Rottlerin enhances IL-1β-induced COX-2 expression through sustained p38 MAPK activation in MDA-MB-231 human breast cancer cells

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Abbreviations: COX-2, cyclooxygenase-2; ROS, reactive oxygen species

Abstract

Cyclooxygenase-2 (COX-2) is an important enzyme in inflammation. In this study, we investigated the underlying molecular mechanism of the synergistic effect of rottlerin on interleukin1ß (IL-1ß)-induced COX-2 expression in MDA-MB-231 human breast cancer cell line. Treatment with rottlerin enhanced IL-1B-induced COX-2 expression at both the protein and mRNA levels. Combined treatment with rottlerin and IL-1ß significantly induced COX-2 expression, at least in part, through the enhancement of COX-2 mRNA stability. In addition, rottlerin and IL-1ß treatment drove sustained activation of p38 Mitogen-activated protein kinase (MAPK), which is involved in induced COX-2 expression. Also, a pharmacological inhibitor of p38 MAPK (SB 203580) and transient transfection with inactive p38 MAPK inhibited rottlerin and IL-1β-induced COX-2 upregulation. However, suppression of protein kinase C δ (PKC δ) expression by siRNA or overexpression of dominant-negative PKC δ (DN-PKC- δ) did not abrogate the rottlerin plus IL-1β-induced COX-2 expression. Furthermore, rottlerin also enhanced tumor necrosis factor- α (TNF- α), phorbol myristate acetate (PMA), and lipopolysaccharide (LPS)-induced COX-2 expression. Taken together, our results suggest that rottlerin causes IL-1β-induced COX-2 upregulation through sustained p38 MAPK activation in MDA-MB-231 human breast cancer cells.

Keywords: breast neoplasms; cyclooxygenase-2; in-

terleukin-1 β ; p38 mitogen-activated protein kinases; RNA stability; rottlerin

Introduction

Cyclooxygenase (COX) is the rate-limiting enzyme that catalyzes arachidonic acid into prostanoids and has two subtypes, COX-1 and COX-2. COX-1 is constitutively expressed in tissues and is thought to be involved in homeostatic prostanoid biosynthesis, while COX-2 is expressed in the cytoplasm and its expression is inducible during inflammatory processes (Griswold and Adams, 1996; Smith et al., 2000; Tsatsanis et al., 2006). According to the several previous reports, COX-2 expression is upregulated by diverse agents and is mediated by Mitogen-activated protein kinases (MAPKs), reactive oxygen species (ROS), AP-1 or NF-KB (Mifflin et al., 2004; Shishodia et al., 2004; Song et al., 2007; Chandramohan et al., 2008; Jaimes et al., 2008; Kim et al., 2009; Chiu et al., 2010). Upregulation of COX-2 has been linked to the progression of tumors, resistance to cell death and the metastatic phenotype of human cancer cells (Chen et al., 2001; Denkert et al., 2004; Brown and DuBois, 2005). The p38 Mitogen-activated protein kinase (MAPK) plays a crucial role in inflammation (Schieven, 2005). Activated p38 MAPK upregulates the production of key inflammatory mediators, including TNF- α , IL-1 β and COX-2, by several independent mechanisms, including direct phosphorylation of transcription factors, direct or indirect mRNA stabilization and enhanced translation of mRNAs in various cell types (Ridlev et al., 1998; Molina-Holgado et al., 2000; Mifflin et al., 2002, 2004; Di Mari et al., 2007).

Rottlerin, which is a pigmented plant compound isolated compound from *Mallotus philippinensis*, was originally identified as a specific inhibitor of the novel protein kinase C (PKC) isoform, PKC δ (Gschwendt *et al.*, 1994; Cross *et al.*, 2000; Kontny *et al.*, 2000; Hsieh *et al.*, 2007). PKC δ activation and translocation are induced by a variety of inflammatory stimuli (Abad *et al.*, 2006). However, some recent studies showed that rottlerin was not effective at inhibiting PKC δ activity *in vitro* and that may display non-specific effects (Soltoff, 2007;



