Protein Tyrosine Nitration in the Mitochondria from Diabetic Mouse Heart

IMPLICATIONS TO DYSFUNCTIONAL MITOCHONDRIA IN DIABETES*

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Illarion V. Turko‡, Li Li§, Kulwant S. Aulak¶, Dennis J. Stuehr‡, Jui-Yoa Chang§, and Ferid Murad‡§§

From the §Department of Integrative Biology and Pharmacology and the ¶Institute of Molecular Medicine, University of Texas Medical School, Houston, Texas 77030 and the ¶Department of Immunology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio 44195

Oxidative stress has been implicated in dysfunctional mitochondria in diabetes. Tyrosine nitration of mitochondrial proteins was observed under conditions of oxidative stress. We hypothesize that nitration of mitochondrial proteins is a common mechanism by which oxidative stress causes dysfunctional mitochondria. The putative mechanism of nitration in a diabetic model of oxidative stress and functional changes of nitrated proteins were studied in this work. As a source of mitochondria, alloxan-susceptible and alloxan-resistant mice were used. These inbred strains are distinguished by the differential ability to detoxify free radicals. A proteomic approach revealed significant similarity between patterns of tyrosine-nitrated proteins generated in the heart mitochondria under different in vitro and in vivo conditions of oxidative stress. This observation points to a common nitration species, which may derive from different nitrating pathways in vivo and may be responsible for the majority of nitryosine formed. Functional studies show that protein nitration has an adverse effect on protein function and that protection against nitration protects functional properties of proteins. Because proteins that undergo nitration are involved in major mitochondrial functions, such as energy production, antioxidant defense, and apoptosis, we concluded that tyrosine nitration of mitochondrial proteins may lead to dysfunctional mitochondria in diabetes.

Protein tyrosine nitration is a common post-translational modification occurring under conditions of oxidative stress in a number of diseases (1, 2). Diabetes is a state of oxidative stress (3, 4). The studies on diabetic mitochondria suggest that diabetes causes dysfunctional mitochondria (5–8). Furthermore, the studies that associate altered free radical status with impaired mitochondrial function provide evidence of protein tyrosine nitration in mitochondria exposed to oxidative stress (9–13), including diabetic mitochondria (14). These observations may have important implications for the pathogenesis of diabetes if the protein targets of nitration, functional consequences of nitration, and pathways for the increase of protein tyrosine nitration in mitochondria were established.

Over the past several years, substantial evidence has been accumulated that major pathways of protein tyrosine nitration in vivo include peroxynitrite (ONOO−) and heme peroxidase-dependent reactions (1). A recent development points to the common nitrating species (nitrogen dioxide, NO2) formed from both pathways, which is responsible for the most nitrotyrosine generated (15, 16). Despite some progress in assessing the putative mechanism of in vivo nitration, the biological relevance of protein tyrosine nitration remains unclear. Very little is known about which specific proteins undergo nitration and whether disease-associated nitration contributes to the appearance of complications or whether it is merely a biomarker, reflecting the presence of complications.

Our hypothesis is that nitration of mitochondrial proteins is a common mechanism by which oxidative stress causes dysfunctional mitochondria. As a source of mitochondria, alloxan-susceptible (ALS)1 and alloxan-resistant (ALR) mice were used. These inbred strains are distinguished by the differential ability to detoxify free radicals (17, 18). In contrast to ALS mice, the ALR mice remain normoglycemic and do not develop diabetes after alloxan (AL) injection. These two closely related strains are especially useful in comparing the susceptibility to damage caused by oxidative stress. The purpose of this study was to identify the nitrated proteins formed in mitochondria under different in vivo and in vitro conditions of oxidative stress and to elucidate the functional outcome of nitration. The data obtained in the present study point to a common nitrating species responsible for the majority of nitrotyrosine formed and concur with the hypothesis that nitration of mitochondrial proteins caused by oxidative stress may result in dysfunctional mitochondria.

