Molecular Changes Evoked by Triethylenetetramine (TETA) Treatment in the Extracelluar Matrix of the Heart and Aorta in Diabetic Rats

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ABBREVIATIONS: AGEs, advanced glycation end-products; CAD, coronary artery disease; Ct, threshold cycle; ECM, extracellular matrix; EC-SOD, extracellular superoxide dismutase; ELISA, enzyme-linked immunosorbent assay; GAG, glycosaminoglycan; HS, heparan sulfate; KHB, Krebs-Henseleit bicarbonate buffer; LV, left ventricle; NO, nitric oxide; $O_2^{\cdot-}$, superoxide anion; PAI-1, plasminogen activator inhibitor-1; PCR, polymerase chain reaction; PKC, protein kinase C; ROS, reactive oxygen species; STZ, streptozotocin; TETA, triethylenetetramine; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor-α.

ABSTRACT

Most diabetic subjects 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 previously reported 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 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-week's 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 (PAI-1), were elevated in untreated diabetic animals and normalized following 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 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 towards normal by TETA treatment of cardiac and arterial structure and function in diabetes.

More than three-quarters of diabetic patients 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 type-2 diabetic patients to a greater extent than in matched controls, and

restored cardiac structure and function towards normal in both groups (Cooper et al.,

2005; Cooper et al., 2004). 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 thought

to be a major function of EC-SOD (Oury et al., 1996). EC-SOD activity is decreased in

arteries of diabetic subjects (Fattman et al., 2003), resulting in increased susceptibility of

vascular cells to effects of $O_2^{\cdot-}$ 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 previously reported that chronic 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 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 that TETA reverses diabetic cardiac disease at least in part through enhanced disposal of extracellular $O_2^{\cdot-}$ 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 (Sigma; 55 mg/kg bodyweight)

in isotonic saline, as described previously (Cooper et al., 2004). Age-matched control

rats were injected with equal volumes of saline (untreated non-diabetic control).

Bodyweights and [glucose]$_{blood}$ were monitored weekly for 16 weeks. After 8 weeks'

diabetes, 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) as triethylenetetramine dihydrochloride (trientine, Fluka) or the equivalent molar dose

of TETA disuccinate (Protemix, Auckland) 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 (12:12-h light-dark cycle; temperature 22.5^{o}C (range: 20-26);

humidity 60% (range 50 - 70)) in like-pairs with *ad libitum* food (Teklad 2018) and

water. Sixteen weeks after STZ injection, rats were anesthetized (halothane, 5%),

appropriately heparinized (200 I.U./kg i.v.) and organs excised. Aortas and cardiac LV

were either perfused or washed free of blood in ice-cold DEPC-treated phosphate-

buffered saline (PBS). Tissues were stored in RNA*later* (Ambion) overnight at 4°C, and

then at -80°C for subsequent RNA isolation. Portions were also stored at -80°C for

parallel protein analyses as well as 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 RNA Midi Kits (Qiagen, Valencia, CA). One µg of total RNA was treated with RQ1 RNase-free DNase (Promega) at 37ºC for 30 min, and then reverse-transcribed with random hexamers and SuperScript^{TM} III Reverse Transcriptase (Invitrogen).

Real-Time Quantitative PCR Analysis. Messenger RNA levels were compared by real-time quantitative PCR (qPCR) with an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). 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 employed 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 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 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 DTT and 10%

(V/V) glycerol) in the presence of a proteinase inhibitor cocktail (Roche). Homogenate

was centrifuged at 13,000g for 20 min at 4°C. The supernatant was isolated, and protein

concentration determined with BCA Protein Assay (Pierce, Rockford, IL). Twenty µg

protein was separated by gel electrophoresis and transferred to a nitrocellulose

membrane. Five µg 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), specific signal was detected with a donkey anti-rabbit IgG-HRP

conjugate (Amersham) and ECL[TM] Plus Western Blotting Detection Reagents

(Amersham) according to the manufacturer's instructions. Epson Perfection 4990

Scanner and SilverFast software (Singapore) were used to scan and evaluate

densitometrically the signal on X-ray film.

Enzyme-linked Immunosorbent Assay (ELISA) for HS. HS levels in LV and aorta

were assayed using an ELISA kit (Seikagaku, Tokyo, Japan). This antibodies used do not

react with other GAGs, including heparin (Yokoyama et al., 1999). Briefly, the assay

procedures were performed as follows: Frozen LV or aortic tissue was homogenized in

ice-cold PBS buffer, homogenate was centrifuged at 13,000g 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, Japan) was added to 100 µL 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

3,000g 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).