Figure 1. Effect of rottlerin on IL-1 β -induced expression of COX-2 protein and mRNA in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with the indicated concentrations of rottlerin in the presence of IL-1 β (5 ng/ml) for 12 h. The cells were lysed and the lysates were analyzed using immunoblotting with anti-COX-2 antibody. Anti-ERK antibody was used to confirm equal loading. (B) Total RNA was prepared and RT-PCR analysis was performed as described in the Methods. (C) MDA-MB-231 cells were treated with the indicated concentrations of IL-1 β in the presence of rottlerin (2.5 μ M) for 12 h. The cells were lysed and the lysates were analyzed using immunoblotting with anti-COX-2 antibody. Anti-ERK antibody was used to confirm equal loading. (D) Total RNA was prepared and RT-PCR analysis was performed as described in the Methods. A ti-ERK antibody was used to confirm equal loading. (D) Total RNA was prepared and RT-PCR analysis was performed as described in the Methods. A ti-ERK antibody was used to confirm equal loading. (D) Total RNA was prepared and RT-PCR analysis was performed as described in the Methods. A representative result is shown; two additional experiments yielded similar results.

Song et al., 2008; Lim et al., 2009; Park et al., 2010).

In this study, we investigated whether rottlerin affects IL-1 β -induced COX-2 expression in breast cancer cells. Combined treatment with rottlerin and IL-1 β -induced COX-2 upregulation is correlated with COX-2 mRNA stability. Sustained activation of p38 MAPK is involved in rottlerin and IL-1 β -induced COX-2 expression. However, the suppression of PKC δ expression by siRNA did not abrogate rottlerin and IL-1 β -induced COX-2 expression. Furthermore, other inflammatory stimuli such as, TNF- α , PMA, and LPS, also upregulate COX-2 expression in the presence of rottlerin.

Results

Rottlerin enhances IL-1 β -induced COX-2 expression in MDA-MB-231 cells

IL-1β is an important inflammatory cytokine that induces COX-2 expression in various cells (Molina-Holgado *et al.*, 2000; Jung *et al.*, 2003). To test whether rottlerin affects IL-1β-induced COX-2 expression, MDA-MB-231 cells were treated with IL-1β (5 ng/ml) and various concentrations (1-5 μ M) of rottlerin for 12 h. As shown in Figure 1A, IL-1β alone slightly induces COX-2 protein expression. Interestingly, co-treatment of MDA-MB-231 cells with rottlerin and IL-1β resulted in a markedly increased COX-2 expression (Figure 1A). To elucidate the relationship between COX-2 protein and COX-2 mRNA in MDA-MB-231 cells, we performed RT-PCR. The levels of COX-2 mRNA also dramatically increased after combined treatment with rottlerin and IL-1 β (Figure 1B). Additionally, incubation with rottlerin (2.5 μ M) enhanced IL-1 β -induced COX-2 protein and mRNA levels at low concentrations of IL-1 β (Figures 1C and 1D).

MAPK signaling pathway activation following rottlerin and IL-1 β treatment

To investigate whether the ERK, JNK, or p38 MAPK pathways are involved in rottlerin and IL-1_β-induced COX-2 expression, we examined whether selective MAPK inhibitors could affect rottlerin plus IL-1_β-induced COX-2 expression. Induction of the COX-2 protein and mRNA expression was dramatically reduced in the presence of p38 MAPK inhibitor (SB 203580), while JNK inhibitor (SP 600125) was ineffective at regulating COX-2 expression. Treatment with MEK1/2 inhibitor (PD 098059) slightly inhibited COX-2 protein and mRNA expression. Because the p38 MAPK inhibitor markedly inhibited COX-2 expression, we assessed the activation of p38 MAPK by detecting its dually phosphorylated form using Western blotting with specific anti-phospho-p38 MAPK antibodies in MDA-MB-231 cells treated with IL-1ß alone, rottlerin alone or rottlerin plus IL-1 β (Figure 2C). IL-1 β (5 ng/ml) treatment induces a strong transient increase in phosphorylated p38 MAPK level, that peaked at 30 min and declined thereafter. Treatment with rottlerin (2.5 µM) alone slightly increases the phosphorylation of p38 MAPK. Interestingly, phosphorylation levels of p38 MAPK were sustained for 90 min after combined treatment with rottlerin and IL-1 β .

