0026-895X/06/7006-2045–2051$20.00
MOLECULAR PHARMACOLOGY
Vol. 70, No. 6
28605/3155411
Mol Pharmacol 70:2045–2051, 2006
Printed in U.S.A.

Molecular Changes Evoked by Triethylenetetramine Treatment in the Extracellular Matrix of the Heart and Aorta in Diabetic Rats

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Received July 9, 2006; accepted September 14, 2006

ABSTRACT

Most patients with diabetes die from cardiac or arterial disease, for which there are limited therapeutic options. Free Cu^{2+} ions are strongly pro-oxidant, and chelatable-Cu^{II} is increased in the diabetic heart. We reported previously that treatment by Cu^{II}-selective chelation with triethylenetetramine (TETA) evokes elevated urinary Cu^{II} in diabetic rats and humans in whom it also improved hallmarks of established left ventricular (LV) disease. Here, we treated diabetic rats with TETA and evaluated its ability to ameliorate Cu^{2+}-mediated LV and arterial damage by modifying the expression of molecular targets that included transforming growth factor (TGF)-β1, Smad4, extracellular matrix (ECM) proteins, extracellular superoxide dismutase (EC-SOD), and heparan sulfate (HS). Eight-weeks of TETA treatment significantly improved cardiac diastolic function but not $[glucose]_{plasma}$ in diabetic animals. LV and aortic mRNAs corresponding to TGF-β1, Smad4, collagen types I, III, and IV, and fibronectin-1, and plasminogen activator inhibitor-1, were elevated in untreated diabetic animals and normalized after TETA treatment. EC-SOD mRNA and protein, and $[HS]_{tissue}$ were significantly decreased in diabetes and restored by drug treatment. Candidate molecular mechanisms by which TETA could ameliorate diabetic cardiac and arteriovascular disease include the suppression of an activated TGF-β/Smad signaling pathway that mediates increased ECM gene expression and restoration of normal EC-SOD and HS regulation. These findings are relevant to the restoration toward normal by TETA treatment of cardiac and arterial structure and function in diabetes.

More than three quarters of patients with diabetes die from various cardiovascular complications (Sicree et al., 2003). We recently demonstrated that the amount of Cu^{II} that can be extracted from the heart via coronary artery perfusion with triethylenetetramine (TETA) is substantively increased in diabetic rats (Cooper et al., 2004). TETA treatment stimulated urinary Cu^{II} excretion in streptozotocin (STZ)-diabetic rats and patients with type-2 diabetes to a greater extent than in matched controls and restored cardiac structure and function toward normal in both groups (Cooper et al., 2004, 2005). Specific underpinning mechanisms relating to Cu^{II} chelation in diseased diabetic cardiac and arterial tissues, however, remain to be elucidated.

Free Cu^{2+} is strongly pro-oxidant in mammalian tissues (Fraústo da Silva and Williams, 2001) and may activate pathways that cause the excessive generation of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$) in diabetic cardiovascular tissues. There is an increased $O_2^{\cdot-}$ production in the heart and arteries of both animals and humans with diabetes or heart failure. Extracellular superoxide dismutase (EC-SOD/SOD3) is a major antioxidant in mammalian tissues. It is highly expressed and active in blood vessels (Marklund, 1984a), and protection of nitric oxide (NO) is believed to be a major function of EC-SOD (Oury et al., 1996). EC-SOD

This work was supported by grants from the Endocore Research Trust; the Foundation for Research Science and Technology, New Zealand; the New Zealand Ministry of Education through the Maurice Wilkins Centre for Molecular Biodiscovery; the Health Research Council of New Zealand; and by Protemix Corporation.

The authors have all declared their association with Protemix Corporation, Auckland, New Zealand, and San Diego, California.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.106.028605.

ABBREVIATIONS: TETA, triethylenetetramine; ECM, extracellular matrix; EC-SOD, extracellular superoxide dismutase; HS, heparan sulfate; LV, left ventricle; NO, nitric oxide; $O_2^{\cdot-}$, superoxide anion; PAI-1, plasminogen activator inhibitor-1; PCR, polymerase chain reaction; ROS, reactive oxygen species; STZ, streptozotocin; TGF-β1, transforming growth factor-β1.

activity is decreased in the arteries of patients with diabetes (Fattman et al., 2003), resulting in increased susceptibility of vascular cells to effects of O_2^- with consequent endothelial dysfunction.

We recently showed that elevation of circulating EC-SOD in type-2 diabetic humans was strongly correlated with an interaction between hemoglobin A_{1c} and $[Cu]_{plasma}$ (Cooper et al., 2005). Furthermore, Cu^{2+} at high concentrations suppressed EC-SOD secretion from cultured vascular smooth muscle cells (Stralin et al., 2003). The extracellular content of EC-SOD in blood vessels is associated with the level of heparan sulfate (HS), a major binding anchor for EC-SOD (Sandstrom et al., 1993). Diabetic status can decrease arterial HS levels (Edwards et al., 2004) and compromise its function by modifying its structure (Vogl-Willis and Edwards, 2004).

