GLYCATION OF MITOCHONDRIAL PROTEINS FROM DIABETIC RAT KIDNEY IS ASSOCIATED WITH EXCESS SUPEROXIDE FORMATION

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Running title: Renal mitochondria in chronic diabetes

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Abstract

Chronic hyperglycemia causes structural alterations of proteins through the Maillard reaction. In diabetes, methylglyoxal (MGO)-induced hydroimidazolones are the predominant modification. In contrast to acute hyperglycemia, mitochondrial respiration is depressed in chronic diabetes. To determine if MGO-derived protein modifications result in abnormalities in mitochondrial bioenergetics and superoxide formation, proteomics and functional studies were performed in renal cortical mitochondria isolated from rats with 2, 6, and 12 mo of streptozotocin (STZ)-induced diabetes. MGO-modified proteins belonged to two pathways: 1) Oxidative phosphorylation, and 2) Fatty acid β-oxidation. Two of these proteins were identified as components of respiratory complex III, the major site of superoxide production in health and disease. Mitochondria from rats with diabetes exhibited a diminution of oxidative phosphorylation. A decrease in the respiratory complex III activity was significantly correlated with the quantity of MGO–derived hydroimidazolone present on mitochondrial proteins in both diabetic and control animals. In diabetes, isolated renal mitochondria produced significantly increased quantities of superoxide, and showed evidence of oxidative damage. Administration of aminoguanidine improved mitochondrial respiration and complex III activity, and decreased oxidative damage to mitochondrial proteins. Therefore, post-translational modifications of mitochondrial proteins by MGO may represent pathogenic events leading to mitochondria-induced oxidative stress in the kidney in chronic diabetes.

Key Words

methylglyoxal, nephropathy, complex III, proteomics
Introduction

Mitochondria are the major source of reactive oxygen species (ROS) in the cell due to continuous generation of superoxide—a by-product of electron transport (9). ROS generated by mitochondria are responsible for the activation of major, independent but interrelated, pathogenic mechanisms for diabetic complications as modeled in endothelial cells exposed to hyperglycemia in vitro (47).

Longstanding diabetes is associated with alterations in mitochondrial metabolism that result in both increased formation of ROS and failure of bioenergetics. In particular, diabetes causes dysfunction of mitochondria in those tissues highly dependent on aerobic metabolism such as heart (16), brain (27) and skeletal muscle (29). The degree of mitochondrial failure has been correlated with the duration of diabetes. Complexes I (66), III and IV of the electron respiratory chain (ERC) are the main mitochondrial targets of hyperglycemia-induced injury.

The development of cardiomyopathy in diabetes is accompanied by both changes in protein expression (67) and post-translational modifications of mitochondrial proteins (66). However, little is known about the nature of mitochondrial dysfunction in the kidney in diabetes, or the responsible structural modifications of mitochondrial proteins (26). In early stages of diabetic nephropathy, ultrastructural changes in renal proximal tubular mitochondria have been found to correlate with disturbances in the main functions of renal tubular cells (25) and typical features of diabetic nephropathy (46, 49). In addition there is ample evidence that oxidative stress is involved in the pathogenesis of diabetic nephropathy (33).

One important explanation for structural modifications of proteins in diabetes is the non-enzymatic glycation of proteins to form advanced glycation end products (AGEs)—the Maillard reaction (6, 44). A recent quantitative study showed higher levels of AGEs on intracellular proteins compared to plasma proteins, and identified dicarbonyl-induced hydroimidazolones as the predominant biomarker in diabetes (64). Inhibition of advanced glycation with aminoguanidine has been shown to attenuate the development and progression of renal injury in humans (5) and experimental diabetes (48, 59), without influencing glycemic control.

Chronic hyperglycemia is thought to alter mitochondrial function through glycation of mitochondrial proteins (26). Levels of methylglyoxal (MGO), a highly reactive α- dicarbonyl
by-product of glycolysis are increased in diabetes (3). MGO readily reacts with arginine, lysine and sulfhydryl groups of proteins (38) and nucleic acids (50), inducing the formation of a variety of structurally identified AGEs. As the major precursor of AGE formation, both in the cell and in the plasma (64), MGO can influence multiple aspects of cellular biology in diabetes (62).

Previous in vitro work in this laboratory demonstrated that MGO has an inhibitory effect on kidney mitochondrial respiration and that MGO-induced modifications are targeted to specific mitochondrial proteins (55). These observations led to the goal of this work—to identify the main mitochondrial protein targets of MGO, the alterations of mitochondrial bioenergetics, and the effect of reversing MGO-activity with aminoguanidine, in the diabetic kidney. The present study demonstrates that MGO-related posttranslational modifications of mitochondrial proteins involve two major mitochondrial pathways: 1) oxidative phosphorylation, and 2) fatty acid β-oxidation. These changes occur concomitantly with progressive decrements of mitochondrial bioenergetics, targeted to complex III, and with increases in the formation of superoxide by mitochondria. Furthermore, the administration of aminoguanidine improves mitochondrial respiration and diminishes oxidative damage to mitochondrial proteins in diabetes.
Materials and methods

Animals

Diabetes was induced by a single tail vein injection of 45 mg/kg of STZ (Sigma-Aldrich, St. Louis, Missouri, USA) in 0.1 mol/L sodium citrate buffer, pH 4.5 to 8 week old male Lewis rats weighing ~150g at the start of the study (Charles River, Wilmington, Massachusetts, USA). Control animals received a vehicle injection. All procedures were performed in compliance with the guidelines of the CWRU Institutional Animal Care and Use Committee. Rats were maintained throughout the study in specific pathogen-free conditions using sterilized cages, feed, and bedding. Animals with plasma glucose higher than 16 mmol/L were classified as diabetic. To maintain body weight and to limit hyperglycemia, 2 IU of ultralente insulin, (Humulin U; Eli Lilly, Indianapolis, Indiana, USA) was administered to diabetic animals 3 times per week in order to prevent ketosis and catabolism. Glycemia was monitored by monthly determination of plasma glucose (Trinder assay kit, Sigma), and measurement of glycated hemoglobin (boronate affinity chromatography (Sigma)) at 2, 6, and 12 months.

