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Participation of various kinases in staurosporine-induced apoptosis of RAW 264.7 cells

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Abstract

Staurosporine induced apoptosis of RAW 264.7 cells, a mouse macrophage-like cell line, as determined by DNA fragmentation, the increase of annexin V-stained cells, and the cleavage of poly(ADP-ribose)polymerase (PARP), a substrate of caspase. Analysis of the increase in the percentage of sub-G_1 cells revealed that the DNA fragmentation occurred in a time- and concentration-dependent manner at 0.021–2.1 μM of staurosporine. Staurosporine induced phosphorylation of p38 mitogen-activated protein kinase (MAPK) but suppressed spontaneous phosphorylation of p44/42 MAPK. The p38 MAPK inhibitor SB203580, the MAPK/extracellular signal-regulated kinase kinase inhibitor PD98059 and the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 potentiated the staurosporine-induced PARP cleavage and DNA fragmentation. The protein kinase A (PKA) inhibitor H-89 potentiated the staurosporine-induced DNA fragmentation without potentiating the PARP cleavage. In contrast, the protein kinase C (PKC) inhibitor Ro-31-8425 suppressed the PARP cleavage and DNA fragmentation. These findings suggested that staurosporine induces apoptosis via the caspase cascade in RAW 264.7 cells. The staurosporine-induced apoptosis is positively regulated by PKC, negatively regulated by p38 MAPK, p44/42 MAPK and PI3K via the caspase cascade, and negatively regulated by PKA without regulation of caspase activation.

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Introduction

Apoptosis is an essential event in several physiological and pathological processes such as development, selection in immune systems, neurodegenerative diseases, host defence against pathogens, and inflammation (Hardy 1999; Mattson et al 2001; Mountz et al 2001; Rathmell & Thompson 2002). The term apoptosis describes a tightly regulated process of cell death characterized by plasma membrane blebbing, chromatin condensation and DNA fragmentation (Leist & Nicotera 1997). During the process of apoptosis, *ced*-3 family proteins are cleaved and activated (Orth et al 1996).

Recently, several target proteins, including poly(ADP-ribose)polymerase (PARP), lamin A, lamin B and actin, have been identified that are cleaved by caspases, *ced*-3 homologues, during the apoptotic process (Porter et al 1997). The activated caspases also cleave the important signalling molecules such as protein kinase C (PKC) δ (Ghayur et al 1996) and mitogen-activated protein kinase kinase 1 (Cardone et al 1997). In contrast, AKT/protein kinase B is inactivated through cleavage by the activated caspases (Bachelder et al 1999). It is generally thought that these cleaved molecules have roles in the modulation of the apoptosis process. In addition, the apoptosis process is also regulated by other intracellular signalling molecules such as p38 mitogen-activated protein kinase (MAPK) (Gálan et al 2000), p44/42 MAPK (Chin et al 1999), PKC (Jun et al 1999) and phosphatidylinositol 3-kinase (PI3K) (Koh et al 1998).

Staurosporine, originally identified as a PKC inhibitor (Tamaoki et al 1986), induces apoptosis of Jurkat cells (Na et al 1996) and human neuroblastoma SH-SY5Y cells (Bijur et al 2000). On the other hand, staurosporine induces production of macrophage inflammatory protein-2 in rat peritoneal neutrophils (Xiao et al 1999) and interleukin-6 (Yamaki & Ohuchi 1999) and prostaglandin E_2 (Yamaki et al 2000) in rat peritoneal macrophages at lower concentrations (2.1–63 nM) than those inducing apoptosis

(100–1000 nM) (Na et al 1996; Bijur et al 2000). The staurosporine-induced production of interleukin-6 (Yamaki & Ohuchi 1999) and prostaglandin E_2 (Yamaki et al 2000) in rat peritoneal macrophages is mediated by activation of PKC and PI3K, and the staurosporine-induced production of macrophage inflammatory protein-2 in rat peritoneal neutrophils is mediated by activation of p38 MAPK and p44/42 MAPK (Xiao et al 1999). However, the modulation mechanism of these kinases by staurosporine and the role of these kinases in staurosporine-induced apoptosis remain to be elucidated. In this study, we analysed pharmacologically the roles of p38 MAPK, p44/42 MAPK, PI3K, PKC and PKA in staurosporine-induced apoptosis of RAW 264.7 cells, a murine macrophage-like cell line, from the view point of caspase activation.

Materials and Methods

Drugs

The drugs used were PD98059 (2′-amino-3′-methoxyflavone) (New England Biolabs Inc., Beverly, MA), LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one), SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole), Ro-31-8425 (2-[8-(aminomethyl) 6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl]-3-(1-methyl-indol-3-yl)maleimide, hydrochloride), H-89 (*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide) (Calbiochem Novabiochem Japan, Tokyo, Japan), calphostin C (2-(12-[2-(benzyloxy)-propyl]-3,10-dihydro-4,9-dihydroxy-2,6,7,11-tetramethoxy-3,10-dioxo-1-perylenyl)-1-methylethylcarbonic acid 4-hydroxyphenyl ester) (BIOMOL Res. Lab., Plymouth Meeting, PA) and staurosporine (9,13-epoxy-1*H*,9*H*-diindolo[1,2,3-gh : 3′,2′,1′ - lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one,2,3,10,11,12,13-hexahydro-10-methoxy-9-methyl-11-(methylamino)-, (9S,10R,11R,13R)-(9CI)) (Kyowa Medex, Tokyo, Japan).

These drugs were dissolved in dimethyl sulfoxide. The final concentration of the vehicle was adjusted to 0.2% (v/v) and the control medium contained the same amount of the vehicle.

Cell culture

RAW 264.7 cells were obtained from the RIKEN Gene Bank (Tsukuba, Japan) and cultured at 37°C under 5% CO_2–95% air in Eagle's minimal essential medium (EMEM) (Nissui Seiyaku, Tokyo, Japan) containing 10% (v/v) fetal bovine serum (Dainippon Pharmaceutical, Osaka, Japan) and 1% (v/v) non-essential amino acid solution (Sigma Chemical Co., St Louis, MO).

Detection of DNA fragmentation by electrophoresis

RAW 264.7 cells (4.0×10^6 cells per 60-mm dish; CORNING Coster Japan, Tokyo, Japan) were incubated at 37°C for 24 h in medium containing staurosporine (2.1 μM). After incubation, cells were washed twice with phosphate-buffered saline (PBS) and lysed by the addition of 0.5 mL of lysis buffer (Tris, 50 mM; EDTA, 10 mM; proteinase K (Sigma), 100 μg mL^{-1}, Triton X-100, 1% (v/v); pH 8.0) at 55°C for 16 h. DNA was extracted twice with phenol–chloroform–isoamylalcohol (25:24:1, v/v), and precipitated with a 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol at −20°C. Precipitated DNA was dissolved in TE buffer (Tris, 1 mM; EDTA, 0.1 mM; ribonuclease I (Sigma), 20 μg mL^{-1}; pH 7.5) and incubated at 37°C for 30 min. Electrophoresis of DNA was performed in 1.4% (w/v) agarose gel at 50 V for 3 h. After electrophoresis, DNA was visualized by ethidium bromide staining and photographed.

