Thioredoxin in human and experimental sepsis*

Stefan Hofer, MD; Claudia Rosenhagen; Hajime Nakamura, MD, PhD; Junji Yodoi, MD, PhD; Christian Bopp, MD; Johannes B. Zimmermann; Meike Goebel, MD; Peter Schemmer, MD, PhD; Karolin Hoffmann, MD; Klaus Schulze-Osthoff, MD, PhD; Raoul Breitkreutz, MD, PhD; Markus A. Weigand, MD, PhD

**Introduction:** Thioredoxin (TRX) is assumed to be beneficial in acute inflammatory diseases because of its potent antioxidant properties and an inhibitory effect on neutrophil evasion into sites of inflammation.

**Objective:** To compare plasma levels of thioredoxin in septic patients and to investigate the role of thioredoxin in a polymicrobial septic mouse model.

**Design and Interventions:** A combined single-center noninterventional clinical observation study and randomized controlled experimental investigation.

**Setting:** Intensive care unit of a university hospital and laboratories of four university hospitals.

**Measurements and Main Results:** To evaluate the role of TRX in sepsis, we measured TRX in plasma of septic patients and compared its levels in survivors and patients who did not survive sepsis. In addition, we examined the effect of neutralization of endogenous TRX as well as of treatment with recombinant TRX in a mouse peritonitis model of cecal ligation and puncture (CLP). We found that the serum plasma levels of TRX were significantly higher in patients with sepsis compared with healthy individuals. Furthermore, nonsurvivors showed even higher TRX levels than survivors of sepsis. The CLP septic mouse model revealed that neutralization of endogenous TRX impaired survival of septic mice, whereas treatment with recombinant TRX after CLP strongly enhanced the survival of mice.

**Conclusions:** Our results therefore demonstrate a critical role for TRX in the septic inflammatory response and suggest TRX as a potential therapeutic target for septic shock. (Crit Care Med 2009; 37:2155–2159)

**Key Words:** thioredoxin; sepsis; reactive oxygen species; migration inhibitory factor; thioredoxin reductase

Thioredoxin (TRX), originally identified in bacteria as an electron donor for ribonucleotide reductase (1), contains a redox-active disulfide/dithiol within the conserved active site sequence Cys-Gly-Pro-Cys and is therefore an important regulator of the redox system. Together with the selenoprotein TRX reductase and nicotinamide adenine dinucleotide phosphate, it operates as a protein disulfide-reducing system (2–7) and plays numerous roles in intracellular and extracellular compartments, including maintenance of the cellular redox environment and modulation of the immune system, for example, by regulating DNA binding of several transcription factors, such as p53, nuclear factor-kappaB, and activator protein-1 (8–10). Therefore, TRX acts not only as an antioxidant, but also as a potent anti-inflammatory molecule.

During the past decade, it has been shown that extracellular levels of TRX are increased in conditions of oxidative stress and inflammation, including acute lung injury, viral infection, autoimmune disease, heart disease, and ischemia-reperfusion injury (11–17). Although these findings suggest that TRX can serve as a marker for oxidative stress in various diseases, the reason for increased TRX levels in such diseases is poorly understood. It has been shown that overexpression of TRX in transgenic mice induced resistance to harmful conditions, including lipopolysaccharide-induced acute hepatitis (18), adriamycin-induced cardiotoxicity (19), proinflammatory cytokine-induced or bleomycin-induced lung injury (20), and cerulein-induced acute pancreatitis (21). Consistent with these results, administration of recombinant TRX prevented ischemic lung injury (22), cerebral infarction (23), and myosin-induced myocarditis (24) in animal models.

Beneficial effects of TRX in those models were ascribed initially to the reducing activity of TRX and its ability to relieve local oxidative stress. Nakamura et al (25) demonstrated a further crucial role of TRX by showing that circulating TRX effectively impaired neutrophil extravasation into sites of inflammation. Because excessive neutrophil extravasation in acute inflammatory diseases leads to tissue damage, elevated levels of circulating TRX might be beneficial in sepsis. Recently, Tamaki et al (26) showed that TRX counteracts the proinflammatory and pro-oxidant effects of macrophage migration inhibitory factor (MIF). Because MIF is an important factor in the pathogenesis of sepsis, inhibition of MIF might be an-

*See also p. 2304.

