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Overexpression of Redox-Active Protein Thioredoxin-1 Prevents Development of Chronic Pancreatitis in Mice

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ABSTRACT

Chronic pancreatitis (CP) is considered to result from repetitive pancreatic injury, and sustained production of various proinflammatory cytokines and chemokines are closely involved in its pathogenesis. Monocyte chemoattractant protein 1 (MCP-1), a member of the CC chemokine family, is believed to contribute to the progression of CP through monocyte/macrophage recruitment. This study aimed to clarify the protective role of thioredoxin-1 (TRX-1), a redox-regulating protein with antioxidative activity, in MCP-1 production and pancreatic fibrosis using a CP model in transgenic mice overexpressing TRX-1 (TRX-1-TG mice) and wild-type C57BL/6 mice. Experimental CP was induced by repeated administration of cerulein and lipopolysaccharide for 6 weeks. In TRX-1-TG mice, pancreatic atrophy was ameliorated, and histologically detectable inflammatory cell infiltration, glandular atrophy, and pseudotubular complex formation were suppressed. Overexpression of TRX-1 also attenuated pancreatic fibrosis and suppressed the activation of pancreatic stellate cells. Serum levels of MCP-1 and pancreatic expression of transforming growth factor-β, platelet-derived growth factor, and MCP-1 were reduced in TRX-1-TG mice compared with levels in wild-type mice. Overexpression of TRX-1 also reduced H_2O_2-induced MCP-1 production in isolated pancreatic acinar cells. These results indicate that TRX-1 can potentially attenuate pancreatic fibrosis via the suppression of oxidative stress and MCP-1-mediated chronic inflammation. *Antioxid. Redox Signal.* 8, 1835–1845.

INTRODUCTION

Chronic pancreatitis (CP) is a chronic progressive disorder characterized by irreversible damage to the pancreas and the development of histological evidence of inflammation and fibrosis. It eventually leads to the destruction and permanent loss of exocrine and endocrine tissue (26). Although the etiology of CP includes various causes such as alcoholic, biliary, hereditary, and idiopathic pancreatitis (27), CP is thought to result from chronic repetitive inflammation and the subsequent repair of pancreatic damage, ultimately followed by the activation of a profibrotic cascade (38). This is the so-called "necrosis–fibrosis sequence theory" (16). Thus, the progression to CP requires the interaction between environmental factors that induce recurrent pancreatic injury and an altered immune response leading to chronic inflammation and fibrosis (39).

The formation of fibrosis in the pancreas is initiated by the differentiation and activation of pancreatic stellate cells (PSCs), which play a pivotal role in the fibrogenesis in CP (3, 38). In response to pancreatic injury and the resultant inflammation, PSCs are activated and transformed from their quiescent phenotype into highly proliferative myofibroblast-like cells that express the cytoskeletal protein α-smooth muscle actin (α-SMA) and produce collagen and other extracellular matrix components (15). PSCs are stimulated by various me-

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diators, including proinflammatory cytokines and growth factors such as tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), IL-6, transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF) (3, 14, 19). Thus, these mediators, which are heavily involved in the pathogenesis of acute pancreatitis (AP), also contribute to the development of CP by repeated PSC activation. Moreover, recent studies have suggested that monocyte chemoattractant protein 1 (MCP-1) plays an important role in the pathogenesis of pancreatic inflammation and fibrosis (20). MCP-1 is a member of the CC chemokine family and exerts strong chemoattractant activity on monocytes and macrophages that produce PDGF and proinflammatory cytokines such as TNF-α and IL-1β, which induce the proliferation and activation of PSCs (43). Therefore, MCP-1 is considered to act as a fibrosis-promoting chemokine in the pathogenesis of CP (20, 43).

Recently, the role of oxidative stress in the development of AP and CP has been clarified (7, 10, 15, 30, 41). In the course of pancreatitis, reactive oxygen species (ROS) are produced by infiltrating inflammatory cells and xanthine oxidase in the endothelial cells (9). As well as disrupting lipid membranes by the peroxidation of fatty acids, ROS act as second messenger molecules and enhance proinflammatory cytokine production by directly activating oxidant-sensitive transcription factors, such as nuclear factor-κB (NF-κB) (1, 42). Accordingly, persistent oxidative stress is considered to be associated with chronic pancreatic inflammation, which influences the proliferation and activation of PSCs.

Thioredoxin-1 (TRX-1) is an endogenous multifunctional protein with antioxidative and anti-inflammatory effects, and contains a redox-active disulfide/dithiol within a highly conserved active site sequence (Cys–Gly–Pro–Cys) (11). TRX-1 is inducible by various types of stresses, and has an important role in host defense mechanisms (21, 22). We recently reported the protective effects of TRX-1 on an experimental model of severe AP induced by cerulein (CER) and lipopolysaccharide (LPS) (24).

Based on accumulating evidence that persistent oxidative stress is involved in the pathogenesis of CP, we hypothesized that TRX-1 attenuates chronic inflammation and subsequent fibrosis of the pancreas. To test this hypothesis, we investigated the protective role of TRX-1 against pancreatic damage and fibrosis using a murine model of CP induced by repeated pancreatic injury.

MATERIALS AND METHODS

Animals

Male wild-type (WT) C57BL/6 mice (20–22 g) were purchased from Japan SLC Inc. (Shizuoka, Japan). The generation of TRX-1 transgenic (TRX-1-TG) mice, in which human TRX-1 (hTRX-1) complementary DNA was inserted between the β-actin promoter and its terminator, has been described previously (36). The presence of the TRX-1 transgene was confirmed by reverse transcription–polymerase chain reaction (RT–PCR) analysis. hTRX-1 transgene expression in the pancreas was confirmed by immunostaining and Western blot analysis, as previously reported (24).

Induction of AP and lipid peroxidation assay

Experimental AP was induced in both WT and TRX-1-TG mice (each group, $n = 10$) by six intraperitoneal injections of CER (Sigma-Aldrich, Tokyo, Japan) at a dose of 50 μg/kg at hourly intervals. LPS was injected at a dose of 5 mg/kg immediately after the six CER injections. Saline was substituted for CER and LPS in both WT and TRX-1-TG mice as controls. The mice were killed 16 h after the first CER injection. Blood samples were collected, and the pancreas were removed, and stored at −80°C until further assays were performed. Lipid peroxidation in the pancreas was evaluated by determining the malondialdehyde (MDA) concentration using a lipid peroxidation assay kit (Calbiochem, La Jolla, CA).