Measurement of DNA fragmentation by flow cytometry

For measurement of DNA fragmentation, RAW 264.7 cells (1×10^6 cells per well of a 12-well plate; CORNING Coster Japan) were incubated at 37°C for various periods in medium in the presence or absence of drugs. After incubation, the cells were washed three times with PBS and detached from the dish by 0.02% (w/v) EDTA in PBS. The cells were then fixed with 70% (v/v) ethanol at −20°C for 3 h, and incubated in PBS containing 50 μg mL^{-1} propidium iodide (Wako Pure Chemical Inc. Osaka, Japan) and 20 μg mL^{-1} ribonuclease I (Sigma) at 37°C for 5 min. Subsequently, the fluorescence intensity of propidium iodide in 10000 cells was analysed by flow cytometry using FACScan (Becton Dickinson), and the percentage of sub-G_1 cells was calculated using LISYS II software.

Measurement of annexin V binding by flow cytometry

For measurement of annexin V binding, RAW 264.7 cells (1×10^6 cells per 1.5-mL tube; CORNING Coster Japan) were incubated at 37°C for 3.25 h in medium in the presence or absence of drugs. After incubation, the cells were washed three times with PBS and stained by propidium iodide and Annexin-V-FLUOS according to the manufacturer's protocol using Annexin-V-FLUOS Staining Kit (Roche Diagnostics GmbH, Mannheim, Germany). Subsequently, the fluorescence intensity of propidium iodide and Annexin-V-FLUOS in 10000 cells was analysed by flow cytometry using FACScan (Becton Dickinson), and the percentages of cells in quadrants were calculated by CELLQuest Software.

Western blot analysis

For Western blot analysis, RAW 264.7 cells (2×10^6 cells per well of a 6-well plate; CORNING Coster Japan) were incubated at 37°C for various periods in medium in the

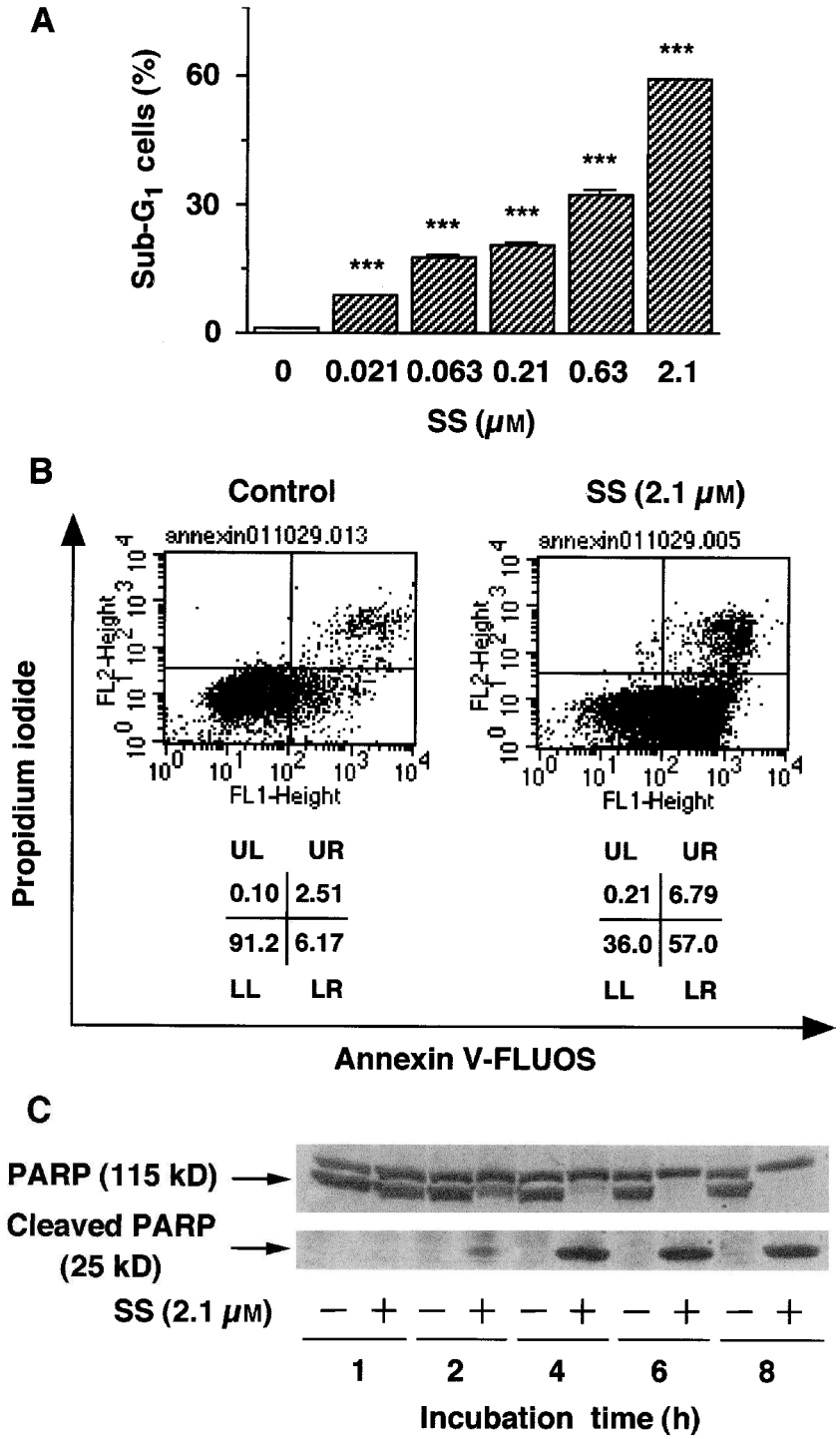

Figure 1 Effects of staurosporine on apoptosis and caspase activation. A. RAW 264.7 cells were incubated at 37°C for 24 h in the presence of the indicated concentrations of staurosporine (SS). Values are the means from 4 samples. $***P < 0.001$ vs control. B. RAW 264.7 cells were incubated at 37°C for 3.25 h in the presence or absence of SS (2.1 μM). UL, upper left quadrant (not cells); UR, upper right quadrant (late apoptotic and necrotic cells); LL, lower left quadrant (viable cells); and LR, lower right quadrant (early apoptotic cells). C. RAW 264.7 cells were incubated at 37°C for the period indicated in the presence (+) or absence (−) of SS (2.1 μM). Higher-molecular-weight band above poly(ADP-ribose)polymerase (PARP) (115 kD) is a non-specific band.