Protocol A: 2 and 12 mo- diabetes model. There were a total of four groups: control and STZ diabetes animals at 2 and 12 mo. 2-, and 12- mo time points were selected specifically to examine the alterations of mitochondrial function in the context of evolution of diabetes.

Protocol B: 6- mo diabetes experiment was performed with control-, experimental diabetic- and aminoguanidine treated diabetic rats. Aminoguanidine was administered in the diet (2.5 g/kg diet) as reported previously for inhibition of diabetic retinopathy (13). A 6- month time point was selected specifically to detect the alterations in mitochondrial bioenergetics induced by diabetes at a time point previously reported to attenuate albuminuria in diabetic rats (48, 59).

Materials

Except as noted all chemical reagents were purchased from Sigma-Aldrich. Lucigenin (bis- N-, ethylacridinium nitrate) was purchased from Molecular Probes (Eugene, Oregon, USA). The MGO-derived imidazole AGE antibody (1H7G5) was described previously (47). Other reagents were of the highest purity available.
Methods

Measurement of mitochondrial respiration

Renal mitochondrial isolation. Mitochondria were isolated from the kidneys of Lewis rats as described previously (55). The tissue was suspended in cold isolation buffer (0.25M sucrose, 2 mM EDTA and 25 mM Tris HCl, pH 7.4), homogenized, and subjected to differential centrifugation. The mitochondria were pelleted at 5000 X g for 10 minutes. The pellet was re-suspended to a final protein concentration of approximately 25 mg/ml in isolation buffer. Using this technique, mitochondria could be stored at 4°C for up to 5 hours without a change in state 3 or state 4 respiratory rates. Proteins were determined using a bicinchoninic acid method (BCA Protein Assay Kit, Pierce, Rockford, Illinois, USA).

Mitochondrial respiration. Respiration assays were performed using a polarographic Clark electrode (Instech Laboratories, Philadelphia, Pennsylvania USA). Reactions were conducted at 25°C in a 750 µL chamber containing 0.5 mg/mL mitochondrial proteins in a respiratory buffer of 125 mM KCl and 5 mM KH₂PO₄ at pH 7.25. State 2 respiration was initiated by adding NADH-linked substrates (10 mM glutamate and 5 mM malate). State 3 respiration was determined by adding 300 nmol ADP. State 4 respiration was defined as oxygen consumed in the presence of the adequate substrates after ADP exhaustion.

2,4-dinitrophenol (DNP)-dependent respiration. Renal mitochondrial ADP-dependent respiration might be altered by diabetes in one of two ways: through an effect on adenine nucleotide translocase and/or ATPase, or by suppressing TCA and ERC. To differentiate these possibilities, renal mitochondrial respiration was measured in the presence of 60 µM DNP. DNP increases O₂ consumption independent of ADP transport or ATP synthesis, but its effect is dependent on the supply of reducing equivalents and ERC components.

NADH oxidase. To determine the ability of the ERC to utilize exogenously added NADH, NADH oxidase was assayed using a protocol developed by Humphries et al (20, 21). Mitochondria were diluted to a protein concentration of 0.2 mg/mL with a hypotonic buffer (10 mM Mops and 0.5 mM EDTA, pH 7.4) and then sonicated for 30 seconds to provide access of NADH and cytochrome c to the ERC. O₂ consumption was monitored after the addition of 9 µM cytochrome c and 0.4 µM NADH.

ERC complexes. To further define the activity of NADH-linked ERC complexes, renal mitochondria are exposed to various experimental conditions (20, 21).
For analysis of complex I, mitochondria (0.5 mg/ml) were diluted to 50 µg/ml in a hypotonic buffer (25 mM KH₂PO₄, 0.5 mM EDTA) and then sonicated. Upon addition of 5 µM antimycin A, 2 mM KCN, 60 µM ubiquinone-1, and 100 µM NADH, consumption of NADH was monitored by spectrophotometer at 340 nm (molar absorptivity, ε = 6200 M⁻¹ cm⁻¹).

To measure complex III activity, mitochondria (0.5 mg/ml) were diluted to 2.5 µg/ml in hypotonic buffer and then sonicated. Upon addition of 40 µM reduced decylubiquinone, 50µM cytochrome c and 2mM KCN, the reduction of cytochrome c was measured at 550 nm (ε=18,500 M⁻¹ cm⁻¹). Reaction was started by the addition of cytochrome c.

Complex IV activity was assayed using an oxygen electrode to record the rate of oxygen consumption upon the addition of 5 mM ascorbate, 250 µM TMPD (N,N,N', N'- tetramethyl- P-phenylenediamine) and 10 µM cytochrome c to 100 µg mitochondrial protein, after sonication.

Detection of superoxide anion in isolated mitochondria

Lucigenin (bis-N-ethylacridinium nitrate) was used to measure superoxide anion production by the mitochondrial ERC (12). Reaction blanks (cuvettes containing all components except mitochondria) were measured first. Renal mitochondria (0.5 mg/mL) were sonicated in respiratory buffer and then diluted to 50 µg/mL in 1 mL of hypotonic buffer (10 mM Mops, 0.5 mM EDTA, pH 7.6) complemented with lucigenin 20 µM (Molecular Probes, Eugene, Oregon, USA). Mitochondrial respiration was started by the addition of 70 µM NADH, as a substrate initiating ERC activity (52). The luminometer (Lumat LB 9501, Berthold, Bundoora, Australia) was set to count every fifth second at 25°C. Rates of superoxide production were expressed as counts per second. Tiron (4,5-Dihydroxy-1, 3-benzene-disulfonic acid) 100 mM was added as a scavenger of superoxide (12, 35).

