Regulation of Prdx6 by Nrf-2 is mediated through aiPLA2 in white matter reperfusion injury

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Abstract

Background

Mechanisms involving upregulation of cytoprotective genes under the control of transcription factors, such as Prdx6 and Nrf2, exist to protect cells from permanent damage and dysfunction under hypoxic conditions. Hypoxia and reperfusion produces overproduction of ROS (reactive oxygen species), which may lead to mitochondrial dysfunction leading to cell death and apoptosis. Here, we explore the hypothesis that Prdx6 protects the spinal cord white matter from hypoxia-reperfusion injury and elucidate the possible mechanism by which Prdx6 elicits its protective effects.

Methods

Briefly, rats were deeply anesthetized with 5% isoflurane and maintained at 1.5%-2.5% isoflurane. A 30 mm section of spinal cord was rapidly removed and placed in cold Ringer's solution (2-4 °C), and then a dorsal column segment was micro-dissected from the spinal cord and adjacent gray matter after longitudinal sectioning. The dissected dorsal column was exposed to hypoxia by perfusing 95% N₂ / 5% CO₂ for 1 h. For reperfusion experiments, the Ringer's was reperfused with 95% O₂ and 5% CO₂ for 2 h and 4 h.

Results

Our results show that the expression of Prdx6 significantly upregulated in white matter after hypoxia compared to the sham group, whereas reperfusion caused a gradual decrease in Prdx6 expression after 2 h and 4 h of reperfusion injury. For the first time, our study revealed the novel expression and localized expression of Prdx6 in astrocytes after hypoxia, and possible communication of astrocytes and axons through Prdx6. Interestingly we observed a gradual increase in Nrf2 expression, which suggests a negative regulation of Prdx6 through Nrf2 signaling. Furthermore, inhibition of aiPLA2 activity of Prdx6 by MJ33 shows that the regulation of Prdx6 by Nrf2 is mediated through aiPLA2 activity.

Conclusion

The present study uncovers a differential distribution of Prdx6 in axons and astrocytes under a hypoxic environment of white matter, and regulation of Prdx6 in hypoxia-reperfusion injury. Our data show that low levels of Prdx6 in reperfusion injury leads to increased inflammation and apoptosis in white matter, therefore the results of this study suggests that Prdx6 has a protective role in spinal hypoxia-reperfusion injury.

Background

Spinal cord ischemia-reperfusion injury is associated with serious clinical manifestations to the central nervous system, including immediate or delayed paraparesis and paraplegia [1]. Although reperfusion is crucial to reestablish a supply of oxygen and nutrients to support cell metabolism and remove potentially

damaging byproducts of cellular metabolism, it can elicit a pathogenetic process that exacerbates injury and leads to profound inflammatory responses [2, 3]. As a result, ischemia-reperfusion injury contributes considerably to the pathology of SCI (spinal cord injury), yet the mechanism behind this injury remains to be fully elucidated. Thus, ischemia-reperfusion injury is a critical medical condition that poses an important therapeutic challenge.

The mechanisms of spinal cord ischemia-reperfusion injury are complex and include hypoxia, oxidative stress, inflammation, apoptosis, and calcium overload, all of which contribute to neuronal cell death [4]. Furthermore, these mechanisms induce several molecules that contribute to spinal cord injury pathology, and identification of these factors may provide targets for therapeutic intervention. One such molecule is Prdx6 (peroxiredoxin-6), an antioxidant protein [5]. It is one of the members of peroxiredoxins family, namely Prdx1-6 [6, 7]. Although peroxiredoxins are extensively expressed in prokaryotes and eukaryotes, and have very similar oxidative stress responses, Prdx6 is known to express at higher levels after injury [8-13]. Interestingly, Prdx6 is the only peroxiredoxin that has bi-functional enzyme activity, peroxidase and aiPLA2 (Ca²⁺-independent phospholipase A2) activity, to protect against oxidative stress and to prevent cells from peroxidation leading to membrane injury [14]. Prdx6 relies on glutathione instead of thioredoxin for the reduction of phospholipid hydroperoxides, and because Prdx6 only has one conserved catalytic cysteine residue, its function differs from the other peroxiredoxins [15].

Notably, an elevated level of Prdx6 was observed in the serum, plasma, and CSF (cerebrospinal fluid) of other traumatic CNS injuries. This demonstrates that Prdx6 may be a useful biomarker for determining the degree of injury following TBI (traumatic brain injury) [8]. Furthermore, studies have shown that Prdx6 can be detected in the CSF following TBI, and CSF analysis of the oxidation of Prdx6 could rapidly determine the prognosis for TBI patients within the first 24 hours of trauma [12]. Yun et al. reported that the expression of Prdx6 is markedly increased in the spinal cord of mice with experimental autoimmune encephalomyelitis compared to other Prdxs [16]. Similarly, Prdx6 has been shown to reduce multiple sclerosis by suppressing inflammation and disruption of the blood brain barrier [16]. There have been few studies concerning Prdx6 in SCI; however, the role of Prdx6 in spinal cord ischemia-reperfusion injury has yet to be determined. In addition, it is unclear how Prdx6 is expressed and regulated in hypoxic white matter injury, which is induced after SCI

In this study, we aimed to investigate the expression and regulation of Prdx6 in white matter reperfusion injury of spinal cord. Here, we explore the hypothesis that Prdx6 protects the spinal cord white matter from hypoxia-reperfusion injury. Our previous study shows that oxidative stress upregulates Prdx6 expression in astrocytes after 24 h of incubation with H₂O₂ (hydrogen peroxide) [17]. We observed downregulation of Prdx6 expression in white matter after reperfusion. In addition, this is the first study that shows that Prdx6 distinctly localized in astrocytes after hypoxia, whereas it is redistributed in astrocytes and axons after reperfusion. Our data show that Prdx6 negatively regulated by Nrf2 and co-localized with Nrf2 in astrocytes. Inhibition of aiPLA2 activity of Prdx6 with MJ33 (1-Hexadecyl-3-(trifluoroethyl)-sn-glycero-2-phosphomethanol lithium) revealed that hypoxia-induced upregulation of

Prdx6, Nrf2, glial fibrillary acidic protein and NF-200 (neurofilament 200) is regulated by Prdx6 and aiPLA2 activity.

