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Research Report

Lipopolysaccharide-induced protein kinase D activation mediated by interleukin-1β and protein kinase C

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ABSTRACT

Protein kinase D (PKD), a newly described serine/threonine kinase, has been implicated in many signal transduction pathways. The present study was designed to determine whether and how PKD is activated in inflammation. The results demonstrated that lipopolysaccharide (LPS, 30 μg/ml) stimulated PKD and protein kinase C (PKC) phosphorylation in spinal neurons within 0.5 h, and the activation reached a maximum at 3 or 8 h and declined at 12 h. The phosphorylation could be inhibited by the selective inhibitors for PKC (100 nM), mainly for PKCα and PKCβ, suggesting the involvement of the PKC pathway. Particularly, PKCα might be critical for LPS-induced PKD activation since the PKCβ inhibitor (100 nM) observed no effect on the phosphorylation of PKD. Furthermore, the expression of interleukin-1β (IL-1β) was significantly induced by LPS within 0.5 h, and reached a maximum at 8 h. IL-1 receptor antagonist inhibited PKD and PKCs activation induced by LPS at a concentration of 50 nM and achieved maximum at 1000 nM. These results demonstrated for the first time that PKD could be activated by LPS in spinal neurons, might via the IL-1β/PKCα pathway. Additionally, immunostaining showed an increase in number of phosphorylated PKD-immunoreactive cells of adult spinal dorsal horn induced by intraplantar injected carrageenan (2 μg/100 μl), and antisense oligodeoxynucleotide to IL-1 receptor type I (50 μg/10 μl, intrathecal injected) inhibited the PKD activation, suggesting an involvement of IL-1β/PKD pathway in inflammation in adult spinal cord.

1. Introduction

Protein kinase D (PKD), also known as protein kinase Cμ (Valverde et al., 1994), is a newly described serine/threonine protein kinase with unique structural, enzymological and regulatory properties that are different from those of the PKC family members. The most distinct characteristics of PKD are the presence of a catalytic domain distantly related to Ca^{2+}-regulated kinases, a pleckstrin homology domain within the regulatory region, and a highly hydrophobic stretch of amino acids in its N-terminal region (Rozengurt et al., 2005). PKD can be activated by a variety of stimuli including biologically active phorbol esters, growth factors, and T- and B-cell receptor agonists via PKC-dependent pathways (Rozengurt et al., 2005).

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Abbreviations: IL-1β, interleukin-1 β; IL-1Ra, interleukin-1 receptor antagonist; IL-1RI, interleukin-1 receptor type I; i.p., intraperitoneal; i.pl., intraplantar; i.t., intrathecal; LPS, lipopolysaccharide; ODN, oligodeoxynucleotide; PKC, protein kinase C; PKD, protein kinase D

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Its activation appears to involve the phosphorylation of Ser-744 and Ser-748 within the activation loop of the catalytic domain (Iglesias et al., 1998) as well as the autophosphorylation of Ser-916 (Matthews et al., 1999; Rozengurt et al., 2005). PKD has been implicated in the regulation of a variety of cellular functions, including signal transduction, membrane trafficking, protein transport and cell survival, migration, differentiation and proliferation (Baron and Malhotra, 2002; Ernest et al., 2005; Hausser et al., 2005; Rozengurt et al., 2005; Sinnett-Smith et al., 2004; Storz et al., 2005; Wong and Jin, 2005). Previous study found that PKD functioned as a direct modulator of vanilloid receptor type 1 (Wang et al., 2004), implying that it might be involved in the regulation of inflammatory responses. It has been reported that PKD is present in neurons of rat dorsal root ganglion (DRG) and cortex (Cabrera-Poch et al., 2004; Wang et al., 2004). However, whether PKD exists and can be activated in spinal neurons is poorly known.

To investigate the neuronal response to inflammation, lipopolysaccharide (LPS), a component of gram-negative bacteria, has been used to incubate with the neurons (Chalimoniuk et al., 2006; Hellstrom et al., 2005; Jeohn et al., 2002). LPS stimulates the innate immunity and inflammation in diverse eukaryotic species by triggering the expression of proteins including cytokines, adhesive proteins, and enzymes that produce low molecular weight proinflammatory mediators (Ulevitch and Tobias, 1995), and activating several pathways of signal transduction in neurons, involving protein kinase C (PKC), protein kinase A (PKA), interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), and so on (Hellstrom et al., 2005; Hou and Wang, 2001; Jeohn et al., 2002; Lastres-Becker et al., 2006). Nevertheless, whether and how PKD is activated by LPS has rarely been studied.

IL-1β is a proinflammatory cytokine, which plays major roles in immunity and inflammation. Its function can be blocked by IL-1 receptor antagonist (IL-1Ra), a competitive inhibitor binding to IL-1 receptors (Arend, 1993), and antisense oligodeoxynucleotide (ODN) to IL-1 receptor type I (IL-1RI), which could down-regulate the expression of IL-1RI and inhibit the effect of IL-1β (Burch and Mahan, 1991; Gayatri et al., 1997). Evidence shows that PKD phosphorylates p53 in HeLa cells, and the phosphorylation can be inhibited by IL-1Ra (Banda et al., 2005), suggesting a relationship between IL-1β and PKD. However, the effect of IL-1β on PKD activation remains unknown.

Thus, the aim of the present study was to determine whether PKD is involved in the LPS-activated pathways, whether the activation of PKD is mediated by IL-1β and PKC in primary spinal neurons, and whether some of these mechanisms exist in adult spinal cord.

