Signal Transduction Cascade in Staurosporine-Induced Prostaglandin E$_2$ Production by Rat Peritoneal Macrophages$^1$

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ABSTRACT

The possible participation of phosphatidylinositol (PI) 3-kinase, p44/42 mitogen-activated protein (MAP) kinases and protein kinase C (PKC) in staurosporine-induced prostaglandin E$_2$ (PGE$_2$) production was investigated pharmacologically in rat peritoneal macrophages. When the cells were incubated in the presence of staurosporine (63 nM), phosphorylation of p44/42 MAP kinases and cytosolic phospholipase A$_2$ (cPLA$_2$) was induced at 15 min and increased until 60 min, whereas PGE$_2$ production and expression of cyclooxygenase-2 (COX-2) protein began to increase at 2 h and increased thereafter. Both PD98059 and U0126, MAP kinase/extracellular signal-regulated kinase (ERK) kinase inhibitors, and LY294002, a PI 3-kinase inhibitor, completely inhibited staurosporine-induced phosphorylation of p44/42 MAP kinases and cPLA$_2$ and PGE$_2$ production. Moreover, U0126 inhibited staurosporine-induced arachidonic acid release at 1 h. Although PD98059 and U0126 at 30 μM partially inhibited staurosporine-induced COX-2 protein expression, they completely inhibited staurosporine-induced PGE$_2$ production. LY294002 at 100 μM did not inhibit staurosporine-induced expression of COX-2 protein. In contrast, Ro-31-8220, a PKC inhibitor, completely inhibited staurosporine-induced PGE$_2$ production and COX-2 protein expression at 8 h but did not inhibit staurosporine-induced phosphorylation of p44/42 MAP kinases and cPLA$_2$. These findings suggest that staurosporine induces PGE$_2$ production by two mechanisms. One is cPLA$_2$ phosphorylation through a signal transduction pathway from PI 3-kinase to p44/42 MAP kinases, by which arachidonic acid, a substrate for COX-1 and COX-2, is increased. The other is COX-2 protein expression, which is induced mainly by activation of PKC and partially by activation of p44/42 MAP kinases; thus, arachidonic acid is metabolized to PGE$_2$.

Prostaglandin E$_2$ (PGE$_2$) is an important chemical mediator of inflammation. At the inflammatory site, macrophages produce a large amount of PGE$_2$ (Humes et al., 1977), which causes pain (Ferreira et al., 1982) and an increase in vascular permeability (Williams and Morley, 1973). For PGE$_2$ production, phospholipase A$_2$ (PLA$_2$) and cyclooxygenase (COX) are responsible, and in the former, two classes of PLA$_2$, the high molecular weight ($M_\text{r}$ 85,000 to 110,000) cytosolic PLA$_2$ (cPLA$_2$) (Chen et al., 1997) and the $M_\text{r}$ 14,000 secretory type II PLA$_2$ (Murakami et al., 1996), are involved. In human monocytes, treatment with anti-sense cPLA$_2$ decreased lipopolysaccharide-induced PGE$_2$ production (Roshak et al., 1999). In the P388D$_1$ macrophage cell line, cPLA$_2$ regulates the activity of secretory type II PLA$_2$ in platelet-activating factor-induced arachidonic acid release (Balsinde and Dennis, 1996). These reports suggest that cPLA$_2$ plays an important role in prostanooid formation in monocytes and macrophages.

Staurosporine, a nonspecific inhibitor of protein kinases (Tamaoki et al., 1986), induces interleukin-8 (IL-8) production in human synovial fibroblasts (Jordan et al., 1996), macrophage inflammatory protein-2 production in rat peritoneal polymorphonuclear leukocytes (Edamatsu et al., 1997; Xiao et al., 1999), and IL-6 production in rat peritoneal macrophages (Yamaki and Ohuchi, 1999). We also described that staurosporine induces arachidonic acid release and PGE$_2$ production in rat peritoneal macrophages (Watanabe et al., 1999). In addition, it has been demonstrated that staurosporine induces PGE$_2$ production via up-regulation of COX-2 protein expression (Moon et al., 1999). At the inflammatory site, COX-2 protein is induced by various inflammatory stimuli such as cytokines and growth factors (Kujubu et al., 1991), and the increase in COX-2 protein expression exacerbates inflammatory responses by producing proinflammatory prostanooids. Therefore, for the suppression of inflammation, inhibitors of COX-2 protein