Recently, several groups reported that the p38 MAPK pathway significantly contributes to COX-2 mRNA stability (Huang et al., 2000; Tsatsanis et al., 2006). We examined the effect of combined treatment with rottlerin and IL-1 β on the COX-2 mRNA stability. To measure COX-2 mRNA stability following rottlerin and IL-1ß treatment, MDA-MB-231 cells were treated with rottlerin (2.5 μ M) and IL-1 β (5 ng/ml) for 12 h to induce COX-2 transcription and then further cultured with or without rottlerin plus IL-1 β in the presence of actinomycin D (2.5) µg/ml). As shown in Figure 2D, treatment with rottlerin plus IL-1 β enhances the half-life of COX-2 mRNA. Together, these data suggest that rottlerin and IL-1ß treatment enhances COX-2 expression at least in part through the sustained activation of p38 MAPK-mediated COX-2 mRNA stability.



Figure 2. Effect of rottlerin on IL-1β-induced phosphorylation of MAPKs and effect of MAPK inhibitors on rottlerin and IL-1β-induced expression of COX-2 in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with 50 μM PD 098059 (MEK1/2 inhibitor), 10 μM SB 203580 (p38 MAPK inhibitor) or 20 µM SP600125 (JNK inhibitor) for 30 min prior to incubation with the indicated concentrations of rottlerin and IL-1ß for 12 h. The cells were lysed and the lysates were analyzed using immunoblotting with anti-COX-2 antibody. Anti-ERK antibody was used to confirm equal loading. (B) Total RNA was prepared and RT-PCR analysis was performed. A representative result is shown; two additional experiments yielded similar results. (C) MDA-MB-231 cells were treated with vehicle, IL-1_β (5 ng/ml), rottlerin (2.5 µM) and IL-1B plus rottlerin for the indicated times. Equal amounts of lysates (40 µg) were subjected to electrophoresis and analyzed using immunoblotting with phospho-p38 MAPK- and p38 MAPK-specific antibodies. (D) MDA-MB-231 cells were treated with 2.5 μM rottlerin and 5 ng/ml IL-1β. After 12 h, each well washed with PBS and treated with 2.5 µg/ml actinomycin D (Act D) in the presence or absence of 2.5 µM rottlerin and 5 ng/ml IL-1β for the indicated times. RT-PCR was performed as described in the Methods.

p38 MAPK signaling pathway is involved in rottlerin plus IL-1β-induced COX-2 expression in MDA-MB-231 cells

To explore the significance of p38 MAPK in rottlerin plus IL-1 β -induced COX-2 expression, we first examined the dose effect of SB 203580 on COX-2 expression. As shown in Figures. 3A and 3B, pretreatment with SB 203580 inhibited rottlerin plus IL-1 β -induced expression of COX-2 protein and mRNA in a dose-dependent manner. To further evaluate that the role of the p38 MAPK in rottlerin plus IL-1 β -induced COX-2 expression, we transiently transfect MDA-MB-231 cells with vector, wild-type p38 MAPK or p38 MAPK-DN (a dominant negative mutant). As shown in Figure 3C, ectopic expression of p38 MAPK-DN decreased COX-2 expression



Figure 3. Effect of p38 MAPK inhibitor and p38 MAPK on rottlerin plus IL-1 β -induced COX-2 expression in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with SB 203580 (1, 5, 10 μ M) for 30 min prior incubation with the indicated concentrations of rottlerin and IL-1 β for 12 h. The cells were lysed and the lysates were analyzed using immunoblotting with anti-COX-2 antibody. Anti-ERK antibody was used to confirm equal loading. (B) Total RNA was prepared and RT-PCR analysis was performed. (C) MDA-MB-231 cells were transiently transfected with vector, p38 wild-type or a p38 dominant negative (DN) mutant and then treated with the indicated concentrations of rottlerin and IL-1 β for 12 h. The cells were lysed and the lysates were analyzed using immunoblotting with anti-COX-2 antibody. Anti-ERK antibody was used to confirm equal loading. A representative result is shown; two additional experiments yielded similar results.

levels in the presence of rottlerin and IL-1 β . These results suggest that rottlerin plus IL-1 β -induced COX-2 expression was modulated, at least, by the p38 MAPK signaling pathway.

