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Lipocalin-prostaglandin D synthase is a critical beneficial factor in transient and permanent focal cerebral ischemia

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Abstract

Prostaglandin D₂ (PGD₂) is the most abundant prostaglandin produced in the brain. It is a metabolite of arachidonic acid and synthesized by PGD₂ synthases (PGDS) via the cyclooxygenase pathway. Two distinct types of PGDS have been identified: hematopoietic (H-PGDS) and lipocalin-type PGDS (L-PGDS). Because relatively little is known about the role of L-PGDS in the central nervous system, here we examined the outcomes in L-PGDS knockout and wildtype (WT) mice after two different cerebral ischemia models, transient middle cerebral artery (MCA) occlusion (*t*MCAO) and permanent distal MCA occlusion (*p*MCAO). In the *t*MCAO model, the MCA was occluded with a monofilament for 90 min and then reperused for 4 days. In the *p*MCAO model, the distal part of the MCA was permanently occluded and the mice sacrificed after 7 days. Percent corrected infarct volume and neurological score were determined after 4 and 7 days, respectively. L-PGDS knockout mice had significantly greater infarct volume and brain edema than did WT mice after *t*MCAO ($P < 0.01$). Similarly, L-PGDS knockout mice showed greater infarct volume and neurological deficits as compared to their WT counterparts after *p*MCAO ($P < 0.01$). Using the two models enabled us to study the role of L-PGDS in both early (*t*MCAO) and delayed (*p*MCAO) ischemic processes. Our findings suggest that L-PGDS is beneficial for protecting the brain against transient and permanent cerebral ischemia. These results provide a better understanding of the role played by the enzymes that control eicosanoid synthesis and how they can be utilized as potential targets to prevent damage following either acute or potentially chronic neurological disorders.

Keywords

cerebral ischemia; middle cerebral artery occlusion; mouse; prostaglandins; PGD₂

INTRODUCTION

Cerebral ischemia is an acute neurological injury resulting from occlusion of blood vessels that supply blood, oxygen, and important nutrients to a given brain region. It initiates an ischemic cascade that can cause severe brain damage and long-term disability in humans. Mechanisms

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involved in development and progression of cerebral ischemia are complex and include excitotoxicity, ionic imbalance, oxidative stress, nitrosylation, and inflammation (Finley Caulfield and Wijman, 2006; Weinberger, 2006). With the failure of glutamate antagonists and antioxidant therapies in clinical trials, attention has been directed toward inflammatory cascades that follow ischemic injury and delayed neuronal cell death. Drugs that combat inflammation may provide an ideal therapeutic intervention (Doré, 2006). Inflammation triggers the activation of phospholipase A₂ and the generation of arachidonic acid from membrane phospholipids (Sapirstein et al., 2005). The arachidonic acid is ultimately converted by cyclooxygenase (COX) and lipoxygenase pathways to prostaglandins, thromboxane A₂, and leukotrienes, collectively termed eicosanoids. Both COX and lipoxygenase pathways have clinical importance in relation to several human disorders (Tomimoto et al., 2002; Doré, 2006).

