

# Proteomics Implicates Peptidyl Arginine Deiminase 2 and Optic Nerve Citrullination in Glaucoma Pathogenesis

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**PURPOSE.** Proteomic analyses of normal and glaucomatous human optic nerve were pursued for insights into the molecular pathology of primary open-angle glaucoma (POAG). Peptidyl arginine deiminase 2 (PAD2), an enzyme that converts protein arginine to citrulline, was found only in POAG optic nerve and was probed further for a mechanistic role in glaucoma.

**METHODS.** Protein identification used liquid chromatography-tandem mass spectrometry. Northern, Western, and immunohistochemical analyses measured PAD2 expression and/or protein citrullination and arginyl methylation in human and mouse optic nerve and in astrocyte cultures before and after pressure treatment. Proteins were identified after anticitrulline immunoprecipitation. In vitro translation of PAD2 was monitored in polyA RNA depleted optic nerve extracts. PAD2 shRNA transfections were evaluated in pressure-treated astrocytes.

**RESULTS.** Western and immunohistochemical analyses confirmed elevated PAD2 and citrullination in POAG optic nerve and decreased arginyl methylation. PAD2 was also detected in optic nerve from older, glaucomatous DBA/2J mice, but not in younger DBA/2J or control C57BL/6J mice. Myelin basic protein was identified as a major citrullinated protein in POAG optic nerve. Pressure-treated astrocytes exhibited elevated PAD2 and citrullination without apparent change in PAD2 mRNA. Addition of exogenous polyA RNA to depleted optic nerve extracts yielded increased PAD2 expression in POAG but not in control extracts. Transfection with shRNA restored PAD2 and citrullination to control levels in pressure-treated astrocytes.

**CONCLUSIONS.** Current results support translational modulation of PAD2 expression and a possible role for the enzyme in POAG optic nerve damage through citrullination and structural disruption of myelination. (*Invest Ophthalmol Vis Sci.* 2006; 47:2508–2514) DOI:10.1167/iovs.05-1499

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Glaucoma is a group of poorly understood neurodegenerative disorders characterized by deformation of the optic nerve head, loss of retinal ganglion cells, and irreversible vision loss in approximately 70 million people worldwide.<sup>1</sup> The risk of glaucoma increases with age, with the disease being 5 to 10 times more prevalent at age 80 than at age 40.<sup>2</sup> Glaucomas are classified as primary when they occur with no known etiology, or as secondary, where a previous illness or injury is contributory. In primary open-angle glaucoma (POAG) most but not all patients exhibit elevated intraocular pressure (IOP) which leads to optic nerve damage, often termed glaucomatous optic neuropathy.<sup>3</sup>

Recent findings suggest that analysis of protein compositional differences between POAG and normal ocular tissues may provide insights into POAG's pathologic mechanisms. For example, in vivo expression of myocilin in optic nerve is reduced in glaucoma as well as under conditions of elevated IOP.<sup>3,4</sup> Elevated pressure on cultured cells from the optic nerve head also modulates expression of several proteins including nitric oxide synthase-2,<sup>5</sup> elastin,<sup>6</sup> cytochrome P4501B1,<sup>7</sup> NCAM-180,<sup>8</sup> and Hsp27.<sup>9</sup> Other in vitro protein expression differences in pressure-treated versus untreated astrocytes have recently been reported in microarray analyses.<sup>10</sup> Previous proteomic studies in our laboratory have shown that cochlin is overexpressed in glaucomatous trabecular meshwork and is possibly involved in POAG by impairing aqueous humor circulation and elevating IOP.<sup>11</sup> Here, we have pursued proteomic analyses of optic nerve and report increased levels of peptidyl arginine deiminase 2 (protein deiminase 2 or PAD2) in POAG optic nerve. PAD2 enzyme activity is modulated by calcium and converts protein arginine to citrulline.<sup>12</sup> We also found that POAG optic nerve exhibits increased citrullination and identified several citrullinated optic nerve proteins, including myelin basic protein. Concomitant with increased citrullination in POAG optic nerve, we observed decreased protein arginyl methylation, suggesting that structural disruption of myelination may contribute to optic nerve degeneration in POAG. The present study also provides in vitro evidence of pressure-induced translational control of PAD2 expression, consistent with a possible role for PAD2 and citrullination in POAG.

## METHODS

### Tissue Procurement

Donor eyes from normal (control) and cadaveric POAG eyes were enucleated within 6 hours of death and obtained from the National Disease Research Interchange and the Cleveland Eye Bank. Glaucomatous eyes that had recorded optic neuropathy and progressive deterioration in visual acuity together with lack of other major central nervous system (CNS) disorders were procured. Acceptable eyes were those that had detailed medical and ophthalmic histories. Controls eyes were from normal donors with no optic neuropathy and no history of eye disease or other major CNS disorders. Twelve glaucomatous and 12

age-matched ( $\pm 4$  years) normal eyes, all from white donors between 55 and 87 years of age were used in this study (brief clinical histories of the optic nerve tissue donors are presented in Supplementary Table S1; all Supplementary Material is available online at <http://www.iovs.org/cgi/content/full/47/6/2508/DC1>). Two additional eyes from different 7-year-old white male donors were used for astrocyte cell culture preparation. Research was conducted according to the tenets of the Declaration of Helsinki. Use of mice followed procedures in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Protein Identification, Western Blot Analysis, Immunohistochemical Analysis, Immunoprecipitation, and Protein Methylation Assays

