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Neuroprotective effects of a nanocrystal formulation of sPLA₂ inhibitor PX-18 in cerebral ischemia/reperfusion in gerbils

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Abstract

The group IIA secretory phospholipase A₂ (sPLA₂-IIA) has been studied extensively because of its involvement in inflammatory processes. Up-regulation of this enzyme has been shown in a number of neurodegenerative diseases including cerebral ischemia and Alzheimer's disease. PX-18 is a selective sPLA₂ inhibitor effective in reducing tissue damage resulting from myocardial infarction. However, its use as a neuroprotective agent has been hampered due to its low solubility. In this study, we test possible neuroprotective effects of PX-18 formulated as a suspension of nanocrystals. Transient global cerebral ischemia was induced in gerbils by occlusion of both common carotid arteries for 5 min. Four days after ischemia/reperfusion (I/R), extensive delayed neuronal death, DNA damage, and increases in reactive astrocytes and microglial cells were observed in the hippocampal CA1 region. PX-18 nanocrystals (30 and 60 mg/kg body wt) and vehicle controls were injected i.p. immediately after I/R. PX-18 nanocrystal injection significantly reduced delayed neuronal death, DNA damage, as well as glial cell activation. These findings demonstrated the effective neuroprotection of PX-18 in the form of nanocrystal against I/R-induced neuronal damage. The results also suggest that nanocrystals hold promise as an effective strategy for the delivery of compounds with poor solubility that would otherwise be precluded from preclinical development.

Keywords

cerebral ischemia/reperfusion; delayed neuronal death; glial activation; PX-18 nanocrystals; sPLA₂ inhibitor; inflammation

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1. Introduction

Phospholipases A₂ (PLA₂) are essential enzymes for maintenance and regulation of cell membrane phospholipids. These enzymes are generally grouped into three broad types, namely, the group IV Ca²⁺-dependent cytosolic PLA₂, the group VI Ca²⁺-independent PLA₂, and the small molecular weight secretory sPLA₂ (Burke and Dennis, 2008; Masuda et al., 2005; Murakami and Kudo, 2002; Sun et al., 2004). Among more than 12 isoforms of sPLA₂ widely distributed in mammalian cells, much attention has focused on the group IIA sPLA₂ (sPLA₂-IIA) because of its role in the pathogenesis of many inflammatory diseases (Murakami and Kudo, 2004). In the peripheral system, this enzyme is regarded as a mediator connecting innate and adaptive immunity, and is upregulated in a number of inflammatory diseases including coronary artery diseases, atherosclerosis, sepsis, arthritis, and infection (Camargo et al., 2008; Ibeas et al., 2008; Kimura-Matsumoto et al., 2008; Krijnen et al., 2006; Leitinger et al., 1999; Mallat et al., 2007; Tietge et al., 2005). Although less is known about the role of sPLA₂ in the central nervous system, up-regulation of sPLA₂-IIA expression and increase in sPLA₂-IIA activity has been reported in rat brain after cerebral ischemia (Adibhatla and Hatcher, 2007b; Lauritzen et al., 1994; Smart et al., 2004). A recent study further demonstrated upregulation of this enzyme in reactive astrocytes in Alzheimer's disease brains as compared with age-matched controls (Moses et al., 2006). Besides fatty acid release, sPLA₂-IIA has been shown to alter membrane functions including stimulation of voltage sensitive Ca²⁺ channels in neurons, potentiation of glutamate excitotoxicity, and induction of neuronal apoptosis (DeCoster et al., 2002; Kolko et al., 2002; Yagami et al., 2002; Yagami et al., 2003). These studies resulted in increasing interest in developing specific inhibitors targeting this type of sPLA₂ (Yagami et al., 2005).