Methods

Animals

Eight-week-old Wistar rats weighing 250-300 g were used for this study. All the animal procedures used in this study were approved by the IACUC (Institutional Animal Care and Use Committee) of the UNMC (University of Nebraska Medical Center). Isolation of spinal cord dorsal column and method of injury is described in our earlier publications [18], and briefly described here. Rats were deeply anesthetized with 5% isoflurane and maintained at 1.5%-2.5% isoflurane and 2-3% oxygen during surgery. A laminectomy was performed to expose the T4-T10 spinal cord. A 30 mm section of spinal cord was rapidly removed and placed in cold Ringer's solution (2-4 °C), after that, a dorsal column segment was micro-dissected from the spinal cord and adjacent gray matter after longitudinal sectioning and placed in Ringer solution bubbled with 95% O₂ and 5% CO₂. 82 dorsal columns (two dorsal columns from each rat, n = 41) were used for this study. There were four groups: sham, hypoxia, reperfusion 2 h, and reperfusion 4 h. Hypoxia was induced by perfusing the Ringer's solution with 95% N₂ and 5% CO₂ for 1 h. For reperfusion injury, the dorsal column was exposed to oxygenated Ringer's solution with 95% O₂ and 5% CO₂ for 2 h and 4 h. For inhibiting aiPLA2 activity of Prdx6, the dorsal columns from all groups were incubated with their respective perfusion of Ringer's solution in the presence of MJ33.

Quantitative real-time PCR (qRT-PCR)

Treated dorsal columns were immediately transferred to RNA/ater™ solution and incubated at 4 °C overnight before transferring them to -80 °C until use. RNA was isolated using QIAGEN's RNeasy Plus Mini Kit. Total RNA (1 µg) was reverse-transcribed to generate cDNA for qRT-PCR (Bio-Rad CFX96 Real-Time PCR Detection System and SYBR Green) using 500 nM primer concentration and 2nd of complementary DNA (cDNA) templates. Table 1 describes the primers used in this study. The relative values were calculated using the delta delta CT method via normalization to β-actin mRNA levels.

Gene	Primer sequence
Prdx6	Forward: 5'-TGACTGGAAGAAGGGAGAGA-3' Reverse: 5'-GATGGGAGCTCTTTGGTGAA-3'
GFAP	Forward: 5'-GAGTGGTATCGGTCCAAGTTT-3' Reverse: 5'-TTGGCGGCGATAGTCATTAG-3'
NF-200	Forward: 5'-CCTCCATGTCCACTCACATAAA-3' Reverse: 5'-CCTGGATCTCTTCTGTCTGTTC-3'
TNF α	Forward: 5'-TGGCGTGTTTCATCCGTTCT-3' Reverse: 5'-CCACTACTTCAGCGTCTCGT-3'
IL-6	Forward: 5'- GTTGCCCTTCTTGGGACTGATG-3' Reverse: 5'- GGCCGGACTCATCGTACTCCTGCTT-3'
β -actin	Forward: 5'- GGAGATTACTGCCCTGGCTCCTAGC-3' Reverse: 5'- GGCCGGACTCATCGTACTCCTGCTT-3'

Immunoblot analysis

Freshly dissected dorsal columns were homogenized after each treatment in ice-cold RIPA (radioimmunoprecipitation) lysis buffer (ThermoFisher Scientific) containing a protease inhibitor cocktail (Pierce, Rockford, IL). Protein was isolated from the tissue homogenate by centrifugation at 10,000 rpm for 15 min at 4 °C, and the protein concentration of the supernatant was measured using a Pierce Coomassie Protein Assay Kit (ThermoFisher Scientific). For nuclear translocation experiments, cytoplasmic and nuclear extracts were prepared using a NE-PER Nuclear and Cytoplasmic Kit (ThermoFisher Scientific). Western blot analysis was performed using standard protocol. After separation on SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis), 15-20 μ g protein was transferred to PVDF (polyvinylidene difluoride) membranes (EMD Millipore Corporation, Billerica, MA) followed by blocking the membrane with 5% non-fat milk in PBS-T (phosphate-buffered saline, pH 7.4 containing 0.1% Tween-20). After that, membranes were incubated with primary antibodies at 4 °C overnight in PBS-T. Next, membranes were washed and probed with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT (room temperature). Bands were detected using Pierce ECL Western Blotting Substrate (ThermoFisher Scientific) according to the manufacturer's instructions. The membranes were analyzed with the Bio-Rad/ChemiDocTM Imaging System and Quantity OneTM software. Band intensity was determined by using Image StudioTM Lite version 5.2 (LICOR Biosciences). For quantitative protein expression, protein of-interest band intensity was normalized with its respective loading control (β -actin).