Induction of CP

To induce chronic pancreatic injury, the mice were subjected to repeated AP episodes, three episodes per week for 6 weeks, as described previously (38). An equivalent amount of saline was injected into both WT and TRX-1-TG mice as controls. The mice were killed 3 days after the final CER treatment. Blood samples and pancreatic tissues were obtained as described above, and pancreatic wet weight was measured.

Morphological examination

Paraffin-embedded tissue samples were sectioned (5 μm), and stained with hematoxylin and eosin or Azan-Mallory. The histopathology of the chronic pancreatic injury was evaluated as previously described (38). Briefly, areas of abnormal architecture were defined and quantified as 0, absent; 1, rare; 2, minimal (<10%); 3, moderate (10%–50%); or 4, severe. Within these areas, the presence of glandular atrophy, fibrosis, and pseudotubular complexes was each scored as 0, absent; 1, minimal (<10%); 2, moderate (10%–50%); or 3, severe. Ten nonoverlapping fields were randomly selected from each mouse to test all parameters. These fields were examined by two independent pathologists in a blind fashion, and a median score was calculated.

Chemical analysis

Protein content was determined to evaluate pancreatic exocrine function, as described previously (13, 17). The hydroxyproline content of the pancreas was measured as an indicator of pancreatic collagen content, as described previously (40). Briefly, pancreatic samples were hydrolyzed in 6 *N* HCl at 130°C for 3 h. The samples were resuspended in 2 ml deionized water and 1 ml 50 m*M* chloramine T in acetate–citrate buffer. Next, 1 ml of 3.15 *M* perchloric acid and 1 ml of Ehrlich's reagent were added, mixed, and the suspension was incubated at 60°C for 20 min. Finally, absorbance was measured at 557 nm, and the values were compared with those of serial dilutions of trans-4-hydroxy-L-proline. The results were expressed in micrograms per gram of tissue.

Immunohistochemistry for α-SMA

For α-SMA staining, sections were deparaffinized and pretreated for heat-induced epitope retrieval for 20 min. En-

dogenous peroxidase activity was quenched with a solution of 0.3% hydrogen peroxide in methanol. After the samples were washed with phosphate-buffered saline, they were treated with 5% fetal bovine serum for 1 h at room temperature to block nonspecific reactions. The sections were then incubated overnight at 4°C with primary mouse monoclonal anti-α-SMA antibody (1:100 dilution; Dako Cytomation, Glostrup, Denmark). After the sections were washed, they were incubated with MOM™ biotinylated anti-mouse immunoglobulin G (IgG) antibody (Vector Laboratories, Burlingame, CA) for 10 min at room temperature, and then with horseradish-peroxidase-labeled streptavidin (ABC elite Kit, Vector Laboratories) for 30 min at room temperature. The reaction products were visualized by treating the sections for 3–5 min with 0.2 mg/ml 3,3′-diaminobenzene tetrahydrochroride in 0.05 *M* Tris-buffered saline (pH 7.4) containing 0.003% hydrogen peroxide. Nuclei were counterstained with methyl green.

Western blot analysis of α-SMA expression

The expression of α-SMA in the pancreas of WT and TRX-1-TG mice treated with repeated CER injections was also investigated by Western blot analysis. To extract cytoplasmic proteins, pancreatic tissue was homogenized in lysis buffer containing 0.1 *M* NaCl, 10 m*M* Tris-HCl, 0.1 m*M* EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, 35 μg/ml pepstatinA, and 10 μg/ml aprotinin, heated for 15 min at 50°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 (10 μg) from each sample was mixed with 2 × sodium dodecyl sulfate (SDS) sample buffer, heated for 5 min at 100°C, and separated by SDS-polyacrylamide gel electrophoresis (PAGE; 15% gel for α-SMA). After SDS-PAGE, the separated proteins were transferred onto polyvinylidene difluoride membranes for 1 h. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature, then washed three times for 5 min each in TBS-T, and incubated overnight at 4°C with primary anti-α-SMA (Dako Cytomation) in TBS-T containing 5% nonfat dry milk. After the membranes were washed three times for 10 min each in TBS-T, they were incubated for 1 h with a secondary goat anti-mouse IgG antibody conjugated with horseradish peroxidase. The membranes were analyzed using the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). The protein signal was quantified by scanning densitometry using a bioimage analysis system.

Reverse transcriptase–polymerase chain reaction (RT–PCR)

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Tokyo, Japan). Total RNA (10 μg) was reverse transcribed into complementary DNA using the SuperScript Preamplification System (Gibco BRL, Rockville, MD). The sequences of the mouse-specific primers for TGF-β, PDGF-B, MCP-1, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were as follows: TGF-β (327 base pairs); sense 5′-GCCCTGGATACCAACTATTGC-3′, anti-sense 5′-GCAGGAGCGCACAATCATGTT-3′, PDGF-B (105 base pairs); sense 5′-AAGCACACCCATGACAAGG-3′, anti-sense 5′-GGGGCAATACAGCAAATAC-3′, MCP-1 (380 base pairs); sense 5′-CCCCACTCACCTGCTGCTACT-3′, anti-sense 5′-GGCATCACAGTCCGAGTCACA-3′, and G3PDH (983 base pairs); sense 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, anti-sense 5′-CATGTAGGCCATGAGGTCCACCAC-3′. Amplification was performed with an automated thermal cycler for 25 cycles for G3PDH, 35 cycles for TGF-β, and 33 cycles for PDGF-B and MCP-1. Each cycle consisted of denaturation for 30 s at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C. Following the final cycle, a 10-min extension step at 72°C was included to ensure full extension of the product. Each PCR product was separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide, and the bands were examined using an automated image analysis system.

