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Mitochondrial Proteomics in Experimental Autoimmune Uveitis Oxidative Stress

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Abstract

Purpose—Photoreceptor mitochondrial oxidative stress is the initial pathologic event in experimental autoimmune uveitis. In this study, the authors determined alterations in retinal mitochondrial protein levels in response to oxidative stress during the early phase of experimental autoimmune uveitis (EAU).

Methods—Retinal mitochondrial fractions during early EAU were prepared and subjected to two-dimensional difference in gel electrophoresis (2D-DIGE). Protein spots showing differential expression were excised and subjected to matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) for peptide identification. Levels of these proteins were also confirmed by Western blot analysis. mRNA expression of these proteins was confirmed by real-time PCR. TUNEL staining was performed to detect apoptosis.

Results—2D-DIGE analysis revealed differential expression of 13 proteins. Ten proteins were overexpressed, including manganese-SOD, α A crystallin, β crystallin, and four proteins were downregulated, including adenosine triphosphate (ATP) synthase, malate dehydrogenase, and calretinin. Increased levels of α A crystallin, β B2 crystallin, MnSOD, and aconitase and decreased levels of ATP synthase were confirmed by Western blot analysis. qPCR also confirmed the increased expression of α A crystallin, β B2 crystallin, MnSOD, and Hsp70. Apoptosis was absent during this phase.

Conclusions—The presence of mitochondrial-specific oxidative stress-related proteins in the early EAU retina along with the downregulation of ATP synthase provides early evidence of stress-related retinal damage. The presence of high levels of α A and β B2 crystallin in the mitochondria may prevent cell death during early EAU.

The experimental autoimmune uveitis (EAU) model has been used extensively to study the immune mechanism and to delineate the tissue damage associated with intraocular inflammation. Although the damage has been attributed to inflammatory cell infiltration, early pathologic changes in the retina occur before leukocytic cell infiltration of the retina and uvea, and the damage appears to be mediated by oxidative stress.^{1–4} The oxidative stress is associated with overexpression of iNOS in the photoreceptor mitochondria.² Peroxynitrite-mediated nitration of the photoreceptor mitochondrial proteins suggests that mitochondrial oxidative stress is the initial event for the retinal damage and amplification of inflammatory processes.¹ Moreover, the retinal DNA damage restricted to mitochondria in the early phase of uveitis supports the role of mitochondrial oxidative stress in retinal damage.⁴

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As a source of reactive oxygen and nitrogen species and also as a primary target of oxidative stress, the mitochondria are highly susceptible to stress-mediated damage. Oxidative and nitrosative stress can alter the functions of the mitochondria by nitrating their proteins, thereby leading to apoptosis of the cells.⁵ However, it is unclear whether mitochondrial oxidative stress modulates the mitochondrial proteins during early EAU. Determination of mitochondrial protein levels can provide insight into the mechanism by which stress alters the functions of the mitochondria in EAU. This can be accomplished using a novel two-dimensional difference in gel electrophoresis (2D-DIGE) in combination with matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS).^{6,7}

In the present study, we determined mitochondrial protein alterations in early EAU retina using the proteomic approach of 2D-DIGE combined with MALDI-TOF MS. We identified significant differences in mitochondrial protein levels between control and EAU retinas. The most notable difference was the suppression of adenosine triphosphate (ATP) synthase despite the upregulation of antioxidant proteins/enzymes and crystallins in early EAU.

Materials and Methods

Induction of EAU

Animal care and use were in compliance with institutional guidelines and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. EAU was induced in 8-week-old B10.RIII mice (Jackson Laboratory, Bar Harbor, ME). Interphotoreceptor retinoid-binding protein (IRBP) peptide, SGIPYIISYLHPGNTILHVD, 25 μ g in phosphate-buffered saline, was emulsified 1:1 vol/vol with Freund's complete adjuvant supplemented with *Mycobacterium tuberculosis* strain H37RA to 2.5 mg/mL. A total of 300 μ L emulsion was injected subcutaneously in each of three sites: base of tail and both thighs (EAU group). The control group consisted of B10.RIII mice injected with normal saline.