PX-18 (2-*N,N*-Bis(oleoyloxyethyl)amino-1-ethanesulfonic acid) (Fig. 1) is a lipid compound shown to offer cytoprotective properties (Franson and Rosenthal, 1989). These earlier studies also demonstrated ability for PX-18 to stabilize membrane, protect mitochondria through inhibition of PLA₂. This type of PLA₂ inhibitor could also inhibit thrombin-stimulated PGE₂ and prostacyclin release in coronary artery endothelial cells (Meyer et al., 2005a), and sPLA₂ in ischemic myocardium (Nijmeijer et al., 2003; Nijmeijer et al., 2008). Its ability to inhibit sPLA₂ and prostaglandin release in human endothelial cells appears to offer promising therapeutic potential as an anti-inflammatory agent (Rastogi et al., 2007). Nevertheless, the wide therapeutic use of this compound has been limited by its low aqueous solubility (Meyer et al., 2005b).

In this study, we investigated the possibility to overcome the solubility problem by formulating the compound as nanocrystals. This type of drug nanocrystals are nanoparticles with particle size <1 μm (typically 200–500 nm) and are comprised of 100% drug without any matrix material (Keck and Müller, 2006). Nanonization of drugs can increase their saturation solubility, which can be explained by the Kelvin and Ostwald-Freundlich equations, as well as their dissolution rate, which is described in the Noyes-Whitney and Prandtl equations (Böhm et al., 1998; Müller and Peters, 1997; Müller and Akkar, 2004). An increase in the bioavailability of drugs formulated as nanosuspensions has been reported (Möschwitzer and Müller, 2006; Müller and Keck, 2004; Müller et al., 2006). Therefore, the ability to formulate drugs or compounds with low solubility as nanocrystals displays a new paradigm in pharmacotherapy and drug delivery. In this study, we test whether PX-18 drug nanocrystals may offer protective effects against neuronal damage after cerebral ischemia, a model well established in our laboratory (Wang et al., 2002).

2. Results

2.1. PX-18 nanocrystals

An average particle size of 41 nm was achieved by applying 20 homogenization cycles at 1500 bar at 5°C. Particle sizes as small as this have been previously reported as nanosuspensions and produced only using a combination technology where the material is lyophilized and subsequently subjected to high pressure homogenization (Möschwitzer and Lemke, 2005). The 1% PX-18 nanosuspension was physically stable over a period of 180 days when stored at 4–6°C (Fig. 2). No changes in particle size and polydispersity index (PI) occurred during the observation period. This protocol ensures a constant quality of the nanosuspension during the *in vivo* study (Fig 2).

2.2. PX-18 is neuroprotective against cerebral I/R-induced DND

Four days after a 5-min CCA occlusion, extensive DND were observed in the hippocampal CA1 subfield (Fig. 3B vs. A). PX-18 administration resulted in a marked reduction of DND (Fig. 3C vs. B). Analysis of the numbers of viable neurons indicated significant differences between I/R in either the I/R+PX-18-30 (30 mg/kg, i.p., $p<0.01$) or I/R+PX-18-60 (60 mg/kg, i.p., $p<0.001$) groups (Fig. 4A).

2.3. PX-18 is neuroprotective against cerebral I/R-induced glial cell activation

Immunohistochemical staining with GFAP showed only few GFAP-positive astrocytes in the sham-operated control groups (Fig. 3D). However, I/R induced an increase in GFAP-positive astrocytes, with small cell bodies and fine cytoplasmic processes flanking the pyramidal neurons around hippocampal CA1 (Fig. 3E). Ischemic animals that were treated with PX-18 showed a marked decrease in reactive astrocytes as compared with the ischemic group (Fig. 3F vs. E).

With isolectin-B4 as a marker, no microglial cells were found in the sham-operated controls (Fig. 3G). However, I/R caused recruitment of microglial cells, which were especially clustered in the CA1 area together with dying neurons (Fig. 3H). Treatment with PX-18 reduced I/R-induced microglial activation (Fig. 3I vs. H).

Analysis of the number of astrocytes indicated significant differences between I/R and I/R+PX-18 ($p<0.001$ for each doses) (Fig. 4B). PX-18 at 60 mg/kg body wt was more effective, as a significant difference was found between I/R+PX-18-60 and I/R+PX-18-30 groups ($p<0.05$). However, the I/R+PX-18-60 group showed no difference from the sham control group ($p<0.05$) (Fig. 4B). Analysis of the numbers of microglial cells in CA1 showed significant differences between the I/R and I/R+PX-18 groups ($p<0.01$ for each doses) (Fig. 4C).