Excessive accumulation of extracellular matrix (ECM) proteins and associated myocardial fibrosis are implicated as pathogenic mechanisms in diabetic heart disease (Marklund, 1992). We reported previously that long-term TETA treatment can regenerate the ECM in diabetic rat hearts (Cooper et al., 2004). TGF-β1 stimulates ECM protein accumulation in diabetic tissues by up-regulating the expression of corresponding genes or down-regulating those for ECM-degrading enzymes (Roberts et al., 1992). There is evidence for a link between redox stress, TGF-β1, and ECM production (Williams, 1998).

The current study investigated molecular mechanisms by which TETA reverses diabetic heart disease in an STZ model. We show here that oral TETA administration significantly improved diastolic function in diabetic rat hearts. We also show that treatment with TETA suppressed the diabetes-evoked up-regulation of TGF-β1/Smad4, collagens I, III, and IV, fibronectin-1, and plasminogen activator inhibitor (PAI)-1 in the LV and aorta of diabetic rats and that it elevated EC-SOD mRNA and protein expression in these tissues. TETA administration was further shown to restore diabetes-induced decreases of HS levels in both heart and aorta. These results support the idea that TETA reverses diabetic cardiac disease at least in part through enhanced disposal of extracellular O_2^- and suppression of increases in ECM gene expression that may result from up-regulation of the TGF-β/Smad signaling pathway.

Materials and Methods

Induction of Diabetes and Drug Treatment. All studies were approved by relevant ethics and regulatory committees. Male Wistar rats, weighing 220 to 250g, were rendered diabetic by a single intravenous tail vein injection of STZ (55 mg/kg body weight; Sigma, St. Louis, MO) in isotonic saline, as described previously (Cooper et al., 2004). Age-matched control rats were injected with equal volumes of saline (untreated nondiabetic control). Body weights and $[glucose]_{blood}$ were monitored weekly for 16 weeks. After 8 weeks, diabetic rats were assigned to one of two groups: untreated diabetic; and TETA-treated diabetic. TETA (20 mg/day-rat) was administered in the drinking water (18 MΩ, Milli Q; Millipore Corporation, Billerica, MA) as triethylenetetramine dihydrochloride (trientine; Fluka, Buchs, Switzerland) or the equivalent molar dose of TETA disuccinate (Protemix, Auckland, New Zealand) for a further 8 weeks. In the HS study, the TETA was administered in the same manner to diabetic and control rats at a dose of 10 mg/day-rat.

Rats were housed [12-h light/dark cycle; temperature, 22.5°C (range, 20–26°C); humidity, 60% (range, 50–70%)] in like pairs with ad libitum food (Teklad 2018; Harlan Teklad, Madison, WI) and water. Sixteen weeks after STZ injection, rats were anesthetized (halothane, 5%), appropriately heparinized (200 IU/kg i.v.), and organs were excised. Aortas and cardiac LV were either perfused or washed free of blood in ice-cold diethyl pyrocarbonate-treated phosphate-buffered saline. Tissues were stored in RNA*later* (Ambion, Austin, TX) overnight at 4°C and then at −80°C for subsequent RNA isolation. Portions were also stored at −80°C for parallel protein analyses and from additional like-treated rats for HS analysis.

Measurement of Cardiac Function in Rats. Cardiac function was determined as previously detailed in isolated perfused working rat hearts (Cooper et al., 2004).

RNA Isolation and cDNA Synthesis. Total RNA was isolated from LV or aortic tissue using RNeasy Midi Kits (QIAGEN, Valencia, CA). One microgram of total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI) at 37°C for 30 min and then reverse-transcribed with random hexamers and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA).

Real-Time Quantitative PCR Analysis. Messenger RNA levels were compared by real-time quantitative PCR with an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Reactions were prepared in the presence of the fluorescent dye SYBR green I. The levels of gene expression of the target sequence were normalized to those of an active endogenous control, 18S ribosomal RNA (18S; Ambion) in the same cDNA sample. Table 1 displays a list of primers synthesized by Invitrogen for each gene analyzed. The primers and TaqMan probe used for type I collagen were proprietary to Applied Biosystems and so are not listed in Table 1. In PCR reactions, 1.5 and 0.25 ng of cDNA and 0.5 and 0.1 μM concentrations of primers were used for amplification of target genes and 18S rRNA, respectively. After PCR amplification, dissociation curves were constructed, and PCR products were subjected to agarose gel electrophoresis to confirm the formation of the specific PCR products. The threshold cycle at which the fluorescent signal reaches a particular value was used as a measure of gene expression. The linear range of dilution for target genes and 18S rRNA showed different slopes, indicating different amplification efficiency for control and target genes, and a standard curve method was therefore used. Analysis of mRNA expression was performed as described in User Bulletin #2 (Applied Biosystems) using standard curves prepared from serially diluted control cDNA samples.

