ERK signaling mediates the induction of inflammatory cytokines by bufalin in human monocytic cells

MASAHIRO KUROSAWA, SATOSHI NUMAZAWA, YOSHIHIRO TANI, AND TAKEMI YOSHIDA
Department of Biochemical Toxicology, School of Pharmaceutical Sciences, Showa University, Tokyo 142-8555, Japan

Kurosawa, Masahiro, Satoshi Numazawa, Yoshihiro Tani, and Takemi Yoshida. ERK signaling mediates the induction of inflammatory cytokines by bufalin in human monocytic cells. Am. J. Physiol. Cell Physiol. 278: C500–C508, 2000.—Treatment of human leukemia THP-1 cells with bufalin, a specific inhibitor of Na+/K+-ATPase, sequentially induces c-fos and inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) gene expressions before the appearance of mature phenotypes of monocytic cells. In this study we examined the signal transduction leading to bufalin-induced gene expressions. Bufalin selectively activated extracellular signal-regulated kinase (ERK), compared with other mitogen-activated protein (MAP) kinase family members. Pretreatment of THP-1 cells with PD-98059, an inhibitor of the ERK-kinase cascade, abolished bufalin-induced c-fos and IL-1β gene expressions, indicating that the ERK-kinase cascade mediates the induction of inflammatory cytokines by bufalin. Inhibition of the Na+/Ca²⁺ exchanger by KB-R7943 and of protein kinase C (PKC) by Ro-31-8220 suppressed ERK activation and gene expressions of c-fos and IL-1β. These findings suggest that Na+/K⁺-ATPase inhibition by bufalin induces calcium influx and thereby activates PKC and ERK. In cells treated with an inhibitor of p38 MAP kinases, SB-203580, bufalin-mediated ERK activation became persistent and the induction of IL-1β and TNF-α expressions was significantly augmented. These results suggest that cross talk in bufalin-mediated ERK activation is negatively regulated by endogenous p38 MAP kinase activations.

interleukin-1β; sodium-potassium-adenosinetriphosphatase; cell differentiation; extracellular signal-regulated kinase

BUFALIN IS ONE OF THE MAJOR active components of a traditional Chinese medicine called Senso or Ch’an Su that is prepared from toad venom extracts. Although the well-known pharmacological actions of bufalin are cardiotoxic effects, bufalin has been shown to induce cell differentiation (23, 24, 36) and apoptosis (30–32) in human leukemia cells under different experimental conditions. Cells treated with bufalin in the absence of serum preferentially undergo apoptosis; meanwhile, cells survive and eventually express differentiated phenotypes in the presence of serum. Ouabain, a typical cardiac glycoside and a specific inhibitor of the Na+/K⁺-ATPase, shows similar effects on leukemia cells, but to a lesser extent (23). The effect of bufalin on cardiac myocytes is due to the inhibition of Na+/K⁺-ATPase and thereby disruption of the cation homeostasis. Analogously, several lines of evidence indicated that the induction of cell differentiation by bufalin is mediated by Na+/K⁺-ATPase inhibition (23).

The mitogen-activated protein (MAP) kinase superfamily of serine/threonine kinases has emerged as an important component of cellular signal transduction. The MAP kinase family members have been implicated in events necessary for proliferation, differentiation, apoptosis, and certain kinds of stress responses (14). Three MAP kinase families, extracellular signal-regulated kinases (ERK), p38 MAP kinases, and c-jun NH₂-terminal kinases (JNK), have been well characterized. These MAP kinases are activated by specific cascades responsible for certain stimuli and eventually induce a variety of cell responses (5, 12, 22).

It has been suggested that the excessive activation of the ERK-kinase cascade, which is generally known to play a role in cell survival, is an event necessary for bufalin-mediated apoptosis (30, 32). Such a hyperexcitation of the ERK-kinase cascade is due to the activation of an upper signal component Ras and the concomitant downregulation of the protein kinase A activity that may negatively regulate the Raf-1 function (30). However, signal transduction leading to bufalin-mediated cell differentiation has not been explored. We report here that ERK activation, similar to apoptosis, plays a critical role in the induction of differentiation of human monocytic leukemia THP-1 cells by bufalin. In addition, we show evidence that p38 MAP kinases and/or their downstream molecules may modulate ERK activity and eventually cell differentiation.

