Metallothionein prevents diabetes-induced cardiac pathological changes, likely via the inhibition of succinyl-CoA:3-ketoacid coenzyme A transferase-1 nitrination at Trp^{374}

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Metallothionein prevents diabetes-induced cardiac pathological changes, likely via the inhibition of succinyl-CoA:3-ketoacid coenzyme A transferase-1 (SCOT) nitration at Trp^{374}. Although SCOT nitration sites were identified at Tyr76, Tyr117, Tyr135, Tyr226, Tyr368, and Trp374, only Tyr76 and Trp374 were demonstrated targets, including superoxide, hydrogen peroxide, and peroxynitrite (3). Intriguingly, cardiac-specific metallothionein (MT) overexpression in transgenic mice protects the heart against superoxide, hydrogen peroxide, and peroxynitrite-induced cardiac degeneration and remodeling. Therefore, the prevention of diabetes-induced cardiac pathological changes by MT may involve the inhibition of SCOT nitration at Trp^{374}.

7 The cysteine-rich protein metallothionein (MT) is a ubiquitously expressed free radical scavenger with a wide range of demonstrated targets, including superoxide, hydrogen peroxide, and peroxynitrite (3). Intriguingly, cardiac-specific MT-overexpressing transgenic mice have a particularly high resistance to the development of diabetic cardiomyopathy for both type 1 and type 2 diabetes conditions (3, 7, 24). Our previous studies revealed that the preventive actions of MT against diabetic cardiomyopathy are mediated mainly by suppression of superoxide generation and its associated nitrosative damage (4).

Among the nitrated proteins identified in the diabetic heart is succinyl-CoA:3-ketoacid CoA transferase (SCOT) (27), an intramitochondrial rate-limiting enzyme. Under normal conditions, SCOT catalyzes conversion of the main ketone body, acetoacetate, into acetoacetyl-CoA, which is subsequently metabolized for energy production by the citric acid cycle (9). Accordingly, SCOT protein is abundant in normal heart, kidney, brain, and muscle and nearly undetectable in normal liver. Hereditary SCOT deficiency leads to ketoadiposis, and patients often present with elevated levels of ketone bodies in serum, especially when undernourished (8, 19).

Studies of diabetic hearts have demonstrated that mitochondrial SCOT activity is decreased compared with that observed in normal hearts (12). Furthermore, Turko et al. determined that the decreased SCOT catalytic activity that occurs in the diabetes; cardiac pathological changes; metallothionein; SCOT; 3-nitrotyrosine

DIABETES HAS BECOME one of the most prolific public health issues worldwide (25). Mechanistic studies have revealed that oxidative and nitrosative stress are major causes of diabetes and its complications (4, 13). Overproduced superoxide has been shown in diabetic individuals to interact with nitric oxide, forming the peroxynitrite (ONOO^-) free radical that mediates biomacromolecule nitration and can ultimately lead to organ dysfunction. The covalent product of tyrosine nitration, 3-nitrotyrosine (3-NT), serves as an index of peroxynitrite-induced protein damage and is used in clinic as a convenient quantitative biomarker of cardiovascular risk (measured in free and protein-bound forms in human plasma) (23). Moreover, overproduction of superoxide and associated peroxynitrite has been implicated in the development of diabetic cardiomyopathy (4, 20, 28). Therefore, a promising approach to prevent the development of diabetic complications will include the prevention of oxidative and nitrosative stress (2).

The well-established and widely used animal model of type 1 diabetes induced by streptozotocin (STZ) has several nitrated molecules in the cardiac tissue. The majority of these nitrated molecules are mitochondrial proteins with known functions in cellular energy metabolism and oxygen oxidation (26, 27, 29). However, whether the nitration of these mitochondrial proteins contributes to the development of diabetic cardiomyopathy remains largely unknown.

