Activation of p38 and c-Jun N-terminal Kinase Pathways and Induction of Apoptosis by Chelerythrine Do Not Require Inhibition of Protein Kinase C*

(Received for publication, November 12, 1999, and in revised form, January 6, 2000)

Rong Yu‡, Sandhya Mandlekar‡, Tse-Hua Tan§, and A.-N. Tony Kong‡¶

From the ‡Department of Pharmaceutics and Pharmacodynamics, Center for Pharmaceutical Biotechnology, College of Pharmacy, University of Illinois, Chicago, Illinois 60612 and the ¶Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030

Chelerythrine, a natural benzophenanthridine alkaloid, has been reported to mediate a variety of biological activities, including inhibition of protein kinase C (PKC). Here we report that chelerythrine induced time- and dose-dependent activation of JNK1 and p38 in HeLa cells, which was mediated by the upstream kinases, MEKK1 and MKK4. However, treatment with two other potent and selective PKC inhibitors, GF-109203X and G66983, or down-regulation of PKC activity by prolonged treatment with phorbol 12-myristate 13-acetate had no effect on JNK1 and p38 activities. Furthermore, under the conditions where JNK1 and p38 were activated, we did not observe any significant inhibitory effect of chelerythrine on the activities of PKC isozymes present in HeLa cells. Interestingly, pretreatment with the antioxidants, N-acetyl-l-cysteine, dithiothreitol, and glutathione, impaired chelerythrine-induced JNK1 and p38 activation. In addition, chelerythrine induced apoptosis that was blocked by the antioxidants and the dominant-negative mutants of MEKK1, MKK4, JNK1, and p38. Together, these results uncover a novel biochemical property of chelerythrine, i.e. activation of MEKK1- and MKK4-dependent JNK1 and p38 pathways through an oxidative stress mechanism, which mediate the induction of apoptosis, but are independent of PKC inhibition.

Mitogen-activated protein kinase (MAPK)1 cascades are evolutionarily conserved in all eucaryotes and play a central role in the cellular responses to various extracellular stimuli (1, 2). The core part of a MAPK pathway, also called MAPK module, consists of three kinases: a MAPK, a MAPK kinase (MEK or M KK), and a MAPK kinase kinase (MEKK) (3, 4). MAPK is activated by the dual phosphorylation on Tyr and Thr residues by a member of MEKs or MKKs, which, in turn, are activated by an upstream MEKK. To date, at least six MAPK members have been identified in mammalian cells. Three of them have been extensively studied: the extracellular signal-regulated protein kinases (ERKs) (5), the c-Jun N-terminal kinases (JNKs, also referred to as stress-activated protein kinases) (6, 7), and the p38s (8). The ERK pathway is predominantly activated by mitogens through a Ras-dependent mechanism (9), and it is required for cell proliferation and differentiation (10). Unlike ERK, however, JNK and p38 are regulated by distinct MAPK modules and are preferentially activated by pro-inflammatory cytokines (11) and various environmental stresses such as UV light (6), γ-irradiation (12), DNA-damaging agents (13), protein synthesis inhibitors (14), heat shock (15), osmotic shock (16), and oxidative stresses (17). Activation of JNK and p38 has been implicated in the regulation of a variety of cellular processes such as T cell activation (18), production of cytokines (19), cell differentiation (20), and apoptotic cell death (21, 22).

Protein kinase C (PKC) represents a family of serine/threonine kinases, which presently consists of at least 11 isozymes (23, 24). According to their structure and cofactor regulation, these isozymes have been classified into three groups as follows: (i) conventional PKCs (α, β, γ, and δ) that are activated by calcium or diacylglycerol; (ii) novel PKCs (θ, ε, η, θ, and μ) that are independent of calcium but responsive to diacylglycerol; and (iii) atypical PKCs (ζ and λ) that do not require either calcium or diacylglycerol for activation. A large number of studies indicate that PKC serves as a central signaling molecule and regulates a variety of cellular processes either directly or through integration with other signaling pathways (23). PKC has been shown to activate Ras-MAPK pathway, leading to cell proliferation (25). Activation of a certain PKC isozyme has also been shown to regulate T cell activation through a JNK-dependent pathway (18). Recently, PKC has been demonstrated to prevent apoptosis in different cell lines, presumably by increasing phosphorylation of Bel-2 (26, 27). Consistent with this notion, inhibition of PKC has been shown to result in apoptosis (28, 29).