Non-steroidal anti-inflammatory drugs, popular medications used to treat pain, heart disease, fever, and inflammation, target the COX pathway (Bishop-Bailey et al., 2006; Premkumar and Raisinghani, 2006). COX 1 and COX 2 catalyze the synthesis of PGH₂, which is then processed by various prostanoid synthetase enzymes to generate PGD₂, PGE₂, PGF_{2α}, prostacyclin (PGI₂), and thromboxane A₂. These prostanoids are lipid mediators that participate in inflammatory responses by binding with G-protein-coupled receptors designated DP, EP, FP, IP, and TP, respectively (Doré, 2006). PGD₂ is highly abundant in the central nervous systems of mice, rats, and humans (Doniach, 1977; Hayaishi, 1991) and is involved in various pathophysiological events such as regulation of the sleep/wake cycle, pain response, hypoxia, seizure, and inflammation (Ujihara et al., 1988; Eguchi et al., 1999; Hayaishi, 2002; Qu et al., 2006; Taniguchi et al., 2007). It is synthesized by hematopoietic prostaglandin D synthetase (H-PGDS) and lipocalin-type PGDS (L-PGDS) (Urade and Hayaishi, 2000; Urade and Eguchi, 2002), and functions by binding to specific receptors with high affinity (DP1) or low affinity (DP2) (Kabashima and Narumiya, 2003; Nagata and Hirai, 2003). In the brain, L-PGDS is expressed in leptomeninges, choroid plexus, and oligodendrocytes (Urade et al., 1993; Beuckmann et al., 2000; Mohri et al., 2006a; Urade and Mohri, 2006), whereas H-PGDS is expressed in amoeboid and ramified forms of microglia (Mohri et al., 2003). L-PGDS also has been shown to play important roles in spinal cord injury, multiple sclerosis, atherosclerosis, Alzheimer disease, and hypertension (Hirawa et al., 2002; Miwa et al., 2004; Grill et al., 2006; Kagitani-Shimono et al., 2006; Kanekiyo et al., 2007).

To our knowledge, no report has described the unique contribution of L-PGDS in cerebral ischemic stroke. The aim of the present study was to determine the pathophysiological role of L-PGDS in cerebral ischemia by investigating the physiological and histological parameters of L-PGDS knockout (L-PGDS^{-/-}) and wildtype (WT) mice subjected to two different cerebral ischemia models: transient middle cerebral artery (MCA) occlusion (tMCAO) and permanent distal MCA occlusion (pMCAO).

MATERIALS AND METHODS

This study was performed in accordance with the NIH guidelines for the use of experimental animals; protocols were approved by the Johns Hopkins Animal Care and Use Committee. Wildtype and L-PGDS^{-/-} C57BL/6 mice were bred and genotyped for this study. The L-PGDS^{-/-} mice were generated as previously reported (Eguchi et al., 1999; Qu et al., 2006). Adult mice (20–28g) were used in these studies.

Anatomical examination of cerebrovasculature

Adult male WT and L-PGDS^{-/-} mice ($n = 3/\text{genotype}$) were deeply anesthetized with halothane before being perfused by cardiac puncture with saline followed by black latex paint. Then the brains were carefully harvested and immersed in 10% formalin for 24 h before examination.

The vessel diameters were evaluated with Metavue software (Meta Imaging Series Software, Downingtown, PA, USA).

Transient focal cerebral ischemia

Transient focal cerebral ischemia was induced by *t*MCAO with an intraluminal filament technique as described (Saleem et al., 2008). Briefly, adult male mice ($n = 9$ WT, 13 L-PGDS^{-/-}) were placed under halothane anesthesia. Body temperature was maintained at $37.0 \pm 0.5^\circ\text{C}$ with a heating pad. Relative cerebral blood flow (CBF) was monitored by laser-Doppler flowmetry (Moor instruments, Devon, England) over the parietal cortex supplied by the MCA. Occlusion of the MCA was accomplished with a 7-0 Ethilon nylon monofilament (Ethicon, Somerville, NJ, USA) coated with flexible silicone and confirmed by a decrease in CBF. During the 90-min occlusion, anesthesia was discontinued, and the animals were transferred to a humidity- and temperature-controlled chamber; animal behavior was also monitored for the entire time period to further confirm the occlusion. Then the mice were re-anesthetized, and the filament was withdrawn. The mice were returned to the chamber for approximately 6 h before being returned to their home cages. Neurological function was measured in each mouse on day 4 after reperfusion according to the following 0–4-point graded scoring system: 0 = no deficit; 1 = forelimb weakness and torso turning to the ipsilateral side when held by tail; 2 = circling to affected side; 3 = unable to bear weight on affected side; and 4 = no spontaneous locomotor activity or barrel rolling, as described previously (Saleem et al., 2008).

