Diabetes-associated nitration of tyrosine and inactivation of succinyl-CoA:3-oxoacid CoA-transferase

ILLARION V. TURKO,1 SISI MARCONDES,1 AND FERID MURAD1,2

1Department of Integrative Biology and Pharmacology, University of Texas Houston Medical School and 2Institute of Molecular Medicine, Houston, Texas 77030

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Turko, Illarion V., Sisi Marcondes, and Ferid Murad. Diabetes-associated nitration of tyrosine and inactivation of succinyl-CoA:3-oxoacid CoA-transferase. Am J Physiol Heart Circ Physiol 281: H2289–H2294, 2001.—High levels of reactive species of nitrogen and oxygen in diabetes may cause modifications of proteins. Recently, an increase in protein tyrosine nitration was found in several diabetic tissues. To understand whether protein tyrosine nitration is the cause or the result of the associated diabetic complications, it is essential to identify specific proteins vulnerable to nitration with in vivo models of diabetes. In the present study, we have demonstrated that succinyl-CoA:3-oxoacid CoA-transferase (SCOT; EC 2.8.3.5) is susceptible to tyrosine nitration in hearts from streptozotocin-treated rats. After 4 and 8 wk of streptozotocin administration and diabetes progression, SCOT from rat hearts had a 24% and 39% decrease in catalytic activity, respectively. The decrease in SCOT catalytic activity is accompanied by an accumulation of nitrotyrosine in SCOT protein. SCOT is a mitochondrial matrix protein responsible for ketone body utilization. Ketone bodies provide an alternative source of energy during periods of glucose deficiency. Because diabetes results in profound re-arrangements in myocardial substrate utilization, we suggest that SCOT tyrosine nitration is a contributing factor to this impairment in the diabetic heart.

STUDIES THAT ASSOCIATE DIABETES WITH IMPAIRED MITOCHONDRIAL FUNCTIONS PROVIDE EVIDENCE OF ALTERED FREE RADICAL STATUS IN MITOCHONDRIA ISOLATED FROM DIABETIC TISSUES (6, 23, 27, 30, 33, 34). NITRIC OXIDE (NO) REGULATES MITOCHONDRIAL FUNCTIONS BY SEVERAL DISTINCT MECHANISMS THAT DEPEND ON NO CONCENTRATION AND REPRESENT EITHER REVERSIBLE OR IRREVERSIBLE EFFECTS (FOR RECENT REVIEWS, SEE REFs. 3, 4, 11, AND 16). IRREVERSIBLE EFFECTS ARE MOSTLY ASSOCIATED WITH PRODUCTION OF PEROXYNITRITE (22), A POTENT OXIDANT THAT IS CAPABLE OF OXIDIZING-NITRATING PROTEINS, LIPIDS, AND DNA (7). ONE POSSIBLE PRODUCT OF ITS REACTION WITH PROTEINS IS 3-NITROTYROSINE (2). IT HAS BECOME RECOGNIZED THAT PROTEIN TYROSINE NITRATION ALTERS THE STRUCTURE AND FUNCTION OF PROTEINS (19) AND MAY ALSO PREVENT TYROSINE PHOSPHORYLATION (21). PEROXYNITRITE IS FORMED BY THE REACTION OF NO AND SUPEROXIDE (22). THE MITOCHONDRIAL RESPIRATORY CHAIN IS A MAJOR SOURCE OF SUPEROXIDE. CONSIDERING THE RECENT EVIDENCE FOR A MITOCHONDRIAL NO SYNTHASE (12, 14), IT IS BECOMING APPARENT THAT INTRAMITOCHONDRIAL PEROXYNITRITE FORMATION (13, 38) CAN TRIGGER MITOCHONDRIAL DYSFUNCTION. THE PRODUCTION OF PEROXYNITRITE AND INCREASED PROTEIN TYROSINE NITRATION HAS BEEN RECENTLY REPORTED IN SEVERAL DIABETIC TISSUES (17, 20, 25, 29, 32, 35, 36). HOWEVER, NEITHER PROTEIN TARGETS OF NITRATION NOR FUNCTIONAL CONSEQUENCES OF PROTEIN NITRATION IN DIABETIC CONDITIONS WERE DESCRIBED.

