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Protein kinase C mediates peroxynitrite toxicity to oligodendrocytes

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ABSTRACT

Peroxyntirite has been suggested to be the potent oxidant causing toxicity to neurons and oligodendrocytes (OLs). Our previous studies have illustrated that intracellular zinc liberation contributes to peroxyntirite toxicity to mature OLs. In this study, we further investigated the signaling pathways involved in this event and identified protein kinase C (PKC) as an important early signaling molecule. We found that a non-selective PKC inhibitor bisindolylmaleimide-1 blocked OL toxicity induced by a peroxyntirite generator SIN-1 and exogenous zinc. The protective effects were due to its inhibition on ERK1/2 phosphorylation and ROS generation. The same phenomenon was also observed in OLs following prolonged treatment with phorbol 12 myristate 13 acetate (PMA), which downregulates the conventional and the novel PKC isoforms (cPKCs and nPKCs). To determine the role of specific PKC isoforms, we found that a specific nPKC inhibitor rottlerin significantly reduced SIN-1- or zinc-induced toxicity, whereas Go6976, a cPKC inhibitor, reduced OL toxicity triggered by zinc, but not by SIN-1 at high concentrations. Rottlerin was more potent than Go6976 to attenuate ERK1/2 phosphorylation and ROS generation induced by SIN-1 or zinc. Surprisingly, zinc only induced phosphorylation of PKC θ , but not PKC δ . Knockdown of PKC θ using lentiviral shRNA attenuated SIN-1- or zinc-induced toxicity. These results suggest that PKC θ might be the major PKC isoform involved in peroxyntirite and zinc toxicity to mature OLs, and provide a rationale for development of specific inhibitors of PKC θ in the treatment of multiple sclerosis and other neurodegenerative diseases, in which peroxyntirite formation plays a pathogenic role.

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Introduction

Oligodendrocytes (OLs) are myelin forming cells in the central nervous system (CNS). They produce myelin sheath to wrap axons and enable the saltatory propagation of action potentials. Destruction of myelin sheath, impediment of remyelination and loss of mature OLs are common features in many neurological diseases including cerebral palsy and multiple sclerosis (Pleasure et al., 2006).

Oxidative stress is one of the important causes for multiple sclerosis (Calabrese et al., 2002). Peroxyntirite, the reaction product of nitric oxide and superoxide, is the most powerful oxidant so far identified (Pacher et al., 2007). It has been suggested that peroxyntirite is the principal toxic species released from reactive microglia causing toxicity to neurons and OLs (Li et al., 2005; Xie et al., 2002). Although it is known that peroxyntirite can cause DNA damage, protein oxidation and lipid peroxidation (Beckman and Koppenol, 1996; Pacher et al., 2007), the cell death mechanisms triggered by peroxyntirite have not been well elucidated. We have previously shown that peroxyntirite toxicity to mature OLs is mediated by intracellular zinc release, activation of extracellular signal-regulated kinase 1/2 (ERK1/2), 12-lipoxygenase (12-LOX) and the formation of

reactive oxygen species (ROS) (Zhang et al., 2006). Since protein kinase C (PKC) is particularly sensitive to redox stress, and is involved in zinc-induced ROS generation (Koh, 2001; Noh et al., 1999), we hypothesize that activation of PKC might be critically involved in the signaling pathways of peroxyntirite toxicity to OLs.

PKC is a family of serine and threonine kinases, which sense the environmental cues and trigger the signaling pathways critical for cell proliferation, migration, differentiation and survival (Giorgi et al., 2010). There are at least 10 PKC isoforms, which are classified into three categories according to the cofactors that are required for the catalytic activity: 1) the conventional or classical PKCs (cPKC: α , β I, β II and γ) are calcium dependent and stimulated by diacylglycerol (DAG); 2) the novel PKCs (nPKC: δ , ϵ , η and θ) are activated by DAG, but are calcium independent; and 3) the atypical PKCs (aPKC: λ , ι and ζ) are insensitive to both calcium and DAG (Newton, 2003). The different isoforms of PKC have been shown to be developmentally regulated and suggested to be involved in the myelin gene expression in OLs (Asotra and Macklin, 1993, 1994; Bhat et al., 1995). However, it is unknown whether activation of the different PKC isoforms can also lead to OL toxicity.

Using primary cultures of mature OLs, we found that the toxicity of OLs induced by SIN-1, a commonly used donor of peroxyntirite, or zinc, is mediated by activation of cPKC and nPKC isoforms. Pharmacological and molecular approaches further indicate that nPKCs, especially PKC θ , may play a predominant role in triggering OL cell death, which is mediated by ERK1/2 phosphorylation and ROS generation.