MATERIALS AND METHODS

Animals and Treatment—The 7-week-old male ALS and ALR mice were obtained from The Jackson Laboratory (Bar Harbor, ME). AL was dissolved in sterile phosphate-buffered saline and injected intraperitoneally at a dose of 60 mg/kg body weight. Control mice received the corresponding volume of buffer. The blood glucose levels were monitored using Accu-Chek Advantage blood glucose monitor and test strips.

1 The abbreviations used are: ALS, alloxan-susceptible; ALR, alloxan-resistant; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight; MPO, myeloperoxidase; ONOO−, peroxynitrite; H2O2, hydrogen peroxide; SCOT, succinyl-CoA3-oxoacid CoA transferase; SOD, superoxide dismutase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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† To whom correspondence should be addressed: Dept. of Integrative Biology and Pharmacology, UT-Houston Medical School, 6431 Fannin, Houston, TX 77030. Tel.: 713-500-7500; Fax: 713-500-0790; E-mail: Ferid.Murad@uth.tmc.edu.

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Tyrosine Nitration in Diabetic Mitochondria

Mitochondria and Treatment—Mitochondria from hearts were isolated using differential centrifugation (14) in 10 mM phosphate buffer (pH 7.2)/0.25 mM sucrose/0.1 mM EDTA supplemented with protease and proteasome inhibitors: 1 mM 2-(aminoethyl)benzenesulfonyl fluoride, 0.5 mM aprotinin, 50 mM bestatin, 15 mM E-64, 20 mM leupeptin, 10 mM pepstatin A, 20 mM protease inhibitors cocktail (Calbiochem), and 20 mM MG-132. Finally, mitochondria were resuspended in 100 mM phosphate buffer (pH 7.2) containing 0.1 mM diethylenetriamine pentaacetic acid. Mitochondria from untreated ALS and ALR mice were treated with either (i) 0.5 mM ONOO⁻ or (ii) 0.5 mM NaN₃/200 μg/ml glucose with 40 ng/ml glucose oxidase (to generate H₂O₂), and 100 mM myeloperoxidase (150 units/ml) at room temperature. Mitochondria were then sonicated and centrifuged at 100,000 × g for 60 min. The high-speed supernatant fraction includes mitochondrial soluble proteins. The high-speed pellet includes mainly mitochondrial membrane proteins. Total protein concentrations in these fractions were determined using the Bio-Rad protein assay with bovine serum albumin as the standard.

Two-dimensional Gel Electrophoresis and Western Blot Analysis—The first dimension was performed using the PROTEAN IEF cell (Bio-Rad). The 7-cm pH 3–10 immobilized pH gradient (IPG) strips were rehydrated with 130 μl of a solution (2 M thiourea, 7 M urea, 4% CHAPS, 0.5% Trition X-100, 0.5% Bioydes 3–10, 1% dithiothreitol, and bromphenol blue) that contained 100 μg of the total protein. Isoelectric focusing was performed in a Mighty V15 min, linearity increased over 2 h, to a maximum of 4,000 V, and then run to accumulate a total of 20,000 Vh. For the second dimension, the IPG strips were equilibrated for 15 min in 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 1% dithiothreitol, and bromphenol blue and then for 15 min in 50 mM Tris-HCl (pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2% iodoacetamide, and bromphenol blue. The strips were then embedded in 0.7% (w/v) agarose on the top of 10% acrylamide slab gels containing a 4% stacking gel. After completion of the second-dimension SDS-PAGE, the proteins were transferred onto polyvinylidene difluoride membrane. Western blot analysis with 12,000 dilution of mouse monoclonal anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) was performed as previously described (14). Control experiments for nitrotyrosine immunoactivity were performed by preincubating (30 min at room temperature) the anti-nitrotyrosine antibody with 4 mM free 3-nitrotyrosine or with 4 mM free t-tyrosine prior to incubation with polyvinylidene difluoride membranes.

