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Targeting optic nerve citrullination in glaucoma: a role for protein-arginine deiminase 2 (PAD2) inhibitors

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CONTENTS

Abstract

Protein-arginine deiminase 2 (PAD2, also known as peptidylarginine deiminase 2), a member of the protein-arginine deiminase (PAD) family, converts protein-bound arginine residues to citrulline, a process known as deimination. Using proteomics, PAD2 was detected in glaucomatous eyes as one of the most abundant proteins in the optic nerve compared to controls. Dysregulation of PAD2, leading to elevated levels and consequent aberrant deimination, was observed in response to pressure *in vitro* and is likely to occur *in vivo* as well. Experimental *in vitro* data indicate that, once initiated, dysregulation of PAD2 is not controllable by pressure regulation alone. Aberrant deimination has detrimental consequences at the cellular and tissue levels, implicating PAD2 as an independent target for intervention for neuroprotection in glaucoma. An account of PADs, their involvement in disease and their inhibitors, including PAD2 inhibitors, is presented here.

Introduction

Glaucoma is a term used to describe a group of late-onset, progressive and irreversible blinding eye diseases. Damage to the optic nerve is characteristic of glaucoma. Open-angle glaucoma, the most common form of glaucoma, affects about 70 million people worldwide (1).

The etiology of glaucoma is poorly understood, and available pharmacological or surgical interventions delay the progression but do not cure the disease. Many forms of glaucoma are associated with an increase in intraocular pressure (IOP). A clear fluid aqueous humor, actively secreted by the ciliary epithelium, serves a vital function in the anterior chamber, providing nutrients and removing excretory products from the cornea. After bathing the corneal endothelium, the aqueous humor exits through the structures of the anterior chamber (2). An imbalance between aqueous humor production and outflow is considered responsible for elevated IOP. Facility of aqueous humor outflow is an important determinant of IOP and obstruction of outflow underlies the disease pathology (2-4). Aqueous humor outflow experiences the greatest resistance at a filter-like region known as the trabecular meshwork (TM) (2).

Age, race, family history and elevated IOP are considered major risk factors for glaucoma (5, 6). However, many patients who develop characteristic glaucomatous neuropathy possess a normal IOP and have what is termed normal-tension or low-tension glaucoma (7, 8). Although lowering pressure has a beneficial effect in some normal-tension glaucoma (NTG) patients (7), it appears that additional pathogenic factors are involved in glaucomatous optic neuropathy in NTG patients (9). It is therefore likely that the identification of pathogenic factors in NTG will provide potential targets for neuroprotection in glaucoma.

Protein-arginine deiminases (PADs) and deimination

PADs (EC 3.5.3.15) are enzymes that catalyze the conversion of protein-bound arginine residues to citrulline (Fig. 1). The process is termed deimination and is often referred to interchangeably as citrullination; the reaction products are referred to as citrullinated proteins or citrulline. Citrulline is an uncommon amino acid. As no known carrier transfer RNA (tRNA) exists for citrulline, it is generally agreed that citrulline is the result of a post-translational modification and is not incorporated into proteins during translation (10, 11). Five PADs have been identified in a variety of mammalian tissues, including PAD1, 2, 3 and 6, which are cytosolic, and PAD4, which exhibits a nuclear localization (12). All PADs possess a high degree of homology (10). PAD4 was recently found to catalyze