presence or absence of drugs. After incubation, the cells were washed three times with PBS and lysed in ice-cold lysis buffer (HEPES, 20 mM; EDTA, 1 mM; NaF, 50 mM; *p*-nitrophenyl phosphate, 2.5 mM; Na_3VO_4, 1 mM; leupeptin, 10 μg mL^{-1}; Triton X-100, 1% (v/v); glycerol, 10% (v/v); pH 7.3), sonicated using a handy sonic

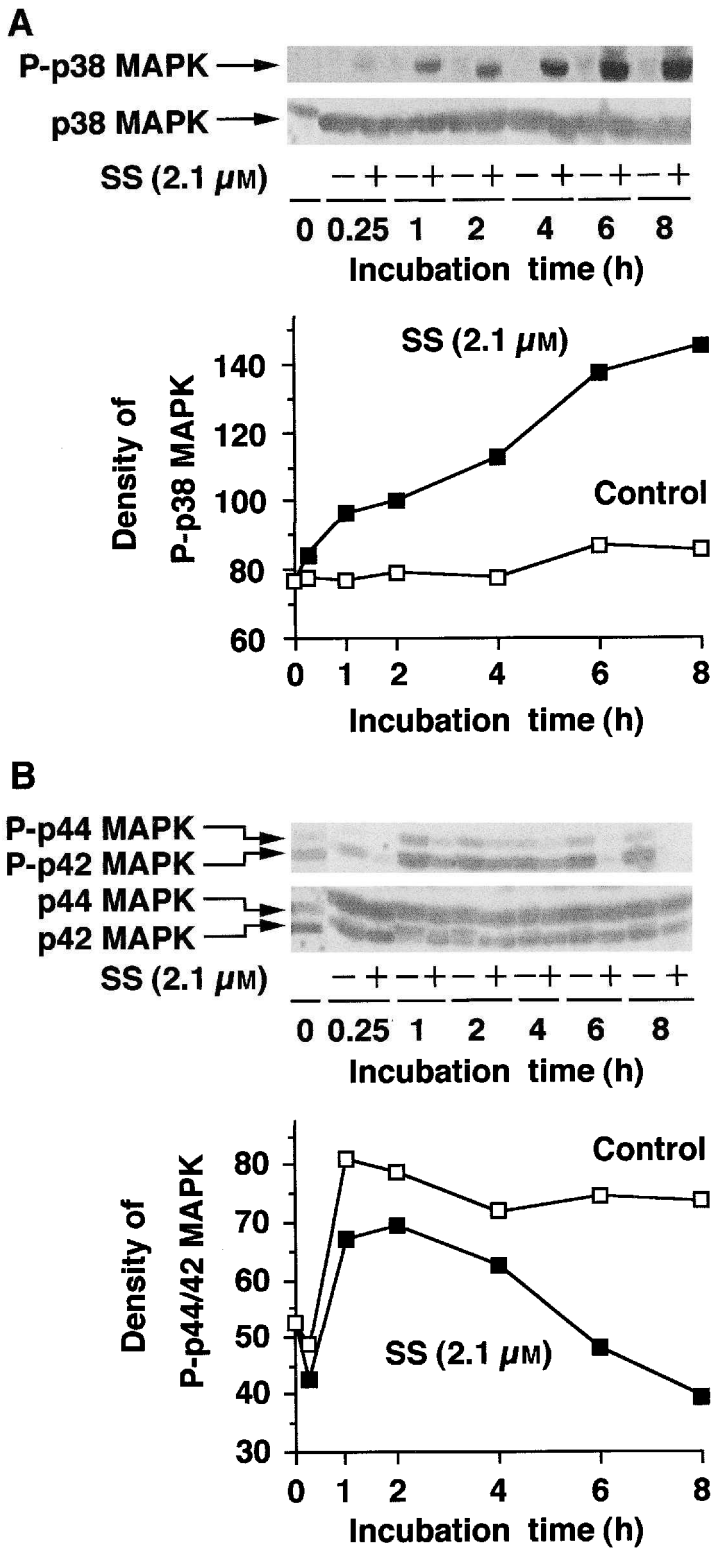

Figure 2 Effects of staurosporine on phosphorylation of p38 MAPK and p44/42 MAPK. RAW 264.7 cells were incubated at 37°C for the period indicated in the presence (+) or absence (−) of staurosporine (SS, 2.1 μM). p38 MAPK and phosphorylated p38 MAPK (P-p38 MAPK) (A), and p44/42 MAPK and phosphorylated p44/42 MAPK (P-p44/42 MAPK) (B) were detected by Western blotting. Each band of P-p38 MAPK (A) and P-p44/42 MAPK (B) was quantified densitometrically.

disrupter (Tomy Seiko Co., Tokyo, Japan) and centrifuged at 12000 ***g*** for 20 min. Protein contents in the supernatant were determined (Bradford 1976) and 50 μg protein was loaded on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. After electrophoresis, the gel was transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Immunoblotting was carried out by using antibodies to PARP (Santa Cruz Biotechnology Inc., Santa Cruz, CA), phosphorylated-p44/42 MAPK (New England Biolabs Inc.), rat MAP kinase R2 (erk1-CT) (Upstate Biotechnology Inc., Lake Placid, NY), phospho-p38 (New England Biolabs Inc.) and p38 (Santa Cruz).

Statistical analysis

The statistical significance of the results was analysed by Dunnett's test for multiple comparisons and Student's *t*-test for unpaired observations.

Results

Induction of apoptosis by staurosporine

Incubation of RAW 264.7 cells for 4 h in the presence of staurosporine (2.1 μM) induced DNA fragmentation as revealed by a ladder-like pattern on electrophoresis (data not shown). At 24 h, control cells showed no DNA fragmentation. Flow cytometry analysis for sub-G_1 cells also demonstrated that staurosporine (2.1 μM) induced DNA fragmentation at 2 h and thereafter (data not shown). The percentage of sub-G_1 cells at 24 h was increased by staurosporine in a concentration-dependent manner from 0.021 to 2.1 μM (Figure 1A). In addition, Annexin-V-FLUOS and propidium iodide double staining analysis revealed that staurosporine (2.1 μM) increased Annexin-V-FLUOS-positive and propidium iodide-negative cells after 3.25 h incubation (Figure 1B).

Induction of caspase activation by staurosporine

Western blot analysis revealed that staurosporine (2.1 μM) induced the cleavage of 115 kDa PARP (p115 PARP) and generated the 25 kDa protein (p25 PARP), a cleaved form of p115 PARP, at 2 h and thereafter (Figure 1C).