Isolation of Retinal Mitochondria

Retinas were isolated from two groups of 48 B10.RIII mice. Each group consisted of 12 day 7 EAU mice and 12 noninjected control mice. The retinas were pooled in each group and were used to separate mitochondrial proteins from cytosolic proteins. Mitochondria were isolated (Mitochondria/Cytosol Fractionation Kit; BioVision Inc., Mountain View, CA),⁸ and suspensions were observed under a microscope to check the efficiency of homogenization. A shiny ring around the cell indicated that it was intact. Thirty-five strokes with a Dounce homogenizer resulted in 90% lysis of the cells. The lysate was spun first for 10 minutes at 700g to remove cellular debris and then at 10,000g for 30 minutes to pellet the mitochondria. The resultant supernatant was saved as the cytosol portion, and the pellet, containing whole mitochondria, was lysed with a mitochondria-specific buffer supplied with the kit. The purity of the fractions was checked by Western blot analysis with a polyclonal antibody against prohibitin, a mitochondrial marker, and the cytoplasmic proteins caspase 3 (Abcam, Cambridge, MA) and calpain (Biovision Inc.). The mitochondrial protein from the control and experimental samples were then subjected to 2D-DIGE analysis.

Two-Dimensional Difference in Gel Electrophoresis

2D-DIGE analysis was carried out by Applied Biomix (Hayward, CA). Briefly, protein assay was accomplished with the use of a protein assay method (Bio-Rad Laboratories, Hercules, CA). Equal quantities of protein from experimental and control retinal mitochondrial samples (3–8 mg/mL) were diluted with the sample 2D cell lysis buffer.

Fluorescent Dye Labeling

Briefly, 30 μ g protein lysate of either sample (control or early EAU retinal mitochondria) was labeled with 1 μ L diluted Cy3 or Cy5 (1:5 diluted with dimethylformamide from 1 nmol/ μ L stock), and the same amount of the pooled standard containing equal amounts of the two samples was labeled with Cy2. The internal standard was used to normalize the Cy3 and Cy5 samples and to compare other gels in the experiments. Each sample was mixed well by vortexing and then kept in the dark on ice for 30 minutes. One microliter of 10 mM lysine was added to each of the samples for quenching. The resultant samples were mixed well by vortexing and kept in the dark on ice for an additional 15 minutes. The three labeled samples were combined (90 μ g) and diluted with 2 $\times$ 2D sample buffer (8 M urea, 4% CHAPS, 20 mg/mL dithiothreitol [DTT], 2% ampholytes (Pharmalytes; GE Health-care, Waukesha, WI), and a trace amount of bromophenol blue). The immobilized pH gradient strips (linear range, 13 cm; pH 3–10) were rehydrated overnight with 100 μ L destreak solution and rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/mL DTT, 1% ampholytes [Pharmalytes; GE Healthcare], and a trace amount of bromophenol blue).

Isoelectric Focusing and Second-Dimensional SDS-PAGE

After the labeled samples were loaded into the strip holder, the 13-cm strip was placed face down, and 1 mL mineral oil was added on top of it. The manufacturer's (Amersham Biosciences, Pittsburgh, PA) protocol was followed, and isoelectric focusing was run in the dark at 20°C. After isoelectric focusing, immobilized pH gradient (IPG) strips were equilibrated in freshly made equilibration buffer 1 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, a trace amount of bromophenol blue, and 10 mg/mL DTT) for 15 minutes with slow shaking, then rinsed in freshly made equilibration buffer 2 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, a trace amount of bromophenol blue, and 45 mg/mL DTT) for 10 minutes with slow shaking. The IPG strips were then rinsed once in the SDS-gel running buffer before they were transferred to gradient SDS-gel (9%–12% SDS-gel prepared using low fluorescence glass plates) and sealed with 0.5% (wt/vol) agarose solution (in SDS-gel running buffer). The SDS-gels were run at 15°C and stopped until the dye front reached the end of the gel.