2.4. PX-18 is neuroprotective against cerebral I/R-induced neuronal DNA damage and degeneration

Four days after a 5-min CCA occlusion, extensive neuronal nuclei damage and neuronal degeneration were observed in the pyramidal neurons in the hippocampal CA1 subfield (Fig. 5B vs. A and E vs. D). PX-18 administration by i.p. injections inhibited cerebral I/R-induced changes in the hippocampal CA1 neurons (Fig. 5C vs. B and F vs. E). To compare the extent of nuclear damage and neuronal degeneration in the CA1 area across treatment conditions, quantitative analysis of the staining was performed. Analysis of DAPI staining revealed significant differences between the I/R and I/R+PX-18 groups ($p<0.001$ for each doses) (Fig. 6A). Analysis of neuronal degeneration also showed significant differences between I/R and I/R+PX-18 groups ($p<0.001$ for each doses) (Fig. 6B). Although PX-18 injected at 60 mg/kg

body wt resulted in slightly more protective effects than that at 30 mg/kg body wt, the differences were not significant ($p>0.05$).

3. Discussion

In this study, PX-18 nanocrystals injected i.p. (30 and 60 mg/kg body wt) 5 min after CCA occlusion/reperfusion were shown to attenuate ischemia-induced delayed neuronal death, an event well established in our laboratory (Wang et al., 2002). PX-18 nanocrystals also reduced ischemia-induced neuronal degeneration, DNA damage, and glial cell activation. Although PX-18 has been demonstrated to be a potent PLA₂ inhibitor in the peripheral system (Franson and Rosenthal, 1997), this is the first indication of the use of PX-18 nanocrystals to effectively ameliorate neuronal damage in the brain.

Nanotechnology holds promise as an effective strategy for the delivery of substances with low aqueous solubility and physico-chemical characteristics that otherwise would have precluded them from preclinical development. Recent investigations have demonstrated different nanoparticle formulations to increase bioavailability of drugs for the CNS (Muller and Keck, 2004b). Examples are studies using liposomes (Ko et al., 2009; Sauer et al., 2005), polymeric nanoparticles (Aktas et al., 2005a; Aktas et al., 2005b; Gao et al., 2006), and different types of lipid nanoparticles (Gessner et al., 2001; Goppert and Muller, 2005; Muller and Keck, 2004a; Muller and Keck, 2004b). In this study, parenteral application of the poorly soluble PLA₂ inhibitor PX-18 was achieved by formulating PX-18 as a suspension of drug nanocrystals. Due to the extremely small particle size of 40 nm, an increase in saturation, as well as dissolution velocity compared to PX-18 bulk material, these nanocrystals offered good bioavailability. Furthermore, the PX-18 nanosuspension also showed good physical stability as the particle size stayed unchanged over an observation period of 180 days. This stability allows a “ready-to-use” formulation over an extended period for the *in vivo* experiments.

