PGJ₂ Provides Prolonged CNS Stroke Protection by Reducing White Matter Edema

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Abstract

Few clinically effective approaches reduce CNS-white matter injury. After early in-vivo white matter infarct, NFkB-driven pro-inflammatory signals can amplify a relatively small amount of vascular damage, resulting in progressive endothelial dysfunction to create a severe ischemic lesion. This process can be minimized by 15-deoxy-A⁻¹₂⁴₄-prostaglandin J₂ (PGJ₂), an analog of the metabolically active PGD₂ metabolite. We evaluated PGJ₂’s effects and mechanisms using rodent anterior ischemic optic neuropathy (rAION); an in vivo white matter ischemia model. PGJ₂ administration systemically administered either acutely or 5 hours post-infarct results in significant neuroprotection, with stereologic evaluation showing improved neuronal survival 30 days post-infarct. Quantitative capillary vascular analysis reveals that PGJ₂ improves perfusion at 1 day post-infarct by reducing tissue edema. Our results suggest that PGJ₂ acts by reducing NFkB signaling through preventing p65 nuclear localization and inhibiting inflammatory gene expression. Importantly, PGJ₂ showed no in vivo toxicity structurally as measured by optic nerve (ON) myelin thickness, functionally by ON-compound action potentials, on a cellular basis by oligodendrocyte precursor survival or changes in ON-myelin gene expression. PGJ₂ may be a clinically useful neuroprotective agent for ON and other CNS infarcts involving white matter, with mechanisms of action enabling effective treatment beyond the currently considered maximal time for intervention.

Introduction

Prolonged CNS ischemia causes microvasculature injury, producing tissue edema, micro-thrombus and hemorrhagic conversion [1]. Post-infarct edema compromises capillary function [1–4]. Edema reduction, independent of other therapy types, is therefore likely to be useful in CNS ischemia treatment.

Much attention focuses on grey matter (GM) ischemia, but relatively little on isolated white matter (WM) infarct. WM comprises 40–44% of the human brain [5], but 13–15% of the rodent brain [6]. The smaller WM volume makes WM ischemia challenging to study using in vivo rodent models. While WM total oxygen requirement is less than that for GM [7], the quantitative vascular differences between the two suggest that WM may be equally sensitive to microvascular injury that results in long-term capillary dysfunction.

The mammalian optic nerve (ON) is a myelinated WM tract possessing all the essential attributes of other CNS WM regions. Unlike other CNS tracts, the ON has an isolated vascular supply [8]. Optic nerve axons derive from the retinal ganglion cell (RGC) neurons whose cell bodies are in the retina of the eye. Nonarteritic anterior ischemic optic neuropathy (NAION) is a sudden idiopathic ON ischemic event in the anterior ON that results in edema and permanent ON damage [9]. A rodent NAION model has been generated [10] (rodent anterior ischemic optic neuropathy; rAION), using laser light activation of intravenous rose Bengal (RB) dye within the target capillary field. The rAION model injuries the smallest ON capillaries, sparing larger vessels, and resulting in ON edema [10]. Previous studies that have revealed early edema-associated capillary changes following CNS ischemic lesions were performed in cortical grey matter or analyzed as part of a complex lesion such as a middle cerebral artery occlusion (MCAO) [11,12]. We utilized the ON to examine microvascular changes following WM ischemia using a newly devised quantitative vessel filling technique.

Prostaglandin J₂ (PGJ₂) is a compound derived from the nonenzymatic modification of prostaglandin D₂ (PGD₂) and has been found to be neuroprotective in a number of systems [13,14]. PGJ₂ binds to and activates peroxisome proliferator-activated receptor-γ (PPAR-γ) and down-regulates nuclear factor kappa B (NFkB) [15]. Both these pathways down-regulate inflammation [16]. Post-infarct PGJ₂ administration is neuroprotective following middle cerebral artery occlusion (MCAO) by non-overlapping NFkB and PPAR-γ dependent and independent
mechanisms [14, 17] as well as being anti-inflammatory when used pre-induction in models of mixed stroke and in non-neuronal systems. PGJ2 reportedly stabilizes vascular capillary flow [for review see [18]], but is also reported to be toxic when tested in vivo against neuronal cells [19], as well as oligodendrocytes and their precursors [20]. However, in vivo analysis is subject to a number of caveats, most notably the enhanced PGJ2 elimination found in vivo and the extended exposure time to high concentrations of PGJ2 found in vivo. Thus the question of whether such a potentially useful compound is too toxic to use for ischemia treatment should be answered in an intact system using reasonable doses and route of administration suitable for a neuroprotectant. We found that PGJ2 is effective in protecting against white matter ischemia-ischemia-induced damage without apparent effect on oligodendrocyte function or oligodendrocyte precursor development post-treatment. Our data suggests that PGJ2 may be a clinically effective, non-toxic in vivo therapeutic adjunct in reducing damage following isolated white matter stroke by directly reducing edema.