MCP-1 levels in serum and isolated pancreatic acinar cells

Serum MCP-1 levels of WT and TRX-1-TG mice of the AP and CP models were determined using a commercial mouse MCP-1 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

To assess the interaction between TRX-1 and MCP-1 production in pancreatic acinar cells, we examined whether MCP-1 production in acinar cells treated with H_2O_2 was influenced by TRX-1 overexpression in the pancreas. Pancreatic acinar cells were obtained from the pancreas of WT and TRX-1-TG mice by collagenase treatment, as described previously (6, 28). These cells (1×10^6 cells/well) were incubated for 4 h at 37°C with various concentrations of H_2O_2. MCP-1 secretion into the incubation medium was measured using a mouse MCP-1 ELISA Kit (R&D Systems). Unstimulated samples were used as controls to estimate the basal rate of MCP-1 production.

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 to compare two groups. The data are expressed as means ± standard errors of the mean (SEM). A p value of < 0.05 was considered statistically significant.

RESULTS

Effects of TRX-1 overexpression on AP

The pancreas of WT mice treated with CER+LPS had the features of AP, which is characterized by marked interstitial edema and extensive infiltration by inflammatory cells. In contrast, TRX-1-TG mice that had been treated in the same way displayed relatively reduced histological inflammation (Fig. 1A). The increase in pancreatic MDA concentrations and serum MCP-1 levels were significantly suppressed in CER+LPS-treated TRX-1-TG mice compared with those of the corresponding WT mice (Fig. 1B and C).

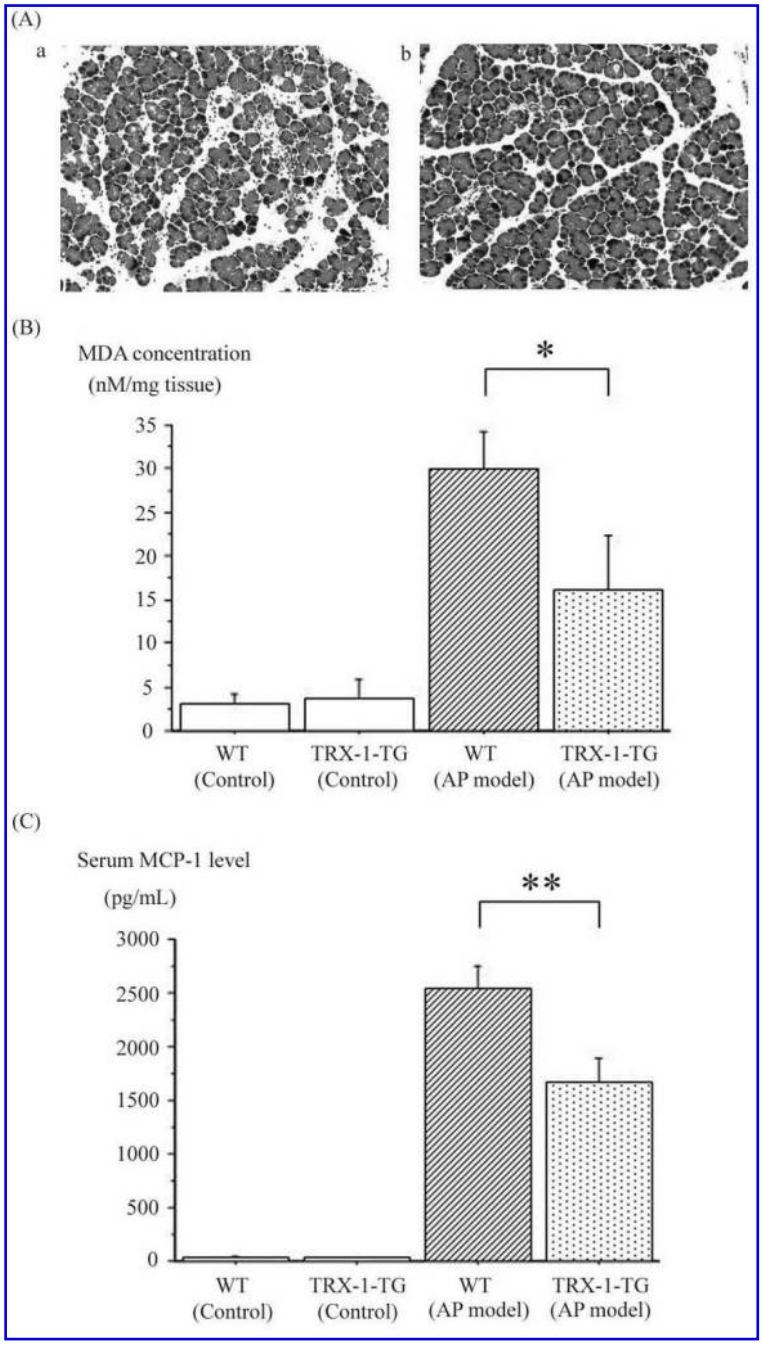

FIG. 1. Histological findings in the pancreas. (**A**) Histological findings in the pancreas of WT and TRX-1-TG mice with AP induced by CER+LPS. Hematoxylin and eosin (H&E)-stained sections; original magnification, ×100. (**a**) In WT mice treated with CER+LPS, severe interstitial edema and extensive infiltration of inflammatory cells into the pancreas were observed. (**b**) In TRX-1-TG mice treated with CER+LPS, pancreatic inflammation was reduced compared with that of WT mice. (**B**) Effects of TRX-1 overexpression on pancreatic MDA concentrations in mice with AP. The increase in pancreatic MDA concentration was significantly suppressed in TRX-1-TG mice with AP compared with that of the corresponding WT mice. *Bars* represent the means ± SEM of five mice. (*$p < 0.05$). (**C**) Serum MCP-1 levels in WT and TRX-1-TG mice with AP. Serum MCP-1 levels were measured 16 h after the first CER injection. There was a significant difference in serum MCP-1 levels between WT and TRX-1-TG mice with AP. *Bars* represent the means ± SEM of 6 mice. (**$p < 0.01$)

