Protective roles of redox-active protein thioredoxin-1 for severe acute pancreatitis

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Thioredoxin (TRX) is an endogenous multifunctional protein that contains a redox-active disulfide/dithiol within a highly conserved active site sequence (Cys-Gly-Pro-Cys) and is found in both prokaryotic and eukaryotic genomes (18). Human TRX was originally identified as an adult T cell leukemia-derived factor that was defined as an IL-2 receptor-α-chain Tac inducer produced by human T cell lymphotrophic virus-1-transformed T cells (40). Several proteins that have a similar active site sequence (Cys-Xxx-Yyy-Cys) are members of the TRX family. TRX consists of two isoforms, TRX-1 and TRX-2. TRX-1 is a cytosolic protein, and TRX-2 is located specifically in the mitochondria. Both TRX-1 and TRX-2 are essential in mammals because knockout mice of each protein are embryonic lethal (23, 32). TRX-1 is a stress-inducible protein that has an important role in protecting host cells from various types of stresses, including viral infection, ischemic insult, and H2O2 exposure (27, 29). Indeed, TRX-1 has scavenging activity for a variety of ROS such as singlet oxygen, hydroxyl radicals, and H2O2 (7, 10). Thus TRX-1 has an important role in maintaining the redox environment of the cell (18). Moreover, TRX-1 has potent anti-inflammatory effects through suppression of neutrophil infiltration in the inflammatory site (27, 28).

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Table 1. Primers used for RT-PCR in pancreatic extracts

<table>
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<tr>
<th>Primer</th>
<th>Sense Sequence</th>
<th>Antisense Sequence</th>
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<tr>
<td>TNF-α (354 bp)</td>
<td>5'-TTTCGTCTACTGAGATGTTGGGTGATGTTGACCC-3'</td>
<td>5'-GATTGAGTAGATGAGTACGAGGGTGACGGG-3'</td>
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<td>L-1β (563 bp)</td>
<td>5'-ATGGCAACTGTTCGGAACACCACTAAT-3'</td>
<td>5'-AGGAAGGATGTTAGAGTTTTCCTTTCATATTT-3'</td>
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<td>IL-6 (638 bp)</td>
<td>5'-ATGACCTGCTTCCTGCAAGAACAGAC-3'</td>
<td>5'-CAGGTAGCTGAGGCTAGATCTC-3'</td>
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<tr>
<td>KC (207 bp)</td>
<td>5'-GGCAATGAGTCTGCCTGATGACGCG-3'</td>
<td>5'-CTTTGGGACACCTTTTTGATCTCT-3'</td>
</tr>
<tr>
<td>G3PDH (983 bp)</td>
<td>5'-AGAAAGGCTGGTGAAGAGTTTGCG-3'</td>
<td>5'-CATGTAGGCCATGAGGTCCACCAC-3'</td>
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KC, keratinocyte-derived chemokine; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

Accordingly, TRX-1 is thought to be involved in the pathophysiology of AP. The present study aimed to elucidate the role of TRX-1 in the host defense mechanism during severe AP. To clarify the possible mechanism of TRX-1 in preventing pancreatic injury and systemic complications, the effects of TRX-1 overexpression in transgenic mice were investigated in a mouse model of severe AP induced with the secretagogue cerulein (CER), a CCK analog, and subsequent lipopolysaccharide (LPS) injection. Therapeutic effects of exogenous recombinant TRX-1 administration after the onset of disease were also investigated. Moreover, to determine the effect of TRX-1 on the secretory response of pancreatic acinar cells, we examined the amylase secretion in isolated pancreatic acinar cells treated with CER in the presence or absence of TRX-1.

MATERIALS AND METHODS

Mice and Reagents. Male wild-type (WT) C57BL/6 mice (20–22 g) were purchased from Japan SLC (Shizuoka, Japan). The generation of TRX-1 transgenic (TRX-1-TG) mice in which human TRX-1 was transplanted was described previously (41). The presence of the TRX-1 transgene was confirmed by RT-PCR analysis (data not shown). Recombinant hTRX-1 (rhTRX-1), anti-hTRX-1 antibodies against hTRX-1 in the pancreas of wild-type (WT; a) and TRX-1-TG (b) mice. rhTRX-1 was assayed using the pancreas of TRX-1-TG mice.