H and E (Hematoxylin and Eosin) staining

H and E staining was performed using a protocol described previously [19]. Briefly, 5 µm longitudinal sections of dorsal columns were deparaffinized with xylene and dehydrated with two changes of 100% alcohol followed by 95%, 70%, and 50% alcohol for 3 min each. Sections were incubated with Harris hematoxylin solution for 8 min, and then washed with running tap water before incubating them with 1% acid alcohol for 30 s. After washing, sections were incubated with 0.2% ammonia water for 1 min, and then counterstained with eosin solution for 1 min. Next, sections were rehydrated with 95% alcohol, and mounted and scanned with a Nikon inverted microscope.

Confocal Microscopy

Histological sections were deparaffinized with two changes of xylene, 5 min each. Sections were rehydrated with two changes of absolute alcohol, 3 min each, followed by 95% alcohol, 70% alcohol, and 50% alcohol for 3 min each. Following rehydration, sections were incubated in 3% H₂O₂ solution in methanol at RT for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed by incubating sections in preheated sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0) at 90 °C for 5 min, and sections were allowed to cool for 20 min. Permeabilization was performed with 0.5% Triton X-100 for 30 min at RT and blocked with 10% FBS (fetal bovine serum) for 1 h at RT. Further sections were incubated with primary antibody (chicken anti-GFAP, 1:500; rabbit anti-NF-200, 1:500; anti-mouse Prdx6, 1:500, rabbit anti-NrF-2, 1:100) overnight at 4 °C. Slides were washed three times with PBS and incubated with their respective secondary antibody (1:500) for 60 min at RT in the dark. For nuclear staining, DRAQ7 (Abcam) was added in secondary antibody and incubated for 60 min at RT. Slides were washed three times with PBS before mounting, and imaged with a Zeiss 710 Meta Confocal Laser Scanning Microscope.

TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining

TUNEL staining was used to assess apoptotic status after reperfusion. The longitudinal sections of dorsal columns stained using an *in situ* cell death detection kit (Roche) according to the manufacturer's instructions. Briefly, tissue sections deparaffinized and rehydrated as described above in the confocal section. After that, tissue sections were incubated with 20 µg/mL protease K in 10 mM Tris/HCl for 30 min at RT. Sections were then incubated with TUNEL reaction mix (enzyme solution + label solution) for 1 h at 37 °C in a dark humidified chamber. After washing, nuclei were counterstained with DAPI (4',6'-diamidino-2-phenylindole). Stained samples were examined with a Zeiss 710 Meta Confocal Laser Scanning Microscope.

Statistical analysis

Statistical analyses performed with GraphPad Prism version 8.0 (GraphPad Software, Inc., San Diego, CA) using ANOVA and Dunnett's multiple comparison test. All data values and error bars were performed in triplicate (n = 3), except for qRT-PCR (n = 5), and are represented as the mean – standard deviation

(SD) of three independent experiments. Differences at the level of $P < 0.05$ considered statistically significant.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary material.

Results

Prdx6 expression is downregulated in white matter after reperfusion

Fig. 1A shows the expression of Prdx6 in WC (whole spinal cord) and in DC (dorsal column) white matter before reperfusion. Our results show that Prdx6 is expressed in both WC and white matter of DC. As expected, the DC shows the expression of GFAP (a marker for astrocytes) but not MAP2 (*microtubule associated protein 2*, a marker for neurons), thus, this indicates an absence of neurons in the DC white matter. Fig. 1B shows the real time gene expression of Prdx6 after 2 h and 4 h of reperfusion. Prdx6 gene expression is significantly upregulated after hypoxia as compared to sham ($***p < 0.001$), while downregulated after 2 h of reperfusion as compared to hypoxia ($^^^p < 0.001$) and sham ($*p < 0.05$). After 4 h of reperfusion, Prdx6 gene expression returned to normal levels compared to sham while it remained downregulated compared to the hypoxia group ($^^^p < 0.001$). Fig. 1C shows increase in protein expression of Prdx6 after hypoxia and this increase gradually decreases after reperfusion 2 h and 4 h as compared to hypoxia. Interestingly, Prdx6 expression is upregulated significantly after 2 h ($***p < 0.001$) and downregulated after 4 h of reperfusion in comparison to sham ($**p < 0.01$).

Gene and protein expression of GFAP and NF-200 after reperfusion

The most abundant cell bodies present in white matter are glial cells and axons, thereof, we were interested to observe the expression of GFAP (a marker for astrocytes) and NF-200 (a marker for axons) after reperfusion-injury, and investigated the correlation of Prdx6 expression with GFAP and NF-200. Real time gene analysis of GFAP expression shows gradual upregulation of its expression in hypoxia after 2 h and 4 h of reperfusion-injury (Fig. 2A). However, GFAP protein shows an upregulation after hypoxia as compared to sham ($***p < 0.001$). Alternatively, GFAP expression shows significant downregulation after reperfusion as compared to hypoxia ($^^^p < 0.001$) (Fig. 2B). A similar pattern was observed with NF-200 (Fig. 2D), while gene expression analysis of NF-200 shows an upregulation in reperfusion (Fig. 2C).

Prdx6 is distinctly localized in astrocytes after hypoxia whereas it is redistributed in astrocytes and axons after reperfusion

We observed expression of GFAP (red, Fig. 3A(a)), NF-200 (green, Fig. 3A(b)), and Prdx6 (blue, Fig. 3A(c)) in the sham group. Fig. 3A(d) shows co-localization of GFAP and Prdx6, suggesting the presence of Prdx6 in astrocytes (pink color, arrowheads). In addition, Prdx6 is co-localized with GFAP in the cell body as well as in the processes, suggesting uniform distribution of Prdx6 in astrocytes (Fig. 3A(g)). Whereas Fig.