ROS generation and NF- κ B activity were not affected by rottlerin plus IL-1 β -induced COX-2 expression

Previous studies have demonstrated that COX-2 induction is stimulated by reactive oxygen species (ROS) generation in several cell lines (Feng et al., 1995; Jaimes et al., 2008). Therefore, we examined whether ROS generation might be involved in rottlerin plus IL-1_β-induced COX-2 expression. As shown in Figures 4A and 4B, pretreatment with antioxidants N-acetylcysteine (NAC) or glutathione (GSH) did not affect rottlerin plus IL-1_B-induced expression levels of COX-2 protein and mRNA. In addition, we tested whether rottlerin plus IL-1β-induced COX-2 expression was associated with NF-kB activity. As shown in Figures 4C and 4D, pretreatment with NF- κ B inhibitor MG132 or BAY 11-7082 did not affect rottlerin plus IL-1β-induced expression levels of COX-2 protein and mRNA. These results suggest that rottlerin plus IL-1β-induced COX-2 expression is independent of ROS generation and NF-kB signaling.



Figure 4. Effect of ROS generation on rottlerin and IL-1 β -induced COX-2 expression in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with GSH (5 mM) or NAC (5 mM) for 30 min prior to incubation with the indicated concentrations of rottlerin and IL-1 β for 12 h. The cells were lysed and the lysates were analyzed using immunoblotting using an-ti-COX-2 antibody. Anti-ERK antibody was used to confirm equal loading. (B) Total RNA was prepared and RT-PCR analysis was performed. A representative result is shown; two additional experiments yielded similar results. (C) MDA-MB-231 cells were treated with MG132 (2 μ M) or BAY 11-7082 (5 μ M) for 30 min prior to incubation with the indicated concentrations of rottlerin and IL-1 β for 12 h. The cells were lysed and the lysates were analyzed using immunoblotting with anti-COX-2 antibody. Anti-ERK antibody was used to confirm equal loading. (D) Total RNA was prepared and RT-PCR analysis was performed. A representative result is shown; two additional experiments.

Rottlerin plus IL-1 β -induced COX-2 expression is independent of PKC δ inhibition

Rottlerin is identified as a putative inhibitor of PKC δ (Gschwendt et al., 1994), thus we examined whether rottlerin plus IL-1β-induced COX-2 expression was directly associated with the inhibition of PKC δ activity. We employed a siRNA duplex against PKC δ mRNA. MDA-MB-231 cells transfected with the control or PKC δ siRNA and treated with IL-1 β (10 ng/ml) for 12 h. Suppression of PKC δ expression by transfection with siRNA did not upregulates IL-1β-induced COX-2 expression in MDA-MB-231 cells (Figure 5A). Additionally, to further confirm the independence of PKC δ activity, MDA-MB-231 cells were transfected with vector or PKC δ dominant negative (DN) mutant and treated with IL-1 β (10 ng/ml) for 12 h. As shown in Figure 5B, ectopic expression of DN-PKC δ did not affect IL-1 β -induced COX-2 expression (Figure 5B). Our results suggest that rottlerin plus IL-1_B-induced COX-2 expression is independent of PKC δ pathway.

Rottlerin enhances TNF- α , PMA- and LPS-induced COX-2 expression in MDA-MB-231 cells

TNF- α , PMA and LPS have been previously shown to induce COX-2 expression (Mitchell *et al.*, 1994; Miralpeix *et al.*, 1997; Huang *et al.*, 2000; Molina-Holgado *et al.*, 2000; Nakao *et al.*, 2002; Shishodia *et al.*, 2004; Woo and Kwon, 2007). We carried out



Figure 5. Rottlerin and IL-1 β -induced COX-2 expression is independent of PKC δ inhibition. (A) MDA-MB-231 cells were transfected with PKC δ siRNA or control siRNA. After transfection, cells were treated with or without the indicated concentrations of IL-1 β for 12 h. The expression levels of COX-2 and PKC δ were analyzed using immunoblotting. Anti-ERK antibody was used to confirm equal loading. A representative study is shown; two additional experiments yielded similar results. (B) MDA-MB-231 cells were transfected with a PKC δ dominant negative (DN) mutant or a vector control. After transfection, cells were treated with or without the indicated concentrations of IL-1 β for 12 h. The expression levels of COX-2 and PKC δ were analyzed using Western blotting. Anti-ERK antibody was used to confirm equal loading. Films corresponding to Western blots probed with anti-COX-2 antibody were exposed for a longer period compared to other antibodies. A representative study is shown; two additional experiments yielded similar results.