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ABBREVIATIONS: PGE$_2$, prostaglandin E$_2$; COX, cyclooxygenase; PLA$_2$, phospholipase A$_2$; cPLA$_2$, cytosolic phospholipase A$_2$; DMSO, dimethyl sulfoxide; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; PI, phosphatidylinositol; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol 13-acetate; IL-8, interleukin-8.
expression might be useful as well as COX-2-specific inhibitors such as NS-398 (Futaki et al., 1994).

This study was intended to clarify pharmacologically the mechanism of action of staurosporine on the activation of cPLA2 and COX-2 protein expression by focusing on the roles of various kinases in rat peritoneal macrophages.

**Materials and Methods**

**Preparation of Rat Peritoneal Macrophages.** A solution of soluble starch (Wako Pure Chemical Inc., Osaka, Japan) and BACTO Peptone (Difco Laboratories, Detroit, MI), 5% each, that had been autoclaved at 120°C for 15 min was injected i.p. into male Sprague-Dawley rats (300–500 g, specific pathogen-free; Charles River Japan Inc., Kanagawa, Japan) at a dose of 5 ml/100 g b.wt. Four days later, the rats were sacrificed by cutting the carotid artery under anesthesia, and the peritoneal cells were harvested (Ohuchi et al., 1985). The rats were treated in accordance with procedures approved by the Animal Ethics Committee of the Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan.

**Macrophage Culture.** The peritoneal cells were harvested from four to five rats in each set of experiments. They were combined, mixed, and suspended in Eagle’s minimum essential medium (Nissui Inc., Tokyo, Japan) containing 10% (v/v) calf serum (Dainippon Pharmaceutical Co., Osaka, Japan), penicillin G potassium (Meiji Seika Co., Tokyo, Japan) (18 μg/ml), and streptomycin sulfate (Meiji Seika Co.) (50 μg/ml) at a density of 1.5 × 10^6 cells/ml of the medium. Two milliliters of the cell suspension was poured into each well of a 6-well plastic tissue culture plate (Corning Coster, Cambridge, MA) or 500 μl of the cell suspension was poured into each well of a 24-well plastic tissue culture plate (Corning Coster). The cells were incubated for 2 h at 37°C. The wells were then washed three times to remove nonadherent cells, and the adherent cells were further incubated for 20 h at 37°C, after which the cells were used for the experiments.

**Drugs Used.** Drugs used were staurosporine (Kyowa Medex, Tokyo, Japan), PD98059 (New England Biolabs, Inc., Beverly, MA), Ro-31-8220 (Calbiochem Novabiochem Japan, Tokyo, Japan), LY294002 (BIOMOL Research Laboratories, Plymouth Meeting, PA), and 12-O-tetradecanoylphorbol 13-acetate (TPA; Sigma Chemical Co., St. Louis, MO). Drugs were dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Co.), an aliquot of each solution was added to the medium, and the final concentration of DMSO in the medium was adjusted to 0.2% (v/v). The control medium contained the same amount of the vehicle. To down-regulate protein kinase C (PKC), the peritoneal cells were incubated for 20 h at 37°C in medium containing TPA at 186 μM (Yabata et al., 1999).

**Viability Assay.** The viability of the macrophages was assessed by the ability of mitochondrial succinate dehydrogenase to cleave 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to the blue compound formazan (Kobayashi et al., 1994). After incubation for 4 h at 37°C with drugs, 10 μl of MTT solution (5 mg/ml) in PBS (137 mM NaCl, 8.1 mM NaHPO_4_, 2.68 mM KCl, 1.47 mM KH_2PO_4_, pH 7.4) was added to each well and the cells were further incubated for 4 h at 37°C. After removal of the medium by aspiration, the resultant colored product was dissolved in 200 μl of DMSO directly added to each well, and the optical density at 570 nm was read on a Microplate Reader (Bio-Rad, Richmond, CA). Treatment with drugs showed no significant changes in the viability of the cells.

