

## Expression and potential role of phospholipase D1 in cryoinjured cerebral cortex of rats

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**Summary.** The expression and potential role of phospholipase D1 (PLD1) were studied in the cerebral cortex of rats after freeze injury. Histopathologically, cryoinjury, by exposing cerebral cortex to a prechilled rod for 1 minute, produced consistent pathological lesions, specifically neuronal death, infiltration of macrophages into the center of the cryoinjury, and reactive astrogliosis at the periphery, which caused the lesion site to become encased. Western blot analysis showed that PLD1 expression in the ipsilateral cerebral cortex increased significantly during days 1 to 3 after cryoinjury and declined slightly at post-injury day 7. PLD1 immunoreactivity was very low in the brains of sham-operated control adults. After cryoinjury, there was substantial PLD1 immunostaining of numerous inflammatory cells in the ipsilateral cortex, which were identical to ED1-positive macrophages. In addition, PLD1 immunoreactivity was increased in some neurons and astrocytes at the periphery of the cryoinjury at post-injury days 3 and 7. These findings suggest that cryoinjury by means of prechilled rods induced consistent histopathological changes in the cerebral cortex. In addition, expression of a cell activation signal, PLD1, was upregulated in macrophages and astrocytes in the ipsilateral cerebral cortex after cryoinjury.

**Key words:** Brain injury, Cryoinjury, Macrophage, Phospholipase D1

### Introduction

Traumatic brain injury is related to several pathophysiological events, including direct mechanical damage, intraparenchymal and subarachnoid hemorrhage, breakdown of the blood–brain barrier, excitotoxicity, and ischemia (McIntosh et al., 1998).

Numerous studies have examined cellular responses to brain injury. In two of the most widely employed models of brain injury, namely fluid percussion and controlled cortical impact injury, the cellular responses include neuronal cell death and axon loss (Sato et al., 2001), activation of microglia, macrophage infiltration (Aihara et al., 1995), and astrocyte reactions (Soares et al., 1995; Baldwin and Scheff, 1996). Even though the aforementioned models have many advantages, cryoinjury using a rod that has been chilled in liquid nitrogen is one alternative for the study of brain injury (Sun et al., 2000). However, the pathological changes that characterize this model remain poorly understood, particularly as regards cell activation factors, such as phospholipases, which are activated temporarily in host and inflammatory cells in injured brain.

Phospholipase D (PLD) has recently been recognized as an important signal transduction enzyme. It is involved in the regulation of diverse cellular processes, such as cell proliferation (Boarder, 1994), apoptosis (Yoshimura et al., 1997; Nakashima and Nozawa, 1999), cell differentiation (Min et al., 1999), membrane trafficking, and secretory events (Cockcroft, 1996). Recently, PLD1 and PLD2, two mammalian isoforms of PLD, have been cloned and characterized at the molecular level (Colley et al., 1997; Frohman et al., 1999). Studies of PLD1 have revealed that expression of this molecule is increased in the hippocampus of rats until 2 weeks after birth (Min et al., 2001) and PLD1 signaling has been described in several areas of the brain, including the cerebral cortex (layer V). Although PLD1 appears to be expressed predominantly in neurons, it is also expressed in glial cells, such as presumed astrocytes and tanycytes (Lee et al., 2000a).

PLD1 plays an important role in neuropathological processes, including ischemic brain injury (Lee et al., 2000b), autoimmune encephalomyelitis (Ahn et al., 2001), and spinal cord injury (Jung et al., 2003). Nevertheless, despite the potential importance of PLD in neurodegeneration of the brain in response to brain injury, little is known about the expression and potential

role of PLD1 protein in the cerebral cortex of injured brains. The aim of this study was to examine the expression pattern and possible role of PLD1 in the course of brain damage after cryoinjury.

## Materials and methods

### Animals

Male Sprague–Dawley rats (200–250 g) were used (Daehan Biolink, Cheongju, Korea). All experimental procedures were conducted with the approval of the Ethics Committee of the Catholic University of Korea and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1996).

### Brain injury and tissue sampling

To injure the brain, the skin and the frontal and occipital bones of rats were removed surgically under ethyl ether anesthesia. A cryoinjury was then created by exposure to a cold probe (3-mm-diameter steel rod chilled in liquid nitrogen) for 1 min, as described previously (Sun et al., 2000). Three rats were sacrificed for immunoblot analysis and immunohistochemistry at each of four time points after surgery (days 0 (sham), 1, 3, and 7).

For immunohistochemistry, rats were perfused through the left ventricle with 4% paraformaldehyde buffered with 0.1 M phosphate buffer (PB, pH 7.2). The brain was removed and immersed in the same fixative for 24 h. For the Western blot analysis, the cryoinjured cerebral cortex was dissected free and frozen until used.