Identification of Nitrated Proteins—Nitrotyrosine-positive spots were identified by matrix-assisted laser desorption ionization/time-of-flight mass spectrometric analysis (MALDI-TOF/MS) of in-gel tryptic digest of nitrotyrosine immunoprecipitated spots. MALDI-TOF-MS was performed using a Voyager-DE STR bioSpectrometry work station (PerSeptive Biosystems). Bradynakin fragment-(1–7) (MH⁺ 757.3997), angiotensin II (MH⁺ 1046.5423), synthetic peptide P14R (MH⁺ 1533.8552), and synthetic peptide fragment-(18–39) (MH⁺ 2468.1985) were used for external calibration. All spectra were also calibrated internally on the trypsin peaks at 842.51 and 2211.0968. Peptide mass fingerprinting data were evaluated using the Mascot software (www.matrixscience.com).

Immuno precipitation with Anti-nitrotyrosine Antibody—High-speed supernatant fractions from ALS mitochondria treated with ONOO⁻ or NO₂⁻/H₂O₂/MPO were precleared with 60 μl of protein A/G Plus-agarose (Santa Cruz Biotechnology) for 1 h at 4 °C. The mixtures were centrifuged to pellet the beads. The supernatant fractions were incubated with 3 μg of mouse monoclonal anti-nitrotyrosine antibody (Upstate Biotechnology) for 16 h at 4 °C. Immune complexes were precipitated from both samples with 60 μl of protein A/G-agarose by rocking overnight at 4 °C. After centrifugation the beads were washed three times with 50 mM Tris-HCl (pH 7.4)/0.5 mM NaCl/0.1% Tween 20. The beads were finally resuspended in sample loading buffer containing SDS and 2-mercaptoethanol, and supernatant fractions were resolved by SDS-PAGE. Westerns were performed using the rabbit anti-succinyl-CoA-3-oxoadipate CoA transferase (SCOT) serum (14), the scCO3 antibody (Santa Cruz Biotechnology). Peroxiredoxin 3 (Peroxiredoxin 3, and SOD2 (Fig. 1, A and B, spots 1–7). Three of them, SCOT, creatine kinase, and peroxiredoxin 3, were also found nitrated in the diabetic mitochondria (Fig. 1C, spots 4–6). The membrane fraction was contaminated with trace amounts of nitrat ed aconitase 2, Grp75, albumin, and creatine kinase (Fig. 2, A and B). Two more proteins in this fraction were identified as a β-subunit of trifunctional protein (Fig. 2, spot 8) and a 24-kDa subunit of NADH-ubiquinone oxidoreductase (Fig. 2, spot 9). Both these proteins are involved in membrane-bound mitochondrial complexes. Several nitrotyrosine-positive spots were observed in the membrane fraction from the diabetic mitochondria. Spot 8 represents the β-subunit of trifunctional protein, the nitrotyrinated protein found in in vitro experiments. Another one was identified (Fig. 2C, spot 10) as a voltage-dependent anion channel 1.

Results

Tyrosine Nitration of Mitochondrial Proteins—We undertook a study to compare the outcome of in vitro and in vivo nitration of mitochondrial proteins. Our in vitro approach includes treatment of purified heart mitochondria from ALS mice with either ONOO⁻ or NO₂⁻/H₂O₂/MPO. Our in vivo approach to generate nitrated proteins includes treatment of ALS mice with AL that caused type I diabetes. In addition, whole mitochondria in all control and treated groups were sonicated and separated into two fractions, which represent soluble mitochondrial proteins and membrane mitochondrial proteins, respectively. Soluble (Fig. 1) and membrane (Fig. 2) mitochondrial proteins were then separately resolved by a two-dimensional PAGE. Following matching of nitrotyrosine immunopositive spots on Western and Coomassie-stained spots on the gel, the gel pieces were manually excised for protein identification by MALDI-TOF/MS. All tyrosine-nitrated spots were excised and processed. However, not all the proteins were identified. The major reason for this was a lack of peptides from particular spots. Probably, several strong nitrotyrosine positive spots seen in Figs. 1 and 2 represent low-abundance mitochondrial proteins, which could not be identified without prefractionation of complex protein mixtures before loading on two-dimensional PAGE.