Measurement of body temperature, blood gases, and mean arterial blood pressure

In a separate cohort of animals ($n = 5$ /genotype), the femoral artery was cannulated for measurement of arterial blood gases and mean arterial blood pressure (MABP) at baseline and at 15-min intervals for 90 min of ischemia and 60 min of reperfusion. Body temperature was determined with a rectal probe at the same time points.

Brain water content

In another cohort of mice ($n = 4$ WT, 5 L-PGDS^{-/-}), brain water content was measured by the wet/dry weight method, as described previously (Wang and Doré, 2007). Mice were deeply anesthetized with halothane and decapitated to remove their brains. Samples were taken from ischemic and nonischemic hemispheres. The brains were weighed wet, oven dried at 100°C for 48 h, and then reweighed. Brain water content (%) was calculated as $(\text{wet weight} - \text{dry weight})/\text{wet weight} \times 100$.

Permanent distal MCAO

The protocol used for *p*MCAO was that described by Majid et al. (Majid et al., 2000) with minor modifications. Briefly, with the mice ($n = 10$ WT, 6 L-PGDS^{-/-}) under halothane anesthesia, a 1.0-cm vertical skin incision was made between the right eye and ear. The temporal muscle was moved, and the temporal bone exposed. Under a surgical microscope, a 2.0-mm burr hole was made just over the MCA, visible through the temporal bone. The main trunk of the distal part of the MCA was directly occluded with a bipolar coagulator, and complete interruption of blood flow at the occlusion site was confirmed by severance of the occlusion site of the MCA. Core body temperature was maintained between 36.5 and 37.5°C during and after the procedures. Animals not circling toward the paretic side after the onset of ischemia and those that developed subarachnoid hemorrhage were eliminated from the study. A successful occlusion was also confirmed by placing the laser-Doppler probe above the temporal ridge to establish that blood flow into the region was terminated. After 7 days, the mice were euthanized and the brains harvested. To determine the neurological deficits caused by this model, a robust 28-point score pattern was used (Wang et al., 2006). Seven days after the *p*MCAO procedure, an experimenter blinded to genotype scored all mice for neurological

deficits. The tests were divided into motor and sensory functions. For motor functions, the tests included: 1) spontaneous activity; 2) symmetry of walking; 3) head/neck movement when suspended by tail; 4) symmetry of forelimbs when suspended by tail; 5) climbing at a 45° angle; and 6) balance on a rod. For sensory function, we used a test in which the vibrissae are stimulated with a cotton-tipped applicator. Each of the seven tests was graded from 1 to 4, establishing a maximum deficit score of 28. Immediately after the testing, the mice were sacrificed for infarct volume analysis.

Quantification of infarct volume in both ischemic stroke models

After neurological assessment, the mice were anesthetized deeply, and the brains were harvested and sliced coronally into five 2-mm thick sections, which were incubated with 1% triphenyltetrazolium chloride in saline for 30 min at 37°C. The area of brain infarct, identified by the lack of triphenyltetrazolium chloride staining, was measured on the rostral and caudal surfaces of each slice and numerically integrated across the thickness of the slice to obtain an estimate of infarct volume (Sigma Scan Pro, Systat, Port Richmond, CA, USA). Volumes of all five slices were summed to calculate total infarct volume over the entire hemisphere and expressed as a percentage of the volume of the contralateral hemisphere. Infarct volume was corrected for swelling by comparing the volumes in the ipsilateral and contralateral hemispheres. The corrected volume of the infarcted hemisphere was calculated as volume of contralateral hemisphere – (volume of ipsilateral hemisphere – volume of infarct) (Doré et al., 2003).

Statistical analysis

The brain sections were imaged and analyzed with SigmaScan Pro 5.0 software (Systat, Inc., Point Richmond, CA). SigmaStat 3.11 was used for statistical analysis. Data are represented as mean ± standard error of the mean. The data for infarction volumes were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple range tests. Neurological deficit scores for *t*MCAO and *p*MCAO were analyzed by the non-parametric Kruskal-Wallis analysis of ranks. Values of $p < 0.05$ were considered to be significant.

