Supplementary Materials and Methods

Proteomic analyses

Briefly, optic nerve tissues were minced with an angled scissor and extracted by homogenization in 100 mM Tris-Cl buffer pH 7.8 containing 5 mM dithiotheritol, 1mM SnCl$_2$, 50 mM NaHPO$_4$, 1mM diethylenetriaminepentaacetic acid, 100 mM butylated hydroxyl toluene and 0.2% SDS. SDS was replaced by 0.1% genapol for extracts where enzymatic determinations were required. Insoluble material was removed by centrifugation (8000 x g for 5 min), and soluble protein quantified by the Bradford assay $^1$. Protein extracts were subjected to SDS-PAGE on 10% gels (Bio-Rad Laboratories, Hercules, CA) and the gels were used either for mass spectrometric proteomic analyses or for Western analyses. For protein identifications, gel slices were excised and digested in situ with trypsin, and peptides were analyzed by liquid chromatography electrospray tandem mass spectrometry using a CapLC system and a quadrupole time-of-flight mass spectrometer (QTOF2, Waters Corporation, Milford, MA). Protein identifications from MS/MS data utilized ProteinLynx™ Global Server (Waters Corporation) and Mascot (Matrix Science) search engines and the Swiss-Protein and NCBI protein sequence databases $^2$.

Western analyses

For these analyses previously described mouse monoclonal antibody (mAb) against PAD2 $^{3,4}$ was used. Mouse mAbs for human myelin basic protein (MBP), myelin proteolipid protein (PLP), myelin associated glycoproteins (MAG) and glial fibrillary acidic protein (GFAP) were procured from Chemicon International unless stated.
otherwise. For quantitative Western analyses, anti-mouse and anti-rabbit secondary antibody linked to 700 nm or 800 nm IR-dyes were used on an Odyssey Infrared Imaging system according to the manufacturer (Li-Cor Biosciences, Lincoln, NB). Polyclonal antibodies (pAbs) to citrulline (Citrulline kit, Upstate Biotechnology), and methyl arginine antibodies (ab412, Abcam) were purchased.

**Protein methylation assays**

Protein methylation assays were performed by measuring incorporation of S adenosyl-L-methyl-$^{14}$C methionine (AdoMet; Sigma Chemical Co. St. Louis, MO). AdoMet ($^{14}$C-labeled; specific activity 50 m Ci/mM) was diluted to yield a concentration of 0.1 mM (100-150 dpm/picomole) and allowed incorporation into the proteins (Ovalbumin) at pH 7.2 following standard protocols $^5$. AdoMet was incubated with Ovalbumin at 37°C for 5 minutes and the reaction was initiated by adding 5µl of protein extract (1mg/ml) and incubated for an additional 5 minutes. The reaction was stopped by adding 0.5 ml of 30% TCA. In control tubes an equivalent amount of ovalbumin instead of tissue extract was added. The mixture was carefully overlayed with ethanol and centrifuged for 15 minutes in a table top clinical centrifuge. The supernatant was decanted and the precipitate was washed three times with 8 ml of TCA solution, once with chloroform: ether: ethanol (1:1:1v/v), and once with ethanol. The precipitates were dissolved in 1 ml of 0.2 M sodium phosphate buffer (pH 7.2) by placing it in a boiling water bath for 5 minutes then transferred into 10 ml of scintillation fluid and counted for radioactivity. One ml of 0.2 M sodium phosphate buffer (pH 7.2) in a tube served as a blank control. The protein
methylase activity was determined for three samples each of equal amounts (10 µg) of tissue extract from control and glaucomatous optic nerve.