Methods

Animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Organization (ACUO) of the University of Maryland at Baltimore (#0906001; Institutional approval A3200-001). All surgery was performed under Ketamine-Xylazine anesthesia, and all efforts were made to minimize suffering. Male Sprague-Dawley (SD) rats (150–200 g) were obtained from Harlan Laboratories, USA.

Optic Nerve Stroke and treatment

ON infarct was induced as previously described [10]. Briefly, intravenous (i.v.) Rose Bengal (RB) (Sigma-Aldrich; 2.5 mg/kg in sterile saline) was administered via tail vein. RB was activated by intraocular optic nerve illumination via 532 nm wavelength laser light by illuminating the optic disk with a 500 μm spot size at 50 mW intensity for 12 seconds. This results in ON ischemia at 1 hour post-induction. Our data suggests that PGJ2 may be a clinically effective, non-toxic in vivo therapeutic adjunct in reducing damage following isolated white matter stroke by directly reducing edema.

In vivo imaging

ON edema and the retina were evaluated in the living animal using a plano-convex contact lens, enabling visualization of the rodent retina and anterior optic nerve (Bernstein et al. 4153-62). Optic nerve fundus images were obtained at baseline and one day post-induction using a digital camera (Nikon D1X). We also visualized and evaluated the retinal cell layers and ON diameter via a spectral domain-optical coherence tomograph (SD-OCT) (Heidelberg Instruments), using the same contact lens. ON edema quantification was performed by measuring the width of the optic nerve shadow (defined as the region between the ends of the retinal image) at the greatest scanned point.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

The proximal 3 mm of the ON was isolated and stored at −80°C. Total ribonucleic acid (RNA) was isolated using the RNeasy micro kit (#74004, Qiagen Inc.) and deoxyribonuclease-1 treated to eliminate genomic DNA contamination. Nerves were frozen on dry ice, then homogenized in lysis buffer using linear acrylamide as a carrier, followed by proteinase K digestion. RNA was analyzed for purity and quality using an Agilent Bio-analyzer. Because of the low yield for individual ON samples, we used the single chimeric primer amplification (SPiA) method (Ovation pico system, Nugen Corp.) to provide non-biased linear amplification of small amounts of mRNA. This reduced animal use, enabling us to compare gene response in individual rats for some studies rather than using pooled mRNA. For ON toxicity studies, RNA preparations were prepared from pooled (n=6 optic nerves), and random primed complementary DNA (cDNA) was generated without an initial linear amplification step. RNA was converted to first strand random primed cDNA via RETR oscript 1710 kit (Ambion Inc.). cDNA was used for qRT-PCR with gene-specific primers using Syber green dye (Bio-Rad Laboratories), with both cyclophilin B and β-Actin expression used as internal controls. Specific mRNA levels were evaluated using the ddCT method of difference subtraction from control gene expression levels. The following primers were used: Tumor necrosis factor alpha (TNFα) (f) accegcaaaagcaagcagg (r) cagaggggattgtcg (interleukin 1-beta (IL-1β) (f) gctagggagccctgtcg (r) gctctgagagacctgacttggca; cyclophilin B (f) tgaagctgacctatc (r) ggtggatgctctgagcttagt. NG2 proteoglycan (f) accagcgctgggtacg (r) tgtggacacgactcggc (127 bp) Myelin basic protein (MBP) (f) gagaggagtctcgtgggatt (r) cggagctgcccacg (138 bp) IL-4: (f) gatgggtctcagcccccacctt (r) ccgtggataccgttcccggtac (72 bp) platelet derived growth factor alpha (PDG Frα) (f) agacagactgecgacttggag (r) tcagctgtcagcctagag (170 bp) Olig1 (f) gacagcgatctgccgatt (r) cggagtgtcgcaggctac (73 bp).

Compound action potentials (CAPs)

CAPs were recorded from rat ONs harvested immediately after CO2 euthanasia. ONs were dissected and immediately submerged in ice-cold (4°C) Locke’s solution composed of (in mM): 136 NaCl, 5.6 KCl, 14.3 NaHCO3, 1.2 Na2HPO4, 2.2 CaCl2, 1.2 MgCl2, 11 mm dextrose, equilibrated continuously with 95% O2, 5% CO2, pH 7.2–7.4. Nerves were pinned to the Sylgard (Dow Corning)-coated floor of a recording chamber (~0.25 ml volume) and superfused (~3–5 ml/min) with oxygenated Locke solution at room temperature, 22-24°C. CAPs were recorded with a glass suction electrode connected to the input stage of an alternating current–coupled differential preamplifier (0.1–1 kHz model DAM-5A, World Precision Instruments). Data were filtered at 2 kHz and sampled at 10 kHz. CAPs were evoked with electrical pulses (0.1–0.5 msec in duration) elicited at 0.2 Hz using a second glass suction electrode. CAPs were digitized via a Digidata 1200 A/D converter (Axon Instruments) and stored on a computer. Ten CAPs were averaged before saving. Data acquisition and storage were controlled via pClamp 9.1 (Axon Instruments) and analyzed with Clampfit 8.2 software (Axon Instruments).