Image Scan and Data Analysis

Image scanning was carried out immediately after SDS-PAGE on a variable mode imager (Typhoon TRIO; Amersham Biosciences, Piscataway, NJ) according to the manufacturer's recommendations. Cy2-, Cy3-, and Cy5-labeled images of each gel were acquired using excitation/emission values of 488/520, 523/580, and 633/670 nm, respectively. The scanned images were then analyzed (Image Quant software, version 5.0; Amersham Biosciences). Protein spot abundance and statistics were performed automatically using extended data analysis software (DeCyder software, version 6.0; Amersham Biosciences) from the two control and EAU samples. Spot detection was carried out automatically using a differential in-gel analysis module. The ratio change of the protein differential expression was obtained from in-gel software analysis (DeCyder; Amersham Biosciences). Proteins from the EAU samples, which showed an increase of more than 20% from the control and those which showed a 20% decrease, were considered significant and were selected for further MALDI-TOF/MS studies.

Spot Picking and Digestion and Mass Spectrometry

Differentially expressed selected protein spots were picked up (Ettan Spot Picker; Amersham Biosciences) after the data analysis (DeCyder software; Amersham Biosciences) and spot picking design. The selected protein spots were subjected to in-gel trypsin digestion, peptide extraction, and desalting, followed by mass spectrometry (MALDI-TOF/MS) analysis to

determine the protein identity. MALDI-TOF/MS analysis was carried out by Applied Biosystems (Foster City, CA).

Western Blot Analysis

Western blot analysis was performed to confirm the results obtained from 2D-DIGE on some of the important and significantly increased or decreased proteins in the retinal mitochondria of EAU mice, which were important and relevant to mitochondrial oxidative stress. The proteins upregulated in 2D-DIGE analyses— α A crystallin, β B2 crystallin, MnSOD, and aconitase 2—and the downregulated protein ATP synthase was chosen for the study. Retinas were isolated from two groups of 12 B10.RIII mice induced with EAU and another two groups of 12 mice serving as noninjected controls. Retinas were pooled from each group for the study. The cytosol and mitochondrial fractions were extracted as in DIGE, as described.

Because α A and β B2 crystallin are mainly cytoplasmic proteins, equal amounts of cytosolic and mitochondrial fractions (50 μ g) were resolved on a Tris-HCl polyacrylamide gels (Ready gels; Bio-Rad Laboratories) at 120 V. For MnSOD and aconitase 2 protein analysis (given that they are both mitochondria-specific proteins), only the mitochondrial fractions were analyzed. After electrophoresis separation, proteins were transferred to polyvinylidene difluoride blotting membranes (Millipore, Billerica, MA). The membranes were blocked in 5% milk in Tris-buffered saline Tween-20 for 1 hour. Membranes were probed with rabbit polyclonal anti- α A crystallin (1:1000; Stressgen, Ann Harbor, MI) mouse monoclonal to β crystallin (1:1000; Stressgen) rabbit polyclonal anti-MnSOD and anti-aconitase 2 and mouse polyclonal ATP synthase C (1:1000; Abcam) overnight at 4°C. After 30-minute incubation with the secondary antibody tagged with horseradish peroxidase, signals were detected with an enhanced chemiluminescence system (Amersham, Cleveland, OH). Membranes were then stripped and reprobed for β -actin (Sigma, St. Louis, MO). Protein band intensity was measured by image densitometry software (Image; Scion, Frederick, MD). The data presented are mean $\pm$ SEM.