2. Result

2.1. *LPS stimulated PKD activation in primary spinal neurons*

PKD expression and phosphorylation at different time in response to LPS stimulation were observed in order to examine whether LPS induces PKD activation in spinal neurons. It was shown that PKD was present in spinal neurons, whereas the levels of PKD expression did not change during the course of LPS stimulation. Phosphorylation of PKD was determined by using two commercially available phospho-PKD-specific antibodies. One of them recognizes the endogenous levels of PKD only when dually phosphorylated at Ser-744 and Ser-748, and the other recognizes PKD only when phosphorylated at Ser-916. By using these antibodies, we observed that LPS (30 μg/ml) induced PKD phosphorylation within 0.5 h, and the activation reached a maximum at 8 h and declined at 12 h (Fig. 1).

2.2. *LPS stimulated PKCs activation in primary spinal neurons*

To examine whether LPS induces PKCs activation in spinal neurons, PKC phosphorylation at different time in response to LPS stimulation was observed. Phosphorylation of PKC (pan) (detected PKCα, β_I, β_{II}, δ, ε and η isoforms only when phos-

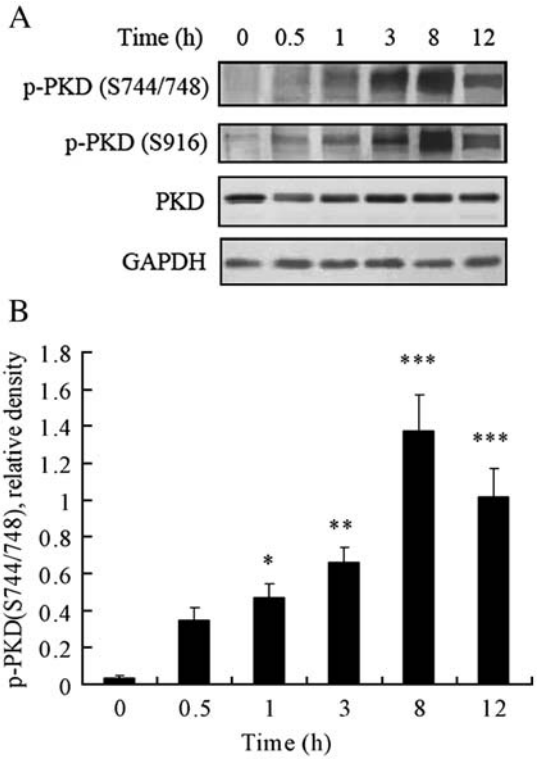

Fig. 1 – LPS time-dependently stimulated PKD activation in spinal neurons. Spinal neurons were incubated with LPS (30 μg/ml) for various times as indicated. PKD activation in cell lysates was analyzed by Western blot analysis using phospho-specific antibodies, which recognize the PKD phosphorylated at Ser-744 and Ser-748 (p-PKD (S744/748)) (first panel) and the PKD phosphorylated at Ser-916 (p-PKD (S916)) (second panel). PKD expression levels were determined by Western blot using a PKD antibody (third panel). PKD expression levels were determined by Western blot using a PKD antibody. (A) Representative immunoblots from three independent experiments were shown. (B) The optical densities of immunoblot bands of protein phosphorylation at Ser-744/Ser-748 for PKD were analyzed by quantitative analysis and expressed as a ratio to that of corresponding GAPDH (n=3). The data were expressed as mean±SEM. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 h group.

phorylated at a carboxy-terminal residue homologous to Ser-660 of PKCβ_{II}), phosphorylation of PKCα/β_{II} (detected PKCα only when phosphorylated at Thr-638 and PKCβ_{II} only when phosphorylated at Thr-641), phosphorylation of PKCγ (detected PKCγ only when phosphorylated at Thr-514), phosphorylation of PKCδ (detected PKCδ only when phosphorylated at Ser-643) and phosphorylation of PKCζ/λ (detected PKCζ only when phosphorylated at Thr-410 and PKCλ only when phosphorylated at Thr-403) were determined by using specific antibodies. It showed that LPS induced PKCs phosphorylation within 0.5 h, and the activation reached a maximum at 3 or 8 h and declined at 12 h (Fig. 2).

2.3. *LPS stimulated PKD activation through a PKC-dependent pathway*

The effect of Ro-32-0432 (a selective cell-permeable PKC inhibitor, principally for PKCα and PKCβ) on PKD activation