The proteins identified are listed in Table I. Seven identical proteins were found nitrated in the soluble fraction from ONOO⁻ and NO₂⁻/H₂O₂/MPO that were precleared with 60 μl of protein A/G Plus-agarose (Santa Cruz Biotechnology) for 1 h at 4 °C. The mixtures were centrifuged to pellet the beads. The supernatant fractions were incubated with 3 μg of mouse monoclonal anti-nitrotyrosine antibody (Upstate Biotechnology) for 16 h at 4 °C. Immune complexes were precipitated from both samples with 60 μl of protein A/G-agarose by rocking overnight at 4 °C. After centrifugation the beads were washed three times with 50 mM Tris-HCl (pH 7.4)/0.5 mM NaCl/0.1% Tween 20. The beads were finally resuspended in sample loading buffer containing SDS and 2-mercaptoethanol, and supernatant fractions were resolved by SDS-PAGE. Westerns were performed using the rabbit anti-succinyl-CoA-3-oxoadipate CoA transferase (SCOT) serum (14), the scCO3 antibody (Santa Cruz Biotechnology). Peroxiredoxin 3 (Peroxiredoxin 3, and SOD2 (Fig. 1, A and B, spots 1–7). Three of them, SCOT, creatine kinase, and peroxiredoxin 3, were also found nitrated in the diabetic mitochondria (Fig. 1C, spots 4–6). The membrane fraction was contaminated with trace amounts of nitrated aconitase 2, Grp75, albumin, and creatine kinase (Fig. 2, A and B). Two more proteins in this fraction were identified as a β-subunit of trifunctional protein (Fig. 2, spot 8) and a 24-kDa subunit of NADH-ubiquinone oxidoreductase (Fig. 2, spot 9). Both these proteins are involved in membrane-bound mitochondrial complexes. Several nitrotyrosine-positive spots were observed in the membrane fraction from the diabetic mitochondria. Spot 8 represents the β-subunit of trifunctional protein, the nitrotyrinated protein found in in vitro experiments. Another one was identified (Fig. 2C, spot 10) as a voltage-dependent anion channel 1.
Immunoprecipitations are traditionally used to reconfirm the correct spot assignments on two-dimensional PAGE and MALDI-TOF identifications. We have immunoprecipitated SCOT, Grp75, and SOD2 with anti-nitrotyrosine antibody from soluble mitochondrial fractions treated with ONOO\(^{-}\) or NO\(_2\)/H\(_2\)O\(_2\)/MPO and proved the identity of these proteins by Western immunoblots with anti-SCOT, anti-Grp75, and anti-SOD2 antibodies, respectively (Fig. 3A). Furthermore, we have screened the MALDI-TOF mass spectra generated for immunoprecipitated nitrated proteins and found direct proof of Grp75 nitration for both treatments used, ONOO\(^{-}\) and NO\(_2\)/H\(_2\)O\(_2\)/MPO (Fig. 3B). The MALDI-TOF mass spectra of the tyrosine-containing tryptic fragment corresponding to residues 188–202 of Grp75 showed a +45 mass unit ion shift from \([M+H]^+\) m/z 1694.8 to \([M+H]^+\) m/z 1739.8. Two additional peaks were also observed and most likely represent a fragmentation and/or chemical reaction of the nitrogroup during the desorption/ionization process. \([M+H]^+\) m/z 1723.8 can represent a loss of one oxygen atom from the nitrogroup and \([M+H]^+\) m/z 1709.8 can represent reduction of the nitrogroup to the amine. The same pattern of MALDI-TOF mass peaks (MH\(^+\), MH\(^+\) + 15, MH\(^+\) + 29, and MH\(^+\) + 45) was published recently for tyrosine-nitrated peptides recovered from nitrated albumin (22) and actin (23).

Protection Against Nitration in ALR Mitochondria—In addition to the ALS mice, ALR mice were used in the present report. ALS and ALR mice are inbred strains. However, in contrast to ALS mice, ALR mice do not develop diabetes after AL injection. The studies reported (17, 18) revealed that the differential ability to detoxify free radicals is an important discriminator that distinguishes these two closely related strains. ALR mice possess constitutively elevated free radical defense status and are a useful control strain to compare susceptibility to free radical-mediated damage. Fig. 4 shows Western blot analysis with anti-nitrotyrosine antibody. Comparison between ALS and ALR groups shows an obvious protection against an in vitro tyrosine nitration in the ALR mitochondria. This effect was observed (i) in both mitochondrial fractions, soluble (Fig. 4A) and membrane (Fig. 4B) and (ii) for both nitration used, ONOO\(^{-}\) and MPO-dependent. Regarding in vivo protein nitration in the diabetic conditions, AL treatment caused an appearance of several nitrated bands in mitochondria from ALS mice (Fig. 4, A and B, lane 4), whereas no nitration was detected in mitochondria from AL-treated ALR mice (Fig. 4, A and B, lane 4). The efficiency of in vitro and in vivo nitration is substantially different in our experiments. In order to visualize in vivo nitrated proteins, a longer film expo-
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Table 1