Detailed methods are provided in Supplementary Materials and Methods, available online. Briefly, for proteomic analyses, proteins were extracted from optic nerve tissue, as reported previously, but with minor modifications.<sup>13</sup> The proteins were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and bioinformatics methods.<sup>11</sup> Western blot analysis was performed on polyvinylidene difluoride (PVDF) membranes according to established protocols,<sup>11,13</sup> and primary antibodies to PAD2, myelin basic protein (MBP), myelin proteolipid protein (PLP), myelin-associated glycoprotein (MAG), glial fibrillary acidic protein (GFAP), citrulline, and methyl arginine. Immunohistochemical analyses to localize PAD2 and citrulline in optic nerve tissue used cadaveric eyes enucleated within 6 hours of death and fixed immediately with calcium acetate buffered 4% *para*-formaldehyde. Immunoprecipitation was performed using antibodies to citrulline and MBP covalently coupled to protein A Sepharose beads with dimethylpimelidate. Protein methylation assays were performed by measuring incorporation of *S* adenosyl-L-methyl-<sup>14</sup>C methionine into ovalbumin using standard protocols.

### RNA Isolation and Quantitation

RNA isolation was performed (TRIzol) with suitable modification of standard protocols (see Supplementary Materials and Methods online). Northern analyses were probed with <sup>32</sup>P-CTP labeled PCR products and, after a 1-hour exposure to a phosphorescence imager (Phosphorimager; GE Healthcare, Piscataway, NJ) screen, was imaged with a variable-mode imager (Typhoon 8600; with Imagequant software; GE Healthcare). Probes for PAD2 (5'-aacctggaggtcagtcctccc-3' and 5'-aacctggaggtcagtcctccc-3'), glyceraldehyde-3-phosphate dehydrogenase (GPDH) (5'-cttcaccaccatggagaagc-3' and 5'-ggcatggactgtggtcatgag-3'), and hypoxanthine guanine ribosyl transferase (HGRT) (5'-gaagactactgtaatgatcagtc-3' and 5'-aaagtctggcctgtatccaacac-3') were generated by PCR for 30 cycles with the indicated primer pairs, <sup>32</sup>P-CTP (9.25 MBq/25  $\mu$ L), and recommended protocols.<sup>14</sup>

### Primary Astrocyte Cultures and Pressure Treatment

Astrocytes from Sprague-Dawley rat (Harlan, Indianapolis, IN) brain cortex were used. Mixed glial cell suspensions were prepared from the third postnatal day (P3) rat brain cortex region according to published procedures,<sup>15</sup> and GFAP-positive cells were obtained by immunopanning<sup>16</sup> (see Supplementary Materials and Methods online).

### Probing Translation with Polyadenylated RNA Depleted Extracts

Assays were performed probing translation of PAD2 on addition of total polyA RNA to optic nerve extracts depleted of mRNAs, PAD2, and GPDH. Extracts were first depleted of polyA RNA with oligo dT-cellulose matrix (BioWorld, Dublin, OH) then depleted of PAD2 and GPDH using mAb and pAb to PAD2 and GPDH, respectively, conjugated to protein A Sepharose beads. Approximately 100  $\mu$ g total protein obtained from each donor was used for each analysis. Approximately 0.15  $\mu$ g total polyA RNA (pooled from two nerves from white

males, aged 79 and 70 years) was added to polyA RNA depleted tissue extracts (per 100  $\mu$ g of extract) and incubated at 37°C for 90 to 120 minutes. <sup>35</sup>S labeled methionine (540 Ci/mmol; MP Biomedical Inc, CA) was used to detect the translated product. Two identical gels (SDS-PAGE), one with <sup>35</sup>S-labeled and the other with cold methionine was subjected to simultaneous side-by-side electrophoresis. The cold methionine gel was blotted to PVDF membrane (Sigma-Aldrich Chemical Co., St. Louis, MO) and probed with PAD2 and GPDH antibodies to determine the identity of the protein bands. Detection was performed with IR-700 or IR-800 dye-coupled secondary antibodies.<sup>12</sup> The radioactive protein bands corresponding to antibody detected counterparts in cold methionine gels were excised and quantified in a scintillation counter (1900CA Tri-Carb; Perkin Elmer, Meriden, CT).