Effects of staurosporine on phosphorylation of p38 MAPK and p44/42 MAPK

Phosphorylation of p38 MAPK was not detected in control cells, but was strongly induced by treatment with staurosporine (2.1 μM) (Figure 2A). The phosphorylation of p38 MAPK by staurosporine was detected at 15 min and thereafter, increasing time dependently until 8 h (Figure 2A). On the other hand, phosphorylation of p44/42 MAPK was observed in control cells, and maximum phosphorylation was detected 1 h after incubation (Figure 2B). In the presence of staurosporine (2.1 μM), the spontaneous phosphorylation of p44/42 MAPK decreased from 15 min to 8 h (Figure 2B).

Effects of the p38 MAPK inhibitor SB203580, the MAPK/extracellular signal-regulated kinase kinase inhibitor PD98059 and the PI3K inhibitor LY294002 on staurosporine-induced apoptosis

The cleavage of PARP induced by staurosporine (2.1 μM) at 6 h was augmented by 10 μM of SB203580 (Figure 3A), 50 μM of PD98059 (Figure 4A) and 50 μM of LY294002 (Figure 5A). In the control cells, SB203580 (10 μM), PD98059 (50 μM) and LY294002 (50 μM) did not induce PARP cleavage (Figures 3A, 4A and 5A). The percentage of sub-G_1 cells was increased time-dependently by treatment with 2.1 μM of staurosporine (Figures 3B, 4B and 5B).

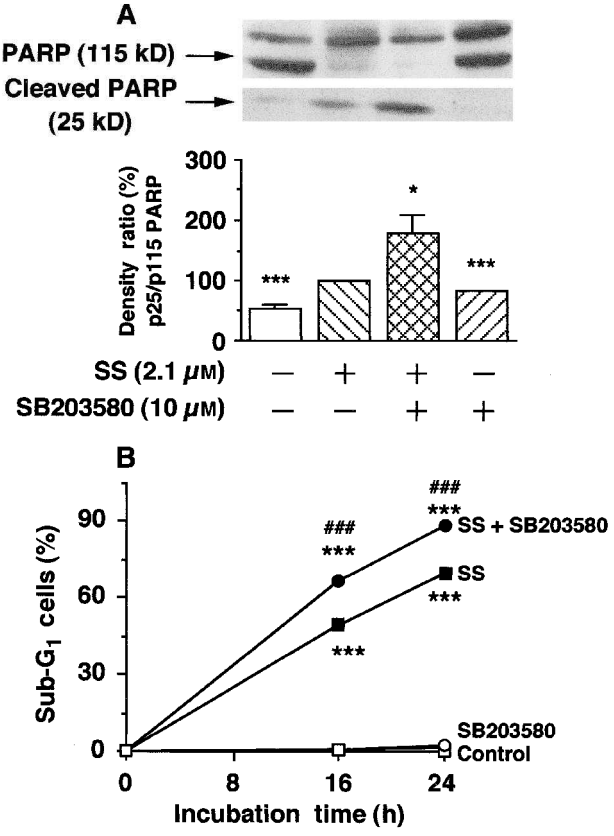

Figure 3 Effects of SB203580, a p38 MAPK inhibitor, on staurosporine-induced DNA fragmentation and PARP cleavage. RAW 264.7 cells were incubated at 37°C for 6 h (A) and for the period indicated (B) in the presence (+) or absence (−) of staurosporine (SS, 2.1 μM) and SB203580 (10 μM). Values are the means±s.e.m. of 3 separate experiments (A) and the means of 4 samples (B). Density ratio in SS control is set to 100% (A). A. $*P < 0.05$, $***P < 0.001$ vs SS (2.1 μM) alone. B. $***P < 0.001$ vs corresponding control or SB203580 alone; $\#\#\#P < 0.001$ vs SS (2.1 μM) alone.

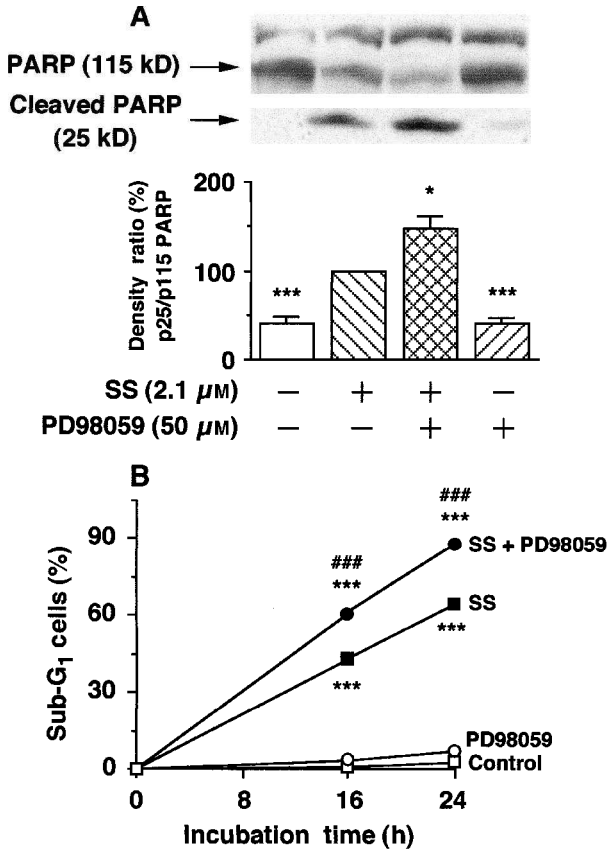

Figure 4 Effects of PD98059, an MEK inhibitor, on staurosporine-induced DNA fragmentation and PARP cleavage. RAW 264.7 cells were incubated at 37°C for 6 h (A) and for the period indicated (B) in the presence (+) or absence (−) of staurosporine (SS, 2.1 μM) and PD98059 (50 μM). Values are the means±s.e.m. of 3 separate experiments (A) and the means of 4 samples (B). Density ratio in SS control is set to 100% (A). A. $^{*}P < 0.05$, $^{***}P < 0.001$ vs SS (2.1 μM) alone. B. $^{***}P < 0.001$ vs corresponding control or PD98059 alone; $^{###}P < 0.001$ vs SS (2.1 μM) alone.