3A(e) and 3A(h) (enlarged image) shows co-localization of NF-200 (green) and Prdx6 (blue) forming a cyan color (arrow heads). Fig. 3A(i) shows superimposed images of Prdx6 (blue), GFAP (red), and NF-200 (green) in the sham group, suggesting the presence of Prdx6 in astrocytes (pink color, yellow arrows) and in axons (cyan color, white arrows) and the circles show axonal endings in astrocytes (Fig. 3A (h)).

We observed expression of GFAP (red, Fig. 3B (a)), NF-200 (green, Fig. 3B (b)) and Prdx6 (blue, Fig. 3B (c)) in the hypoxia group. Interestingly, hypoxia induced the movement of Prdx6 into astrocytes as indicated by distinct co-localization of Prdx6 (blue) with GFAP (red) (pink color, yellow arrows) (Fig. 3B (i)). Unlike the sham group, we observed major co-localization of Prdx6 with GFAP in the cell body of astrocytes and limited co-localization was observed in the processes (Fig. 3B (g)). However, most of the Prdx6 is accumulated in astrocytes in the hypoxia group; we observed very low levels of co-localization of Prdx6 (blue) with NF-200 (green) (cyan color, white arrows) (Fig. 3B (h)). In addition, we observed a distinct co-localization of Prdx6, GFAP, and NF-200 where axons were making end feet to the astrocytes (white color, red arrows), suggesting possible communication of astrocytes and axons through Prdx6 (Fig. 3B (i)).

After 4 h of reperfusion, we observed co-localization of Prdx6 with GFAP (red) and NF-200 (green) suggesting a substantial movement of Prdx6 from astrocytes to axons (Fig. 3C (i)). Like hypoxia, distinctive co-localization of Prdx6 with GFAP was observed in the cell body of astrocytes and limited co-localization was observed in astrocytes processes (Fig. 3C (g)). However, unlike the sham group, the Prdx6 was characteristically co-localized with NF-200 at the periphery of axons (Fig. 3C (h)).

Reperfusion unable to protect hypoxia induced tissue damage and apoptosis

Next, we wanted to investigate if Prdx6 expression and its characteristic distribution in white matter after reperfusion have any correlation with apoptosis. Histopathological evaluation shows a well-organized structure and healthy looking cell bodies in the sham group (Fig. 4A (a)). Contrarily, hypoxic white matter indicated shrunken cell bodies, pronounced vacuolation, and disrupted tissue. In addition, characteristic reactive astrocytosis, indicated by abundant eosinophilic (pink) cytoplasm, was also observed in the hypoxia group (Fig. 4A (b)). An increased axonal separation (asterisk) and enhanced vacuolation was observed in reperfusion group, which suggests that reperfusion further exacerbates the hypoxia-induced tissue damage (Fig. 4A (c)).

Analysis of pro-inflammatory genes, TNF α and IL-6, showed that reperfusion further increased the hypoxia-induced inflammation in white matter (Fig. 4B and 4C). A significant continued upregulation of TNF α was observed in 2 h and 4 h of reperfusion as compared to the sham ($***p < 0.001$) and hypoxia group ($^^^p < 0.001$) (Fig. 4B). Similarly, significant upregulation of IL-6 was observed in hypoxia, 2 h, and 4 h of reperfusion as compared to sham ($***p < 0.001$), however, only 2 h of reperfusion shows upregulation of IL-6 in comparison to the hypoxia group ($^p < 0.05$) (Fig. 4C).

Apoptosis in white matter was examined by TUNEL staining, which measures the DNA fragmentation as an indication of apoptosis (Fig. 4D). Our results show that the number of TUNEL-positive cells (green) that represented fragmented DNA was markedly increased in the hypoxia group compared to the sham

group. The number of TUNEL-positive cells was increased further in the reperfusion group compared to the sham as well as the hypoxia group (Fig. 4D). Altogether, these results suggest that reperfusion exacerbates the hypoxia induced inflammation, tissue damage, and apoptosis.

Nrf2 negatively regulates Prdx6 expression in hypoxia-reperfusion injury

The antioxidant genes, including Prdx6, are tightly regulated by ARE (antioxidant response elements), and Nrf2 is a major trans-activator of antioxidant genes in response to hypoxia, inflammation, and oxidative stress. It is known that Nrf2 activates the transcription of Prdx6 [20], however, its relation with Prdx6 in the white matter of spinal cord is not known. Our data shows that expression of Nrf2 is significantly upregulated in hypoxia and reperfusion groups as compared to the sham group (Fig. 5A). The gradual upregulation of Nrf2 protein expression in hypoxia and reperfusion groups is correlated with increased inflammation and oxidative stress in 2 h and 4 h of reperfusion-injury. Interestingly, expression of Prdx6 is gradually decreased with 2 h and 4 h of reperfusion, which suggests that Nrf2 negatively regulates the expression of Prdx6 in the white matter of the spinal cord.

Furthermore, upregulation of Nrf2 was observed in cytosolic and nuclear fraction of the hypoxia group as compared to the sham group, suggesting a nuclear translocation of Nrf2 in hypoxia (Fig. 5B). However, an upregulation of Nrf2 was observed only in cytosolic fraction of the reperfusion group and expression of Nrf2 remained unchanged in nuclear fraction as compared to the sham group (Fig. 5B). This suggest that the prolonged oxidative stress in the reperfusion group moves back the hypoxia-induced nuclear translocation of Nrf2 to the cytosol, therefore the cytosolic fraction majorly contributes to the increased expression of Nrf2 in the reperfusion group.