### Western blot analysis

The lesioned cerebral cortex was dissected free, homogenized in lysis buffer (20 mM HEPES, pH 7.2; 1% Triton X-100; 1% deoxycholate; 0.1% SDS; 150 mM NaCl; 10 µg/ml leupeptin; 10 µg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride) and then centrifuged. Proteins were resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis (8% SDS-PAGE) and blotted on to a Tropifluor™ polyvinylidene fluoride (PVDF) membrane. The membrane was probed with anti-PLD1 antibody diluted in blocking solution. The antibody used in the present study has been characterized previously (Lee et al., 2000a,b). Immunoreactive bands were visualized using horseradish peroxidase-conjugated anti-rabbit IgG and enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). Densitometric analysis was performed using an Eagle Eye TMII still video system (Stratagene, La Jolla, CA). Measurement of the optical density of bands was repeated five times per animal. The immunoblot was normalized to a beta-tubulin-immunoreactive band. The relative value of the control was arbitrarily defined as 1.

### Immunohistochemistry

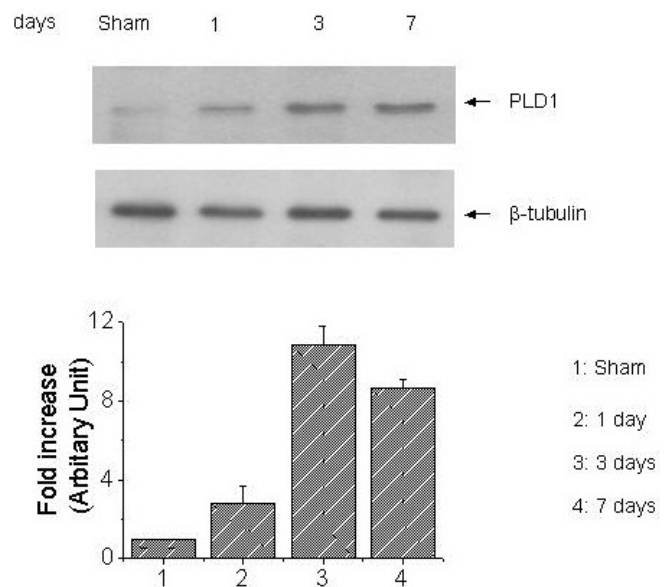
Sections (5 µm) of paraffin-embedded cerebral cortex were deparaffinized and allowed to react with affinity-purified anti-PLD1 antibody. To identify astrocytes and macrophages, rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (1:800) (Dako, Copenhagen, Denmark) and ED1 (anti-rat macrophage antibody; Serotec, London, UK) were used, respectively. Immunoreactivity was visualized with an avidin–biotin peroxidase reaction (Vector Elite, Vector Labs, Burlingame, CA). Peroxidase was developed with diaminobenzidine (DAB; Vector). Sections were counterstained with hematoxylin prior to mounting.

To colocalize PLD1 and glial markers in the same section, PLD1-immunopositive tissue was bleached in 0.3% hydrogen peroxide in distilled water and then reacted with a second primary antibody, either anti-ED1 or anti-GFAP, using the protocol described above with DAB as the chromagen. The colocalization of both antigens in a single cell was readily apparent.

## Results

### Increased expression of PLD1 in cryoinjured cerebral cortex

Western blot analysis revealed that the level of PLD1 expression in the ipsilateral cerebral cortex increased by post-injury day 1 (Fig. 1, lane 2), increased



**Fig. 1.** Western blot analysis of phospholipase D1 (PLD1) protein expression in the cerebral cortex. **Upper panel:** A representative Western blot for PLD1. Lane 1, sham-operated. Lanes 2–4, post-injury days 1, 3, and 7, respectively. **Lower panel:** Bar graph of results of densitometric data analysis (mean±SEM, n=3 rats/group). There is a 3-fold increase at day 1, an 11-fold increase at day 3, and a 9-fold increase at day 7 compared with the control (arbitrarily normalized to 1).