Effects of TRX-1 overexpression on CP

Histologically, the acinar units were tightly packed, and Azan–Mallory-stained connective tissue was localized only around the ducts and small blood vessels in the pancreas of control WT and TRX-1-TG mice (Fig. 2A and B). In the pancreas of WT mice treated with repeated CER injections, the acinar units were atrophic, and some acinar units appeared to have redifferentiated into tubular complexes at the time of sacrifice (Fig. 2C). Moreover, an increased number of inflammatory cells were distributed within the matrix, and thick bands of extracellular matrix had been deposited around the acinar units (Fig. 2D). Fatty changes were also observed focally, and islets were morphologically preserved. In contrast, in the pancreas of TRX-1-TG mice of the CP model, all histological markers of pancreatic damages such as glandular atrophy, pseudotubular complexes, fibrosis, and inflammatory cell infiltration were obviously attenuated (Fig. 2E and F). Histological scores for pancreatitis in WT mice were significantly higher than those in TRX-1-TG mice (Figs. 3A: abnormal architecture: 3.00 ± 0.37 vs. 1.90 ± 0.31, respectively, $p < 0.05$; 3B: glandular atrophy: 2.20 ± 0.39 vs. 1.20 ± 0.36, respectively, $p < 0.05$; 3C: pseudotubular complex: 2.20 ± 0.39 vs. 1.20 ± 0.33, respectively, $p < 0.05$; 3D: fibrosis: 2.10 ± 0.35 vs. 1.20 ± 0.25, respectively, $p < 0.05$; 3E: total score: 9.50 ± 1.38 vs. 5.30 ± 1.41, respectively, $p < 0.05$).

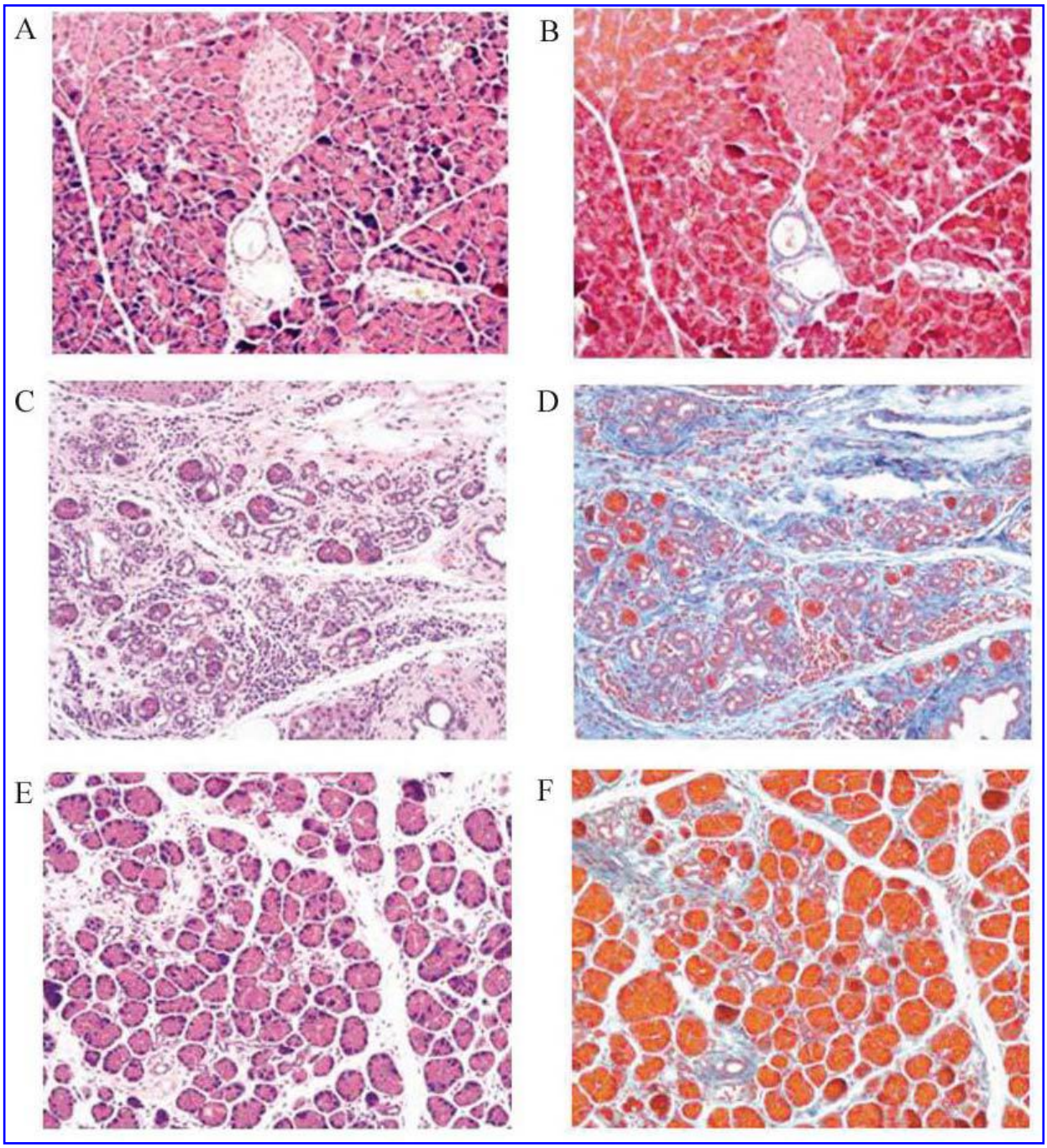

FIG. 2. Histological findings in the pancreas of WT and TRX-1-TG mice with CP induced by recurrent AP. (**A**, **C**, **E**) H&E-stained sections, (**B**, **D**, **F**) Azan-Mallory-stained section, original magnification, ×200. (**A**, **B**) In the pancreas of untreated WT and TRX-1-TG mice, the acinar units were tightly packed, and collagen deposition was localized only around the pancreatic ducts and small blood vessels. (**C**, **D**) In WT mice treated with repeated AP episodes for 6 weeks, marked acinar atrophy and intralobular and interlobular fibrosis with inflammatory cell infiltration were observed. (**E**, **F**) In treated TRX-1-TG mice, these changes were obviously reduced.

Effects of TRX-1 overexpression on pancreatic atrophy, fibrosis, and exocrine function

A marked decrease in pancreatic wet weight, which reflects pancreatic atrophy after chronic injury, was noted in WT mice with CP, whereas this decrease was significantly attenuated in TRX-1-TG mice with CP (Fig. 4A).