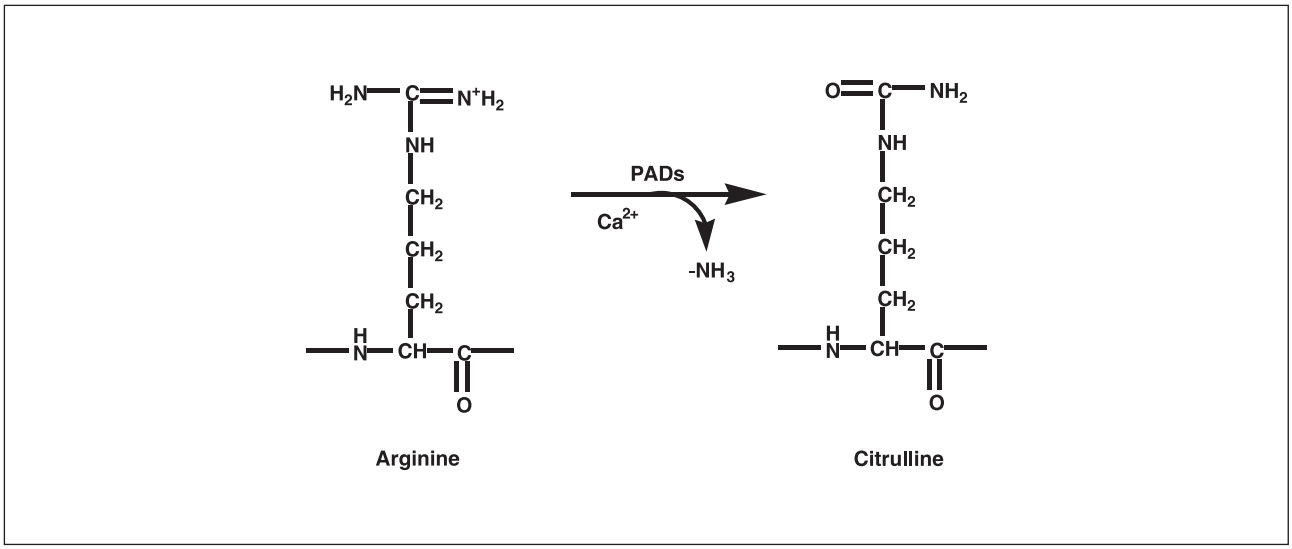

Fig. 1. Schematic representation of protein-arginine deiminase (PAD) activity. Protein-bound arginine is converted by PADs in the presence of calcium ions to citrulline, leading to the formation of ammonia, as depicted. PAD2 is the major enzyme catalyzing this reaction in neuronal tissue.

reverse methylation or demethylation, as well as deimination of proteins (13, 14). PAD2 (protein-arginine deiminase 2, or peptidylarginine deiminase 2) is found predominantly in neuronal tissues (15). The sequence homology and genomic organization of the known PAD enzymes in mammalian and nonmammalian species have been reviewed elsewhere (10).

All PAD enzymes require calcium ions for their activity, generate ammonia as a byproduct (Fig. 1) and are unable to convert free L-arginine. The enzymes known to convert free L-arginine to L-citrulline, generating nitric oxide (NO), are the nitric oxide synthases (NOS; EC 1.14.13.39), found in eukaryotes, including mammalian cells. Unlike the PADs, NOS does not require calcium ions for its activity. Arginine deiminase (EC 3.5.3.6) in bacteria also generates free citrulline. Detailing these activities is beyond the scope of this review. However, elevated IOP was shown to increase NOS2 activity (16, 17). The NOS enzymes have been implicated in neurotoxicity (18) and neurological diseases such as amyotrophic lateral sclerosis (ALS) (19, 20). NOS inhibitors, notably 6-aminoguanidine and nipradilol, have been evaluated as intervention strategies for glaucoma (17, 21-23) and have been proposed for pharmacological neuroprotection in glaucoma (24).

PADs and disease

PADs and deimination have been implicated in autoimmune rheumatoid arthritis (25). Elevated PAD2, citrullinated proteins and/or citrulline have been detected and implicated in human neurological diseases, with or without ocular manifestations, most prominently in multiple sclerosis (15), autoimmune encephalomyelitis (26), Alzheimer's disease (27, 28), ALS (20) and glaucoma (29, 30), opening up the prospect for the possible utilization of pan-PAD inhibitors and/or highly specific PAD2 inhibitors in the treatment of these diseases.

A prokaryotic enzyme that deiminates proteins, as well as free L-arginine, in the absence of calcium ions has been identified in *Porphyromonas gingivalis*. This enzyme is not related to the vertebrate PADs and shares sequence homology with bacterial arginine deiminases. Inhibitors of this enzyme have been considered for the treatment of periodontitis (31).