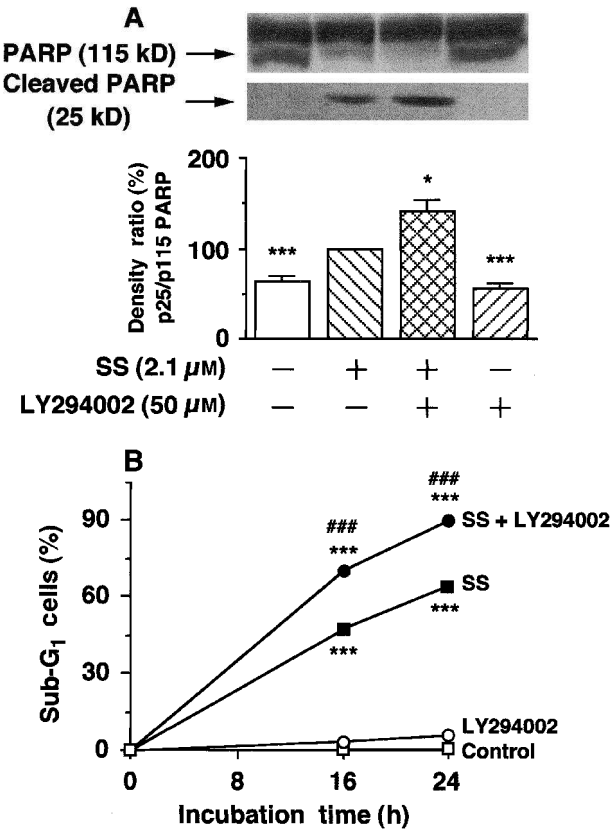

Figure 5 Effects of LY294002, a PI3K inhibitor, on staurosporine-induced DNA fragmentation and PARP cleavage. RAW 264.7 cells were incubated at 37°C for 6 h (A) and for the period indicated (B) in the presence (+) or absence (−) of staurosporine (SS, 2.1 μM) and LY294002 (50 μM). Values are the means±s.e.m. of 3 separate experiments (A) and the means of 4 samples (B). Density ratio in SS control is set to 100% (A). A. $^{*}P < 0.05$, $^{***}P < 0.001$ vs SS (2.1 μM) alone. B. $^{***}P < 0.001$ vs corresponding control or LY294002 alone; $^{###}P < 0.001$ vs SS (2.1 μM) alone.

SB203580 (10 μM), PD98059 (50 μM) and LY294002 (50 μM) further increased the percentage of sub-G_1 cells 16 and 24 h after treatment with staurosporine (Figures 3B, 4B and 5B). At 24 h, 3 μM SB203580, 15 μM PD98059 and 15 μM LY294002 also increased the percentage of sub-G_1 cells significantly ($P < 0.05$) (data not shown). These findings suggest that p44/42 MAPK, p38 MAPK and PI3K participate in suppressing the staurosporine-induced apoptosis.

Effects of Ro-31-8425, a PKC inhibitor, on staurosporine-induced apoptosis

The staurosporine-induced cleavage of PARP at 6 h was partially suppressed by 10 μM Ro-31-8425 (Figure 6A). The percentage of sub-G_1 cells was also decreased by 10 μM Ro-31-8425 at 16 and 24 h (Figure 6B). Ro-31-8425, at 3 μM, decreased the percentage of sub-G_1 cells significantly ($P < 0.05$) at 24 h (data not shown). The effect of calphostin C, another specific PKC inhibitor, on staurosporine-induced apoptosis was also examined. It was shown that calphostin C, at 1 and 3 μM, inhibited the staurosporine-induced increase in the percentage of sub-G_1 cells in a concentration-dependent manner. After 24 h incubation, about 30% of staurosporine-induced increase in the percentage of sub-G_1 cells was significantly ($P < 0.001$) suppressed by calphostin C (3 μM) (data not shown). These findings suggest that PKC participates in the promotion of apoptosis induced by staurosporine.

Effects of H-89, a PKA inhibitor, on staurosporine-induced apoptosis

Although the staurosporine-induced cleavage of PARP was not affected by H-89 (15 μM) (Figure 7A), the per-

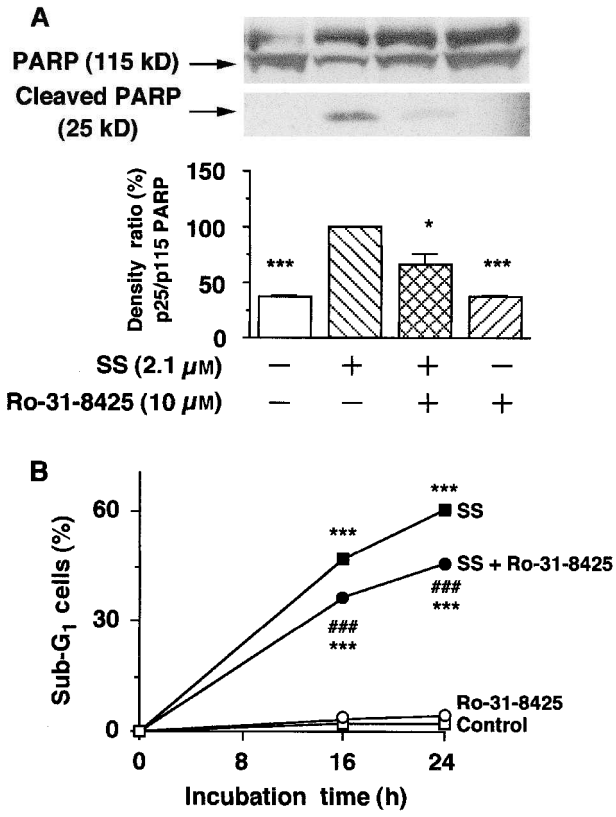

Figure 6 Effects of Ro-31-8425, a PKC inhibitor, on staurosporine-induced DNA fragmentation and PARP cleavage. RAW 264.7 cells were incubated at 37°C for 6 h (A) and for the period indicated (B) in the presence (+) or absence (−) of staurosporine (SS, 2.1 μM) and Ro-31-8425 (10 μM). Values are the means±s.e.m. of 3 separate experiments (A) and the means of 4 samples (B). Density ratio in SS control is set to 100% (A). A. $^{*}P < 0.05$, $^{***}P < 0.001$ vs SS (2.1 μM) alone. B. $^{***}P < 0.001$ vs corresponding control or Ro-31-8425 alone; $^{\#\#\#}P < 0.001$ vs SS (2.1 μM) alone.

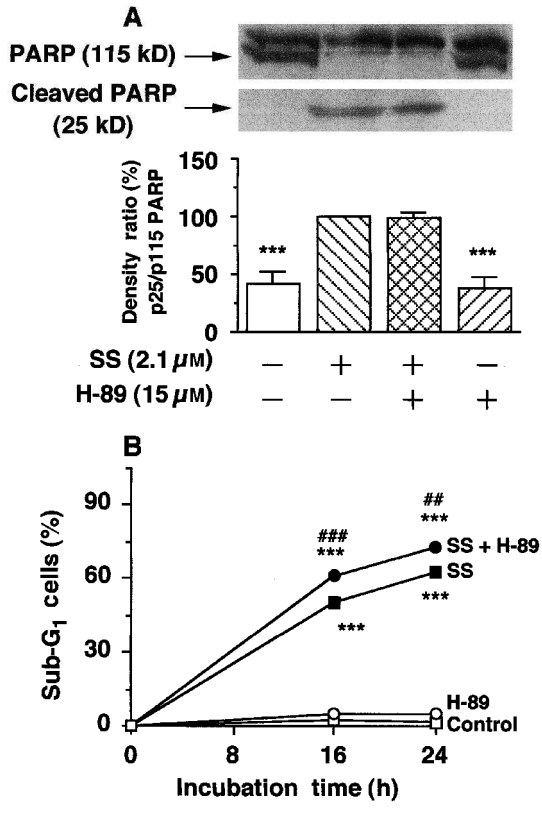

Figure 7 Effects of H-89, a PKA inhibitor, on staurosporine induced DNA fragmentation and PARP cleavage. RAW 264.7 cells were incubated at 37°C for 6 h (A) and for the period indicated (B) in the presence (+) or absence (−) of staurosporine (SS, 2.1 μM) and H-89 (15 μM). Values are the means±s.e.m. of 3 separate experiments (A) and the means of 4 samples (B). Density ratio in SS control is set to 100% (A). A. $^{***}P < 0.001$ vs SS (2.1 μM) alone. B. $^{***}P < 0.001$ vs corresponding control or H-89 alone; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs SS (2.1 μM) alone.

centage of sub-G_1 cells was significantly increased at 16 and 24 h (Figure 7B). These findings suggest that PKA negatively regulates staurosporine-induced apoptosis without affecting caspase activity.