Pancreatic fibrosis was quantified by measuring the hydroxyproline content of the pancreas. The amount of hydroxyproline in the pancreas of WT mice with CP was signifi-

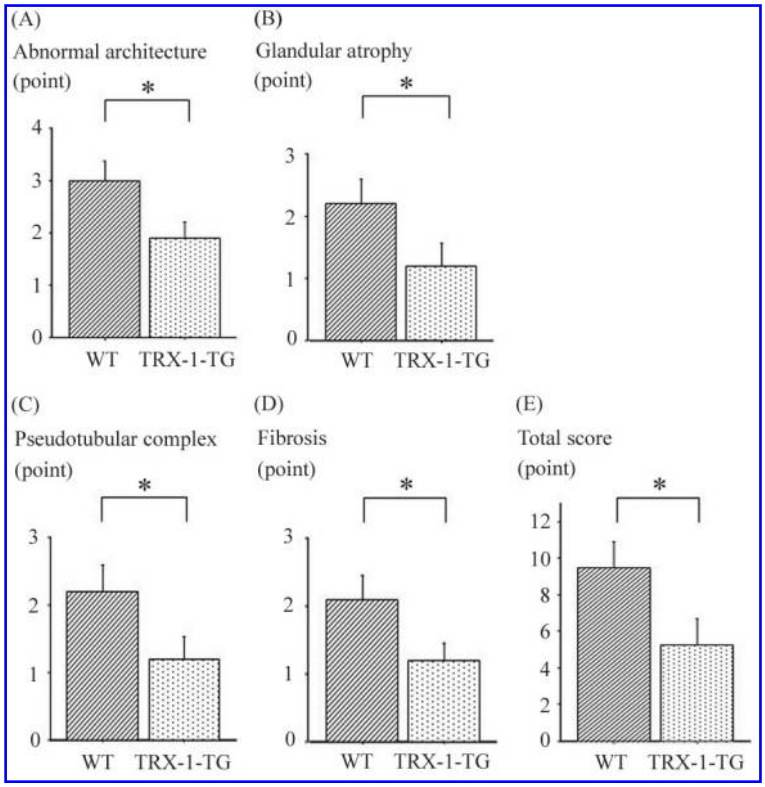

FIG. 3. Effects of TRX-1 overexpression on histological scores. Compared with those of WT mice with CP, TRX-1-TG mice had less abnormal architecture (**A**), less glandular atrophy (**B**), fewer pseudotubular complexes (**C**), and less fibrosis (**D**). Total scores were also significantly reduced in TRX-1-TG mice (**E**). *Bars* represent the means ± SEM of 5 mice. (*$p < 0.05$)

cantly higher than that observed in control mice. In contrast, the pancreatic hydroxyproline content of TRX-1-TG mice with CP was significantly reduced (Fig. 4B).

The pancreatic protein content, corrected by pancreatic volume, was examined to evaluate pancreatic exocrine function. The intrapancreatic protein content of WT mice with CP was significantly reduced to 57.7% of that in control mice, reflecting marked injury to the pancreatic exocrine gland. In contrast, the decrease in protein content was ameliorated (82.3% of the control) in TRX-1-TG mice with CP (Fig. 4C).

Effects of TRX-1 overexpression on PSC activation

The activation of PSCs was studied by immunostaining for α-SMA. In the normal pancreas, α-SMA staining is seen mainly in blood vessel walls (data not shown). Repeated CER injections induced a marked proliferation of α-SMA-positive activated PSCs in the fibrotic areas (Fig. 5A-a). However, the numbers of α-SMA-positive cells were reduced in TRX-1-TG mice with CP (Fig. 5A-b). Western blot analysis showed that α-SMA expression was significantly lower in TRX-1-TG mice with CP than in WT mice with CP (Fig. 5B).

Expression of TGF-β, PDGF, and MCP-1 genes in the pancreas

Pancreatic expression of TGF-β, PDGF, and MCP-1 mRNAs was elevated in WT mice with CP. However, the expression of these mediators was significantly suppressed in TRX-1-TG mice (Fig. 6).

Effects of TRX-1 overexpression on MCP-1 production in serum and isolated pancreatic acinar cells

Serum MCP-1 levels were significantly increased in WT mice with CP. This increase was significantly suppressed in TRX-1-TG mice with CP (Fig. 7).

The treatment of isolated pancreatic acinar cells with H_2O_2 stimulated MCP-1 production in a dose-dependent manner. However, treatment with a high dose (500 μ*M*) of H_2O_2 decreased MCP-1 production through cytotoxicity. MCP-1 production was significantly inhibited in pancreatic acini obtained from TRX-1-TG mice compared with that in acini from WT mice when these cells were treated with 200 μ*M* H_2O_2 (Fig. 8).

DISCUSSION

In this study, we have shown that TRX-1 plays an inhibitory role in the development of pancreatic fibrosis in a murine model of CP. The major findings of this study are as follows. First, the overexpression of TRX-1 suppressed oxidative stress, as determined by MDA levels, and ameliorated acute pancreatic injury. Second, the overexpression of TRX-1 attenuated the pancreatic fibrosis induced by repetitive AP episodes, through the suppression of PSC activation. Third, the overexpression of TRX-1 significantly reduced H_2O_2-induced MCP-1 production *in vitro*.

CP is the result of repetitive episodes of pancreatic inflammation and necrosis, followed by pancreatic regeneration (23,