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Results

The pan PKC inhibitor bisindolylmaleimide 1 protected against SIN-1 or zinc induced toxicity to mature OLs

Activation of PKC has been found to mediate zinc induced ROS generation and neuronal toxicity (Noh et al., 1999). We have previously shown that peroxynitrite toxicity to mature OLs is mediated by intracellular zinc release and the subsequent ERK1/2 phosphorylation and ROS generation (Zhang et al., 2006). Therefore, it is likely that PKC activation also participates in peroxynitrite toxicity to mature OLs. Treatment of mature OLs with SIN-1 (0.5 mM), a peroxynitrite generator, or ZnCl₂ (100 μM) for 30 min caused a significant increase in PKC activity, and the increase was completely blocked by the pan PKC inhibitor, bisindolylmaleimide-1 (Bis-1, 10 μM) (Fig. 2A). Bis-1 dose-dependently attenuated SIN-1 or zinc induced toxicity to mature OLs. The EC₅₀ for Bis-1 in reducing SIN-1 or zinc toxicity is about 2 μM. Bis-1 at 10 μM completely blocked OL toxicity induced by SIN-1 and ZnCl₂ (Figs. 2B and C).

Fig. 1. The purity of cultures of mature OLs. A, Following 2 weeks incubation in BDM plus T3 and CNTF culture medium, cells were fixed with 4% paraformaldehyde and immunostained with antibodies against MBP and GFAP, markers for mature OLs and astrocytes, respectively. Few GFAP positive astrocytes (AS; green labeled cells shown with arrows) were present in cultures of mature OLs (red). Nuclei were stained with DAPI (blue). B, Isolectin, a selective marker for microglia (MG; green staining cells shown with arrows), was used to detect the contamination of MG in cultures of mature OLs (red). Nuclei were stained with DAPI (blue).

Fig. 2. The pan PKC inhibitor Bis-1 blocked PKC activation and mature OL toxicity induced by SIN-1 and ZnCl₂. A, Mature OLs were exposed to a peroxynitrite donor SIN-1 (0.5 mM) or ZnCl₂ (100 μM) in the absence or presence of Bis-1 for 30 min, and then lysed for assaying the PKC activity. **, $p < 0.01$ and ***, $p < 0.001$ were obtained when the SIN-1 and the zinc treated groups were compared with the control group. ##, $p < 0.01$ was obtained when the Bis-1 treated groups were compared with SIN-1 alone and ZnCl₂ alone groups. A representative experiment of three that were performed is shown. B, OLs were exposed to SIN-1 (0.5 mM) in the absence or presence of Bis-1 for 2 h, and the toxicity was assessed at 24 h. Bis-1 dose-dependently protected against SIN-1 induced toxicity. *, $p < 0.05$ and **, $p < 0.01$ were obtained when the Bis-1 treated SIN-1 groups were compared with the SIN-1 alone group. A representative experiment of six that were performed is shown. C, OLs were exposed to ZnCl₂ (100 μM) in the absence or presence of Bis-1 for 2 h, and the toxicity was assessed at 24 h. Bis-1 dose-dependently protected against ZnCl₂ induced toxicity. *, $p < 0.05$ and ***, $p < 0.001$ were obtained when the Bis-1 treated ZnCl₂ groups were compared with the zinc alone group. A representative experiment of six that were performed is shown.

Bis-1 attenuated SIN-1 or ZnCl₂ induced ERK1/2 phosphorylation and ROS generation

To determine whether the protective effect of the pan PKC inhibitor is due to its inhibition of ERK1/2 phosphorylation, OLs were treated with SIN-1 or ZnCl₂ for 60 and 120 min, and the effect of Bis-1 on ERK1/2 phosphorylation was assessed by western blot analysis. SIN-1 (0.5 mM) induced a significant ERK1/2 phosphorylation at 2 h, which was almost completely blocked by Bis-1 at 10 μM (Fig. 3A). Similarly, ZnCl₂ (100 μM) induced ERK1/2 phosphorylation was also attenuated by Bis-1 (Fig. 3B).

Fig. 3. Bis-1 attenuated SIN-1 and ZnCl₂ induced ERK1/2 phosphorylation and ROS generation in OLS. A–B, OLS were treated with SIN-1 (0.5 mM) or ZnCl₂ (100 μM) in the absence or presence of Bis-1 for 60 min and 120 min, and then lysed for western blot analysis. The phosphorylation of ERK1/2 induced by SIN-1 (A) or ZnCl₂ (B) was attenuated by Bis-1 at 10 μM. The expression of ERK1/2 was unaltered in all the treatment conditions, and β-actin was used as a loading control. A representative experiment of three that were performed is shown. C–D, OLS were treated with SIN-1 (0.5 mM) or ZnCl₂ (100 μM) in the absence or presence of Bis-1 for 2 h, and then exposed to DCF for 30 min. ROS generation induced by SIN-1 (C) or ZnCl₂ (D), as indicated by the fold increase of DCF fluorescence intensity (F.I.) was significantly attenuated by Bis-1 at various concentrations. ***, $p < 0.001$ was obtained when the Bis-1 treated SIN-1 (C) and ZnCl₂ groups (D) were compared to the untreated SIN-1 (C) and ZnCl₂ groups (D), respectively. A representative experiment of six that were performed is shown.