RESULTS

Effects of transient MCA occlusion and reperfusion

It has been observed that differences in size and vascular territory between gene-deleted and WT mice may interfere with the outcomes of ischemic pathophysiology. Therefore, to have a first look at potential gross anatomical changes, we examined large cerebral vessels in WT and L-PGDS^{-/-} mice after identical injection of black latex paint. No differences in the vascular diameter were observed between the two groups (Fig. 1). Next, we monitored the impact of *t*MCAO on physiologic parameters of the WT and L-PGDS^{-/-} mice to determine whether differences existed that could potentially affect stroke outcome. No significant differences were observed in CBF, MABP, or body temperature (Fig. 2) or in blood gas concentrations (pH, PaCO₂, and PaO₂; Table 1) between WT and L-PGDS^{-/-} mice. All of these parameters were within normal ranges in both WT and L-PGDS^{-/-} mice. However, the mean infarct size of mice lacking L-PGDS was significantly larger ($52.0 \pm 5.1\%$; $P < 0.01$) than that of WT mice after 90 min of *t*MCAO and 4 days of reperfusion (Fig. 3A and B). Furthermore neurological deficit scores of the L-PGDS^{-/-} mice (2.6 ± 0.2) were significantly greater ($P < 0.01$) than those of the WT mice (1.8 ± 0.2) (Fig. 3C), indicating greater dysfunction.

To further substantiate our outcomes regarding the role of L-PGDS, we evaluated water content in each hemisphere of the WT and L-PGDS^{-/-} mice (Fig. 4). When the contralateral hemispheres were compared, the water content of the L-PGDS^{-/-} mice did not differ substantially from that of the WT mice. However, the water content of the ipsilateral hemispheres was significantly

higher ($P < 0.01$) in the LPGDS^{-/-} mice than in the WT mice, suggesting that disruption of the blood-brain barrier is greater in the absence of L-PGDS.

Effects of permanent distal MCA occlusion

Because the *p*MCAO model produces small infarcts that are confined to the cortex, we used this model to assess delayed ischemic effects in L-PGDS^{-/-} mice. Here, we observed 100% survival rates up to 7 days in the mice subjected to *p*MCAO. After 7 days of permanent occlusion, L-PGDS^{-/-} mice had significantly larger ($27.0 \pm 5.4\%$, $P < 0.01$) infarct size than did their counterpart WT mice (Fig. 5A and B). Furthermore, *p*MCAO produced neurological deficit scores that were higher ($P < 0.01$) in L-PGDS^{-/-} mice (16.8 ± 0.7) than in WT (11.8 ± 1.1) mice; (Fig. 5C). These observations further support the likelihood that L-PGDS plays a beneficial role in *t*MCAO.

DISCUSSION

In the present study, we investigated the effect of L-PGDS on transient and permanent ischemia-induced brain injury by comparing pathophysiological outcomes in WT and L-PGDS^{-/-} mice. Using two different models of ischemia enabled us to evaluate the role of L-PGDS not only in short-term reperfusion injury but also in long-term neurological outcomes of permanent ischemia. We found that in both MCAO models, neurological dysfunction and percent corrected infarct volume of the ipsilateral hemisphere were significantly higher in the knockout mice than in the WT mice. Brain water content after *t*MCAO also was significantly higher in L-PGDS^{-/-} mice than in their WT counterparts, despite the fact that the physiological parameters measured (MABP, blood chemistry) and the water content of the contralateral hemisphere were not significantly different between the two groups and were within the normal ranges. Our results demonstrate that the absence of L-PGDS exacerbates ischemic brain injury, suggesting that L-PGDS and likely the generation of PGD₂ would be beneficial in an ischemic paradigm. Consequently, as COX-2 inhibitors are being tested to limit the production of prostaglandins, researchers should keep in mind that a downstream pathway such as the one modulated by L-PGDS could also be beneficial; therefore it would be best if novel drugs did not inhibit this pathway and perhaps should be designed to activate it.