Real-Time PCR

Quantitative real-time PCR (iCycler; Bio-Rad Laboratories) was performed to quantitate the gene expression of the proteins differentially expressed by 2D-DIGE. Some of the important proteins associated with oxidative stress, such as MnSOD, α A crystallin, β B2 crystallin, and Hsp70, were selected for the assay. Retinas were isolated from three groups of B10.RIII mice, each consisting of six controls and six mice induced with EAU on day 7 after immunization, as described. The retinas were pooled together from each group, the RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), and the cDNA template was generated (Omniscript RT kit; Qiagen, Valencia, CA). Each 25- μ L PCR reaction mixture contained a master mix (SYBR Green I; Bio-Rad Laboratories), 0.5 μ M gene-specific primers for MnSOD, α A crystallin, β B2 crystallin, Hsp70, and the cDNA template. GAPDH was used as the normalizing gene because it was constant in our experimental conditions. The sequences of the primers for α A and β B2 crystallins are shown in Table 1. Primers of MnSOD and Hsp70 were obtained from Superarray Biosciences (Frederick, MD). PCR reactions were performed in triplicate, along with GAPDH. The specificity of the PCR amplification products was checked with dissociation melting-curve analysis.

The threshold cycle (Ct) difference between the experimental and control groups was calculated and normalized to GAPDH, and the increase (x -fold) in mRNA expression was determined by the $2^{-\Delta\Delta C_t}$ method.⁹ Statistical analysis of $\Delta\Delta C_t$ was performed with a Student's t -test for three independent samples, with significance set as $P < 0.05$, and was compared between the EAU and control groups.

Detection of Apoptosis in Early EAU Retina

EAU was induced in six B10.RIII mice, as described. Six mice injected with saline were used as controls. The mice were killed on day 7 after injection, and eyes were fixed in 4% formalin and embedded in paraffin. Five micrometers of sections of 12 early EAU eyes and 12 control eyes were taken to determine the pupillary-optic nerve plane and were stained for terminal transferase dUTP nick end labeling (TUNEL) staining. The TUNEL procedure was performed using a detection kit (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit; Chemicon, Temecula, CA) according to manufacturer's instructions. The sections were then stained with peroxidase substrate and diaminobenzidine and counterstained with hematoxylin and eosin (H&E) and were viewed by light microscopy. Phosphate-buffered saline (PBS) was used in place of TdT enzyme as the negative control, and positive slides provided with the kit were used as positive controls.

Results

2D-DIGE and Protein Identification

Before 2D-DIGE analysis, the mitochondrial and cytosolic fractions were checked for purity by their specific markers—prohibitin, a mitochondrial marker, and cytoplasmic markers caspase 3 and calpain—by Western blot analysis. The results showed that there were no interorganelle contaminants (see Fig. 3A). Figure 1A shows the differentially expressed proteins in the retinal mitochondria during early EAU compared with the control eyes.

Data analysis software (DeCyder software, version 6.0; Amersham Biosciences) codetected and differentially quantified the protein spots in the images after matching, quantitation, and statistical analysis between the two gels and directly provided the ratio of spot density of EAU/control. Among 1000 protein spots, statistical analysis performed from two individual DIGE experiments revealed 13 proteins were differentially expressed by 20% fold change in the mitochondria of early EAU compared with matching controls. A similar differential expression of proteins was observed in the two sets of experiments. Figure 1B shows the images of selected proteins identified by the analysis software as having either increased or decreased levels of expression in the mitochondria. The differentially expressed proteins were identified using mass spectrometry and were identified as 13 different proteins by MALDI-TOF MS analysis. These were MnSOD, α A crystallin, β B2 crystallin, lamin B1, syntaxin-binding protein, fructose biphosphate aldolase, aspartate aminotransferase, aconitase hydratase, and mitochondrial stress 70 protein, which were upregulated by 126%, 115%, 95%, 56%, 33%, 32%, 32%, 24%, and 21%, respectively, whereas ATP synthase, calretinin, guanine nucleotide-binding protein, and malate dehydrogenase were downregulated by 100%, 46%, 30%, and 23% respectively, from the controls. The 3D views of data analysis software (DeCyder software, version 6.0; Amersham Biosciences) showing significant differential expression of proteins in the mitochondria during early EAU are presented in Figure 2. Two additional proteins were downregulated in the EAU samples compared with controls, and they were identified as a 36-kDa protein and adult male hypothalamus cDNA by the MALDI-TOF/MS analysis (Fig. 1B). Because these were not relevant to mitochondrial oxidative stress, they were not chosen for further studies.