Prdx6 and Nrf2 are co-localized in hypoxia-reperfusion injury in the white matter of the spinal cord

As we have shown earlier that Prdx6 is localized with astrocytes and axons in the sham group, we observed a co-localization of Prdx6 (green) and Nrf2 (blue) with GFAP (red) (white color, yellow arrows). This suggests a possible association of Prdx6 and Nrf2 with astrocytes. In addition, most of the co-localization of Prdx6 and Nrf2 in astrocytes were observed around the nucleus (Fig. 5C, sham). In the hypoxia group, co-localization of Prdx6 (green) and Nrf2 (blue) was increased compared to the sham group and was majorly associated with reactive astrocytes (GFAP, red) (Fig. 5C, Hypx). Unlike the hypoxia group, Nrf2 expression was observed in astrocytes as well as other cell bodies after reperfusion. Compared to hypoxia, Prdx6 (green) and Nrf2 (blue) co-localization with GFAP (red) was more evenly distributed in astrocytes after reperfusion (Fig.5C, 4H-R).

Inhibition of aiPLA2 enzyme activity of Prdx6 prevents hypoxia-induced upregulation of Prdx6 and Nrf2

Hypoxia causes tissue acidification and reperfusion injury involves a “pH paradox”, where it returns from acidotic to physiologic pH that causes rapid cell death [21]. It is reported that Prdx6 exhibits maximal aiPLA2 activity at an acidic pH and maximum peroxidase activity at neutral pH [22]; therefore, we further evaluated if negative regulation of Prdx6 by Nrf2 is mediated through its aiPLA2 activity. A serine

“protease” inhibitor, MJ33, inhibited the aiPLA2 activity. Our data show that expression of Prdx6 in hypoxia returned to normal levels whereas it remains upregulated in reperfusion injury after inhibition of aiPLA2 activity (Fig. 6A). Looking at the expression of Nrf2, we observed no change in expression after hypoxia and an upregulation at 2 h of reperfusion compared to the sham group (Fig. 6B). Interestingly, a significant downregulation of Nrf2 was observed after 4 h reperfusion compared to after 2 h of reperfusion (Fig. 6B). Therefore, we observed a correlation of Nrf2 expression and Prdx6 expression after MJ33 treatment, suggesting that the negative regulation of Prdx6 observed earlier by Nrf2 (Fig. 1C and Fig. 5A) is mediated through aiPLA2 activity of Prdx6.

Next, we evaluated the expression of GFAP and NF-200 after MJ33 treatment. We observed no significant change in expression of GFAP in the hypoxia group, whereas 2 h and 4 h of reperfusion showed an upregulation of GFAP compared to the sham group (Fig. 6C). Conversely, we observed significant downregulation of NF-200 in the hypoxia group and significant upregulation in the 4 h reperfusion group compared to the sham group (Fig. 6D). We observed significant upregulation of NF-200 in the 2 h as well as 4 h of reperfusion groups compared to the hypoxia group (Fig. 6D), which is an indication of increased axonal damage after inhibition of aiPLA2 activity of Prdx6. Together, these results suggest that hypoxia-induced upregulation of Prdx6, Nrf2, GFAP, and NF-200 are regulated by Prdx6 aiPLA2 activity.

Discussion

Reperfusion of ischemic tissue, although necessary to re-establish delivery of oxygen and nutrients to support cell metabolism and remove potentially damaging byproducts, could paradoxically induce and exacerbate tissue injury and necrosis [21, 23]. Nevertheless, reperfusion is amenable to therapeutic intervention [24]. Following SCI, Prdx6, a member of the peroxiredoxin family, is detected in the CSF [8, 12]. However, generally considered as a protective gene, the role of Prdx6 in SCI is not known. Therefore, this study investigated the hypothesis that Prdx6 protects spinal cord white matter from hypoxia-reperfusion injury. Here, we report a novel movement of Prdx6 into astrocytes after exposing white matter to hypoxia, and its redistribution after reperfusion.

Prdx6 is a moonlighting protein that harbors aiPLA2 activity in addition to its glutathione peroxidase function [25, 26]. Based on its activity to scavenge H_2O_2 and other hydroperoxides, Prdx6 is expected to have a role in antioxidant defense. Supporting this idea, Prdx6 reduces oxidative stress and counteracts ROS generation in models of Alzheimer’s disease [27], and reduces inflammation and protects against disruption to the blood-brain barrier in a model of multiple sclerosis [16]. In contrast, Prdx6 signaling through TLR4 (toll like receptor 4) increases after an ischemic stroke, leading to NF- κ B-mediated inflammation and apoptosis [28, 29]. Also, Prdx6 has been shown to inhibit neurogenesis through downregulation of WDFY₁-mediated TLR4 signal [30]. Moreover, the pharmacological inhibition of aiPLA2 activity of Prdx6 reduces inflammation in an experimental stroke model [31]. This evidence makes the role of Prdx6 controversial in neurodegenerative diseases, which seems to be linked to the differential expression of Prdx6 and its activity in various neurodegenerative disease models. Previously, oxidative stress has been shown to induce Prdx6 expression in astrocytes cultures of spinal cord [17, 32]. Similarly,

ischemia-reperfusion has been shown to cause increased expression of Prdx6 around hippocampal blood vessels [33] and following mid-to-moderate TBI [8]. We observed a downregulation of Prdx6 gene and protein expression as compared to hypoxia in the white matter of spinal cord (Fig. 1), which is correlated with the downregulation of protein expression of GFAP and NF-200 after reperfusion (Fig. 2). Of note, the downregulation of Prdx6 and NF-200 protein expression that we observed after 4 h of reperfusion compared to the sham group suggests a potential role of Prdx6 in axons.