### The shRNA Treatment of Primary Astrocytes

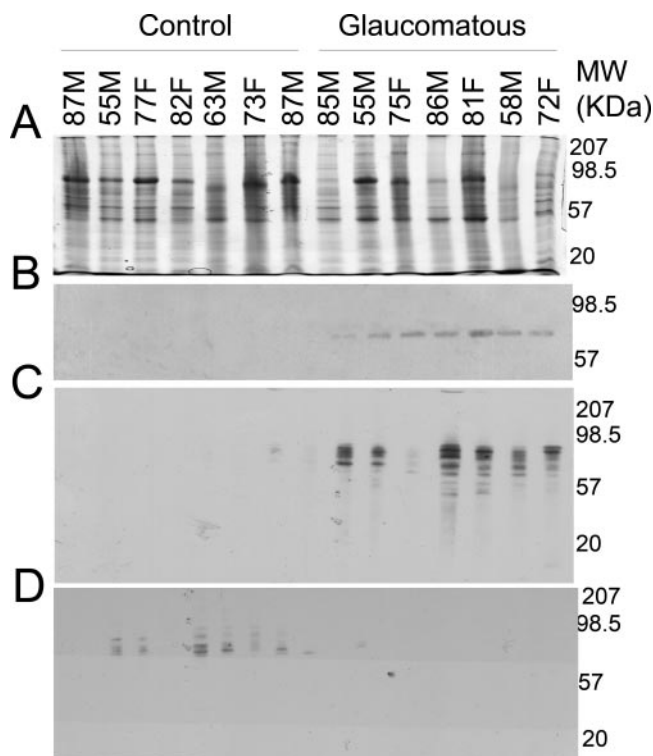
For treatment of rat cortex astrocytes, shRNA against PAD2 (RHS1764-9219528) was procured (pShag Magic Ver. 2.0 vector; Open Biosystems, Huntsville, AL). This shRNA contains the sequence (5'-TGCTGTTGACAGTGAGCGACAGCCTTGACTCATTTGGAAATAGTGAAGCCACAGATGTATTCCAAATGAGTCAAGGCTGGTGCCTACTGCCTCGGA-3') from the coding region of PAD2. For a negative control, we used a nonsilencing shRNA sequence cloned into RHS1703 (pShag Magic 2.0; Open Biosystems), verified to contain no homology to known mammalian genes. Approximately 30% confluent cells (~3000 primary astrocytes)<sup>17</sup> were transfected (SuperFect; Qiagen, Valencia, CA) transfection reagent, purified vector (5  $\mu$ g) DNA (Qiagen), and the manufacturer's recommended protocols. The posttransfected astrocytes were selected on geneticin (10  $\mu$ g/ $\mu$ L). The primary astrocytes were subjected to pressure (40 mm of Hg) and transfected with shRNA on plates immediately after they were brought to normal atmospheric pressure.

## RESULTS

### Detection of Peptidyl Arginine Deiminase 2 in Glaucomatous Optic Nerve

Protein extracts from eight POAG and eight control optic nerve donor tissues were separated on SDS-PAGE, gel slices were excised from the top to the bottom of the gel (Fig. 1A) and proteins were identified using well-established mass spectrometric and bioinformatics methods.<sup>13</sup> Two additional donor tissues not shown in Figure 1A (white females, POAG and control, aged 72 and 73 years) were also subjected to proteomic analyses. Overall, 250 proteins were identified, of which 68 were detected only in glaucomatous optic nerve (see Supplementary Table S2 online). Apparent proteome differences must be verified because the lack of detection by LC-MS/MS does not necessarily mean the absence of protein expression. Notably, PAD2 was detected in four of eight glaucomatous optic nerves by mass spectrometry (see Supplementary Table S2 online), and subsequent immunoblot analysis verified its presence in seven of seven glaucomatous tissue but did not detect it in any normal optic nerve tissue (Fig. 1B). Western analyses of five additional glaucomatous tissues also detected PAD2. Overall, we found PAD2 to be uniquely associated with 12 of 12 POAG donor optic nerves by proteomic and Western analyses combined but in none of 12 normal controls devoid of other neurodegenerative disorders. However, by immunoblot we detected PAD2 in optic nerve from two of seven human donors exhibiting other CNS disorders but without glaucoma (data not shown), a finding consistent with reports of increased PAD2 in several neurodegenerative diseases.<sup>18,19</sup>

Based on these findings from human optic nerve, we probed PAD2 expression in an established animal model of glaucoma, the DBA/2J mouse. This mouse line exhibits increased IOP at approximately 6 to 8 months of age, with progressive damage to the optic nerve and hearing loss.<sup>20</sup>



**FIGURE 1.** Elevated PAD2 levels and citrullination in glaucomatous optic nerve. (A) Representative SDS-PAGE of human optic nerve protein (~10 µg per lane) from POAG and control donors (Coomassie blue staining). Gel slices were excised and proteins identified by LC MS/MS (see Supplementary Table S2 online). (B) Representative Western analyses with monoclonal anti-PAD2 of protein extracts from human optic nerve demonstrating the presence of ~72-kDa protein uniquely in glaucomatous tissues. (C) Western analyses with rabbit polyclonal antibody to citrulline (10 µg protein per lane). Before applying antibody, membrane immobilized protein was treated with 2,3-butanedione monooxime and antipyrine in a strong acid atmosphere enabling chemical modification of citrulline into ureido groups and ensures detection of citrulline-containing proteins regardless of neighboring amino acid sequences. (D) Western analyses with mouse monoclonal antibody to protein methylarginine (10 µg protein per lane). Protein was extracted from the optic nerve of white cadaveric donor eyes; age and gender are indicated.

Western analyses detected PAD2 and citrullination in the optic nerve of 8- to 12-month-old DBA/2J mice, but not in DBA/2J mice at 5 months of age, nor in 5- to 12-month-old control C57BL/6J mice, which do not exhibit increased IOP (Fig. 2).