The cysteine-rich protein metallothionein (MT) is a ubiquitously expressed free radical scavenger with a wide range of demonstrated targets, including superoxide, hydrogen peroxide, and peroxynitrite (3). Intriguingly, cardiac-specific MT-overexpressing transgenic mice have a particularly high resistance to the development of diabetic cardiomyopathy for both type 1 and type 2 diabetes conditions (3, 7, 24). Our previous studies revealed that the preventive actions of MT against diabetic cardiomyopathy are mediated mainly by suppression of superoxide generation and its associated nitrosative damage (4).

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hearts of STZ-induced diabetic rats is accompanied by increased levels of nitrotyrosine in the SCOT proteins (27). A similar phenomenon was also observed in the alloxan-induced mouse model of diabetes (26). The role of nitration in decreased SCOT activity in humans was confirmed by Rebrin et al. (21). By investigating the posttranslational modifications that occurred in proteins during the aging process, tryptophan was identified as a specific in vivo target of nitration and shown to increase SCOT activity. Subsequently, Wang et al. identified four 3-NT residues (Tyr4, Tyr76, Tyr126, and Tyr174) in SCOT and showed that Tyr4 and Tyr76 are very important for the nitration-induced decrease in activity (31). The present study was designed to investigate the mechanism by which nitration of specific sites in SCOT exert changes in its activity and contribute to pathological processes, particularly in diabetic cardiomyopathy. The diabetes-related differential panel of nitrated proteins in cardiac tissues was identified using wild-type (WT) and MT-TG STZ-induced mice. First, Western blotting after one-dimensional electrophoresis (1-DE) of cardiac proteins showed that the majority of nitrated proteins were ~58 kDa. Separation by two-dimensional electrophoresis (2-DE) and Western blotting revealed that three of the spots ~58 kDa were nitrated SCOT. Furthermore, whereas the total SCOT protein expression was not significantly different between the two diabetic mouse strains, the WT mice showed significantly higher amounts of its nitrated form and significantly lower SCOT activity. Mass spectroscopy (MS) revealed that the nitration levels of SCOT Tyr76 and Trp374 sites were significantly higher in the WT diabetic hearts, but only Trp374 nitration was significant attenuated by MT. On the basis of these results, we theorize that MT may protect the heart from diabetes-related changes by preventing nitration of SCOT Trp374, thereby preserving its enzyme activity for mitochondrial energy metabolism.

**EXPERIMENTAL PROCEDURES**

**Diabetes model.** MT-TG mice were produced from the FVB strain (WT genotype) and have been previously well characterized (4, 5, 24). Both the MT-TG and WT mice were maintained in the same cages with free access to rodent diet and tap water.

To induce the diabetes model, 8-wk-old male mice, as used before (4, 5, 24, 30), were given a single intraperitoneal injection of 150 mg/kg body wt of STZ dissolved in sodium citrate buffer (pH 4.5, Sigma-Aldrich, St. Louis, MO). Control (nondiabetic) mice were given a corresponding volume of sodium citrate without STZ. Three days after the STZ treatment, whole blood glucose was detected using a SureStep Complete Blood Glucose Monitor (Sigma), and mice with glucose levels >250 mg/dl were considered diabetic (4, 5). At 2, 4, 8, and 16 wk after diabetes onset, 10 mice of each group were euthanized with pentobarbital sodium, and the heart tissues were collected for analysis.

**Histopathological examination.** Heart tissues were fixed with 10% formalin and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin-eosin (H&E) and Sirius red (to detect collagen) and examined under a light microscope (4, 5).

**Superoxide measurement.** Heart tissues were embedded in OCT compound and frozen at −20°C. Tissue sections (10 μm) were immediately processed by applying the dihydroethidium molecular probe (2 × 10−6 mol/l; Sigma-Aldrich) and incubated at 37°C for 30 min in the dark. Superoxide-specific staining was observed by fluorescence microscopy (excitation wavelength 488 nm, emission wavelength 610 nm) (4).