*PLD expression in cryoinjured cerebral cortex in rats*

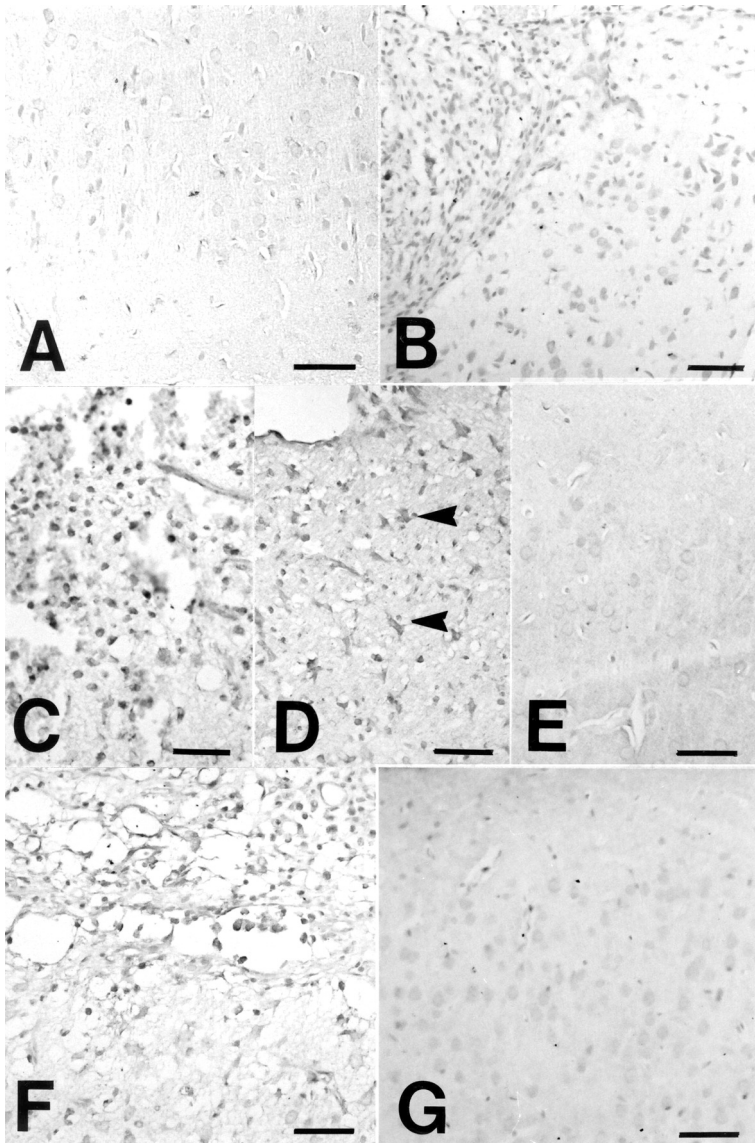
significantly (~9 fold) by day 3 (Fig. 1, lane 3), and decreased slightly by day 7 (Fig. 1, lane 4).

*Immunohistochemistry*

In sham-operated controls, there was no typical PLD1 immunostaining in the cerebral cortex. As shown in a previous study (Lee et al., 2000a), there was little (if any) PLD1 immunoreactivity in adult rats (Fig. 2A). Negative controls (primary antisera omitted) were immunonegative for PLD1 (Fig. 2B). At post-injury day 3, some inflammatory cells in the ipsilateral cortex were positive for PLD1 and appeared to be identical to macrophages (Fig. 2C) and some vessels and astrocytes at the periphery of the cryoinjury had moderately intense

PLD1-immunoreactivity (Fig. 2D). Some glial cells and neurons were PLD1-immunopositive, and there was less intense immunoreactivity in the contralateral cerebral cortex (Fig. 2E) compared to the ipsilateral cortex (Fig. 2D). These observations suggest that cryoinjury induces an increase in the expression of PLD1 in the aforementioned cell types at the periphery of cryoinjured ipsilateral cerebral cortex and may indirectly have the same effect in the contralateral cortex. At post-injury day 7, there was PLD1 immunoreactivity in the ipsilateral cortex (Fig. 2F) and less intense PLD1 immunoreactivity in the contralateral cortex (Fig. 2G).

To examine which cells express PLD1 protein in the cryolesion, PLD1 immunostaining was carried in sections that were adjacent to those used for ED1



**Fig. 2.** Immunostaining for PLD1 in the cerebral cortex of a sham-operated control rat (A) and rats with cryolesions at post-injury days 3 (C-E) and 7 (F, G). In the sham-operated control, there are few PLD1-immunopositive cells in the cerebral cortex (A). There is no immunostaining in negative controls from which primary antisera have been omitted (B). At post-injury day 3, some inflammatory cells in the ipsilateral cortex are positive for PLD1 and are identical to macrophages (C), while some vessels and astrocytes (D) at the periphery of the cryolesion are also PLD1-immunopositive. However, some glial cells and neurons are PLD1-immunopositive in the contralateral cerebral cortex (E), showing that cryolesioning induces an increased expression of PLD1 in the aforementioned cell types in the ipsilateral cortex and indirectly in the contralateral cortex. At post-injury day 7, PLD1 immunoreactivity is observed in the ipsilateral cortex (F) and less intense immunoreactivity is also seen in the contralateral cortex (G). A-F are counterstained with hematoxylin. Scale bar: 50  $\mu$ m.



immunostaining. In the center of the cryoinjury at post-injury day 3, PLD1-immunopositive cells (Fig. 3A, C) were also immunostained with ED1 (Fig. 3B) and/or GFAP (Fig. 4D). At post-injury day 7, the pattern of PLD1 immunostaining was largely similar to that at post-injury day 3 (data not shown).