ON vascular filling quantification

Quantitative ON vascular analysis was performed using tissue from terminally anesthetized (surgical plane anesthesia) rats. Animals were placed on a warming pad (~38°C) and transcardially perfused sequentially with the following heated (~38°C)
solutions: 120 ml heparinized saline (50 units/ml) with 2 µg/ml atropine sulfate (Sigma Chemicals) and 100 µM adenosine (HAAS solution), 50 ml fluorescein-conjugated bovine serum albumen (FITC-BSA) with 2% dissolved gelatin (300 bloom, Sigma Chemicals) in HAAS solution, 20 ml FITC-BSA with 4% dissolved gelatin in HAAS solution. The descending aorta was clamped during perfusion. The ascending aorta was clamped under pressure at perfusion’s end and the carcass immersed in ice dissolved gelatin in HAAS solution. The descending aorta was dissolved gelatin (PVDF) membrane, and detected using secondary antibodies coupled to horseradish peroxidase (Vector labs) and developed using Lumi-light substrate (Roche Laboratories). Antibodies used included the NFκB- p65 subunit (sc372, Santa Cruz Biotechnol- gy, CA), an antibody that cross-reacts with p50/p65-NFκB subunits p65/p50 (#4764, Cell Signaling), and inducible nitric oxide synthase (iNOS) (#160862, Cayman Chemical). β-Actin (#A5441, Sigma) and Lamin C (#4777, Cell Signaling) were used as loading controls [22].

Retinal ganglion cell neuron (RGC) Stereology

Stereology was performed on flat-mounted whole retinae 30 days post-induction, using StereoInvestigator software (Micro- brightfield Inc.) on a Nikon E800 microscope with a motor-driven stage driven by the stereological software to generate random fields of up to 30 cell nuclei for counting. A minimum of 1000 cells Brn 3a(+) were counted per retina, which is greater than the number required by the Schmitz-Hof equation [23] for statistical validity.

ON-oligodendrocyte precursor Stereology

Confocal images were acquired in tiled z-stacks on an LSM-510 duo microscope (Zeiss software, Zeiss Inc.). Using Imaris software (Bitplane Inc.), an isosurface was created within the z-stack on GFAP+ (Cy3 channel) regions of the ON to calculate tissue volume. The isosurface was used to mask the NG2-Cy5 channel, setting all voxels outside the mask to zero. Both NG2(+)/GFAP(+) (considered to be astrocyte precursors) and NG2(+)/GFAP(−) (oligodendrocyte precursors) were detected. NG2(+)/GFAP(+) (cell body and processes) cells with a DAPI positive nucleus present within the masked volume were counted as positive oligodendro- cye precursors.

Transmission electron microscopy

Sections of optic nerve prepared as above for vascular imaging were further analyzed after embedding in Durcupan resin (Electron Microscopy Sciences, PA). Specimens were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, 2 mM calcium chloride in 0.1 M Phosphate buffer (pH 7.4), and post-fixed with 1% osmium tetroxide, 1% potassium ferrocyanide in 0.1 M phosphate buffer. Specimens were washed and dehydrated using increasing ethanol washes, followed by 1% uranyl acetate and finally infiltration with Durcupan resin and polymerized at 60°C overnight. Ultratrin (70 nm) sections were cut and collected using a Leica UC6 ultramicrotome (Leica Microsystems, Inc., Bannock- burn, IL), counterstained with uranyl acetate and lead citrate, and examined in a transmission electron microscope (Technai T12, FEI Inc.) operated at 80 kV. Digital images were acquired using an AMT bottom mount CCD camera and AMT600 software (Advanced Microscopy Techniques, MA).

Myelin quantification was performed on 3 optic nerves/treatment group, 30 days post-treatment. 5 axons from each size family (small, medium, large diameter axons) were identified at low magnification, and myelin thickness was measured at the narrowest point for each axon at high (30–40,000×) magnification. Myelin values were averaged for each group.

Results

PGJ2 reduces ON edema

Naive rats have an intraocular ON segment that is flat, with a reddish hued border (Figure 1B). Retinal veins (Figure 1B: Rv) are of normal caliber. One day after rAION-induced infarct, the ONs...
of vehicle treated animals (Figure 1D) are edematous and increased in size (Figure 1D), with whitening and blurred margins, compared with naive animals (compare Figure 1D, white arrows, with Figure 1B). Retinal veins are engorged, with edema blocking vein outlines at the ON margin (Figure 1D, black arrow). The ON head one day post-infarct in animals treated acutely with PGJ2, is reduced in size, with reduced edema (compare Figure 1F, PGJ2 treated, with vehicle treated, Figure 1D).