Mitochondrial proteins in nitrotyrosine immunoreactive gel spots in Figs. 1 and 2

| Spot | Protein                                      | Mass (kDa) | Peptide matches | Sequence coverage | Score (MOWSE) | Accession number (GI) |
|------|----------------------------------------------|------------|----------------|-------------------|---------------|-----------------------|
| 1    | Aconitase 2, mitochondrial                    | 85.5       | 13             | 21                | 178 [66]      | 18079339             |
| 2    | Grp75, precursor                              | 73.7       | 8              | 17                | 102 [66]      | 2119726              |
| 3    | Albumin                                      | 68.7       | 10             | 20                | 122 [66]      | 19705431             |
| 4    | Succinyl-CoA:3-oxoacid CoA transferase (SCOT) | 56.0       | 5              | 17                | 89 [66]       | 18266680             |
| 5    | Creatine kinase, sarcomeric mitochondrial precursor | 47.4 | 8 | 27 | 121 [66] | 125313 |
| 6    | Peroxiredoxin 3                              | 28.1       | 5              | 21                | 88 [62]       | 6680690              |
| 7    | Superoxide dismutase 2 (SOD2), mitochondrial precursor | 24.7 | 3 | 21 | 61 [53] | 7305511 |
| 8    | Trifunctional protein, β-subunit              | 51.4       | 11             | 25                | 165 [66]      | 20824854             |
| 9    | NADH-ubiquinone oxidoreductase, 24-kDa subunit, precursor | 27.3 | 9 | 44 | 123 [63] | 20178012 |
| 10   | Voltage-dependent anion channel 1 (VDAC-1)    | 32.3       | 7              | 46                | 137 [68]      | 6755963              |

Functional Analysis

Functional analysis of the tyrosine-nitrated proteins was performed for a SCOT and for a membrane-bound mitochondrial protein complex (Complex I). SCOT is the rate-limiting enzyme in ketolysis (21). Ketolysis occurs in the mitochondrial matrix and is the process of ketone body acetoacetate conversion to acetyl-CoA. Recently, we found that SCOT underwent tyrosine nitration in the rat heart following streptozotocin administration (14). To our knowledge, it was the first study to identify increased tyrosine nitration of a specific mitochondrial protein in a rat model of diabetes. Now, it has been confirmed using a mouse model of diabetes as well as by in vitro nitration. Using anti-SCOT antibody we found the same level of SCOT expression in ALS and ALR mitochondria (Fig. 5A). This finding permitted normalization of measured SCOT activity to total protein (Fig. 5B).

Complex I is located in the inner mitochondrial membrane. This is one of the energy metabolism enzymes that catalyzes the transport of electrons from NADH to ubiquinone for ATP synthesis. Complex I consists of more than 40 subunits (24), including a 24-kDa subunit, which was found nitrated. We were not able to obtain antibodies specific to the 24-kDa subunit of Complex I to verify expression levels of this protein in the ALS and ALR mitochondria. Instead of this, we used a method of quantitative proteomics. This approach is based on the use of light (non-deuterated) and heavy (deuterated) acrylamide to alkylate cysteine residues in proteins before their separation by two-dimensional PAGE and subsequent analysis by MALDI-TOF/MS. To estimate the relative amount of a 24-kDa subunit in ALS and ALR mitochondria, the ALS mitochondria were labeled with the light acrylamide and the ALR mitochondria were labeled with heavy acrylamide. Heavy acrylamide has 3 deuteriums instead of 3 protons and is 3 mass units heavier than light acrylamide. From the chemical point of view, there is no difference between light and heavy forms of acrylamide. This results in equal efficiency of alkylation in separate samples and, after mixing, the co-migration of the same proteins from separate samples on two-dimensional PAGE (19, 20). The 24-kDa subunit was then in-gel digested with trypsin and identified using MALDI-TOF/MS. The following quantitative profiling was accomplished by comparing a ratio between the intensities of the light and heavy mass components of the cysteine-containing peptide.