EXPERIMENTAL PROCEDURES

Materials. Bufalin was isolated from chloroform extracts of a Chinese toad venom preparation (Senso, Shibata Pharmaceutical, Tokyo, Japan) by repetitive chromatography on silica gel and a LiChroprep RP-18 Lobar column (Merck, Darmstadt, Germany), as described previously (23). PD-98059 and Ro-31-8220 were purchased from Calbiochem (La Jolla, CA). a-Hydroxyfarnesyl phosphonic acid (a-HFPA) was purchased from Cayman (Ann Arbor, MI). KB-R7943 was kindly provided by H. Nakagawa (Kanebo, Osaka, Japan). SB-203580 was a gift from SmithKline Beecham (King of Prussia, PA). Polyclonal anti-ERK, anti-phospho-p38 MAP kinase, anti-phospho-JNK, and monoclonal anti-phospho-ERK antibodies for immunoblot analysis were purchased from New England Biolabs (Beverly, MA). [γ-32P]ATP and

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[α-32P]dCTP were from The Institute of Isotopes of the Hungarian Academy of Sciences, Hungary. Other chemicals were of the highest grade commercially available.

Cell culture. THP-1 cells (28) were obtained from Riken Cell Bank (Tsukuba, Japan). Cells were maintained in an RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 20 mM HEPES, 0.2% sodium bicarbonate, and penicillin-streptomycin. Cells were seeded at $1 \times 10^5$ cells/ml and maintained in continuous logarithmic growth in a humidified 5% CO₂ atmosphere at 37°C. To determine cell adhesion, cells attached to the substrate were washed twice with PBS and treated with 0.25% trypsin/0.02% EDTA for counting. Phagocytic activity was determined by measuring the cells that engulfed carboxylate latex particles (average diameter, 0.75 µm, 2.5% solids-latex; Polysciences, War- rington, PA). Cells were incubated with latex particles (2 µl/ml) at 37°C for 5 h. After washing twice with PBS, cells containing more than five latex particles were scored as phagocytic cells.

Northern blot analysis. Total RNA was isolated from the cells by acid guanidinium thiocyanate-phenol-chloroform extraction as described by Chomczynski and Sacchi (2). Northern blot analysis was carried out as described previously (24). Probes used were the 1.7-kb EcoRI/PstI fragment of a human c-fos cDNA purified from pSPT-fos cDNA plasmid (29) (JCRB), the 1.1-kb PstI insert of a human interleukin-1β (IL-1β) cDNA purified from IL-1 X-14 plasmid (21) and the 0.82-kb EcoRI insert of a human tumor necrosis factor-α (TNF-α) cDNA purified from pUC-RI-4 large plasmid (26) (American Type Culture Collection), and the 0.5-kb insert of a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA purified from GD5 plasmid (20).

Immunoblot analysis. Cells were lysed in boiling SDS-sample buffer. Denatured proteins were separated on a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Pall Biosupport Division, Port Washington, NY) at 120 mA for 1 h. The membrane was incubated with 0.2% casein-based I-Block (Tropix, Bedford, MA) dissolved in 20 mM Tris-HCl (pH 7.6) containing 137 mM NaCl and 0.1% Tween 20 (TTBS) for 1 h at room temperature, washed with TTBS (3 × 15 min), and incubated for 1 h with primary antibody dissolved in the blocking solution overnight at 4°C. After washing, the membrane was incubated for 1 h with respective horseradish peroxidase-linked secondary antibodies. Immunoreactive proteins were detected by the enhanced

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**Fig. 1.** Differentiation of THP-1 cells by bufalin. Cells were treated with bufalin (Bu) at a concentration of 10 or 30 nM as indicated for 4 days (A, B) or for times indicated (C). A: Viable cell number, cell adhesion, and phagocytic activity are illustrated as means ± SE (n = 4). B: Photographs of cells incubated in absence (control) or presence of bufalin (30 nM) were taken under a light microscope (original magnification, ×200). C: Expressions of genes encoding c-fos, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) in cells treated with bufalin were examined by Northern blot analysis. The GAPDH expression was also analyzed to assess equal loading.
chemiluminescence system (Amersham-Pharmacia Biotech, Buckinghamshire, UK).