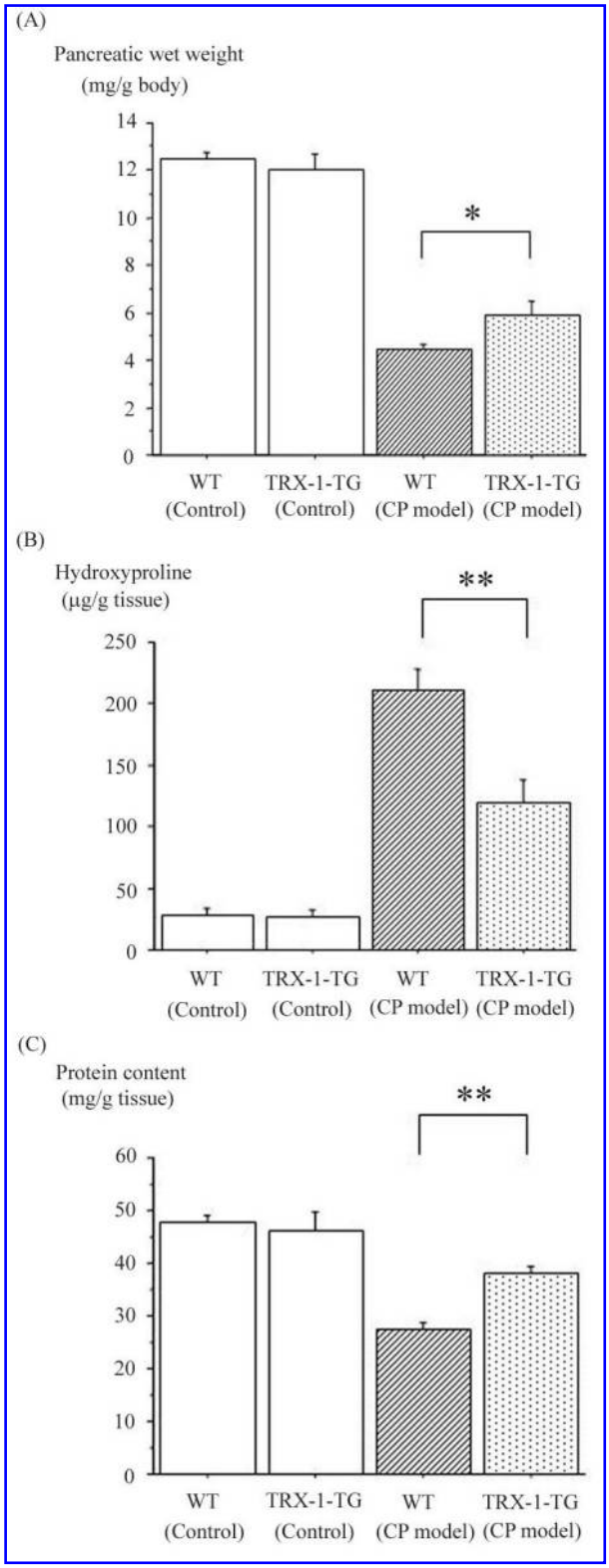

FIG. 4. Effects of TRX-1 overexpression on the severity of CP. The severity of CP was evaluated by pancreatic wet weight (**A**), pancreatic hydroxyproline content (**B**), and pancreatic protein content (**C**). All indicators of disease severity were significantly attenuated in TRX-1-TG mice with CP as compared with those of the corresponding WT mice. *Bars* represent the means ± SEM of 5 mice. (**$p < 0.01$, *$p < 0.05$)

38). The inflammation and necrosis arising from recurrent AP cause scarring in the periductal areas, and subsequent obstruction of the ductules and stasis within the duct, leading finally to acinar atrophy and fibrosis (35). It has been reported that a combination treatment with CER and LPS induces more severe pancreatic inflammation in an experimental model than treatment with CER only (8), and seems to be more suitable for the study of pancreatic fibrogenesis and treatment. Accordingly, in the present study, we induced experimental AP by treatment with CER and a subsequent injection of low-dose (5 mg/kg) LPS. In the AP model, overexpression of TRX-1 suppressed the effects of oxidative stress, as determined by MDA levels, and attenuated acute pancreatic injury, as described in our recent report (24). In the CP model induced by repeated CER+LPS injection, all WT mice displayed the histopathological and functional features of advanced CP, characterized by marked acinar atrophy and fibrosis. These results suggest that repeated severe acute injury induces intense pancreatic fibrogenesis and acinar atrophy, and support the necrosis–fibrosis sequence hypothesis. These pathological changes in the pancreas were strikingly reduced in the TRX-1-TG mice, possibly due to the amelioration of each acute injury, as observed in the transgenic mice with AP.

In the present study, the MDA concentration in WT and TRX-1-TG mice with CP was not significantly elevated compared to control mice (data not shown). We suppose that it is probably because pancreatic samples were obtained 3 days after the last CER injections. Indeed, the pancreatic MDA concentration in AP model reached peak value at 12–24 h and then declined, and was finally normalized 3 days after the CER+LPS injections. Accordingly, our results suggest that although oxidative stress induced by a single AP episode may not persist for a long time, repetitive injury is involved in the pathogenesis of CP and suppression of each oxidative stress is important for attenuation of chronic pancreatic injury as shown in mice overexpressing TRX-1.

Accumulating evidence suggests that PSCs play a central role in pancreatic fibrogenesis in response to various injurious stimuli (19). PSCs proliferate in response to PDGF and are activated in response to TGF-β (4, 31, 33, 34). Proliferating and activated PSCs express α-SMA and their synthesis of extracellular matrix is accelerated, including collagen type I and III, and fibronectin. Potential sources of cytokines in the inflamed pancreas are activated mononuclear cells and macrophages for TGF-β and PDGF (29, 32), platelets for TGF-β and PDGF (18), and injured acinar cells and PSCs themselves for TGF-β (3, 34). In the present study, the overexpression of TRX-1 suppressed the pancreatic expression of the TGF-β and PDGF genes. Although the sources of the cells in which overexpression of TRX-1 suppresses cytokine production have not been clarified, this suppression of cytokine release in TRX-1-TG mice might result in a decrease in PSC activation and subsequent pancreatic fibrosis.

The recruitment of inflammatory cells, including polymorphonuclear cells, monocyte/macrophages, and lymphocytes, to the inflammatory site is a crucial event in the pathogenesis of pancreatitis (43). MCP-1, a member of CC subfamily of chemokines, exerts strong chemoattractant activity on monocytes and macrophages (37). Therefore, MCP-1 is thought to act as a fibrosis-promoting chemokine through the recruit-