### Increased Citrullination and Decreased Methylation in Glaucomatous Optic Nerve

Western analyses of POAG optic nerve showed increased PAD2, increased citrullination but decreased protein arginyl methylation relative to the normal control (Figs. 1B–D). Decreased arginyl methylation (Fig. 1D) concomitant with increased citrullination (Fig. 1C) is consistent with the conversion of arginine to citrulline before methylation (Fig. 1B). However, to determine whether the decreased levels of methylated proteins in POAG optic nerve could be due to down-regulation of protein methylation activities, we performed Western analyses for both protein arginine *N*-methyltransferase 1 (PRMT1) and coactivator-associated arginine methyltransferase 1 (CARM1). Normalized to GPDH, the expression levels of CARM1 and PRMT1 appear to be comparable in control and POAG optic nerve (see Supplementary Fig. S1 online). We also

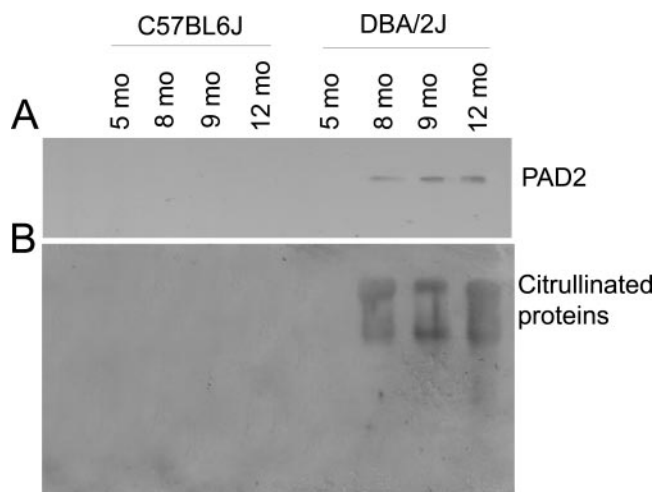
assayed methyltransferase activity in S-AdoMet-depleted crude tissue extracts, by using radiolabeled S-AdoMet and found essentially identical activities ( $40,075 \pm 1,515$  and  $40,615 \pm 2,061$  cpm, respectively) in control and glaucomatous optic nerve tissue (three independent experiments). Decreased methylation could also be due to demethylination of methylated protein arginines, and this possibility cannot be ruled out. In any event, the observed citrullination is due to increased deiminase activity, and the decreased level of methylated arginine is not due to lack of protein methyltransferase (see Supplementary Fig. S1 online).

### Immunohistochemical Localization of PAD2 and Citrullinated Proteins

Immunohistochemical analyses showed localization of PAD2 in the lamina cribrosa region of POAG optic nerve (Figs. 3A, 3C) along with citrullinated proteins (Figs. 3B, 3D). The lamina cribrosa region of the optic nerve is shown schematically in Supplementary Figure S2 online. Identically treated control and glaucomatous optic nerve showed clear differences in citrullinated protein content in the lamina cribrosa region (Figs. 3B, 3D). Normal control optic nerve exhibited much less immunohistochemical reactivity for citrullinated proteins.

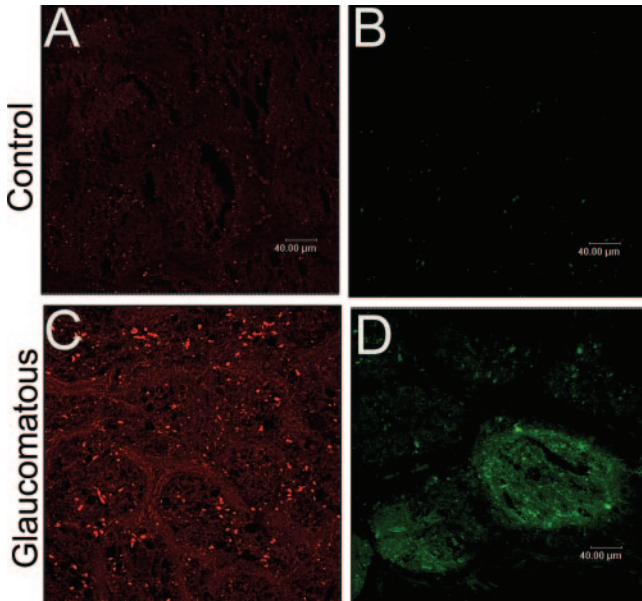
### Identification of Citrullinated Proteins in POAG Optic Nerve

Proteins in POAG optic nerve were immunoprecipitated with anticitrulline antibody for protein identification (Figs. 4A, 4B). The most intense citrulline immunoreactive component in these immunoprecipitations (IPs) was identified by mass spectrometric and Western blot analysis as MBP, suggesting that this is a major citrullinated protein in POAG optic nerve (Figs. 4C, 4D). To confirm this finding, anti-MBP was also used to immunoprecipitate proteins from normal and POAG optic nerve and subsequently probed with anticitrulline antibody. More citrullinated MBP was observed in POAG than in normal optic nerve extracts (Fig. 4). Mass spectrometric and Western analyses of anticitrulline immunoprecipitation products also detected citrullinated myelin proteolipid protein and myelin associated glycoprotein in POAG optic nerve (Fig. 4D). Mass spectrometry also detected myelin P0 protein and myelin oligodendrocyte protein in the anticitrulline IP. Other proteins



**FIGURE 2.** Western blot analysis using PAD2 and citrulline antibodies. (A) Anti-PAD2 Western analyses of control (C57BL/6J) and DBA/2J mice optic nerve at indicated ages (in months). (B) Anti-citrulline Western analyses of mouse optic nerve.