Chelerythrine is a natural benzophenanthridine alkaloid that displays a wide range of biological activities such as anti-platelet (30), anti-inflammatory (31), and antibacterial effects (31). Most notably, chelerythrine exhibits anti-tumor property (32) and induces apoptosis in murine lymphoma (33) and human leukemia (28, 29). Thus, chelerythrine has the potential for drug development against cancers. Since chelerythrine was described previously to be an inhibitor of PKC (32), the biological effects of chelerythrine have been presumably ascribed to the inhibition of PKC; however, a number of recent studies indicate that PKC-independent mechanisms are involved (34–

---

*This work was supported in part by National Institutes of Health Grants R01-CA73647 (to A.-N. T. K.) and R01-AI38649 (to T.-H. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Dept. of Pharmaceutics and Pharmacodynamics, Center for Pharmaceutical Biotechnology, M/C870, College of Pharmacy, University of Illinois, 900 S. Ashland Ave., Chicago, IL 60612. Tel.: 312-413-9646; Fax: 312-413-9303; E-mail: KongT@uic.edu.

‡ The abbreviations used are: MAPK, mitogen-activated protein kinase; PKC, protein kinase C; MKK, MAPK kinase; MEKK, MAPK kinase kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated protein kinase; PMA, phorbol 12-myristate 13-acetate; DTT, dithiothreitol; NAC, N-acetyl-l-cysteine; HEK293, human embryonic kidney (293) cell line; H3CDFS, dithioldihydrofluorescein; DAPI, 4,6-ianidino-2-phenyindole; GST, glutathione S-transferase; ATRF2, activating transcription factor 2; MBP, myelin basic protein; HA, hemagglutinin; X-gal, 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
38. In this study, we investigated the involvement of MAPKs and their relationship with PKC in chelerythrine-treated HeLa cells. As reported below, chelerythrine strongly stimulated JNK1 and p38, but not ERK2, activities. Interestingly, chelerythrine did inhibit the PKC activity under the conditions where JNK1 and p38 were activated. Furthermore, blocking JNK and p38 pathways suppressed chelerythrine-induced apoptosis. Thus, activation of MAPK pathways represents a novel biochemical effect of chelerythrine.

MATERIALS AND METHODS

Cell Culture, Antibodies, and Chemicals—HeLa cells (human cervical squamous carcinoma) were obtained from ATCC (Manassas, VA). Cells were cultured at 37 °C and 5% CO2 in minimum essential medium supplemented with 10% fetal bovine serum, 2.2 g/liter sodium bicarbonate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were normally starved overnight in serum-free medium before treatment, unless otherwise indicated. Histidine-tagged JNK1 protein and polyclonal antibodies against ERK2, MEKK1, and SEK1 were purchased from the Santa Cruz Biotechnology Inc. (Santa, Cruz, CA). Rabbit anti-p-p38 polyclonal antibody was purchased from New England Biolabs Inc. (Beverly, MA). Monoclonal antibodies against different isozymes of PKC were purchased from Transduction Laboratories (Lexington, KY). Mouse anti-HA monoclonal antibody (12CA5) was purchased from Roche Molecular Biochemicals. Rabbit anti-JNK1 antisera (Ab101) was described previously (12). Chelerythrine was purchased from ALEXIS® (San Diego, CA). PKC substrate, histone H1, PKC inhibitors, GF-109203X and Gö6983, and GST-SEK1 fusion protein were purchased from Calbiochem. Fluorescence dye, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA), was purchased from Molecular Probes, Inc. (Eugene, OR). [γ-32P]ATP (6000 Ci/mmol) was purchased from NEN Life Science Products. Myelin basic protein (MBP), catalase, aminotriazole, N-acetyl-l-cysteine (NAC), hydrogen peroxide (H2O2), phorbol 12-myristate 13-acetate (PMA), dithiothreitol (DTT), glutathione (GSH), and 4',6'-idamidino-2-phenylindole (DAPI) were purchased from Sigma.