We confirmed in vivo changes in post-infarct ON edema by spectral domain-optical coherence tomography (SD-OCT). SD-OCT utilizes infra-red laser imaging to identify subtle alterations in tissue density, thickness and disruptions. SD-OCT can reveal cross-sectional tissue features and resolve questions concerning sub-surface structural changes in living tissue. SD-OCT data are shown in Figure 1. The retina in naive eyes is visualized as a laminated structure (Figure 1A, ret). The ON diameter is indicated (Figure 1A, white arrows). One day post-rAION in vehicle treated animals (Figure 1C) there is increased opacity of the intra-retinal axonal layer and ON diameter expansion (Figure 1G, arrows). In contrast, SD-OCT of rAION-induced PGJ2-treated animals show reduced ON edema and diameter (Figure 1E) compared with vehicle treated rats (arrows Figure 1E, compare with 1C). To confirm these findings, we quantified the changes in ON edema, using the SD-OCT. ON widening was compared in animals either treated with PGJ2 (n = 5 animals) or vehicle (n = 5 animals) (Figure 1G). There was reduced ON edema 1 d post-rAION induction in the PGJ2-treatment group (mean ON width 535.8 ± 108 microns), compared with vehicle-treated animals (649.8 ± 150.5 microns), while there were no differences in baseline ON widths of either group (372.8 ± 13.3 PGJ2 treated v. 378.6 ± 22.2 vehicle-treated) These results confirm that PGJ2 treatment reduces ON edema following infarct.

PGJ2 improves capillary perfusion by reducing post-infarct edema

We quantitatively evaluated capillary perfusion volume in the ON proximal segment (the region directly affected by the ON infarct). Capillary volumes were compared post-infarct in vehicle vs. PGJ2-treated eyes. We used the capillary volume of the uninfarcted eye of each animal as an internal control to eliminate individual variation in capillary density, which can be considerable.

Our analysis revealed that infarct-related primary ON damage is associated with decreased capillary perfusion, which occurs in the anterior 1 mm of the ON and is the site of the rAION primary lesion [24]. This region of the naive ON has a dense anastomotic capillary network with limited arterial perfusion or venous drainage (Figure 2A) [8].

The naive ON has an extensive capillary network that is relatively uniformly distributed throughout the tissue (Figure 2A). Four hours post-rAION there is a detectable but minimal capillary dropout in both the vehicle (Figure 2C) and PGJ2-treated (Figure 2D) ONs. No significant differences were found in contralateral (uninduced) ONs between PGJ2- and vehicle-treated animals (data not shown), when compared with naive contralateral eyes. One day post-rAION, vehicle-treated animals showed a

Figure 1. SD-OCT and retinal color photos of naive, and rAION-induced vehicle and PGJ2 -treated eyes. A,B. Naive eye. By OCT, the retina is flat against the back of the eye. The ON shadow is narrow (arrows and white line). Retinal photo reveals the ON is flat against the retina, with no protrusion. Retinal veins (RV) are of narrow caliber. C,D. rAION-vehicle treated. ON shows increased diameter by OCT analysis (arrows), compared with the naive eye (compare white line lengths). The retinal photo reveals a swollen and edematous ON (delineated by the white arrows). Crossing vessels are obscured in the edematous nerve (black arrow). E,F. rAION- PGJ2 treatment. The ON shadow by OCT imaging is smaller, compared with the vehicle treated animal (compare arrows and underlying diameter line in OCT photos of naive, and vehicle- and PGJ2-treated, rAION induced eyes). ON edema in PGJ2-treated animals is reduced in the color photo and is similar to the naive eye. Retinal veins (RV) engorgement is reduced. G. SD-OCT quantification. The optic nerve diameter is equivalent in both naive and uninduced PGJ2-treated animals. One day post-induction, the ON diameter is increased in both groups. ON diameters of PGJ2-treated animals are narrower, compared with vehicle-treated animals. The PGJ2-associated edema reduction is statistically significant (** p < 0.05; n = 7), Bonferroni multiple comparison test.

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severe loss of capillary vascular perfusion (Figure 2E), compared with PGJ2-treated animals (Figure 2F). Quantified mean perfusion of vehicle treated ON capillaries was only 25% of filling compared with contralateral (naive control) nerve of the same animal (1.0 of filling), and expressed on the Y axis as a fractional vessel volume (OD/OS). PGJ2-treated animals show significantly more patent capillaries at one day than treated animals (Mann-Whitney U test, p<0.03). There is minimal loss of capillary patency in both vehicle- (panel C) and PGJ2-treated (panel D) nerves. E and F: ON capillary filling 1 day post-induction. There is significant loss of vascular patency in ONs of vehicle treated animals (panel E). ONs in PGJ2-treated animals (panel F) reveal considerably more patent vasculature at one day. Scale bar: 100 microns.

Figure 2. Microvascular analysis of naive and infarcted ONs at 4 hours and 1 day post-induction. A. FITC-BSA vascular imaging of naive ON. Filled capillaries are relatively uniformly distributed throughout the nerve, communicating with peripheral vasculature. B. Quantitative capillary analysis of 1 day post-rAION-induced ON, in vehicle- and PGJ2-treated animals. Infarcted nerves were compared with the contralateral (naive control) nerve of the same animal (1.0 of filling), and expressed on the Y axis as a fractional vessel volume (OD/OS). PGJ2-treated animals show significantly more patent capillaries at one day than vehicle treated animals (Mann-Whitney U test, p<0.03). C and D: ON capillary filling 4 hours post-induction. There is minimal loss of capillary patency in both vehicle- (panel C) and PGJ2-treated (panel D) nerves. E and F: ON capillary filling 1 day post-induction. There is significant loss of vascular patency in ONs of vehicle treated animals (panel E). ONs in PGJ2-treated animals (panel F) reveal considerably more patent vasculature at one day. Scale bar: 100 microns.

