Increased Protein Nitration in Mitochondrial Diseases: Evidence for Vessel Wall Involvement

Gaetano Vattemi†, Yehia Mechref§, Matteo Marini†, Paola Tonin†, Pietro Minuzz†, Laura Grigoli†, Valeria Guglielm§, Iveta Klouckova§, Cristiano Chiamulera¶, Alessandra Meneguzzi¶, Marzia Di Chio¶, Vincenzo Tedesco¶, Laura Lovato†, Maurizio Degan¶, Guido Arcaro¶, Alessandro Leci¶, Milos V. Novotny§, and Giuliano Tomelleri††

Mitochondrial diseases (MD)1 are heterogeneous disorders because of impairment of respiratory chain function leading to oxidative stress. We hypothesized that in MD the vascular endothelium may be affected by increased oxidative/nitrative stress causing a reduction of nitric oxide availability. We therefore, investigated the pathobiology of vasculature in MD patients by assaying the presence of 3-nitrotyrosine in muscle biopsies followed by the proteomic identification of proteins which undergo tyrosine nitrination. We then measured the flow-mediated vasodilatation as a proof of altered nitric oxide generation/bioactivity. Here, we show that 3-nitrotyrosine staining is specifically located in the small vessels of muscle tissue and that the reaction is stronger and more evident in a significant percentage of vessels from MD patients as compared with controls. Eleven specific proteins which are nitrated under pathological conditions were identified; most of them are involved in energy metabolism and are located mainly in mitochondria. In MD patients the flow-mediated vasodilatation was reduced whereas baseline arterial diameters, blood flow velocity and endothelium-independent vasodilatation were similar to controls. The present results provide evidence that in MD the vessel wall is a target of increased oxidative/nitrative stress. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.002964, 1–10, 2011.

From the †Department of Neurological Sciences and Vision, Section of Clinical Neurology, University of Verona, Italy, ‡Department of Chemistry, Indiana University, Bloomington, Indiana, 47405, ¶Department of Biomedical and Surgical Sciences, Section of Internal Medicine, University of Verona, Italy, and ||Department of Medicine and Public Health, Section of Pharmacology, University of Verona, Verona, Italy

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1 The abbreviations used are: MD, mitochondrial diseases; mtDNA, mitochondrial DNA; RNS, reactive nitrogen species; NO, nitric oxide; 3-NT, 3-nitrotyrosine; FMD, flow-mediated vasodilatation; nNOS, neuronal nitric oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS; UEA-I, Ulex europaeus agglutinin-I; CFA, common femoral artery.

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**EXPERIMENTAL PROCEDURES**

Patients—Available muscle biopsy specimens from 16 patients with mitochondrial respiratory chain dysfunction were evaluated; the same patients were then recalled and invited to participate in this study. The characteristics of our patient population are summarized in Table I. The clinical diagnosis was based on established criteria (16). mtDNA abnormalities were identified in 11 patients; 3 had myoclonic epilepsy with ragged-red fibers (MERRF) and the A8344G mutation, 4 had mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) and the A3243G mutation, 1 had maternally inherited diabetes and deafness (MIDD) and the A3243G mutation, and 3 had chronic progressive external ophthalmoplegia (CPEO) and a single deletion. Two patients (patient 15 and 16) had histological [ragged red fibers (RRFs) and cytochrome c oxidase (COX) deficiency] and biochemical (complex IV deficiency) signs of mitochondrial myopathy (patient 12 had 5% of RRFs and COX deficiency in 30% of the fibers; patient 14 had 3% of RRFs). In these five patients, mitochondrial myopathy (patient 12 had 5% of RRFs and COX deficiency) and biochemical (complex IV deficiency) signs of mitochondrial myopathy were acquired and analyzed using the NIH Scion Image analysis program in biopsy specimens from seven patients (2, 5, 7, 8, 10, 13, and 15) and 75 controls. The comparison of the relative staining was carried out by two independent, blinded investigators. The total number of blood vessels and the number of 3-Nitrotyrosine-positive blood vessels were quantitatively analyzed in biopsy specimens from seven patients (2, 5, 7, 8, 10, 13, and 15) and 75 controls. A hallmark of these nitrogen species is the conversion of tyrosine to 3-nitrotyrosine (3-NT), whether free or part of a polypeptide chain (11, 12). This nitration of tyrosine can compromise the functional and/or structural integrity of target proteins (13–15).