**Immunoprecipitations**

Antibody-coupled protein A beads were used for all immunoprecipitations (IPs). About 100 µg of protein A sepharose CL-4B beads (Amersham Pharmacia Biotech, CA) was coupled with 100 µg antibody (citrulline or MBP) using dimethylpimelimidate (DMP). The antibody-bead suspension was subjected to addition of 25 mg of DMP and incubated at room temperature in 50 mM sodium borate buffer pH 8.3 for 2 hour, the addition of 25 mg DMP to the suspension was repeated 4 times. Rabbit pAb against human MBP, procured from Dako Corporation was used for IP and mouse human MBP mAb was used for Western detection. Antibody-conjugated beads were washed and incubated for 2 hour with 200 mM ethanolamine pH 8.0. Antibody beads were finally washed with phosphate buffered saline pH 7.4 and incubated with protein extracts (~100 µg) prepared in 100 mM Tris-Cl buffer pH 7.5, 50 mM NaCl and 0.01% genapol. For IP with anti-citrulline, the protein extract in a total volume of 10 µl (2-2.5 µg/µl) was treated with 2 µl of acidified FeCl₃ containing 2,3-butanedione monooxime and antipyrine provided in the citrulline kit for 90 minutes. Time period of 90 minutes was found optimal and prevents formation of insoluble materials. Following incubation the volume was raised to 500 µl using 100 mM Tris-Cl buffer pH 8.0, 50 mM NaCl and 0.01% genapol and incubated with 100 µg of anti-citrulline coupled beads for 1 hour at room temperature. The MBP IP was performed by incubating 100µg antibody-coupled beads with ~100 µg protein extract in 500 µl of 100 mM Tris-Cl buffer pH 7.5, 50 mM NaCl and 0.01% genapol for 1 hour. After
incubation the beads were recovered by centrifugation at 2500 x g for 5 minutes and washed with 3 x 500 µl of 100 mM Tris-Cl buffer pH 7.5, 100 mM NaCl and 0.02% genapol. The beads were boiled with 30 µl Laemmli buffer\textsuperscript{6} for 2 minutes and separated on a 10% SDS-PAGE. The gels were subjected to either Western blot analyses or Coomassie blue staining with subsequent LC MS/MS of excised gel bands.

**Histochemical Analyses**

Immunohistochemical analyses to localize PAD2 in optic nerve tissue were performed with cadaver eyes enucleated within six hours of death and fixed immediately with calcium acetate buffered 4% para-formaldehyde. Paraffin embedded tissue was blocked and sectioned (12 µm) in 2% BSA in phosphate buffered saline (PBS), then incubated with 10 ng anti-PAD2 antibody\textsuperscript{4} overnight at 4°C and subsequently with 10 ng Alexa 594 conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for one hour at room temperature. For immunohistochemical analysis of citrulline containing proteins, a kit from Upstate Biotechnology was used. Briefly, the tissue sections after de-paraffinization were subjected to 2,3-butanedione monooxime and antipyrine treatment in a strong acid atmosphere for 3 hours followed by five washes with 2% BSA in PBS. For detection of citrulline, Alexa 488 conjugated secondary antibody was used. The nuclei were stained with TOPRO-3. The treatment of tissue with 2,3-butanedione monooxime and antipyrine in a strong acid atmosphere enables chemical modification of citrulline into ureido groups and ensures detection of citrulline-containing proteins regardless of neighboring amino acid sequences\textsuperscript{7}. Processing steps, in a strong acid environment and antipyrine, however, makes TOPRO-3 nuclear staining (or any
other nuclear stain) less pronounced. Sections sealed with vectashield and were analyzed either with a Leica TCP2 scanning confocal microscope or with a Nikon EFD-3 fluorescence microscope attached to a CCD camera. Rat brain astrocytes were subjected to immunohistochemical analysis in a similar fashion.

