oxidative stress in many diseases. TRX is involved in infection by various viruses as well as HTLV-1, and in a variety of human cancers [14–17]. Autoimmune diseases including rheumatoid arthritis (RA) and Sjögren's syndrome are associated with increased levels of TRX [18,19]. In addition, acutely elevated circulating TRX appears to be beneficial to reduce inflammation. For example, circulating TRX markedly blocks migration of neutrophils into the lipopolysaccharide-stimulated air pouch in mice with elevated TRX levels [20].

In this study, we measured the serum levels of TRX in asthmatic patients to investigate its clinical roles. Increased serum TRX levels were observed in patients with asthma attacks and associated with loss of lung functions and eosinophil activation.

2. Materials and methods

2.1. Study subjects

We collected blood samples from 48 patients with bronchial asthma (Table 1). Asthma was diagnosed according to the criteria of the American Thoracic Society [21]. None of the patients with asthma had complications of other lung diseases, or had a history suggesting systemic viral infection, tumors, or autoimmune diseases. Disease severity of asthma and severity

of asthma attack were classified according to the NHLBI/WHO Workshop on the Global Strategy for Asthma (GINA guidelines) [22]. All patients with atopic asthma showed a positive radioallergosorbent test (RAST) (specific immunoglobulin E (IgE) $\geq$ class 3), a positive skin prick test to one or more common inhalant allergens, or elevated total serum IgE level. All patients were treated with inhaled β_2 agonist when required. Pulmonary function was monitored in all patients by measuring the expiratory peak flow rate and FEV_{1.0}%. Informed consent was obtained from each subject for participation in the study, and the clinical research ethics committee of our university approved this study.

2.2. Serum TRX measurement

Serum TRX was measured using a sensitive sandwich ELISA kit (Fujirebio, Inc., Tokyo, Japan) [15]. All measurements were made in duplicate, and the average value was adopted.

2.3. Measurement of serum ECP levels

After the blood sampling using specially designed sampling tubes for ECP measurement (vacutainer tube with SST gel; Beckton Dickinson, San Jose, CA), samples were left at room temperature for 1–2 h. The serum was obtained by centrifugation to measure ECP with an ECP Radioimmunoassay kit (Pharmacia, Uppsala, Sweden).

2.4. Evaluation of pulmonary function

Immediately after the blood sampling, FEV_{1.0}% and percentage of predicted peak expiratory flow (%PEF) were evaluated using CHESTAC-65V (CHEST, Tokyo, Japan). FEV_{1.0} was expressed as the percentage of forced vital capacity (FEV_{1.0}%).

2.5. Statistical analysis

Normally distributed data were expressed as mean $\pm$ standard deviation. When the data did not show a normal distribution, data were presented as medians with 25 and 75% interquartiles. Differences among groups were tested by parametric or nonparametric test as appropriate as indicated in each figure or table. Comparisons of the serum TRX levels in subgroups were tested by the paired or the unpaired Student's *t*-test or the one-way analysis of variance (ANOVA) for normally distributed data and by the Mann–Whitney *U*-test or the Kruskal–Wallis test for not normally distributed data. In case of significant difference, the Kruskal–Wallis test was followed by the Mann–Whitney *U*-test with Bonferroni correction. The relationships between these variables, which were normally distributed, were examined with the Pearson's correlation coefficient. Correlations between serum TRX levels and other parameters were determined by the Spearman's rank correlation coefficient when the data were not normally distributed. Statistical significance was defined as $P < 0.05$. However, in the Mann–Whitney *U*-test with Bonferroni correction, *P*-value of 0.0166 or less was regarded as significant (Bonferroni correction for three groups).

3. Results

3.1. Serum levels of TRX

To study the clinical significance of TRX in asthma, we measured the serum levels of TRX in asthmatic patients with and without attacks. There was no significant correlation between the serum TRX levels and age in all three groups (all subjects, non-attack subjects and attack subjects) (Table 2). In addition, no significant differences in the serum TRX levels were found in patients with asthma in term of sex, smoking history, state of atopy or receiving inhaled corticosteroid (Table 3). Furthermore, the serum TRX levels showed no significant differences among subgroups according to the disease severity of asthma (Table 4). As shown in Fig. 1A, the TRX levels in patients with asthma were

significantly elevated in patients with mild (34.63 [28.40–42.73] ng/ml, $n = 19$, $P = 0.0064$) and moderate attack (38.83 [35.14–50.80] ng/ml, $n = 7$, $P = 0.0017$) compared with those during the asymptomatic period (26.62 [21.78–32.63] ng/ml, $n = 30$) (Kruskal–Wallis test, $P = 0.00090$). We further examined serum TRX in identical patients during both attacks and asymptomatic periods. The TRX levels during attacks were significantly higher than those during remission ($n = 8$, $P = 0.0048$) (Fig. 1B).