Confirmation of Differential Protein Expression in the Mitochondria of Early EAU by Western Blot Analysis

To test whether the proteins were truly differentially expressed in early EAU, we examined the expression of α A crystallin, β crystallin, MnSOD, aconitase, and ATP synthase in the cytosolic and mitochondrial extract from EAU and control samples. Western blot analysis results showed that α A and β crystallin proteins were markedly increased in the cytosol fraction of EAU retinal samples compared with the nonimmunized controls. The mitochondrial fraction

of the controls did not show any bands, whereas the EAU samples showed a distinctive band around 20 kDa for α A crystallin and around 26 kDa for β crystallin. (Fig. 3B). Protein expression of MnSOD and aconitase 2 also showed marked increases in EAU mice compared with control animals. MnSOD was detected at the expected molecular mass of approximately 26 kDa and aconitase at approximately 85 kDa. However, the expression of ATP synthase was decreased in the EAU mitochondrial extract compared with the controls (Fig. 3C). Densitometric analysis, followed by statistical analysis, was performed for three different experiments. Data are presented in Figures 3B and C.

Gene Expression of Proteins Differentially Expressed in EAU Mitochondria

A significant increase was noted in the mRNA of α A crystallin, β B2 crystallin, MnSOD, and Hsp70 in the EAU retina compared with controls. α A crystallin was upregulated 30.24-fold, and β B2 crystallin was increased by 41.2-fold. MnSOD and HSP70 were upregulated 2.39- and 4.92-fold, respectively (Fig. 4).

Absence of Apoptotic Cells in the Retina during Early EAU

Paraffin-embedded serial sections were obtained from 12 early EAU eyes and 12 control eyes and subjected to H&E staining. From each eye, six sections were stained, the retina was extensively studied at the pupillary-optic nerve plane, and a representative section from the control and EAU eye was chosen. On day 7 during early EAU, the retina showed no apoptosis and was similar to the control animals (Figs. 5A, B). Numerous TUNEL-positive cells were seen in the positive control slides (Fig. 5C), and TUNEL staining was totally abolished when the TdT enzyme was replaced with PBS (not shown).

Discussion

In the present study, analysis of proteins in the mitochondria of the EAU retina revealed a marked increase in the levels of MnSOD, α A crystallin, and β B2 crystallins, among a few others, suggesting MnSOD and crystallins may not only respond to stress, they may also offer protection against the stress insult. The role of MnSOD in the oxidative stress response has been extensively studied; MnSOD protects cells from stress by converting superoxide to H_2O_2 , thus preventing superoxide from interacting with nitric oxide to form the highly reactive peroxynitrite.¹⁰

The present study also resulted in the novel finding that expression of α A crystallin and β B2 crystallin was increased by approximately 95% and 115%, respectively, in early EAU retinal mitochondria compared with controls. The inner photoreceptor segments are packed with mitochondria because of their high metabolic requirements. Our previous reports had already shown upregulation of α A crystallin in the photoreceptor inner segments—the site of oxidative stress—thus indicating that increased expression of α A crystallin in the mitochondria could occur mainly in the photoreceptors. We had previously shown the protective function of α A crystallin and the photoreceptor use of this crystallin to prevent damage and subsequent apoptosis caused by oxidative stress.¹¹ The current finding of crystallins in the mitochondria further supports their protective effect in mitochondrial oxidative stress.

Studies on the α A knockout mouse have clearly demonstrated the protective role of α A crystallin in intercepting the apoptotic processes by binding to nitrated cytochrome *c* and the processed p24 subunit of caspase 3 and have proved to be an efficient inhibitor of photoreceptor apoptosis.¹¹ In the present study the increased expression of α A crystallin in the photoreceptor mitochondria and the absence of apoptotic cells further explains its protective role in mitigating mitochondrial oxidative stress and suggests that it might bind to cytochrome *c* and prevent its release into the cytosol, thereby preventing apoptosis. However, further studies are required

to confirm this finding. Given that α A is a molecular chaperone, it is also likely that α A exerts its protective effect by binding to other proteins in the mitochondria during oxidative stress.