Discussion

In staurosporine-induced apoptosis, development of membrane asymmetry, caspase activation and DNA fragmentation are reported in Chinese hamster ovary cells (Godard et al 1999), oligodendrocytes (Scurlock & Dawson 1999) and prostate cancer cells (Marcelli et al 2000). A similar process has been reported for tumour necrosis factor α-induced apoptosis of bovine glomerular endothelial cells (Messmer et al 1999) and Fas-induced apoptosis in human lymphocytes (Ogura & Handa 2000). In RAW 264.7 cells, development of membrane asymmetry (Figure 1B), activation of caspase (Figure 1C), fragmentation of DNA (Figure 1A) and activation of p38 MAPK (Figure 2A) were induced by staurosporine. In BJAB human B lymphoma cells, staurosporine induces an activation of p38 MAPK, like Fas, via the cleavage of the Ste20-like protein kinase Mst1 by caspases (Graves et al 1998). Because staurosporine-induced apoptosis was enhanced by the p38 MAPK inhibitor SB203580 (Figure 3), it was suggested that the staurosporine-induced apoptosis is negatively regulated by activation of p38 MAPK. However, in RAW 264.7 cells, Jun et al (1999) reported that the inhibition of p38 MAPK by SB203580 partially inhibits apoptosis induced by sodium nitroprusside, and Callsen & Brune (1999) reported that the inhibition of p38 MAPK by SB203580 has no effect on apoptosis induced by *S*-nitrosoglutathione. Thus, the role of p38 MAPK in apoptosis seems to vary with the type of apoptosis inducer.

In RAW 264.7 cells, apoptosis induced by serum deprivation is rescued by transforming growth factor β1 (TGF-β1) through activation of p42 MAPK (Chin et al 1999). Activation of p44/42 MAPK inhibits the release of cytochrome c from mitochondria and activation of caspase

(Erhardt et al 1999). Activation of MEK, upstream of p44/42 MAPK, inactivates the pro-apoptotic Bcl-2 family protein Bad (Scheid & Duronio 1998). These reports suggest that p44/42 MAPK activated by survival factors, including serum and TGF-β1, is anti-apoptotic. On the other hand, p44/42 MAPK activated directly by apoptosis-inducing agents such as nitric oxide (Mohr et al 1998), tumour necrosis factor-α (Chin et al 1998) and hyperoxia (Petrache et al 1999) is reported to have pro-apoptotic activity in RAW 264.7 cells. In RAW 264.7 cells, the phosphorylation of p44/42 MAPK in the absence of staurosporine (Figure 2B) might be caused by serum stimulation, as reported by Flamigni et al (2001) in human ECV304 cells. This serum-induced phosphorylation of p44/42 MAPK was moderately inhibited by staurosporine treatment (Figure 2B), indicating that staurosporine does not activate p44/42 MAPK in RAW 264.7 cells. Because the staurosporine-induced apoptosis was enhanced on inhibition of p44/42 MAPK activation by the MEK inhibitor PD98059 (Figure 4), the serum-induced activation of the MEK-p44/42 MAPK pathway is suggested to negatively regulate the staurosporine-induced apoptosis. Therefore, our findings support the hypothesis that under conditions of apoptosis where p44/42 MAPK is not activated, such as in staurosporine-induced apoptosis in RAW 264.7 cells, p44/42 MAPK has an anti-apoptotic function.

PI3K is activated by lysophosphatidic acid in murine macrophages (Koh et al 1998) and by growth factors in human vascular endothelial cells (Zeng et al 2000). In this study, we showed that the inhibition of PI3K by LY294002 enhanced the staurosporine-induced apoptosis in RAW 264.7 cells (Figure 5). This finding suggests that PI3K activated by staurosporine has an anti-apoptotic activity in RAW 264.7 cells. Previously, we reported that staurosporine-induced production of interleukin-6 (Yamaki & Ohuchi 1999) and prostaglandin E_2 (Yamaki et al 2000) in rat peritoneal macrophages was also inhibited by LY294002, suggesting that the activation of PI3K by staurosporine is important for the production of interleukin-6 and prostaglandin E_2. However, Wan et al (2002) reported that staurosporine reduced the phospho-Akt level in Ishikawa cells; namely, staurosporine inhibits PI3K and induces apoptosis. The discrepancy might be due to the difference in cell type. It is also possible that the LY294002-sensitive kinases, but not PI3K, such as DNA-dependent protein kinase (Izzard et al 1999), play an important role in staurosporine-induced changes of cellular functions.

Inhibition of PKC by Ro-31-8425 suppressed the staurosporine-induced apoptosis in RAW 264.7 cells (Figure 6). The inhibition also suppresses *Actinobacillus actinomycetemcomitans*-induced apoptosis of murine macrophage cell line J774.1 (Nonaka et al 1998). Although these findings suggest pro-apoptotic activity of PKC, the mechanism by which PKC enhances apoptosis remains to be elucidated. The PKC family consists of 11 isozymes that seem to share contradictory roles in apoptosis. Moreover, their activation depends on various factors, such as requirements of calcium, diacylglycerols and phospholipids, molecular locations (Newton 1995) and cleavage by caspases (Emoto et al 1995). Among the PKC isozymes, PKCδ (which is cleaved and activated by caspase) is especially important for apoptosis (Ghayur et al 1996). The activation of PKCδ causes the activation of caspase (Basu & Akkaraju 1999) that, in turn, induces apoptosis in RAW 264.7 cells (Jun et al 1999). As we have reported (Yamaki & Ohuchi 1999; Yamaki et al 2000), staurosporine seems to activate some kind of PKC isozyme in rat peritoneal macrophages. It is possible that staurosporine activates PKCδ by caspase activation to induce apoptosis, because the PKC inhibitors Ro-31-8425 (Figure 6) and calphostin C (data not shown) inhibited staurosporine-induced apoptosis. It is also possible that the inhibition of PKC by Ro-31-8425 or calphostin C increases the staurosporine-induced activation of p38 MAPK, thus inhibiting staurosporine-induced apoptosis. Actually, inhibition of PKCζ activates p38 MAPK (Berra et al 1997) and the overexpression of some kind of PKC isozyme suppresses sodium nitroprusside-induced p38 MAPK activation (Jun et al 1999). Therefore, it is possible that Ro-31-8425 and calphostin C inhibit PKCζ, which is hardly inhibited but rather activated by staurosporine (Yamaki et al 2000), and thus activate p38 MAPK and inhibit staurosporine-induced apoptosis.