Quantitation of the western blots by densitometry showed that the ratio of the phosphorylated versus the total ERK1/2 at 2 h following ZnCl₂ treatment was increased up to 6-fold, when compared to the early time points. In the presence of Bis-1, it was reduced to $40 \pm 15\%$ of the levels in zinc alone treated group ($n = 3$, $p < 0.01$). We next examined the effect of Bis-1 on SIN-1 or zinc induced ROS generation by quantitative analysis of DCF fluorescence. SIN-1 (0.5 mM) caused a 15-fold increase of ROS generation, as indicated by the enhanced DCF fluorescence intensity, and the ROS generation was dose-dependently reduced by Bis-1 treatment (Fig. 3C). Similarly, ZnCl₂ (100 μM) also dose-dependently induced ROS generation, which was almost completely blocked by Bis-1 at each of the three concentrations (1, 3 and 10 μM) (Fig. 3D).

Expression of various PKC isoforms in mature OLS and their downregulation by prolonged PMA treatment

To determine which PKC isoforms are expressed in mature OLS, we used PKC isoform specific primers and isoform selective antibodies to assess the mRNA and the protein levels of the various PKC isoforms. Reverse transcriptase PCR revealed that all the PKC isoforms, including the cPKCs (α , β and γ), the nPKCs (η , ϵ , δ and θ), and the aPKCs (ι/λ and ζ), are expressed in mature OLS, with a particularly high expression of PKC β (Fig. 4A). Similar to the findings by Asotra and Macklin (1994), we found that the conventional PKC α and PKC β are highly expressed in mature OLS, whereas no expression of PKC γ was detected by western blot (Fig. 4B). Among the novel PKC isoforms, PKC θ and PKC ϵ were highly expressed, the expression of PKC δ was intermediate and the PKC η expression was undetectable (Fig. 4B). The expression of the atypical PKC ι , PKC λ and PKC ζ was also found in mature OLS (Fig. 4B). It is known that prolonged treatment

with PMA can cause downregulation of several PKC isoforms by ubiquitination and proteasomal degradation (Leithe et al., 2003). After 24 h pretreatment with PMA, both the cPKCs (α and β) and the nPKCs (θ , ϵ and δ) were downregulated, whereas the expression of aPKCs (ι/λ and ζ) was not affected (Fig. 4B). This result is consistent with the fact that PMA binds to the conventional and the novel PKCs, but not to the atypical PKCs (Steinberg, 2008).

Prolonged treatment of OLS with PMA attenuated SIN-1 or zinc induced toxicity, ERK1/2 phosphorylation and ROS generation

At 24 h after PMA pretreatment, OLS were found to be completely resistant to SIN-1 (0.5 mM) or zinc (100 μM) induced toxicity (Fig. 5A), suggesting that activation of cPKC and nPKC, but not aPKC, is involved in the toxicity of OLS. Consistent with the results obtained by using Bis-1 (Fig. 3), SIN-1 and zinc induced ERK1/2 phosphorylation was also dramatically reduced by PMA pretreatment (Figs. 5B and C). OLS pretreated with PMA for 24 h partially reduced ROS generation induced by SIN-1 (Fig. 5D), whereas the ROS generation induced by zinc was almost completely blocked (Fig. 5E). These results suggest that both zinc dependent and independent mechanisms are involved in SIN-1 induced ROS generation in mature OLS.

Effects of selective inhibition of the different PKC isoforms on OL toxicity induced by SIN-1 and zinc

Considering the fact that Bis-1, as well as the prolonged treatment with PMA, can reduce the activity of both cPKCs and nPKCs, it is unclear how cPKCs and nPKCs can differentially contribute to SIN-1 or zinc induced toxicity to OLS. Go6976 and rottlerin are the commonly

Fig. 4. Expression of various PKC isoforms in mature OLs. A, RNA samples isolated from OLs were analyzed by reverse transcriptase PCR using the primer pairs (see Table 1) specific for rat PKC α , β , γ , η , ϵ , δ , θ , λ and ζ . The products were run on a 1% agarose gel impregnated with ethidium bromide. The bands were visualized under UV light. A representative experiment of three that were performed is shown. B, Mature OLs were treated with PMA (100 nM) for 24 h and then lysed to detect the expression of various PKC isoforms. The isoform specific antibodies were used to compare the expression of each PKC specific isoform with and without PMA prolonged treatment. β -actin was used as a loading control for lysates from control (CON) and PMA pretreated cells. A representative experiment of three that were performed is shown.

used cPKC and nPKC inhibitors, respectively (Goekjian and Jirousek, 1999; Gschwendt et al., 1994). Go6976 at 3 μ M significantly attenuated OL toxicity induced by SIN-1 at 0.5 mM, but not at 1 mM (Fig. 6A). Rottlerin at 3 μ M attenuated OL toxicity induced by SIN-1 at both concentrations (Fig. 6A). Go6976 and rottlerin significantly attenuated zinc toxicity to mature OLs (Fig. 6B). These results suggest that both the conventional and the novel PKC isoforms contribute to the toxicity of OLs induced by exogenous zinc. However, the involvement of PKCs in SIN-1 induced toxicity to OLs appears to be dependent on the concentrations of SIN-1 used. At relatively low concentration, SIN-1 toxicity is mediated by both cPKC and nPKC isoforms, whereas at high concentration, the toxicity of SIN-1 seems to be mediated by activation of the novel PKCs only.