We investigated the pathobiology of vasculature in MD first, by assaying the presence of 3-NT in muscle biopsies followed by the proteomic identification of proteins that undergo tyrosine nitration, and then, by measuring the flow-mediated vasodilatation (FMD) as a proof of altered NO generation/bioactivity.

**Table I**

| Patient | Sex | Age (years) | Age at onset | Clinical phenotype | mtDNA mutation |
|---------|-----|-------------|--------------|--------------------|----------------|
| 1       | M   | 44          | 40           | MERRF              | A8344G         |
| 2       | M   | 53          | 47           | MERRF              | A8344G         |
| 3       | M   | 46          | 36           | MERRF              | A8344G         |
| 4       | M   | 42          | 41           | MELAS              | A3243G         |
| 5       | M   | 30          | 18           | MELAS              | A3243G         |
| 6       | M   | 53          | 35           | MELAS              | A3243G         |
| 7       | M   | 16          | 6            | MELAS              | A3243G         |
| 8       | M   | 38          | 37           | MIDD               | A3243G         |
| 9       | F   | 50          | 46           | CPEO               | Single deletion|
| 10      | M   | 59          | 53           | CPEO               | Single deletion|
| 11      | F   | 67          | 54           | CPEO               | Single deletion|
| 12      | M   | 35          | 33           | Mitochondrial myopathy | Not yet identified |
| 13      | F   | 57          | 54           | Mitochondrial myopathy | Not yet identified |
| 14      | M   | 40          | 36           | Mitochondrial myopathy | Not yet identified |
| 15      | M   | 64          | 62           | Mitochondrial myopathy | Not yet identified |
| 16      | M   | 53          | 50           | Mitochondrial myopathya | Not yet identified |

* Mitochondrial myopathy and diabetes mellitus.

In the study that was approved by the Internal Reviewing Board of our Institution.

**Histology and Histochemistry—** Muscle samples were snap frozen in liquid nitrogen-cooled isopentane. Serial 8-μm-thick cryosections were stained with hematoxylin and eosin, modified Gomori trichrome, ATPase (pH 4.3, 4.6, and 10.4), succinate dehydrogenase (SDH), cytochrome c oxidase (COX), NADH-TR, Periodic Acid Schiff, Sudan black, and acid phosphatase.

**Immunohistochemistry and Confocal Immunofluorescence Microscopy—** Immunohistochemical studies were performed on 6.5-μm-thick transverse muscle sections using fluorescence method with a well-characterized rabbit polyclonal antibody to 3-NT (Upstate Biotechnology). Immunohistochemistry for neuronal nitric oxide synthase (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) was also done on serial 6.5-μm-thick cryosections. Postcutting fixation was not done on sections. Controls were muscle biopsies from subjects who were ultimately deemed to be free from muscle diseases.

Double immunofluorescence was performed using antibody to 3-NT and eNOS in combination with (a) a mouse monoclonal antibody to smooth muscle actin, as a marker of vascular smooth muscle cells and (b) a biotinylated *Ulex europaeus* agglutinin-I (UEA-I), as an endothelial cell marker. The reaction was examined by confocal fluorescence microscope (LSM510, Zeiss).

To control staining specificity the primary antibody was omitted or replaced with nonimmune sera at the same concentration. Two independent, blinded investigators evaluated the relative staining.

**Quantification of 3-Nitrotyrosine-positive Blood Vessels—** For quantification studies, double immunofluorescence was performed with antibody to 3-NT in combination with biotinylated UEA-I, as described. The total number of blood vessels and the number of 3-NT-positive blood vessels were quantitatively analyzed in biopsy specimens from seven patients (2, 5, 7, 8, 10, 13, and 15) and 75 controls. The comparison of the relative staining was carried out by two independent, blinded investigators.