Statistical Analysis. Data are expressed as means ± SEM. We planned comparisons for

each gene between control and diabetic groups to verify impact of diabetes, and between

diabetic and TETA-treated diabetic groups to measure effect of the drug. The unpaired

Student's t-test was hence used to determine the significance of between-group gene

expression differences (Prism, v4.02). Mixed linear effect models were fitted by

restricted maximum likelihood using SPlus v7.0.2 (Insightful) to analyze $(-dP_{LV}/dt)_{mean}$.

One-way ANOVA with post-hoc Tukey's-test was used to determine the significance of

between-group HS concentration differences (Prism, v4.02). P values of < 0.05 have

been considered significant, and n values indicate numbers 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 bodyweights of different groups of rats were similar at the time

of STZ injection. Bodyweight 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, nor 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 non-diabetic rats (4.48 ± 0.012 x 10^{-3} *vs*. 2.71 ± 0.015 x 10^{-3}; $P < 0.001$), showing that they had developed cardiac hypertrophy. This ratio was significantly restored towards normal in diabetic rats that received 8-week oral TETA treatment (4.18 ± 0.078 x 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 ($-dP_{LV}/dt)_{mean}$ towards 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 previously reported (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 expression of mRNAs corresponding to major ECM proteins. Expression of collagen I (Fig. 2A), III (Fig. 2B), IV (Fig. 2C), fibronectin-1 (Fig. 2D) and PAI-1 (Fig. 2E) in LV were elevated in diabetes and these increases were suppressed 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 non-diabetic 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. 3A-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

(PKC)- and advanced glycation endproduct (AGE)-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, when

compared with matched control values (Fig. 4A, B). However, 8-weeks' TETA treatment

significantly restored EC-SOD mRNA levels in these diabetic tissues, by 2.8- and 1.8-

fold, respectively (Fig. 4A, B). Interestingly, EC-SOD protein expression level in

diabetic aorta was lower than that in non-diabetic aorta, which was significantly

increased by TETA treatment (Fig. 4C, 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 significantly lower in diabetic rats than matched controls. TETA treatment

significantly increased HS levels in both tissues (Fig. 5A, B), although mean values were

still lower than those in non-diabetic 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 the 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 upregulation 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 multi-factorial,

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 over-accumulation 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

previously showed that collagen III protein is substantively elevated in LV of diabetic

rats and also normalized by TETA treatment (Cooper et al., 2004).

TGF-β1, a potent fibrogenic factor, stimulates collagen synthesis in cultured

stellate cells, and over-expression 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).

Interestingly, 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 (Askari et al., 2004; Brewer et al., 2004; Brewer et al., 2003).

Tetrathiomolybdate was also found to suppress nuclear factor kappa B, which in turn

controls transcription of many angiogenic cytokines (Brewer, 2005), and to inhibit

chronic 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 thought 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). Interestingly, 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 the results suggest that two feed-forward cycles of

reciprocal stimulation between TGF-β1 and PAI-1 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.

EC-SOD was said to act, at least in part, via down-regulation of the profibrotic

TGF-β pathway. Consistently, EC-SOD null mice 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 TNF-α also affects EC-SOD (Marklund, 1992), but we

did not observe changes in TNF-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.

Alternatively, suppression of EC-SOD in diabetic LV and aorta could result from

activation of the TGF-β/Smad signaling pathway.

We previously reported that plasma EC-SOD was significantly higher in diabetic

patients 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. Since 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 EC-SOD and

vascular HS. This observation is consistent with evidence that EC-SOD bound in arterial

walls is decreased in diabetic patients (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 diabetic

patients (Cooper et al., 2005). Not only can HS localize EC-SOD to improve vascular

ROS disposal, but it also has anti-atherogenic 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.

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In summary, we propose that TETA treatment attenuates extracellular Cu^{2+}-

evoked cardiac and arterial disease, at least in part by suppressing 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 expression of EC-SOD, the sole antioxidant enzyme known to

scavenge extracellular $O_2^{\cdot\cdot}$, 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.

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Footnotes

The authors have all declared their association with Protemix Corporation,

Auckland, New Zealand and San Diego, CA, USA.