**Measurement of PGE2 Concentrations.** Rat peritoneal macrophages (0.75 × 10^6 cells) were incubated at 37°C for the periods indicated in 0.5 ml of medium in each well of a 24-well plastic tissue culture plate. The conditioned medium was collected and centrifuged at 1500g and 4°C for 5 min, and PGE2 concentrations in the supernatant fraction were radioimmunoassayed (Ohuchi et al., 1985). PGE2 antiserum was purchased from PerSeptive Diagnostics (Cambridge, MA).

**Western Blot Analysis of p44/42 Mitogen-Activated Protein (MAP) Kinases, cPLA2, and COX-2.** For Western blot analysis of p44/42 MAP kinase and cPLA2, 3.0 × 10^5 peritoneal macrophages were incubated in each well of a 6-well plastic tissue culture plate at 37°C for the periods indicated in medium containing no calf serum in the presence or absence of drugs. For Western blot analysis of COX-2, 3.0 × 10^6 peritoneal macrophages were incubated in each well of a 6-well plastic tissue culture plate at 37°C for the periods indicated in medium containing 10% calf serum (v/v) in the presence or absence of drugs. After incubation, the cells were washed three times with PBS and lysed in ice-cold lysis buffer (20 mM HEPES, 1% (v/v) Triton X-100, 1 mM EDTA, 50 mM NaF, 2.5 mM p-nitrophenyl phosphate, 1 mM Na_3VO_4_, 10 μg/ml leupeptin, and 10% (v/v) glycerol, pH 7.3). Proteins in the cell lysate were separated by electrophoresis on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. Immunoblotting was carried out by using antibodies to COX-2 (C-20) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), cPLA2 (C-20) (Santa Cruz Biotechnology Inc.), phospho-p44/42 MAP kinase (Thr202/Tyr204) (New England Biolabs Inc., Beverly, MA), and rat MAP kinase R2 (erk1-CT) (Upstate Biotechnology Inc., Lake Placid, NY). In some experiments, phosphorylation of p44/42 MAP kinase was determined by gel shift assay.

**Measurement of Radioactivity Released from [3H]Arachidonic Acid-Labeled Macrophages.** The peritoneal cells (1.5 × 10^6 cells/well) were incubated in each well of a 12-well plastic tissue culture plate at 37°C for 2 h. The wells were then washed three times to remove nonadherent cells, and the adherent cells were further incubated for 20 h at 37°C, after which the cells were used for the experiments.

**Statistical Analysis.** The statistical significance of the results was analyzed by Dunnett’s test for multiple comparisons and Student’s t test for unpaired observations.

**Results**

**Time Course of PGE2 Production and COX-2 Protein Expression.** When rat peritoneal macrophages were incubated in the presence of 63 nM staurosporine, PGE2 production in the conditioned medium began to increase at 2 h, and thereafter increased time dependently until 8 h (Fig. 1A). In the absence of staurosporine, PGE2 concentrations in the conditioned medium did not increase (Fig. 1A).

Western blot analysis revealed that staurosporine at 63 nM increased COX-2 protein levels at 2 h, reaching a maximum at 8 h (Fig. 1B). No increase in COX-2 protein levels was observed when rat peritoneal macrophages were incubated without staurosporine (Fig. 1B).

**Time Course of Phosphorylation of p44/42 MAP Kinases and cPLA2.** By treatment with staurosporine (63 nM), phosphorylation of p44/42 MAP kinases was induced 15 min after incubation and continued to increase time dependently until 60 min after incubation (Fig. 2A). Without treatment with staurosporine, no phosphorylation of p44/42 MAP kinases was observed during the incubation period (Fig. 2A). Phosphorylation of cPLA2 was also observed at 15 min and increased time dependently until 60 min after incubation with staurosporine (Fig. 2B). No phosphorylation of cPLA2 was induced without staurosporine treatment (Fig. 2B).
Effects of PD98059 and U0126, MAP Kinase/ERK Kinase (MEK) Inhibitors, on Staurosporine-Induced Phosphorylation of p44/42 MAP Kinases and cPLA2, COX-2 Expression, and PGE2 Production. Staurosporine-induced phosphorylation of p44/42 MAP kinases at 15 min was suppressed by PD98059 at a concentration of 1 μM or greater (Fig. 3A). Staurosporine-induced PGE2 production at 8 h was also inhibited by PD98059 in a concentration-dependent manner (Fig. 4A), and staurosporine-induced phosphorylation of cPLA2 at 45 min was inhibited by PD98059 at a concentration of 1 μM or greater (Fig. 3B). However, PD98059 at 10 μM did not suppress the staurosporine-induced expression of COX-2 protein at 8 h (Fig. 4B), at which concentration PD98059 inhibited staurosporine-induced PGE2 production completely (Fig. 4A). At 30 μM, PD98059 partially suppressed the staurosporine-induced COX-2 protein expression (Fig. 4B).