Immunocomplex kinase assay. Cells (2 × 10^6) were washed with ice-cold PBS containing 50 mM NaF, 30 mM Na_2P_2O_7, and 100 µM Na_3VO_4, and lysed in 0.5 ml of the lysis buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 50 mM NaF, 30 mM Na_2P_2O_7, 100 µM Na_3VO_4, 50 µg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride, 2 µg/ml leupeptin, and 1% Triton X-100] for 30 min at 0°C. The cell lysate was centrifuged at 15,000 rpm for 15 min. The supernatant (400 µl) was incubated with 2 µg of anti-ERK antibodies (Santa Cruz) for 1 h at 4°C and 20 µl of protein A-agarose for an additional 2 h at 4°C. The immunocomplex was washed twice with 1 ml of the lysis buffer containing 0.5 M NaCl and twice with 1 ml of the kinase buffer (20 mM Tris-HCl, pH 7.6, 10 mM MgCl_2, 2 mM MnCl_2, 1 mM dithiothreitol, 100 µM Na_3VO_4, 1 mM EGTA). The kinase reaction was carried out with 10 µl of the kinase buffer, 2 µg of myelin basic protein (MBP), and 5 µCi of [γ-^32P]ATP at 30°C for 15 min. The reaction was stopped by the 2× SDS sample buffer and boiling for 5 min. Phosphorylated MBP was separated by 13% SDS-PAGE, fixed, dried, and analyzed with a Fuji BAS 3000 image analyzer (Fuji, Kanagawa, Japan).

RESULTS

Differentiation of THP-1 cells by bufalin. Bufalin induced growth arrest (Fig. 1A) and the concomitant increase in the number of adherent and phagocytic cells in a time-dependent (Fig. 1C) and dose-dependent (Fig. 1A) manner in human monocylic THP-1 cells. Bufalin at a concentration of 30 nM also induced the sustained c-fos gene expression in THP-1 cells observed as early as 6 h, and lasting at least up to 48 h after treatment (Fig. 1C). In addition, expressions of IL-1β and TNF-α, inflammatory cytokines that were produced by mature monocytes, increased (Fig. 1C) in conjunction with functional markers of monocytic differentiation such as phagocytosis (Fig. 1A) and cell adhesion (Fig. 1, A and B). Therefore, we utilized c-fos, IL-1β, and TNF-α gene
expressions as markers of cell differentiation in the following study.

Effect of the inhibitor of Na⁺/Ca²⁺ exchanger. Bufalin binds to the myocyte Na⁺-K⁺-ATPase and inhibits its pumping function, thus leading to transient accumulation of intracellular Na⁺. The Na⁺/Ca²⁺-exchange system functions in its reverse mode to decrease cytoplasmic Na⁺ and concomitantly to increase Ca²⁺ concentration in myocardial cells. These bufalin-mediated changes in the cation concentration are thought to be the basis of its primary action on cardiac muscle. We asked whether the influx of calcium ion by the Na⁺/Ca²⁺ exchanger was involved in the machinery of bufalin-mediated differentiation of THP-1 cells. KB-R7943 is a recently developed specific inhibitor for the exchanger and blocks the calcium influx more potently than the efflux (11, 33). Pretreatment of cells with KB-R7943 at a concentration sufficient to block the Na⁺/Ca²⁺ exchanger (34) inhibited bufalin-mediated c-fos and IL-1β gene expressions to about 50% of the control (Fig. 2, A and B). These results suggest that influxed Ca²⁺ may function as a second messenger in bufalin-mediated signal transduction. To ensure this notion, we utilized calcium ionophore A-23187. The forced increase in the calcium concentration in cells resulted in a dose-dependent increase in IL-1β and TNF-α expressions (Fig. 2C). However, the ionophore did not induce functional differentiation markers, such as cell adhesion and phagocytosis (data not shown).