Fig. 1. Expression of human (h) thioredoxin (TRX)-1 protein in TRX-1 transgenic (TG) mice. A: immunohistochemical staining using antibody against hTRX-1 in the pancreas of wild-type (WT; a) and TRX-1-TG (b) mice. hTRX-1 was diffusely expressed in the pancreas of TRX-1-TG mice. Original magnification ×400. B: Western blot showing the constitutive expression of hTRX-1 protein (12 kDa) in the pancreas, lung, and the liver of TRX-1-TG mice. rhTRX-1, recombinant human TRX-1.
Western blot analysis. Expression of hTRX-1 protein in the pancreas, lung, and liver of TRX-1-TG mice was investigated by Western blotting. Expression of iNOS and IkBα in the pancreas of WT and TRX-1-TG mice treated with saline or CER + LPS was also investigated by Western blotting (2 h after the last CER injection for iNOS and 30 min for IkBα). For cytoplasmic protein extraction, tissues were homogenized in lysis buffer containing 0.1 mol/l NaCl, 10 mmol/l Tris-HCl, 0.1 mmol/l EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, 35 μg/ml pepstatin A, and 10 μg/ml aprotinin, heated for 15 min at 30°C, and centrifuged at 14,000 rpm for 5 min at 4°C. Aliquots of the supernatant were stored at −80°C until use. Protein concentrations were measured using a BCA protein assay kit (Pierce, Rockford, IL). Cytoplasmic protein (30 μg) from each sample was mixed with 2× SDS sample buffer, heated for 5 min at 100°C, and separated by SDS-PAGE (18% gel for hTRX-1 and 10% gel for iNOS and IkBα). After SDS-PAGE, separated proteins were transferred onto polyvinylidene difluoride membranes for 1 h. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h at room temperature, washed three times for 5 min each in TBS-T, and incubated with a primary anti-hTRX-1 antibody, anti-iNOS antibody (BIOMOL Research Laboratory, Plymouth Meeting, PA) or anti-IkBα antibody (Cell Signaling Technology, Beverly, MA), in TBS-T containing 5% nonfat dry milk overnight at 4°C. After being washed three times for 10 min each in TBS-T, the membranes were incubated with a secondary goat anti-mouse IgG antibody (for hTRX-1 protein) or a secondary goat anti-rabbit IgG antibody (for iNOS and IkBα protein) conjugated with horseradish peroxidase for 1 h. The membranes were analyzed by the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). The protein signal was quantified by scanning densitometry using a bioimage analysis system.

Semiquantitative PCR. Pancreatic cytokine or chemokine gene expression was investigated using semiquantitative RT-PCR. Pancreatic tissue samples were collected 2 h after the last CER injection. Total RNA was extracted using an RNaseasy Mini Kit (Qiagen, Tokyo, Japan). Total RNA (10 μg) was reverse transcribed into cDNA using the Super Script Preamplification System (GIBCO-BRL, Rockville, MD). Sequences of mouse-specific primers for TNF-α, IL-1β, IL-6, KC, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) are listed in Table 1. Amplification was performed with an automated thermal cycler for 25 cycles for G3PDH, 35 cycles for TNF-α, IL-1β, and KC, and 37 cycles for IL-6. Each cycle consisted of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C. The final cycle included a 10-min extension step at 72°C to ensure full extension of the product. Each PCR product was electrophoresed on a 1.5% agarose gel containing ethidium bromide, and the bands were examined using an automated image analysis system.

Pharmacological kinetics of rhTRX-1. A group of WT mice were intraperitoneally injected with 2 mg/kg rhTRX-1. They were killed 1, 3, 6, 12, or 24 h after the injection. Pancreatic tissues were homogenized in lysis buffer. hTRX-1 concentrations of sera and pancreatic tissues were measured by ELISA.