Western blot detection of post-translational modifications of mitochondrial proteins by oxidative pathway

One hallmark of the oxidation status of proteins is the immunodetection of carbonyl groups. An OxyBlot™ Protein Oxidation Kit (Intergen), was used to test for the presence of these moieties. The carbonyl groups in the mitochondrial proteins (7µg/µl) were derivatized to 2,4-dinitrophenylhydrazone (DNPH) by reaction with 2,4- dinitrophenylhydrazine (DNP). For negative controls, control solution was added instead of the DNPH solution. The DNP-
derivatized protein samples were separated by polyacrylamide gel electrophoresis under antioxidant conditions (NuPAGE Bis-Tris 4-12%, Invitrogen) followed by blotting onto the nitrocellulose membrane. The membrane was probed with a primary antibody (dilution 1:150), specific to the DNP moiety of the proteins. Binding of this antibody was detected by incubating the membrane with a 1:600 dilution of an anti rabbit IgG conjugated to horseradish peroxidase. After incubation with a chemiluminescent substrate, primary antibody bound to DNP moiety was observed by autoradiography. To ensure equal loading among lanes, the membranes were stained with Ponceau S (Sigma) for 3 minutes until the bands appeared, then washed three times in PBS-T buffer. The intensities of the bands were visually confirmed to be similar across the lanes.

**Western blot detection of specific post-translational modifications of mitochondrial proteins by the Maillard reaction**

Mitochondrial proteins were loaded at 14 µg/lane on duplicate 4-12% NuPAGE gels (Invitrogen, Carlsbad, California, USA). One gel of the duplicate was stained with Coomassie blue to assess the size distribution of mitochondrial proteins and to ensure equal loading among lanes. The second gel was electroblotted on a nitrocellulose membrane and probed with a monoclonal antibody to MGO-derived imidazole AGE (1H7G5) at 1:6000 (47). The specific sites of antibody binding were detected with a 1:40,000 dilution of anti-mouse IgG conjugated to HRP. After adding the chemiluminescent substrate (Pierce), MGO-modified proteins were observed by autoradiography. Densitometric evaluation of the blots was carried out using the VersaDoc imaging system and Quantity One software (BioRad).

**Immunoprecipitation of mitochondrial proteins**

Fifty µL of mitochondrial pellet was resuspended in 900 µL of lysis buffer (20 mM Tris pH 8.0, 1 mM EDTA, 200 mM NaCl and 1% Triton X100) sonicated and ultracentrifuged at 150,000 X g for 30 minutes. The collected supernatant with solubilized proteins was subjected to an immunoprecipitation procedure using Seize™ X Protein A Kit (Pierce), with the modification that we use protein G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, California, USA), because of their higher binding affinity for mouse antibodies. Briefly, ~100 µg of solubilized proteins were incubated overnight with 5µg of purified MGO-derived AGE.
imidazole antibody linked to the protein G support. Immunoprecipitated antigen was eluted, and then subjected to one-dimensional PAGE as described.

2-D electrophoresis of mitochondrial proteins

Mitochondrial protein samples were prepared for 2-D electrophoresis using a protocol described by Lopez (39) and Molloy (43) with the following modifications. Briefly, 350 µL of mitochondrial pellet (22µg mitochondrial protein/µl) was resuspended in 350 µL rehydration buffer containing 7 M urea, 2 M thiourea, 1% CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]1-propanesulfonate), 1% Triton X-100, sonicated, and then ultracentrifuged at 150,000 X g for 30 minutes to remove particulate matter. The samples were diluted with rehydration buffer to a protein concentration of 1 µg/µL. DTT (64 mM) and Bio-Lyte 3/10 Ampholyte (1%) were added to the collected supernatant. The supernatant with solubilized mitochondrial proteins was used to rehydrate 7 cm, ReadyStrip IPG strips (BioRad). Strips were rehydrated passively with 180 µg mitochondrial proteins. Isoelectric focusing was performed at 20,000 V h in a PROTEAN IEF cell (BioRad). Strips were equilibrated in 1) NuPAGE LDS Sample Buffer, 10% NuPAGE sample Reducing Agent, and then 2) 1 X NuPAGE LDS Sample Buffer, 125 mM Iodoacetamide (Invitrogen). The proteins resolved in IPG strips were applied to second dimension gels (NuPAGE Novex 4-12% Bis-Tris ZOOM Gel, Invitrogen). One gel was used for Coomassie stain and another one was electroblotted, probed with antibody (1H7G5) and visualized as above. To adequately separate the approximately 1000 proteins theorized to be present in mitochondria, the same sample was applied to three narrow-range overlapping gradient strips (pI 3-6, 5-8, 7-10), in order to improve protein separation by expanding a small pI range across the entire width of the gel (7 cm).

Identification of MGO-modified proteins

The MGO-modified proteins were identified by overlapping the appropriate Coomassie stained 1D- and 2D-gel with specific membranes probed with anti-MGO derived imidazole antibody. Proteomic identification of MGO-modified proteins was performed as previously described (28). Spots were excised, destained, washed, and dehydrated before reduction with dithiothreitol and alkylation with iodoacetamide. The protease trypsin was driven into the gel pieces by rehydrating them in 30 µL 20 ng/µL trypsin in 50 mM ammonium bicarbonate on ice
for 10 min. Any excess trypsin solution was removed and 20 µL 50 mM NH₄HCO₃ added. The sample was digested overnight at room temperature. The resulting peptides were extracted in two aliquots of 30 µL 50% acetonitrile/5% formic acid, combined, and evaporated to <20 µL for LC-MS analysis.