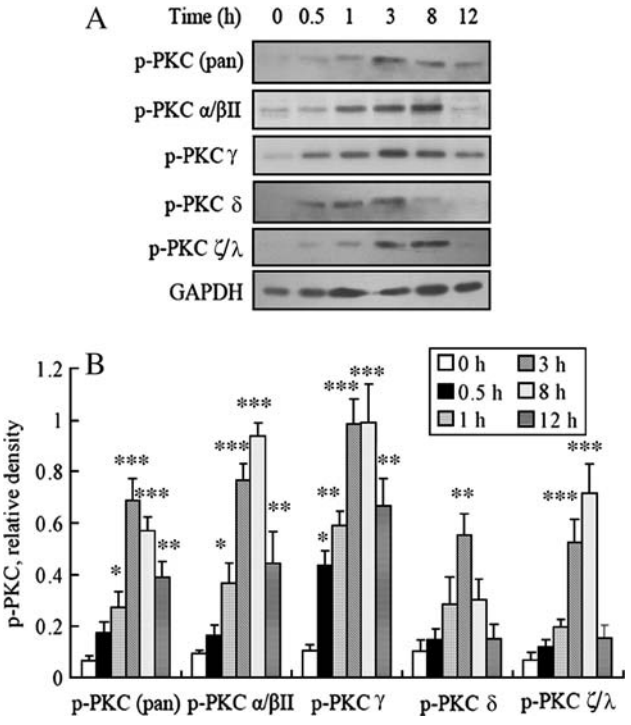

Fig. 2 – LPS time-dependently stimulated PKCs activation. Spinal neurons were incubated with LPS (30 μg/ml) for various times as indicated. PKC activation in cell lysates was analyzed by Western blot analysis using phospho-specific antibodies, which recognize PKCα, β_I, β_{II}, δ, ε and η phosphorylated at Ser-660 (p-PKC (pan)) (first panel), PKCα/β_{II} phosphorylated at Thr-638 and Thr-641 (p-PKCα/β_{II}) (second panel), PKCγ phosphorylated at Ser-514 (p-PKCγ) (third panel), PKCδ phosphorylated at Thr-643 (p-PKCδ) (fourth panel) and PKCζ/λ phosphorylated at Thr-410 and Thr-403 (p-PKCζ/λ) (fifth panel). (A) Representative immunoblots from three independent experiments were shown. (B) The optical densities of immunoblot bands were analyzed by quantitative analysis and expressed as a ratio to that of corresponding GAPDH (n=3). The data were expressed as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. 0 h group.

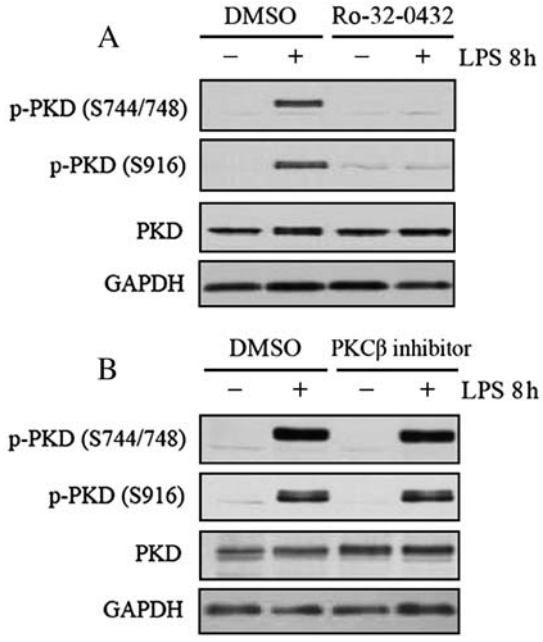

Fig. 3 – PKCs but not PKCβ were involved in LPS-induced PKD activation. The selective PKC inhibitor Ro-32-0432 (100 nM) (A), PKCβ inhibitor (100 nM) (B) or DMSO (Me_2SO, the solvent for Ro-32-0432 and PKCβ inhibitor) was added to the culture medium at 7 h following LPS treatment and incubated for 1 h. PKD activation and expression in cell lysates were analyzed by Western blot as described in the legend for Fig. 1. Representative immunoblots from three independent experiments were shown. p-PKD (S744/748), the PKD phosphorylated at Ser-744 and Ser-748; p-PKD (S916), the PKD phosphorylated at Ser-916.

stimulated by LPS was examined to assess the role of PKC isoforms in LPS-induced PKD activation in spinal neurons. Ro-32-0432 (100 nM) was added to the culture medium at 7 h following LPS treatment and incubated for 1 h. The same amount of DMSO (Me_2SO, the solvent for Ro-32-0432) was added in control cells and also incubated for 1 h. Compared to the DMSO control, Ro-32-0432 almost completely blocked PKD phosphorylation induced by LPS (Fig. 3A), suggesting that PKCs, probably PKCα or PKCβ, were involved in the LPS-induced PKD activation in spinal neurons.

To further investigate which isoform of PKCs participated in PKD activation, the PKCβ inhibitor (3-(1-(3-Imidazol-1-ylpropyl)-1*H*-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione) (100 nM) was added to the culture medium at 7 h following LPS treatment and incubated for 1 h. The result showed no effect on LPS-induced PKD activation (Fig. 3B). These data implied that PKCα might be the principal PKC isoform necessary for LPS-induced PKD activation in spinal neurons.

2.4. *IL-1β mediated LPS-induced PKD activation*

The changes of IL-1β expression induced by LPS in primary spinal neurons were detected by Western blot. The expression of IL-1β was elevated from 0.5 h and reached a maximum at 8 h and declined at 12 h following LPS stimulation (Figs. 4A, B).

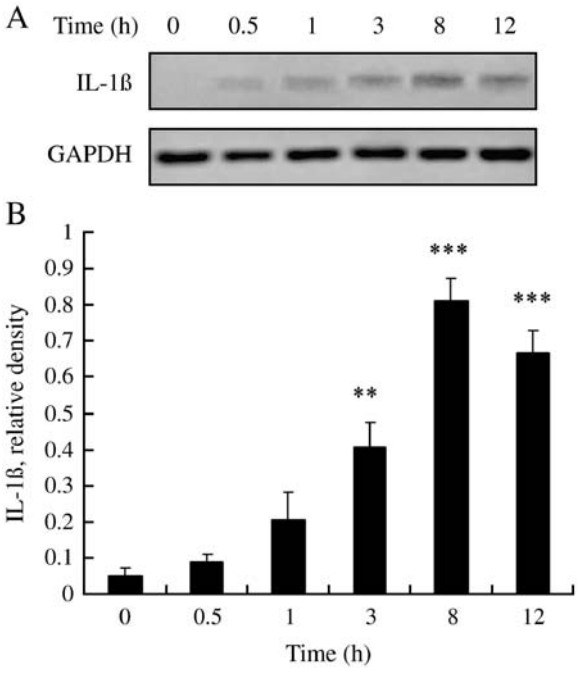

Fig. 4 – LPS time-dependently stimulated IL-1β expression in spinal neurons. Spinal neurons were incubated with LPS (30 μg/ml) for various times as indicated. IL-1β expression in cell lysates was analyzed by Western blot analysis. (A) A protein band of ~20 kDa for IL-1β was detected. Representative immunoblots from three independent experiments were shown. (B) The optical densities of immunoblot bands were analyzed by quantitative analysis and expressed as a ratio to that of corresponding GAPDH ($n=3$). The data were expressed as mean ± SEM. $^{**}P<0.01$ and $^{***}P<0.001$ vs. 0 h group.