From the Department of Anesthesiology (SH, CR, CB, JBZ, MAW), University of Heidelberg, Heidelberg, Germany; Department of Preventive Medicine (HN), The Tazuke Kofukai Medical Research Institute, Kitano Hospital, Osaka, Japan; Department of Biological Responses (JY), Institute for Virus Research, Kyoto University, Kyoto, Japan; Institute of Transfusion Medicine and Immunology (MG), Mannheim, Germany; Department of Surgery (PS, KH), University of Heidelberg, Heidelberg, Germany; Interfaculty Institute for Biochemistry (KS-O), University of Tübingen, Tübingen, Germany; Department of Anesthesiology (RB), University of Frankfurt, Frankfurt, Germany; and Department of Anesthesiology (MAW), Justus-Liebig University, Giessen, Germany.

Drs. Hofer and Rosenhagen contributed equally to the work.

Drs. Breitkreutz and Weigand share senior authorship and contributed equally to the work.

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For information regarding this article, E-mail: markus.weigand@chiru.med.uni-giessen.de

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other mechanism of TRX action. These results suggest that TRX has important roles not only as an antioxidant, but also as an anti-inflammatory molecule.

Little is known, however, about the role of TRX in sepsis. The aims of this study were therefore two-fold: 1) to assess TRX levels in plasma of septic patients and 2) to investigate whether TRX is beneficial in a mouse sepsis model.

**MATERIALS AND METHODS**

**Patients.** After approval of the institutional review board and informed consent from the closest relatives of the patients, we selected two cohorts of consecutive subjects consisting of a group of 24 sepsis patients and an additional group of 14 patients with seven survivors and seven nonsurvivors from sepsis. Patients were prospectively enrolled in the study within the first 24 hours after the onset of septic shock according to the criteria of the American College of Chest Physicians/Society of Critical Care Medicine conference (27). In brief, patients were considered to have sepsis if they had clinical and/or microbiologic evidence of infection as the likely cause of systemic inflammation. Systemic inflammation was indicated by the presence of at least two of the following criteria: a) body temperature >38°C or <36°C; b) heart rate >90 bpm; c) respiratory rate >20 breaths/min or Paco2 >32 mm Hg; and d) leukocyte count >12,000 cells/mm³, <4,000 cells/mm³, or >10% immature forms. Septic shock was defined as sepsis associated with a) hypotension persisting despite adequate fluid resuscitation, as manifested by systolic blood pressure <90 mm Hg, a reduction of ≥40 mm Hg from baseline in the absence of other causes for hypotension, use of dopamine >5 µg/kg·min⁻¹, or any dose of norepinephrine, or b) organ hypoperfusion, characterized by lactic acidosis, oliguria (<30 mL/hr), or acute alteration in mental status unexplained by other conditions. On enrollment in the study, all patients were mechanically ventilated. The severity of illness was estimated using the Acute Physiology and Chronic Health Evaluation II score (28). Blood samples were drawn from indwelling central venous catheters at the time of hospitalization. All patients were sequentially studied for 28 days or until death. As controls, blood samples of 34 healthy volunteers were examined.

**Determination of TRX in Plasma.** We performed an enzyme-linked immunosorbent assay (Redox Bio Science, Kyoto, Japan) for the detection of TRX in plasma, as published previously (17). The assay yielded linear values of up to 1000 ng/mL of the TRX standard supplied by the manufacturer. Corrected plasma TRX concentrations were obtained in relation to the plasma hemoglobin levels (17). Accordingly, all samples were measured at least in duplicate. Because red blood cells contain high concentrations of TRX (17), samples with hemolysis (n = 4), as detected by visual inspection, were excluded from the analysis. For internal reference and comparison with septic patients, TRX levels were measured in 34 healthy donors. TRX levels in healthy controls were mean ± SEM 87.2 ± 13.5 ng/mL (upper 95% confidence interval [CI]: 114.7, lower 95% CI: 59.7) and comparable to published reference values of arterial or venous TRX levels from our laboratory (29). Presumably due to different lot charges of the enzyme-linked immunosorbent assay or other unknown reasons, TRX levels in our healthy individuals known reasons, TRX levels in our healthy individuals were elevated corrected plasma TRX concentrations compared with healthy individuals.