Treatment with rhTRX-1 for experimental AP. Another group of WT mice was intraperitoneally injected with 30 mg/kg LPS immediately after six hourly CER injections (50 μg/kg) for induction of AP. As a therapeutic protocol, the mice were then intraperitoneally injected with 2 mg/kg rhTRX-1 three times at 3-h intervals just after completion of the CER administration. BSA (2 mg/kg) was used as a control. Tissues were removed 24 h after the first CER injection for histological examination.

Isolation of pancreatic acinar cells and amylase assay. To assess the interaction between TRX-1 and secretory response of pancreatic acinar cells, we studied whether amylase secretion from acinar cells treated with CER could be influenced either by TRX-1 overexpression in the pancreas or treatment with rhTRX-1. Pancreatic acinar cells were obtained from the pancreas of WT or TRX-1-TG mice by collagenase treatment as described previously (2, 3, 34, 49, 52). These cells were incubated with various concentrations of CER (10−12–10−7 M) for 30 min at 37°C. Moreover, acinar cells obtained from WT mice were incubated with 10−6 M CER in the presence or absence of various concentrations of rhTRX-1 (0.1, 1.0, and 10 μM) for 30 min at 37°C. Amylase activity in the incubation medium was measured using an α-Amylase Assay Kit (Wako, Osaka, Japan). Unstimulated samples served as controls to estimate the basal rate of secretion.
Fig. 3. **A**: histological findings of the pancreas and extrapancreatic organs 24 h after the first CER injection. (hematoxylin and eosin (H&E)-stained section). 

- **a**: In sham-treated WT mice, histological findings of the pancreas were normal. In WT mice treated with CER + LPS, focal hemorrhage and acinar cell necrosis (b) and moderate to severe interstitial edema and extensive infiltration of inflammatory cells of the pancreas (c) were observed. 
- **e**: Marked inflammatory cells infiltrated the pulmonary cavity, and the alveolar walls were thickened. 
- **g**: Lobules of the liver were disorganized with vacuolization of hepatocytes. 
- **d**: In TRX-1-TG mice treated with CER + LPS, inflammation in the pancreas was decreased compared with WT mice. 

**B**: effects of hTRX-1 overexpression on histological AP inflammatory scores. TRX-1-TG mice treated with CER + LPS had less edema (a), inflammatory cell infiltration (b), and acinar necrosis (c) of the pancreas than WT mice treated with CER + LPS. Bars represent the means ± SE of 6 mice. **P < 0.01.
Statistical analysis. Differences between more than two groups were evaluated by one-way ANOVA. Where appropriate, Student’s t-test and the Mann-Whitney U-test were used for comparisons of two groups. The cumulative survival rate was calculated using the Kaplan-Meier method, and survival curves were compared by the log-rank test. The data were expressed as means ± SE. A P value < 0.05 was considered statistically significant.

RESULTS

hTRX-1 expression in transgenic mice. Expression of the hTRX-1 transgene was confirmed by immunohistochemistry and Western blotting. Immunohistochemistry showed an abundant expression of hTRX-1 in pancreatic acinar cells and in Langerhans cells in TRX-1-TG mice, and Western blot demonstrated the constitutive expression of hTRX-1 protein in the pancreas, lung, and the liver of TRX-1-TG mice (Fig. 1, A and B). Moreover, the serum and pancreatic hTRX-1 levels in TRX-1-TG mice were 126 ng/ml and 399 ng/mg protein, respectively.

Resistance of TRX-1-TG mice with CER + LPS-induced AP. The survival rate was 94% (14/15) in WT mice at 8 days after treatment with CER + LPS (10 mg/kg). When a higher dose of LPS (30 mg/kg) was administered in combination with CER, the survival rate decreased to 33% (5/15) in WT mice. Therefore, combined administration of CER + LPS (30 mg/kg) was employed for the induction of severe AP in the following experiments. Notably, the survival rate after treatment with CER + LPS (30 mg/kg) in TRX-1-TG mice (13/15; 87%) was significantly higher than in WT mice, suggesting that TRX-1-TG mice are resistant to the induction of severe AP (Fig. 2A). Serum levels of amylase and lipase in TRX-1-TG mice treated with CER + LPS were significantly lower than those in WT mice (Fig. 2B). Histologically, WT mice treated with vehicle had normal histology of the pancreas (Fig. 3Aa), lung, and liver (data not shown). WT mice treated with CER + LPS had the features of severe AP, characterized by focal hemorrhage and acinar cell necrosis (Fig. 3Ab). Marked interstitial...