The changes of time course were in line with those of PKD activation, suggesting some relationship between IL-1β and PKD in LPS-stimulated spinal neurons.

To further investigate the effects of IL-1β on LPS-induced PKD activation, spinal neurons were incubated with LPS and IL-1Ra together for 8 h. IL-1Ra was observed to inhibit PKD activation in a concentration-dependent manner. It inhibited PKD activation at a concentration of 50 nM and achieved maximum at 1000 nM (Fig. 5). The levels of PKD expression did not change. These data indicated that IL-1β was involved in LPS-induced PKD activation in spinal neurons.

2.5. *IL-1β was involved in LPS-induced PKC activation in spinal neurons*

We had demonstrated that PKCs and IL-1β were both involved in LPS-induced PKD activation. To determine whether there is a relationship between PKCs and IL-1β, the effect of IL-1Ra on PKCs activation induced by LPS was examined. Phosphorylation of PKC (pan), PKCα/β_{II}, PKCγ, PKCδ and PKCζ/λ was determined by using specific antibodies. It showed that IL-1Ra significantly inhibited the phosphorylation of PKC (pan) and PKCα/β_{II} at a concentration of 50 nM and achieved maximum at 1000 nM (Fig. 6). Other isoforms of PKC phosphorylation

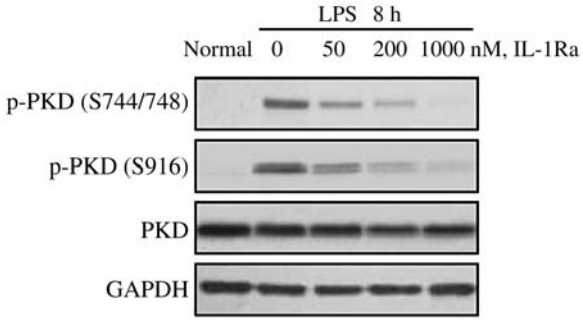

Fig. 5 – IL-1Ra dose-dependently inhibited PKD activation induced by LPS in spinal neurons. Spinal neurons were exposed to LPS (30 μg/ml) and IL-1Ra (with the different concentrations as indicated) for 8 h. PKD activation and expression in cell lysates were analyzed by Western blot as described in the legend for Fig. 1. Representative immunoblots from three independent experiments were shown. p-PKD (S744/748), the PKD phosphorylated at Ser-744 and Ser-748; p-PKD(S916), the PKD phosphorylated at Ser-916.

were not affected. On the basis of these data, we hypothesized that, in primary spinal neurons, IL-1β induced by LPS might trigger PKCs (mainly PKCα/β_{II}) activation and influence PKD activation mostly through PKCα-dependent pathway.

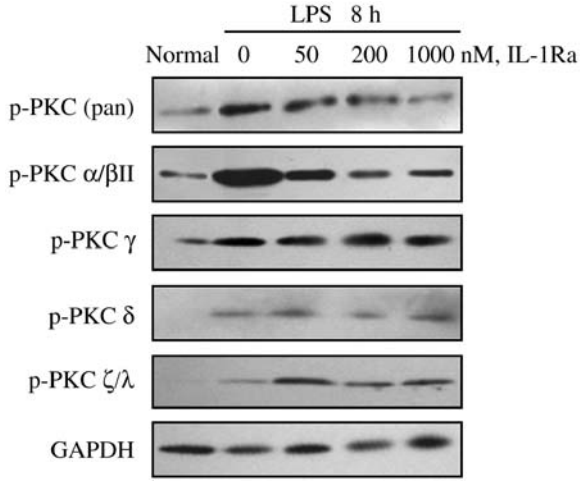

Fig. 6 – IL-1Ra inhibited LPS-induced PKCs activation in spinal neurons. Spinal neurons were exposed to LPS (30 μg/ml) and IL-1Ra (with the different concentrations as indicated) for 8 h. PKCs activation in cell lysates was determined by Western blot analysis using phospho-specific PKC antibodies, which recognize PKCα, β_I, β_{II}, δ, ε and η phosphorylated at Ser-660 (p-PKC (pan)) (first panel), PKCα/β_{II} phosphorylated at Thr-638 and Thr-641 (p-PKCα/β_{II}) (second panel), PKCγ phosphorylated at Ser-514 (p-PKCγ) (third panel), PKCδ phosphorylated at Thr-643 (p-PKCδ) (fourth panel) and PKCζ/λ phosphorylated at Thr-410 and Thr-403 (p-PKCζ/λ) (fifth panel). Representative immunoblots from three independent experiments were shown.

2.6. *IL-1β was involved in peripheral inflammation induced-PKD activation in the spinal cord of adult rats*

To examine whether these mechanisms exist in adult spinal cord, some in vivo studies were performed. Antisense and sense ODN to IL-1RI (50 μg/10 μl) was intrathecal (i.t.) injected respectively, once daily for 3 days. On the 3rd day following i.t. injection, carrageenan (2 μg/100 μl) was intraplantar (i.pl.) injected to produce peripheral inflammation. Three hours later, rats were sacrificed and the expression of phosphorylated PKD in the spinal cord was detected by immunohistochemistry, using the antibodies recognized PKD when phosphorylated at Ser-916. It showed an increase in number of phosphorylated PKD-immunoreactive cells of ipsilateral spinal dorsal horn induced by carrageenan, mainly limited in the superficial layers, and antisense ODN to IL-1RI inhibited the PKD activation (Fig. 7), suggesting an involvement of IL-1β/PKD pathway in inflammation in adult spinal dorsal horn. As a control, sense ODN showed no significant effect (data not shown).