Phospholipases A₂ are ubiquitous in mammalian cells and play an important role in maintenance of membrane phospholipids. These enzymes are also responsible for production of a number of lipid mediators. The presence of multiple isoforms of PLA₂ in different cellular systems in mammalian tissues (Masuda et al., 2005) including the brain (Molloy et al., 1998) makes elucidation of the involvement of specific PLA₂ subtypes in neurodegenerative diseases a difficult and challenging endeavor (Sun et al., 2004). Up-regulation of sPLA₂-IIA mRNA expression has been reported in rat brain after transient global and focal ischemia (Lauritzen et al., 1994; Lin et al., 2004). Our study further demonstrated up-regulation of the inflammatory sPLA₂-IIA in reactive astrocytes in response to focal cerebral I/R (Lin et al., 2004). An increase in sPLA₂ activity was also observed after photochemically induced focal cerebral ischemia (Yagami et al., 2002). Similarly, exposure of cultured astrocytes to oxygen glucose deprivation (OGD) also caused an increase in sPLA₂ expression and activity (Gabryel et al., 2007). Although the exact damaging effects of sPLA₂-IIA to neurons have not been clearly elucidated, earlier studies reported its ability to potentiate Ca²⁺ influx through the L-type voltage-sensitive Ca²⁺ channels (Yagami et al., 2003). sPLA₂ has also been shown to enhance glutamate signaling, liberating arachidonic acid (AA) and production of excessive prostaglandin D₂ (PGD₂), which are factors leading to neuronal apoptosis after cerebral I/R (DeCoster et al., 2002; Kolko et al., 1996; Kolko et al., 1999; Rodriguez De Turco et al., 2002; Yagami et al., 2002). Consequently, it is reasonable to hypothesize that release of sPLA₂-IIA from reactive astrocytes during I/R may constitute an important source of injury to neurons.

Experimental data have indicated an important role for inflammatory cascades during I/R (Ishibashi et al., 2002). Cerebral ischemia induces inflammatory responses in the brain that is associated with the induction of a variety of cytokines. Pro-inflammatory cytokines are involved in transcriptional up-regulation of sPLA₂-IIA in astrocytes. Actually, cytokines such

as IL-1 β and TNF α are linked to increased oxidative stress, altered lipid metabolism and subsequently ischemia-induced neuronal death (Adibhatla and Hatcher, 2007a; Muralikrishna Adibhatla and Hatcher, 2006). Treatment with the TNF α antibody or IL-1 β receptor antagonist significantly attenuated infarction volume, sPLA₂-IIA protein expression, and PLA₂ activity while restoring the phosphatidylcholine levels after I/R (Adibhatla and Hatcher, 2007a).

Due to the inflammatory properties of sPLA₂-IIA, there is substantial interest in developing inhibitors specific to this type of PLA₂ (Oslund et al., 2008; Touaibia et al., 2007). Several studies have demonstrated the neuroprotective effects of sPLA₂-IIA inhibitors, including the indole analog, indoxam (Touaibia et al., 2007; Yagami et al., 2002), and the non-steroidal, anti-inflammatory drug S-2474 (Yagami et al., 2005). Results from this study further demonstrate that PX-18, when formulated as nanocrystals, can offer protective effects in rescuing neurons from I/R-induced neuronal damage.

4. Materials and methods

4.1 PX-18 nanocrystal preparation, characterize and administration

Richard Berney (RBA Pharma, LLC) supplied the PX-18 (BES Dioleate). PX-18 is a white waxy solid with a molecular mass of 742.2 and is almost insoluble in water. Müller and Pardeike (Department of Pharmaceutics, Biopharmaceutics and NutriCosmetics, Freie Universität Berlin, Germany) prepared the PX-18 drug nanocrystals. The PX-18 nanosuspension was produced by high-pressure homogenization using a Micron LAB 40 (APV Deutschland GmbH, Germany). To ensure the stability of the nanosuspension for injection, 1% (w/w) PX-18 was suspended in a solution containing 1% (w/w) of the surfactant Tween 80 (Sigma, Germany), 2.5% (w/w) of the isotonic agent glycerol (Caelo, Germany), and 96.5% (w/w) water. The suspension was pre-homogenized by applying two cycles at 150 bar, two cycles at 500 bar, and two at 1000 bar. The homogenization process was completed by 20 cycles at 1500 bar. The production temperature was 5°C. The nanosuspension was diluted 1:1 with a solution containing 1% (w/w) Tween 80, 2.5% (w/w) glycerol and 96.5% (w/w) water for injection to obtain a 0.5% (w/w) (5 mg/ml) PX-18 nanosuspension. A solution containing only the Tween 80 and glycerol was used for the vehicle control.