3.2. Relation between serum TRX and pulmonary function tests in patients during attacks

The pulmonary function tests were performed immediately after the blood sampling. Although all subjects measured %PEF at home, FEV_{1.0}% and %PEF were not evaluated using CHESTAC-65V in four of them. Therefore, these four subjects were excluded. The serum TRX levels inversely correlated with both FEV_{1.0}% ($r = -0.44$, $P = 0.039$) and %PEF ($r = -0.49$, $P = 0.020$) in patients during attacks ($n = 22$) (Fig. 2A and B). No correlations between the serum TRX levels and both FEV_{1.0}% ($r = 0.086$, $P = 0.66$) and %PEF ($r = 0.013$, $P = 0.51$) were observed in asymptomatic patients.

3.3. Relation between serum TRX and parameters of eosinophil activation

Eosinophils are the primary effector cells in the mechanism of asthma. Serum levels of ECP are known as markers for eosinophil [23]. Serum ECP is elevated in accordance with asthma exacerbations [23]. For this reason, it is of paramount interest to clarify their relation with TRX. We initially examined the relation between serum TRX and peripheral eosinophil counts. Neither %eosinophils nor eosinophil counts correlated with the TRX levels ($rs = 0.0030$, $P = 0.98$ and $rs = 0.035$, $P = 0.79$, respectively). We measured the serum ECP concentration using radioimmunoassay. There was a significant correlation between TRX and ECP levels in sera from asthmatic patients ($n = 56$, $rs = 0.32$, $P = 0.016$) (Fig. 3).

Table 2
Relation between the serum TRX levels and age in patients with asthma

	Serum TRX					
	All subjects		Non-attack		Attack	
	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value
Age	−0.044	0.77	−0.234	0.22	0.185	0.36

Since all data were normally distributed, correlations were conducted using Pearson's correlation test.

Table 3

Comparisons of the serum TRX levels with sex, smoking history, state of atopy, and inhaled corticosteroid treatment

	All subjects (<i>n</i> = 48)		Non-attack (<i>n</i> = 30)		Attack (<i>n</i> = 26)	
	Serum TRX (ng/ml)	<i>P</i> -value	Serum TRX (ng/ml)	<i>P</i> -value	Serum TRX (ng/ml)	<i>P</i> -value
Sex (M/F)	29.75 (25.76–37.75)/ 30.86 (21.65–37.60)	0.53	28.92 (24.57–37.33)/ 23.71 (20.06–31.75)	0.23	39.59 ± 14.18/38.08 ± 12.04	0.6
Smoking (NS/S)	29.60 (23.03–37.30)/ 32.30 (27.63–41.64)	0.25	27.42 ± 7.84/29.81 ± 5.13	0.57	37.91 ± 12.09/42.72 ± 16.98	0.4
Atopy (NA/A)	30.95 ± 11.64/ 32.96 ± 12.65	0.61	27.11 ± 8.38/28.17 ± 7.12	0.64	38.77 ± 10.72/38.87 ± 14.25	0.88
ICS (NICS/ICS) ^a	29.57 ± 9.52/ 34.30 ± 13.72	0.19	26.77 ± 6.63/28.62 ± 8.10	0.62	36.57 (27.02–42.96)/36.81 (31.16–43.98)	0.2

Definition of abbreviations: M, male; F, female; NS, non-smoking; S, smoking; NA, non-atopy; A, atopy; ICS, inhaled corticosteroid. Normally distributed data were expressed as mean ± standard deviation and calculated by the unpaired Student's *t*-test. When the data did not show a normal distribution, data were presented as medians with 25 and 75% interquartiles in parentheses and calculated by the Mann–Whitney *U*-test.

^a Not receiving inhaled corticosteroid/receiving inhaled corticosteroid.

4. Discussion

In this study, we investigated the clinical relevance of serum TRX in bronchial asthma. We found increased levels of serum TRX in patients with asthma attacks compared with those in remission. This increase correlated with the degree of airway obstruction as assessed by FEV_{1,0}% and %PEF. Serum TRX levels also correlated with serum ECP. This is the first study to report the clinical relevance of TRX in the pathogenesis of asthma.