Western Blot Analysis. Frozen aorta was homogenized in ice-cold lysis buffer [50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM $MgCl_2$, 50 mM EDTA, 2 mM dithiothreitol and 10% (v/v) glycerol] in the presence of a proteinase inhibitor cocktail (Roche, Indianapolis, IN). Homogenate was centrifuged at 13,000*g* for 20 min at 4°C. The

TABLE 1
Sequences of oligonucleotide primers used for real-time PCR analysis

Gene	Primer
Collagen III	
Forward	5′-TCCCCTGGAATCTGTGAATC-3′
Reverse	5′-TGAGTCGAATTGGGGAGAAT-3′
Collagen IV	
Forward	5′-GGGTTTTCCCTTCTTTTCGT-3′
Reverse	5′-CATGGTGAATCGCTGTAA-3′
Fibronectin-1	
Forward	5′-GAGGCCACCATCACTGGTT-3′
Reverse	5′-AGTGCGATGACATAGATGGTGTA-3′
PAI-1	
Forward	5′-AGAGCCAATCACAAGGCACT-3′
Reverse	5′-AGGCAAGTGAGGGCTGAAG-3′
TGF-β1	
Forward	5′-GGAAAGGGCTCAACACCTG-3′
Reverse	5′-CAGTTCTTCTCTGTGGAGCTGA-3′
Smad4	
Forward	5′-GGTCCGTAGGTGGAATAGCC-3′
Reverse	5′-CGGAGGTCGTCTACACCAAT-3′
EC-SOD	
Forward	5′-CTTGGGAGAGCTTGTCAGGT-3′
Reverse	5′-CACCAGTGACAGGTTGCAGA-3′

supernatant was isolated, and protein concentration was determined with BCA Protein Assay (Pierce, Rockford, IL). Twenty micrograms of protein was separated by gel electrophoresis and transferred to a nitrocellulose membrane. Five micrograms of rat brain tissue extract (Stressgen Bioreagents, Canada) was used as a positive control. Western blots were performed using a rabbit anti-EC-SOD (Phoenix Pharmaceuticals, Belmont, CA), specific signal was detected with a donkey anti-rabbit IgG-horseradish peroxidase conjugate (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and ECL Plus Western Blotting Detection Reagents (GE Healthcare) according to the manufacturer's instructions. Epson Perfection 4990 Scanner and SilverFast software (Epson, Singapore) were used to scan and evaluate densitometrically the signal on X-ray film.

Enzyme-Linked Immunosorbent Assay for HS. HS levels in LV and aorta were assayed using an enzyme-linked immunosorbent assay kit (Seikagaku, Tokyo, Japan). The antibodies used do not react with other glycosaminoglycans, including heparin (Yokoyama et al., 1999). In brief, the assay procedures were performed as follows: frozen LV or aortic tissue was homogenized in ice-cold phosphate-buffered saline, homogenate was centrifuged at 13,000*g* for 20 min at 4°C, and supernatant was isolated and protein concentrations determined (BCA). Then, 10 μl of 20 mg/ml actinase E (Kaken Pharmaceuticals, Tokyo, Japan) was added to 100 μl of supernatant and incubated at 55°C for 20 h to digest tissue and release bound heparan sulfate from proteins. After incubation, samples were boiled for 5 min, cooled, and centrifuged at 3000*g* for 10 min. Then samples were assayed according to manufacturer's instruction using a 96-well plate. After assay reactions, the plate was read at 450 nm using a microplate reader (SpectraMax 340; Molecular Devices, Sunnyvale, CA).

Statistical Analysis. Data are expressed as means ± S.E.M. We planned comparisons for each gene between control and diabetic groups to verify the impact of diabetes and between diabetic and TETA-treated diabetic groups to measure the effect of the drug. The paired Student's *t* test was hence used to determine the significance of between-group gene expression differences (Prism, version 4.02; GraphPad Software Inc., San Diego, CA). Mixed linear effect models were fitted by restricted maximum likelihood using SPlus v7.0.2 (Insightful) to analyze $(-dP_{LV}/dt)_{mean}$. One-way analysis of variance with post hoc Tukey's test was used to determine the significance of between-group HS concentration differences (Prism, version 4.02). *P* values of <0.05 have been considered significant, and *n* values indicate the number of replicates.

Results

Selective Cu^{II} Chelation Improved Cardiac Parameters in Diabetic Rats. Hyperglycemia occurred within 2 days after STZ administration in all experiments, during which hyperglycemia persisted equivalently in both untreated and TETA-treated diabetic rats. The mean body weights of different groups of rats were similar at the time of STZ injection. Body weight gain over the 16-week experimental period in TETA-treated diabetic rats was significantly higher than in untreated diabetic rats ($P < 0.05$) (Fig. 1A). Blood glucose concentrations did not differ among groups at the time of STZ injection or between TETA-treated and untreated diabetic rats over the 16-week experimental period (Fig. 1B, and data not shown), indicating that the TETA-mediated Cu^{II} chelation did not decrease blood glucose levels in diabetic rats.