Fig. 6A shows that peptide containing two cysteine residues, YHIQVCTTTPCMRLR, from the 24-kDa subunit is represented by two isotopic envelopes that are separated by 6 mass units (3 mass units per each cysteine residue). The first isotopic envelope represents the 24-kDa subunit from ALS mitochondria. The second isotopic envelope represents the 24-kDa subunit from ALR mitochondria. ALS and ALR samples were mixed at 1:1 ratio. Because the intensities of [M + H]+ m/z 1807.78 and [M + H]+ m/z 1813.83 are equal, we concluded that the expres-
Fig. 4. Western blots of in vitro and in vivo protein nitration in the heart mitochondria from ALS and ALR mice. Control untreated (lane 1), ONOO⁻-treated (lane 2), NO₂⁻/H₂O₂/MPO-treated (lane 3), and AL-treated (lane 4) samples are shown for mitochondrial soluble (A) or membrane (B) proteins. 10 μg of each sample was separated by SDS-PAGE and probed on Western blots with a monoclonal anti-nitrotyrosine antibody. To reveal light bands in the mitochondria from AL-treated ALS mice, the longer film exposure of lane 4 is also shown for mitochondrial soluble (C) and membrane (D) proteins. The molecular mass standards in kDa are shown on the left.

Fig. 5. Nitrated of SCOT. A, Western blot analysis with anti-SCOT antibody shows similar SCOT expression in the ALS and ALR heart mitochondria. B, SCOT catalytic activities before and after indicated treatments in the ALS and ALR heart mitochondria. The data are expressed as percentage of control. All measurements were performed for n = 6 animals. *, p < 0.05 compared with the control value.

Fig. 6. Nitrated of Complex I. A, isotopic envelopes for a peptide from 24-kDa subunit that contains two cysteine residues. ALS and ALR mitochondrial pellets were alkylated with light and heavy acrylamide, respectively. It appears that a peak [M + H]⁺ (m/z 1807.78) corresponds to a tryptic peptide YHQVCTTTPCMCLR 122YHIQVCTTTPCMCLR [M + H]⁺ (m/z 1665.78) modified with light acrylamide (+ 71.08 mass unit per each cysteine residue). A peak [M + H]⁺ (m/z 1813.83) corresponds to the same peptide modified with heavy acrylamide (+ 74.08 mass unit per each cysteine residue). Relative heights of these peaks give a relative abundance of the 24-kDa subunit in the ALS and ALR mitochondria. B, Complex I catalytic activities before and after indicated treatments in the ALS and ALR heart mitochondria. The data are expressed as percentage of control. All measurements were performed for n = 6 animals. *, p < 0.05 compared with the control value.

**DISCUSSION**

It is generally accepted that oxidative stress can lead to dysfunctional mitochondria, and dysfunctional mitochondria may generate further free radicals contributing to the oxidative stress, thus accelerating cellular dysfunction in chronic and age-related diseases. Of particular relevance are reports of tyrosine nitration of mitochondrial proteins associated with oxidative stress (9–14). Our hypothesis is that nitration of mitochondrial proteins is a common mechanism by which oxi-
ative stress causes dysfunctional mitochondria. Here we compared the patterns of nitrated proteins in heart mitochondria generated with ONOO- or MPO-dependent reactions in vitro to those generated in heart mitochondria in vivo by diabetes. Because the local environment of the targeted tyrosine residue may also play a key role in determining the rate of nitration and eventually the final outcome of the reaction (16, 25), mitochondrial soluble and mitochondrial membrane proteins were analyzed separately. The two-dimensional PAGE patterns of in vitro nitrated proteins were found remarkably similar for ONOO- and MPO-dependent reactions in both soluble (Fig. 1, A and B) and membrane (Fig. 2, A and B) mitochondrial fractions. Our data are consistent with an earlier observation (26) that ONOO- and the NO2-/H2O2/MPO mixture nitrated many of the same protein bands in rat heart homogenate. This tempted us to support a conclusion (15, 16) that a single nitrating species (presumably NO2-) may be formed, which is common for both reactions. The conclusion is further supported by the notion that mitochondrial proteins from ALR mice were protected against nitration. The mitochondria have a multicomponent antioxidant defense system, and though identification of a specific component responsible for observed protection was beyond the scope of this study, the similar protection against nitration, no matter which nitrating reaction was used to cause nitration, points to a single nitrating species involved.