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Figure 3C with 3D). At high magnification, the naïve ON capillary shows intact mitochondria and the presence of small vacuoles (Figure 3E). In comparison, vacuoles fusing with the cytoplasmic membrane are consistently present in the capillaries of rAION-induced endothelial cells (Figure 3F), which co-localize with reduced and absent areas of capillary patency. C–F: ultrastructural analysis using TEM. C: Naïve ON capillary, magnification 4400 ×. An endothelial cell nucleus (N) is adjacent to the capillary lumen (L). D: ON capillary 1 day post-induction, magnification 4400 ×. The endothelial cell nucleus (N) is lobulated and enlarged, and protruding into the lumen (L), which is reduced in diameter. E: Naïve ON capillary, magnification 15000 ×. Mitochondria are intact and there are small vacuoles in the endothelial cytoplasm, adjacent to the lumen (L). The nucleus (N) is normal in appearance. F: ON capillary 1 day post-induction, magnification 15000 ×. The endothelial cell nucleus (N) has changed appearance. Large cytoplasmic vacuoles, emptying on both sides of the cell membrane, suggest enhanced fluid transit from the lumen and increased extracellular fluid. Scale bars: A. 50 microns. B. 10 microns. C,D, E,F.

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and 3 kDa dextran accumulation is limited to the lesion in the anterior ON (Figure 5D, arrows), without leakage in the posterior ON, suggesting that gross BBB breakdown is limited to the region of the anterior ON without general loss of endothelial competency.

RGC neurons die after ischemic axonopathy [25]. Following rAION induction in the rat, 90% of RGC loss occurs by 30 days [26]. Unbiased stereology of Brn3a (+) RGCs was performed 30 days post-induction in naïve, vehicle, and PGJ2-treated animals (Figure 6).

Both immediate and delayed (5 hr post-induction) PGJ2 administration reduced rAION-induced RGC loss compared with vehicle controls (Figure 6A–C). The patterns of RGC- and axonal loss were also compared (Figure 6D–F) with those of naïve retina and ON.

RGCs in naïve retina are present as a monolayer of Brn3a (+) cells (Figure 6A, naïve, and inset, below). rAION-induced vehicle treated animals showed extensive regional loss of Brn3a(+) immunopositive cells (Figure 6B rAION-vehicle, and inset below). Systemic post-induction PGJ2 treatment improved overall RGC numbers at 30 days after infarct, compared with vehicle treated animals (Figure 6C, 15d-PGJ2, inset below).

We also compared the ON structure in the different treatment groups. Axons from naïve ONs are of different diameters, (Figure 6D, AxB) in thinly septated bundles (Figure 6D, Sep).

ONs from rAION-induced vehicle-treated animals (Figure 6E) show diffuse as well as focal axonal loss and scarring (Figure 6E, AxB), with increased septal thickness (Figure 6E, Sep; compare with 6D). In contrast, ONs of rAION-induced PGJ2-treated animals (Figure 6F) have structural characteristics intermediate to that of the naïve and vehicle-treated animals, with a milder diffuse axonal loss of axon bundles (Figure 6F, AxB), and fewer areas of complete loss (Figure 6F, compare with 6D and 6E).

Retinal stereological analysis 30 days post-infarct reveal extensive RGC loss in vehicle-treated animals (Figure 6G, vehicle: 55±12%, n = 12 animals), compared with naïve eyes (compare naïve and vehicle, Figure 6G). Systemic PGJ2 administration effectively reduced RGC loss when administered immediately post-induction (Figure 6G, PGJ2: 38±14%, n = 10 animals). Since tissue edema is a gradual phenomenon, we hypothesized that PGJ2 could provide protection against the ischemia-related compartment effect even when administered at times later than the current maximum time limit used in thrombolytic interventions in stroke, i.e. 4.5 hours. To assess this we treated with PGJ2 5 hours post-insult. PGJ2 is nearly as effective in reducing RGC loss at 5 hours post-induction as when administered immediately after rAION induction (Figure 6G, PGJ2 at 5 hours: 35±12% additional RGCs (n = 7 animals) vs. 38±14% additional RGCs in animals given PGJ2 immediately post-infarct). The neuroprotec-
The effective effect was statistically significant at both treatment times, compared with vehicle (ANOVA, p<0.05).