Diabetes causes cardiac dysfunction and failure in STZ-diabetic rats (Cooper et al., 2004) and humans (Struthers and Morris, 2002). Diabetic rats showed a significantly higher ratio of cardiac mass to body mass than that of nondiabetic rats ($4.48 \pm 0.012 \times 10^{-3}$ versus $2.71 \pm 0.015 \times 10^{-3}$; $P < 0.001$), showing that they had developed cardiac hypertrophy. This ratio was significantly restored toward normal in diabetic rats that received 8-week oral TETA treatment ($4.18 \pm 0.078 \times 10^{-3}$; $P < 0.05$). Sixteen-week diabetic rats also had lowered $(-dP_{LV}/dt)_{mean}$ ($P = 0.017$), whereas 8-week TETA treatment improved $(-dP_{LV}/dt)_{mean}$ toward normal ($P = 0.022$) (Fig. 1C). Thus, consistent with our previous report (Cooper et al., 2004), diabetes caused cardiac hypertrophy and diastolic dysfunction that were substantively ameliorated by TETA treatment. These findings indicate that biochemical changes reported herein reflect structural and functional cardiac changes induced by diabetes and TETA treatment similar to those we reported previously (Cooper et al., 2004).

ECM Protein Expression (LV and Aorta). To investigate the molecular mechanisms by which TETA improved cardiac structure and function in diabetic rats, we analyzed the expression of mRNAs corresponding to major ECM proteins. Expression of collagen I (Fig. 2A), III (Fig. 2B), and IV (Fig. 2C), fibronectin-1 (Fig. 2D), and PAI-1 (Fig. 2E) in LV were elevated in diabetes, and these increases were sup-

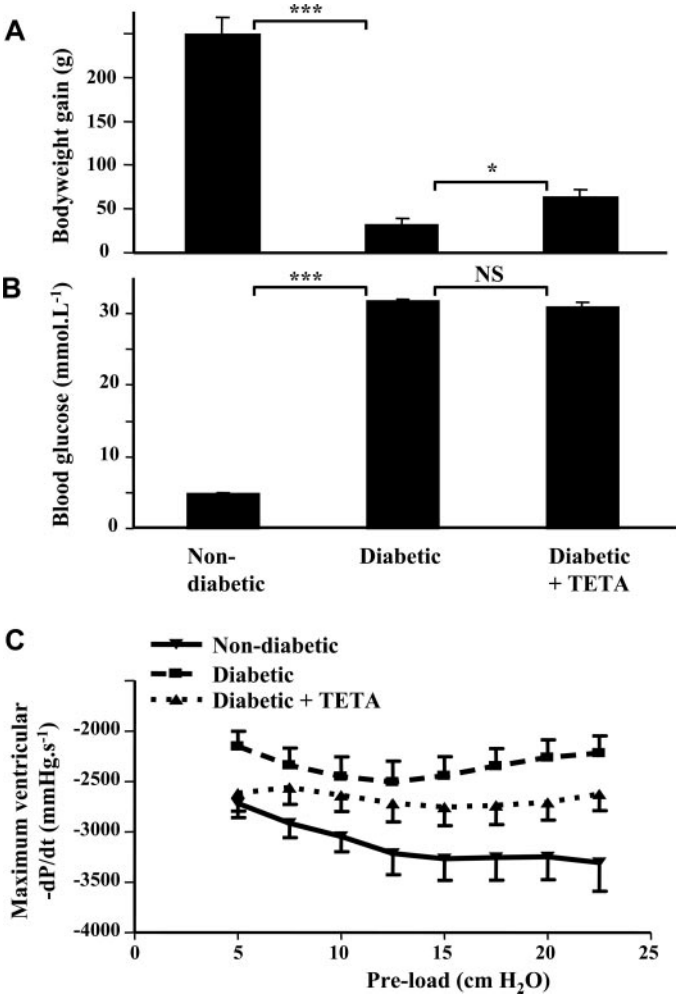

Fig. 1. Effect of TETA treatment on body weight gain (A), blood glucose (B), and $(-dP_{LV}/dt)_{mean}$ (C) with increasing preload in isolated perfused working hearts ($n = 9$–14 per group). Eight weeks after diabetes induction with STZ, rats were treated with TETA (orally) for a further 8 weeks. *, $P < 0.05$; ***, $P < 0.001$; NS, not significant. In C, $(-dP_{LV}/dt)_{mean}$ values were significantly lower in diabetes ($P = 0.017$), and this effect was partially ameliorated by TETA treatment ($P = 0.022$).

pressed by TETA treatment. Expression of collagens III (Fig. 2F) and IV (Fig. 2G), and fibronectin-1 (Fig. 2H) in the aortas of diabetic rats were also elevated, and these increases were also suppressed to normal by TETA treatment.