RECENTLY, WE FOUND THAT THE MITOCHONDRIAL PROTEIN SUCINYL-COA:3-OXOACID COA TRANSFERASE (SCOT) UNDERGOES TYROSINE NITRATION IN THE RAT HEART AND KIDNEYS FOLLOWING ENDOTOXIN ADMINISTRATION (26). SCOT IS THE RATE-DETERMINING ENZYME IN KETOLYSIS. KETOLYSIS OCCURS IN THE MITOCHONDRIA OF MANY EXTRAHEPATIC ORGANS AND IS THE PROCESS OF KETONE BODY ACETOACETATE CONVERSION TO ACETYL-COA (24). DURING PERIODS OF LIMITED AVAILABILITY OF CARBOHYDRATES OR WHEN CARBOHYDRATES CANNOT BE USED EFFECTIVELY, KETOGENESIS ENABLES FAT-DERIVED ENERGY TO BE USED BY SUCH ORGANS AS THE HEART, KIDNEY, SKELETAL MUSCLE, AND BRAIN. THE IMPAIRED UTILIZATION OF KETONE BODIES IN DIABETES (18, 28) AND DECREASED SCOT ACTIVITY (8, 15) HAVE BEEN REPORTED, BUT THE MOLECULAR MECHANISM(S) UNDERLYING THIS IMPAIRMENT HAS NOT BEEN DESCRIBED.

WE THEREFORE EVALUATED SCOT NITRATION IN DIFFERENT TISSUES FROM DIABETIC AND CONTROL RATS. FURTHER STUDIES PRESENTED HEREIN ADDRESSED CHANGES IN SCOT CATALYTIC ACTIVITY AS WELL AS ITS CORRELATION WITH SCOT NITRATION IN THE HEART AND KIDNEY. THE DATA SUPPORT THE VIEW THAT TYROSINE NITRATION OF SCOT MIGHT EXPLAIN THE DECREASED SCOT CATALYTIC ACTIVITY IN THE DIABETIC HEART.

MATERIALS AND METHODS

Animals and treatment. Male Sprague-Dawley rats (median body weight 250 g) were from Harlan (Indianapolis, IN). Animals were maintained on standard rat chow and tap water ad libitum. Streptozotocin (STZ; Sigma) was dissolved in sterile 0.1 M citrate buffer (pH 4.5) and injected intraperitoneally at a dose of 60 mg/kg body wt. Control rats received the corresponding volume of citrate buffer. The blood glucose levels were monitored using Accu-Chek Advantage blood glucose monitor and test strips (Boehringer-Mannheim; Indianapolis, IN). The STZ-
treated rats that showed fasting blood glucose levels above 350 mg/dl, in addition to loss of weight and polyuria, were considered as having insulin-dependent diabetes mellitus. Diabetic rats and age-matched control rats were euthanized at 1, 4, or 8 wk after STZ injection.

**Tissue extracts.** Frozen tissues were homogenized in 50 mM sodium phosphate buffer (pH 7.2)-0.15 M NaCl containing protease inhibitors (100 µM benzamidine, 0.005 U/ml aprotinin, 20 µM leupeptin, 15 µM pepstatin A, and 8 µM antipain). Homogenates were centrifuged for 10 min at 1,500 g, and the supernatant fractions were centrifuged again for 60 min at 100,000 g. The supernatant fractions from the high-speed centrifugation were considered to contain the total soluble protein of the tissue (i.e., cytoplasm and mitochondrial matrix).

**Preparation of mitochondria.** Mitochondria from the heart and kidney were isolated using differential centrifugation. Fresh tissues from control and STZ-treated rats were minced with scissors and homogenized in a glass homogenizer with a motor-driven Teflon pestle in 10 mM phosphate buffer (pH 7.2)-0.5 mM EDTA-0.25 M sucrose. After centrifugation at 750 g for 10 min, the supernatant fractions were centrifuged for 10,000 g for 20 min. Pellets were washed twice with 10 mM phosphate buffer (pH 7.2)-0.5 mM EDTA-0.25 M sucrose. After the final wash, mitochondria were resuspended in 50 mM phosphate buffer (pH 7.2), and aliquots were quickly frozen in liquid nitrogen.