Effect of the protein kinase C inhibitor. It is obvious that the increase in intracellular Ca²⁺ affects the various signal-transducing modules. Protein kinase C (PKC) is one of the well-known components in calcium-mediated signal transduction. Thus we tested the role of PKC in bufalin-induced cell differentiation. Pretreatment of cells with the selective PKC inhibitor Ro-31-8220 (3, 4) suppressed bufalin-induced IL-1β expressions in a dose-dependent manner (Fig. 3A). Ro-31-8220 at a concentration of 2 µM suppressed IL-1β induction by 14% (Fig. 3, B and C). In addition, the

Fig. 3. Effect of Ro-31-8220 on bufalin-induced c-fos and IL-1β gene expressions in THP-1 cells. A: Cells were pretreated with 0.1% DMSO (−) or Ro-31-8220 (Ro) for 1 h at concentrations indicated and then treated with 30 nM bufalin (Bu) for 48 h. Northern blot analysis was carried out as described in legend to Fig. 1. B: Cells were pretreated with 0.1% DMSO or 2 µM Ro-31-8220 for 1 h and then treated with 30 nM bufalin for the times indicated. IL-1β and c-fos transcripts were analyzed as described in legend to Fig. 1. C: Cells were pretreated with 2 µM Ro-31-8220 for 1 h followed by bufalin treatment for 12 (c-fos) or 48 h (IL-1β). Transcripts of c-fos and IL-1β were analyzed by Northern blot analysis, quantified with the image analyzer, and normalized with respective GAPDH expression. Bufalin-induced levels were set at 100%. Data are means ± SE from three independent experiments.
increased c-fos expression by bufalin was also suppressed by 30% (Fig. 3, B and C). Effective concentrations of Ro-31-8220 on bufalin-mediated responses coincided well with those reported to inhibit the TPA-induced responses (1).

Effect of inhibitors of the ERK-kinase cascade. Previous reports have suggested that the ERK-kinase cascade plays an important role in bufalin-induced apoptosis (30). Therefore, we next examined whether the ERK-kinase cascade was also involved in the induction of cell differentiation by bufalin. PD-98059 specifically inhibits activation of MAP kinase/ERK kinase (MEK), an upstream kinase of ERK; thus it is believed to be a selective inhibitor of the ERK-mediated cascade. Pretreatment of cells with PD-98059 at a concentration reported to inhibit association of MEK and c-Raf (6) suppressed bufalin-induced c-fos and IL-1β expressions almost completely (Fig. 4A).

Ras is an upstream component of the ERK-kinase cascade in the growth factor-generated signal transduction (17). It is also reported that Ras is activated in the course of bufalin-mediated apoptosis (30). Therefore, we examined the role of Ras in bufalin-induced THP-1 cell differentiation by using α-HFPA, a cell-permeable farnesyltransferase inhibitor (7). Preincubation of cells with α-HFPA at a concentration known to inhibit the Ras function (7) showed virtually no effect on bufalin-induced c-fos and IL-1β expressions (data not shown).

MAP kinase activation by bufalin. ERK activation was examined by immunoblot analysis using the phosphospecific antibody that recognizes phosphorylated Thr-202 and Tyr-204 of activated ERK1 and ERK2 (Fig. 4B). Because experiments were conducted in the presence of serum, the phosphorylated ERK2 was detectable in unstimulated cells. Bufalin induced an increase in ERK2 phosphorylation, peaking at 1 h after treatment. However, the increased ERK2 phosphorylation was transient and returned to the basal level by 3 h. ERK1 was also activated by bufalin in a time course similar to ERK2 but to a lesser extent (Fig. 4B). Pretreatment of cells with Ro-31-8220 cancelled bufalin induction of ERK phosphorylation (Fig. 4C). In addition, bufalin failed to induce ERK phosphorylation in cells treated with KB-R7943 (Fig. 4D). These results suggest
that ERK activation by bufalin is a downstream event of Ca\(^{2+}\) influx and PKC activation.