PGD₂ is considered to be the most abundant prostaglandin present in the brain (Abdel-Halim et al., 1977). It is well known that inflammation increases PGD₂ synthesis in the central nervous system (Hetu and Riendeau, 2005; Mohri et al., 2006b). Published reports indicate that two PGDS enzymes (H-PGDS and L-PGDS) regulate the formation of PGD₂, which acts mainly through two different G-protein-coupled receptors, namely DP1 (Boie et al., 1995) and CRTH2 (chemo-attractant homologous receptor expressed on TH2 cells), also known as DP2 (Hirai et al., 2001). Recently, we showed that the DP1 receptor plays a protective role in ischemia/reperfusion injury (Saleem et al., 2007). However, it is important to determine the role of brain L-PGDS during ischemic stroke because it might constitute a mechanism by which brain PGD₂ biosynthesis can be regulated independently of other prostaglandins. It has been suggested that during the course of inflammation, a shift occurs from initial dominance of PGE₂ toward PGD₂ biosynthesis during later stages of inflammation, a response that may contribute to resolution of inflammation (Gilroy et al., 2003). The proposed function of PGD₂ in the resolution phase of inflammation makes this compound a distinctive member of the prostanoid family.

Recent studies have indicated that variation in the cerebrovasculature of animals affects stroke outcome in global and focal cerebral ischemia (Kitagawa et al., 1998; Zhen and Doré, 2007). Here, we compared the cerebrovascular anatomy of WT and L-PGDS^{-/-} mice and found no obvious difference between the two groups, signifying that differences in outcome did not stem from differences in structure. Further, it was important to evaluate the effect of L-PGDS

absence on physiological parameters such as CBF, MABP, body temperature, and blood gases (pH, PaCO₂ and PaO₂) because the maintenance of physiological parameters reduces secondary disease or conditions that develop in the course of a primary disease (Goldstein and Hankey, 2006). The laser-Doppler method is a widely used method for CBF measurement (Harada et al., 2005; Shah et al., 2006; Saleem et al., 2007). We chose this method to measure CBF because it offers the advantage of determining temporal changes in CBF in real time. We did not detect any significant differences between the two genotypes during the occlusion or during the early reperfusion in the *t*MCAO model. Future work will be required to comprehensively assess the role of L-PGDS in absolute blood flow and brain-vessel regulation, but here, no obvious differences in the gross superficial cerebrovasculature or in the relative blood flow were noted between the WT and the L-PGDS^{-/-} mice.

The transient focal ischemia model produces large infarcts that significantly affect the striatum, extend to the cortical region, and often result in high mortality rates, particularly in certain knockout mice. Therefore, it is sometimes difficult to study long-term or delayed ischemic responses with this model. The *p*MCAO model used here provides an advantage because the damage is limited to the distal cortical region of the MCA territory, and the mice can be evaluated at later time points. Using the *p*MCAO model, we were able to study the role of L-PGDS in shaping the distal cortical damage of mice following permanent stroke and to evaluate neurological deficits 7 days after ischemic insult. The resulting data support the neuroprotective role of L-PGDS in stroke, as observed with the *t*MCAO model.

PGD₂ also plays an important role in induction of brain edema (Asano et al., 1985; Taniguchi et al., 2007). The physiological and molecular mechanisms of brain edema formation after ischemia/reperfusion-induced brain injury are multifaceted (Xiao, 2002). The pathobiology of ischemia-elicited cerebral edema includes a cytotoxic component (secondary to post-ischemic failure of the Na⁺/K⁺ ATPase and other ATP-dependent transporters) and a vasogenic component (secondary to breakdown of the blood-brain barrier, with leakage of plasma proteins into extracellular space), although several other mechanisms, such as disruption of Ca²⁺ signaling, inflammatory mediators, neurohumoral responses, vascular endothelial growth factor, and upregulation of water channels, are now strongly implicated in ischemia-induced edema (Gerriets et al., 2004; Hosomi et al., 2005). In general, the distinction between cytotoxic edema and vasogenic edema is that the latter needs blood flow to cause swelling. In the present study, we compared the percent brain water content in WT and L-PGDS^{-/-} mice to determine the amount of brain edema. Our results indicate that absence of L-PGDS leads to a significant increase in brain water content in the ipsilateral hemisphere but has no significant effect on the contralateral hemisphere. At present, it is uncertain to what extent all of these pharmacological properties associated with PGD₂ are relevant to cerebral ischemia.