Similar to the effect of PD98059, the staurosporine-induced phosphorylation of p44/42 MAP kinases at 15 min (Fig. 5A), phosphorylation of cPLA2 at 45 min (Fig. 5B), and PGE2 production at 8 h (Fig. 5C) were suppressed by U0126 in a concentration-dependent manner. But the staurosporine-induced COX-2 protein expression was not completely suppressed by U0126 at 30 μM (Fig. 5D).

Effects of U0126 on the Staurosporine-Induced Release of Radioactivity from [3H]Arachidonic Acid-Labeled Macrophages. Staurosporine (63 nM) increased the release of radioactivity from [3H]arachidonic acid-labeled macrophages when determined at 1 h (Fig. 6). Staurosporine-induced increase in the release of radioactivity from [3H]arachidonic acid-labeled macrophages when determined at 1 h (Fig. 6). Staurosporine-induced increase in the release of radioactivity from [3H]arachidonic acid-labeled macrophages was suppressed by U0126 in a concentration-dependent manner (Fig. 6).

Effects of PD98059 and U0126, MAP Kinase/ERK Kinase (MEK) Inhibitors, on Staurosporine-Induced Phosphorylation of p44/42 MAP Kinases and cPLA2,
Effects of LY294002, a Phosphatidylinositol (PI) 3-Kinase Inhibitor, on Staurosporine-Induced Phosphorylation of p44/42 MAP Kinases and cPLA2, PGE2 Production, and COX-2 Protein Expression.

Staurosporine-induced phosphorylation of p44/42 MAP kinases was suppressed by LY294002 in a concentration-dependent manner (Fig. 7A). LY294002 also suppressed staurosporine-induced cPLA2 phosphorylation, but a maximum 50% inhibition was attained at 100 μM (Fig. 7B). In contrast, LY294002 did not suppress staurosporine-induced COX-2 protein expression at 8 h (Fig. 8B) but inhibited PGE2 production at 8 h in a concentration-dependent manner (Fig. 8A).

Effects of Ro-31-8220, a Specific Inhibitor of PKC, on Staurosporine-Induced Phosphorylation of p44/42 MAP Kinases and cPLA2, PGE2 Production, and COX-2 Protein Expression.

Staurosporine-induced phosphorylation of p44/42 MAP kinases was not suppressed by Ro-31-8220 at 1 to 10 μM (Fig. 9A). Phosphorylation of cPLA2 induced by staurosporine also was not suppressed by Ro-31-8220 (data not shown). However, staurosporine-induced COX-2 protein expression (Fig. 9B) and PGE2 production (Fig. 9A) were suppressed dose dependently, and complete inhibition was induced at 10 μM Ro-31-8220.

Effects of PKC Down-Regulation on Staurosporine-Induced Phosphorylation of p44/42 MAP Kinases, COX-2 Protein Expression, and PGE2 Production.

Rat
peritoneal macrophages were treated with TPA (816 nM) for 20 h to induce down-regulation of PKC. In the PKC down-regulated macrophages, TPA (47 nM) treatment failed to induce PGE$_2$ production (Fig. 10), phosphorylation of p44/42 MAP kinases (Fig. 11A), and COX-2 protein expression (Fig. 11B). In contrast, staurosporine (63 nM) induced PGE$_2$ production (Fig. 10), phosphorylation of p44/42 MAP kinases (Fig. 11A), and COX-2 protein expression (Fig. 11B).