TGF-β1 and Smad4 Expression (LV and Aorta). To further characterize the mechanism by which TETA inhibited the accumulation of ECM proteins in diabetic rats, we analyzed the mRNA expression of genes involved in the TGF-β/Smad signaling pathway, which elicits stimulation of collagen production and plays a pivotal role in fibrogenesis (Sharma and Ziyadeh, 1995). Expression levels of mRNAs corresponding to TGF-β1 (Fig. 3A) and Smad4 (Fig. 3B) were increased by 1.9- and 1.4-fold, respectively, in LV tissue of diabetic rats compared with nondiabetic controls. In aortic tissue, TGF-β1 (Fig. 3C) and Smad4 (Fig. 3D) mRNA expression levels were similarly increased by 3.3- and 1.5-fold, respectively; TETA treatment significantly reversed the increased expression of these mRNAs in both LV and aorta of diabetic rats (Fig. 3, A–D).

EC-SOD Expression (LV and Aorta). EC-SOD is the only antioxidant enzyme known to be present in several extracellular compartments (Marklund, 1984b). Activation of the TGF-β/Smad signaling pathway reportedly suppresses EC-SOD expression (Marklund, 1992). Moreover, diabetes-induced oxidative stress was reported to stimulate TGF-β-mediated matrix synthesis in renal glomeruli through activation of protein kinase C- and advanced glycation end product-mediated processes (Akahori et al., 2005). Here, we showed that EC-SOD mRNA levels in LV and aorta from diabetic rats at 16 weeks were significantly decreased by 2.2- and 2.1-fold, respectively, compared with matched control values (Fig. 4, A and B). However, 8 weeks of TETA treatment significantly restored EC-SOD mRNA levels in these diabetic tissues by 2.8- and 1.8-fold, respectively (Fig. 4, A and B). It is interesting that EC-SOD protein expression level in diabetic aorta was lower than that in nondiabetic aorta, which was significantly increased by TETA treatment (Fig. 4, C and D).

HS Concentrations (LV and Aorta). HS is the major binding anchor of EC-SOD in extracellular compartments (Sandstrom et al., 1993). It can bind and localize EC-SOD in the extracellular matrix of blood vessels, where it eliminates ROS and sustains NO bioavailability (Marklund, 2002). HS concentrations in both LV (Fig. 5A) and aorta (Fig. 5B) were

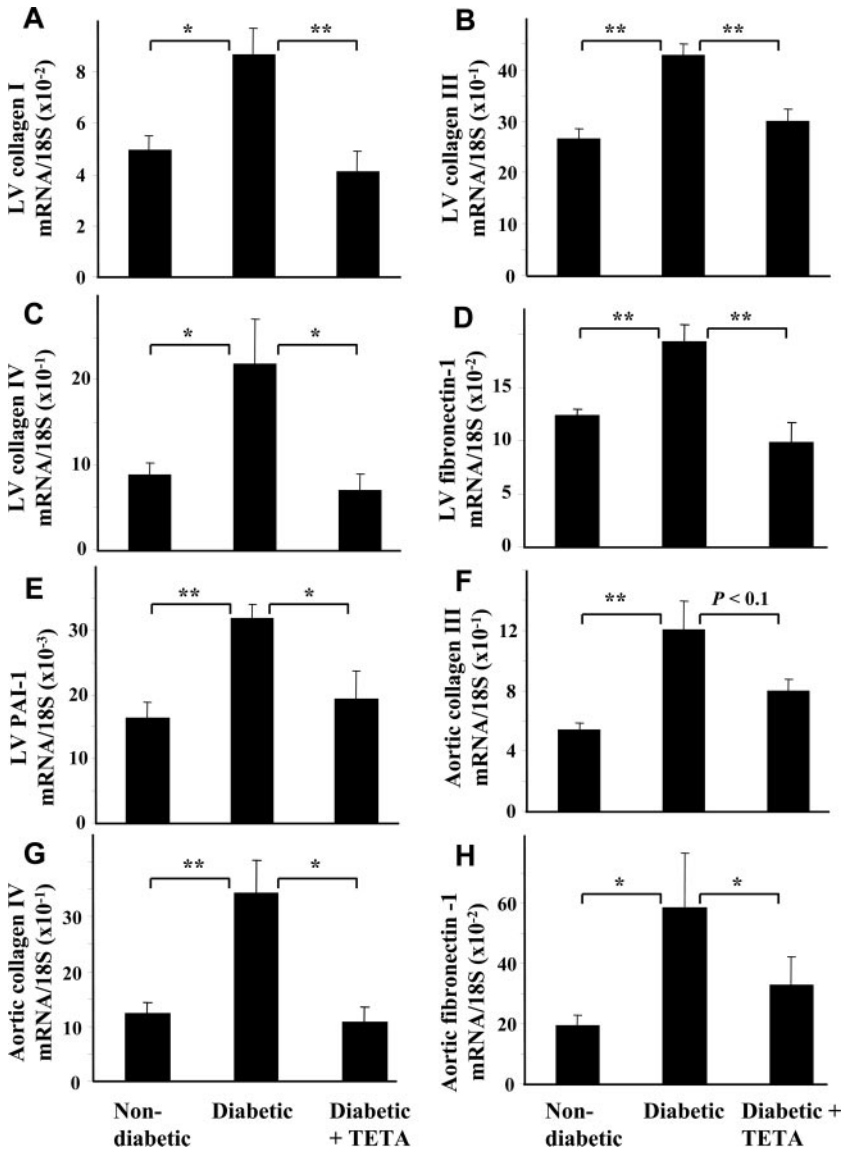

Fig. 2. TETA restored to normal collagen I (A), collagen III (B), collagen IV (C), fibronectin-1 (D), and PAI-1 (E) mRNA levels in LV and collagen III (F), collagen IV (G), and fibronectin-1 (H) mRNA levels in the aorta of STZ-diabetic rats ($n = 5$ per group). *, $P < 0.05$; **, $P < 0.01$.