**Protein extraction.** Heart tissues were minced and homogenized in ice-cold lysis solution [7 M urea, 2 M thiourea, 2% CHAPS, 40 mM Trizma base, 2% immobilized pH gradient (IPG) buffer, 40 mM DTT, 1% protease inhibitor]. Centrifugation (15,000 g, 15 min) was performed to collect the protein-containing supernatant, and the total protein concentration was measured by Bradford protein assay (Bio-Rad, Hercules, CA).

**1-DE and 2-DE.** For 1-DE, 15 μg of total heart proteins was mixed with tris-glycine loading buffer, boiled for 5 min, and loaded onto 10.6% gels for resolution by electrophoresis using tris-glycine running buffer. For 2-DE, 400 μg protein samples was incubated with rehydration solution (8 M urea, 2% CHAPS, 0.5% IPG buffer, bromophenol blue) and then centrifuged at 15,000 g for 15 min at 4°C to collect the protein-containing supernatant for subsequent application to 13 cm, IPG strips with pH gradient 3–10 (GE Healthcare, Uppsala, Sweden). After overnight incubation at room temperature, the strips were subjected to first-dimension isoelectric focusing (IEF) using an Etan IPGphor 3 system (GE Healthcare). The processed IPG strips were stored at −80°C until the second-dimension sodium dodecyl sulfate (SDS)-polyacrylamide gel (10.6%) electrophoresis (PAGE) was carried out using an SE 600 Ruby cooled vertical electrophoresis device (GE Healthcare). For this step, the IPG strips were first equilibrated in an aqueous solution (50 mM Tris, 6 M urea, 30% glycerol, 2% SDS, 55 mmol/l DTT, pH 8.8) by incubation for 15 min and then in a solution of the same composition but with the DTT replaced by 110 mmol/l iodoacetamide. The electrophoresis was carried out at 20 mA for 30 min followed by 30 mA until the bromophenol blue dye front had migrated off the lower end of the gel. The gels were then stained with ethyl violet-zincon (EZ) before imaging, based on a published method (26).

**Immunoprecipitation of nitrated proteins with anti-3-NT.** Heart tissues were homogenized in lysis buffer (PBS and 1% protease inhibitor cocktail [Sigma]). Centrifugation (15,000 g, 15 min at 4°C) was performed to collect the protein-containing supernatant, which was subsequently incubated with 7 μg of anti-3-NT antibody (Millipore, Watford, UK) overnight at 4°C. Immune complexes were collected by incubating with Protein G Plus-Agarose (GE Healthcare) for 2 h at 4°C and washed three times with PBS. The immunoprecipitated proteins were recovered by resuspension in loading buffer, and the protein was detected by Western blotting.

**Western blotting.** Electrophoresis-resolved proteins were transferred to a polyvinylidene difluoride membrane, which was rinsed briefly in tris-buffered saline (TBS), blocked in blocking buffer (5% milk) for 1 h, and washed three times with TBS containing 0.05% Tween 20. The membranes were incubated with primary antibodies [1:5,000 anti-3-NT antibody (Millipore); 1:1,000 anti-SCOT antibody (ab105320; Abcam, Cambridge, MA); 1:5,000 anti-connective tissue growth factor (CTGF) antibody (ab6992; Abcam); 1:1,000 anti-transforming growth factor-β1 (TGF-β1) antibody (ab64715; Abcam)] for 2 h, washed three times with TBS containing 0.05% Tween 20, and reacted with the appropriate secondary antibody (horseradish peroxidase conjugated) for 1 h. Antibody-antigen complexes were detected using enhanced chemiluminescence staining (Amersham, Buckinghamshire, UK) (4, 5) and analyzed with the ImageMaster 2D Platinum 6.0 software.