Our immunofluorescence results highlight several findings that contrast with available data for other ischemic-reperfusion injuries. Prdx6 has been shown to express primarily in astrocytes in the rat cerebral cortex with negligible expression in neurons and microglia. In another study, Prdx6 is primarily located in the spinal cord neurons [34]. We observed the expression of Prdx6 in astrocytes as well as in axons in the white matter of spinal cords under basal conditions (Fig. 3A). Interestingly, a distinct localisation of Prdx6 in astrocytes was observed in hypoxia, which is evident by the co-localization of Prdx6, GFAP, and NF-200 at axons end feet in astrocytes (Fig. 3B). Because reperfusion facilitates reoxygenation and returns tissues to physiologic pH, we observed expression of Prdx6 in astrocytes as well as in axons after reperfusion (Fig. 3C). However, unlike the sham group, where Prdx6 was present in thin axons in large quantities, expression of Prdx6 in reperfusion is largely associated with the cell membrane of axons. These observations can be explained by the regulation of Prdx6 enzymatic activities through its subcellular localization. At an acidic pH, when aiPLA2 is maximal, Prdx6 binds to reduced phospholipids, whereas at cytosolic pH, it binds only to oxidized phospholipids [22]. Importantly, the binding of Prdx6 to oxidized phospholipids requires the translocation of Prdx6 from the cytosol to the cell membrane [25, 35]. Aside from subcellular localization, the enzymatic activities of Prdx6 can be regulated by interactions with other proteins. Our data show that TNF α was highly increased after reperfusion, which is responsible for, along with other factors, increased apoptosis observed after 4 h of reperfusion (Fig. 4). Similarly, a significant upregulation of TNF α and downregulation of Prdx6 was observed after contusion SCI [34]. In addition, TNF- α -RNAi-LV resulted in the increase of Prdx6, which corresponded to the recovery of motor function in SCI rats [34]. These results suggest that the low levels of Prdx6 in reperfusion induce pro-inflammatory genes and apoptosis, which is regulated by its cellular and subcellular localization. Therefore, Prdx6 showed a protective role in white matter injury of the spinal cord.

Next, we were interested in studying the interaction of Prdx6 and Nrf2, which is a major trans-activator of antioxidant genes, in response to hypoxia, inflammation, and oxidative stress [36]. Our data show that Nrf2 negatively regulates Prdx6 in reperfusion injury of white matter (Fig. 5A). Although Nrf2 activity is cytoprotective, Nrf2 activation beyond a certain threshold can be detrimental, which is the case in reperfusion injury. This notion is supported further by the nuclear translocation of Nrf2, which is strictly regulated by the direct interaction of inhibitory protein KEAP1 (Kelch-like ECH-associated protein 1). Oxidative stress causes Nrf2 to dissociate from KEAP1 and, subsequently, to translocate into the nucleus, which results in its binding to ARE and the transcription of downstream target genes [37, 38]. We observed nuclear translocation of Nrf2 in the hypoxia group, which explains the upregulation of Prdx6, however, nuclear translocation of Nrf2 was not observed in reperfusion (Fig. 5B). Moreover, confocal

studies show the co-localization of Prdx6 with Nrf2 (Fig. 5C), which suggests the interaction of Prdx6 with Nrf2 and, thereby, regulation of Prdx6 by Nrf2.

Furthermore, the role of aiPLA2 activity of Prdx6 in white matter reperfusion injury was investigated by inhibiting it with a serine “protease” inhibitor, MJ33 [39]. Previous reports suggested important physiological roles of aiPLA2 activity in the turnover of phospholipids, repair of peroxidized cell membranes, and activation of NOX2 (NADPH oxidase 2) [40]. We observed inhibition of hypoxia-induced upregulation of Prdx6 after MJ33 treatment, whereas expression of Prdx6 in reperfusion injury remains upregulated (Fig. 6A). These results could be attributed to the differential activity of aiPLA2 at different pH levels. In basal conditions (sham), a major fraction of intracellular Prdx6 is localized to the cytosol where the neutral pH supports neither aiPLA2 binding to membranes nor its enzymatic activity [40, 41]. Under acidic conditions (hypoxia), aiPLA2 activity increased by increased binding of Prdx6 to phospholipids. However, the cytosolic Prdx6 does bind to oxidized substrate at a pH of 7 (reperfusion) so that oxidation of cell membrane phospholipids facilitates binding to Prdx6 and subsequent aiPLA2 activity. In fact, aiPLA2 activity with oxidized substrates is similar at acidic and neutral pH [40], which suggests the role of this enzyme in the repair of peroxidized cell membranes. Nrf2 protein expression shows a similar pattern as observed with Prdx6 protein after MJ33 treatment (Fig. 6B), suggesting that aiPLA2 activity of Prdx6 is involved in the Nrf2-mediated regulation of Prdx6. In addition, our data show that hypoxia-induced upregulation of GFAP and NF-200 is regulated by Prdx6 aiPLA2 activity (Fig. 6C and 6D). Interestingly, inhibition of aiPLA2 activity of Prdx6 significantly increased the expression of NF-200 in reperfusion injury as compared to the sham group (and hypoxia). Indeed, the abnormal hyperphosphorylation of neurofilaments leads to the loss of their stabilizing task and results in axonal damage in the brain and white matter [42]. Together, these results suggest that Prdx6-aiPLA2 activity is harmful to astrocytes and axons in white matter.