**RESULTS**

**Extracellular Thioredoxin is Elevated in Nonsurvivors of Septic Patients.** To investigate the role of TRX in sepsis, we first measured the plasma levels of TRX in 24 severe septic patients (9 women, 15 men; mean age 57.4 ± 5.3 yrs) and 34 healthy controls (14 women, 20 men; mean age 42.2 ± 6.1 yrs). Septic patients had an Acute Physiology and Chronic Health Evaluation II score of 25.2 ± 3.4 (mean ± SEM) and suffered from abdominal infection (n = 13), pneumonia (n = 6), or trauma (n = 5). As shown in Figure 1A, we found that patients with severe sepsis displayed markedly elevated corrected plasma TRX concentrations com-
pared with healthy individuals (control 87.2 ± 13.5 ng/mL vs. sepsis 161.6 ± 14.0 ng/mL; p < 0.001).

Because extracellular TRX was increased in plasma of septic patients, we next evaluated whether TRX levels differed between survivors and nonsurvivors of sepsis within the first 24 hours after onset of septic shock. Figure 1B shows that nonsurvivors had significantly higher TRX levels than survivors (nonsurvivors 269.4 ± 61.7 ng/mL vs. survivors 100 ± 17.2 ng/mL; p < 0.03).

Neutralization of Extracellular Thioredoxin is Deleterious in Experimental Sepsis. To further investigate the contribution of TRX to disease outcome in sepsis, we used a septic mouse model and blocked the activity of endogenous TRX with neutralizing antibodies following CLP. In two independent experiments, 20 female C57BL/6 mice were subjected to CLP and treated by an intraperitoneal injection of a thioredoxin-neutralizing rabbit antibody or control serum (each 200 µL of 2 mg/mL) immediately and 6 hours after CLP. Survival was monitored every 4 hours. Anti-TRX, antithioredoxin.

To evaluate whether a delayed treatment with TRX after onset of bacterial sepsis was still sufficient for protection, we subjected 14 female C57BL/6 mice to CLP and then treated the mice intraperitoneally with 50 µg TRX or albumin 4 hours after CLP, followed by additional injections 12, 24, and 48 hours later. Although survival was slightly impaired compared with animals treated immediately after CLP (14% survival control group, 57% survival TRX group), TRX still exerted a strong survival-promoting effect (Fig. 3C). These results therefore demonstrate that TRX treatment reduces mortality in experimental sepsis.

DISCUSSION

TRX is a ubiquitous thiol protein, extracellular levels of which are increased...
under conditions of oxidative stress and inflammation. The present study demonstrates that plasma TRX levels were significantly higher in patients with sepsis compared with healthy volunteers. Furthermore, TRX levels in nonsurvivors of sepsis were even higher than in survivors, thus indicating a pivotal role of TRX in sepsis. Neutralization of TRX in the CLP mouse model proved to be deleterious, whereas intraperitoneal injection of TRX starting directly after CLP and 4 hours later markedly improved survival of mice. These findings strongly suggest that TRX has a therapeutic effect in experimental sepsis.

An imbalance in the redox state is known to induce TRX production (34). Although previous studies showed that TRX is a good marker of oxidative stress (11–17, 34–37), only little data exist about TRX levels in septic patients. Thioredoxin is released from cells on oxidative stress (38, 39). Therefore, the elevated plasma levels of TRX most likely reflect the increased oxidative stress in septic patients. Our findings of elevated TRX levels in nonsurvivors compared with survivors further indicate that nonsurvivors suffer from a strong oxidative stress, which cannot be counteracted even by increased TRX production. This situation might be similar to HIV infection in which high plasma levels of TRX have been particularly found in those patients who die early during autoimmune deficiency syndrome progression (17).