It was previously shown that TRX is associated with various diseases [14]. Increased TRX levels have been reported in patients with hepatitis C virus (HCV) chronic hepatitis [15] and human immunodeficiency virus infection [24]. HCV-infected patients with higher serum TRX levels exhibit progression of liver fibrosis and resistance to interferon therapy [15]. The prognosis of acquired immunodeficiency syndrome (AIDS) patients with elevated plasma TRX and low CD4 T-cell counts is poor [25]. The cells infected with HTLV-1, Epstein–Barr virus, or human papilloma virus highly express TRX, suggesting its important role in the coexistence of host cells and viruses [14]. Studies with a variety of human primary tumors have shown the

overexpression of TRX in tumors including hepatocellular carcinoma (HCC), squamous cell carcinoma, cervix cancer, pancreatic cancer, and colon cancer, compared with the levels in normal tissue [14]. Interestingly, serum or plasma levels of TRX are elevated in patients with hepatocellular and pancreatic ductal carcinoma, suggesting that TRX can be a useful clinical parameter in these cancers [16,17]. In collagen diseases like RA and Sjögren's syndrome, TRX is also highly expressed at the inflamed foci [18,19]. For example, the TRX levels are increased in synovial fluid compared with plasma samples in patients with RA [18]. Although the serum levels of TRX did not reflect the disease severity of asthma, we showed that serum levels of TRX were significantly higher in patients with both mild and moderate attacks than those in remission. Furthermore, the TRX levels significantly increased depending on the degree of asthma attacks. Although we did not show evidence for TRX levels in healthy subjects, the serum concentrations reported by other investigators, who measured serum TRX by the same ELISA kit as we used, ranged from 1.3 to 50.7 ng/ml with a median of 24.9 ng/ml [15]. Since the present findings for the TRX levels during mild and moderate attacks were medians of 34.63 and 38.83 ng/ml, respectively, the TRX levels

Table 4

Comparisons of the serum TRX levels with severity of asthma

	Intermittent	Mild persistent	Moderate persistent	<i>P</i> -value
All subjects	29.97 (21.79–39.82) (<i>n</i> = 21)	28.16 (23.72–37.33) (<i>n</i> = 20)	34.72 (31.54–37.71) (<i>n</i> = 7)	0.64 ^a
Non-attack	27.08 ± 7.00 (<i>n</i> = 12)	28.21 ± 7.70 (<i>n</i> = 16)	(<i>n</i> = 2)	0.83 ^b
Attack ^c	41.86 ± 17.15 (<i>n</i> = 10)	38.09 ± 11.89 (<i>n</i> = 10)	35.04 ± 3.60 (<i>n</i> = 6)	0.20 ^d

Normally distributed data were expressed as mean ± standard deviation. When the data did not show a normal distribution, data were presented as medians with 25 and 75% interquartiles in parentheses.

^a Calculated by the Kruskal–Wallis test.

^b Calculated by the unpaired Student's *t*-test.

^c Disease severity of asthmatic patients with attack was evaluated in their asymptomatic period.

^d Calculated by the one-way analysis of variance (ANOVA).