Conclusions

Altogether, our data showed that low levels of Prdx6 in reperfusion injury led to increased inflammation and apoptosis in white matter. In addition, the activity of Prdx6 in white matter is regulated by its cellular distribution and by possible interactions of Prdx6 with TNF α and Nrf2. The regulation of Prdx6 by Nrf2 is mediated through aiPLA2 activity, and inhibition of Prdx6 aiPLA2 activity can reduce astrocytes and axon damage. Thereby, inhibition of aiPLA2 activity of Prdx6 could be a strategy to improve recovery in SCI (Fig. 7).

Abbreviations

Ca²⁺-independent phospholipase A2 (aiPLA2), antioxidant response elements (ARE), complementary DNA (cDNA), 4',6-diamidino-2-phenylindole (DAPI), dorsal column (DC), fetal bovine serum (FBS), glial fibrillary acidic protein (GFAP), hematoxylin and eosin (H and E), Institutional Animal Care and Use Committee (IACUC), Kelch-like ECH-associated protein 1 (KEAP1), *microtubule associated protein 2* (MAP2), 1-Hexadecyl-3-(trifluoroethyl)-sn-glycero-2-phosphomethanol lithium (MJ33), NADPH oxidase 2 (NOX2),

neurofilament 200 (NF-200), nuclear factor erythroid 2–related factor 2 (Nrf2), phosphate-buffered saline (PBS-T), peroxiredoxin-6 (Prdx6), polyvinylidene difluoride (PVDF) quantitative real-time PCR (qRT-PCR), radioimmunoprecipitation (RIPA), reactive oxygen species (ROS), room temperature (RT), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), spinal cord injury (SCI), traumatic brain injury (TBI), toll like receptor 4 (TLR4), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), University of Nebraska Medical Center (UNMC), whole spinal cord (WC)

Declarations

Ethical Approval and Consent to participate: The study was performed according to the ethical principles of the University of Nebraska Medical center and was approved by the local ethics committees.

Consent for publication: Not Applicable.

Availability of supporting data: The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary material.

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Authors' contributions: AD designed and performed experiments in white matter injury model, performed rest of the in vitro experiments, analyzed and interpreted the data, discussions about hypoxia, wrote and revised the manuscript. SA conceived, designed and supervised the project, performed some experiments, analyzed data and was responsible for data interpretation and writing and revising the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Expression of Prdx6 after hypoxia-reperfusion injury in the white matter of spinal cord. A Protein expression of Prdx6 (25 kDa), GFAP (50 kDa), and MAP2 (c = 74 kDa, d = 70 kDa) is shown in WC (whole cord) and DC (dorsal column) of white matter. β-actin (42 kDa) was used as loading control. B Real-time PCR analysis showing gene expression of Prdx6 after hypoxia-reperfusion injury. The sham was used as control and β-actin was used as internal control. 2h-R and 4h-R represents 2 h and 4 h reperfusion injury. Error bars represent the mean ± SD (n = 5). *p < 0.05 vs. sham; ***p < 0.001 vs. sham; ^^^p < 0.001 vs. hypoxia (Hypx). C Upper panel: representative cropped western blots showing protein expression of Prdx6 in white matter after 2 h and 4 h of reperfusion injury. Lower panel: densitometry analysis of Prdx6 normalized with loading control β-actin. The values were again normalized to the sham values, which corresponded to 100%. Error bars represent the mean ± SD (n=3). **p < 0.01 vs. sham; ***p < 0.001 vs. sham; ^^^p < 0.001 vs. hypoxia (Hypx).

Figure 2

Gene and protein expression of GFAP and NF-200 after reperfusion. A Real-time PCR analysis showing expression of GFAP after hypoxia-reperfusion injury. The sham was used as control and β -actin was used as internal control. 2h-R and 4h-R represents 2 h and 4 h reperfusion injury. Error bars represent the mean $\pm$ SD (n=5). *p < 0.05 vs. sham; ***p < 0.001 vs. sham; ^^p < 0.001 vs. hypoxia (Hypx); ^^p < 0.001 vs. Hypx. B Upper panel: representative cropped western blots showing protein expression of GFAP (50 kDa) in white matter after 2 h and 4 h of reperfusion injury. Lower panel: densitometry analysis of GFAP normalized with loading control β -actin (42 kDa). The values were again normalized to the sham values,

which corresponded to 100%. Error bars represent the mean $\pm$ SD ($n = 3$). *** $p < 0.001$ vs. sham; ^^^ $p < 0.001$ vs. hypoxia (Hypx). C Real-time PCR analysis showing expression of NF-200 after hypoxia-reperfusion injury. Error bars represent the mean $\pm$ SD ($n = 5$). *** $p < 0.001$ vs. sham; ^^^ $p < 0.001$ vs. Hypx. D Upper panel: representative cropped western blots showing protein expression of NF-200 in white matter after 2 h and 4 h of reperfusion injury. Lower panel: densitometry analysis of NF-200. Error bars represent the mean $\pm$ SD ($n = 3$). *** $p < 0.001$ vs. sham; ^ $p < 0.05$ vs. hypoxia (Hypx); ^^^ $p < 0.001$ vs. Hypx.