Analyses were performed on a Finnigan LCQ-Deca ion trap mass spectrometer system with a Protana microelectrospray ion source interfaced to a self-packed 10 cm X 75 µm id Phenomenex Jupiter C18 reversed-phase capillary chromatography column. The microelectrospray ion source was operated at 2.5 kV. The digest was analyzed using the data dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide mol wt and product ion spectra to determine amino acid sequence in successive instrument scans. This mode of analysis produces approximately 1000 collisionally induced dissociation (CID) spectra of ions ranging in abundance over several orders of magnitude. Not all CID spectra are derived from peptides.

The data were analyzed by using all CID spectra collected in the experiment to search the NCBI non-redundant database with the search program TurboSequest. All matching spectra were verified by manual interpretation. The interpretation process was also aided by additional searches using the programs Mascot and Fasta.

**Statistical methods.** Between group differences were determined by ANOVA, or t test, with significance calculated by the method of Tukey, using JMP 5.0.1 (SAS Inc, Cary, North Carolina, USA). Bivariate linear regression analysis was performed for continuous variables, calculating ρ for the fitted line. Differences were considered significant at p <.05.
Results

Glycemic control

At every time point plasma glucose (not shown) and glycated hemoglobin levels were significantly higher in rats with STZ diabetes compared to controls. At 2 mo, glycated hemoglobin was 8.9 ± 1.1% in diabetes compared to 2.1± 0.9% in controls; 6 mo, diabetes 9.9 ± 1.0 %, controls 3.8 ±0.8 %; 12 mo diabetes 11.4±1.0 %, controls 5.8 ±0.5, respectively. Treatment with aminoguanidine did not alter plasma glucose or glycated hemoglobin levels (8.9±2.3%).

12 mo diabetes causes diminution of renal mitochondrial respiration

ADP-dependent NADH-linked respiration

The effect of the diabetic state on NADH-linked state 3 (ADP-dependent) respiratory rates was evaluated by using intact cortical renal mitochondria given glutamate and malate as substrates. Isolated mitochondria respond with a characteristic increase in the rate of oxygen consumption after the addition of ADP (state 3), and a leveling off of consumption with ADP exhaustion (state 4). Mitochondria isolated from 2 mo control rats had a state 3 respiratory rate of 210.33 ± 31.74 natom O •mg mitochondrial protein •min⁻¹, a state 4 respiratory rate of 39.33 ±4.58 natom O •mg mitochondrial protein •min⁻¹, and a respiratory quotient (RQ, state 3/state 4) of 5.36 ±0.46 with glutamate and malate as substrates. Data ranges were similar to previously published observations (54, 55, 58).

Two mo diabetes did not alter renal cortical mitochondrial respiration, Figure 1. In contrast, 12- mo diabetes caused a significant inhibition of state 3 respiratory activity in the presence of NADH-linked substrates (p = 0.0003), compared with age-matched controls (Figure 1A). Mitochondria isolated from 12 mo diabetic animals exhibited a significant increase in respiratory state 4 (p < 0.0001), Figure 1B. The respiratory quotient (RQ), the ratio of state 3 to state 4, reflects the respiratory function of the inner mitochondrial membrane, and provides a measure of mitochondrial integrity. RQ declines with aging, and 12 mo diabetes (p< 0.0001) (Figure 1C).
DNP-dependent NADH-linked respiration

Uncoupling agents collapse the proton gradient, thereby promoting maximum rates of mitochondrial respiration dependent on TCA and ERC activities, and independent of ATP synthase and ADP translocase. Renal mitochondria obtained from animals with 12-months of chronic hyperglycemia demonstrated a significant inhibition of uncoupled respiration compared to control (p=0.002) (Figure 2A). This inhibition of uncoupled respiration was of the same magnitude as the inhibition of ADP-dependent respiration (~20%), supporting the idea that long-term diabetes exerts its effect on the TCA or ERC rather than on the ATP synthase and ADP translocase.

NADH oxidase assay

The NADH oxidase assay (Figure 2B) tests the ability of the ERC to utilize exogenously added NADH. 12 mo diabetes was associated with significant inhibitions of ERC activity by 19% compared to control (p=0.001). ERC activity was not affected by 2 mo diabetes.

To further define the activity of NADH-linked ERC complexes, 12 mo control or diabetic renal mitochondria were exposed to various experimental conditions (20, 21). The activities of complexes I and IV of the ERC were not affected by chronic hyperglycemia. Studies of complex III revealed a 20% decrease in the activity in diabetic mitochondria isolated from 12 mo diabetic rats (p=0.0006) (Table 1).

Diabetic mitochondria are a source of oxidative stress

We measured superoxide production by renal mitochondria isolated from normal and diabetic kidney. Superoxide production was quantitated for a period of 75 seconds in each individual mitochondrial sample oxidizing NADH. As expected, control mitochondria respond with an increase in the rate of superoxide production upon the addition of NADH and the initiation of the ERC activity, consistent with the accepted concept that 1-4% of oxygen consumed is released as free radicals in normal conditions. Figure 3A shows a representative experiment contrasting superoxide production by control and diabetic mitochondria. Mitochondria from diabetic animals produced superoxide at increased rates. Tiron scavenged superoxide produced by both control and diabetic mitochondria. Data for individual experiments used to generate curves similar to those shown in Figure 3A were analyzed at 55 seconds.
Mitochondria from animals with 12 mo of diabetes showed a 19.8% increase in superoxide production compared to their matched controls (p= 0.0003, not shown).