**Electroelution and in-solution digestion.** A gel DNA elution kit (Bio-Rad) was used following the manufacturer’s protocol. Briefly, 40 bands of SCOT were electroeluted from the gel using elution buffer (25 mM Tris, 0.25 M glycine, 0.1% SDS) and 250 μA for 60 min. Protein fractions (1.5 ml for each) were collected, concentrated by vacuum centrifugation (final volume of 200 μl), mixed with 600 μl of ice-cold acetone, and incubated at −20°C for 2 h. Centrifugation (14,000 g, 10 min) was used to harvest the protein-containing pellets, which were then dried by vacuum centrifugation (supernatants were discarded). The dried proteins were dissolved in 50 mM NH4HCO3, reduced by incubation with 10 mM DTT at room temperature for 1 h, and alkylated by incubation with 40 mM iodoacetamide for 30 min at
room temperature in the dark. Trypsin (Promega, Madison, WI) dissolved in 25 mM NH$_4$HCO$_3$ was added, followed by protein enzyme (at a 500:1 ratio). Digestion was performed at 37°C overnight.

Dried samples were dissolved in 0.1% trifluoroacetic acid, desalted by ZipTip C$_{18}$ column (Millipore), and dried using a SpeedVac. Finally, formic acid (0.1% vol/vol) was added, and the samples were analyzed by MS.

**In-gel digestion.** Protein samples were resolved on a 10.6% polyacrylamide gel and stained with ethyl violet-zincon (26). Gel spots of interest were excised, minced, and extensively processed by incubation with destaining solution (10% acetic acid, 30% ethanol) for at least 3 h. The gel samples were then dehydrated by acetonitrile, reduced by 10 mM DTT (45 min, 56°C), alkylated by 55 mM iodoacetamide (30 min, 25°C, in dark), and rehydrated by trypsin.
solution (12.5 ng/µl in 25 mM ammonium bicarbonate, 5 mM CaCl2, 45 min, on ice). Excess solution was removed, and gel samples were incubated overnight at 37°C. Peptides were extracted from the gel samples by incubation for 15 min in 25 mM ammonium bicarbonate and 15 min in 5% formic acid. Then, supernatants from all fractions of a single sample were pooled, dried using a SpeedVac, dissolved in 0.1% formic acid, and desalted on by ZipTip C18 column.

**Nanoelectrospray ionization-tandem mass spectrometry.** In-gel tryptic digests (4 µl) were applied to on-line nanoflow liquid chromatography (nLC) system EASY-nLC (Proxeon Biosystems, Odense, Denmark, now part of Thermo Fisher Scientific, Bremen, Germany) equipped with 10-cm capillary columns (internal diameter 75 µm) filled with 3 µm of Reprosil-Pur C18-A2 resin (Dr. Maisch, Ammersbach-Entringen, Germany). The flow gradient consisted of 10–30% acetonitrile in 0.1% formic acid delivered at a flow rate of 200 nL/min for 45 min, 30–100% acetonitrile in 0.1% formic acid delivered at a flow rate of 200 nL/min for 1 min, and 100% acetonitrile in 0.1% formic acid delivered at a flow rate of 200 nL/min for 10 min. The eluate was electrospayed through the Proxeon nano-ESI-MS/MS (nanoelectrospray ionization-tandem mass spectrometry) source to the LTQ Velos Pro dual-pressure linear ion trap mass spectrometer (Thermo Fisher Scientific). Using the full ion scan mode over the m/z range of 200–1800, collision-induced dissociation (CID) was performed in the linear ion trap using a 4.0-Th isolation width and 35% normalized collision energy with helium as the collision gas. Five dependent MS/MS scans were performed on each ion using dynamic exclusion. In addition, the precursor ion that had been selected for CID was dynamically excluded from further MS/MS analysis for 30 s.

**Data processing and database search.** The MS/MS spectra were processed using Proteome Discoverer software (version 1.3; Thermo Fisher Scientific, Waltham, MA). The Swiss-Prot protein sequence database (release 54.5; taxonomy selection *Mus musculus*) was searched using a concatenated forward-decoy sequence approach by the Mascot search engine (Matrix Science Mascot 2.3). The search parameters were 1.5 Da mass error tolerance for precursor ions, 0.8 Da mass error tolerance for fragment ions, carbamidomethylation (C) as the fixed modification, oxidation (M) and nitration (Y, W) as the variable modifications, 2 as the number of missed cleavages, P < 0.05 as the significance threshold, and ESI-TRAP as the type of instrument.