PAD2-directed deimination has been shown to be associated with kainate-induced neurodegeneration in rat brain (32, 33). The potential exploitation of PADs in the treatment of different diseases has therefore received widespread attention. A number of issued patents and patent applications have centered on the application of PADs and PAD autoantibodies in rheumatoid arthritis, either as biomarkers or as therapeutic intervention strategies (34, 35). PAD1 and PAD3 have been proposed as targets in skin diseases (Tables I and II), including psoriasis (36). PAD-like proteins (having about 40% homology with PADs) have been proposed as possible contraception targets (37, 38). A PAD3-like protein has also been proposed as a target for certain cancers (39). Finally, PAD2 has been proposed as a target for the treatment of multiple sclerosis (40, 41) and glaucoma (42).

Biological consequences of deimination and deimination substrates

The consequences of protein deimination may vary widely and depend on the protein substrate undergoing deimination, as well as the location where these substrates are being deiminated. Deimination appears to inhibit cell proliferation, leading to cell cycle arrest and apoptosis (43, 44). In neuronal systems (brain and the optic nerve), myelin contains several arginine-rich pro-

Table I: Known protein substrates of protein-arginine deiminases (PADs).

Protein	PAD activity	Disease	Ref.
Keratin	PAD1	Psoriasis	53
Myelin basic protein (MBP)	PAD2	Multiple sclerosis	60
Glial fibrillary acidic protein (GFAP)	PAD2	Alzheimer's disease	28
GFAP	PAD2	Multiple sclerosis	59
Vimentin	PAD2	Alzheimer's disease	28
Trichohyalin	PAD3	Skin disease	63
Histones (H2A, H3 and H4)	PAD4	Rheumatoid arthritis	56,57
Filaggrin	PAD4	Rheumatoid arthritis	64
Fibrinogen	PAD4	Rheumatoid arthritis	64

Table II: Known inhibitors of protein-arginine deiminases (PADs).

Inhibitor	Target PAD activity	Disease	Remarks	Ref.
Paclitaxel	PAD2	Multiple sclerosis	Antitumor agent; LD_{50} in mice is 128 mg/kg	60
Interferon beta	PAD2	Multiple sclerosis	Perhaps an indirect effect at the gene expression level	60
Monoiodoacetate	PAD3	Skin disease		65
PCMB	PAD3	Skin disease		65
Bz-N(G)-monomethyl-Arg‡	PAD4	Rheumatoid arthritis	Synthetic arginine analogue	58
Bz-N(G),N(G)-dimethyl-Arg‡	PAD4	Rheumatoid arthritis	Synthetic arginine analogue	58
shRNA (several)	PAD2	Rheumatoid arthritis, glaucoma, multiple sclerosis	Specific shRNA against NCBI accession numbers NM_007365, NM_007365	29
BAPTA-AM	PAD2	Primary open-angle glaucoma	Useful for controlling the intracellular Ca concentration. Acetoxymethyl ester derivative of BAPTA	30
F-Amidine	PAD4	Rheumatoid arthritis	Inactivate covalent modification of Cys645	66
Cl-Amidine	PAD4	Rheumatoid arthritis	Inactivate covalent modification of Cys645	67
2-Chloroacetamidine	PAD4	Rheumatoid arthritis	Inactivate covalent modification of Cys645	67-69
shRNA (several)	PAD4		Specific shRNA against NCBI accession numbers NM_012387, NM_012387, NM_012387, NM_012387	
Antipain	PAD*	Periodontitis	Formation of a hemicetal adduct between the aldehyde group of the inhibitor and the active site	31
Thiourea	PAD*			31
Cysteine	PAD*	Periodontitis		31
Leupeptin	PAD*	Periodontitis		31

*PAD from *Porphyromonas gingivalis* that converts protein-bound arginines and free arginines, does not require calcium for activity and is homologus to bacterial arginine deiminase that acts on free arginines; ‡The methylated Arg derivatives are very poor or inactive PAD4 inhibitors and many investigators now do not consider them as PAD4 inhibitors.

teins that are susceptible to deimination (45), including myelin basic protein (MBP), which has been detected as a major citrullinated protein in the brain of multiple sclerosis patients (46) and the optic nerve of patients with primary open-angle glaucoma (POAG) (29).