3. Discussion

The major findings of the present study were that LPS activated PKD in primary spinal neurons, might via the IL-1β/PKCα pathway. We observed that PKD could be phosphorylated at Ser-744/Ser-748 and Ser-916 in spinal neurons in response to LPS. Inhibition of the activation of PKCα and PKCβ by the selective inhibitors significantly reduced PKD activation by LPS. PKCα might be critical for mediating PKD activation by LPS since the PKCβ inhibitor observed no effect on the phosphorylation of PKD. Furthermore, IL-1Ra inhibited LPS-induced PKD and PKCs activation. Additionally, in adult spinal dorsal horn, a similar mechanism of IL-1β/PKD pathway in inflammation also existed.

PKD not only is a direct diacylglycerol target but also lies downstream of PKCs in a novel signal transduction pathway implicated in the regulation of multiple fundamental biological processes (Rozengurt et al., 2005). In addition to the biologically active phorbol esters, a variety of regulatory pep-

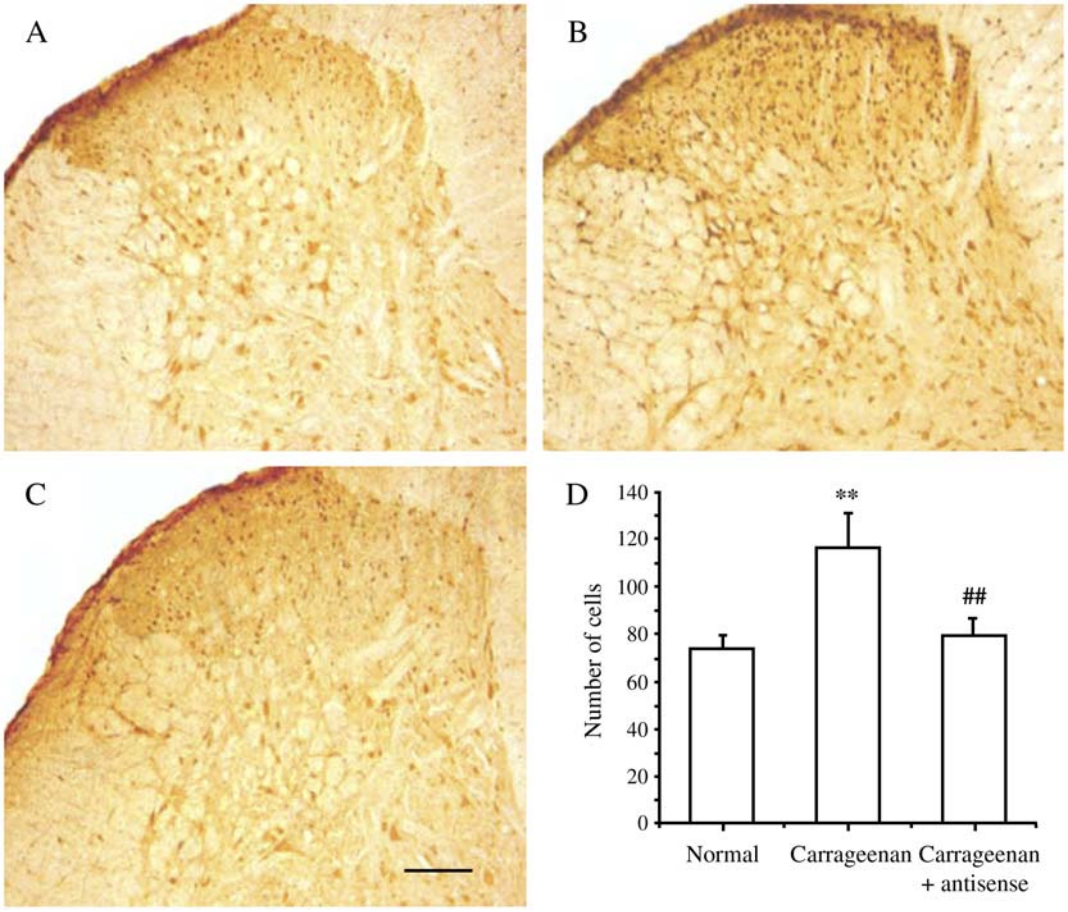

Fig. 7 – Antisense ODN to IL-1RI inhibited the expression of phosphorylated PKD induced by carrageenan in the spinal cord detected by immunohistochemistry. Antisense ODN (50 μg/10 μl) was i.t. injected once daily for 3 days. On the 3rd day following i.t. injection, carrageenan was i.pl. injected. Three hours later, rats were sacrificed and the expression of phosphorylated PKD in the spinal cord was detected using the antibodies recognized PKD when phosphorylated at Ser-916. Images were shown for phosphorylated PKD immunostaining in the ipsilateral spinal dorsal horn of normal group (A), carrageenan group (B) and carrageenan plus antisense group (C). PKD-immunoreactive cells were mainly limited in the superficial layers of the spinal dorsal horn. The results were quantified and demonstrated (D). Data were represented as mean ± SEM ($n=6$). $^{**}P<0.01$ vs. normal group; $^{\#\#}P<0.01$ vs. Carrageenan group. Scale bar = 200 μm.

tides, including G-protein-coupled receptor ligands (e.g. bombesin, vasopressin and thrombin) or growth factors (e.g. epithelial growth factor, vascular endothelial growth factor), induced PKD activation in fibroblast vascular smooth muscle cells and endothelial cells (Rozengurt et al., 2005; Tan et al., 2003; Wong and Jin, 2005; Zugaza et al., 1997). PKD has been found to be present in neurons of nervous system, including the DRG and cortex of rat (Cabrera-Poch et al., 2004; Wang et al., 2004). However, so far, few studies have reported on the expression and regulation of PKD in spinal neurons. In this study, our results showed that PKD could be well expressed in primary spinal neurons, and stimulation of spinal neurons with LPS leads to an activation of PKD. These results suggested that PKD activation is one of the signaling events in spinal neurons in response to LPS stimulation.