**Diabetic mitochondria are a target of oxidative stress**

The formation of carbonyl groups is associated with protein oxidation. Using an OxyBlot™ immunodetection method, mitochondrial proteins over a broad range of molecular masses are shown to be targets of progressive oxidative modifications with aging and duration of diabetes. Four bands, consistently present in all samples (> 62, 62, 49 and ~ 38 kDa, arrows, Figure 3B) were quantitated using densitometry. 12 mo diabetes resulted in an increase in mitochondrial protein oxidation (p<0.05 for >62 kDa band, and p<0.001 for 62 kDa band) (Figure 3C).

**Diabetes causes carbonyl-induced modifications of mitochondrial proteins**

Western blot analysis using antibody against MGO-imidazole revealed the presence of three protein bands, at ~14, 28, and 38 kDa. However, the intensity of staining for the 38 and 14 kDa proteins were not significantly modified in diabetes compared to control (Figure 4A). In contrast the signal intensity of ~28 kDa band was markedly increased in all mitochondria isolated from animals with diabetes (Figure 4A). The proteins at ~14 and 38 kDa revealed positive staining in the absence of the antibody (not shown), the result of either endogenous peroxidase activity, or the presence of trace levels of contaminating metals in buffers used for routine Western blotting procedures (7).

To circumvent these issues, immunoprecipitation of those proteins having MGO derived imidazole epitopes was performed. This approach identified only those proteins of ~28kDa mol wt. As demonstrated by immunoprecipitation (Figure 4B), the intensity of MGO induced modifications increased with the duration of diabetes.

Signal intensity of the 28 kDa band obtained from each 12- month animal was measured using densitometry. There was a significant negative correlation between the signal intensity and complex III activity in both control (ρ= 0.77, p< 0.03) and diabetic animals (ρ= 0.70, p< 0.05) (Figure 4C).

*Aminoguanidine improves the mitochondrial respiration and complex III activity, and diminishes the oxidative modifications of mitochondrial proteins*
Renal mitochondria showed a significant inhibition of state 3 respiratory activity in the presence of NADH-linked substrates at 6 mo-diabetes, compared with their age-matched controls (p<.01) (Figure 5A). Figure 5B shows that mitochondria isolated from rats with 6 mo diabetes exhibited a significant increase in respiratory state 4 (p = 0.005), and a decline in respiratory quotient (RQ) (p< 0.01) (Figure 5C). Treatment with aminoguanidine resulted in an improvement of state 3 respiration as shown in Figure 5A, although aminoguanidine did not normalize respiratory state 4 (Figure 5B), and had no influence on RQ (Figure 5C). Importantly, aminoguanidine treatment normalized the diabetes-induced decline in complex III activity. (Figure 5D).

Western blot analysis revealed the presence of three MGO-imidazole containing proteins, at ~14, 28, and 38 kDa. Similar to the 12- mo time point, the 38 and 14 kDa proteins were not significantly modified in 6 mo diabetes compared to control (Figure 6A). In contrast the signal intensity of ~28 kDa band was significantly increased in mitochondria isolated from 6 mo diabetic animals. As expected, aminoguanidine treatment was associated with a significant decrease in MGO-induced modifications of proteins running at 28kD (p= 0.0004), Figure 6B.

Mitochondrial proteins are a target of oxidative modifications as early as 6 mo of diabetes as demonstrated by Figure 7A and B. Diabetes resulted in an increase in mitochondrial protein oxidation apparent in the density of the >62, 62, and 49 kDa bands (p=0.005, p=0.01, p=0.003, respectively). Aminoguanidine treatment reversed this pattern, decreasing oxidation associated with diabetes towards the normal level, Figures 7A and B.

**Identification of MGO-modified proteins**

Figure 8A shows a representative 5-8 pI gel from a series of 2D gel electrophoresis analyses of proteins in mitochondria isolated from the cortical kidney of 12 mo diabetic animals. Repetitive analysis of these samples gave similar and reproducible patterns of protein expression for each type of sample.

Subsequent LC/MS analysis permitted unambiguous identification of the proteins. The spots in Figure 8A that are indicated by the arrows are proteins containing MGO-derived imidazole epitopes, as was found after Western blot analysis (Figure 8B).

Digestion and analysis of these spots (Figure 8A) identified the proteins with corresponding NCBI accession numbers, mo wts and pIs shown in Table 2. Commercial
antibodies against these proteins are not available yet, so their identity was not confirmed by immunoprecipitation. However, these observations were established after repeated separation of proteins using both one-dimensional and two-dimensional gel electrophoresis.
Discussion

The present study describes for the first time, a direct relationship between the formation of intracellular AGEs on renal mitochondrial proteins, the decline in mitochondrial function, and the formation of ROS.

Non-enzymatic modifications of proteins by glucose to form AGEs has been linked to the development of diabetic nephropathy (5, 17, 24, 57, 69). The kidney may be uniquely vulnerable to this process because it is the target both of AGEs that form within renal cells and AGEs excreted in the urine. In addition, the main marker of Maillard-induced alterations in diabetes is the presence of MGO-induced hydroimidazolone on intracellular proteins (64).

In vitro studies in this laboratory have demonstrated that mitochondrial proteins are subcellular targets of dicarbonyls, particularly the highly reactive compound MGO. In renal mitochondrial suspension, MGO attaches to mitochondrial proteins, forms MGO-derived imidazole AGEs, and nearly instantaneously inhibits mitochondrial oxygen consumption by the ERC (55). There is evidence that MGO plays a role in protein structural changes and diabetic complications. MGO is markedly elevated in plasma from patients with diabetes (31, 40). Circulating MGO levels are correlated with the level of glycemia (3, 41) and associated with diabetic complications (45) including diabetic nephropathy (3).