In this study one of the beta crystallins, β B2 crystallin, earlier described as a structural protein,¹² was localized in the mitochondria with high levels of expression during early EAU. Recent reports have shown that apart from its structural functions, β B2 crystallin is very sensitive to stress and other factors.¹³ However, the nonstructural functions, which appear to be very important, have not been elucidated in detail. β B2 crystallin was also shown to stabilize other proteins during oxidative stress conditions through its high β -sheet content and to ensure that storage levels of cytoplasmic Ca^{2+} are maintained.¹⁴ β B2 crystallins were also found to bind to calcium and to maintain calcium homeostasis.¹⁵ However, its localization in the mitochondria has not been reported. Our present findings reveal for the first time the presence of β B2 crystallin in high levels in the photoreceptor mitochondria during early EAU, suggesting that it might help stabilize mitochondrial proteins and calcium homeostasis during oxidative stress.

Although α and β crystallins are primarily cytosolic proteins, α B crystallins were recently found to be translocated to the mitochondria during ischemic reperfusion in the heart. It was found to be elevated threefold in the mitochondria, where it was phosphorylated and modulated mitochondrial damage.^{16,17} Similarly, in our study, alpha and beta crystallin might have translocated during mitochondrial oxidative stress in the photoreceptors. However, further studies have to be conducted to prove their function in the mitochondria.

Heat shock proteins such as mitochondrial Hsp70 have been shown to play an important role in facilitating import into and folding of assembly of nuclear-encoded proteins in the mitochondrial matrix.^{18–21} The changes in their expression reflect adaptive responses that indicate underlying oxidant stress that can damage proteins. Upregulation of Hsp70 is one mechanism through which cells adapt to environmental stress. Mitochondrial Hsp70 replaces damaged mitochondrial proteins and plays a role in mitochondrial calcium regulation and mitochondrial biogenesis.^{22,23} Results from our 2D-DIGE analysis in the present study revealed increased expression of mtHsp70 in the mitochondria in EAU animals, indicating that it might also play an important role in regulating mitochondrial function and in protective and repair mechanisms during early EAU.

Despite the increased levels of SOD, crystallins, and Hsp70 in the mitochondria in early EAU, there was evidence of early molecular damage in the form of downregulation of ATP synthase, malate dehydrogenase, calretinin, and guanine nucleotide-binding proteins indicating impaired mitochondrial function and altered tissue oxygen metabolism.²⁴ The markedly decreased protein level of ATP synthase in the retinal mitochondria during early EAU in the present study indicates loss of ATP synthase activity and decreased cellular ATP levels, which reflect oxidative damage in the mitochondria. ATP synthases maintain mitochondrial morphology and mitochondrial membrane potential.²⁵ Loss of ATP synthase could be a pathologic event occurring during early EAU. Similarly decreased levels of ATP synthase were observed in a hepatic mitochondrial proteome after troglitazone treatment in SOD heterozygous mouse and in the hepatic mitochondria after acetaminophen treatment in mice caused by oxidant stress in the mitochondria.^{26,27}

The expression of aconitase hydratase, one of the most sensitive markers of oxidant stress in mitochondria because of its [4Fe-4S] clusters,²⁸ was upregulated in our study, suggesting that oxidative stress in the mitochondria altered its expression during early EAU. Aconitase is a potential biomarker for mitochondrial oxidative stress because of its inactivation by oxidants. Its expression was altered and its function was attenuated in the mitochondria from troglitazone-treated SOD[±] mice.^{29,30} The presence of oxidative stress in the mitochondria also

causes the elevation of other intramitochondrial antioxidant defenses, such as fructose biphosphate aldolase A.³¹ In the present study, this protein was increased in early EAU.