Our study suggests that genetic deletion of L-PGDS exacerbates ischemic reperfusion injury without affecting body temperature, MABP, blood pH, PaO₂, or PaCO₂. In addition, loss of L-PGDS led to significantly more cortical damage than that observed in WT mice following distal *p*MCAO. These results provide the first evidence that L-PGDS is important for the resolution of brain injury following transient and permanent brain ischemia.

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List of Abbreviations

CBF	cerebral blood flow
COX	cyclooxygenase
MABP	mean arterial blood pressure
MCA	middle cerebral artery
pMCAO	permanent middle cerebral artery occlusion
tMCAO	transient middle cerebral artery occlusion
WT	wildtype

Fig. 1.

Genetic deletion of L-PGDS does not significantly affect cerebral arterial vasculature in mice. Photographs showing the cerebral arterial vasculature of (A) WT and (B) L-PGDS^{-/-} mice. (C) Macroscopic analysis of cerebral arterial vasculature did not reveal differences in the circle of Willis or major cerebral arteries between L-PGDS^{-/-} and WT mice.

Fig. 2.

Absence of L-PGDS does not affect the monitored physiological parameters. Relative cerebral blood flow (CBF, A), core body temperature (B), and mean arterial blood pressure (MABP, C) were recorded at baseline (ctrl), immediately at induction of ischemia (immed), and at 15-min intervals during ischemia and 1 h of reperfusion. Change in CBF was recorded as a percent of baseline.

Fig. 3. Deletion of L-PGDS enhances ischemic brain injury and neurological dysfunction after transient ischemia. (A) Photographs of infarcted brain slices from WT (left) and L-PGDS^{-/-} (right) mice. (B) Hemispheric infarct volume, shown as a percent of total hemisphere volume and corrected for brain swelling, was significantly larger in L-PGDS^{-/-} mice than in WT mice after 90 min of ischemia and 4 days of reperfusion. (C) Neurological scores assessed 4 days after ischemia were significantly higher in L-PGDS^{-/-} mice than in WT mice, indicating greater neurological dysfunction. * $P < 0.01$.

Fig. 4. Water content in ischemic and non-ischemic hemispheres of WT and L-PGDS^{-/-} mice 4 days after transient middle cerebral artery occlusion. * $P < 0.01$.

Fig. 5. Deletion of L-PGDS enhances ischemic brain injury and neurological dysfunction after permanent distal middle cerebral artery occlusion. (A) Representative photographs of infarcted brain slices from WT (left) and L-PGDS^{-/-} (right) mice. (B) Cortical infarct volume, shown as a percent of total cortical volume and corrected for swelling, was significantly larger in L-PGDS^{-/-} mice than in WT mice after 7 days. (C) Neurological scores assessed 7 days after ischemia were significantly higher in L-PGDS^{-/-} mice than in WT mice, indicating greater neurological dysfunction (* $P < 0.01$).

Table 1
Effect of MCAO on physiological parameters in WT and L-PGDS^{-/-} mice

Parameter	Wildtype Mice			L-PGDS ^{-/-} Mice		
	Baseline	1 h MCAO	1 h Reperfusion	Baseline	1 h MCAO	1 h Reperfusion
pH	7.37 ± 0.02	7.34 ± 0.01	7.35 ± 0.01	7.34 ± 0.01	7.34 ± 0.01	7.33 ± 0.01
PaCO ₂	38.7 ± 1.2	40.3 ± 1.1	39.7 ± 1.5	39.1 ± 1.1	39.4 ± 1.0	39.5 ± 1.0
PaO ₂	108 ± 2	128 ± 4	112 ± 5	111 ± 5	127 ± 7	114 ± 3