Protein identifications were validated only if they satisfied the following three requirements: 1) having a score that was significant (P < 0.05) with cut-off criteria, 2) one peptide having a score of >35 or two peptides having a score of >30, and 3) being identified in at least two of the three runs. Proteins that were identified by a set or subset of peptides used for identification of another protein were not taken into account. By integrating the m/z values in ion chromatograms with high-performance liquid chromatography retention times in chromatogram form, the peak area for each site was obtained. Relative quantification was obtained by comparing peak area of nitrated to nitrated plus nonnitrated peptides.

To analyze the important role of Tyr76, Trp374, and Tyr135 sites, a 3-D model of mouse SCOT was created with AutoDock 4.0 software.
using the 3-D structure of 3OXO chain A, which shares 84.6% identities with our query sequences, as template.

SCOT catalytic activity measurement. SCOT catalytic activity was measured spectrophotometrically by monitoring the absorbance changes at 313 nm at room temperature, which was an indicator of acetocetyl-CoA formation. The reaction mixture consisted of 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 0.2 mM succinyl-CoA, 0.1–10 mM lithium acetocetate, and 4 mM iodoacetamide. The catalytic reaction was initiated by addition of 300 µg of protein. The absorbance changes were recorded from 0 to 3 min (17, 32). The activity of SCOT was calculated as percentage of the control.

Statistical analyses. Statistical calculations were performed with Prism 5 software (GraphPad, San Diego, CA). All data are expressed as means ± SD. Comparison between two groups was performed by t-test. Comparisons among multiple groups were performed by one-way analysis of variance (ANOVA) as well as by two-way ANOVA. Statistical significance was considered when the P value of ≤0.05.

RESULTS

Diabetes-induced cardiac structural alterations and prevention by MT. Three days of STZ treatment successfully induced diabetes in both MT-TG and WT mice. At 8–16 wk after diabetes onset, H&E staining revealed that the hearts of WT diabetic mice had significantly more histopathological abnormalities than those of the MT-TG diabetic mice (Fig. 1A). The cardiac abnormalities included derangement of the myocardial structure, myofibrillar discontinuation, and foci showing necrotic cell death and fibrosis (Fig. 1A). Sirius red staining confirmed that the significant increase of fibrosis occurred in only the hearts of WT diabetic mice (Fig. 1B). In addition, the cardiac fibrosis in WT diabetic mice was further confirmed by Western blot detection of the profibrotic markers, TGFβ1 (Fig. 1F) and CTGF (Fig. 1G), which were absent in the MT-TG mice. These results are consistent with our previous studies that showed the significantly different structural abnormalities, accompanied by cardiac dysfunction, in WT diabetic mice but not in MT-TG diabetic mice (4, 5, 30).

MT inhibition of diabetes-induced superoxide and 3-NT generation in the heart. To determine the association of oxidative and nitrosative stress with diabetes-induced pathological changes, superoxide and 3-NT generation were assessed by fluorescent staining and Western blot, respectively. As shown in Fig. 1C, increased superoxide generation was detected in the hearts of WT diabetic mice at week 8 after induction and was absent in the hearts of MT-TG diabetic mice.

Since superoxide interacts with nitric oxide to form peroxynitrite, we measured 3-NT accumulation as an index of protein nitration. Western blotting revealed a significant increase in 3-NT accumulation in the hearts of WT diabetic mice (at weeks 2, 4, and 8 postinduction), which were located predominantly in the groups of proteins with molecular masses of ~58 kDa and were close to the sizes observed in our previous study (4). The 3-NT accumulation in the hearts of WT diabetic mice was highest at weeks 4–8 (Fig. 1D). Therefore, we compared 3-NT accumulation in the hearts of WT and MT-TG diabetic mice at week 8 and detected no 3-NT accumulation in the hearts of MT-TG diabetic mice (Fig. 1E).