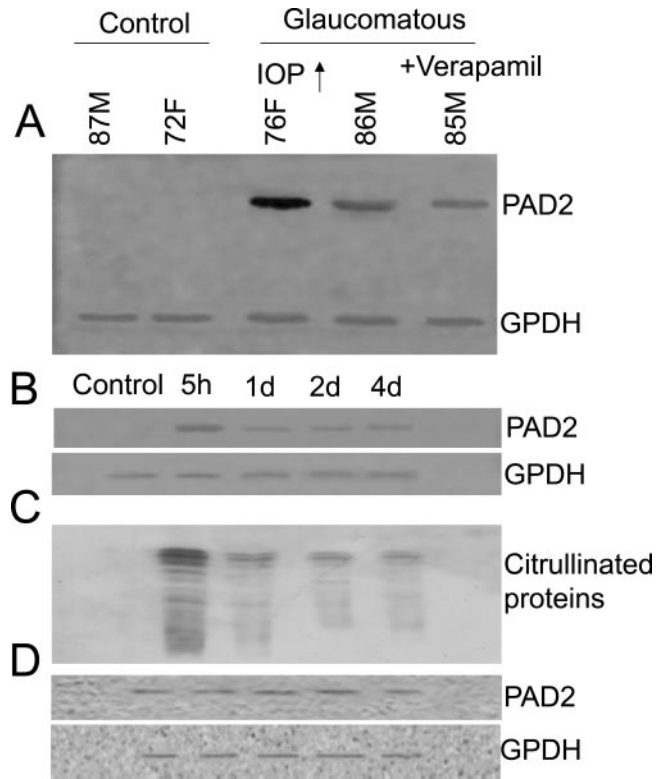


**FIGURE 3.** Immunohistochemical localization of PAD2 and citrullinated proteins in the optic nerve. Control (82-year-old male) and glaucomatous (84-year-old male) optic nerve scanning confocal images are shown for the white tissue donors. (A, C) Anti-PAD2 staining (secondary conjugated with AlexaFluor 594) images; PAD2 immunoreactivity was predominantly observed in glaucomatous optic nerve. (B, D) Control and glaucomatous optic nerve images stained with anti-citrulline antibodies (secondary conjugated with AlexaFluor 488). Citrulline immunoreactivity was predominantly observed in glaucomatous optic nerve.

identified in the anticitrulline IP are listed in Supplementary Table S3 online.

**Pressure and PAD2 Expression In Vivo and In Vitro**

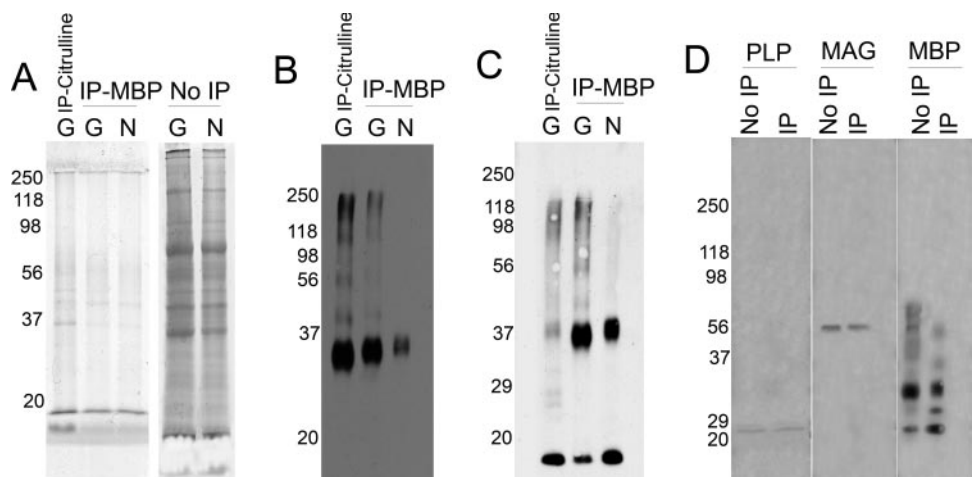
Apparent high levels of PAD2 were observed in donors with elevated IOP (Fig. 5A). Optic nerve from a 76-year-old female POAG donor with high IOP but no surgical or pharmacological intervention, and without head or eye injury from a fatal automobile accident, was found to exhibit a very high level of PAD2. Other donor eyes that had undergone either surgical or combined surgical and pharmacological intervention to relieve elevated IOP were also analyzed for PAD2. Notably, we observed that POAG optic nerve PAD2 remained detectable after