After inflammation, excessive uptake of Ca^{2+} and eventual overload increase the mitochondrial production of reactive oxygen species, which may induce the opening of the mitochondrial permeability transition pore, the release of cytochrome *c*, and the inhibition of ATP production, ultimately leading to cell death.^{32–35} The decreased expression of calretinin in this study indicated altered calcium regulation in the retinal mitochondria and, thus, mitochondrial dysfunction. Another protein with altered expression was the mitochondrial aspartate aminotransferase, which was increased to approximately 32% compared with controls. A common enzyme found in the mitochondria of all cells, aminotransferase plays an important role in amino acid metabolism and is a critical component of the malate-aspartate shuttle, which transports reducing equivalents across the inner mitochondrial membrane.³⁶ Decreased expression of malate dehydrogenase was also detected in the mitochondria, suggesting altered mitochondrial function.

To confirm the 2D-DIGE findings, we also performed Western blot analysis on some of the significant and important oxidative stress-related proteins such as α A crystallin, β crystallin, MnSOD, aconitase 2, and ATP synthase. There was a significant alteration in the expression of these proteins during early EAU, thus confirming our 2D-DIGE results. Real-time PCR also confirmed these results, indicating that there was an increase in the gene transcription of α A, β B2, MnSOD, and Hsp70 during early EAU. Increased expression of α A and β B2 crystallins has been previously reported from our laboratory.¹¹

Our previous reports have shown increased expression of iNOS and peroxynitrite in the retina, primarily localized to the inner segments of the photoreceptors.² High levels of iNOS in the mitochondria could damage mitochondrial DNA, as we reported earlier.⁴ Mitochondrial DNA represents a critical target for oxidative damage. Once damaged, mitochondrial DNA can amplify oxidative stress by decreased expression of critical proteins important for electron transport, leading to a vicious circle of reactive oxygen species and organellar dysregulation that eventually triggers apoptosis.^{37,38}

In the present study we used a novel proteomic technique, 2D-DIGE, which is more sensitive than conventional 2D electrophoresis, to analyze retinal mitochondrial proteomic changes and to discover novel insights into mitochondrial responses during the early phase of EAU. The gel-to-gel variance that often misleads quantitative comparison of protein expression levels is minimized by the multiplexing approach of 2D-DIGE, which uses different protein samples independently labeled with three spectrally resolved fluorescent dyes (Cy2, Cy3, and Cy5).

The present study extends previous observations of occurrences of mitochondrial oxidative stress in the retina during early EAU, before leukocyte infiltration. However, it shows that mitochondrial oxidative stress alters protein expression in the mitochondria, thus altering their functions. In addition to CuZn SOD, there was increased expression of crystallins, particularly α A, a small heat shock protein known to be upregulated in the retina in mitochondrial oxidative stress. Further functional studies on the photoreceptor mitochondria during early EAU are required to dissect the mechanism of retinal mitochondrial damage at molecular levels.

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Figure 1.

Differential expression of retinal mitochondrial proteins during early experimental autoimmune uveitis (EAU). Mitochondria were isolated from the retinas of B10.RIII mice induced with EAU on day 7 after immunization and from the control animals. The protein extracts from EAU and control samples were differentially labeled with Cy3 and Cy5 and resolved using a single 2D gel. An internal standard containing equal amounts of each mitochondrial sample labeled with Cy2 was also used. After electrophoresis, the gel was illuminated with excitation wavelengths of the cyanine dyes. The gels were also stained with Coomassie blue (A). Colocalization of the EAU and control sample proteins labeled with different cyanine dyes after 2D-DIGE analysis (B). The two images from both samples were overlaid, and the gel was scanned using a highly sensitive typhoon imager. Then it was processed by analysis software to codetect and differentially quantify the protein spots in the images. *Red spots* represent upregulated proteins in the EAU, and *green spots* indicate downregulated proteins. Fifteen differentially expressed proteins that were identified by MALDI TOF-MS are circled as follows: (upregulated proteins) 1, MnSOD; 2, aconitase hydratase; 3, fructose biphosphate aldolase; 4, α A crystallin; 5, β B2 crystallin; 6, lamin B1; 7, syntaxin-binding protein; 8, mtHsp70; (downregulated proteins) 9, aspartate aminotransferase;

10, ATP synthase; 11, calretinin; 12, malate dehydrogenase; 13, guanine nucleotide-binding protein; 14, 36-kDa protein; 15, adult male hypothalamus cDNA.