**Catalytic activity of SCOT.** Mitochondria were disrupted by sonication in 50 mM sodium phosphate buffer (pH 7.2)-0.15 M NaCl containing protease inhibitors (100 µM benzamidine, 0.005 U/ml aprotinin, 20 µM leupeptin, 15 µM pepstatin A, and 8 µM antipain). The disrupted mitochondria were centrifuged for 60 min at 100,000 g. The supernatant fractions from the high-speed centrifugation were considered to contain the total soluble protein of the mitochondria and were used for measurements of the SCOT catalytic activity. SCOT catalytic activity was measured using the assay procedure described by Williamson et al. (39). Briefly, the incubation mixture contained (in mM) 50 Tris-HCl (pH 8.0), 0.1 succinyl-CoA, 0.2 acetoacetate, 5 MgCl₂, 4 iodoacetamide, and high-speed supernatant fractions (50–100 µg of total protein). SCOT catalytic activity was measured spectrophotometrically by following the formation of acetoacetoy-CoA (the forward direction) at 313 nm. SCOT catalytic activity was normalized to the total protein in high-speed supernatant fractions, because Western blot analysis of these fractions with anti-SCOT antibodies revealed similar amounts of SCOT.

**Western blot analysis.** After separation by reducing SDS-PAGE on 10% gels, proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad), which were blocked with 5% nonfat milk in 20 mM Tris·HCl (pH 7.2)-0.3 M NaCl-0.1% Tween 20 (TBS/T). To detect nitrotyrosine-containing proteins, the membranes were incubated with 1:2,000 dilution of mouse monoclonal anti-nitrotyrosine antibodies (1 mg/ml, Upstate Biotechnology). Rabbit polyclonal anti-SCOT antibody was generated against the KGP-KFEKRIERL peptide (BioSource International; Hopkinton, MA) and was used (1:5,000 dilution of serum) to detect SCOT. After being washed with TBS/T, immunoreactive proteins were detected using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

**Nitrotyrosine immunoprecipitation.** High-speed supernatant fractions (1 mg) from heart mitochondria of the 8-wk diabetic rats were precleared with 20 µl of protein A/G Plus-Agarose (Santa Cruz Biotechnology) for 1 h at 4°C. The mixture was centrifuged to pellet the beads, and the supernatant fraction was incubated with 3 µg of mouse monoclonal anti-nitrotyrosine antibody (Upstate Biotechnology) for 16 h at 4°C. Immune complexes were precipitated with 20 µl of protein A/G Plus-Agarose by rotating the suspension for 1 h at 4°C. After centrifugation, the beads were washed twice with TBS/T. The beads were finally resuspended in sample loading buffer containing SDS and 2-mercaptoethanol, and the supernatant fraction was resolved by SDS-PAGE. Western blot analysis was performed using the rabbit anti-SCOT serum.

**Determination of TBARS in mitochondria.** To measure thiobarbituric acid-reactive substances (TBARS), mitochondria were disrupted by sonication. A mitochondrial suspension of 300 µl (4 mg of total protein/ml) was mixed with 100 µl of a 10% solution of polyoxyethylene ester W-1 (nondetergent), 100 µl 75% trichloroacetic acid, and 500 µl of an aqueous solution containing 0.6% 2-thiobarbituric acid. The reaction mixture was heated in boiling water for 30 min and centrifuged, and the absorbance was measured at 532 nm against a reaction mixture blank. The amounts of TBARS were calculated using malondialdehyde bis(dimethyl acetal) as a standard.

**Protein concentration.** Total protein concentrations were determined using the Bio-Rad Protein Assay with bovine serum albumin as the standard.

**Statistical analysis.** The results were analyzed using unpaired Student’s t-test or, where applicable, ANOVA followed by Bonferroni’s modified t-test. P values < 0.05 were considered statistically significant. Data are presented as means ± SE.

**RESULTS**

**SCOT nitration in normal and diabetic rat tissues.** Figure 1A shows the immunodetection of SCOT and tyrosine-nitrated SCOT in high-speed supernatant fractions from normal and diabetic rat tissues 8 wk after STZ administration. Our data are consistent with those previously published (10, 39) and show the highest level of SCOT protein exists in the heart and