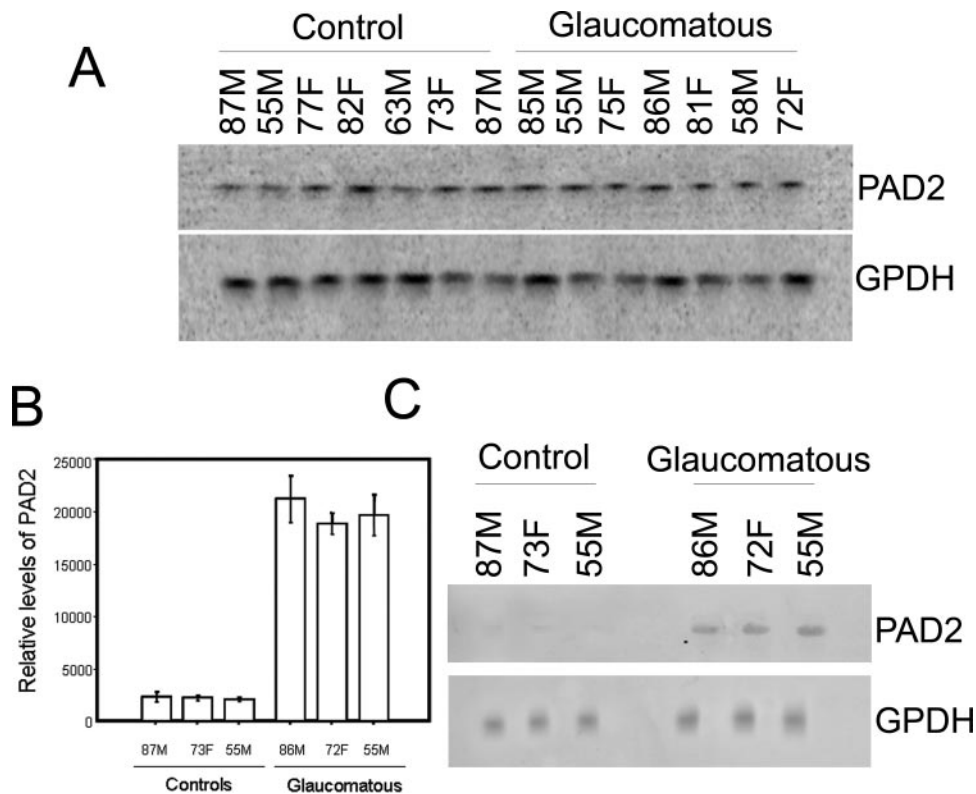


**FIGURE 5.** Elevated level of PAD2 and citrulline in response to pressure. (A) Representative Western analyses with anti-PAD2 and anti-GPDH of human optic nerve demonstrating the presence of PAD2 relative to GPDH control immunoreactivity. Protein extracted from the optic nerve of cadaveric white donor eyes of the indicate age and gender. All glaucomatous donors had elevated IOP and were subjected to surgical intervention except 76F. 85M also received verapamil, a calcium modulator. (B) Representative Western analyses with anti-PAD2 of rat brain astrocytes subjected to 40 mm Hg pressure for 5 hours and then returned to atmospheric pressure for up to 4 days, as indicated. (C) Representative Western blot analysis with anti-citrulline of protein extracts (5 μg) from astrocytes subjected to elevated pressure as in (B). (D) Representative Northern analyses of total RNA (~2 μg) isolated from pressure-treated or untreated astrocytes as in (B).

surgical or pharmacological intervention and after the IOP returned to normal. Results from two such glaucomatous donors (86M and 85M) subjected to trabeculectomy with the 85M donor also receiving verapamil (a calcium modulator) are pre-

**FIGURE 4.** Immunoprecipitation of human optic nerve proteins. (A) Coomassie blue detection of immunoprecipitation (IP) products from glaucomatous (G) and normal (N) optic nerve proteins with anti-citrulline or anti-MBP and of glaucomatous and normal optic nerve extracts without IP (15 μg). (B) Western detection with anti-citrulline of anti-citrulline or anti-MBP IP products from glaucomatous and normal optic nerve extracts. (C) Western detection with anti-MBP of anti-citrulline or anti-MBP IP products from glaucomatous and normal optic nerve extracts. (D) Western detection with antibodies to myelin proteolipid protein (PLP), myelin-associated glycoprotein (MAG), and MBP of anti-citrulline IP products from glaucomatous optic nerve.





**FIGURE 6.** Translational modulation of PAD2. (A) Representative Northern analyses of total RNA (5  $\mu$ g) isolated from normal control and glaucomatous human optic nerve. Donor age and gender are indicated. (B) In vitro translation of PAD2 (measured as dissociations per minute) was monitored in polyA RNA, PAD2, and GPDH depleted normal control and glaucomatous optic nerve extracts. Radioactive PAD2 (relative to GPDH) is shown. (C) Parallel Western blot analysis of in vitro translation products in (B) using anti-PAD2 and anti-GPDH with 700-IR coupled secondary antibodies. Gray-scale images are from infrared scanning. Donor age and gender are indicated.

sented in Figure 5A. Although both (86M and 85M) show lower levels of optic nerve PAD2 compared with those without intervention (76F), their PAD2 level is still high compared with normal controls. In the verapamil-treated eye (85M), the PAD2 level appeared lower than in the other POAG eyes, with or without intervention (86M and 76F).