Effects of Go6976 and rottlerin on SIN-1 and zinc induced ERK1/2 phosphorylation and ROS generation

Because both Go6976 and rottlerin were shown to protect against SIN-1/ZnCl₂ induced toxicity to mature OLs, we speculated that these inhibitors would attenuate ERK1/2 phosphorylation and ROS generation. Similar to the effect of Bis-1 on the phosphorylation of ERK1/2 induced by SIN-1 and zinc (Figs. 3A and B), rottlerin (3 μ M) and Go6976

(3 μ M) also reduced ERK1/2 phosphorylation in OLs induced by SIN-1 and ZnCl₂ (Fig. 7A). Notably, a better inhibition was provided by rottlerin than Go6976. Rottlerin also showed a greater inhibitory effect than Go6976 on SIN-1/zinc induced ROS generation (Figs. 7B–E). Go6976 at 1 and 3 μ M significantly attenuated ROS generation induced by SIN-1 at 0.5 mM, but not at 1 mM (Fig. 7B). Rottlerin at 1 and 3 μ M reduced ROS generation induced by SIN-1 at both concentrations (Fig. 7C). Similarly, Go6976 from 0.1 to 3 μ M reduced ROS generation by 50%, whereas rottlerin dose-dependently attenuated ROS generation and a complete inhibition was obtained by rottlerin at 3 μ M (Figs. 7D and E). These results suggest that nPKCs might be the major PKC isoforms involved in SIN-1 and zinc toxicity to OLs.

Downregulation of PKC θ reduced OL toxicity induced by zinc and SIN-1

Although rottlerin is commonly used as a selective inhibitor for PKC δ (Gschwendt et al., 1994), the high sequence homology between PKC δ and PKC θ suggests that PKC θ may be also inhibited by rottlerin (Baier and Wagner, 2009; Boschelli, 2009). It has been documented that PKC activity is under control of its phosphorylation (Newton, 2010; Parekh et al., 2000), therefore, we examined whether zinc can affect the phosphorylation of PKC θ and PKC δ in mature OLs. At 2 h after zinc treatment, there is a 3-fold increase of PKC θ phosphorylation in mature OLs. The phosphorylation was completely blocked by rottlerin (3 μ M), but not affected by Go6976 (3 μ M) (Fig. 8A). Bis-1 (10 μ M) slightly attenuated ERK1/2 phosphorylation. Surprisingly, we found that the phosphorylation of PKC δ was not increased at 2 h following zinc treatment (Fig. 8A). There was no difference in the ratio of the phosphorylated and the total PKC δ in all the treatment conditions (n=3). This result suggests that PKC θ , but not PKC δ , is activated by zinc treatment. To further test the role of PKC θ in the signaling pathways of OL cell death, we used RNA interference to specifically downregulate the expression of PKC θ in mature OLs. Infection of OLs with shPKC θ lentiviral particles for 4 days caused a dramatic downregulation of PKC θ , when compared to the non-infected and the GFP shRNA lentiviral particle infected controls (Fig. 8B). Downregulation of PKC θ significantly reduced zinc and SIN-1 induced toxicity to mature OLs (Figs. 8C and D). Although PKC ϵ is also expressed in mature OLs, the involvement of PKC ϵ in the cell death pathways of OLs is likely to be excluded, because the IC₅₀ of rottlerin for PKC ϵ is up to 80–100 μ M (Gschwendt et al., 1994). Taken together, these results suggest that PKC θ might be the major nPKC isoform involved in the OL toxicity induced by SIN-1/zinc.

Discussion

It is well documented that PKCs are particularly sensitive to redox modification and the altered PKC activity can regulate diverse cellular functions, including proliferation, differentiation, survival and death (Giorgi et al., 2010). Although numerous studies have suggested that various PKC isoforms are involved in neuronal survival and death (Aras et al., 2009; Miller et al., 2009; Nelson and Alkon, 2009), the role of PKCs in oxidative stress induced toxicity to OLs has not been elucidated. In this study, we found that PKCs are important signaling proteins involved in the cell death pathways triggered by the potent oxidant, peroxynitrite. Consistent with our previous findings that peroxynitrite toxicity to mature OLs is partially mediated by intracellular zinc liberation (Zhang et al., 2006), the cell death induced by both SIN-1, a donor of peroxynitrite, and zinc is triggered by activation of cPKC and nPKC isoforms. However, the involvement of different PKC isoforms in SIN-1 induced toxicity to OLs is dependent on the concentrations of SIN-1 used. At high concentration, the toxicity of SIN-1 seems to be mediated by the novel PKCs, but not by the conventional PKCs. PKC θ is likely the major PKC isoform contributing to peroxynitrite-/zinc-induced toxicity to OLs.