Figure Legends

Fig. 1. Effect of TETA treatment on A: bodyweight gain; B: blood glucose; and C: (-

$dP_{LV}/dt)_{mean}$ with increasing preload in isolated perfused working hearts (n = 9-

14/group). Eight weeks after diabetes-induction with STZ, rats were treated with TETA

(p.o.) 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 ($P = 0.022$). STZ, streptozotocin.

Fig. 2. TETA restored to normal A: collagen I; B: collagen III, C: collagen IV; D:

fibronectin-1; and E: PAI-1 mRNA levels in LV, as well as F: collagen III; G: collagen

IV; and H: fibronectin-1 mRNA levels in the aorta of STZ-diabetic rats ($n = 5$/group). *P

< 0.05, **$P < 0.01$. STZ, streptozotocin.

Fig. 3. Inhibition by TETA of diabetes-induced over-expression of A: TGF-β1; and B:

Smad4 mRNA levels in LV, as well as C: TGF-β1; and D: Smad4 mRNA levels in aorta

of STZ-diabetic rats ($n = 5$/group). *$P < 0.05$, **$P < 0.01$. STZ, streptozotocin.

Fig. 4. TETA restored EC-SOD mRNA levels in A: LV and B: aorta; and C: a

representative Western analysis 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 non-diabetic control was

designated as 100%. N.D., non-diabetic; Dia., diabetic; Dia.+T., TETA-treated diabetic;

P.C., positive control (rat brain tissue extract, Stressgen). $*P < 0.05$, $**P < 0.01$, $***P <$
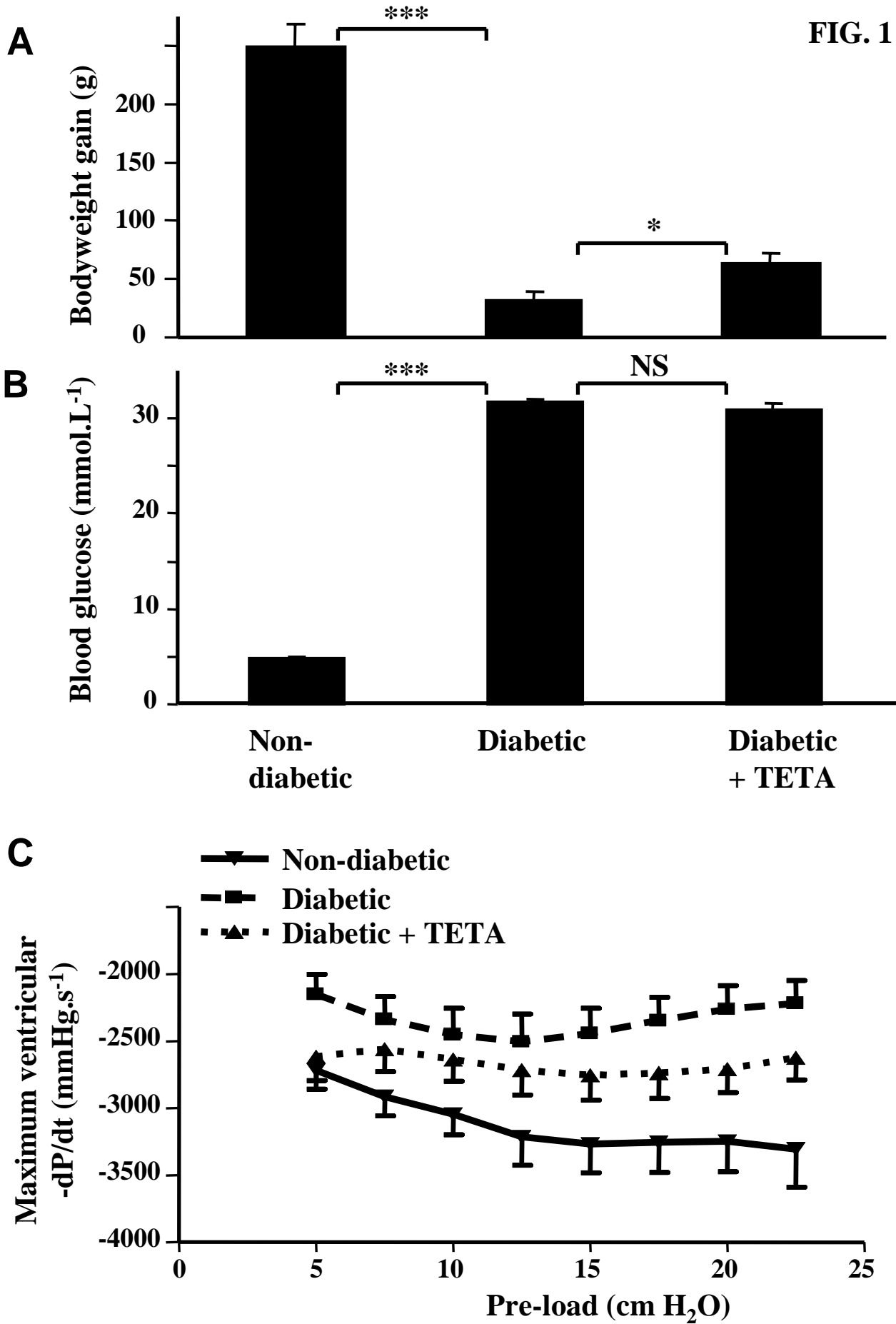
0.001. STZ, streptozotocin**.**