PKD can be activated via PKC-dependent and -independent pathways (Cabrera-Poch et al., 2004; Lemonnier et al., 2004; Rozengurt et al., 2005; Tan et al., 2003; Wong and Jin, 2005; Zugaza et al., 1997). In this study, we found that the PKC inhibitors Ro-32-0432 markedly inhibited LPS-induced PKD activation, suggesting that the PKC pathway is involved in LPS-induced PKD activation in spinal neurons. PKC isoforms (principally α, β_I, β_{II}, γ) are expressed in spinal neurons (Akinori, 1998). However, which isoforms of PKC could be activated by LPS in primary spinal neurons had rarely been reported. Herein, we observed that LPS time-dependently stimulated PKCα, β, γ, δ, η and λ activation. Many PKC isoforms, such as PKCα, PKCβ, PKCδ, PKCε, PKCη and PKCθ, can activate PKD (Rozengurt et al., 2005; Tan et al., 2003; Wong and Jin, 2005). To address which isoform of PKC mediating LPS-induced PKD activation in spinal neurons, we applied the approach of exclusion. First, the treatment with Ro-32-0432, which principal inhibited PKCα and PKCβ, blocked LPS-induced PKD activation, suggesting that PKCα or PKCβ was involved in LPS-induced PKD activation. Next, the fact that the PKCβ inhibitor did not affect LPS-induced PKD activation excluded the involvement of PKCβ, implying that LPS-induced PKD activation might be mediated by PKCα in spinal neurons. However, because of lacking a specific PKCα inhibitor, additional studies using dominant negative expression, siRNA knockdown or overexpression of PKCα on LPS-induced PKD activation in spinal neurons would be necessary to further determine the role of PKCα.

The PKC isoforms that mediate PKD activation may vary in response to the specific cellular stimuli in different cell types (Rozengurt et al., 2005; Tan et al., 2003). For example, activation of PKD can occur through activation of PKCδ by thrombin in aortic smooth muscle cells (Tan et al., 2003), PKCε by lipid raft disruption in PC12 cells (Cabrera-Poch et al., 2004), and the constitutively active mutant of PKCη in COS-7 cells (Brandlin et al., 2002), and PKCθ in T cells (Yuan et al., 2002). Similar to our results, Wong and Jin (2005) demonstrated that PKCα-mediated PKD activation was stimulated by vascular endothelial growth factor in endothelial cell. And Li et al. (2004) reported that the role of PKD in neurotensin secretion from BON endocrine cells was mediated by PKCα and PKCδ. PKD appears to be a scaffold protein and binds with some PKC isoforms such as PKCη and PKCθ, participating diverse intracellular signaling pathways (Brandlin et al., 2002; Rykx et al., 2003; Yuan et al., 2002); therefore, it is possible that PKD forms a complex with PKCα mediating LPS-induced immune or inflammatory response in spinal neurons. However, to date, the direct interaction and exact relationship between PKCα and PKD has rarely been reported and requires further investigation.

Furthermore, we found that LPS-induced PKD and PKC activation was inhibited by IL-1Ra in spinal neurons. The major cellular sources of IL-1β production in the central nervous system (CNS) are glial cells, predominantly microglia. However, several studies have provided evidence for the neuronal expression of IL-1β: neurons in the arcuate and preoptic regions of the hypothalamus (Huitinga et al., 2000; Watt and Hobbs, 2000), the CA1 field of the hippocampus (Bhat et al., 1996), the frontal cortex, the cerebellum, the brainstem, the locus coeruleus (Berkenbosch et al., 1992) and the spinal cord (Yang et al., 2004). Previous studies reported that LPS increased IL-1β level in cultured central neurons (Chiou et al., 2006; Hellstrom et al., 2005). Consistent with that, we detected that LPS time-dependently induced IL-1β production in spinal neurons. PKC was found to play a mediating role in the IL-1β signaling pathway (Hou et al., 2003; Obreja et al., 2002). In this study, we observed that IL-1Ra inhibited the phosphorylation of PKCs (particularly PKCα/β_{II}) and PKD induced by LPS in spinal neurons. As mentioned above, LPS-induced PKD activation might be mediated by PKCα. Therefore, we presumed that IL-1Ra inhibited PKD phosphorylation through inhibiting PKCα phosphorylation, suggesting IL-1β signaling induced by LPS via PKCα-mediated downstream activation of PKD.

Additionally, immunostaining was used to examine whether these mechanisms exist in adult spinal cord. Antisense ODN to IL-1RI has been reported to successfully down-regulate the expression of IL-1RI and inhibit the effect of IL-1β (Burch and Mahan, 1991; Gayatri et al., 1997). Our previous study also demonstrated that antisense ODN could inhibit inflammation induced by carrageenan (not published). In the present study, antisense ODN reduced carrageenan-induced phosphorylation of PKD, suggesting an involvement of IL-1β/PKD pathway in inflammation in adult spinal dorsal horn. However, further studies using selective PKC inhibitor are needed to investigate whether PKCα is also involved in the pathway.