Fig. 5. Prolonged treatment with PMA attenuated OL toxicity, ERK1/2 phosphorylation and ROS generation induced by SIN-1 and zinc. **A**, Following 24 h pretreatment with PMA (100 nM), OLs were exposed to SIN-1 and ZnCl₂ for 2 h, and the toxicity was assessed at 20–24 h. Prolonged treatment with PMA significantly blocked SIN-1 or ZnCl₂ induced toxicity to OLs (***, $p < 0.001$). **B**, Following 24 h pretreatment with PMA, OLs were exposed to SIN-1 (0.5 mM) for 60 min and 90 min, and then lysed for western blot analysis. PMA prolonged treatment attenuated SIN-1 induced phosphorylation of ERK1/2. A representative experiment of three that were performed is shown. **C**, Following 24 h pretreatment with PMA, OLs were exposed to ZnCl₂ (200 μM) for 60 min and 120 min, and then lysed for western blot analysis. PMA prolonged treatment attenuated ZnCl₂ induced phosphorylation of ERK1/2. A representative experiment of three that were performed is shown. **D–E**, OLs were pretreated with PMA for 24 h, and then exposed to SIN-1 or ZnCl₂ for 2 h. After washing with EBSS, cells were exposed to DCF for 30 min. ROS generation induced by SIN-1 (**D**) or ZnCl₂ (**E**) was significantly attenuated by prolonged PMA treatment (*, $p < 0.05$ and ***, $p < 0.001$). A representative experiment of six that were performed is shown.

The PKC family proteins are composed of an N-terminal regulatory domain and a C-terminal catalytic domain linked by a flexible hinge region (Stabel and Parker, 1991). The C-terminal catalytic domain is highly conserved among all the PKC isoforms, whereas the N-terminal regulatory domain determines the specificity of the various PKC isoforms. The C1 and C2 domains in the N-terminal region of the classical PKCs bind to DAG and calcium, respectively. The novel PKCs lack the C2 homologous domain, and the C2 and part of the C1 domains are absent in the atypical PKCs. Because of these structural differences, the novel PKCs are known to be calcium independent, and the atypical PKCs are insensitive to both DAG and calcium (Giorgi et al., 2010). Using the PKC isoform specific antibodies, we found that the classical PKC α and PKC β , the novel PKC θ , PKC δ and PKC ϵ , and the atypical PKC ζ and PKC λ are expressed in mature OLs. Prolonged

treatment with PMA, a commonly used analog of DAG, downregulates PKC α , β , θ , δ and ϵ , but not PKC ζ and λ . Because the pan PKC inhibitor and the prolonged PMA treatment completely prevent mature OLs from SIN-1 or zinc induced toxicity, we speculated that activation of the classical PKCs (PKC α and PKC β) and the novel PKCs (PKC θ , PKC δ and PKC ϵ) contributes to the toxicity of OLs induced by peroxynitrite or zinc.

It is known that both the N-terminal and the C-terminal regions of PKCs can be redox regulated (Gopalakrishna and Jaken, 2000). Oxidation of the cysteine-rich zinc finger motifs in the N-terminal domain can liberate free zinc and activate the kinases. However, at high concentration, oxidative agents can directly react with the C-terminal catalytic region and cause inactivation of the kinase (Giorgi et al., 2010). Our results showed that the inhibitors of both cPKCs and

Fig. 6. Go6976 and rottlerin attenuated SIN-1 and zinc induced toxicity to OLs. **A.** Mature OLs were exposed to SIN-1 in the presence of Go6976 or rottlerin for 2 h, and the toxicity was assessed at 20–24 h. Go6976 attenuated OL toxicity induced by SIN-1 at 0.5 mM, but not at 1 mM. Rottlerin reduced OL toxicity induced by SIN-1 at both concentrations. **, $p < 0.01$ and ***, $p < 0.001$ were obtained when the Go6976 or the rottlerin treated SIN-1 groups were compared with the respective SIN-1 alone groups. **B.** Mature OLs were exposed to ZnCl₂ in the presence of Go6976 or rottlerin for 2 h, and the toxicity was assessed at 20–24 h. ***, $p < 0.001$ was obtained when the Go6976 or the rottlerin treated zinc groups were compared with the zinc alone groups. A representative experiment of six that were performed is shown.

nPKCs attenuated OL toxicity induced by SIN-1 at 0.5 mM, but only the inhibitor of nPKCs, but not the inhibitor of cPKCs, reduced SIN-1 induced toxicity at higher concentrations. SIN-1 or peroxynitrite induced PKC activation may occur simultaneously with zinc liberation from PKC, or is caused by zinc released from mitochondria and the other zinc binding proteins, such as metallothioneins and zinc finger proteins (Hao and Maret, 2005; Maret, 2008; Zhang et al., 2007). Recently, an elegant study has demonstrated that mutations in a zinc finger protein ZFP191 can disrupt the myelinating function of differentiated OLs (Howng et al., 2010). We speculate that the release of zinc from ZFP191 may inhibit its function and reduce the expression of an array of myelin-related genes. To support the hypothesis that zinc can directly activate PKC in OLs, we found exogenous zinc can also cause PKC activation, and the toxicity of zinc is blocked by the PKC inhibitors. Inhibition or degradation of PKCs by PMA attenuates SIN-1 or zinc induced ERK1/2 phosphorylation and ROS generation, suggesting PKCs are the early signaling molecules in the cell death pathways. It is unclear how PKCs are activated by zinc. The fact that exogenous zinc induced toxicity can be blocked by both the classical and the novel PKC inhibitors, suggesting that zinc entry may also affect the homeostasis of the intracellular calcium and the membrane phospholipids, although the generation of ROS may in turn activate PKCs and form a positive feedback loop, which may occur in the absence of the cofactors (Giorgi et al., 2010).