Comparison of the patterns of nitrated proteins generated in the diabetic mitochondria in vivo revealed some obvious similarity with in vitro nitrations for both mitochondrial fractions (Figs. 1 and 2). At least four proteins, SCOT, creatine kinase, peroxiredoxin 3, and trifunctional protein, were found nitrated in all treatments used. Several nitrotyrosine immunopositive spots, which were not identified, also have the same positions in all treatments used. Taken together, these data also suggest a common nitrating species, which may derive from different nitrating pathways and be responsible for the majority of nitrotyrosine formed. However, in addition to the strong similarities, we also observed some clear differences between in vitro and in vivo patterns, especially for membrane proteins. This presumably reflects an additional in vitro reaction(s), which was not mimicked by the in vitro treatments used in this study. Two-dimensional PAGE separation in combination with MALDI-TOF/MS identification is a powerful proteomic approach to analyze a pattern of nitrated proteins in specific conditions. However, this experimental approach also has some limitations, especially in respect to the resolution of low-abundance and hydrophobic proteins (27). There are about 2,000 proteins in mitochondria, many of which are membrane-bound or have a low molecular weight and/or have very alkaline pI/s. We were not able to match several nitrotyrosine immunopositive spots on Western blots to particular Coomassie-stained spots on gels. We also probably lost some nitrated proteins based on their properties. These experimental limitations may contribute to some inconsistency between the in vitro and in vivo studies.

Apart from the mechanism of tyrosine nitration, its biological significance is a subject of great interest. Protein tyrosine nitration alters the structure and function of proteins and may prevent tyrosine phosphorylation (1, 2, 28). In the present study, we used two closely related strains of mice, ALS and ALR, that possess different abilities to dissipate free radical-mediated adverse processes (17, 18). We demonstrate here that in vitro tyrosine nitration of SCOT or Complex I in heart mitochondria from ALS mice results in a decrease of their catalytic activity. The observation is consistent with recent publications that show the vulnerability of SCOT and Complex I to the conditions favorable for protein nitration (12, 14, 29, 30). Studies with heart mitochondria from ALR mice revealed that ALR mitochondria possess some specific features that protect proteins against tyrosine nitration. Furthermore, the protection against nitration also protects the functional properties of these proteins, at least with SCOT or Complex I. This is well correlated with in vitro data obtained for diabetic hearts from ALS and ALR mice. Although in vivo, NO2/Peroxynitrite formation and nitration of SCOT in the diabetic hearts from ALS mice, there was no nitration or inactivation of SCOT in the hearts from ALR mice. For Complex I, we observed neither significant inactivation nor nitration in the in vitro experiments performed.

In summary, the remarkable similarity between patterns of tyrosine-nitrated proteins generated in heart mitochondria under different in vitro and in vivo conditions of oxidative stress points to a common nitrating species responsible for the majority of nitrotyrosine formed. We also provide direct evidence that nitration affects properties of some mitochondrial proteins. The alterations in activity of other nitrated proteins, if any, warrant further investigation. Because specific proteins nitrated in vitro under diabetic conditions are involved in major mitochondrial functions, such as energy production (SCOT and creatine kinase), antioxidant defense (peroxiredoxin 3), and apoptosis (voltage-dependent anion channel-1), this study supports a general conclusion that nitration of mitochondrial proteins may cause dysfunctional mitochondria.

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