Discussion

This study suggested that staurosporine induces PGE$_2$ production via two different mechanisms. One is stimulation of arachidonic acid release by cPLA$_2$ phosphorylation through activation of PI 3-kinase followed by phosphorylation of p44/42 MAP kinases; the other is induction of COX-2 protein mainly by activation of PKC and partially by activation of p44/42 MAP kinases.

cPLA$_2$ is activated by phosphorylation; phosphorylated cPLA$_2$ has four to five times the activity of nonphosphorylated cPLA$_2$ (Lin et al., 1993). In this paper, we described that cPLA$_2$ is phosphorylated 15 min after treatment with staurosporine and the phosphorylation continues to increase until 45 min (Fig. 1B). This is consistent with the finding that staurosporine induces arachidonic acid release at 1 h in rat peritoneal macrophages (Watanabe et al., 1990). As shown in Fig. 1, COX-2 protein expression and PGE$_2$ production were increased by staurosporine 2 h after incubation. These findings suggest that the arachidonic acid released by activated cPLA$_2$ is converted into PGE$_2$ by staurosporine-induced COX-2. Before the expression of COX-2 by staurosporine, it is possible that the released arachidonic acid is converted into PGE$_2$ by pre-existing COX-1. Western blot analysis demonstrated that COX-1 protein is detected in rat peritoneal macrophages before stimulation with TPA or thapsigargin (Yamashita et al., 1997).

The MAP kinase cascade, including p44/42 MAP kinases is an important signal transduction pathway for the stimulation of arachidonic acid metabolism; it participates in cPLA$_2$ phosphorylation in Chinese hamster ovary cells (Lin et al., 1993) and COX-2 gene expression in NIH 3T3 cells (Xie and Herschman, 1996). It is also reported that the MAP kinase cascade is activated by PKC in Cos cells (Berra et al., 1995).
and by PI 3-kinase in mouse lymph node T cells (Eder et al., 1998). Therefore, we examined the roles of PI 3-kinase, p44/42 MAP kinases, and PKC in the staurosporine-induced increase in arachidonic acid metabolism.

The PI 3-kinase inhibitor LY294002 and the MEK inhibitors PD98059 and U0126 suppressed staurosporine-induced phosphorylation of p44/42 MAP kinases (Figs. 3A, 5A, and 7A), but the PKC inhibitor Ro-31-8220 had no effect (Fig. 9C), suggesting that the MAP kinase cascade is activated by PI 3-kinase but not by PKC in rat peritoneal macrophages. LY294002, PD98059, and U0126 also suppressed staurosporine-induced cPLA2 phosphorylation (Figs. 3B, 5B, and 7B). Therefore, it is possible that staurosporine activates PI 3-kinase and transduces signals to activate p44/42 MAP kinases, thus phosphorylating cPLA2. However, it is also possible that PI 3-kinase regulates staurosporine-induced PGE2 production by some unknown pathway other than a PI 3-kinase → p44/42 MAP kinases → cPLA2 pathway, because staurosporine-induced PGE2 production was completely suppressed by the PI 3-kinase inhibitor LY294002 (Fig. 8A), although staurosporine-induced phosphorylation of cPLA2 was only partially inhibited (Fig. 7B).

Fig. 9. Effects of Ro-31-8220 on staurosporine-induced phosphorylation of p44/42 MAP kinases and PGE2 production and COX-2 protein expression. Rat peritoneal macrophages were incubated at 37°C for 15 min to determine phosphorylation levels of p44/42 MAP kinases (C) or for 8 h to determine PGE2 production (A) and COX-2 protein expression (B) in the presence or absence of staurosporine (SS) (63 nM) and the indicated concentrations of Ro-31-8220. A, PGE2 concentrations in the conditioned medium were determined by radioimmunoassay. N.D., not detectable. Values are the means ± S.E. from four samples. Statistical significance: **P < .01; ***P < .001 versus SS (63 nM) alone. COX-2 (B) and p44/42 MAP kinases (C) were detected by Western blot analysis. The results were confirmed by repeating two independent sets of experiments in the presence or absence of staurosporine (SS) (63 nM) and the indicated concentrations of Ro-31-8220. p44/42 MAP kinases were detected by Western blot analysis. The results were confirmed by repeating two independent sets of experiments.