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**Fig. 1.** Western immunoblots using anti-succinyl-CoA:3-oxoacid CoA-transferase (SCOT) and anti-nitrotyrosine antibodies. A: 20 µg of high-speed supernatant fractions from various normal [streptozotocin (STZ)−] and 8-wk diabetic (STZ+) rat tissues were loaded per lane. Proteins transferred to polyvinylidene difluoride (PVDF) membrane were analyzed with rabbit polyclonal anti-SCOT antibody or with monoclonal anti-nitrotyrosine antibody. B: mitochondrial high-speed supernatant fraction from 8-wk diabetic heart (lane 1) and a protein that was immunoprecipitated with monoclonal anti-nitrotyrosine antibody from the same supernatant fraction (lane 2). Proteins transferred to PVDF membrane were analyzed with rabbit polyclonal anti-SCOT antibody.
kidney. An important observation for these two tissues is that levels of SCOT expression were not different between control and diabetic groups. Other tested tissues show either substantially less or barely detectable amounts of SCOT protein, and even longer exposures (not shown) did not reveal SCOT nitration in these tissues. Basal SCOT nitration was only detected in the heart and kidney. Furthermore, nitration of SCOT in the heart was clearly increased 8 wk after STZ treatment, whereas nitration of SCOT in the kidney remained unchanged. Figure 1B shows immunoprecipitation of nitrated SCOT from diabetic heart 8 wk after STZ administration using anti-nitrotyrosine antibody and following Western blot analysis of immune complexes using anti-SCOT antibody. Control experiments for nitrotyrosine immunoreactivity (26) were also performed by preincubating (30 min at room temperature) the anti-nitrotyrosine antibody with 4 mM free 3-nitrotyrosine or with 4 mM free l-tyrosine before use in Western blot analysis (data not shown). The combination of such negative and positive controls with immunoprecipitation data (Fig. 1B) indicates that the tyrosine-nitrated protein was indeed SCOT.

Figure 2 shows a time course of SCOT nitration in heart during progression of diabetes. An increase in nitration was observed at 4 wk after STZ treatment and increased further after 8 wk. In contrast, the amount of SCOT protein in heart samples remained unchanged.

**SCOT catalytic activity.** Basal nitration of SCOT was found in both the heart and kidney, but the diabetes-associated increase in SCOT nitration was found only in the heart (Fig. 1A). We measured SCOT catalytic activity in mitochondrial high-speed supernatant fractions from both tissues to understand whether an increase in tyrosine nitration may affect the activity of SCOT. Figure 3 shows that SCOT catalytic activity was significantly lower in mitochondria from diabetic hearts after 4 and 8 wk of STZ treatment. It was decreased 24% after 4 wk (111 ± 2.0 nmol·min⁻¹·mg⁻¹ in diabetic group vs. 147 ± 3.8 nmol·min⁻¹·mg⁻¹ in age-matched control group) and decreased 39% after 8 wk (102 ± 6.0 nmol·min⁻¹·mg⁻¹ in diabetic group vs. 167 ± 0.7 nmol·min⁻¹·mg⁻¹ in age-matched control group). The activity of SCOT in kidney did not statistically differ between control and diabetic groups at any time selected (Fig. 3).

**Mitochondrial lipid peroxidation.** Protein tyrosine nitration and lipid peroxidation are both free radical-mediated chemical processes. As a measure of lipid peroxidation, the thiobarbituric acid test was used. This test estimates the amount of malondialdehyde precursors, including hydroperoxides and endoperoxides. Figure 4 shows the formation of TBARS in the heart and kidney mitochondria from control and diabetic tissue samples 4 and 8 wk after STZ administration. A small significant decrease in TBARS (10–16% compared with controls, with \( P \) in the range from 0.031 to 0.046) was observed for the samples from the diabetic heart and kidney. This decrease was approximately the same after 4 or 8 wk of STZ administration.

**DISCUSSION**

The chemical modification of proteins in diabetes has been recently reviewed (1). An important issue raised is whether oxidative damage to proteins may cause a progression of the disease or it is merely a secondary indicator reflecting the tissue damage and the presence of complications. Although oxidative protein modifica-
tion has been implicated to be a deleterious component of diabetes, little is known about the nature and extent of the protein modification or about the mechanisms linking these modifications to the pathogenic process. Formation of 3-nitrotyrosine is a marker or index of increased generation of reactive nitrogen and oxygen species. Diabetes-associated formation of tyrosine-nitrated proteins was observed in pancreatic islet β-cells (32) and in the diabetic placenta (25), platelets (35), kidney (20, 36), and orbit (29). However, specific protein targets remain to be identified. Obviously, identification and functional studies of proteins that undergo nitration in diabetic conditions is necessary.