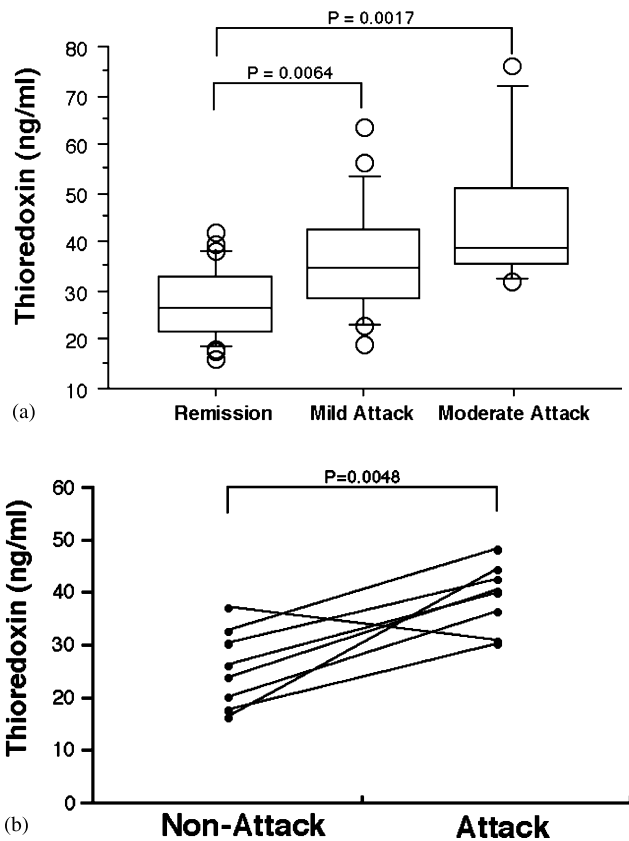


Fig. 1. (A) The serum levels of TRX in patients with bronchial asthma with mild ($n = 19$) and moderate ($n = 7$) attacks, and without attack ($n = 30$). The serum TRX levels of patients with mild (34.63 [28.40–42.73] ng/ml, $n = 19$, $P = 0.0064$) and moderate (38.83 [35.14–50.80] ng/ml, $n = 7$, $P = 0.0017$) attacks were significantly higher than those of non-attack subjects (26.62 [21.78–32.63] ng/ml, $n = 30$) (Kruskal–Wallis test, $P = 0.00090$). (B) Increase in the serum TRX levels in patients with asthma before and after attack ($n = 8$; paired t -test, $P = 0.0048$).

during asthma attacks tend to be higher than those in remission or normal controls. These findings suggest that the serum TRX level is increased in connection with asthma attacks.

It is unclear whether the levels of TRX can be changed in parallel with other clinical parameters. In HIV-infected patients, the increase in plasma TRX levels correlates with altered surface expression of CD62L, CD38, and CD20 on peripheral blood mononuclear cells that occur in later stages of HIV infection [24]. Significant correlations are observed in serum levels between TRX and markers of hepatic fibrosis such as ferritin, hyaluronic acid, IV collagen, and P-III-P [15]. In contrast, Miyazaki et al. [16] studied the relation between the serum levels of TRX and α -fetoprotein in patients with HCC, and found no significant correlation between the markers. Interestingly, four HCC cases with the normal level of α -fetoprotein exhibited increased levels of serum TRX, whereas 12 HCC cases with a high concentration of α -fetoprotein had normal serum TRX

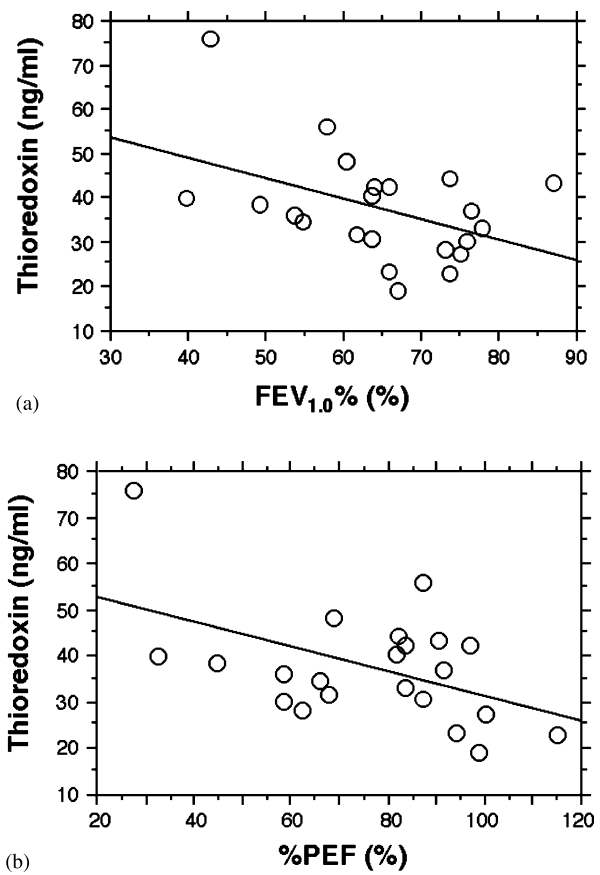


Fig. 2. Relation between the serum TRX levels and pulmonary function in patients with asthma during attack. There were significant negative correlation between serum TRX levels and both FEV_{1.0}% and %PEF in patients during attack ($n = 22$, Pearson's correlation: (A) $r = -0.44$, $P = 0.039$ and (B) $r = -0.49$, $P = 0.020$, respectively).