PKC Mutants—pCMV-Flag-p38(AGF), were described previously (22). Expression vectors for mutant plasmid, pcDNA3-Flag-MKK4(ala), was a gift from Dr. Roger J. Silvio Gutkind (National Institutes of Health, Bethesda). MKK4 gene, OR). [32P]ATP (6000 Ci/mmol) was purchased from NEN Life Science Products. Myelin basic protein (MBP), catalase, aminotriazole, N-acetyl-l-cysteine (NAC), hydrogen peroxide (H2O2), phorbol 12-myristate 13-acetate (PMA), dithiothreitol (DTT), glutathione (GSH), and 4',6'-idamidino-2-phenylindole (DAPI) were purchased from Sigma.

Immunocomplex Kinase Assays of MAPK, SEK1, and MEKK1 Activities—After treatments, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested in cell lysis buffer containing 10 mM Tris-HCl (pH 7.1), 50 mM NaCl, 50 mM NaF, 100 μM Na3VO4, 5 μM ZnCl2, 2 mM iodoacetate, 1 mM phenylmethylsulfonfyl fluoride, and 0.1% Triton X-100. Cell lysates were homogenized by passing through a 23-gauge needle three times. The homogenate were sonicated at 4°C for 30 min before centrifugation at 12,500 × g for 15 min at 4°C. Kinase activities of JNK1, p38, ERK2, SEK1, and MKK4 were determined by SDS-PAGE and visualized by autoradiography.

Flow Cytometric Analysis of Intracellular Peroxides—After treatment with different agents, H2DCFDA (5 μM) was added to the medium and incubated at 37°C for 30 min as described previously (39). Cells were washed with ice-cold PBS and resuspended on the plate. Following centrifugation at 1000 × g for 5 min, cell pellets were resuspended in PBS containing 2% fetal bovine serum and 5 μM H2DCFDA. The fluorescence intensities of H2DCFDA of more than 10,000 viable cells from each sample were analyzed by using a Becton Dickinson FACScan flow cytometer with excitation and emission settings of 488 and 525 nm, respectively. Prior to data collection, propidium iodide was added to the sample to gate out dead cells.

Nuclear Staining Assays—After treatments, floating cells were collected by centrifugation at 2000 × g for 15 min, and attached cells were first trypsinized and then harvested by centrifugation. Apoptotic cells with condensed or fragmented nuclei were visualized by DAPI staining as described previously (42). Briefly, cells were washed once with ice-cold PBS before fixing in a solution of methanol:acetic acid (3:1) for 30 min. Fixed cells were placed on slides. After evaporation of fixing solution, cells were stained with 1 μg/ml DAPI for 15 min. The nuclear morphology of cells was examined by a fluorescence microscope.

Western Blot Analysis of JNK, p38, and PKC Isozymes—Approximately 107 cells were lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 for 30 min on ice. Cell lysates were vortexed and cleared by centrifugation at 12,500 × g for 20 min. DNA in supernatant was extracted with an equal volume of neutral phenol:chloroform:i-soyamin alcohol mixture (25:24:1) at room temperature for 15 min and precipitated with 2 volumes of 100% ethanol and one-tenth volume of 3 M sodium acetate (pH 5.2) overnight at −20°C. The DNA precipitates were spun down at 12,500 × g for 20 min and washed once with 70% ethanol. The air-dried DNA pellets were incubated with 5 μg/ml DNase-free RNase in a 40-μl Tris-EDTA buffer (pH 8.0) at 37°C for 2 h. Fragmented DNA was resolved on 1.5% agarose gels in the presence of 0.5 μg/ml ethidium bromide.

Role of MAPKs in Chelerythrine-induced Apoptosis

To assay the individual PKC isozyme activity, different isozymes were immunoprecipitated directly from cell lysates with their respective antibodies as described previously (41). The immunoprecipitates were washed four times with a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100. PKC activity was analyzed by electrophoresis as described above and quantitated with a PhosphorImager (AMBIS, Inc., San Diego, CA). The results were expressed as a percentage, relative to solvent-treated controls.

Flow Cytometric Analysis—After treatments, floating cells were collected by centrifugation 3000 × g for 10 min, and attached cells were trypsinized and then harvested by centrifugation. Apoptotic cells with condensed or fragmented nuclei were visualized by DAPI staining as described previously (42). Briefly, cells were washed once with ice-cold PBS before fixing in a solution of methanol:acetic acid (3:1) for 30 min. Fixed cells were placed on slides. After evaporation of fixing solution, cells were stained with 1 μg/ml DAPI for 15 min. The nuclear morphology of cells was examined by a fluorescence microscope.