2-DE analysis of nitrated proteins. Figure 2, A–C, shows representative 2-DE gels of heart proteins from nondiabetic

<table>
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<tr>
<th>Start</th>
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<th>Nitrated Site</th>
<th>m/z</th>
<th>Retention Time</th>
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</table>

Letters in boldface are the nitrated residue (amino acid): tyrosine amino group of Tyr(Y) and tryptophan amino group of Trp(W). SCOT, succinyl-CoA:3-ketoacid CoA transferase-1. Peptide identifications were accepted at 1.5 Da precursor-ion mass accuracy and >95% probability as determined by the Mascot algorithm.

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WT mice, diabetic WT mice at week 8 postinduction, and diabetic MT-TG mice at week 8 postinduction. No significantly different changes were observed among the three groups. However, 3-NT antibody Western blot analysis of the 2-DE gels of heart proteins from the nondiabetic WT mice (Fig. 2D), diabetic WT mice at week 8 postinduction (Fig. 2E), and diabetic MT-TG mice at week 8 postinduction (Fig. 2F) showed that the numbers and levels of nitrated proteins in the basic region of gels were significantly higher for the diabetic WT mice. In contrast, only slight differences in the extent of nitration were observed between the nondiabetic WT mice and the diabetic MT-TG mice at week 8 postinduction.

Identity of nitrated proteins. Considering the previously identified nitrated proteins in diabetic hearts (11, 15, 18, 26, 27, 32), the proteins that showed significant differences in nitrination levels in the 2-DE gels of heart proteins from WT and MT-TG mice were further characterized by tryptic digestion and LC-MS/MS analysis. The MS/MS spectra for the nitrated and unnitrated peptides shown in Fig. 4 (A–L) were used to identify the nitrated amino acids and their positions. The identified nitrated peptide sequences are listed at the top of the top panel for each peptide.
MT prevents nitrination of SCOT and preserves its catalytic activity. Figure 3A shows the Western blot detection of SCOT protein in the diabetic hearts at various times after diabetes induction. The diabetic WT mice showed only a slight decrease in SCOT cardiac expression at weeks 4–8 postinduction. Figure 3B shows the anti-SCOT antibody Western blot detection of anti-3-NT immunoprecipitants. The diabetic WT mice showed significantly increased nitrated SCOT in the cardiac tissue at week 8 postinduction, which was distinct from the results in the diabetic MT-TG mice at week 8 postinduction.

To determine whether the nitrated SCOT retains its catalytic function, SCOT catalytic activity was compared between the WT and MT-TG mice. SCOT catalytic activity was significantly reduced in the hearts of diabetic WT diabetic mice at weeks 4–12 postinduction, with the lowest level observed at week 4 postinduction (Fig. 3C). In contrast, SCOT catalytic activity was significantly reduced in the hearts of diabetic MT-TG mice only at week 4 postinduction (P < 0.05 vs. age-matched nondiabetic MT-TG mice).

Identification of and effect of MT on the SCOT nitrated sites. MS analysis of the electroelution-enhanced SCOT identified five nitrated tyrosines (Tyr76, Tyr117, Tyr135, Tyr226, and Tyr368) and one nitrated tryptophan (Trp374), as evidenced by a +45 mass unit shift (the typical feature of NO modification (14)) and presented in Table 1. Distinct peak profiles existed for the nitrated and nonnitrated Tyr76, Tyr135, and Trp374 residues (Fig. 4A) for the cardiac samples from diabetic WT mice at week 8 postinduction. Diabetes induced quantitative increases in Tyr76 nitration at weeks 4–8 postinduction and in Trp374 nitration at week 4 postinduction but induced a consistent quantitative decrease in Tyr135 nitration from weeks 2–8 postinduction (Fig. 5). Overexpression of cardiac MT led to significantly reduced Trp374 and Tyr135 nitration (Fig. 5). The 3-D modeling revealed that Tyr76 and Trp374 are located at the edge of the active site pocket (Fig. 6), indicating that these two sites play an important role in SCOT activity.