In summary, in the present study we found, to our knowledge never been reported, that PKD could be activated by LPS and periphery inflammation in primary spinal neurons and spinal dorsal horn, respectively. We hypothesized an IL-1β/PKCα pathway in LPS-induced PKD activation. Thus, the present study identified PKD as a new component in inflammation-induced intracellular signaling pathway in spinal neurons, and this may implicate PKD in mediating inflammation.

4. Experimental procedures

4.1. *Materials*

Anti-phospho PKD antibodies (p-PKD (S744/748) and p-PKD (S916)), anti-PKD antibody and anti-phospho PKC antibodies (p-PKC (pan), p-PKCα/β_{II}, p-PKCγ, p-PKCδ, p-PKCζ/λ) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-IL-1β antibody and recombinant rat IL-1Ra were from R&D System, Inc. (Minneapolis, MN). LPS and Carrageenan

were from Sigma (Poole, UK). Ro-32-0432 and PKCβ inhibitor (3-(1-(3-Imidazol-1-ylpropyl)-1*H*-indol-3-yl)-4-anilino-1*H*-pyrrole-2, 5-dione) were from Calbiochem (San Diego, CA). Cell culture reagents were obtained from Invitrogen (Paisley, UK).

4.2. *Cell cultures and treatment*

The care and use of animals in these experiments followed the guideline and protocol approved by the Animals Care and Use Committee of Shanghai Medical College, Fudan University. All efforts were made to minimize the number of animals used and their suffering. Primary cultures of spinal cord neurons were prepared from 17-day-old Sprague–Dawley rat embryos. The fetuses were removed from timed-pregnant females immediately after sacrificed by cervical dislocation. Upon removal fetuses were transferred to chilled, sterile Hanks' buffered salt solution and were then killed by decapitation. The entire spinal cord was removed by an anterior approach (Jiang et al., 2006). Cells were dissociated and plated at a density of 10^5 cells per well into 24-well tissue culture plates previously coated with 0.1% poly-D-lysine (Sigma, St. Louis, MO, USA). The cells were maintained in serum-free Neurobasal medium containing 2% B27 supplement (Invitrogen). Cultures were kept at 37 °C in a humidified atmosphere of 5% CO_2/95% air, half of the medium was changed to fresh serum-free medium twice a week. Following 10 days in culture under these conditions, >85% of the cells in culture were neurons as assessed by immunostaining with polyclonal antibodies against 4′,6′-diamino-2-phenylindole (DAPI, Fig. 8), which stained the nuclei of neurons, and the glial fibrillary acidic protein, which stained the astrocytes (data not shown). On the 10th day, lipopolysaccharide (30 μg/ml), Ro-32-0432, PKCβ inhibitor or IL-1Ra was added.

4.3. *Western blot analysis*

Cells were harvested and lysed in a buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 5 mM EGTA, 20 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM $NaVO_3$, 10 mM NaF and proteinase inhibitor mixture. The protein concentrations in the lysates were determined using the Bradford method (Bio-Rad). Equal amounts of protein (30 μg) were subjected to SDS-PAGE and electrotransferred to Immun-Blot PVDF membranes. Membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 2 h at room temperature. Membranes were then probed with a primary antibody against the specific protein, washed four times with 0.2% Tween 20 in TBS and then subjected to a second incubation with rabbit anti-goat or goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:1000; Santa Cruz) in TBS/5% BSA for 1 h at 37 °C. The signal was detected by an enhanced chemiluminescence method (ECL kit, Santa Cruz), and exposed to Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA). The intensity of the bands was captured and analyzed using GeneSnap Image Analysis Software (Syngene, UK). The primary antibodies were polyclonal anti-phospho PKD antibodies (p-PKD (S744/748) and p-PKD (S916)) (1:1000; Cell Signaling Technology), a polyclonal anti-PKD antibody (1:1000; Cell Signaling Technology), polyclonal anti-phospho PKC antibodies (p-PKC (pan), p-PKCα/β_{II}, p-PKCγ, p-PKCδ, p-PKCζ/λ) (1:1000; Cell Signaling Technology) and a polyclonal anti-IL-1β antibody (1:200; R&D System). Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) is a catalytic enzyme, constitutively expressed in almost all tissues at high levels and was used here as a loading control. The non-phosphorylated form of the proteins and GAPDH was done at the same blots, and the phosphorylated forms were on a sister membrane.

4.4. *Intrathecal administration and antisense ODN*

Intrathecal administration was performed on adult male Sprague–Dawley rats (Experimental Animal Center, Shanghai Medical College of Fudan University, China) weighing 200–220 g. Chronically indwelling i.t. catheters were implanted into the subarachnoid space of lumbar enlargement of the rats for ODN administration. An i.t. catheter (PE-10 tube) was inserted through the gap between the L4 and L5 vertebrae and extended to the subarachnoid space of the lumbar enlargement (L4 and L5 segments) under sodium pentobarbital (40 mg/kg) anesthesia by intraperitoneal (i.p.) injection. The catheter was filled with sterile normal saline (approximately 4 μl), and the outer end was plugged. The external end of the tube was passed subdermally and secured to the back of the neck where an