**PGJ₂ exerts ON neuroprotection by inhibiting the classical NFKB pathway**

Previous studies of PGJ₂’s mechanisms of action suggest that PGJ₂ works by mixed mechanisms. These include peroxisome proliferator-activated receptor gamma (PPARγ) activation and NFKB inhibition. In order to identify ON-NFKB activation and its inhibition by PGJ₂, we examined p65 (NFKB) expression and nuclear localization in vivo via a novel immunohistochemical technique (Figure 7A). Because κB sites (the target of the NFKB signaling complex) are numerous throughout the nuclear genome [27], NFKB cellular content is consequently quite high for a transcription factor. We localized the NFKB-p65 subunit by using immunohistochemical precipitation of nickel-diaminobenzidine (Ni-DAB), a black product that quenches 4',6-diamidino-2-phenylindole (DAPI) fluorescence. Nuclei were stained in situ following NFKB immunolocalization with DAPI. The presence of p65-associated Ni-DAB quenches the DAPI signal relative to the concentration of intra-nuclear p65. Differences in nuclear p65 localization and concentration can therefore be evaluated by...
differential DAPI fluorescence when other immunostaining factors are kept constant (Figure 7A, panels 1–4).

We compared nuclear fluorescence levels between the different treatment strategies, as well as total cellular and nuclear Ni-DAB signal levels. Using this new technique, our data reveals that NFκB-p65 subunit expression is diffusely present in ON glial and vascular cells in the WM of the un-infarcted, vehicle-treated ON (Figure 7A, panel 1). There is considerable quenching of the DAPI nuclear signal even in naïve uninduced nerves (Figure 7A, panel 1 arrows). In vehicle treated animals 1 day post-rAION induction, there is a noticeable increase in overall p65 expression, with tissue darkening (Figure 7A, panel 2). In vehicle treated animals 1 day post-PGJ2 treatment, there is an increased p65 intercellular Ni-DAB signal, concurrent with strong DAPI fluorescence (Figure 7A, panel 3), compared with naïve ON (compare vehicle to naïve in panels 3 and 1, and compare insets). rAION-induced ONs in PGJ2 treated animals also show increased intracellular p65 signal (Figure 7A, panel 4 inset), but conversely, also show increased DAPI nuclear fluorescence (Figure 7A, panel 4; note blue color intensity). These data suggest that while total cellular p65 increases in infarcted PGJ2-treated ON tissues, there is decreased p65 nuclear localization. This result is supported by the strong perinuclear p65 signal in the same tissues (arrowheads, Figure 7A, panel 4), indicating that NFκB is sequestered in the cytoplasm in these cells. Thus, PGJ2 administration results in strong p65 up-regulation independent of rAION treatment. ON infarct also results in additional p65 up-regulation. The increased p65 expression is not translocated to the nucleus in PGJ2-treated animals.

To independently confirm this finding, we compared well-documented p65 downstream transcript targets via qRT-PCR (Figure 7B). ON total RNA was isolated from rAION-induced and contralateral un-induced nerves from individual animals 1 day post-rAION. Animals were treated with either vehicle, or PGJ2 (n = 5/group). First strand cDNA was prepared by random priming and analyzed for IL-1β and TNFα expression. IL-1β and TNFα mRNA upregulation occurred 1 day post-infarct induction (Figure 7B). PGJ2 administration significantly reduced ON levels.
of both cytokines (Figure 7B; Students t-test, p<0.05). These results suggest that PGJ$_2$ administration inhibits the classical NFkb-associated inflammatory pathway activated after ischemia.

We also evaluated NFkB-related p65 protein levels using western blot analysis (Figure 7C). Protein homogenates were prepared from the first 2 mm of ON and ONs were pooled (n = 3) to achieve sufficient detection concentration. Four conditions were evaluated: 1) 1 day post-rAION induction (I) or uninduced (U) ONs treated with either PGJ$_2$ or vehicle (veh). p65 expression increased slightly in vehicle-treated ON post-infarct, compared with uninduced (naive) control ON. PGJ$_2$ administration greatly increased p65-ON expression, regardless of rAION status, compared with naive or vehicle (Figure 7C; compare PGJ lanes with vehicle and naive).

We confirmed PGJ$_2$’s effects on p65 expression in corpus callosum (CC); another WM tract, by western analysis (Figure 7D). Tissues were dissected from both naive- and 1 day post-PGJ$_2$-treated animals. Western analysis revealed PGJ$_2$-associated upregulation of p65 (Figure 7D, p65, compare naive and vehicle-treated with PGJ$_2$-treatment). Analysis using a polyclonal antibody that recognizes both p50 and p65 subunits also revealed p65 upregulation only in PGJ$_2$-treated CC (Figure 7D; p50/p65 row). PGJ$_2$ treatment also reduced inducible nitric oxide synthase (iNOS), a protein known to be controlled by NFkB activity (Figure 7D, iNOS), compared with naive or vehicle treated animals. All these data confirm that PGJ$_2$ administration upregulates p65 and blocks it from entering the nucleus in both CNS white matter structures, the ON and CC.