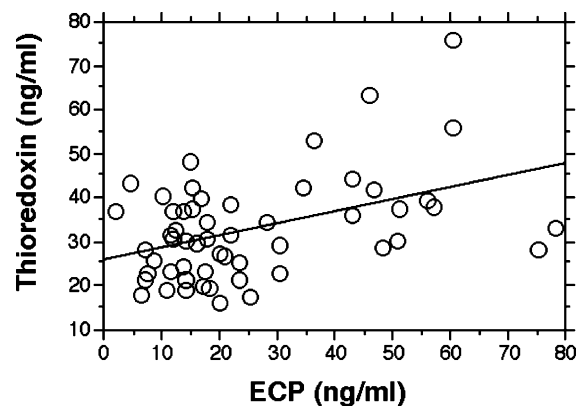


Fig. 3. Relation between the serum ECP and the serum TRX levels. Significant correlation between the ECP and the TRX levels in serum of asthmatic patients was shown ($n = 56$, Spearman's rank correlation, $r_s = 0.32$, $P = 0.016$).

levels. These observations suggest that serum TRX can be a parallel or additional marker depending on each disease. We showed that serum TRX levels during asthma attacks inversely correlate with the FEV_{1.0}% and %PEF. We also investigated the relationship

between serum TRX levels and serum ECP levels. Significant correlation was observed with ECP, a marker for eosinophil activation, but not with peripheral eosinophil counts or %eosinophils. The present results imply that serum levels of TRX definitely reflect airway obstruction and eosinophil activation in patients with asthma.

Regarding the significance of serum TRX levels in the pathogenesis of asthma, two different hypotheses are suggested. First, TRX may act as a scavenger of oxidants. TRX has been demonstrated to possess protective activities against oxidative stress in various models of diseases including atherosclerosis [26], reperfusion injury [27], and diabetes [28]. Since asthma is characterized by prominent production of ROS from inflammatory cells, TRX possibly plays a role to remove those oxidants. The second hypothesis is that TRX exhibits cytokine- or chemokine-like activity including activation of eosinophils [12–14]. Eosinophil functions such as cytotoxicity [29], migration [13], and release of major basic protein [30] are enhanced by TRX in vitro. Furthermore, TRX is a unique chemoattractant for neutrophils, monocytes, and T-cells as well as eosinophils [12,13]. However, leukocyte transmigration from blood vessels is likely to be impaired in in vivo models where the level of chemoattractants is elevated in circulation. Intravenous injection of IL-8 reduces polymorphonuclear leukocytes' accumulation in the tissue [31]. Leukocyte migration is also impaired in transgenic mice with high circulating levels of IL-8 [32] or monocyte chemoattractant protein-1 [33], which is sufficient to induce chemotaxis in vitro. Consistent with the effect of chemokines, Nakamura et al. [20] recently reported that circulating TRX efficiently blocks migration of neutrophils into the lipopolysaccharide-stimulated air pouch. In that study, TRX did not alter the expression of adhesion molecules on endothelium, suggesting its direct effect on neutrophils. We showed here that serum TRX levels were elevated in patients with asthma attacks. It is possible that high levels of circulating TRX decrease the ability of eosinophils to migrate into inflamed sites.

An important issue is that the elevation of circulating TRX is acute or chronic. The chronic elevation of TRX is harmful in HIV infection. The plasma TRX chronically elevated is highly associated with the shortening of survival in patients with AIDS [25]. In contrast, acute elevation of TRX appears to be beneficial. For example, injection of a bolus of TRX prevents ischemic reperfusion injury [27]. We observed higher TRX levels during asthma attacks, which are suggested to be an acute elevation and may be helpful to immune system. One explanation for the present results is that the circulating TRX acts as a down regulator that suppresses eosinophil extravasation, through prominent eosinophil infiltration in the airway during asthma attack, to minimize

the allergic inflammation. The action of TRX may be elicited either by the disappearance of the local chemokine gradients or deactivation of eosinophils with a loss of reactivity to other chemokines. Indeed, we found that pretreatment of TRX reduced eotaxin-induced eosinophil chemotaxis in vitro (manuscript in preparation).

In summary, we showed that elevated levels of serum TRX were observed in patients with asthma attacks, and that the serum TRX levels correlated with the clinical parameters of asthma on attack as well as marker for eosinophil activation. These findings suggest that the serum TRX level reflects both the status of asthma attack and the degree of eosinophilic inflammation, and that sheds light on developing a new strategy to treat asthma.

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