Fig. 4. A: effects of hTRX-1 overexpression on myeloperoxidase (MPO) activity of the pancreas and the lung of mice with AP. MPO activity was significantly lower in the pancreas (a) and the lung (b) of TRX-1-TG mice treated with CER + LPS than in WT mice. Bars represent the means ± SE of 6 mice. **P < 0.01. B: effects of hTRX-1 overexpression on malondialdehyde (MDA) concentration in the pancreas of mice with AP. Pancreatic MDA concentration was significantly lower in TRX-1-TG mice treated with CER + LPS than in WT mice. Bars represent the means ± SE of 6 mice. **P < 0.01. C: Western blot analysis of inhibitor of κB-α (IκB-α) protein in the cytoplasm of the pancreas in AP mice. a: A marked decrease in IκB-α protein was observed in WT mice treated with CER + LPS but not in TRX-1-TG mice. b: Quantitative data are shown. Bars represent the means ± SE of 5 experiments. **P < 0.01.
edema and extensive infiltration of inflammatory cells were also observed (Fig. 3Ac). Moreover, many inflammatory cells infiltrated the pulmonary alveolar cavity, and the alveolar walls were thickened (Fig. 3Ag). In contrast, TRX-1-TG mice treated with CER + LPS had reduced histological inflammation in these organs compared with WT mice (Fig. 3, Ad, Af, and Ah). Histological scores of pancreatitis induced by CER + LPS in TRX-1-TG mice were significantly lower than those in WT mice (Fig. 3B: a, edema: 2.70 ± 0.11 vs. 1.80 ± 0.16, \( P < 0.01 \); b, infiltration: 2.10 ± 0.16 vs. 1.40 ± 0.11, \( P < 0.01 \); and c, necrosis: 1.85 ± 0.17 vs. 0.75 ± 0.10, \( P < 0.01 \)).

**MPO activity.** WT mice treated with CER + LPS had markedly elevated MPO activity in the pancreas and lung. The increase of MPO activity of both organs was significantly smaller in TRX-1-TG mice than in WT mice (\( P < 0.01 \); Fig. 4A).

**MDA concentration and IkB-α degradation in the pancreas.** Pancreatic MDA concentrations were significantly increased by CER + LPS administration in both WT and TRX-1-TG mice. The increase was significantly smaller in TRX-1-TG mice than in WT mice (\( P < 0.01 \); Fig. 4B). Moreover, there was marked degradation of IkB-α protein in the pancreas of WT mice treated with CER + LPS. In contrast, degradation of IkB-α protein was almost abolished in TRX-1-TG mice (Fig. 4C).

**Cytokine/chemokine messages and iNOS expression in the pancreas.** Pancreatic mRNA expression of TNF-α, IL-1β, IL-6, and KC was elevated in WT mice treated with CER + LPS. However, gene expression was significantly suppressed in TRX-1-TG mice compared with WT mice (Fig. 5B: a, TNF-α: \( P < 0.01 \); b, IL-1β: \( P < 0.05 \); c, IL-6: \( P < 0.05 \); d, KC: \( P < 0.05 \)). iNOS expression was also upregulated in WT mice treated with CER + LPS. Its expression was significantly lower in TRX-1-TG mice than in WT mice (\( P < 0.05 \); Fig. 5B).