PGJ$_2$ is not toxic to oligodendrocytes in vivo

We evaluated PGJ$_2$’s long-term associated in vivo effects on neuronal and oligodendrocyte function by four methods: 1) Analysis of myelinated axonal conduction speed via compound action potential (CAP) analysis [Jimura 33-37]. This was compared to un-infarcted naive- and PGJ$_2$-treated ONs (i.e., 100 μg/kg PGJ$_2$ at 1, 7 and 30 days post-administration, (Figure 8A). 2) qPCR analysis of mRNA from animals given 100 μg/kg PGJ$_2$ eight days prior to analysis (n = 3 animals/group; total 6 ONs/group) (Figure 8B). 3) TEM-ultrastructural analysis and quantification of myelin thickness of ON-axons from naive vs. PGJ$_2$-treated (100 μg/kg) animals (Figure 9A and B). 4) Quantitative analysis of the NG2 (+) expressing oligodendrocyte precursors (NG2(+)/GFAP(−)) in ONs from naive vs. PGJ$_2$-treated (100 μg/kg) animals 30 days post-treatment (Figure 9C and D).

ON-CAPs from naive animals generate three amplitude peaks corresponding to the relative axon diameters present in rat ON (Figure 8A-naive). Slight apparent differences in ON transmission speed were seen between naive and treated animals 8 d post-treatment) animals but interneural differences are also present when different naive animals are compared with each other (D. Weinreich, unpublished data) (see inset, Figure 8A). Slight CAP variations were also apparent in ONs from animals given 400 μg/kg PGJ$_2$ eight days prior to testing, compared with naive controls (compare naive, 100 and 400 μg/kg values; inset, Figure 8A). Thus, data from PGJ$_2$-treated ONs were all within the naive range group (D. Weinreich, unpublished data).

q-PCR of OM mRNA from animals treated 8 days post-100 μg/kg also revealed little change in housekeeping genes, or myelin-related genes typically associated with mature oligodendrocytes (MBP and CNPase; Figure 8B). Analysis of genes associated with immature- or precursor oligodendrocyte function (Olig-1 and PGDFRα; Figure 8B) also showed little change in treated animals, compared with vehicle controls.

TEM-analysis of ON ultrastructure was compared from naive- vs PGJ$_2$-(100 μg/kg) treated ONs 30 days after administration (Figure 9A). Axons in the naive ON are tightly packed into septated bundles that are most easily seen at low magnification (Figure 9A top panels). Axonal neurofilaments and laminated oligodendrocyte myelin are clearly visible at higher magnification (Figure 9A, lower panels). No ultrastructural changes were qualitatively detectable in the ON-myelin sheaths of animals treated with PGJ$_2$, compared with naive. We also quantified myelin sheath thickness in the ON axons from animals 30 days post-PGJ$_2$ administration (Figure 9B, as measured by total thickness of the myelin sheath at the most compact point detectable). There was little difference in average myelin thickness of any axon type (small, medium, or large diameter fibers) (Figure 9B; compare mean myelin thickness for each group) in the rat ON.

Oligodendrocyte precursors have been reported to be more sensitive to PGJ$_2$ toxicity in vivo than mature oligodendrocytes [20]. We evaluated NG2(+)/GFAP(−) oligodendrocyte precursor cells 30 days post-treatment, in the ONs of naive- and PGJ$_2$-treated (100 μg/kg) animals (Figure 9C) [28]. In the rodent ON, NG2(+) cells that are either GFAP(+) or GFAP(−) are distributed throughout the nerve sections (Figure 9C). Similar NG2(+) expression patterns were seen in ONs from naive and PGJ$_2$-treated animals (30 days post-treatment). Stereological analysis of ON-NG2(+) cell numbers revealed similar numbers of NG2(+) cells in both vehicle- and PGJ$_2$-treated animals 30 days post-administration (Figure 9D). Thus, PGJ$_2$ does not result in the long-term loss of oligodendrocyte precursors in vivo.

Discussion

Our study shows that PGJ$_2$ reduces ON infarct damage when administered immediately after rAION injury and even 5 hours post-injury. PGJ$_2$ therefore provides an extended window of stroke treatment opportunity, as measured by 30 day neuronal survival. An important mechanism of action appears to be edema reduction, which can improve infarcted ON tissue perfusion after injury.

The current ON-infarct model demonstrates many of the responses of CNS white matter-specific damage, including pro-inflammatory signals such as TNFα [29] and IL-1β [30]. These cytokines are associated with increased BBB permeability [31]. Tissue edema in the ON-restricted space can produce a compartment syndrome that compromises otherwise uninjured capillary lumens in the infarct penumbra. Compartment syndromes have been demonstrated to play a major role in the extension of damage in lesions involving grey matter [32,33].