The strategy to focus on MGO-derived imidazole modifications of mitochondrial proteins, through the use of the specific anti-imidazole AGE antibody, yielded six proteins in mitochondria from rats with 12 mo of diabetes. These proteins are known to be involved in two specific mitochondrial metabolic pathways associated with energy production: 1) oxidative phosphorylation, and 2) fatty acid β-oxidation.

Four of the MGO-modified proteins are components of the ERC. Two of the modifications were not associated with functional changes. Thus, the G chain of F1-ATPase was shown to possess MGO-derived imidazole motifs, although there were no changes in the phosphorylation apparatus. In addition, the MGO-induced hydroimidazolone epitope present on the 30 kDa subunit of complex I was not associated with a change in the maximal activity of complex I.

In contrast, both proteomic and functional studies show that complex III is the key target of MGO-induced modifications. Complex III spans the central part of the respiratory
chain, catalyzing electron transfer from ubiquinol to oxidized cytochrome c. This study shows that two out of eleven complex III components are modified by MGO in diabetes: core protein 1 and cytochrome c1. Based on the crystal structure of complex III, both proteins are exposed to the mitochondrial intermembrane space, which is conceptually more accessible to MGO from the cytoplasm. More than that, MGO may be formed within the intermembrane space in close proximity to these proteins, by spontaneous dephosphorylation and decomposition of dihydroxyacetone phosphate, a compound of the glycerol-3-phosphate shuttle (51). Owing to this design, MGO may readily attach to the arginine residue of these two proteins. The role of core protein 1 in mitochondrial respiration is uncertain, but it may play a role in complex III assembly (23) (4) and in vectorial proton translocation activity (11, 53). Cytochrome c1 channels the electrons from the iron-sulfur protein (FeS) to cytochrome c. The tight interaction between the two type 1 hemes located on these two cytochromes suggests a direct heme-to-heme transfer and it is mainly stabilized by electrostatic forces between amino acid residues (22, 65). A potential target is the arginine residue in the 166 position (Arg 166 in yeast corresponds to Arg 102 in mammals). This arginine seems to be essential for cytochrome c1 function, since it is highly conserved in phylogenesis, lies in the loop of the cytochrome c binding site of complex III, and direct mutagenesis of this site results in loss of function (1). Therefore we hypothesize that in diabetes, acquired modification of essential arginine residues of cytochrome c1 by MGO present in the intermembrane space may interfere with catalytic activity of these proteins. Imidazolone-modified cytochrome c1 may not be able to transfer electrons to cytochrome c, resulting in more reduced proximal components of complex III, increased pressure at the Qo site of complex III where the electrons may be donated to molecular oxygen and form superoxide (34, 36).

This manuscript has relied heavily on the use of an immunochemical method as a common procedure for the analysis of an MGO- induced protein glycation product imidazole, using a well-characterized monoclonal antibody against a specific AGE structure. However, this immunochemical method is restricted to detection and semiquantitation of a single marker compound of glycation. Yet, MGO- induced protein glycation products in vivo are heterogeneous. Therefore the choice of this antibody is based on a robust literature implicating MGO- induced AGE-imidazole as the major quantitative and qualitative modifications in the glomeruli in patients with diabetes (64). However, other MGO- induced AGE products may be formed in high yields in long- term diabetes on mitochondrial proteins. More then that, because
of steric constraints, not all AGE epitopes on a protein may be available for the interaction with the antibody. AGE immunoassays used in this study must be considered to yield semiquantitative results only.

The decline in renal mitochondrial respiration in diabetes in response to complex I-related substrates, localizes the defect to complexes I, III and IV. In addition, a decline of ERC activity (as demonstrated by the decreased utilization of exogenous NADH in diabetes) parallels the global diminution of mitochondrial respiration. In vitro, the ERC is much more sensitive to MGO modification than the TCA (55). Thus, the presence of MGO-induced imidazole epitopes on critical protein components of complex III may explain specific inhibition of complex III in diabetes. Furthermore, inhibition of MGO-induced modifications with aminoguanidine restores mitochondrial ERC function. An important negative finding is the absence of MGO-modifications of protein components of TCA (Table 2). In the cell, MGO formation is mainly external to the mitochondria (62). Because components of the ERC are localized to the mitochondrial inner membrane, they are likely to have increased vulnerability to modification by MGO in contrast to the TCA situated in the mitochondrial matrix.

The magnitude of the inhibition of complex III demonstrated in this study is sufficient to account for the diminution of whole mitochondrial respiration seen in the kidney in chronic diabetes. Metabolic expression of an existing defect in oxidative phosphorylation is dependent on a biochemical threshold that varies across very large ranges according to the tissue (56, 61). Normal kidney mitochondria have an excess of available complex III and demonstrate a high biochemical threshold after experimental inhibition (56). In contrast in long-term diabetes, the diminution of complex III activity parallels the magnitude of the decline in oxidative phosphorylation. We speculate that the threshold curve might be modified in chronic diabetes by both posttranslational modifications and a decrease in the quantity of components of complex III as previously shown in another highly oxidative tissue, the heart (67).

Numerous reports have demonstrated that oxidative stress induced by diabetes plays a major role in the development of diabetic nephropathy (30). However, there is no consensus about the origin of mitochondrial ROS in diabetes. Our results suggest that the selective diabetes-related alterations in complex III may enhance the production of ROS. Electron transfer among the components of complex III occurs via a bifurcation of electron flow mediated by motion of the iron-sulfur protein (FeS) between cytochrome c1 and cytochromes b (36).
Following abstraction of an electron by FeS, the remaining ubisemiquinone donates the second electron to cytochromes b. The electron from FeS sequentially reduces cytochrome c1, then cytochrome c via a docking interaction facilitated by small subunits of complex III. Thus, impaired electron transfer in the FeS-cytochrome c1-cytochrome c loop of complex III, due to loss of catalytic activity of cytochrome c1, would lead to an increased relative reduction of cytochromes b and ubisemiquinone with generation of ROS via direct electron leak to molecular oxygen (68). The inhibition of complex III has been shown to be responsible for the creation of 70-80% of superoxide in the ubiquinone cycle (8) or by triggering a reverse electron transfer to complex I (37). This study clearly demonstrates that treatment with an inhibitor of AGE-induced posttranslational modifications of complex III components improves mitochondrial respiration and complex III activity and diminishes the oxidative damage to mitochondrial proteins.