**Measurement of eNOS-positive Vessel Wall—** For quantification studies, digital photomicrographs of eNOS-positive blood vessels were acquired and analyzed using the NIH Scion Image analysis program in biopsy specimens from seven patients (see previous section) and seven controls that were matched for age, sex, and biopsied muscle. The total area of the vessel wall and the area of the eNOS-positive vessel wall were blindly measured for each subject.
(n = 2–9 slides/subject). The ratio between the eNOS immunoreactive area and the total area was obtained for each vessel wall by dividing these values.

**Assay of NOS Activity**—NOS activity assay was performed by measuring (3H) L-arginine (Amersham Biosciences) to (3H) L-citrulline conversion with a NOS assay kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer’s instructions. Muscle biopsies from eight patients (2, 5, 7, 8, 9, 10, 13, and 15) and 11 controls who were matched for age, sex, and biopsied muscle were evaluated. All measurements of NOS activity were normalized for total proteins. Protein concentration was determined with the Bradford method.

Muscle tissue was homogenized with a lysis buffer (Tris-HCl 25 mM, EDTA 1 mM, EGTA 1 mM). For the nNOS and eNOS determination the supernatants were incubated for 60 min at 37 °C in a reaction buffer (Tris-HCl 25 mM, tetrahydrobiopterin 5 μM, flavin adenine mononucleotide 1 μM, flavin adenine dinucleotide 1 μM), and in the presence of NADPH 10 mM, (3H) L-arginine 50μCi/μl, and CaCl2 6 mM. As the inducible isoform of NOS is calcium-independent, the determination of iNOS activity was performed in the absence of CaCl2. The reaction was stopped with 10 mM of phosphoric acid and the equilibrated, which specifically binds to arginine, was added to the sample reaction. Following centrifugation,3H L-citrulline content in the eluate was quantified in a β-counter (Beckmann). Control reactions were performed with iNOS and nNOS positive controls and with L-MNNA for negative controls.

**SDS-PAGE Electrophoresis, Two-dimensional Gel Electrophoresis and Immunoblot Analysis**—The expression of nNOS and eNOS was evaluated by one-dimensional immunoblotting as described (15). Coomassie Blue staining was used to assess protein loading. Bands were quantified densitometrically using the Quantity One software (Bio-Rad).

The presence of nitrated proteins was investigated by two-dimensional immunoblot. For two-dimensional gel electrophoresis, frozen human muscle was homogenized in lysis buffer (7 M urea, 2 M thiourea, 4% w/v 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid, 40 mM Tris-base, and 65 mM dithioerythritol). Protein load was 60 μg for analytical gels and 120 μg for gels to transfer. The IEF (first dimension) was carried out on nonlinear wide-range immobilized pH gradients (pH 3–10; 18 cm long IPG strips) and the second dimension on 6–18% T polyacrylamide linear gradient gels. Analytical gels were stained with ammonical silver nitrate. Gels to transfer were blotted on 0.2 μm nitrocellulose membranes (BioRad) for 16 h (Trans-Blot Cell; BioRad, Hercules, CA), and stained with 0.2% Ponceau S. Protein spots were excised from two-dimensional gels, destained in 25 mM ammonium bicarbonate, 40% ethanol, and washed with acetonitrile. Trypsin digestion was performed according to our previously published procedure (19). Briefly, gel pieces were placed in Eppendorf tubes and cut into smaller (less than 1 mm in each dimension) pieces. Gel pieces were covered with 200 μl of 200 mM ammonium bicarbonate buffer prepared in 40% acetonitrile and incubated at 37 °C for 30 min to destain spots. The solution was then removed from the tube and discarded. Next, gel pieces were completely dehydrated by drying in an Eppendorf Vacufuge concentrator (Brinkmann Instruments, Westbury, NY) for 20 mins. Twenty microliters of 20 μg/ml trypsin solution (in 36 mM ammonium bicarbonate, 9% acetonitrile) were then added to the dried gel pieces. After gel pieces were rehydrated, 50 μl of 40 mM ammonium bicarbonate prepared in 9% acetonitrile were added. Samples were incubated for 18 h at 37 °C prior to removing the liquid from gel pieces and transferring to a new prewashed tube. Trypsin digests (−70 μl) were dried in the Vacufuge concentrator and reconstituted in 10 μl of water. These solutions were immediately subjected to LC-MS/MS analysis.