It is interesting to note that the OL toxicity induced by SIN-1 at high concentrations seems to be mediated by activation of the novel PKC isoforms, but not the classical PKCs. Among the novel PKC isoforms, we found that PKC θ , PKC δ and PKC ϵ are depleted by the prolonged PMA treatment and the expression of PKC η is undetectable

in mature OLs. These results suggest that the novel PKC isoforms involved in SIN-1 or peroxynitrite toxicity to mature OLs might be either the individual or the combination of PKC θ , PKC δ and PKC ϵ . The low affinity of rottlerin to PKC ϵ (80–100 μ M) (Gschwendt et al., 1994) suggests that it is unlikely that this novel PKC isoform is involved in peroxynitrite toxicity to mature OLs. Moreover, activation of PKC ϵ is generally thought to be protective against oxidative stress induced injury (Barnett et al., 2007; Shirai et al., 2008). Conversely, PKC δ has been shown to cause cell death in a variety of oxidative and pathological conditions (Murriel and Mochly-Rosen, 2003). Hydrogen peroxide (H₂O₂) and depletion of glutathione (GSH), the endogenous antioxidant, have been shown to activate PKC δ and induce cell death in culture (Domenicotti et al., 2003; Majumder et al., 2001). Activation of PKC δ is also found to trigger apoptotic cell death in the animal models of ischemia, Parkinson's disease, Alzheimer's disease and several other neurological diseases (Kanthasamy et al., 2003). It is demonstrated that activation of PKC δ occurs via its cleavage by caspase-3 at the hinge region between the regulatory and the catalytic domains resulting in the generation of a persistently active 41-kDa catalytic fragment (Kanthasamy et al., 2003). Therefore, PKC δ is likely not activated and involved in a more aggressive, necrotic cell death. In this study, we found that zinc does not induce PKC δ phosphorylation, consistent with our previous findings that the cell death of mature OLs induced by peroxynitrite and zinc is possibly non-apoptotic (Zhang and Rosenberg, 2004; Zhang et al., 2006). This result indicates that PKC δ may not be involved in peroxynitrite and zinc induced toxicity to mature OLs.

PKC θ is structurally the closest PKC isoform compared to PKC δ , the catalytic domains of PKC θ and PKC δ are 76% identical (Boschelli, 2009). Although PKC θ is mainly expressed in lymphocytes, skeletal muscle, and platelets (Hayashi and Altman, 2007), we found that the levels of the mRNA and the protein expression of PKC θ in mature OLs are compatible to those of PKC δ and PKC ϵ . Our results showed that zinc induced a significant phosphorylation of PKC θ , which was almost completely blocked by rottlerin. Surprisingly, PKC δ is not phosphorylated by zinc, again indicating that it might not be involved in zinc induced toxicity to mature OLs. Knockdown of PKC θ renders OLs resistant to zinc and SIN-1 induced toxicity, suggesting activation of PKC θ is involved in the cell death pathway. It has been reported that mouse with targeted gene deletion of PKC θ is resistant to the induction of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (Salek-Ardakani et al., 2005; Tan et al., 2006). Although inhibition of lymphocytes proliferation and infiltration to the CNS in PKC θ knockout mice is attributable to the inability of EAE induction (Salek-Ardakani et al., 2005; Tan et al., 2006), lack of PKC θ activation in mature OLs may also contribute significantly to the protective effects against oxidative stress induced injury to mature OLs. This result suggests that selective PKC θ inhibitors can be used as therapeutic agents to prevent OL toxicity in multiple sclerosis, traumatic brain and spinal cord injury and the other neurological diseases in which peroxynitrite formation may play an important role.

Experimental methods

Materials

SIN-1 was obtained from Cayman Chemical Co. (Ann Arbor, MI). Go6976 and rottlerin were obtained from Tocris Bioscience (Missouri, USA). 2',7'-dichlorofluorescein diacetate (DCF) was purchased from Molecular Probes, Inc. (Eugene, OR). All the culture materials were purchased from Gibco Life Technologies (Grand Island, NY). Platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and ciliary neurotrophic factor (CNTF) were from Peprotech (Princeton, NJ). All other reagents were obtained from Sigma (St. Louis, MO).

Fig. 7. Go6976 and rottlerin attenuated SIN-1 and zinc induced ERK1/2 phosphorylation and ROS generation in OLs. **A**, OLs were treated with SIN-1 or ZnCl₂ (Zn) in the presence of Go6976 or rottlerin for 2 h, and then lysed for western blot. Rottlerin has a better inhibitory effect than Go6976 on ERK1/2 phosphorylation induced by SIN-1 or ZnCl₂. A representative experiment of six that were performed is shown. **B–C**, Mature OLs were exposed to SIN-1 in the presence of Go6976 (**B**) or rottlerin (**C**) for 2 h, and then exposed to DCF for 30 min. ROS generation was significantly attenuated by Go6976 and rottlerin. *, $p < 0.05$ and **, $p < 0.01$ were obtained when the Go6976 (1 μM and 3 μM) and the rottlerin (1 and 3 μM) treated groups were compared with the SIN-1 alone groups. A representative experiment of six that were performed is shown. **D–E**, Mature OLs were exposed to ZnCl₂ in the presence of Go6976 (**D**) or rottlerin (**E**) for 2 h, and then exposed to DCF for 30 min. ROS generation was significantly attenuated by Go6976 and rottlerin. **, $p < 0.01$ and ***, $p < 0.001$ were obtained when the Go6976 (0.1, 1 and 3 μM) and the rottlerin (0.1, 1 and 3 μM) treated groups were compared with the zinc alone groups. A representative experiment of six that were performed is shown.