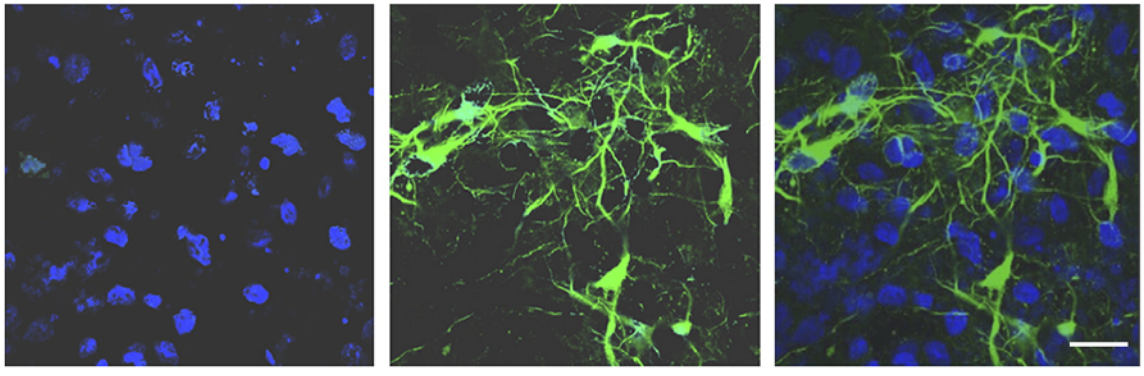

Fig. 8 – Primary spinal neurons were observed on the 10th day of culture by immunostaining. The nuclei of neurons were stained with DAPI (blue), and the skeletons were stained with β-tubulin (green). Scale bar=20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

incision had been made to allow exit. The animals were allowed to recover from the implantation surgery for 3 days prior to any experiment, and monitored daily after surgery for signs of motor deficiency. Those that showed any neurological deficits resulting from the surgical procedure were excluded from the experiments. The location of the distal end of the i.t. catheter was verified at the end of every experiment by injection of Pontamine Sky Blue via the i.t. catheter.

Down-regulation of IL-1RI was ensured via i.t. delivery of antisense ODN specifically complementary to a segment of the sequence of IL-1RI mRNA. The sequence of antisense ODN was: 5′-CACTTTCATATTCTCCAT-3′. The sense ODN to IL-1RI (5′-ATGGAGAATATGAAAGTG-3′) was used as the control. These two kinds of ODNs were fully phosphorothioated, with the antisense ODN sequence, proved to be effective and specific previously (Burch and Mahan, 1991; Gayatri et al., 1997). They were used at a dose of 50 μg dissolved in 10 μl of nuclease-free normal saline per injection per rat, and each i.t. injection of ODN was followed by 5 μl normal saline flush, once daily for 3 days as a pretreatment before carrageenan injection.

4.5. *Periphery inflammation*

On the 3rd day following i.t. injection, periphery inflammation was induced by i.pl. injection of carrageenan (λ-carrageenan, Sigma, 2 μg/100 μl of normal saline (0.9% NaCl)) into unilateral hind paw of non-anesthetized rats. The inflammation, which appeared shortly after injection in the form of redness, edema and hyper-responsiveness to noxious stimuli was limited to the injected paw and lasted about 72 h. The rats were used to perform the experiments at 3 h following the injection of carrageenan, corresponding to the peak inflammatory response (Hargreaves et al., 1988; Pertovaara et al., 1998).

4.6. *Immunohistochemistry*

Rats were given an overdose of urethane (1.5 g/kg, i.p.) and perfused through the ascending aorta with 200 ml of normal saline followed by 300 ml 4% paraformaldehyde in 0.1 M phosphate-buffered (PB, pH 7.4). The L4/5/6 segments of spinal cord were then removed, postfixed in the fixative solution for 4 h at 4 °C and immersed in 30% sucrose in PB for 24–48 h at 4 °C for cryoprotection. Frozen sections (30 μm) were cut and collected in cryoprotectant solution (0.05 M PB, 30% sucrose, 30% ethylene glycol) and then stored at −20 °C until use.

Free-floating tissue sections were processed for phosphorylated PKD protein immunocytochemistry by the Avidin–Biotin method. Sections were removed from storage at −20 °C and placed directly into 0.1 M PB for 10 min. Following three 15 min rinses in 0.01 M PBS, the sections were preincubated for 30 min at room temperature in a blocking solution of 3% normal goat serum in 0.01 M PBS with 0.3% Triton-X 100 (NGST). The sections were then incubated in anti-phosphorylated PKD (S916) polyclonal antibody (1:1000; Cell Signaling Technology) diluted in 1% NGST at 4 °C for 48 h. The incubated sections were washed three times in 1% NGST and incubated in biotinylated goat anti-rabbit immunoglobulin G (lgG) (1:200, Vector Laboratories, Burlingame, CA) for 1 h at room temperature, washed three times in 1% NGST and incubated for 1 h in avidin–biotin–peroxidase complex (1:100, Vector Laboratories) at room temperature. Finally, the sections were washed three times in 0.01 M PBS, and phosphorylated PKD-like immunoreactive product was visualized by catalysis of 3,3-diaminobenzidine (DAB) by horseradish peroxidase in the presence of 0.01% H_2O_2. The sections were then mounted, dehydrated and covered. To test the specificity of the primary antibody, controls were performed, including the substitution of normal rabbit sera for the primary antibody and omission of the primary antibody. None of these controls showed any sign of immunohistochemical reaction. The sections were observed and analyzed using Leica Q500IW image analysis system. For the analysis of the spinal cord segments, the number of cells for phosphorylated PKD immunoreactivity was calculated for each section. For each animal, 10 sections were taken from the L4–L6 spinal cord segments and the mean value was calculated. The investigator responsible for image analysis was blind to the experimental condition of each rat.

4.7. *Statistical analysis*

All data presented in this study were representative of at least three independent experiments and expressed as mean ± SEM and analyzed by SPSS 11.5. An analysis of variance (ANOVA) with repeated measures followed by S-N-K test was used for post hoc analysis for differences between groups. $P<0.05$ was considered significant.

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