significantly lower in diabetic rats than in matched controls. TETA treatment significantly increased HS levels in both tissues (Fig. 5, A and B), although mean values were still lower than those in nondiabetic controls. TETA-mediated elevations were correspondingly greater in aortic than LV tissue (Fig. 5), consistent with the hypothesis that its renormalizing effects on cardiac HS content could mainly reflect increases in HS content of coronary arteries.

Discussion

In this study, we reconfirmed that TETA significantly improved cardiac structure and function in diabetic rats. Consistent with our previous report (Cooper et al., 2004), TETA ameliorated diabetes-evoked LV hypertrophy and diastolic dysfunction without lowering blood glucose (Fig. 1). Here, we have shown for the first time that Cu^{II} chelation suppressed diabetes-evoked up-regulation of mRNAs corresponding to several ECM proteins and inhibited diabetes-induced activation of the TGF-β1/Smad signaling pathway. TETA treatment also caused robust increases in mRNA and protein expression of EC-SOD. Furthermore, HS was partially restored by TETA treatment in the heart and aorta of diabetic rats.

Although the pathogenesis of diabetic cardiovascular disease is multifactorial, tissue fibrosis is one of its main pathological hallmarks, and excessive accumulation of ECM is a key consequence thereof. Diabetes causes overproduction of cardiac ECM, which contributes to diastolic dysfunction, and ECM overaccumulation has been reported in the diabetic rat heart (Martin et al., 2005). Studies have shown that collagen IV and fibronectin coalesce around smooth muscle cells in the aortic media (Sista et al., 2005). In this study, mRNAs corresponding to these major ECM components were increased in experimental diabetes (Fig. 2). TETA normalized levels of collagen IV and fibronectin-1 mRNAs in diabetic rats (Fig. 2). Consistent with this finding, we showed previously that collagen III protein is substantively elevated in LV of diabetic rats and is normalized by TETA treatment (Cooper et al., 2004).

TGF-β1, a potent fibrogenic factor, stimulates collagen synthesis in cultured stellate cells, and overexpression of TGF-β1 in transgenic mice caused hepatic fibrosis (Sanderson et al., 1995). TGF-β-induced collagen production in cultured cardiac fibroblasts was increased by elevated glucose, indicating that TGF-β signaling pathway may play a major role in cardiac fibrosis and dysfunction (Martin et al., 2005). In the current study, we present in vivo evidence for an inhibitory effect of TETA on diabetes-induced over-expression of TGF-β1 and Smad4 mRNA in LV and aorta (Fig. 3). It is interesting that inhibition of TGF-β expression has been reported to be associated with the inhibitory effect of both lung and liver fibrosis by copper-lowering therapy with tetrathiomolybdate (Brewer et al., 2003, 2004; Askari et al., 2004). Tetrathiomolybdate was also found to suppress nuclear factor κB, which in turn controls transcription of many angiogenic cytokines (Brewer, 2005), and to inhibit long-term inflammation (Omoto et al., 2005). We suggest that this inhibition of mRNA expression of TGF-β1 and one of its major downstream signaling components, Smad4, may be an important mechanism by which TETA ameliorates diabetes-induced cardiac and arterial diseases.

PAI-1 is the primary inhibitor of plasminogen activator in vivo and is believed to promote tissue fibrosis (Schnaper et al., 1995). Previous studies have shown that PAI-1, whose promoter contains Smad-binding elements, is significantly induced by TGF-β via Smad activity (Stroschein et al., 1999). PAI-1 mRNA expression was induced by various inflammatory agents, including TGF-β (Venugopal et al., 2004). It is interesting that PAI-1 has been shown to directly control TGF-β expression and thereby ultimately regulate ECM production in diabetes (Nicholas et al., 2005). Here, we have provided in vivo evidence for the elevated expression of PAI-1 mRNA in diabetic LV, which was ameliorated by TETA treatment (Fig. 2). All of the results suggest that two feed-forward cycles of reciprocal stimulation between TGF-β1 and PAI-1