The particle size of the PX-18 nanosuspension was measured by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instruments, UK). PCS yields the mean particle diameter and the polydispersity index (PI). Using PCS measurements and the time dependent changes of the intensity of light scattered by the nanoparticles, an autocorrelation function was calculated by a correlator. This calculated correlation function was fitted to a theoretical correlation function. In case of a monodisperse particle size distribution, the fitted correlation function equals exactly the calculated correlation function. With increasing width of the particle's size distribution, the deviation between the fitted correlation function and the calculated correlation function is also increased. The deviation between the calculated correlation function and the fitted correlation function was determined and the PI served as a measure of the width of the particle size distribution. A monodisperse particle size distribution would give a PI of 0; whereas, a broad particle size distribution with an unknown shape would give a PI of 1.

4.2 Induction of global forebrain ischemia to gerbils and preparation of brain samples

Adult male Mongolian gerbils (60–80 g body wt) (Charles River, Wilmington, MA) were housed in the Small Animal Facilities of the University of Missouri. Gerbils were given free access to water and lab chow and maintained at 22 $\pm$ 2°C with a constant humidity under a 12:12 hr light: dark cycle. Gerbils were randomly divided into 4 groups, namely, sham (n = 10), I/R (n = 11), I/R+PX-18 (30 mg/kg; n = 11), and I/R+PX-18 (60 mg/kg; n = 11). Both

common carotid arteries were occluded for 5 minutes followed by reperfusion under anesthesia with isoflurane (2.5%), nitrous oxide (70%), and oxygen (30%) (Wang et al., 2002). During the operation, animals were kept at 37°C using a heating block. Sham-operated gerbils underwent the same procedures except that the carotid arteries were not occluded. The presence of communicating arteries in gerbils was detected by monitoring the decrease in regional cerebral blood flow (rCBF) before and after clamping the bilateral CCA using a laser doppler blood flow monitor (MBF3D, Moor Instruments, Axminster, Devon, UK). Gerbils that showed a decrease in rCBF of less than 80% were excluded from the subsequent analysis (Wang et al., 2002). The University of Missouri Animal Care and Use Committee (Protocol #1741) approved the animal protocol. Experiments were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

PX-18 (5 mg/ml) nanocrystals were prepared in 1% Tween 80, and 2.5% glycerol in water. The clear suspension was injected i.p. (30 and 60 mg/kg body weight) immediately after reperfusion. Sham controls and I/R controls were similarly injected with the preparation without PX-18. Four days after ischemia, gerbils were transcardially perfused with heparinized saline (30 ml) and then with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4, 100 ml). The brains were then post-fixed in the same fixative for 3 days. Brain tissues were then embedded in paraffin for histochemical and immunohistochemical examinations. Six micron thick coronal sections were cut at the dorsal hippocampal area.

4.3 Histochemical and immunohistochemical staining

4.3.1. Assessment of neurons, astrocytes and microglial cells—Cresyl violet staining was used to estimate the ischemic neuronal damage. GFAP immuno-histochemistry and isolectin-B4 staining were used to show astrocytes and microglial cells. Protocols for these staining have been described previously (Wang et al., 2002).

4.3.2. Fluoro-Jade B staining to identify neuronal degeneration—Fluoro-Jade B is a novel polyanionic fluorescein derivative, which sensitively and specifically binds to degenerating neurons (both apoptotic and necrotic). Its high affinity to degenerating neurons with green fluorescence has made it a useful marker for neurodegeneration. Fluoro-Jade B staining to examine ongoing damage was performed according to the protocol described by Schmued et al (Schmued et al., 1997).

4.3.3. DAPI staining to identify nuclear DNA damage—The fluorescent dye 4',6-diamidine-2'-phenylindole (DAPI; Roche Molecular Chemicals), which intercalates specifically into adenine-thymidine base pairs of DNA, was used to identify nuclear DNA damage in neurons (Hara et al., 1999). Deparaffinized and hydrated sections were immersed in DAPI (0.1 µg/ml) in phosphate-buffered saline (PBS) for 20 min at room temperature, and then examined under fluorescence at 340 nm.