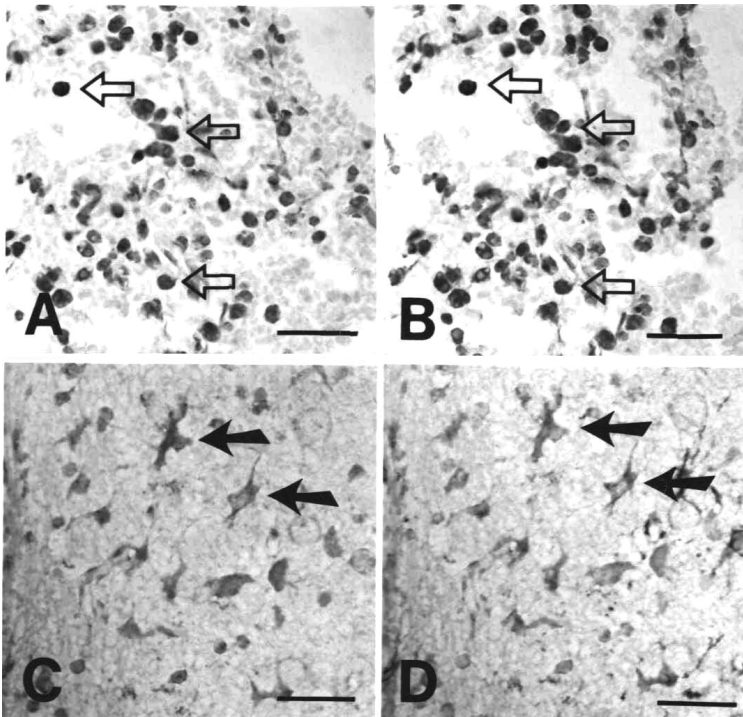
## Discussion

In the present study, we used a unilateral cryoinjury to induce consistent brain damage and subsequent neurodegeneration, as used previously to study rat autoimmune encephalomyelitis (Sun et al., 2000). Based on the histological changes that occurred over the time following the induction of the lesion, this method may be used to study the mechanism of neurodegeneration in cases where controlled cortical impact devices are not available to create brain lesions.

This study is the first to report that PLD1 expression in the cerebral cortex changes after brain injury. Specifically, we found that PLD1 protein expression is upregulated significantly after cryoinjury, suggesting that PLD1 plays a role in the response of tissues to damage or in the process of repair that follows brain injury. The phenotype of upregulated cells that express PLD1 in the brain has been documented in the developing (Min et al., 2001) and adult rat brain (Lee et al., 2000a). There is general agreement that PLD1 plays a role in cell division (Min et al., 1999, 2001) and formation of cannabinoid receptor agonist, anandamide (Di Marzo et al., 1996).

The functional role of macrophages remains unresolved and these cells may play a beneficial or detrimental role in the injured brain (Prewitt et al., 1997; Morganti-Kossmann et al., 2002). Evidence that supports a detrimental role includes the finding that cytotoxic molecules such as tumor necrosis factor alpha are secreted from activated macrophages (Sun et al., 2000); inhibition of these cytotoxic molecules ameliorates brain damage. Contrary to the aforementioned negative effect of macrophages, these cells are one of the most important types of cells that participate in the remodeling of damaged brain. In a previous study, it was suggested that macrophages are required to clear cell debris from lesioned areas, because damaged red blood cells and myelin debris contain inhibitors of axonal growth, such as Nogo (Prewitt et al., 1997). In addition, transplantation of macrophages into spinal cord lesions can ameliorate spinal cord injury in rat models (Rapalino et al., 1998).

Taking everything into consideration, it appears that cryoinjury by means of exposure of the cortex to a prechilled rod for 1 minute produced consistent pathological lesions in the cortex. These were characterized by neuronal death, the appearance of macrophages in the center of the cryolesion, and reactive astrogliosis at the periphery, which caused the lesion site to become encased. In addition, the increased expression of PLD1 protein in lesions following cryoinjury originated mainly from ED1-expressing macrophages and astrocytes. Therefore, PLD would appear to play an important role in the activation of macrophages and



**Fig. 3.** Identification of PLD1-immunopositive cells in the ipsilateral cerebral cortex of cryoinjured rats at post-injury day 3. PLD1-immunopositive cells (open arrows) (A) are also immunopositive for ED1 (anti-rat macrophage antibody; open arrows) (B). At the periphery of the cryoinjury, PLD1-positive process-bearing cells (arrows in C) are also immunopositive for glial fibrillary acidic protein (arrows in D). Immunostaining in A, B, and C, D is in the same tissue section. Sections in A-D are counterstained with hematoxylin. Scale bar: 30  $\mu$ m.

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astrocytes, both of which play important roles in the pathogenesis of brain injury.

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