Fig. 10. Effects of down-regulation of PKC by TPA pretreatment on staurosporine-induced PGE2 production. Rat peritoneal macrophages were incubated at 37°C for 20 h in the presence of TPA (816 nM). The cells were then washed and further incubated at 37°C for 8 h in the presence (+) or absence (−) of staurosporine (SS) (63 nM) or TPA (47 nM). PGE2 concentrations in the conditioned medium were determined by radioimmunoassay. Values are the means ± S.E. from four samples. Statistical significance: ***P < .001 versus no stimulation without TPA pretreatment; †††P < .001 versus no stimulation with TPA pretreatment. The results were confirmed by repeating two independent sets of experiments.

There are conflicting reports on the activation of p44/42 MAP kinases by PI 3-kinase. In Chinese hamster ovary cells, the activation of p44/42 MAP kinases by platelet-derived growth factor is dependent on PI 3-kinase, but in Swiss 3T3 cells, the activation depends not on PI 3-kinase but on PKC (Duckworth and Cantley, 1997). PKC-dependent activation of MAP kinase cascade is also reported in Cos cells (Berra et al., 1995). However, in mouse lymph node T cells, the MAP kinase cascade is activated by PI 3-kinase (Eder et al., 1998). In this study, we demonstrated that PI 3-kinase, but not PKC, plays a significant role in staurosporine-induced phosphorylation of p44/42 MAP kinases in rat peritoneal macrophages. Thus, the role of PI 3-kinase and PKC in the activation of p44/42 MAP kinases seems to vary with type of cell.

In contrast, the staurosporine-induced COX-2 protein expression was suppressed by the PKC inhibitor Ro-31-8220 (Fig. 9B), but not by the PI 3-kinase inhibitor LY294002 (Fig. 8B). The MEK inhibitors PD98059 and U0126 partially inhibited the staurosporine-induced COX-2 protein expression (Figs. 4B and 5D). According to Beltman et al. (1996), the PKC inhibitor Ro-31-8220 inhibits MAP kinase phosphatase-1, which inactivates p44/42 MAP kinases. However, as shown in Fig. 9, Ro-31-8220 inhibited the staurosporine-induced COX-2 protein expression without affecting the phosphorylation of p44/42 MAP kinases. Therefore, inhibition of MAP kinase phosphatase-1 might not be involved in the mechanism for the inhibition by Ro-31-8220 of the stauro-
Therefore, it is suggested that aPKC responsible for the staurosporine-induced expression of COX-2 protein. In support of this, in IL-1-stimulated rat peritoneal macrophages were incubated at 37°C for 20 h in the presence of TPA (816 nM). The cells were then washed and further incubated at 37°C for 15 min to determine phosphorylation levels of p44/42 MAP kinases (A) and for 8 h to determine COX-2 protein expression (B) in the presence (+) or absence (−) of staurosporine (SS) (63 nM) or TPA (47 nM). p44/42 MAP kinases (A) and COX-2 protein expression (B) were detected by Western blot analysis. The results were confirmed by repeating two independent sets of experiments.

Staurosporine-induced expression of COX-2 protein was abrogated completely by the PKC inhibitor, Ro-31-8220 (Fig. 9B), but only slightly inhibited by down-regulation of PKC (Fig. 11B). Down-regulation of PKC by TPA treatment occurs specifically in the cPKC and nPKC isoforms (Yahata et al., 1999). On the other hand, Ro-31-8220 can inhibit all isoforms of PKC in the cPKC and nPKC isoforms (Yahata et al., 1999). Therefore, it is suggested that aPKCζ, which is not down-regulated by TPA treatment (Yahata et al., 1999) but inhibited by Ro-31-8220 (Limatola et al., 1994), is exclusively responsible for the staurosporine-induced expression of COX-2 protein. In support of this, in IL-1-stimulated rat renal mesangial cells, activation of aPKCζ induces NF-κB-dependent expression of COX-2 protein (Rzymkiewicz et al., 1996).