To our knowledge, this is the first study to identify the increase of tyrosine nitration of a specific protein in diabetes. We found that the increase in SCOT tyrosine nitration in the heart of STZ-treated rats (Fig. 2) is accompanied by the decrease in SCOT catalytic activity (Fig. 3). These data may explain the earlier observation (15) that SCOT catalytic activity is diminished ~50% in the rat heart 2 mo after STZ administration. Despite some basal SCOT nitration in the kidney, diabetes progression was not accompanied by increased SCOT nitration (Fig. 1), and SCOT catalytic activity in the kidney remained unchanged (Fig. 3).

Fenselau and Wallis (8) also found a decrease in SCOT catalytic activity in rat skeletal muscle following STZ administration. We did not observe SCOT nitration in the skeletal muscle, but we did observe a decrease in SCOT protein per milligram of soluble mitochondrial protein after STZ administration (Fig. 1). This may be an explanation of the data of Fenselau and Wallis (8).

SCOT is located in the mitochondrial matrix. Taking into account the sources of NO and superoxide in the mitochondria and the higher solubility and stability of reactive nitrogen and oxygen species in the hydrophobic phase, mitochondria could be a favorable place for nitrotyrosine producing reactions under conditions when prooxidative forces take over antioxidant defenses. This may also promote lipid peroxidation in mitochondria. Published data on the mitochondrial lipid peroxidation in diabetic rats are incomplete and conflicting. It has been suggested that the level of lipid peroxidation increases (37), remains the same (9), or decreases (33). Because the activity of antioxidant enzymes, availability of nonenzymic antioxidants, and unsaturation of lipids vary broadly in diabetes, a part of the confusion is probably due to different conditions used: mitochondria were from different tissues and at various stages of development of diabetes. We observed significant decreases in the level of mitochondrial lipid peroxidation (Fig. 4) in the heart and kidney of diabetic versus age-matched control rats after 4 and 8 wk of diabetes. An important issue is that mitochondrial lipid peroxidation did not correlate with SCOT nitration under conditions used in the present study. The in vivo nitrating agent(s) is also unresolved (reviewed in Ref. 19). Meanwhile, peroxynitrite remains a possible candidate. Peroxynitrite formation can result in mitochondrial lipid peroxidation; however, it should be noted that other agents may also be involved. The absence of a simple correlation between SCOT nitration and lipid peroxidation in the diabetic mitochondria may imply that these processes require activation of several pathways.

SCOT catalyzes the formation of acetoacetyl-CoA from acetoacetate. This is the rate-determining step of ketone body conversion into acetyl-CoA, which subsequently enters the citric acid cycle. Cardiac muscle is able to produce energy from a wide range of substrates.

Fig. 4. Levels of lipid peroxidation during the progression of diabetes. Thiobarbituric acid-reactive substances (TBARS) were measured in sonicated mitochondria from the heart (A) and kidney (B) of diabetic and normal age-matched rats. Data are means ± SE (expressed as pmol TBARS formed/mg total protein); n = 6 rats in each group. *P values are 0.046 and 0.040 for 4- and 8-wk heart and 0.031 and 0.033 for 4- and 8-wk kidney, respectively, and are significantly lower than the controls.

Fig. 5. Schematic representation of the pathways of myocardial substrate metabolism.
(Fig. 5), including fatty acids, glucose, lactate, and ketone bodies. Accumulating evidence has implicated changes in myocardial energy substrate use as contributing to diabetes-associated cardiomyopathies (reviewed in Ref. 31). Hearts from animals with experimental diabetes have been shown to have impaired myocardial glucose and lactate uptake and a greater use of free fatty acids (Fig. 5). The myocardial citric acid cycle does not appear to be altered in diabetes (5, 27, 31). Our finding that SCOT undergoes tyrosine nitration and shows lower catalytic activity in the diabetic heart is consistent with the shift in the source of acetyl-CoA for the citric acid cycle in diabetic hearts (reviewed in Ref. 31).

In summary, we have described for the first time that a specific protein, SCOT, is tyrosine nitrated in the diabetic heart. Tyrosine-nitrated SCOT exhibits decreased catalytic activity and is probably an important contributing factor to the abnormalities in myocardial energy metabolism observed in diabetic heart.

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REFERENCES