Aminoguanidine is the prototype of an efficient dicarbonyl scavenging agent that prevents the formation of AGEs from dicarbonyl precursors (63). In pharmacologic doses, aminoguanidine has been shown to delay dramatically the development of chronic complications of diabetes (13, 48, 59). Aminoguanidine is a potent inhibitor of the inducible form of nitric oxide synthase with an IC50 of 31 µM (2), and can act as an antioxidant in vivo by decreasing oxidant- induced ROS production and lipid peroxidation products (18). However, the kinetics of scavenging lipid peroxidation products are 1000 fold slower than for dicarbonyls (63). In addition, hydroxyl radicals are scavenged only at very high concentrations (1-10 mM) (70). Therefore the achievable concentrations of aminoguanidine in kidney (740 nmol/g in the whole kidney), are too low to inhibit nitrosylation of mitochondrial proteins (2) or to exert direct antioxidant effects (48). In contrast, aminoguanidine was shown to have a prooxidant effect (63). In summary aminoguanidine’s extend beyond the scavenging of dicarbonyls. Because of non-specific effects, the actions of this agent on mitochondrial ROS production must be interpreted with great caution in long-term models of diabetes. A stronger study would be to demonstrate the effects of MGO in an experimental model in which complete ablation of MGO production is possible, such as glyoxalase I transgenic animals. Experiments in these models are in the planning phase at present in our laboratory.

Existing literature suggests that the MGO-imidazolone modification identified on enzymes of fatty acid β-oxidation are not likely to alter function. The active site architecture of mitochondrial enoyl CoA hydratase (crotonase) reveals the presence of one glutamic acid as the
main catalytic structure (14) rather than a more MGO-susceptible amino acid (such as arginine). Crotonase catalyzes the hydration of 2-trans-enoyl-CoA intermediates of fatty acid oxidation (15), and is readily upregulated in the presence of high levels of substrates as described in the diabetic heart (67). Electron transfer flavoprotein is a necessary electron acceptor for many mitochondrial dehydrogenases including those involved in fatty acid degradation. The identification of MGO-induced modifications in the β-oxidation pathway is likely to reflect a generalized up-regulation of these enzymes, in response to high levels of substrates.

The findings reported in this paper extend knowledge of the central role played by mitochondria in diabetes from acute hyperglycemia to a model of chronic disease. In cell culture, acute hyperglycemic conditions cause increased mitochondrial superoxide formation as a result of increased substrate, increased availability of reducing equivalents provided by the TCA to ERC, and mitochondrial membrane hyperpolarization (47). There is a threshold above which even a small increase in mitochondrial potential gives rise to a significant increase in hydrogen peroxide production (32). ROS production may derive from MGO induced-post-translational modification of mitochondrial enzymes. Gredilla et al demonstrated that complexes I and III are the main sites of superoxide production by intact renal mitochondria (19). The direct correlation between the intensity of MGO-induced modifications and the inhibition of complex III in the kidney in chronic diabetes, is consistent with prior work localizing ROS production to complex III in intact (10, 34, 60), aged (42) and ischemic-reperfused (36) heart mitochondria and in diabetic liver (34).

In conclusion, in chronic diabetes, mitochondrial proteins are prone to post-translational modifications due to glycation and oxidation. Carbonyl-induced modifications are targeted to key protein components of major mitochondrial cycles, such as oxidative phosphorylation and β-oxidation. In these studies, there is a statistically significant negative correlation between the extent of MGO induced modifications and mitochondrial bioenergetics in the kidney of rats with long-term diabetes. In addition, functionally damaged mitochondria give rise to increased quantities of superoxide and oxidative damage to mitochondrial proteins. At 6 months an inhibitor of advanced glycation restores functional and structural alterations of renal mitochondria. Therefore this study provides early evidence in support of the hypothesis that glycation of mitochondrial proteins may be the primary cause of mitochondrial dysfunction and oxidative stress in chronic diabetes.
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References


Table 1: Effect of 12 m diabetes on electron respiratory chain activity  Mitochondria were sonicated and enzyme activity was determined as described in Material and Methods. Values are expressed as nmol/min/mg of mitochondrial proteins and represent the Mean ± SD

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Table 2: Mitochondrial proteins identified in MGO-derived imidazole immunoreactive spots in Figure 7

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Figure Legends

Figure 1: Diabetes is associated with diminution of ADP-dependent NADH-linked respiration. Respiratory assays were performed with 0.5 mg/ml mitochondrial proteins resuspended in respiratory buffer with NADH-linked substrates (glutamate 10mM, malate 5mM) at 25°C. A. State 3 respiration was initiated with the addition of 0.3 mM ADP. B. State 4 respiration was the rate of oxygen consumption following depletion of ADP. C. Respiratory quotient is defined as the ratio between respiratory State 3/State 4. Box plots show quartiles of the data. *, significantly different comparing control and diabetes; †, significantly different comparing 2 with 12 mo of control and diabetes. C, Control (n=8); D, Diabetes (n=8).