RNA isolation and quantitation
Total RNA from optic nerve was isolated using TRIZOL with modification of the protocol recommended by the supplier (Invitrogen Inc., Carlsbad, CA). The optic nerve from donor eyes was carefully excised and minced into small pieces first using scissors and then a scalpel. Prior to use, tissue was washed with diethylpyrocarbonate (DEPC) water and all solutions were prepared in DEPC water. The minced tissue was placed in a glass homogenizer with 1 ml TRIZOL per 100 mg of tissue and homogenized in a glass homogenizer DUALL 20 (Kimble Kontes Glass Co, Vineland, NJ) with 10 stroke cycles each at room temperature and after freezing with liquid nitrogen for 1 min for 40 cycles. This RNA was extracted with chloroform, isoamylalcohol and precipitated with sodium citrate/sodium chloride and isopropanol. The RNA from astrocytes was isolated following the standard recommended TRIZOL protocol. The final air-dried RNA precipitate was suspended in DEPC water, spectrophotometrically quantified and stored at –80°C until use. For relative quantification, about 1 µg of RNA after separation on a 5% polyacrylamide gel in TAE buffer was subjected to Northern blotting using standard protocols. It was probed with 32P-CTP labeled PCR products and after 1 hour exposure to a Molecular Dynamics Phosphorimager screen, imaged using a Typhoon 8600 variable mode imager with Imagequant software. Probes for PAD2 (5’- aaacctggaggtcagtcccc-3’
and 5’- aaacctggaggtcagtcccc-3’), GPDH (5’-cttcaccaccatggagaaggc-3’ and 5’-ggcatggacttgtgtcatgag-3’) and HGRT (5’-gaagagctactgtgcatgac-3’ and 5’-aaagtctgtgctgtatatcaac-3’) were generated by PCR for 30 cycles using the indicated primer pairs, $^{32}$P-CTP (9.25 MBq/25µl) and recommended protocols.

**Western analysis of PAD2 and Citrulline in the mouse optic nerve**

DBA/2J Mice were procured from The Jackson Laboratory (Bar Harbor, ME) and bred to generate the animals used in this study. Mice were sacrificed with carbon dioxide and optic nerve tissue was dissected. All procedures were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic Foundation. Protein was extracted from optic nerve tissue by homogenization in 100 mM Tris-Cl buffer pH 7.5 containing 5 mM dithiotheritol, 1 mM SnCl$_2$, 50 mM NaHPO$_4$, 1 mM diethylene-triaminepentaacetic acid, 100 mM butylated hydroxy toluene and 0.5% SDS. Insoluble material was removed by centrifugation (8000 x g for 5 min), and soluble protein quantified by the Bradford assay. Western blot analyses were performed with 5 µg protein extract, 4-20% gradient gels (Invitrogen Inc, CA), electroblotting to PVDF membrane and probing with monoclonal PAD2 antibody or polyclonal anti-citrulline antibody.

**Primary astrocyte cultures and pressure treatment**

Astrocytes from Sprague Dawley rats (Harlan, Indianapolis, Indiana) brain cortex were used for these studies. Mixed glial cell suspensions were prepared from the P3 rat cortex regions following published procedures from which enriched GFAP positive cells were obtained using immunopanning. The astrocytes were exposed to a pressure of 40 mm
of Hg for five hours\textsuperscript{11,12}. Briefly, the cells plated in six well plates (Costar, Cambridge, MA, USA) at a density of $3\times10^3$ cells/well and grown to semiconfluence in 2 days were incubated with serum free medium overnight. A closed pressurized chamber (5% carbon dioxide) equipped with a manometer was used to subject the cells to elevated pressure. Cells were placed in the chamber and the pressure was elevated to 40 mm Hg. The chamber was subsequently placed in a tissue culture incubator at 37°C. Control cells from identical passage of cell lines were simultaneously incubated in a tissue culture incubator at atmospheric pressure at 37°C. The cells were incubated for 5h or 1-4 days after pressure treatment. After incubation, cells were trypsinized and subjected to culture or Western analyses. For culture, the cells were plated on a cover slip and allowed 16 hours recovery period and subjected to immunohistochemistry using mouse monoclonal PAD2 and rabbit polyclonal GFAP antibodies. The cells were permeabilized with 200 μl of 0.2% Triton X-100 in phosphate buffer saline pH 7.5 for 1 hour after fixation with 4% paraformaldehyde for 1 hour. Western analyses were performed using antibodies to PAD2 and citrulline as described above.

References