Figure 2.

Increased and decreased presence of proteins in the retinal mitochondria in early EAU, as identified by MALDI-TOF/MS. Proteins are as follows: (upregulated) 1, MnSOD; 2, aconitase hydratase; 3, fructose biphosphate aldolase; 4, α A crystallin; 5, β B2 crystallin; 6, lamin B1; 7, syntaxin-binding protein; 8, mtHsp70; (downregulated) 9, aspartate aminotransferase; 10, ATP synthase; 11, calretinin; 12, malate dehydrogenase; 13, guanine nucleotide-binding protein. 3D views of data analysis software showing significant differential expression of the selected 13 proteins in the mitochondria during early EAU. Comparative analysis of the protein spot intensities using the data analysis software. Selected spots are displayed as 3D images. Spot boundary of selected proteins is displayed in yellow.

Figure 3.

Increased protein expression of α A crystallin, β crystallin, Mn-SOD, and aconitase and decreased protein level of ATP synthase in the mitochondrial extracts of early EAU mice. Mitochondria and cytosol were fractionated from the retinas of control (nonimmunized) and EAU mice. The purity of each fraction was tested using a mitochondrial marker, prohibitin, and two cytoplasmic markers, caspase 3 and calpain (A). Fractionated mitochondria and cytosolic proteins from control and early EAU mice were analyzed for α A crystallin, β crystallin (B), MnSOD, aconitase and ATP synthase (C). Western blot analysis showed a significant increase in α A crystallin ($P < 0.001$), β crystallin ($P < 0.001$), MnSOD ($P < 0.0001$), and aconitase ($P < 0.005$) in the EAU retinal mitochondria, whereas ATP synthase was decreased in EAU mitochondria ($P < 0.02$).

Figure 4.

Gene expression of α A crystallin, β B2 crystallin, MnSOD, and Hsp70 in early EAU. Gene expression of α A and β B2 was significantly upregulated in EAU retinas during day 7 after immunization, with increases of 30.24-fold and 41.2-fold, respectively. MnSOD and Hsp70 was also significantly upregulated with increases of 2.39-fold and 4.92-fold, respectively. mRNA was quantitated by real-time PCR analysis using gene-specific primers and was normalized to GAPDH. The relative multiple of change in mRNA expression was determined using the method $2^{-\Delta\Delta C_t}$. Experiments were conducted in triplicate. Statistical analysis was performed using *t*-tests. *** $P < 0.0001$; ** $P < 0.005$; * $P < 0.02$.

Figure 5.

Absence of apoptotic cells in the retina during early EAU on day 7 after injection. Paraffin sections were subjected to TUNEL staining with an apoptosis detection kit. Diaminobenzidine was used as the chromogen. Sections were counter-stained with H&E. Absence of TUNEL-positive cells in early EAU retina (**A**) and control (**B**). TUNEL-positive cells in the section of positive control (**C**).

Table 1Gene-Specific Primers of αA and $\beta B2$ Crystallins and GAPDH Used for Real-Time PCR Analysis

Genes	Forward Primer	Reverse Primer
<i>αA crystallin</i>	5 -ACAACGAGAGGCAGGATGAC-3	5 -AGGGGACAACCAAGGTGAG-3
<i>$\beta B2$ crystallin</i>	5 -GGCTACGAGCAGGCTAATTG-3	5 -CCTTGTAATCCCCCTTCTCC-3
<i>GAPDH</i>	5 -TGCACCACCAACTGCTTA-3	5 -GGATGCAGGGATGATGTTC-3

Using the primer pairs listed in the table, quantitative (real-time) PCR was performed on retinas obtained from B10.RIII mice induced with EAU and from control mice. *GAPDH* served as the housekeeping gene for normalization. The specificity of each primer set was verified by melting-curve analysis.