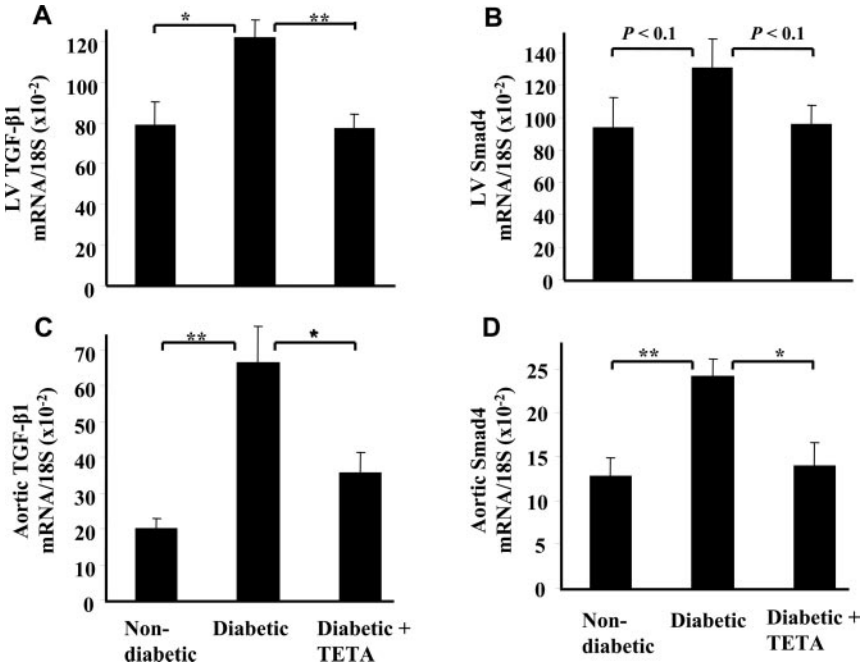

Fig. 3. Inhibition by TETA of diabetes-induced overexpression of TGF-β1 (A), and Smad4 (B) mRNA levels in LV and TGF-β1 (C) and Smad4 (D) mRNA levels in aorta of STZ-diabetic rats ($n = 5$ per group). *, $P < 0.05$; **, $P < 0.01$.

may perpetuate the fibrotic response in diabetic heart disease. Diabetes-evoked activation of both TGF-β1 and PAI-1, which regulate each other's expression, may create a self-stimulatory cycle that enhances ECM accumulation and tissue fibrosis.

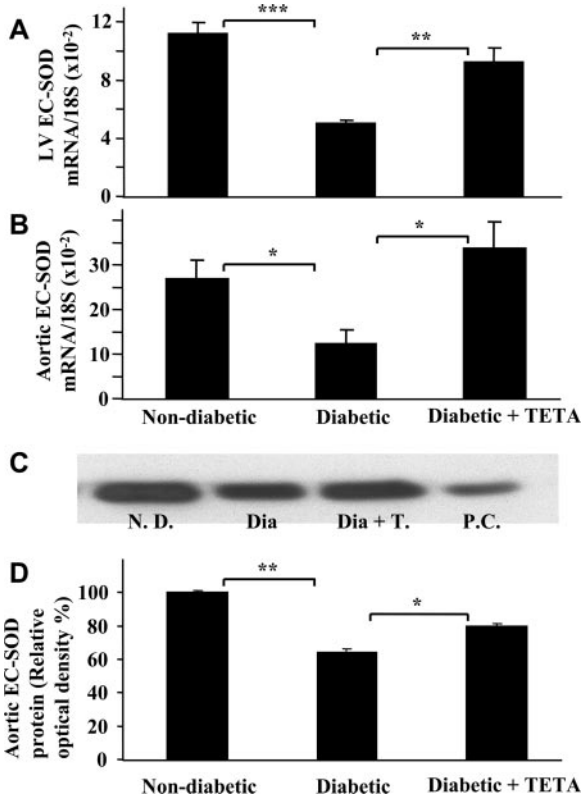

Fig. 4. TETA restored EC-SOD mRNA levels in LV (A) and aorta (B), and a representative Western analysis (C) in aorta (protein) of STZ-diabetic rats (n = 5/group). The calculation of protein restoration is shown in D: quantitative densitometric evaluation of EC-SOD protein in the aorta. The density of nondiabetic control was designated as 100%. N.D., nondiabetic; Dia., diabetic; Dia.+T., TETA-treated diabetic; P.C., positive control (rat brain tissue extract; Stressgen). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

EC-SOD was said to act, at least in part, via down-regulation of the profibrotic TGF-β pathway. EC-SOD-null mice consistently displayed increased susceptibility to inflammation and pulmonary fibrosis, suggesting that one mechanism by which EC-SOD protects against pulmonary fibrosis is by inhibiting inflammation (Fattman et al., 2003). Here, we found that TETA treatment increased EC-SOD mRNA and artery-associated protein, whereas it suppressed mRNAs corresponding to collagens I, III, and IV and fibronectin-1 in LV and aortic tissue from diabetic rats. TETA-evoked suppression of the activated TGF-β/Smad pathway and suppression of elevated mRNAs corresponding to ECM proteins may be associated with restoration of EC-SOD mRNA and protein. There have been prior suggestions that tumor necrosis factor-α also affects EC-SOD (Marklund, 1992), but we did not observe changes in tumor necrosis factor-α in heart and aorta of diabetic rats in this study (data not shown). Our results support the idea that EC-SOD, acting as a TGF-β1 antagonist, may disrupt the vicious cycle of TGF-β1 over-production in cardiovascular fibrosis. On the other hand, suppression of EC-SOD in diabetic LV and aorta could result from activation of the TGF-β/Smad signaling pathway.