Effect of the p38 inhibitor. Figure 5 illustrates the effects of a specific inhibitor for activated p38 MAP kinases on bufalin-induced IL-1\(\beta\) and TNF-\(\alpha\) expressions. It is surprising that pretreatment of cells with SB-203580 augmented the bufalin-mediated cytokine expressions at concentrations selective to p38 MAP kinases (16, 27). The maximum effect of SB-203580 was observed at a concentration of 5 \(\mu M\), which enhanced IL-1\(\beta\) and TNF-\(\alpha\) gene expressions 6.3- and 4.0-fold, respectively, compared with bufalin alone (Fig. 5B). In accordance with the cytokine expressions, SB-203580 augmented the cell-substrate adherence induced by bufalin. Bufalin alone did not induce cell adhesion significantly, as determined 48 h after treatment; however, pretreatment with SB-203580 induced a great increase in the number of cells attached to the substrate (Fig. 5, C and D). These results strongly suggest that the inhibition of endogenous p38 MAP kinase activities enhances bufalin-induced cell differentiation. To determine action of SB-203580 on bufalin-induced differentiation signals, ERK phosphorylation was analyzed in cells treated in combination with bufalin and SB-203580. Although SB-203580 alone showed virtually no effect on ERK phosphorylation (data not shown), it markedly enhanced the effect produced by bufalin treatment (Fig. 6A). A strong signal of ERK phosphorylation was observed as early as 30 min and was sustained up to 48 h. Furthermore, the immunocomplex kinase assay revealed that SB-203580 actually augmented bufalin-mediated ERK activation (Fig. 6B). These observations suggest that p38 MAP kinases negatively regulate ERK activities that may function as a central module in bufalin-induced cell differentiation.

Fig. 5. Inhibition of p38 MAP kinases enhances bufalin-mediated differentiation of THP-1 cells. A: Cells were pretreated with 0.1% DMSO or SB-203580 (SB) for 1 h at concentrations as indicated and then treated with 30 nM bufalin for 24 h. Northern blot analysis using the IL-1\(\beta\) and TNF-\(\alpha\) cDNA probes were carried out as described in legend to Fig. 1. B: Steady-state levels of IL-1\(\beta\) and TNF-\(\alpha\) mRNAs in cells treated with SB-203580 and bufalin as described in A were quantified and normalized as described in legend to Fig. 2. Data are expressed as a percentage of the bufalin-induced level and are means \(\pm\) SE from three independent experiments. C: Cells were pretreated with 0.1% DMSO or 5 \(\mu M\) SB-203580 for 1 h and then treated with 30 nM bufalin for 48 h. The culture media were removed and adherent cells were examined under a light microscope (original magnification, \(\times 200\)). D: Cells were treated as described in C and viable cell number and cell adhesion were examined. Data are means \(\pm\) SE (n = 4).
BUFFALIN INDUCTION OF CYTOKINES BY ERK ACTIVATION

The known biochemical action of buffalin is the inhibition of Na\(^{+}\)-K\(^{+}\)-ATPase activity. Buffalin induces an increase in the intracellular Na\(^{+}\) concentration and a concomitant influx of Ca\(^{2+}\) by the action of the Na\(^{+}\)/Ca\(^{2+}\) exchanger. These mechanisms are the basis of the positive inotropic effect of cardiotonic steroids on isolated heart preparations (35); buffalin has been shown to have a similar effect (8). On the other hand, our previous studies have demonstrated that differentiation-inducing activities of cardiotonic steroids correlated well with the Na\(^{+}\)-K\(^{+}\)-ATPase inhibitory abilities in human leukemia cells (23). In addition, murine-derived leukemia cells display much less susceptibility to the action of buffalin than human cells (23). Such species-dependent differences are related to the lower binding affinity between cardiotonic steroids and the α-subunit of murine Na\(^{+}\)-K\(^{+}\)-ATPase (9, 18). Furthermore, ouabain-resistant THP-1 cells are less susceptible to buffalin (23), indicating that the primary target of the cardiotonic steroid in cell differentiation is Na\(^{+}\)-K\(^{+}\)-ATPase. Although there is no direct evidence indicating that inhibition of Na\(^{+}\)-K\(^{+}\)-ATPase is involved in buffalin-mediated apoptosis, it is postulated that the enzyme inhibition could also be a primary action (30). It has been reported that an excessive activation of the ERK-kinase cascade may function during buffalin induction of apoptosis (30); however, signal transductions that elicit cell differentiation are still obscure. In this study we thus attempted to clarify the downstream module of Na\(^{+}\)-K\(^{+}\)-ATPase inhibition by using selective inhibitors for signal transducers.