Six microvolumes of trypsin digested gel pieces were loaded on a PepMap300 C18 cartridge (5 μm, 300 Å, Dionex, Sunnyvale, CA) then separated on an in-house packed 150 mm × 75 μm i.d. pulled-tip nano-column packed with C12 Jupiter Proto-90 Å (Phenomenex, Torrance, CA). Sample elution was attained using a 15-min gradient from 100% to 65% solvent A, 97:3:0.1 water/acetoni/tric Imam/acidic solvent B was 0.1% formic acid in acetonitrile) at 250 nl/min using an Ultimate 3000 nano-LC system (Dionex, Sunnyvale, CA). From the end of the column, ions were electrosprayed directly to an linear trap quadrupole (LTQ) FT mass spectrometer (ThermoElectron, San Jose, CA), which recorded mass spectra and data-dependent tandem mass spectra of the peptide ions. MSMS spectra were searched against protein sequences for Homo sapiens in the Swiss-Prot database using a licensed copy of MASCOT for peptide sequence determination and subsequent protein identification. A protein was reported to be found in a spot only when three or more peptides obtained a score at or above the “identity or extensive homology” score (95% confidence).

The score for an MS/MS match is based on the absolute probability (P) that the observed match between the experimental data and the database sequence is a random event. Ions score is −10Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 30 indicate identity or extensive homology (P < 0.05). Also, Expect which is the number of times we would expect to obtain an equal or higher score, purely by chance was employed. The lower this expectation value is, the more significant the results are.

Peak list was generated using our in-house developed software (TurboRAW2Mg). This utility, described in details in Reference 20, is available as part of ProtQuant, which can be acquired freely from http://nccg.indiana.edu. The parameters used to extract peaks in this utility are MW 600–45,000; absolute total ion intensity threshold 100; minimum ion count 5; retention time tolerance (min) 1; precursor tolerance (ppm) 20; and peak tolerance (ppm), 200. The peak list generated using this utility was searched using MASCOT 2.0 search engine employing trypsin as the enzyme with 2 possible missed cleavages. Because the samples were reduced and alkylated, carbamidomethyl fix modification of cystine residues was used. No variable modification was used. Mass tolerance of precursor ion was set to 2 Da, whereas that of the fragment ion was set to 0.8 Da. Uniprot-sprot database released on September 10, 2008 was used with Homo sapiens subset of Uniprot-sprot which has 20328 sequences.

**Immunoprecipitation and Immunoblotting**—To validate the correct identification of nitrated proteins, immunoprecipitation of the putative modified proteins including vimentin, desmin, ATP synthase β-chain subunit, protein disulfide-isomerase A3, voltage-dependent anion channel 1 and MnSOD, and Western blotting with 3-NT antibody were performed. Briefly, frozen muscles were homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA, 0.5% deoxycholic acid, pH 8.0, with protease inhibitors).
Aliquots corresponding to 0.5 mg of protein were first precleared by incubation with protein A-agarose (Roche Applied Science) for 1 h at 4 °C and then incubated with the relevant antibody overnight at 4 °C on an orbital shaker. Protein A-agarose was added for 3 h at 4 °C and following centrifugation, the immunoprecipitated material was washed three times with lysis buffer. Following addition of two-dimensional buffer, proteins were resolved by two-dimensional gel electrophoresis, blotted on nitrocellulose membrane, and then incubated with the antibody to 3-NT. Proteins were detected as previously described.