**Pharmacokinetics of rhTRX-1.** Serum hTRX-1 levels of WT mice given rhTRX-1 (2 mg/kg) were 609.4, 154.4, 50.9, 17.6, and 2.3 ng/ml at 1, 3, 6, 12, and 24 h, respectively. hTRX-1 levels in the pancreatic tissues were 24.5, 2.3, 0.4, \(<0.1\), and \(<0.1\) ng/mg protein at 1, 3, 6, 12, and 24 h, respectively. The half-life of rhTRX-1 was 3.1 h in the serum and 3.4 h in the pancreatic tissue.

**Effects of rhTRX-1 administration on CER + LPS-induced AP in WT mice.** Based on the results obtained from the pharmacokinetic study of rhTRX-1, we administered 2 mg/kg rhTRX-1 intraperitoneally three times at 3-h intervals to CER + LPS-injected WT mice. Exogenous administration of rhTRX-1 improved the survival of CER + LPS-injected WT mice from 33% (5/15) to 80% (12/15) (Fig. 6A). Histological inflammation of the pancreas and lung was remarkably attenuated in WT mice treated with rhTRX-1 compared with untreated WT mice (Fig. 6, B and C: a, edema: 2.70 ± 0.11 vs. 2.25 ± 0.12, \( P < 0.05 \); b, infiltration: 2.10 ± 0.16 vs. 1.50 ± 0.12, \( P < 0.01 \); c, necrosis: 1.85 ± 0.17 vs. 1.40 ± 0.13, \( P < 0.05 \)). Moreover, the elevation of both serum amylase and lipase levels was significantly reduced by rhTRX-1 administered 24 h after the first CER injection (\( P < 0.01 \); Fig. 6D).

**Fig. 5.** A: cytokine and chemokine gene expression in the pancreas of WT and TRX-1-TG mice treated with CER + LPS. Tumor necrosis factor (TNF)α (a), interleukin (IL)-1β (b), IL-6 (c), and keratinocyte-derived chemokine (KC; d) mRNA levels in the pancreas relative to the mean of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) in the same mice are shown. Bars represent the means ± SE of 6 mice. After treatment with CER + LPS, increase of cytokine and chemokine expression in TRX-1-TG mice was significantly lower than in WT mice. *\( P < 0.05 \) and **\( P < 0.01 \). B: quantitative data of Western blot analysis for inducible nitric oxide synthase (iNOS) expression in the pancreas of mice with AP. Upregulation of iNOS expression in WT mice treated with CER + LPS was reduced in TRX-1-TG mice. Bars represent the means ± SE of 5 experiments. *\( P < 0.05 \).

**Effect of TRX-1 on CER-stimulated amylase secretion from pancreatic acinar cells.** In accord with previously reported findings, biphasic secretory response that peaks with 10⁻¹⁰M was observed in acinar cells obtained from WT mice when they were treated with increasing concentrations of CER. Similar changes were also observed in acinar cells obtained from TRX-1-TG mice (Fig. 7A). Moreover, the simultaneous incubation of cells from WT mice with various concentrations of rhTRX-1 did not significantly inhibit amylase secretion induced by CER stimulation (10⁻¹⁰M; Fig. 7B).

**DISCUSSION**

The present study clearly demonstrated that TRX-1 has an important role in the protection of the development of severe AP. The major findings of this study are as follows. First, rhTRX-1 overexpression in transgenic mice attenuated the severity of experimental AP. Second, exogenous administration of rhTRX-1 had therapeutic effects against experimental AP. It is currently widely accepted that AP is triggered by aberrant premature activation of digestive enzymes within the...
pancreatic acinar cells and subsequent autodigestion of the pancreas (39). The following progression of AP is associated with infiltration of various inflammatory cells that produce many cytokines and ROS (13). Moreover, severe AP is often accompanied by bacterial translocation from the digestive tract, resulting in systemic inflammatory response syndrome with multiple organ dysfunction (35). Thus endotoxin, a LPS present in the gram-negative bacterial wall, is thought to be strongly involved in the progression of AP leading to severe disease (14). Indeed, LPS elicits NF-κB activation and induces
SOD and glutathione content, possibly because of their exhaustion of the antioxidant defenses (46). Indeed, a depletion of pancreatic AP, the increased production of ROS is thought to overwhelm the amount of antioxidants; however, during the development of conditions, they are believed to be captured by a sufficient tors in the development of pancreatic injury (9, 36). Although experimental AP suggests that ROS are important aggravating fac-
cations, and mortality rates of AP patients (48).

endotoxin levels correlated with the severity, systemic compli-
results are consistent with a previous report in which serum correlated with the concentration of LPS administered. These inflammatory reaction. As expected, the survival rate inversely...