MBP is one of the most abundant proteins of the myelin sheath and functions in maintaining the stability of the sheath (47-49). Citrullinated MBP exhibits altered properties relative to the unmodified protein, including a lower net positive charge which disrupts its tertiary structure, as well as the ability to interact with lipids and maintain a compact myelin sheath (49, 50). Deimination also decreases the ability of MBP to aggregate large unilamellar vesicles (LUVs) (50), a process important for adhesion between intracellular surfaces of myelin. Citrullinated MBP exhibits increased susceptibility to autocatalytic cleavage (51) and cathepsin D proteolysis (49), which may generate immunodominant peptides, leading to sensitization of T-cells and an autoimmune response in demyelinating diseases.

Decreased protein arginyl methylation concomitant with increased PAD2 immunoreactivity and protein deimination were observed in the optic nerve in POAG (29). Using synthetic peptides that contain either arginine or methylated arginine residues, human PAD2, PAD3 and PAD4 enzymes and PAD enzymes from several mouse tissues have been shown *in vitro* to convert only non-methylated protein L-arginine to protein L-citrulline, but not methylated protein L-arginine. The finding of the interference of arginine methylation in deimination is also supported by experiments involving amino acid analysis of bovine histones with or without treatment with PADs (52).

In skin diseases and rheumatoid arthritis, citrullinated proteins generate autoantibodies, often eliciting an autoimmune response (53, 54), and PAD3 has been implicated in skin diseases, including psoriasis (53).

Histones act as a substrate for deimination and have the potential to play a role in a wide range of diseases (11). PAD4 has been suggested to play a role in reversing protein methylation, which has a role in relatively long-term cellular signaling (11, 13).

In the neuronal system, PAD2 is the major enzyme that undergoes dysregulation in the diseased state (55). A number of proteins, such as fibrinogen, vimentin and glial fibrillary acidic protein (GFAP), have been found to be substrates for deiminase activities (Table I). Several of these protein substrates may be involved in cell adhesion, cell migration and cell-cell communication. Whereas PAD4 is nuclear and expected to alter transcriptional events, modification of histones is suggestive of translational modulation (56, 57).

The modification of MBP, GFAP and vimentin (Table I) by PAD2 is suggested to affect cell signaling, cell adhesion and cell migration. Molecular mechanisms involving deimination may play a role in glaucomatous neuropathy. The presence of multiple citrullinated proteins in POAG optic nerve, including MBP, and possibly also myelin proteolipid protein (PLP) and myelin-associated glycoprotein (MAG), among others (29), would appear likely to disrupt myelination. The deimination of optic nerve head matrix proteins such as vimentin (28) may weaken their anchorage and result in overall weakness at the level of the optic nerve head. The deimination may cause changes in the dynamics of myelin components and may also cause disruption of the optic nerve head matrix protein framework, which may initiate or contribute to glaucomatous neuropathy (29).

Identification of PAD2 in glaucomatous optic nerve

Elevated PAD2 was identified in glaucomatous optic nerve but not in controls during proteomic analyses (29). Optic nerves from a total of 12 normal and 12 glaucomatous cadaver eyes were subjected to proteomic or Western blot analyses. About 8 samples in each group (glaucomatous and control) were subjected to liquid chromatography-tandem mass spectrometry, leading to the identification of PAD2 as one of the most abundant proteins in glaucomatous eyes, while being absent in control eyes. Subsequent Western bot and/or immunohistochemical analyses of all 24 samples confirmed the presence of PAD2, and its reaction products, citrullinated proteins, were observed in human POAG optic nerve, but not in controls.

PAD inhibitors targeting optic nerve deimination and PAD2 in glaucoma

As stated before, all PADs share a high degree of homology. This poses a challenge to develop specific activity-based inhibitors. Previously, methylated derivatives of arginines (Table II) had been postulated as PAD inhibitors (58). However, many investigators discovered that they were very poor inhibitors. Decreased arginine methylation concomitant with elevated deimination was found in the glaucomatous optic nerve head; in the same study, elaborate investigation did not reveal impairment of the cellular methylation machinery in the excised optic nerve (29). This observation would be consistent with the idea that deimination precedes methylation of arginines, or that deimination prevents subsequent methylation of the same arginines. Deimination has been shown to occur only on nonmethylated arginines (52). Taken together with the observations in glaucomatous optic nerve of increased deimination and decreased methylation, this suggests elevated and faster deimination of the arginines. The unmodified protein-bound arginines are competing modification candidates for methylation and deimination machinery. A number of small-molecule inhibitors have been developed for PADs (Table II).