DNA Fragmentation Assays—Approximately 107 cells were lysed in a buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 for 30 min on ice. Cell lysates were vortexed and cleared by centrifugation at 12,500 × g for 20 min. DNA in supernatant was extracted with an equal volume of neutral phenol:chloroform:i-soyamin alcohol mixture (25:24:1) at room temperature for 15 min and precipitated with 2 volumes of 100% ethanol and one-tenth volume of 3 M sodium acetate (pH 5.2) overnight at −20°C. The DNA precipitates were spun down at 12,500 × g for 20 min and washed once with 70% ethanol. The air-dried DNA pellets were incubated with 5 μg/ml DNase-free RNase in a 40-μl Tris-EDTA buffer (pH 8.0) at 37°C for 2 h. Fragmented DNA was resolved on 1.5% agarose gels in the presence of 0.5 μg/ml ethidium bromide.

Western Blot Analysis of JNK, p38, and PKC Isozymes—After treatment, cell lysates were prepared as described above. Twenty five μg of total protein, as determined by Bradford assay, was resolved on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane using the semidyallization method with 5% non-fat dry milk in TBS (20 mM Tris-HCl (pH 7.4), 8 g/liter NaCl, and 0.2 g/liter KCl) for 1 h at room temperature. The membrane was then incubated with 1 μg/ml of the indicated primary antibodies in TBS containing 3% non-fat milk at 4°C overnight. Membrane was washed three times with TBS and blocked with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. After
Itantly (11, 17, 43). We therefore measured the activities of p38 and ERK2 following treatment of HeLa cells with various concentrations of chelerythrine. Similar to JNK1, p38 activity was strongly induced by chelerythrine in a dose-dependent manner (Fig. 1C). Western blotting showed that such induction was not due to the increase of protein levels of p38 (Fig. 1C). When ERK2 activity was measured, a strong induction was detected in PMA-treated cells but not in chelerythrine-treated cells (Fig. 1D). To exclude the possibility that ERK2 activity was measured at an unfavorable time point after treatment with chelerythrine, we also performed a time course study. But no ERK2 activation was observed (data not shown). These results revealed that chelerythrine preferentially induced JNK1 and p38 activation, but had no effect on ERK2 activity.

Chelerythrine-induced JNK1 and p38 Activation Is Mediated by MEKK1 and MKK4—Because incubation of chelerythrine with the immunoprecipitated JNK1 or p38 did not stimulate their kinase activities (data not shown), we speculate that there is the involvement of upstream kinases. Previous studies have shown that activation of JNK by various stimuli is mediated by MEKK1. We therefore examined the effects of chelerythrine on MEKK1 activity. HeLa cells were treated with chelerythrine (2.5 μM) for the indicated times. Endogenous MEKK1 was immunoprecipitated with a specific antibody, and kinase activity was assayed with a kinase-inactive GST-SEK1 fusion protein as substrate. Chelerythrine induced MEKK1 activation in a time-dependent fashion (Fig. 2A). The stimulated MEKK1 activity was detected as early as 15 min after treatment, preceding JNK1 activation shown in Fig. 1B.

Because MEKK1 is unable to phosphorylate JNK directly, we next examined the activation of MKK4 or SEK1, a kinase that has been shown to interact specifically with MEKK1 and activate JNK1 (44). As shown in Fig. 2B, chelerythrine also stimulated MKK4/SEK1 kinase activity, which appeared at 15 min and peaked 1 h after chelerythrine treatment. Thus, both MEKK1 and MKK4 are activated upon treatment with chelerythrine. Furthermore, cotransfection with a dominant-negative mutant MEKK1(KR) or MKK4(ala) impaired chelerythrine-induced JNK1 activation (Fig. 2C) as well as p38 activation (Fig. 2D). These results provide strong evidence that the upstream kinases, MEKK1 and MKK4, mediate the activation of JNK1 and p38 by chelerythrine.