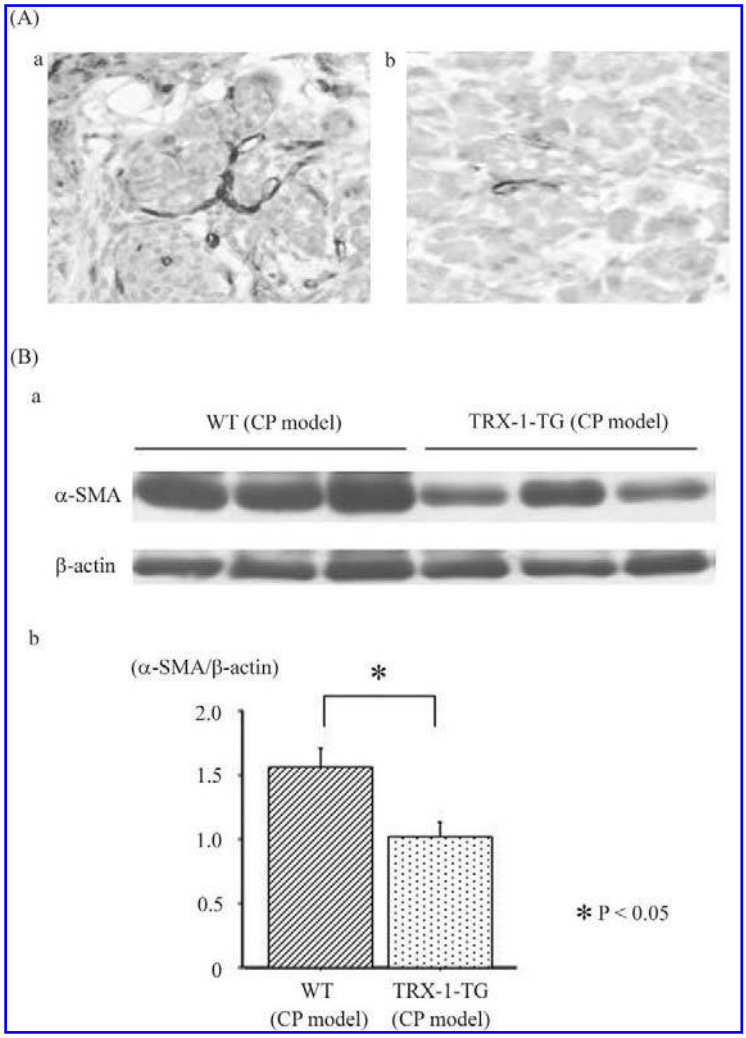

FIG. 5. Immunostaining for α-SMA. (**A**) An increased number of α-SMA-positive PSCs was observed in WT mice with CP (**a**), whereas only a few α-SMA-positive cells were present in TRX-1-TG mice with CP (**b**). Original magnification, ×400. (**B**) Western blot shows that α-SMA expression was significantly lower in TRX-1-TG mice than in WT mice of the CP model. *Bars* represent the means ± SEM of three experiments. (*$p < 0.05$)

ment of monocytes and macrophages, which secrete various proinflammatory cytokines and chemokines such as TGF-β and PDGF, and the resultant activation of PSCs. In the course of pancreatitis, MCP-1 is induced from injured acinar cells, monocytes/macrophages, and other damaged pancreatic tissues, such as endothelial cells and smooth muscle cells (43). In this study, we have shown that the overexpression of TRX-1 significantly reduced serum MCP-1 levels in mice with AP and H_2O_2-induced MCP-1 production from isolated pancreatic acinar cells. Treatment with a blocker of MCP-1 synthesis was reported to improve AP in mice (6). Moreover, serum MCP-1 levels and gene expression of MCP-1 were significantly lower in TRX-TG mice than in WT mice. Recently, anti-MCP-1 gene therapy has been shown to attenuate CP in rats (43). Our results and those of previous studies suggest that the local and systemic production of MCP-1 is one of the crucial regulators in the development of pancreatitis and that suppression of MCP-1 production is a promising strategy for the treatment of both AP and CP. The present data indicating that serum MCP-1 levels in WT mice with AP were 10-fold higher than those of WT mice with CP also demonstrate the importance of anti-MCP-1 therapy, especially in the early stages of the disease.

MCP-1 production in the pancreas is regulated by NF-κB activation at the transcriptional level (5). We recently reported that in the pancreas of a murine AP model, TRX-1 overexpression *in vivo* reduced the oxidative stress and the expression of proinflammatory cytokines and a chemokine by suppressing NF-κB activation (24). Accordingly, the inhibition of MCP-1 production by TRX-1 overexpression might be partly due to the suppression of NF-κB activation.

Recent studies have reported the usefulness of antioxidants for the treatment of experimental CP with fibrosis (10, 41). We previously reported that the overexpression of TRX-1 decreased thioacetamide-induced hepatic fibrosis and bleomycin-induced lung fibrosis in mice through a reduction in acute phase injury and oxidative stress (12, 25). Interestingly, TRX-1 has a direct inhibitory effect on the proliferation of hepatic stellate cells, the characteristic features of which are very similar to those of PSCs (4). In agreement with the results of our study, Apte *et al.* reported that antioxidants prevented PSC activation and collagen production by rat PSCs stimulated with a prooxidant complex or ethanol (2). Thus, evidence for the beneficial effects of antioxidants in the pathophysiology of CP has been accumulating. In future, further detailed experiments and clinical trials will be required