Figure 3

Dynamic changes in Prdx6 protein expression in spinal cord white matter. 3A sham group the representative confocal images of triple staining with anti-GFAP (red), anti-NF-200 (green), and anti-Prdx6 (blue). Prdx6 is co-localized with GFAP in astrocytes (A(d), pink color marked with yellow arrowheads) and with NF-200 in axons (A(e), cyan color marked with white arrowheads) Scale = 20 μ m. 3B Hypoxia group an increased localization of Prdx6 within astrocytes was observed (B(d), pink color marked with yellow arrowheads). Also, Prdx6 protein expression spatially overlapped minimally with dystrophic axons and axonal endbulbs (B(e), cyan color marked with white arrows) Scale = 20 μ m. 3C Reperfusion group Prdx6 is majorly co-localized with GFAP in astrocytes (C(d) pink color marked with yellow arrowheads) but also show co-localization with NF-200 in axons (C(e) cyan color marked with white arrows.) f: merge image of GFAP, NF-200, and Prdx6, yellow arrowhead shows the expression of Prdx6 in astrocytes and white arrowhead demonstrates the expression of Prdx6 in axons. g, h, and i: shows the zoom in images of d, e, and f respectively. Circles demonstrate the probable communication of axons and astrocytes through Prdx6. Scale = 10 μ m.

Figure 4

Reperfusion injury unable to protect hypoxia induced tissue damage and apoptosis. A Histopathologic evaluation of the white matter of the spinal cord stained with hematoxylin and eosin. a: in the sham group, most of the cells are oligodendrocytes (arrowheads), but there are a few normal astrocytes as well (arrows). Here, cells are identified primarily by their nuclear characteristics (blue) because cell bodies or cellular processes cannot be distinguished with routine H and E stain. b: hypoxic section shows white

matter with reactive astrocytosis (arrows) with abundant eosinophilic (pink) cytoplasm. c: reperfusion 4 h group demonstrates pronounced vacuolation (arrowheads) and disrupted tissue pattern (arrows). B Real-time PCR analysis showing gene expression of TNF α after hypoxia-reperfusion injury. The sham group was used as control and β -actin was used as internal control. 2h-R and 4h-R represents 2 h and 4 h reperfusion injury. Error bars represent the mean $\pm$ SD (n = 5). ***p < 0.001 vs. sham; ^^^p < 0.001 vs. hypoxia (Hypx). C Real-time PCR analysis showing gene expression of IL-6 after hypoxia-reperfusion injury. Error bars represent the mean $\pm$ SD (n = 5). ***p < 0.001 vs. sham; ^p < 0.05 vs. Hypx. D TUNEL assay shows DNA fragmentation in longitudinal sections of spinal cord dorsal columns. The green color indicates TUNEL staining and blue color shows DAPI staining of nucleus. The cyan color indicates the apoptotic TUNEL positive cells (TUNEL +ve) and the blue color shows healthy cells (TUNEL -ve). Scale: 50 μ m.

Figure 5

Nrf2 negatively regulates Prdx6 expression in hypoxia-reperfusion injury. A Western blot analysis of Nrf2 protein expression after hypoxic-reperfusion injury. Upper panel: representative cropped western blots showing protein expression of Nrf2 in white matter after 2 h and 4 h of reperfusion injury. Lower panel: densitometry analysis of Nrf2 normalized with loading control β -actin. The values were again normalized to the sham values, which corresponded to 100%. Error bars represent the mean $\pm$ SD (n = 3). ***p < 0.001 vs. sham; ^^p < 0.001 vs. hypoxia (Hypx). B Nuclear translocation of Nrf2 under hypoxia and reperfusion injury. Upper panel: representative cropped western blots showing cytosolic and nuclear expression of Nrf2 in white matter. Lower panel: densitometry analysis of Nrf2 normalized with loading control β -actin. The values were again normalized to the sham values, which corresponded to 100%. Error bars represent the mean $\pm$ SD (n = 3). ***p < 0.001 vs. sham; ^p < 0.05 vs. hypoxia (Hypx). ^^p < 0.001 Hypx. C The representative confocal images of triple staining with anti-GFAP (red), anti-Prdx6 (green), and anti-Nrf2 (blue) in sham, hypoxia (Hypx), and 4 h reperfusion (4h-R) dorsal columns. DRAQ5™, a far-red fluorescent DNA dye, was used as counterstain to label the nucleus (pseudo-color gray). White arrow shows the co-localization of GFAP, Prdx6, and Nrf2 (merged white color) and yellow arrow shows the co-localization of Prdx6 and Nrf2 (merged cyan color). Scale = 20 μ m.

Figure 6

Inhibition of aiPLA2 enzyme activity of Prdx6 prevents hypoxia-induced upregulation of Prdx6 and Nrf2. aiPLA2 enzyme activity of Prdx6 was inhibited with MJ33, and protein expression of Prdx6 was increased A, Nrf2 B, GFAP C, and NF-200 D was analyzed. Upper panels show representative blots and lower panels show their respective densitometry analysis. β -actin was used as loading control. Error bars represent the

mean $\pm$ SD (n = 3). *p < 0.05 vs. sham; **p < 0.01 vs. sham; ***p < 0.001 vs. sham; ^^p < 0.01 vs. hypoxia (Hypx); ^^p < 0.001 vs. Hypx.

Figure 7

Schematic representation of the role of Prdx6 in white matter injury. Hypoxia induced the movement of Prdx6 into astrocytes whereas reperfusion redistributes it in astrocytes and axons. Low levels of Prdx6 in reperfusion injury leads to increased inflammation and apoptosis in white matter, thus Prdx6 protects white matter against hypoxic-reperfusion injury.

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