Flow-mediated Vasodilatation and Echo-Doppler Analysis—Endothelial function was investigated according to the model of FMD using a high-resolution ultrasound echo Doppler (Esaote AU5) with a 7.5 MHz linear transducer. The axial resolution of this probe was 0.05 mm and ultrasonic calipers accurate to 0.05. Each patient was investigated in a controlled environment at 22 °C. After patients rested for 15 min in the supine position, systolic, diastolic, mean blood pressure, and heart rate were determined every 2 min by an oscillometric recorder (Dinamap, model 845, Critikon, Tampa, FL) positioned on the right arm. To assess the basal values of each hemodynamic test, we considered the average of three blood pressure determinations and heart rate recordings. We measured common femoral (CFA) and brachial (BA) artery diameters and flow velocity at a fixed position: 1 cm before CFA bifurcation and 1 cm above the elbow fold. Hemodynamic measurements, related to FMD, were obtained 30 s and 1, 2, 4, 6, and 8 min after the beginning of distal hyperemia and were related to the flow velocity increase at the level of investigated arteries. Thirty minutes after, endothelium-independent vasodilatation (i.e. induced by a nitric oxide donor) was tested; changes in brachial and femoral artery diameter were recorded 3 and 5 min following the sublingual administration of nitroglycerin (300 μg).

Diameter variations were expressed as percentage of the basal diameter. The area under the curve was calculated with the trapezoidal method (21). Two observers carried out echo-doppler investigations and hemodynamic measurements.

Echo-Doppler analysis of the carotid arteries was performed to evaluate the presence of atherosclerotic lesions and the intima-media thickness.

Statistical Analysis—The χ-square test was used for the comparisons of 3-NT in blood vessels of patients and controls. The Student’s t test for unpaired comparison was used for ratio values of eNOS-positive vessel wall. One-way ANOVA was used to compare two variables in patients and controls when indicated. A p value of 5% or less was considered as statistically significant.

RESULTS

Immunohistochemistry and Confocal Fluorescence Microscopy—Nitrotyrosine was detected in the wall of small blood vessels (inner diameter ranging from 5 to 100 μm), which were located within the interfascicular septa, from the patients’ muscle biopsy whereas no staining was observed in the examined capillaries (Fig. 1). A partial colocalization of 3-NT was observed to endothelium (labeled with UEA-1), as well as to smooth muscle cells (labeled with actin) of blood vessels, (Fig. 1). 3-NT staining was stronger in the patients than in controls.
and a quantitative analysis, performed in 7 out of 16 patients, showed that nitrotyrosine positive vessels were significantly different in patients and controls, thus providing evidence of increased RNS production (Table II). The striking data was the similar, or even higher, percentage of 3-NT positive vessels in the younger patients as compared with our controls over 70. In the oldest patients this percentage reached almost 65, three times more than what observed in aged-matched controls.

The nNOS immunoreactivity was restricted to the sarcolemma of the muscle fibers and the staining was comparable in patients and controls (data not shown). Immunolabeling for eNOS was observed in the vessel wall from the patients’ and controls’ muscle biopsy. However, the staining was stronger in the patients than in controls (Fig. 2) and the image analysis of eNOS-positive blood vessels, performed in 7 out of 16 patients, revealed that the stained area for eNOS—expressed as the ratio of the eNOS immunoreac-

| Age (year) range | Patients | | | | Controls | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Subjects (n) | Total vessels (n) | NT+ vessels (n) | NT-vessels (n) | NT+ vessels (%) | Subjects (n) | Total vessels (n) | NT+ vessels (n) | NT-vessels (n) | NT+ vessels (%) | p value |
| 0–9 | 8 | 174 | 6 | 168 | 3.4 |
| 10–19 | 11 | 206 | 10 | 196 | 4.8 | <0.001 |
| 20–29 | 11 | 207 | 10 | 197 | 4.8 |
| 30–39 | 11 | 266 | 13 | 253 | 4.9 | <0.001 |
| 40–49 | 11 | 287 | 37 | 250 | 12.9 |
| 50–59 | 9 | 236 | 39 | 197 | 16.5 | <0.001 |
| 60–69 | 10 | 322 | 59 | 263 | 18.3 | <0.001 |
| >70 | 4 | 117 | 40 | 77 | 34.2 |

**Table II**
Quantification of 3-nitrotyrosine-positive (NT+) blood vessels. The total number of blood vessels and the number of 3-nitrotyrosine-positive (NT+) and 3-nitrotyrosine-negative (NT-) blood vessels were quantitatively analyzed in biopsy specimens from seven patients and seventy-five control subjects.