To explore in vitro whether pressure induces PAD2 and subsequent citrullination, primary rat cortex astrocyte cultures were subjected to an increase in pressure by 40 mm Hg for 5 hours and then restored to atmospheric pressure. The short-term elevated pressure led to increased PAD2 in astrocytes that was still detectable after 4 days at atmospheric pressure by Western blot analysis (Fig. 5B) and immunohistochemistry (see Supplementary Fig. S3 online). Increased citrullination was also observed in the astrocytes concomitant with pressure treatment and remained detectable after 4 days at atmospheric pressure (see Supplementary Fig. S3C online). In contrast to pressure-induced changes in PAD2 protein expression, by Northern blot analysis PAD2 mRNA levels did not significantly change in astrocytes subjected to pressure (Fig. 5D). These observations were replicated in astrocytes derived from a 7-year-old human optic nerve head (data not shown).

### Translational Control of PAD2 Overexpression

To further probe whether increased PAD2 expression in vivo is due to increased PAD2 mRNA, total RNA from normal human and POAG optic nerve were subjected to Northern blot analysis. The amount of the PAD2 transcript normalized to that of GPDH was found to be very similar between seven control and seven glaucomatous donors (Fig. 6A), suggesting PAD2 overexpression in POAG optic nerve may be translationally regulated. Additional experiments supporting translational control of PAD2 expression were performed with normal and POAG optic nerve extracts depleted of both polyadenylated RNA and the PAD2 and GPDH proteins (Fig. 6B, 6C). These depleted extracts lack translation capability without exogenous mRNA.

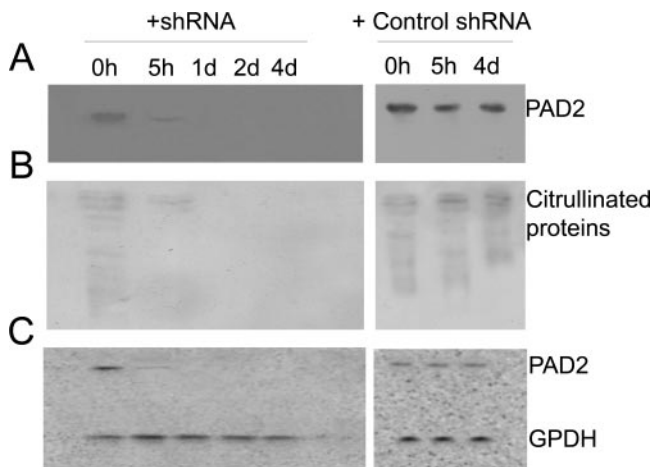
On addition of exogenous polyadenylated RNA to the depleted extracts, a large increase in PAD2 expression (relative to GPDH) was observed in the POAG extracts but not in the control extracts (Fig. 6B, 6C).

### Downregulation of Citrullination with PAD2 shRNA

Increased PAD2 and citrullination were observed in astrocytes subjected to pressure even after restoration of atmospheric pressure (Fig. 5). As a possible approach to reducing pressure-induced citrullination, we tested the effect of lowering PAD2 mRNA in vitro in astrocytes. Primary culture astrocytes were subjected to pressure and transfected with shRNA immediately after they were brought to atmospheric pressure. We found that astrocytes treated with a PAD2 specific shRNA (but not with a nonspecific shRNA) exhibited reduced PAD2 expression (Fig. 7A) and reduced citrullination (Fig. 7B) as a consequence of degradation of the mRNA transcript (Fig. 7C). Although some residual citrullination was observed, PAD2 mRNA was completely removed by shRNA within the sensitivity of detection (Fig. 7C). We did not observe any difference in cell morphology due to this reduction in mRNA, although immunohistochemical analysis showed that PAD2 was reduced in astrocytes treated with shRNA (see Supplementary Figs. S4B, S4C online) compared with pressure-treated group without shRNA (see Supplementary Figs. S3B, S3C online).

### DISCUSSION

Classic proteomic methods initially detected PAD2 in the optic nerve of glaucomatous but not normal human donors. Subsequently, PAD2 was found to be uniquely associated with glaucomatous human optic nerve by Western and immunohistochemical analyses of additional POAG and normal donor tissues. Western blot analysis also demonstrated the presence



**FIGURE 7.** Transfection with shRNA restored PAD2 and citrullination to control levels in pressure-treated astrocytes. Astrocytes were subjected to 40 mm Hg then transfected with PAD2 shRNA and analyzed for PAD2 expression and citrullination. The control is a nonsilencing shRNA sequence. (A) Anti-PAD2 Western blot analysis; (B) anti-citrulline Western blot analysis; (C) Northern blot analysis of total RNA for PAD2 mRNA.

of PAD2 in optic nerve from the DBA/2J glaucomatous mouse at ages 8 to 12 months, but not in younger DBA/2J mice that do not exhibit elevated IOP nor in optic nerve from control C57BL/6J mice. Proteomic analyses identified many other proteins in human optic nerve (see Supplementary Table S2 online); however, the significance of other proteins detected only in glaucomatous optic nerve remains to be determined.