Western blotting and RT-PCR to test the effect of rottlerin on TNF- α -, PMA- and LPS-induced COX-2 expression in MDA-MB-231 cells. As shown in Figures 6A, 6B and 6C, treatment with rottlerin enhances TNF- α -, PMA- and LPS-induced COX-2 protein and mRNA expression. Pretreatment with a p38 inhibitor decreases COX-2 protein and mRNA expression after treatment with rottlerin plus TNF- α , PMA or LPS. These results show that rottlerin enhances COX-2 expression induced by multiple reagents.

Discussion

In the present study, we demonstrate for the first time that treatment of breast cancer cells with rottlerin in combination with IL-1ß markedly induces COX-2 expression. It has been reported that rottlerin is a specific inhibitor of PKC δ (Gschwendt et al., 1994), However, some recent studies showed that rottlerin might not act directly on PKC δ , but instead elicit cellular changes that mimic those caused by the direct inhibition of PKC δ (Gschwendt et al., 1994; Soltoff, 2007; Song et al., 2008; Lim et al., 2009; Park et al., 2010). Therefore, we tested the possibility that rottlerin could enhance IL-1_β-induced COX-2 expression in human breast cancer cells. In this study, we demonstrate that co-treatment with rottlerin enhances IL-1β-induced COX-2 expression through sustained p38 MAPK activation in MDA-MB-231 cells. However, our results indicate that PKC $\boldsymbol{\delta}$ is not involved in



Figure 6. Effect of rottlerin on TNF- α -, PMA-, LPS-induced COX-2 expression in MDA-MB-231 cells. MDA-MB-231 cells were treated with TNF- α (A), PMA (B) or LPS (C) in the presence of rottlerin (2.5 μ M) or were pretreated with SB203580 (10 μ M) and then treated with TNF- α plus rottlerin for 12 h. The cells were lysed and the lysates were analyzed using immunoblotting with anti-COX-2 antibody. Anti-ERK antibody was used to confirm equal loading. Total RNA was prepared and RT-PCR analysis was performed as described in the Methods. A representative study is shown; two additional experiments yielded similar results.

rottlerin plus IL-1 β -induced COX-2 upregulation. This view is supported by the following findings: (a) suppression of PKC δ expression with siRNA did not upregulates IL-1 β -induced COX-2 expression and (b) overexpression of DN-PKC δ did not upregulates IL-1 β -induced COX-2 expression.

COX-2 is aberrantly overexpressed in many human cancers such as colon, lung, breast, and prostate cancer (Smith et al., 2000; Brown and DuBois, 2005). COX-2 gene expression is regulated by transcriptional and post-transcriptional mechanisms; the relative contribution of each mechanism is dependent on the stimulus, the cellular environment and the particular cell type. Studies that examined COX-2 transcription have shown that NF-κB. nuclear factor of interleukin-6 (NF-IL-6), cAMP response element (CRE), and AP1 were commonly or individually involved in the regulation of COX-2 expression (Dannenberg et al., 2001; Dempke et al., 2001). In addition, post-transcriptional regulation of COX-2 expression has been mainly attributed to stabilization of the COX-2 mRNA by signaling proteins (p38 MAPK, ERK, PI3K/PKB), RNA binding proteins (HuR, TTP) and AU-rich elements in the