Given its central role in reversing arginine methylation, the involvement of PAD4 (11, 13) in many neurological processes cannot be ruled out. However, as stated before, PAD2 appears to be most prevalent and perhaps most important in neurological systems (15, 26, 28-30, 59, 60). The dysregulation of PAD2 in neurological diseases may involve multiple factors and multiple levels. PAD2 regulation has been found to undergo aberrant transcription (55) in the brain in multiple sclerosis and aberrant translation (29) in glaucomatous eyes.

Inhibition of PADs, including PAD2, can be achieved at the transcriptional and translational levels, as well as directly at the activity level. To determine the level of inhibition of PAD2 and the effect of PAD2 inhibition, *in vitro* and *in vivo* models have been used. Cultured astrocytes have been utilized as an *in vitro* model system (29, 30) and DBA/2J mice as an *in vivo* model system (29) for glaucoma, while ND4 mice have been used as a model system (61) for multiple sclerosis. Specific inhibitors of PADs are difficult to design due to their sequence similarity and similar active site, but the use of inhibitory RNA has shown promise. Despite a degree of regulation at the translational level, PAD2 short hairpin RNA (shRNA) produced a high degree of downregulation of PAD2 and consequent protein deimination in pressure-treated astrocytes (Fig. 2). shRNA molecules against PAD2 have been successful in downregulating the PAD2 message, and their activity for providing neuroprotection in glaucoma is the subject of a patent (29, 42).

shRNA molecules for PAD2 and PAD4 are readily available in viral vectors from several commercial vendors. At the activity level, using astrocytes as a model system, calcium chelators such as BAPTA-AM have been shown to act as PAD2 inhibitors and reduce *in vitro* the protein deimination that occurs in response to elevated pressure (30). Several other activity-based synthetic small-molecule inhibitors have been developed for PAD2 and PAD4 (Table II), the efficacy of which remains to be investigated in glaucoma, multiple sclerosis and other neurological diseases. In the future, *in vivo* animal models, for example, the DBA/2J mouse (29, 62) and the ND4 mouse (61), will allow us to determine the efficacy of different PAD2 inhibitors as neuroprotectants for glaucoma and multiple sclerosis, respectively. Preliminary animal