A pronounced systemic inflammatory reaction during various infections (25). Accordingly, in the present study, we induced severe AP in mice by treatment with a CCK analog, CER, and subsequent LPS injection. In this experimental model of AP, the pancreas of WT mice was severely destroyed with a strong inflammatory reaction. As expected, the survival rate inversely correlated with the concentration of LPS administered. These results are consistent with a previous report in which serum endotoxin levels correlated with the severity, systemic complications, and mortality rates of AP patients (48).

Extensive evidence obtained from several models of exper-
mental AP suggests that ROS are important aggravating fac-
tors in the development of pancreatic injury (9, 36). Although

Nitric oxide (NO) also acts as an important intracellular and intercellular messenger in inflammatory responses (38). A high concentration of NO is produced by iNOS, which is mainly induced in neutrophils and macrophages during the inflammatory process (47). Excessive NO generation is implicated in the pathophysiology of AP, because it decreases blood pressure and induces organ ischemia, leading to pancreatic tissue injury (8). In the present study, the increase in iNOS expression in the pancreas of AP mice was significantly smaller in TRX-1-TG mice than in WT mice. Induction of iNOS is also regulated predominately at the transcriptional level through NF-κB-dependent mechanisms (42). Accordingly, TRX-1 overexpression might have inhibited iNOS expression by suppressing NF-κB activation in the present study.

We also demonstrated that exogenous administration of rhTRX-1 ameliorated experimental AP. Especially, neutrophil
infiltration in the pancreatic tissue was significantly reduced in mice treated with rhTRX-1. TRX-1 has dual regulatory effects on leukocyte movement. Although it acts in a lower dose as a chemoattractant by itself for neutrophils and monocytes in vitro, it shows desensitizing effects in a higher dose against chemokine-induced chemotaxis of neutrophils and macrophages (4). Indeed, intravenous administration of rhTRX-1 directly suppresses LPS-induced leukocyte (mainly neutrophils) infiltration in the mouse air pouch model (28). Accordingly, acute elevation of circulating TRX-1 caused by injection of rhTRX-1 appears to show beneficial effects against inflammation of the body. Indeed, intravenous administration of rhTRX-1 decreased bleomycin-induced lung injury (19) or ischemic reperfusion injury (33, 53). Moreover, exogenously administered TRX-1 is considered to act as an antioxidant (18). Indeed, extracellular TRX-1 scavenges H2O2 together with peroxiredoxin IV (secreted form of peroxiredoxin; see Ref. 18). On the other hand, rTRX-1 permeates the cell membrane and enters the cytosol, and scavenges intracellular ROS (21). Thus, although the precise mechanism underlying TRX-1 entry in the cells has yet to be elucidated, exogenously administered rhTRX-1 might exert its therapeutic effects on AP both outside and inside the cells.

In the present study, TRX-1 did not directly affect the CER-stimulated amylase secretion in pancreatic acinar cells. This result is in agreement with a previous report that antioxidants such as glutathione, SOD, and catalase did not affect the amylase secretion in pancreatic acinar cells (54) and suggests that the protective mechanism of TRX-1 against AP is not the result of an inhibitory effect on pancreatic secretory function.

In conclusion, exogenous administration and overexpression of redox-active protein TRX-1 significantly reduced the severity of experimental AP in mice, which indicated a protective function. This result is in agreement with a previous report that antioxidants such as glutathione, SOD, and catalase did not affect the amylase secretion in pancreatic acinar cells (54) and suggests that the protective mechanism of TRX-1 against AP is not the result of an inhibitory effect on pancreatic secretory function.

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