COX-2 mRNA 3'-UTR (Lasa et al., 2000). IL-1ß and p38 MAPK-mediated stabilization of the COX-2 mRNA has been previously reported (Dean et al., 1999). Studies using pharmacological inhibitors of MAPKs demonstrated the role of p38 MAPK in rottlerin plus IL-18-induced COX-2 upregulation in MDA-MB-231 cells (Figure 3A). Furthermore, the suppression of rottlerin plus IL-1β-induced COX-2 expression by overexpression of the DN-p38 MAPK (Figure 3C) is consistent with the concept that the p38 MAPK signaling pathway is important for regulating both COX-2 transcription and mRNA stability. One of the interesting findings from this study is that treatment with rottlerin enhances TNF-a-, PMA- and LPS-induced COX-2 protein and mRNA expression (Figure 6) as well as IL-1β-induced COX-2 expression. These observations suggest that rottlerin-mediated TNF-a-, PMA-, LPS-, or IL-1β-induced COX-2 upregulation via the p38 MAPK signaling pathway is a common response.

ROS play an important role in inflammatory processes as mediators of injury. Feng *et al.* (1995) reported that oxidant stress is a specific and important inducer in COX-2 expression induced by IL-1, TNF- α and LPS. However, in our data, pretreatment with an ROS scavenger did not suppress rottlerin plus IL-1 β -induced COX-2 expression (Figure 4). Another interesting finding from this study is that NF- κ B is not involved in rottlerin plus IL-1 β -induced COX-2 upregulation.

In conclusion, the present study shows that COX-2 expression is regulated by rottlerin. We have provided sufficient evidence showing that p38 MAPK signaling pathways play a vital role in the regulating COX-2 expression after combined treatment with rottlerin and IL-1 β in breast carcinoma cells, which may contribute to a better understanding of inflammation and cancer.

Methods

Cells and materials

MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and grown in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS), 20 mM HEPES and 100 μ g/ml gentamicin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Recombinant interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) proteins were purchased from R&D systems (Minneapolis, MN). MG132, BAY 11-7082 and PMA were obtained from Calbiochem (San Diego, CA). Rottlerin, PD 98059, SB 203580, SP 600125, LY 294002 and wortmannin were purchased from BIOMOL (Plymouth Meeting, PA). Actinomycin D, glutathione (GSH), N-acetylcysteine (NAC) and lipopolysaccharides (LPS) were purchased from Sigma-Aldrich (St. Louis, MO).

Anti-COX-2 antibody was obtained from the Cayman Chemical Company (Ann Arbor, MI); anti-phospho-JNK, anti-JNK, anti-phospho-p38 MAPK, anti-phospho-ERK, anti-phospho-AKT and anti-AKT antibodies from Cell Signaling (Danvers, MA); and anti-PKC δ antibody was purchased from BD Transduction LaboratoriesTM (San Diego, CA).

Western blotting

Cellular lysates were prepared by suspending 1.5×10^6 cells in 100 µl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM MgCl₂, 0.1% Triton X-100, 25 mM MOPS (4-morpholinepropane-sulfonic acid), 100 µM phenylmethylsulfonyl fluoride and 20 µM leupeptin, adjusted to pH 7.2), disrupted by sonication and extracted at 4°C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Detection of specific proteins was carried out with an ECL Western blotting kit (Millipore Corp.) according to the manufacturer's instructions.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA). Single-strand cDNA was synthesized from 2 μ g of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI). The cDNA for COX-2 was amplified by PCR with specific primers: 5'-CCGGAC AGGATTCTATGGAGA-3' (sense) and 5'-GAAGTGCTGG GCAAAGAATGC-3' (antisense). PCR amplification was carried out as follows: 1 × (94°C, 3 min); 25 × (94°C, 45 s; 55°C, 45 s; 72°C, 45 s); and 1 × (72°C, 10 min). PCR products were analyzed using agarose gel electrophoresis and visualized by ethidium bromide.

Transfection and small interfering RNA (siRNA)

MDA-MB-231 cells were seeded at a density of 1×10^5 cells/well in six-well culture plates the day before transfection to achieve 50-60% confluence. Wild-type p38 MAPK, a dominant negative (DN) p38 MAPK mutant or an empty vector were transfected into cells using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The SMART pool siRNA against PKC δ used in this study was obtained from Dharmacon. Control siRNA-A, a negative control for experiments using targeted siRNA, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transfected with siRNA oligonucleotides using OligofectamineTM Reagent (Invitrogen).

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