4.3.4. Quantitative assessment and data analysis—Neuronal damage, degeneration, and glial activation were quantified by counting the number of live neurons and fluorescent, or immunoreactive positive cells of Fluoro-Jade B, DAPI, GFAP, and Isolectin-B4 within a defined CA1 area (320 × 100µm) as described previously (Wang et al., 2002). Each side of the hippocampi were viewed per high magnification (magnification 400 ×) using the Bioquant Image Analysis System (Bioquant True Colors Windows 95 Software, version 2.50, Nashville, TN).

4.4 Statistical treatment of data

Data (mean $\pm$ SEM) were subjected to one-way ANOVA followed by Newman-Keuls post-hoc tests using the GraphPad Prism program version 4.0. A p value of less than 0.05 was considered statistically significant.

Abbreviations

CCA, common carotid arteries
I/R, ischemia/reperfusion
DND, delayed neuronal death
GFAP, glial fibrillary acidic protein
rCBF, regional cerebral blood flow
ROS, reactive oxygen species
DAPI, 4',6-diamidino-2'-phenylindole
sPLA₂-IIA, group IIA secretory phospholipase A2
AA, arachidonic acid
PGD2, prostaglandin D2
PX-18, 2-[N,Nbis(2-oleoyloxyethyl)amine]-1-ethanesulfonic acid

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Chemical Structure of PX-18

Fig. 1.
Chemical structure of PX-18

Fig. 2. Average particle diameter and polydispersity index (PI) of the 1% PX-18 nanosuspension immediately after production (day 0), and after 30 days and 180 days of storage at 4–8°C.

Fig. 3.

The effects of PX-18 (30 mg/kg, i.p., injected immediately after I/R) on neuronal survival, astrocytic and microglial activation in the hippocampal CA1 area at 4 days after a 5-min CCA occlusion in gerbils. Representative photomicrographs depicting neurons (cresyl violet, A–C), astrocytes (GFAP, D–F), and microglial cells (isolectin B4, G–I). The experimental design and staining procedures were described in the Experimental Procedures. (Magnification, 400 ×)

Fig. 4.

Histograms depicting the number of neurons (A), astrocytes (B) and microglial cells (C) in the hippocampal CA1 area in sham ($n = 10$), ischemia ($n = 11$), ischemia+PX-18, 30 mg/kg ($n = 11$), and ischemia+PX-18, 60 mg/kg ($n = 11$) groups. See Experimental Procedures for experimental design and description for cell counting. Data represent means $\pm$ SEM. One-way ANOVA revealed significant differences among groups ($p < 0.0001$ for neurons, astrocytes and microglia). Neuman-Keuls multiple comparison tests revealed significant differences between sham and I/R, as well as between I/R and I/R+PX-18 groups, but comparison between I/R+PX-18-30 and I/R+PX-18-60 groups showed a significant difference only for astrocytes ($p < 0.05$). See text for details on pairwise comparisons.

Fig. 5. The effects of PX-18 (30 mg/kg, i.p., injected immediately after I/R) on nuclear damage and neuronal degeneration in the hippocampal CA1 area at 4 days after a 5-min CCA occlusion in gerbils. Representative photomicrographs depicting DAPI (A–C) and Fluoro-Jade-B (D–F) staining. The experimental design and staining procedures were described in Experimental Procedures. (Magnification, 400 ×)

Fig. 6.

Histograms depicting the number of DAPI (A) and Fluoro-Jade B (B) staining in the hippocampal CA1 area in sham ($n = 10$), ischemia ($n = 11$), ischemia+PX-18, 30 mg/kg ($n = 11$), and ischemia+PX-18, 60 mg/kg ($n = 11$) groups. See Experimental Procedures for experimental design and description for cell counting. Data represent means $\pm$ SEM. One-way ANOVA revealed significant differences among groups ($p < 0.0001$ for each). Neuman-Keuls multiple comparison tests revealed significant differences between sham and I/R, as well as between I/R and I/R+PX-18 groups, but comparison of I/R+PX-18-30 and I/R+PX-18-60 groups showed no significant differences. See text for details on pair wise comparisons.