Collectively, these results suggested that the observed loss of SCOT activity might be predominantly related to increased Tyr76 and Trp374 nitration and that MT protection against diabetes-inhibited SCOT activity might be related to the prevention of diabetic Trp374 nitration in SCOT.

DISCUSSION

The relationship between diabetic complications and oxidative and/or nitrosative stress is believed to be related to increased superoxide generation and 3-NT accumulation (2, 16). In this study, we confirmed that high levels of superoxide and 3-NT accompany development of cardiac remodeling in the late stage of diabetes. Two to eight weeks after induction of diabetes, both superoxide generation and 3-NT accumulation were significantly evident, but the event had disappeared at 16 wk after induction. This finding may indicate that both oxidative and nitrosative stress play important roles in initiating the cardiac pathological changes at the early stages of diabetes and that nitrosative stress does not contribute substantially at the later stages.

Reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) are generated under various physiological and pathological conditions. Therefore, under normal conditions, ROS, including superoxide and hydrogen peroxide, may not damage biomacromolecules. However, when superoxide reacts with nitric oxide to form high levels of RNS, such as peroxynitrite, damage to proteins, lipids, and DNA may occur (22). Among the effects caused by peroxynitrite, protein nitration has attracted a great deal of research attention because it may cause dysfunction of the target protein (33) and promote disease-related complications such as those of diabetes (18, 21, 31).

MT is a promising protective agent of diabetes-induced pathological changes. Importantly, diabetes patients with polymorphisms in the MT gene are more susceptible to the development of various diabetic complications (10, 34). We considered that MT protection from diabetes-induced pathological

Fig. 5. Relative quantification of nitrated to total Tyr76, Tyr135, and Trp374 expression. A: enhanced nitrated Tyr76 in diabetic WT and MT-TG mice at weeks 4 and 8 postinduction. B: at week 4 postinduction, Tyr135 nitration was relatively high in diabetic mice, and MT averted the nitration. C: at week 4 postinduction, enhanced nitrated Trp374 was present in diabetic WT mice, and MT averted the enhancement at weeks 4 and 8 postinduction. Data are presented as means ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001.
changes might be attributed to its antioxidant function, theoriz-
ing that it might reduce nitrosative damage, based on our
previously published data showing that diabetic MT-TG mice
had significantly less 3-NT accumulation in the heart at 2–4 wk
of diabetes (4). In the present study, we have further demon-
strated the significant increase in 3-NT accumulation in the
hearts of diabetic WT mice (at weeks 2, 4, and 8 of diabetes),
which were located predominantly in the groups of proteins
with molecular masses of ∼58 kDa and which was similar to
the results of our previous study (4). The observation that the
3-NT accumulation in the hearts of diabetic WT mice (at
week 8 of diabetes) was not significantly different from that in
the age-matched nondiabetic controls further suggested that
MT protects the heart from diabetes-induced 3-NT accumula-
tion at the early (2 wk) stage of disease.

Comparison of protein nitration profiles in the hearts of
diabetic mice with and without overexpression of the MT gene
allowed us to uncover which nitrated proteins might play
critical roles in the development of diabetic cardiomyopathy
and the MT protective mechanism. One of these differentially
nitrated proteins was identified as SCOT, a key enzyme in
ketone body conversion into energy that occurs in extrahepatic
organs (32). Under the diabetic condition, glucose is not
effectively absorbed, leaving the ketone body as the main
energy source. When SCOT is nitrated, its structure is changed,
which impacts the protein function (12, 31).