PAD2 converts arginine to citrulline, and we observed increased protein citrullination and decreased protein arginine methylation in POAG optic nerve. Recently, PAD2, directed citrullination was associated with kainite-induced neurodegeneration in rat brain.<sup>18,19</sup> PAD2 predominantly occurs in neuronal tissues<sup>21</sup>; however, five protein deiminases have been identified in a variety of tissues, including protein deiminase-1, -2, -3, and -6 which are cytosolic and protein deiminase 4 which exhibits nuclear localization.<sup>22</sup> PAD4 was recently found to catalyze reverse methylation or demethylation as well as deimination of proteins.<sup>23,24</sup> PADs have been implicated in demyelinating diseases,<sup>21</sup> and citrullination has been implicated in diseases such as autoimmune rheumatoid arthritis,<sup>25</sup> multiple sclerosis,<sup>21</sup> and amyotrophic lateral sclerosis.<sup>26</sup>

The consequences of citrullination are many and varied. Notably, myelin contains several arginine-rich proteins that are susceptible to citrullination,<sup>27</sup> including MBP which we detected as a major citrullinated protein in POAG optic nerve. MBP is one of the most abundant proteins of the myelin sheath and functions in maintaining the stability of the sheath.<sup>28</sup> Citrullinated MBP exhibits altered properties relative to the unmodified protein, including a lower net positive charge, which disrupts its tertiary structure and ability to interact with lipids and maintain a compact myelin sheath.<sup>29,30</sup> Citrullination also decreases the ability of MBP to aggregate large unilamellar vesicles (LUVs),<sup>29</sup> a process essential for adhesion between intracellular surfaces of myelin. Citrullinated MBP exhibits increased susceptibility to cathepsin D proteolysis, which may generate immunodominant peptides leading to sensitization of T-cells for the autoimmune response in demyelinating diseases.<sup>30</sup> Citrullination also appears to inhibit cell proliferation, leading to cell cycle arrest and apoptosis.<sup>31,32</sup> Such mechanisms may all play a role in glaucomatous neuropathy. The presence of multiple citrullinated proteins in POAG optic nerve, including MBP, myelin proteolipid protein, and myelin-

associated glycoprotein among others, would appear likely to disrupt myelination. Citrullination of optic nerve head matrix proteins may weaken their anchorage and overall weakness at the level of optic nerve head. We hypothesize that citrullination causes changes in the dynamics of myelin components and also may cause disruption of the optic nerve head matrix protein framework that may initiate or contribute to glaucomatous neuropathy.

A variety of factors trigger PAD2 expression. Calcium imbalance has been implicated in eliciting PAD2 activity,<sup>19</sup> and perhaps calcium influences the increased PAD2 observed in myelinating immature oligodendrocytes.<sup>33</sup> Increased IOP in glaucoma often is associated with influx of calcium (e.g., from ischemia), resulting in increased intracellular calcium.<sup>34</sup> Notably in myelin, calcium concentration plays an important role in modulating several protein interactions,<sup>28,35</sup> including for example MBP interaction with calmodulin, which citrullination can disrupt.<sup>36</sup> In POAG, events triggered by intraocular pressure, including fluctuations in optic nerve intracellular calcium concentration, may increase the level of PAD2 and citrullination. Indeed, our *in vivo* results from POAG donors (Fig. 5A) and the DBA/2J glaucomatous mouse model (Fig. 2) support elevated IOP as a possible contributing factor to PAD2 expression. Our *in vitro* results (Fig. 5B) also suggest that pressure induces PAD2 expression in astrocytes; however, competing factors in the pressure treated astrocyte model may contribute to the observed results. Others have shown in astrocytes that hypoxia induces PAD2 expression, citrullination, and elevated intracellular calcium concentration.<sup>34,37</sup> Accordingly, fluctuations in gas composition during the 5-hour astrocyte pressure treatment used in the present study may have altered O<sub>2</sub> and CO<sub>2</sub> concentrations, resulting in hypoxia and/or media pH changes that impacted PAD2 expression.

Our observations are consistent with posttranscriptional control of PAD2 expression. In a preliminary analysis, optic nerve-derived RNA (pooled from two donors each: control and glaucomatous) used in a microarray analysis revealed changes in mRNA levels for 1923 proteins between control and glaucomatous optic nerve tissue; however, PAD2 was not among them. (Supporting microarray data have been deposited in the National Center for Biotechnology's GEO database, accession number GSE2387.) Optic nerve PAD2 mRNA levels appeared to be very similar between control and glaucomatous donors *in vivo* (Fig. 6A) and between pressure-treated and untreated astrocytes *in vitro* (Fig. 5D). However, glaucomatous optic nerve extracts depleted of polyadenylated RNA, PAD2 and GPDH, exhibited a significant increase in PAD2 expression (relative to GPDH) on addition of equal amounts of polyadenylated RNA, with no comparable increase in control extracts (Fig. 6B, 6C). These *in vitro* data suggest that the overexpression of PAD2 in glaucomatous tissue is primarily controlled at the translational level. However, *in vivo*, a lower normal steady state expression level could result from an increased degradation rate as well as from a decrease in the rate of translation. We have shown that *in vitro* targeted degradation of PAD2 mRNA with shRNA in pressure-treated astrocytes leads to a decrease in PAD2 and citrullination. The therapeutic potential of such targeted approaches remains to be evaluated in appropriate animal models of glaucoma. Whether PAD2 expression and citrullination cause neurodegeneration in POAG or are consequential to damage in glaucomatous optic nerve also remains to be determined. In any event, the present results implicate optic nerve PAD2-directed citrullination in the pathogenesis of glaucoma.



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