Oligodendrocyte culture

Primary cultures of OLs were prepared by shaking off the progenitor cells from mixed glial cell cultures as previously described (Wang et al., 2004; Zhang et al., 2006). Briefly, mixed primary glial cultures were prepared from 2 to 3 day-old Sprague–Dawley rats by dissociation of the brains after they were dissected from the pups. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin in poly-lysine coated 75 cm² flasks incubated in 95% air/5% CO₂, at 37 °C. The media were changed 3 times per week until the cells were confluent (7–10 days). The flasks were then shaken for 1 h on an orbital shaker (200 rpm) at 37 °C to remove microglia. They were then changed to new media and shaken

overnight. The OL progenitor cells were detached from the astrocyte layer and were resuspended and seeded onto poly-ornithine coated plates [96-well (2×10^5 cells/plate), 24-well (4×10^5 cells/plate)] in a basal chemically-defined medium (BDM) [DMEM with 1 mg/ml bovine serum albumin (BSA), 50 μg/ml apo-transferrin, 5 μg/ml insulin, 30 nM sodium selenite, 10 nM biotin, 10 nM hydrocortisone] plus 10 ng/ml of both PDGF and bFGF. The cells were maintained in BDM and the medium was half-changed 3 times per week. For culturing mature OLs, OLs at day 7 were changed to BDM plus 3,3',5-triiodo-L-thyronine (T₃) (15 nM) and CNTF (10 ng/ml) and were half-changed 3 times per week for 2 weeks. At this stage, more than 95% of the cells were mature OLs (Baud et al., 2004; Wang et al., 2004) and were used for the experiments. The contamination of astrocytes and microglia was each around 2% as shown in Fig. 1.

Fig. 8. Downregulation of PKCθ attenuated OL toxicity induced by zinc. **A**, OLs were treated with ZnCl₂ in the presence of Bis-1, Go6976 (Go) or rottlerin (Ro) for 2 h, and then lysed for western blot. ZnCl₂ caused a significant increase of PKCθ phosphorylation, which was completely blocked by rottlerin, but not by Go6976. Bis-1 also slightly attenuated the phosphorylation of PKCθ. There was no phosphorylation of PKCδ following zinc treatment. A representative experiment of three that were performed is shown. **B**, OLs were infected with Lentiviral PKCθ shRNA particles for 4 days and then lysed for detection of the expression of PKCθ. Transfection with PKCθ shRNA caused a dramatic reduction of the expression of PKCθ, when compared to the GFP shRNA transfected cells. Prolonged treatment with PMA also completely blocked the expression of PKCθ. A representative experiment of three that were performed is shown. **C–D**, OLs were infected with Lentiviral PKCθ shRNA particles for 4 days and then treated with ZnCl₂ (**C**) or SIN-1 (**D**) for 2 h. The toxicity was assessed at an additional 24 h. Transfection with GFP shRNA had no effect on zinc or SIN-1 induced toxicity. However, transfection with PKCθ shRNA significantly attenuated cell death induced by ZnCl₂ or SIN-1. *, $p < 0.05$ and **, $p < 0.01$ were obtained when PKCθ shRNA transfected groups were compared with GFP shRNA transfected and the control groups following zinc or SIN-1 treatment. A representative experiment of three that were performed is shown.

PKC activity assay

The activity of PKC in mature OLs following oxidative stress was measured using PKC activity assay kit from Assay Designs, Inc (Ann Arbor, MI). In brief, OLs in 24-well plates were treated with SIN-1 (0.5 mM) or ZnCl₂ (100 μM) in the absence and presence of bisindolylmaleimide 1 (Bis-1, 10 μM) for 30 min and then lysed with protein lysis buffer. Whole cell lysates were adjusted to the equal protein concentration with lysis buffer and the same volume of each sample was added to the ELISA plate, which is precoated with crebtide, a substrate that can be readily phosphorylated by PKC. ATP was added to each well to initiate reaction at 30 °C for 90 min. After emptying the contents of each well, phosphospecific substrate antibody was added and incubated for 1 h. The phosphorylated crebtide was quantified following manufacturer's instructions.

Toxicity assay

The survival of cells after various treatments in 96-well plates was evaluated by visual inspection using phase contrast microscopy and quantified by Alamar Blue (Trek Diagnostic Systems, Inc., Westlake, OH), a viability assay that was previously described and validated by cell counting using trypan blue exclusion (Back et al., 1999). In all experiments, the culture plates were first washed twice with Hank's balanced salt solution (HBSS) containing 0.1% BSA and then placed in

Earle's balanced salt solution (EBSS, which is composed of 116 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, 26 mM NaHCO₃, 1 mM NaH₂PO₄ and 5.5 mM D-glucose). OLs were treated with SIN-1 or ZnCl₂ for 2 h, washed twice with HBSS containing 0.1% BSA, and then placed in BDM with T3 and CNTF. Inhibitors of PKCs were applied 15 min before and during cell exposure to SIN-1 or ZnCl₂. After cells were incubated for 20–24 h, the culture medium was replaced with EBSS plus a 1:100 dilution of Alamar Blue. After 2 h exposure, the fluorescence of the Alamar Blue solution in each well in the plates was read at room temperature using the Spectra MAX Gemini XS microplate reader with excitation wavelength at 530 nm and emission wavelength at 590 nm. The data from Alamar Blue assays matched the results from visual inspection.