Figure 2: Diabetes is associated with diminution of ERC activity. A. Uncoupled respiration was initiated by the addition of 60 µM DNP to 0.5 mg/ml mitochondrial proteins resuspended in respiratory buffer with NADH-linked substrates (glutamate 10mM, malate 5mM) at 25°C. The results are expressed as percentage of the age-matched controls. Mean ± SD. * significant differences between paired values. B. NADH-oxidase assay was performed with 0.2 mg/ml mitochondrial proteins in a hypotonic buffer as described in Materials and methods. Oxygen consumption was monitored after the addition of 9 µM cytochrome c followed by 0.4 µM NADH. The results are expressed as percentage of the age-matched controls. Box plots show quartiles of the data. * significantly different comparing control and diabetes. C, Control (n=8); D, Diabetes (n=8).

Figure 3: Cortical renal mitochondria are both the source and the target of ROS in chronic diabetes. A. Representative experiment demonstrating that cortical renal mitochondria isolated from 12 mo diabetic animals (squares) form higher levels of superoxide than their age-matched controls (circles). Mitochondria were sonicated and diluted to 50 µg/ml in experimental buffer as described in the Material and Methods section. Respiration was started by the addition of 70µm of NADH (arrow) in the presence of 20 µM lucigenin. Tiron (100 mM) was added at 55 seconds (arrow). B. Mitochondrial proteins were prepared for carbonyl analysis and ~14 µg from each sample was separated by SDS-PAGE. A representative membrane is shown. C. Densitometric analysis of the amount of MGO-induced modification to mitochondrial proteins in 12-mo
animals. Diamond plots indicate mean ±SD of the data. * significantly different comparing control and diabetes. C, Control (n=8). D, Diabetes (n=8).

**Figure 4: Mitochondrial proteins isolated from diabetic kidneys are target of MGO- induced modifications.** A. 14 µg of mitochondrial proteins were separated by SDS- PAGE, electroblotted on nitrocellulose membrane and probed with a monoclonal antibody to MGO- derived imidazole AGE (1H7G5). B. Immunoprecipitation of MGO- modified proteins with the antibody 1H7G5. C. Correlation between the quantity of MGO- induced modification present on mitochondrial proteins and the inhibition of complex III activity in 12- month animals (Control (n=8), ρ = .77, p<.03; Diabetes (n=8) ρ = .70, p<0.05).

**Figure 5: Inhibition of advanced glycation restored the diabetic- induced alterations in mitochondrial function.** Respiratory assays were performed with 0.5 mg/ml mitochondrial proteins resuspended in respiratory buffer with NADH- linked substrates (glutamate 10mM, malate 5mM) at 25°C. A. State 3 respiration was initiated with the addition of 0.3 mM ADP. B. State 4 respiration was the rate of oxygen consumption following depletion of ADP. C. Respiratory quotient is defined as the ratio between respiratory State 3/State 4. D. Ubiquinone-cytochrome c reductase activity of mitochondrial complex III was assayed as described. Box plots show quartiles of the data. * significantly different comparing control and diabetes, † significantly different comparing diabetes and aminoguanidine. C, Control (n=5). D Diabetes (n=5). A, Diabetes treated with aminoguanidine (n=5).

**Figure 6: Inhibition of advanced glycation diminishes the diabetic- induced MGO- modifications on mitochondrial proteins.** A. 14 µg of mitochondrial proteins were separated by SDS-PAGE, electroblotted on nitrocellulose membrane and probed with a monoclonal antibody to MGO-derived imidazole AGE (1H7G5). B. Densitometric analysis of the amount of MGO- induced modification of mitochondrial proteins at 6,mo. Diamond plots indicate mean±SD of the data. * significantly different comparing control and diabetes, † significantly different comparing diabetes and aminoguanidine. C, Control (n=5). D Diabetes (n=5). A, Diabetes treated with aminoguanidine (n=5).
Figure 7: Inhibition of advanced glycation diminishes oxidative modifications on mitochondrial proteins. A. Mitochondrial proteins were prepared for carbonyl analysis and ~14 µg from each sample was separated by SDS-PAGE. The depicted membrane is representative of eight experiments. B. Densitometry analysis of the four bands indicated by the arrows. Diamond plots indicate mean±SD of the data. * significantly different comparing control and diabetes, † significantly different comparing diabetes and aminoguanidine. C Control (n=5). D Diabetes (n=5). A, Diabetes treated with aminoguanidine (n=5).

Figure 8: Two-dimensional gel pattern for MGO-modified proteins in cortical renal mitochondria from rats with 12 months of diabetes. A. Coomassie blue stained gel. B. Membrane probed with an antibody against MGO-derived imidazole AGE (1H7G5). Arrows indicate the MGO-modified proteins. See Table 2.
Figure 1 A, B, C
Figure 2 A, B

O2 consumption (% matched control)

A.

B.

Figure 2 A, B
Figure 3 A

A.

Time (seconds)

0 20 40 60 80 100

(RFU) Relative Fluorescence Units

0 100 200 300 400

Diabetes

Control

NADH

Tiron

Figure 3 A
Figure 3 B, C

Band Density (Arbitrary Units)

- >62 kDa
- 62 kDa
- 49 kDa
- 38 kDa

Control vs. Diabetes
Figure 4

A.

B.

C. Band Density (Arbitrary Units) vs. Complex III (nmol/mg/min)
Figure 5 A,B

O₂ consumption (natoms/mg/minute)

A. State 3

B. State 4

Control Diabetes DM + Aminoguanidine

Figure 5 A,B
Figure 5 C,D

C. Control Diabetes DM + Amino-guanidine

D. Complex III (nmol/mg/min)

Figure 5 C,D
Figure 6 A,B

**A.**

- C
- D
- A

**B.**

**BAND DENSITY**

(Arbitrary Units)

<28 kDa

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*†*
Figure 7 A,B

BAND DENSITY (Arbitrary Units)

Control  Diabetes  DM + Amino-guanidine

Figure 7 A,B
Figure 8