In the ON-infarct model, direct blockade by platelet-fibrin thrombi provide a relatively small contribution to overall ischemia. Large ON vessels occasionally showed small, adherent thrombi that did not block the lumen. Most of the perfusion loss appears to result from progressive edema, which further restricts capillary perfusion in the local vascular bed. This is demonstrable by the minimal capillary dropout at 4 hours, either with or without treatment, compared with the much greater loss of perfusion at one day post-induction (see Figure 2). The progressive edema in vehicle-treated animals compromises additional capillaries in otherwise unaffected tissue. These progressive compensatory mechanisms continue after 4 hours, evidenced by both adequate perfusion of the ON at 4 hours post-rAION, and PGJ$_2$’s ability to block edema when given five hours post-induction. The current model provides an advantage in that development and reduction of WM edema can be directly observed.
In addition to its antagonistic effects on NFκB, PGJ2 is a peroxisomal proliferator-activated receptor gamma (PPARγ)- and Nrf2-agonist [34,35]. All of these distinct activities in white matter may contribute to PGJ2’s anti-inflammatory properties. The calculated initial blood concentration after administration of 100 µg/kg PGJ2 injection is approximately 4.9 µM, based on rat blood volume [36]. Similar prostaglandins can enter all rat tissues [37], but with significantly reduced CNS penetration relative to skin and internal organs. Based on this, we presume that the exposure of endothelial cells is likely to be much higher than that seen by other CNS components. Thus, it is not surprising that the CNS vasculature is a primary site of PGJ2 interaction.

While PGJ2 administration reduces expression of the NFκB classical pathway-related genes TNFα and IL-1β, there are a large number of NFκB-related transcriptional sites present in the genome. This large copy number and the strong intracellular NFκB expression enables us to utilize immunohistochemical NFκB-Ni-DAB quenching of DAPI nuclear signal. Our data show increased p65 cellular signal following infarct, in vehicle treated animals, with typical nuclear localization, which is coupled to the increase in downstream transcription of related genes. This was confirmed by western analysis. However PGJ2 administration by itself elicits even more intracellular p65 expression than ON infarct alone. This is demonstrable by increased Ni-DAB signal, in both injured and uninjured ON, of PGJ2-treated animals. But while there is increased NFκB staining in PGJ2-treated animals, there is reduced DAPI quenching, detectable by both increased DAPI nuclear fluorescence in PGJ2-treated ONs and increased NFκB perinuclear signal. These data confirm that PGJ2 treatment blocks p65 translocation to the nucleus. NFκB activation is typically associated with IL-1β and TNFα upregulation in CNS infarct, but is significantly reduced with PGJ2 treatment, despite an overall increase in cellular NFκB. Thus, the net effect of PGJ2 in vivo is to reduce p65-mediated signaling by retaining it in the cytoplasm.

To confirm the generality of PGJ2’s mechanisms of action in CNS white matter, we compared protein expression in CC of naïve and PGJ2-treated animals (Figure 7D). CC responses to PGJ2 are similar to those seen in ON. Interestingly, CC-p65 subunit levels increase while the p50 subunit of NFκB remains constant. This behavior has previously been observed in rat CNS endothelial cells after in vivo hypoxia/reperfusion injury [38]. Upregulation of iNOS expression following ON infarct is a relatively late phenomenon [39], which may be related to the post-infarct macrophage influx seen in rAION [24], and confirmed for clinical NAION [40].

Significant controversy revolves around PGJ2’s potential neurotoxicity. A number of reports have focused on in vivo administration to cell cultures [20,41]. These systems can respond differently in their sensitivity compared to what is seen in vivo. PGJ2 also may be neuroprotective at low concentration and induce apoptosis at higher doses [17,20]. Concentration at the site of action may also be important, since prostaglandins can be highly lipophilic and be absorbed by capillary membranes. We did not find in vivo PGJ2 oligodendrocyte toxicity, as measured by ON-myelin gene expression, alterations in myelin thickness, toxicity to oligodendrocyte precursors (both by qRT-PCR and immunohistochemistry), or changes in overall ON function when PGJ2 was administered in doses similar to those used in our neuroprotection assays. These data suggest that systemically administered PGJ2, when given in doses that produce measurable neuroprotection against focal axonal ischemia, is relatively nontoxic to ON white matter in vivo.

A major problem facing clinicians treating CNS infarcts is the current short treatment time window; currently limited to 4.5 hours or less for ‘clot buster’ drugs [42]. This may be due to the greater sensitivity of the gray matter-neuronal cell body component to ischemia. Since the majority of ON capillaries in the ON-white matter CNS tract are still patent at 4 hours post-induction, the time window for WM-infarct treatment may be much longer than currently considered. WM infarct therapy may
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also be complementary to other neuroprotective strategies. PGJ₂’s neuroprotective effect is seen even when given 5 hours post-induction. The differential nature of CNS tissue injury following infarct, coupled to the large WM contribution to human brain mass, suggest that a neuroprotectant such as PGJ₂ that protects at a vascular level should be considered as a useful addition to grey matter neuroprotective approaches in order to achieve a maximally effective therapeutic rescue for infarcted CNS tissue.

Disclosure

The use of PGJ₂ for NAION has been granted US patent #8,106,096 to the University of Maryland-Baltimore.

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Author Contributions
Conceived and designed the experiments: SLB, JDJ, ACP, DW. Performed the experiments: JDN, YG, BJS, SLB, DW. Analyzed the data: SLB, JDJ, ACP, DW. Wrote the paper: SLB, JDJ.

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