A) p44 MAPK p42 MAPK

SS (63 nM) − + − + −
TPA (47 nM) − + − + −

B) COX-2

SS (63 nM) − + − + −
TPA (47 nM) − + − + −

Pretreatment with TPA

Fig. 11. Effects of down-regulation of PKC by TPA pretreatment on staurosporine-induced phosphorylation of p44/42 MAP kinases and COX-2 protein expression. Rat peritoneal macrophages were incubated at 37°C for h in the presence of TPA (816 nM). The cells were then washed and further incubated at 37°C for min to determine phosphorylation levels of p44/42 MAP kinases (A) and for h to determine COX-2 protein expression (B) in the presence (+) or absence (−) of staurosporine (SS) (63 nM) or TPA (47 nM). p44/42 MAP kinases (A) and COX-2 protein expression (B) were detected by Western blot analysis. The results were confirmed by repeating two independent sets of experiments.

Staurosporine-induced expression of COX-2 protein expression. Because staurosporine induces phosphorylation of other MAP kinases such as c-Jun N-terminal kinase (Yao et al., 1997) and p38 MAP kinase (Xiao et al., 1999), of which activation causes increase in the expression of COX-2 gene (Xie and Herrschman, 1995) and COX-2 protein (Ridley et al., 1997), it is possible that several different MAP kinase pathways are partially involved in the staurosporine-induced COX-2 protein expression. But these findings indicate that PKC plays a significant role in the expression of COX-2 protein induced by staurosporine.

Staurosporine-induced expression of COX-2 protein was abrogated completely by the PKC inhibitor, Ro-31-8220 (Fig. 9B), but only slightly inhibited by down-regulation of PKC (Fig. 11B). Down-regulation of PKC by TPA treatment occurs specifically in the cPKC and nPKC isoforms (Yahata et al., 1999). On the other hand, Ro-31-8220 can inhibit all isoforms of PKC, including aPKCζ (Limatola et al., 1994). Therefore, it is suggested that aPKCζ, which is not down-regulated by TPA treatment (Yahata et al., 1999) but inhibited by Ro-31-8220 (Limatola et al., 1994), is exclusively responsible for the staurosporine-induced expression of COX-2 protein. In support of this, in IL-1-stimulated rat renal mesangial cells, activation of aPKCζ induces NF-κB-dependent expression of COX-2 protein (Rzymkiewicz et al., 1996).

The mechanism for the aPKCζ activation by staurosporine at lower concentrations has not been clarified. However, it is reported that staurosporine induces translocation of cPKC (Wolf and Bagnioli, 1988), nPKC (Sawai et al., 1997), and aPKC (O’Connell et al., 1997) from the cytosol to the membrane as the PKC activator TPA does (Kraft and Anderson, 1983). In addition, it is also reported that staurosporine, at less than 100 nM concentrations, inhibits cPKC and nPKC but does not inhibit aPKC (Geiges et al., 1997). Therefore, aPKC might play a significant role in staurosporine-induced events at lower concentrations.

Additional investigation is necessary to clarify the target molecules of staurosporine, because such molecules seem to be very important for the induction of many cellular functions in a variety of cells. For example, staurosporine induces neurite outgrowth of PC12 cells (Hashimoto and Hagino, 1989), macrophage inflammatory protein-2 production in rat peritoneal neutrophils (Edamatsu et al., 1997; Xiao et al., 1999), and IL-6 production in rat peritoneal macrophages (Yamaki and Ohuchi, 1999). It is reported that Ras activates PI 3-kinase (Sjolander et al., 1991) and PKC (Dominguez et al., 1992). Therefore, Ras might be a candidate for the target molecules of staurosporine. In NIH 3T3 cells, staurosporine activates the stress-responsive protein kinases, Krs-1 and Krs-2 (Taylor et al., 1996), which are related to Ste20p encoding a serine/threonine kinase that acts upstream of MEK kinase in the pheromone-responsive MAP kinase pathway and other pathways in yeast. Therefore, Krs-1 and Krs-2 might regulate activation of the PI 3-kinase → p44/42 MAP kinases pathway by staurosporine.

In conclusion, our findings suggest that staurosporine induces PGE2 production by two independent pathways, a PI 3-kinase → p44/42 MAP kinases pathway and a PKC → COX-2 pathway.

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