Because staurosporine potentiates cyclic AMP-dependent promoter activity in PC12 cells (Sasaki et al 1995), we examined the role of PKA in staurosporine-induced apoptosis of RAW 264.7 cells. As shown in Figure 7B, staurosporine-induced increase in the percentage of sub-G_1 cells was potentiated by the PKA inhibitor H-89, suggesting that PKA suppresses staurosporine-induced apoptosis of RAW 264.7 cells. The suppressive role of PKA is also reported in nitric oxide-induced apoptosis of RAW 264.7 cells (Messmer et al 1995). Although inhibition of PKA by H-89 increased the percentage of sub-G_1 cells (Figure 7B), caspase activation was not affected (Figure 7A). However, downregulation of caspase activity by PKA is reported in apoptosis of smooth muscle cells (Orlov et al 1999). The mechanism of the caspase-independent potentiation of apoptosis by H-89 remains to be clarified.

Although the effects of kinase inhibitors examined in this study were relatively small, it has been proved that staurosporine-induced apoptosis is negatively regulated by p38 MAPK, p44/42 MAPK and PI3K, and positively regulated by PKC via regulation of caspase activity. PKA also negatively regulates the apoptosis via a caspase-independent pathway.

Conclusions

In RAW 264.7 cells, staurosporine induces apoptosis through the activation of caspase and fragmentation of DNA. Staurosporine-induced apoptosis is negatively regulated by p38 MAPK, p44/42 MAPK and PI3K, and positively regulated by PKC via regulation of caspase activity. PKA also negatively regulates the apoptosis via a caspase-independent pathway. The PKC isoform that participates in staurosporine-induced apoptosis remains to be identified.

References

Bachelder, R. E., Ribick, M. J., Marchetti, A., Falcioni, R., Soddu, S., Davis, K. R., Mercurio, A. M. (1999) p53 inhibits α6β6 integrin survival signaling by promoting the caspase 3-dependent cleavage of AKT/PKB. *J. Cell Biol.* **147**: 1063–1072

Basu, A., Akkaraju, G. R. (1999) Regulation of caspase activation and *cis*-diamminedichloroplatinum(II)-induced cell death by protein kinase C. *Biochemistry* **38**: 4245–4251

Berra, E., Municio, M. M., Sanz, L., Frutos, S., Diaz-Meco, M. T., Moscat, J. (1997) Positioning atypical protein kinase C isoforms in the UV-induced apoptotic signaling cascade. *Mol. Cell. Biol.* **17**: 4346–4354

Bijur, G. N., De Sarno, P., Jope, R. S. (2000) Glycogen synthase kinase-3β facilitates staurosporine- and heat shock-induced apoptosis. Protection by lithium. *J. Biol. Chem.* **275**: 7583–7590

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254

Callsen, D., Brune, B. (1999) Role of mitogen-activated protein kinases in *S*-nitrosoglutathione-induced macrophages apoptosis. *Biochemistry* **38**: 2279–2286

Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., Frisch, S. M. (1997) The regulation of anoikis: MEKK-1 activation requires cleavage by caspases. *Cell* **90**: 315–323

Chin, B. Y., Choi, M. E., Burdick, M. D., Strieter, R. M., Risby, T. H., Choi, A. M. (1998) Induction of apoptosis by particulate matter: role of TNF-α and MAPK. *Am. J. Physiol.* **275**: L942–L949

Chin, B. Y., Petrache, I., Choi, A. M., Choi, M. E. (1999) Transforming growth factor β1 rescues serum deprivation-induced apoptosis via the mitogen-activated protein kinase (MAPK) pathway in macrophages. *J. Biol. Chem.* **274**: 11362–11368

Emoto, Y., Manome, Y., Meinhardt, G., Kisaki, H., Kharbanda, S., Robertson, M., Ghayur, T., Wong, W. W., Kamen, R., Weichselbaum, R., Kufe, D. (1995) Proteolytic activation of protein kinase C δ by an ICE-like protease in apoptotic cells. *EMBO J.* **14**: 6148–6156

Erhardt, P., Schremser, E. J., Cooper, G. M. (1999) B-Raf inhibits programmed cell death downstream of cytochrome c release from mitochondria by activating the MEK/Erk pathway. *Mol. Cell. Biol.* **19**: 5308–5315

Flamigni, F., Facchini, A., Giordano, E., Tantini, B., Stefanelli, C. (2001) Signaling pathways leading to the induction of ornithine decarboxylase: opposite effects of p44/42 mitogen-activated protein kinase (MAPK) and p38 MAPK inhibitors. *Biochem. Pharmacol.* **61**: 25–32

Gálan, A., Garciá-Bermejo, M. L., Troyano, A., Vilaboa, N. E., De Blas, E., Kazanietz, M. G., Aller, P. (2000) Stimulation of p38 mitogen-activated protein kinase is an early regulatory event for the cadmium-induced apoptosis in human promonocytic cells. *J. Biol. Chem.* **275**: 11418–11424

Ghayur, T., Hugunin, M., Talanian, R. V., Ratnofsky, S., Quinlan, C., Emoto, Y., Pandey, P., Datta, R., Huang, Y., Kharbanda, S., Allen, H., Kamen, R., Wong, W., Kufe, D. (1996) Proteolytic activation of protein kinase C δ by an ICE/CED 3-like protease induces characteristics of apoptosis. *J. Exp. Med.* **184**: 2399–2404

Godard, T., Deslandes, E., Lebailly, P., Vigreux, C., Sichel, F., Poul, J. M., Gauduchon, P. (1999) Early detection of staurosporine-induced apoptosis by comet and annexin V assays. *Histochem. Cell Biol.* **112**: 155–161

Graves, J. D., Gotoh, Y., Draves, K. E., Ambrose, D., Han, D. K., Wright, M., Chernoff, J., Clark, E. A., Krebs, E. G. (1998) Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. *EMBO J.* **17**: 2224–2234

Hardy, K. (1999) Apoptosis in the human embryo. *Rev. Reprod.* **4**: 125–134

Izzard, R. A., Jackson, S. P., Smith, G. C. (1999) Competitive and noncompetitive inhibition of the DNA-dependent protein kinase. *Cancer Res.* **59**: 2581–2586