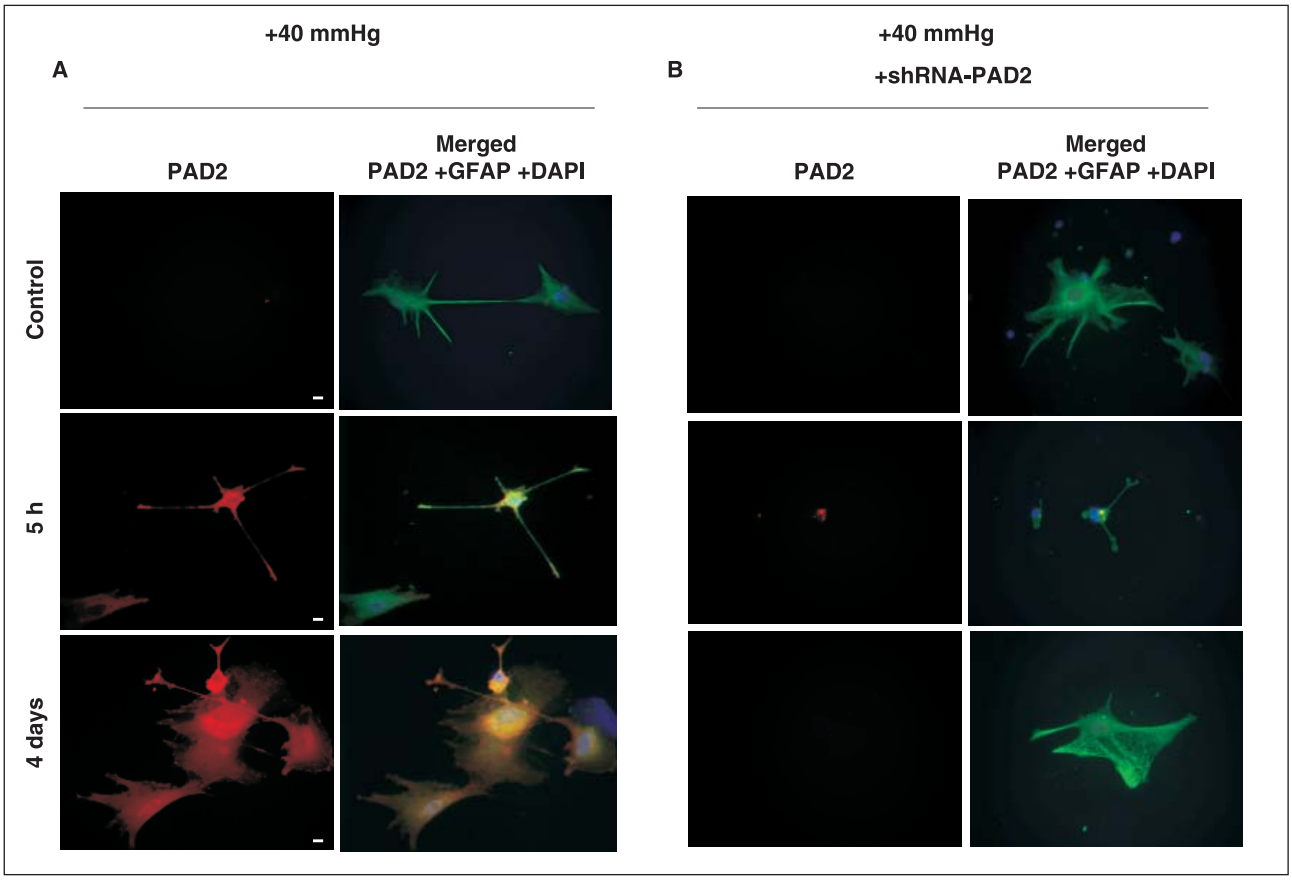

Fig. 2. Immunohistochemical analysis of protein-arginine deiminase 2 (PAD2) downregulation in isolated rat cortical astrocytes. **A.** Astrocytes were incubated with mouse monoclonal anti-PAD2 and rabbit polyclonal anti-GFAP antibodies and DAPI (4',6-diamidino-2-phenylindole) with pressure (40 mmHg for 5 h) or without pressure (atmospheric pressure for 5 h; control, upper panel) for 5 h or 4 days. **B.** Astrocytes were incubated with anti-PAD2 and anti-GFAP antibodies and DAPI after subjecting them to a pressure of 40 mmHg for 5 h and treatment with short hairpin RNA (shRNA) against the coding region of the PAD2 gene in a plasmid vector, or control (atmospheric pressure for 5 h; upper panel) and incubated for 5 h or 4 days. Bar = 40 μm.

studies indicate that shRNA, small interfering RNA (siRNA) and small-molecule inhibitors of PAD2 may act as potential neuroprotectants in glaucomatous neuropathy.

Conclusions

PADs have been implicated in a number of fundamental biological processes, including the reversal of long-term methylation signals. PADs, and in particular PAD2, have been implicated in several neurological diseases with or without vision loss, and specifically in glaucoma. It is apparent that dysregulation of PAD2 is critical in that it renders protein deimination aberrant, leading to unintended and detrimental biological consequences in glaucoma and other neurological diseases. Calcium modulation may have limited efficacy for controlling PAD2. Several calcium-modulating drugs are being tested for modulation of PAD2 activity with the aim of using the best subset of such drugs for the treatment of glaucoma in the DBA/2J mouse model. Small inhibitory RNA, its high specificity and modes of specific delivery hold promise for developing siRNA and shRNA inhibitors for PADs in these diseases. A number of synthetic small-molecule inhibitors have shown promise. With the growing spectrum of such inhibitors, it is likely that high specificity and efficacy may be achieved. Elucidation of the early events in dysregulation will help the advancement of our understanding of these diseases and the development of more effective intervention. In the meantime, the present arsenal of PAD inhibitors holds promise for neuroprotection in glaucoma, and more rigorous animal studies are needed to confirm their suitability for human use.

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