**Fig. 2.** Immunofluorescence images of eNOS-positive blood vessels. A, and B, showing the stronger staining for eNOS in the vessel wall from a patient with MELAS (patient 4) as compared with two pair-matched control subjects (C, and D).
tive area out of the total area of each vessel—was significantly higher in the patients than in controls (Fig. 3), thus suggesting an increased expression of the enzyme.

Immunostaining for iNOS largely produced no positive signal in either control or patient blood vessels. A positive staining for iNOS was seen in a few blood vessels from patients with MELAS (Fig. 4).

**SDS-PAGE Electrophoresis and Immunoblot Analysis**—By immunoblotting, eNOS was increased in the patients’ muscles as compared with controls, whereas the expression of nNOS was similar in patient and control muscles (Fig. 5).

**Assay of NOS Activity**—Total NOS activity, the calcium-dependent activity of the constitutive isoforms of NOS (eNOS and nNOS) as well as the calcium-independent activity of iNOS, were increased in the patients’ muscle biopsies compared with control muscles (Fig. 6). However, the differences were not statistically significant.

**Identification of Specific Nitrated Proteins in MD Patients**—By two-dimensional gel electrophoresis followed by immunoblot analysis with antibody to 3-nitrotyrosine, 11 immunoreactive spots were identified exclusively in the patients as compared with matched controls (Fig. 7A). The spots that were still present in the blot after reducing treatment with sodium dithionite were considered false positive. The 11 spots specifically observed in MD were identified by mass spectrometry analysis as listed in Table III (see also supplemental Table 1).

Immunoprecipitation of six (vimentin, desmin, ATP synthase β-chain subunit, protein disulfide-isomerase A3, volt-
age-dependent anion channel 1, and MnSOD) out of the 11 proteins and Western blotting with the antibody to 3-NT revealed expected spots corresponding to the above proteins in MD patients’ muscle, but not in the controls’ muscle (Fig. 7B). These results ensure that the proteins identified by mass spectrometry are indeed the ones nitrated.

**Flow-mediated Vasodilatation and Echo-Doppler Analysis**—A statistically significant difference in FMD (AUC, minutes 0–8) of BA and CFA was observed between patients and controls whereas no differences in endothelium-independent vasodilatation (AUC, minutes 0–5) were present (Fig. 8). The arterial diameters at baseline and the increase in blood flow-velocity were similar in patients and controls, thus indicating that differences were not related to variations of baseline vascular geometry and driving force for endothelium-dependent vasodilatation (Table IV). Blood pressure and heart rate, measured every 2 mins throughout the hemodynamic tests, did not vary significantly within...
DISCUSSION

The major result from the present study is the recognition of the vessel wall as a target of oxidative and nitrative stress in patients affected with mitochondrial respiratory chain dysfunction. In addition, our data indicate that increased oxidative and nitrative stress may reduce NO bioactivity in the vasculature of MD patients.

We identified 3-NT immunoreactivity in the small blood vessels of MD patients regardless from the clinical phenotype and the genetic mutation, and the amount of 3-NT was significantly higher in the patients’ muscle samples as compared with control muscles. The increased 3-NT expression, observed by immunohistochemistry and confocal immunofluorescence microscopy in the muscle biopsies from all the studied patients, was specifically located in the endothelium and smooth muscle cells of the vessel wall. 3-NT results from nitration of free tyrosine or protein tyrosine residues through one of the two relevant nitration pathways that operate in vivo, namely peroxynitrite and heme peroxidase-dependent nitration (11, 12). Therefore, 3-NT accumulation reflects a loss of balance between oxidant formation and antioxidant defense mechanisms, known as oxidative and nitrosative stress (22, 23).