Jun, C. D., Oh, C. D., Kwak, H. J., Pae, H. O., Yoo, J. C., Choi, B. M., Chun, J. S., Park, R. K., Chung, H. T. (1999) Overexpression of protein kinase C isoforms protects RAW 264.7 macrophages from nitric oxide-induced apoptosis: involvement of c-Jun *N*-terminal kinase/stress-activated protein kinase, p38 kinase, and CPP-32 protease pathways. *J. Immunol.* **162**: 3395–3401

Koh, J. S., Lieberthal, W., Heydrick, S., Levine, J. S. (1998) Lysophosphatidic acid is a major serum noncytokine survival factor for murine macrophages which acts via the phosphatidylinositol 3-kinase signaling pathway. *J. Clin. Invest.* **102**: 716–727

Leist, M., Nicotera, P. (1997) The shape of cell death. *Biochem. Biophys. Res. Commun.* **236**: 1–9

Marcelli, M., Marani, M., Li, X., Sturgis, L., Haidacher, S. J., Trial, J. A., Mannucci, R., Nicoletti, I., Denner, L. (2000) Heterogeneous apoptotic responses of prostate cancer cell lines identify an association between sensitivity to staurosporine-induced apoptosis, expression of Bcl-2 family members, and caspase activation. *Prostate* **42**: 260–273

Mattson, M. P., Duan, W., Pedersen, W. A., Culmsee, C. (2001) Neurodegenerative disorders and ischemic brain diseases. *Apoptosis* **6**: 69–81

Messmer, U. K., Lapetina, E. G., Brune, B. (1995) Nitric oxide-induced apoptosis in RAW 264.7 macrophages is antagonized by protein kinase C- and protein kinase A-activating compounds. *Mol. Pharmacol.* **47**: 757–765

Messmer, U. K., Briner, V. A., Pfeilschifter, J. (1999) Tumor necrosis factor-α and lipopolysaccharide induce apoptotic cell death in bovine glomerular endothelial cells. *Kidney Int.* **55**: 2322–2337

Mohr, S., McCormic, T. S., Lapetina, E. G. (1998) Macrophages resistant to endogenously generated nitric oxide-mediated apoptosis are hypersensitive to exogenously added nitric oxide donors: dichotomous apoptotic response independent of caspase 3 and reversal by the mitogen-activated protein kinase kinase (MEK) inhibitor PD 098059. *Proc. Natl Acad. Sci. USA* **95**: 5045–5050

Mountz, J. D., Hsu, H. C., Matsuki, Y., Zhang, H. G. (2001) Apoptosis and rheumatoid arthritis: past, present, and future directions. *Curr. Rheumatol. Rep.* **3**: 70–78

Na, S., Chuang, T. H., Cunningham, A., Turi, T. G., Hanke, J. H., Bokoch, G. M., Danley, D. E. (1996) D4-GDI, a substrate of CPP32, is proteolyzed during Fas-induced apoptosis. *J. Biol. Chem.* **271**: 11209–11213

Newton, A. C. (1995) Protein kinase C: structure, function, and regulation. *J. Biol. Chem.* **270**: 28495–28498

Nonaka, K., Ishisaki, A., Muro, M., Kato, S., Oido, M., Nakashima, K., Kowashi, Y., Nishihara, T. (1998) Possible involvement of protein kinase C in apoptotic cell death of macrophages infected with *Actinobacillus actinomycetemcomitans*. *FEMS Microbiol. Lett.* **159**: 247–254

Ogura, M., Handa, S. (2000) Induction of apoptosis by novel synthesized acylamides of human lymphocytes. *Biochim. Biophys. Acta* **1483**: 111–118

Orlov, S. N., Thorin-Trescases, N., Dulin, N. O., Dam, T. V., Fortuno, M. A., Tremblay, J., Hamet, P. (1999) Activation of cAMP signaling transiently inhibits apoptosis in vascular smooth muscle cells in a site upstream of caspase-3. *Cell Death Differ.* **6**: 661–672

Orth, K., O'Rourke, K., Salvesen, G. S., Dixit, V. M. (1996) Molecular ordering of apoptotic mammalian CED-3/ICE-like proteases. *J. Biol. Chem.* **271**: 20977–20980

Petrache, I., Choi, M. E., Otterbein, L. E., Chin, B. Y., Mantell,

L. L., Horowitz, S., Choi, A. M. (1999) Mitogen-activated protein kinase pathway mediates hyperoxia-induced apoptosis in cultured macrophage cells. *Am. J. Physiol.* **277**: L589–L595

Porter, A. G., Ng, P., Janicke, R. U. (1997) Death substrates come alive. *Bioessays* **19**: 501–507

Rathmell, J. C., Thompson, C. B. (2002) Pathways of apoptosis in lymphocyte development, homeostasis, and disease. *Cell* **109**: S97–S107

Sasaki, K., Tsukada, T., Adachi, I., Yamaguchi, K. (1995) Staurosporine potentiates cAMP-mediated promoter activity of the vasoactive intestinal polypeptide gene in rat pheochromocytoma PC12 cells. *Biochem. Biophys. Res. Commun.* **214**: 1114–1120

Scheid, M. P., Duronio, V. (1998) Dissociation of cytokine-induced phosphorylation of Bad and activation of PKB/akt: involvement of MEK upstream of Bad phosphorylation. *Proc. Natl Acad. Sci. USA* **95**: 7439–7444

Scurlock, B., Dawson, G. (1999) Differential responses of oligodendrocytes to tumor necrosis factor and other pro-apoptotic agents; role of ceramide in apoptosis. *J. Neurosci. Res.* **55**: 514–522

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., Tomita, F. (1986) Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135**: 397–402

Wan, X., Yokoyama, Y., Shinohara, A., Takahashi, Y., Tamaya, T. (2002) PTEN augments staurosporine-induced apoptosis in PTEN-null Ishikawa cells by downregulating PI3K/Akt signaling pathway. *Cell Death Differ.* **9**: 414–420

Xiao, Y. Q., Someya, K., Morita, H., Takahashi, K., Ohuchi, K. (1999) Involvement of p38 MAPK and ERK/MAPK pathways in staurosporine-induced production of macrophage inflammatory protein-2 in rat peritoneal neutrophils. *Biochim. Biophys. Acta* **1450**: 155–163

Yamaki, K., Ohuchi, K. (1999) Participation of protein kinases in staurosporine-induced interleukin-6 production by rat peritoneal macrophages. *Br. J. Pharmacol.* **127**: 1309–1316

Yamaki, K., Yonezawa, T., Ohuchi, K. (2000) Signal transduction cascades in staurosporine-induced prostaglandin E_2 production by rat peritoneal macrophages. *J. Pharmacol. Exp. Ther.* **293**: 206–213

Zeng, G., Nystrom, F. H., Ravichandran, L. V., Cong, L. N., Kirby, M., Mostowski, H., Quon, M. J. (2000) Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. *Circulation* **101**: 1539–1545