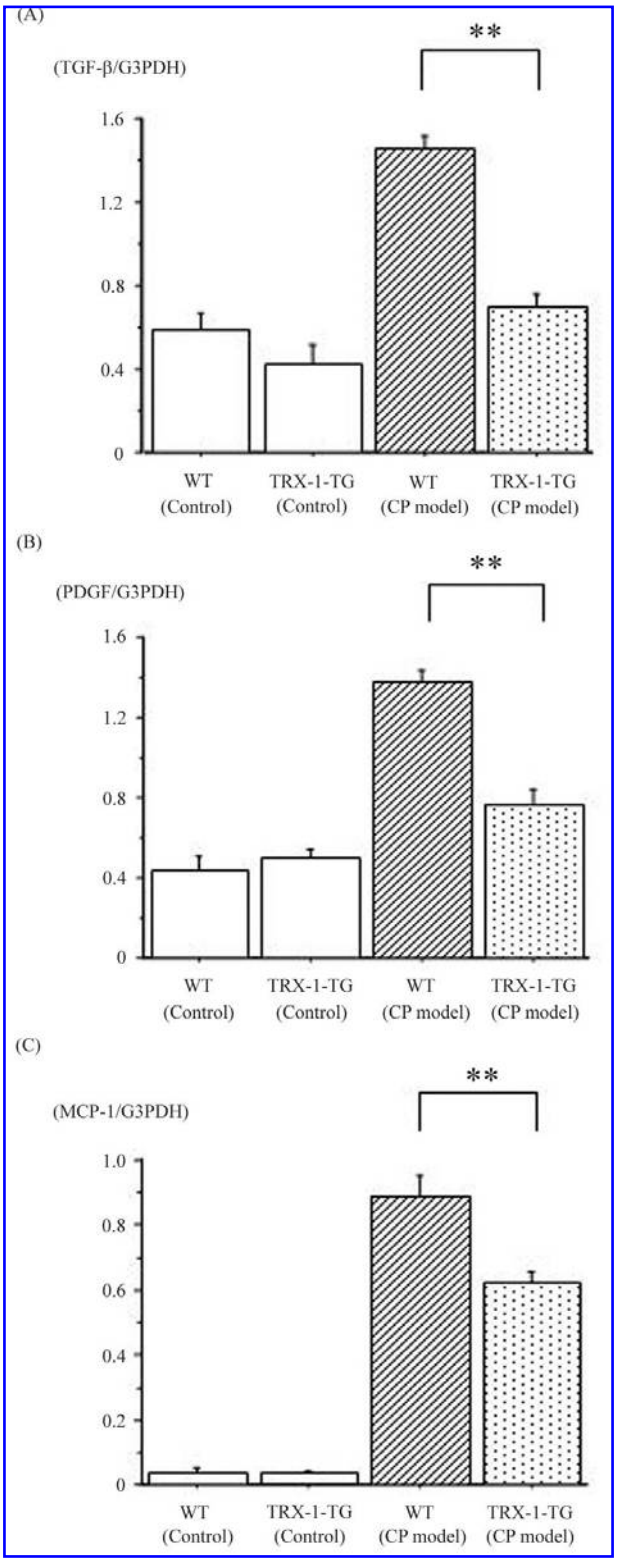

FIG. 6. Expression of cytokine and chemokine genes in the pancreas of WT and TRX-1-TG mice with CP. (**A**) TGF-β, (**B**) PDGF, and (**C**) MCP-1 mRNA levels in the pancreas relative to the mean level of G3PDH in the same mice are shown. Increases in the expression of these mediators in TRX-1-TG mice with CP were significantly lower than those in WT mice with CP. *Bars* represent the means ± SEM of 5mice. (**$p < 0.01$)

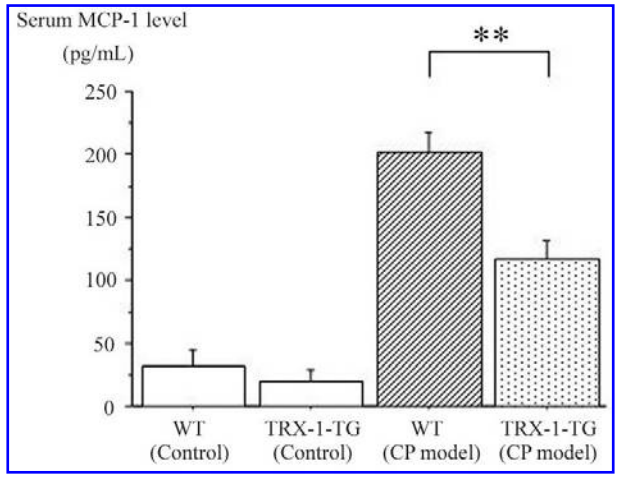

FIG. 7. Serum MCP-1 levels in WT and TRX-1-TG mice with CP. Serum MCP-1 levels were measured 3 days after the final CER treatment. There was a significant difference in the serum MCP-1 levels of WT and TRX-1-TG mice with CP. *Bars* represent the means ± SEM of 5 mice. (**$p < 0.01$)

to confirm the potential clinical applications of antioxidants, including TRX-1.

In summary, the overexpression of TRX-1 reduced H_2O_2-induced MCP-1 production by pancreatic acini *in vitro* and pancreatic fibrosis induced by chronic injury *in vivo*. These

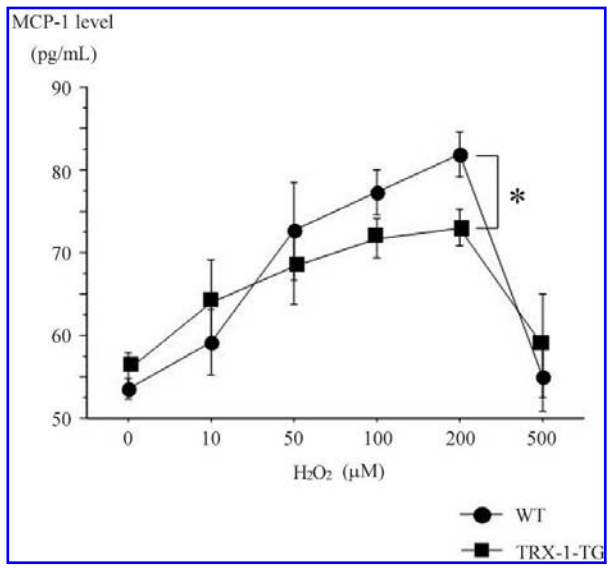

FIG. 8. Effects of TRX-1 overexpression on MCP-1 production in isolated pancreatic acini. Freshly isolated pancreatic acini (1×10^6 cells/well) were treated with various concentrations of H_2O_2 for 4 h. MCP-1 levels in culture supernatants were determined by ELISA. MCP-1 levels were significantly reduced in pancreatic acini obtained from TRX-1-TG mice compared with those from WT mice when these cells were treated with 200 μ*M* H_2O_2. Five replicates were performed for each treatment in the experiment. The results shown are means ± SEM. (*$p < 0.05$)

results indicate a protective role for TRX-1 in the development of pancreatic fibrosis and suggest that TRX-1 might be a new therapeutic strategy for the treatment of pancreatic fibrosis.

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ABBREVIATIONS

AP, acute pancreatitis; α-SMA, α-smooth muscle actin; CER, cerulein; CP, chronic pancreatitis; ELISA, enzyme-linked immunosorbent assay; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; hTRX-1; human thioredoxin-1; IgG, immunoglobulin G; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; MDA. malondialdehyde; NF-κB, nuclear factor-κB; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet derived growth factor; PSCs, pancreatic stellate cells; ROS, reactive oxygen species; RT–PCR, reverse transcription–polymerase chain reaction; SDS, sodium dodecyl sulphate; SEM, standard error of the mean; TBS-T, Tris-buffered saline with 0.1% Tween-20; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; TRX-1, thioreodoxin-1; TRX-1-TG, thioredoxin-1 transgenic; WT, wild type.

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