After showing the increased levels of nitrotyrosine, we performed a detailed proteomic analysis on muscle biopsy from our MD patients in order to characterize the proteins that undergo a tyrosine nitration. We identified 11 specific proteins that are nitrated under pathological conditions and that can be grouped into classes based on their recognized functions: energy metabolism, cytoskeletal, chaperone, antioxidant, and ion channel (Table II).

### Table III

| Protein Function         | Spot | Protein                                           | Accession number |
|--------------------------|------|---------------------------------------------------|------------------|
| ENERGY METABOLISM        | 8    | ACONITATE HYDRATASE                               | Q99798           |
|                          | 3    | ATP SYNTHASE β-CHAIN SUBUNIT                      | P06576           |
|                          | 6    | PYRUVATE DEHYDROGENASE E1 COMPONENT SUBUNIT BETA  | P11177           |
|                          | 7    | SUCCINATE DEHYDROGENASE [UBIQUINONE] FLAVOPROTEIN SUBUNIT | P31040         |
|                          | 5    | UBIQUINOL-CYTOCHROME-C REDUCTASE COMPLEX CORE PROTEIN 1 | P31930         |
|                          | 9    | CREATIN KINASE                                    | P06732           |
| CYTOSKELETAL             | 2    | DESMIN                                            | P17661           |
|                          | 1    | VIMENTIN                                          | P08670           |
| CHAPERONE                | 4    | PROTEIN DISULFIDE-ISOMERASE A3                    | P30101           |
| ANTIOXIDANT              | 11   | MnSOD                                             | Q6LEN1           |
| ION CHANNEL              | 10   | VOLTAGE-DEPENDENT ANION CHANNEL 1                 | (P21796)         |

### Table IV

| VARIABLE | Median (range) | Subjects with MD (n = 16) | Control Subjects (n = 16) |
|----------|----------------|---------------------------|---------------------------|
| Basal arterial diameter (mm) | 4.20 (3.10–5.20) | 4.20 (3.50–5.20) |
| Increase in blood flow velocity (%) | 129 (8–1000) | 156 (15–531) |

| VARIABLE | Median (range) | Subjects with MDs (n = 16) | Control Subjects (n = 16) |
|----------|----------------|-----------------------------|---------------------------|
| Basal arterial diameter (mm) | 7.50 (5.60–10.20) | 8.20 (5.60–10.90) |
| Increase in blood flow velocity (%) | 178 (-21–1160) | 163 (-6–940) |

FIG. 8. Flow-mediated vasodilatation and endothelium-independent vasodilatation of brachial and common femoral artery. Box and whisker plots show the percentage increase in arterial diameters as a function of time (area under the curve, AUC) induced by distal hyperemia (flow-mediated vasodilatation) and by the sublingual administration of nitroglycerin. Data are from 16 patients with mitochondrial diseases and 16 pair-matched controls.

Each subject (data not shown). No differences were observed between patients and controls for the prevalence of carotid artery stenosis or increased intima/media thickness.
of acute oxidative stress the nitration of mitochondrial pro-
teins is a reversible dynamic process, because of a mech-
anism not yet identified, thus suggesting that nitration might
be a cellular signaling mechanism (26, 27).

The identification of several proteins that have an impor-
tant role in energy metabolism offers, therefore, a potential
rationale for the biological dysfunction of blood vessels in
MD patients. Tyrosine nitration has been detected in several
pathological conditions including neurodegenerative and in-
flammatory diseases (28). The biological significance of
modifying tyrosine residues by nitration is under investiga-
tion, with several potential consequences such as altera-
tions in the secondary structure, function, and proteolytic
removal, which could deeply modulate the cellular function
(13–15). Published data strongly indicate the utility of nitro-
tyrosine as a potential biological marker to determine risk
associations with cardiovascular diseases (29).