We reported previously that plasma EC-SOD was significantly higher in patients with diabetes than that in matched control subjects and that TETA treatment suppressed this elevation and restored circulating EC-SOD to normal (Cooper et al., 2005). Here, we have shown that arterial HS content was lower in rats with insulin-deficient diabetes than in matched controls, which is perhaps the cause of lower artery-bound EC-SOD. One possible explanation for these observations may lie, at least in part, in responses of arterial HS. Because most EC-SOD is bound in blood vessel walls (Marklund, 1984a), a minor release of EC-SOD into the blood may cause a significant increase in circulating EC-SOD, thereby generating a negative association between serum

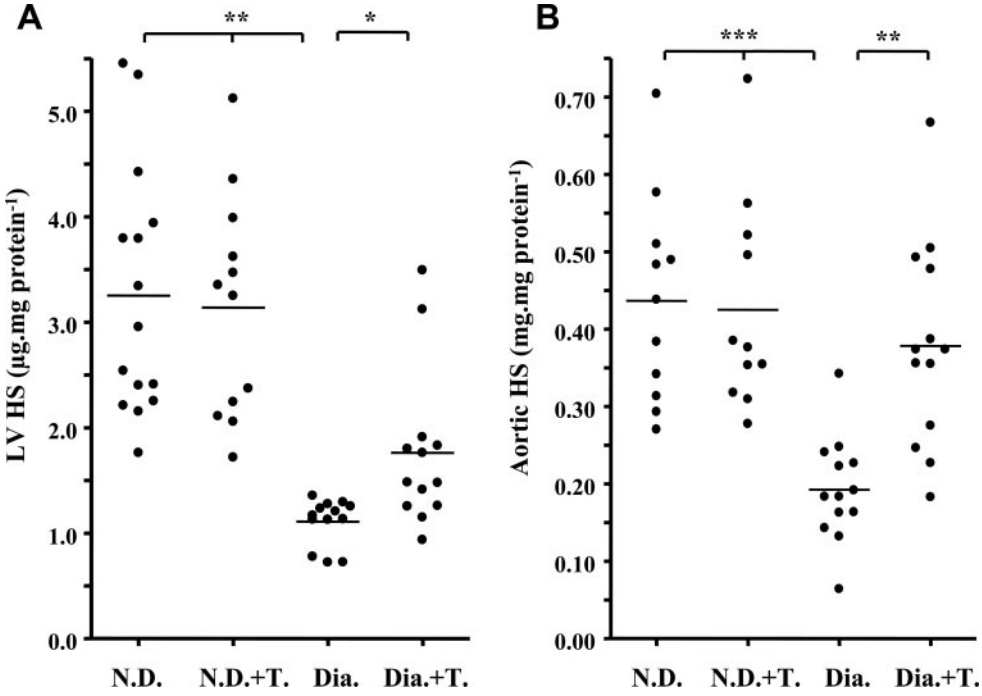

Fig. 5. TETA partially restored HS levels in LV (A) and aorta of diabetic rats (B) (n = 11–14 per group). N.D., nondiabetic; N.D.+T., TETA-treated nondiabetic; Dia., diabetic; Dia.+T., TETA-treated diabetic. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

EC-SOD and vascular HS. This observation is consistent with evidence that EC-SOD bound in arterial walls is decreased in patients with diabetes (Ciechanowski et al., 2003; Fattman et al., 2003). TETA's ability to restore HS levels in blood vessels may be a factor underlying our observation that TETA treatment suppresses the elevated serum EC-SOD in patients with diabetes (Cooper et al., 2005). Not only can HS localize EC-SOD to improve vascular ROS disposal, but it also has antiatherogenic properties (Sivaram et al., 1995) to down-regulate fibroblast growth factor (Nugent et al., 1993) and to inhibit arterial smooth muscle cell proliferation (Castellot et al., 1981). TETA may also exert its effects in diabetic heart failure, at least in part, through vascular HS modulation.

In summary, we propose that TETA treatment attenuates extracellular Cu^{2+}-evoked cardiac and arterial disease, at least in part by suppressing the activation of the TGF-β/Smad signaling pathway and PAI-1 that would otherwise evoke increased ECM protein production and associated cardiac and arterial fibrosis. In addition, TETA treatment robustly stimulates the expression of EC-SOD, the sole antioxidant enzyme known to scavenge extracellular $O_2^{\cdot-}$, thereby enhancing the potential for its disposal (Fig. 4). TETA also beneficially modified vascular HS, which was compromised by diabetes. Whether TETA affects HS synthesis or consumption is also under investigation. We also expect that the results reported here may well be relevant to cardiovascular diseases associated with other underlying conditions, such as hypertensive heart disease, ischemic cardiomyopathy, and ageing, in which there are metabolic perturbations similar to those in diabetes.

Acknowledgments

We thank C. A. Tse, K. H. Liu, and V. Tintinger for editorial and technical assistance.

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