Recently, Callister et al (40) measured plasma TRX levels in children with meningococcoc septice shock (MSS). In contrast to our finding, the authors detected persistently lower TRX levels in patients with MSS than in the uninfected controls. There are several possible explanations for these differences. Previous studies identified genetic polymorphisms that influence the outcome of MSS (41); therefore, it is conceivable that the low TRX levels in MSS are because of a genetic disposition. Furthermore, although the patients of our study suffered from polymicrobial peritonitis and pneumonia, patients with MSS are exposed to high endotoxin concentrations. Nevertheless, it is interesting to note and consistent with our data that also in MSS nonsurviving children showed higher plasma TRX levels than survivors, although only a relatively small number of patients were compared in this study (40). Therefore, plasma TRX levels are presumably still a valid indicator for oxidative stress in MSS.

The anti-inflammatory effects of TRX are not completely understood. Recently, the proinflammatory cytokine MIF has been identified as a member of the TRX superfamily. MIF, however, differs from TRX by increasing oxidative stress and downregulating antioxidant responses. Furthermore, it was demonstrated that expression of TRX and MIF is regulated in an inverse manner (26, 42). Tamaki et al (26) showed that administration of TRX could efficiently attenuate colonic inflammation. Because this beneficial effect of TRX was associated with a strong downregulation of MIF, it was suggested that suppression of MIF production may be even more important than suppression of tumor necrosis factor-α and interferon-γ (26). Because MIF is involved in the pathogenesis of sepsis (31), it is conceivable that the anti-inflammatory effects of TRX may be at least partially because of an MIF-counteracting mechanism.

TRX has been reported to promote neutrophil chemotaxis and inhibit neutrophil extravasation (25, 43). At first view, these paradoxical effects of TRX presumably depend on the site of TRX administration—i.e., locally or into the bloodstream—and the presence of a chemotactic gradient. Nakamura et al (25) found that circulating TRX suppresses inflammation by downregulating neutrophil extravasation into sites of inflammation. This anti-inflammatory effect, however, requires blood concentrations of TRX in the range of 1000 ng/mL. Nevertheless, the plasma concentrations of TRX in the nonsurviving septic patients were well below 1000 ng/mL, suggesting that endogenous TRX production might have been insufficient to confer survival in these patients.

To evaluate the effects of TRX in sepsis, we subjected mice to CLP and neutralized endogenous TRX with anti-TRX antibodies. Indeed, inhibition of TRX significantly impaired survival, suggesting endogenous TRX to have a key role as protective factor in sepsis. Recently, it has been published that adjunctive treatment of patients with high-dose sodium selenite reduces mortality rate in patients with severe sepsis or septic shock (44). Because TRX reductase is a selenoprotein, the beneficial effect of selenite could have been mediated by the TRX system. The protective effect of TRX was supported by our finding that administration of recombinant TRX at doses of 50 μg per intraperitoneal injection significantly improved survival in the CLP model. In this respect, it is worth mentioning that intraperitoneal injection of 50 μg TRX into mice can maintain plasma levels of >1000 ng/mL for at least 3 hours (24), which is sufficient to suppress neutrophil extravasation (25). For a potential clinical use, such a therapeutic window of TRX administration is highly relevant. Therefore, we investigated whether delayed administration of TRX was still able to confer survival of mice. Indeed, although the beneficial effect of TRX was reduced compared with animals treated immediately after CLP, administration of TRX 4 hours after the onset of sepsis still exerted a protective effect.

In summary, we show that the extraacellular levels of TRX are elevated in patients with severe sepsis. In a septic mouse model, administration of TRX promoted survival, whereas neutralization of TRX impairs survival. Taken together, our data suggest that TRX plays an important role in sepsis and should therefore be further investigated as a potential therapeutic option.

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