After showing the increased levels of nitrotyrosine and the
presence of specific nitrated proteins in MDs, we addressed
the relevance of nitric oxide synthase enzymes in the produc-
tion of nitric oxide (NO). NO generation is catalyzed by three
distinct isoforms of nitric oxide synthase (NOS) (30). Two
isoforms are constitutively expressed, one in endothelial cells
eNOS) and the other in the brain (nNOS) and both are calci-
um-dependent; the third isoform, inducible (iNOS), is regu-
lated primarily at the transcriptional level and is calcium-
independent (30). Via immunoblot analysis we documented an
up-regulation of eNOS in muscle biopsies of MD patients and
by confocal immunofluorescence microscopy we demonstrat-
ed its location specifically in the vessel wall. The distri-
bution of eNOS-positive immunostaining was similar in pa-
tients and controls. The image analysis of eNOS-positive
blood vessels, performed in muscle biopsy from seven con-
trols and seven MD patients with clinical heterogeneous pres-
entation using the NIH Scion Image analysis program,
showed that eNOS-positive area was significantly higher in
the patients. These data confirm the increased expression of
the protein as demonstrated by immunoblot and support the
view that an overactivation of eNOS could occur and be
responsible for the increased levels of nitric oxide. Unlike
eNOS, we did not detect in blood vessels any significant
abnormal immunoreactivity of the other two isoforms of NOS
including nNOS and iNOS; nNOS expression was similar in
the patients’ and in controls’ muscle thus suggesting that
these enzymes are not casually involved in the production of
nitrating species. Unfortunately, the data on quantitative
measurement of eNOS activity are not so straightforward.
The increased activity of the total and of the constitutive isoforms
of NOS in MD patients’ muscle was not statistically significant
in contrast with the clear demonstration of an increased ex-
pression. This discrepancy may be because of the biochemical
assay that does not distinguish the activities of the two
constitutive isoforms of NOS. Alternatively, the uncoupling of
eNOS resulting from the oxidative and nitrative stress could
lead to a significant impairment of NO production which, in
turn, could concur to injure the flow mediated vasodilatation.

Once we proved that in MDs the vessel walls are involved
in oxidative and nitrative stress, we evaluated the endothe-
lial function in vivo by measuring FMD in the same MD
patients. This noninvasive functional test has been used to
investigate the release and bioactivity of NO in the periph-
eral vasculature, because vasodilatation is specifically pre-
vented by the administration of endothelial NO synthase
inhibitors (31).

The reduced FMD observed in these patients is accom-
panied by a preserved vasodilatory response to the sub-
lingual administration of nitroglycerin, thus indicating that
structural changes in the vascular wall or reduced relaxant
capacity of smooth muscle cells are unlikely responsible for
the altered vascular response. Therefore, we hypothesize
that the decreased NO bioactivity, because of the formation
of reactive nitrogen species, provide an adequate explana-
tion of our findings and the nitrotyrosine accumulation in
the blood vessels of these MD patients strongly sustains this
view (11, 12).

The increased oxidative and nitrative stress and the mod-
ified vascular function in MD may be of relevance for the
pathophysiology of these disorders and may also give clues
for the understanding of the biochemical mechanism impli-
cated in endothelial dysfunction. A blunted or absent in-
crease in shear stress and NO-induced vasodilatation in
peripheral arteries may result in increased vascular resis-
tance and inadequate blood supply, further worsening the
energetic imbalance in skeletal muscle because of ineffec-
tive ATP generation. Recent data from experimental models
indicate that mitochondrial dysfunction is associated with
vascular alterations including increased blood pres-
sure, atherosclerosis, and premature aging (32, 33). More-
over, mtDNA mutations have been associated with in-
creased blood pressure also in humans (34–37). The
accumulation of nitrotyrosine, not only within the vascular
wall of MD patients, but also, to some extent, in the small
vessels from all, but the youngest control subjects, sug-
gests that mitochondrial dysfunction may anticipate the
unavoidable fate of age- and oxidation-related dysfunc-
tional endothelium that is observed in the general popula-
tion and in experimental models (13, 38, 39).

This article contains supplemental Table S1.
†† To whom correspondence should be addressed: Department of
Neurological Sciences and Vision, Section of Clinical Neurology, Poli-
clinico G.B. Rossi, P.le L.A. Scuro 10, 37134 Verona. Phone: +39-
045-8124461; Fax: +39-045-585933; E-mail: giuliano.tomelleri@
univr.it.

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