Western blot analyses

At various times after SIN-1 or zinc treatment, OLs were placed on ice. Following medium aspiration, cells were washed once with ice-cold phosphate-buffered saline, and lysed with lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM glycerolphosphate, 1 mM Na₃VO₄, and 1 mM phenylmethylsulfonyl fluoride. An aliquot of cell lysate was removed for later protein determination. Cell lysate was mixed with Laemmli buffer, boiled for 5 min, and stored at –20 °C. Equal amounts of protein were separated by 4–12% SDS-PAGE and electrotransferred to

Table 1
Primer sequences of mouse PKC isoforms.

PKC isoforms	Forward	Reverse
α	5'-cccattccagaaggagatga-3'	5'-ttcctgtcagcaagcatcac-3'
β	5'-tccctgatcccaaaagtga-3'	5'-aactgaaccagccatcac-3'
δ	5'-cagaccaaggaccactgtt-3'	5'-gcataaaacgtagccggta-3'
γ	5'-accagggtcatctacagg-3'	5'-cttcctcatcttccccatca-3'
ϵ	5'-gaggactggattgacctgga-3'	5'-atctctgcagtgaggagcagt-3'
η	5'-catccccacacaagttcaacg-3'	5'-atatttcgggttgaggacc-3'
λ	5'-tatggcttcagcgttgactg-3'	5'-cctttgggtccttggtaga-3'
θ	5'-atggacaaccccttctacc-3'	5'-gcggatgtctctctcactc-3'
ζ	5'-aagtgggtggacagtgaagg-3'	5'-cagcttctctccatctcttg-3'

a polyvinylidene difluoride membrane. Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h and then incubated overnight at 4 °C with the primary antibodies for phosphorylated ERK1/2, total ERK1/2 (Cell Signaling, Beverly, MA) and PKC isoform specific antibodies (BD Biosciences). After washing with TBST, the membrane was incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Bio-Rad Laboratories, Hercules, CA). The membranes were washed again as above and visualized by enhanced chemiluminescence (ECL) according to the manufacturer's protocol (PerkinElmer Life Sciences).

Measurement of ROS generation

Intracellular free radical generation was evaluated with DCF (Molecular Probes, Eugene, OR) as we previously described (Wang et al., 2004; Zhang et al., 2006). Briefly, following treatment with SIN-1 or ZnCl₂ with/without PKC inhibitors for 2 h, cells in 96-well plates were loaded with DCF (20 μ M) for 30 min in EBSS (95% air/5% CO₂, 37 °C). After the loading solution was removed, cells were washed and incubated in EBSS, and the fluorescence in each well was measured and recorded in the fluorescence plate-reader as described above (with excitation at λ = 480 nm and emission at λ = 530 nm and temperature set to 37 °C).

Reverse transcriptase PCR (RT-PCR) and PCR analysis

Total RNA was isolated from mature OLs using RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA synthesis from total RNA was performed by ReverAid First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD) using 1 μ g total RNA and 1 μ l oligo (dT)₁₈ following manufacturer's instructions. 1 μ l of each cDNA synthesized in the reverse transcriptase reaction was used for PCR amplification in the presence of 1 U Taq DNA polymerase in Tag buffer, 0.2 mM each of dNTPs, and 1 μ M of each primer. The PKC isoform specific primers were listed in Table 1. The PCR amplification reaction used a three-step program (30 s at 95 °C, 30 s at 60 °C, and 45 s at 72 °C) for a total of 30 cycles. The PCR product was run on 1.5% agarose gel and visualized under UV light.

shRNA transfection

Mature OLs were infected with PKC θ shRNA lentiviral particles (sc-36247v, Santa Cruz Biotechnology) in the presence of polybrene (2 μ g/ml) for 24 h. Polybrene was used to enhance the binding of lentiviral particles to cell membranes. After replacing the lentiviral particle containing medium with the normal culture medium (BDM plus T3 and CNTF), cells were incubated for another 3 days and then treated with ZnCl₂ (150 μ M) or SIN-1 (1 mM) for 2 h. The toxicity was assessed at 20–24 h later using Alamar Blue assay. The lentiviral particles of copGFP (sc-108084, Santa Cruz Biotechnology) were used as a negative control. The transfection efficiency was around 50% when the GFP fluorescence was examined.

Statistics

Statistical significance was assessed using ANOVA with the Tukey-Kramer *post-hoc* multiple comparison test. Statistical analysis was performed using the Instat program from GraphPad Software (San Diego, CA). Representative experiments are shown unless noted otherwise. Experiments were performed with triplicate samples, and the data are expressed as mean $\pm$ SD. All experiments were repeated at least 3 times.

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