Accumulated evidence has indicated that the transient increase in intracellular calcium concentration leads leukemia cells to undergo cell differentiation (25). Under such experimental conditions, activation of the ERK-kinase cascade and the concomitant increase in the c-fos expression have been observed (19). As demonstrated in this study, KB-R7943 that inhibits the Na\(^{+}\)/Ca\(^{2+}\) exchanger-operated calcium influx suppressed buffalin induction of c-fos and IL-1β expressions, suggesting that the increased calcium mobilization contributes (if it is not entirely responsible) to the buffalin induction of cell differentiation. Induction of IL-1β and TNF-α expressions by calcium ionophore further supports the idea that a calcium influx may trigger buffalin-mediated cellular signals. On the other hand, A-23187 did not induce phagocytosis and cell-substrate adherence, suggesting that the transient increase in intracellular Ca\(^{2+}\) is an event important to the terminal differentiation of THP-1 cells. Similar effects of A-23187 on THP-1 cells have been reported previously (10).

Increased calcium ion may activate various signal transducers. The classical type of the PKC (cPKCa, cPKCb1, cPKCb2, and cPKCy) is the well-demonstrated class of signal module activated by increased Ca\(^{2+}\) (25). These PKC isoforms have been shown to be involved in cell proliferation, differentiation, and apoptosis. This study demonstrated that the submicromolar range of Ro-31-8220, which preferentially inhibits the cPKC rather than other types of PKC (13), suppressed the expression of differentiation markers induced by buffalin, suggesting involvement of cPKC in the buffalin-mediated signal transduction. It has been reported that PKC directly interacts with and activates c-Raf and, as a consequence, transduces the signal to the ERK-kinase cascade (15). The transient activation of ERK by buffalin was sensitive to KB-R7943 and Ro-31-8220. Moreover, PD-98059, which inhibits association of c-Raf and MEK, was capable of blocking buffalin induction of c-fos and IL-1β gene expressions, indicating that Na\(^{+}\)-K\(^{+}\)-ATPase inhibition may lead to sequential activation of cPKC → c-Raf → MEK → ERK. The fact that inhibition of the Ras function by α-HPPA showed virtually no effect on buffalin induction of c-fos and IL-1β expressions further supports the assumption described above. Other MAP kinase family members, p38 MAP kinases and JNK, seem to be less sensitive to buffalin as determined by phosphospecific antibodies.

DISCUSSION

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indicating that bufalin preferentially and selectively activates the ERK-kinase cascade among the MAP kinase families during cell differentiation.

Meanwhile, the basal phosphorylation state of p38 MAP kinases was relatively high compared with ERK or JNK. That SB-203580 significantly augmented bufalin induction of the ERK activation and cytokine expressions was surprising and suggests that the basal activity of the p38 MAP kinases modulates either ERK dephosphorylation or the upstream module of the ERK-kinase cascade. In addition, p38 MAP kinase activities could negatively regulate THP-1 cells to undergo differentiation. To our knowledge, such a synergistic activation of ERK by the p38 MAP kinase inhibitor has not been reported to date. Molecular mechanisms governing the cross talk between the ERK and the p38 MAP kinases modulates either ERK dephosphorylation or the upstream module of the ERK-kinase cascade. In addition, p38 MAP kinase activities dephosphorylation or the upstream module of the ERK-kinase cascade. In addition, p38 MAP kinase activities have been reported to date. Molecular mechanisms governing the cross talk between the ERK and the p38 MAP kinase families during cell differentiation.

In conclusion, our data show that Na+-K+-ATPase inhibition by bufalin induces activation of the ERK-kinase cascade, an event necessary for differentiation of THP-1 cells. Our data also suggest that the Na+-Ca2+-exchanger-operated transient Ca2+-influx sequentially mobilizes PKC, the ERK-kinase cascade, c-fos, and other specific gene expressions. It is noteworthy that bufalin utilizes the ERK-kinase cascade as a central signal module during not only cell differentiation but also apoptosis. Further studies regarding cross talk between MAP kinases could elucidate machinery deciding cell differentiation and apoptosis.

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Address for reprint requests and other correspondence: M. Kurosawa, Dept. of Biochemical Toxicology, Showa Univ. School of Pharmaceutical Sciences, 1–5–8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan (E-mail: kuromasa@pharm.showa-u.ac.jp).

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REFERENCES


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