Fig. 5. TETA partially restored HS levels in A: LV; and B: aorta of diabetic rats ($n = 11$-

14/group). N.D., non-diabetic; N.D.+T., TETA-treated non-diabetic; Dia., diabetic;

Dia.+T., TETA-treated diabetic. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

TABLE 1

Sequences of oligonucleotide primers used for real-time PCR analysis

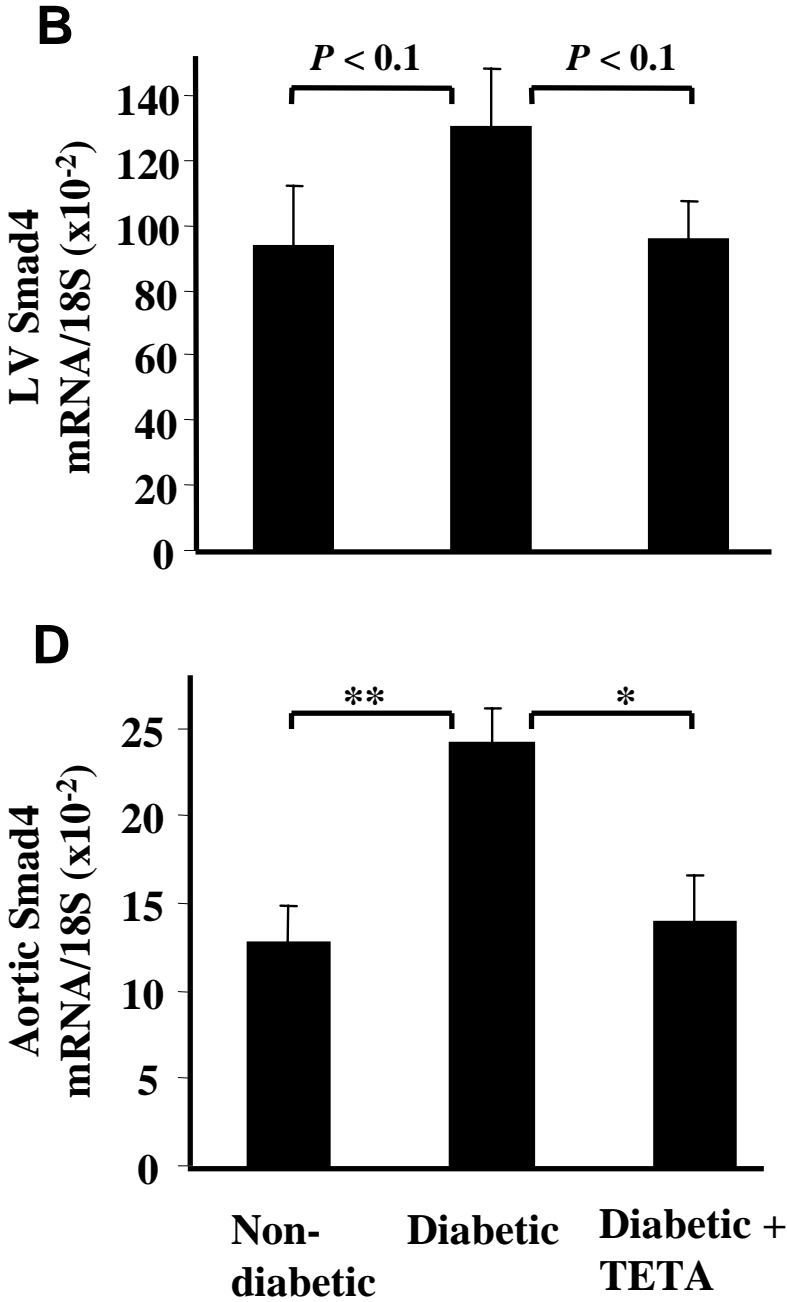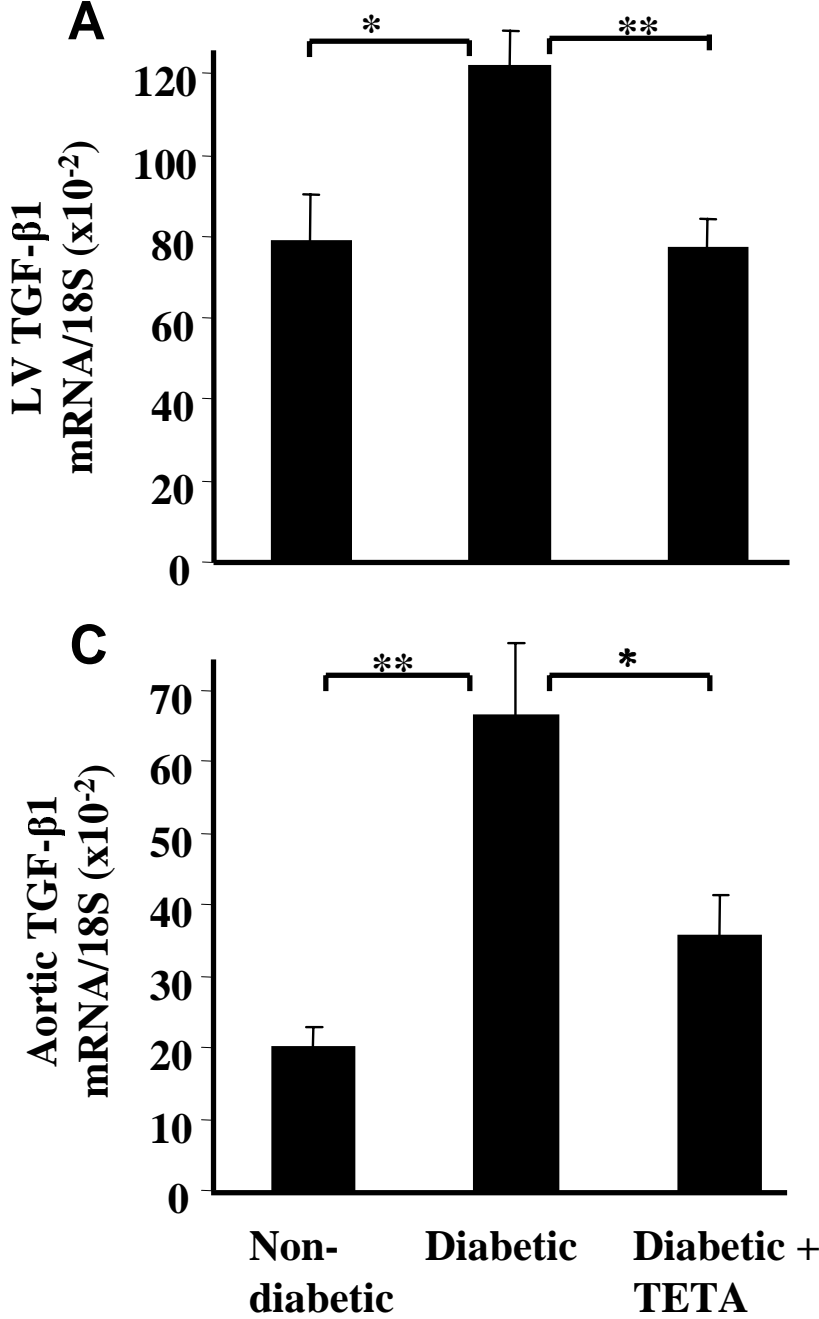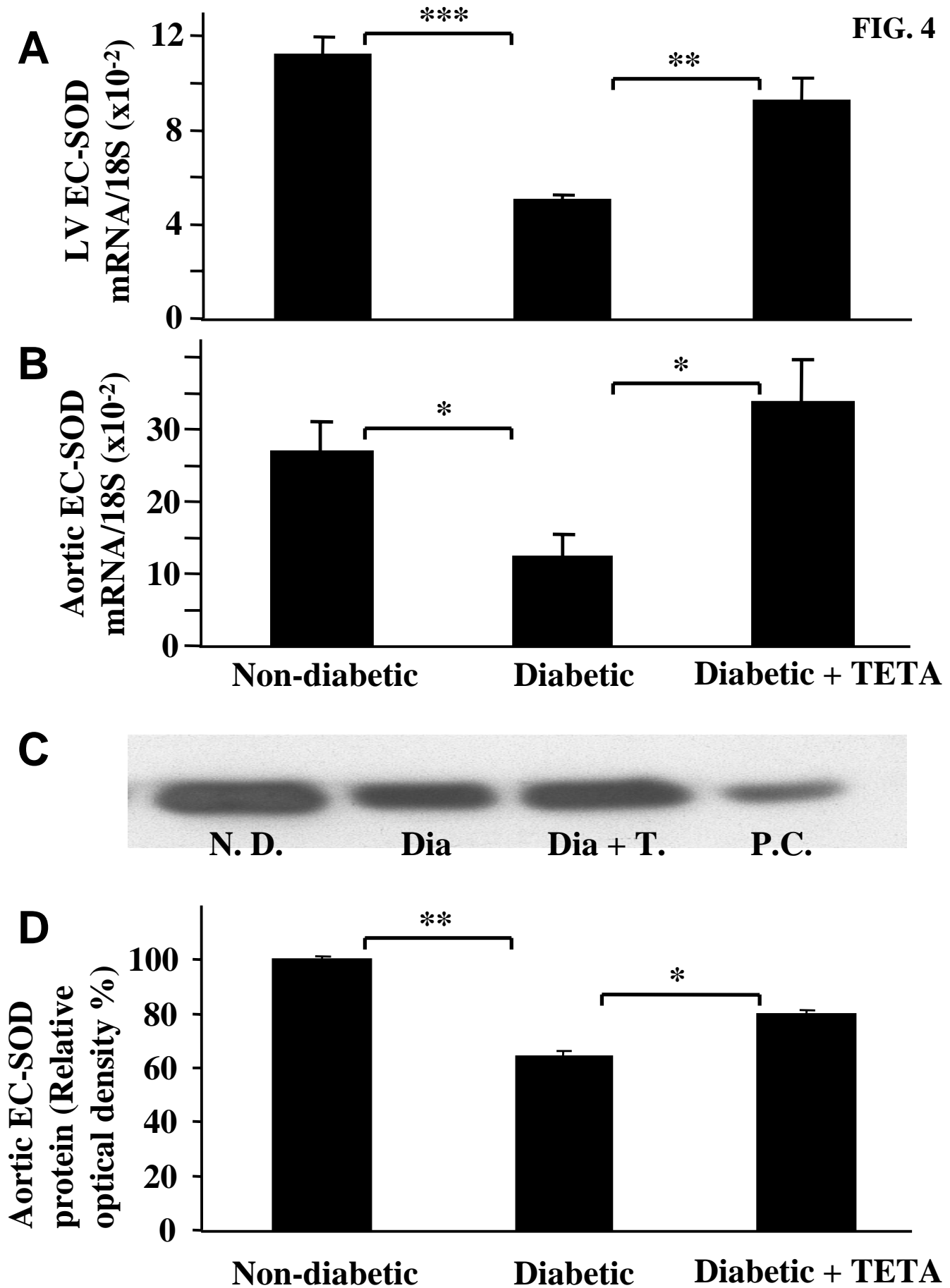| Gene	Forward primer (5' – 3')	Reverse primer (5' - 3')
Collagen III	TCCCCTGGAATCTGTGAATC	TGAGTCGAATTGGGGAGAAT
Collagen IV	GGGTTTTCCCTTCTTTTCGT	CATGGTGAATCGCTGTAA
Fibronectin-1	GAGGCCACCATCACTGGTT	AGTGCGATGACATAGATGGTGTA
PAI-1	AGAGCCAATCACAAGGCACT	AGGCAAGTGAGGGCTGAAG
TGF-β1	GGAAAGGGCTCAACACCTG	CAGTTCTTCTCTGTGGAGCTGA
Smad4	GGTCCGTAGGTGGAATAGCC	CGGAGGTCGTCTACACCAAT
EC-SOD	CTTGGGAGAGCTTGTCAGGT	CACCAGTGACAGGTTGCAGA

FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5