The first reports of SCOT nitration involved heart and
kidney tissues of STZ- and endotoxin-treated rats, with detec-
tion carried out by staining with an anti-3NT antibody (17, 27).
In those two studies, the SCOT nitration level was found to be
increased, but the SCOT activity level was found to be de-
creased, and this observation held true for both the heart and
kidney. The decreased activity was ascribed to nitration, since
the total expression of SCOT protein was unchanged. How-
ever, in subsequent studies, age-related changes in the amount
of SCOT protein and nitration content were found to be
significantly different in the kidney and heart (1, 21). In partic-
ular, the amount of SCOT in the heart remained un-
changed with age, although the nitration level was significantly
increased with age (21). In the kidney, the total amount of
SCOT was decreased with age, whereas the nitration level of
SCOT remained unchanged with age (1).

In support of these previous findings, we have demonstrated
in the present study that MT prevents SCOT nitration and also
preserves SCOT activity under diabetic conditions. Some pre-
vious studies have also suggested that diabetic nitration of
SCOT may play an important role in the development of
diabetic cardiomyopathy (4, 21). However, an appropriate
animal model was not available to show whether or not
preventing SCOT nitration would significantly prevent patho-
genic progression.

In the present study, SCOT was found to be significantly
nitrated in the hearts of diabetic WT mice at week 8 postin-
duction but not in the hearts of age-matched diabetic MT-TG
mice, which indicates that MT prevention of diabetic cardio-
myopathy may be mediated by suppression of SCOT nitration
and is consistent with the indications of a previous study (6). In
addition, Wang et al. reported that a +45 mass unit ion shift
(−NO2) occurred at Tyr4 and Tyr76 of SCOT in cardiac tissues
from the db/db mouse model (8). Furthermore, the authors
employed a human recombinant SCOT to further determine
that both of these Tyr sites were readily nitrated upon incuba-
tion with peroxynitrite. In another study, when both Tyr4 and
Tyr76 were mutated in vitro, the recombinant SCOT became
resistant to peroxynitrite-induced nitration while maintaining
its catalytic activity (31). The current study investigated the in
vivo situation and demonstrated that cardiac SCOT in diabetic
mice was nitrated at Tyr76, Tyr135, and Trp374 residues. Rela-
tive quantification further revealed that, as diabetes progressed
in STZ-induced mice, the nitrated Tyr76 and Trp374 levels
increased, with a particularly robust increase occurring at week
4 after induction. Considering these novel in vivo results in
conjunction with the in vitro results from Wang et al. (31), we
theorized that the observed SCOT activity loss was due to
Tyr76 and Trp374 nitration.

Furthermore, in the current study, 3-D modeling of the
SCOT protein indicated that both Tyr76 and Trp374 are located
at the edge of the active site pocket. This novel finding
supports the theory that Tyr\textsuperscript{76} and Trp\textsuperscript{374} play important roles in determining SCOT activity, and which complements the finding by Wang et al. that Tyr\textsuperscript{76} determines the activity of recombinant human SCOT (31). However, when the diabetic WT and MT-TG mice were compared in the present study, the results did not indicate a determinant role for Tyr\textsuperscript{76} nitration in the induction of diabetes-induced pathological changes. Therefore, we propose that MT protection of diabetes-induced pathological changes is related predominantly to the prevention of Trp\textsuperscript{374} nitration, according to our novel observation that MT substantially attenuates Trp\textsuperscript{374} nitration in diabetic mice at weeks 4 and 8 postinduction.

In summary, during the development of diabetes, oxidative stress can cause SCOT nitration at Tyr\textsuperscript{76} and Trp\textsuperscript{374}, along with induction of several pathological changes in the heart. MT prevention of diabetes-induced SCOT nitration at Trp\textsuperscript{374} through its antioxidant function is most likely the principal mechanisms responsible for its prevention of diabetes-induced pathological changes in the heart. However, further study of its expression, using in vitro expression systems and site mutation, are needed to verify the important role of SCOT and its nitrated sites.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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