Treatment with Other PKC Inhibitors or Down-regulation of PKC with PMA Does Not Affect JNK and p38 Activation by Chelerythrine—Chelerythrine has been described to be an inhibitor of PKC (32) and also strongly activated JNK and p38 as demonstrated above. We wondered whether inhibition of PKC might serve as a general signal to initiate JNK or p38 pathway. To test this, we examined the effects of two other potent PKC inhibitors, GF-109203X and Go6983. Previous studies have shown that GF-109203X selectively inhibits the isozymes, α, β, γ, δ, and ε (45), and Go6983 selectively inhibits α, β, γ, and δ, and ζ PKC isozymes (46). Unlike chelerythrine, treatment of HeLa cells with GF-109203X or Go6983 did not stimulate JNK1 activity (Fig. 3A). Furthermore, pretreatment with these two inhibitors had little effect on chelerythrine-induced JNK1 activation (Fig. 3B). We also examined the effect of down-regulation of PKC on JNK1 activity. HeLa cells were pretreated with PMA (100 nM) for 36 h, prior to challenge with chelerythrine for different times. The result in Fig. 3C shows that prolonged treatment with PMA had no effect on JNK1 activity and did not affect chelerythrine-induced JNK1 activation either. Similar results were obtained when p38 activity was measured (Fig. 3D). These data indicate that inhibition of PKC does not necessarily lead to JNK or p38 activation and raise the possibility that chelerythrine-induced JNK1 and p38 activation is inde-
were determined with the anti-HA antibody by Western blotting. GST-ATF2-(1–96) as substrate. The protein levels of exogenous p38 were assayed for activity with GST-c-Jun-(1–79) as substrate. The protein activity was measured by immunocomplex assays using His-JNK1 as substrate. C, suppression of JNK activation by dominant-negative mutants of MEKK1 and MKK4. HeLa cells were transfected with an expression vector encoding HA-tagged JNK1 and an empty vector or a vector encoding a dominant-negative kinase mutant, MEKK1(KR) or MKK4(ala). Twenty four hours after transfection, cells were stimulated with chelerythrine (CHE, 2.5 \mu M) for 2 h. Exogenous JNK1 was immunoprecipitated with the HA-specific monoclonal antibody (12CA5) and assayed for kinase activity with GST-c-Jun-(1–79) as substrate. The protein levels of exogenous JNK1 were measured with the anti-HA antibody by Western blotting. D, suppression of p38 activation by dominant-negative mutants of MEKK1 and MKK4. HeLa cells were transfected with a plasmid encoding HA-tagged p38\alpha and MEKK1(KR) or MKK4(ala) expression vector as in C. Following stimulation with 2.5 \mu M chelerythrine for 2 h, exogenous p38\alpha was immunoprecipitated with anti-HA monoclonal antibody and assayed for kinase activity with GST-ATF2-(1–96) as substrate. The protein levels of exogenous p38 were determined with the anti-HA antibody by Western blotting.

Chelerythrine Does Not Inhibit PKC Activity at Concentrations That Activate JNK1 and p38—To define further the role of PKC in chelerythrine-induced JNK and p38 activation, we examined the effect of chelerythrine on PKC activity. GF-109203X and Go6983 were also included in this experiment. As expected, GF-109203X and Go6983 inhibited PKC activity when added to cell culture medium (Fig. 4A); however, at the concentrations that strongly induced JNK1 and p38 activation, chelerythrine did not show any inhibitory effect on PKC activity. To exclude the possibility that the lack of inhibitory effect of chelerythrine is due to the dissociation of the compound from PKC during the isolation procedure, we conducted the in vitro assays. Total PKC was isolated from HeLa cells and incubated with different concentrations of agents tested. PKC activity was determined by the phosphorylation of histone H1. As shown in Fig. 4B, GF-109203X and Go6983 showed strong inhibition on PKC activity, whereas the effect of chelerythrine was dose-dependent. No significant inhibition was observed for chelerythrine at the concentrations less than 25 \mu M. Similar results were obtained when PKC activity was assayed in the presence of activators, phosphatidylserine and dieolein (data not shown).

Considering the diversity and the different nature of PKC isoforms, we further measured the effect of chelerythrine on individual isozyme activity. Western blotting showed that HeLa cells expressed six PKC isozymes, \( \alpha, \gamma, \delta, \epsilon, \zeta, \) and \( \lambda \) (or \( \iota \)), a human homologue of \( \lambda \), and prolonged treatment with PMA (100 nM, for 36 h) dramatically down-regulated conventional PKCs (\( \alpha \) and \( \gamma \)) and novel PKCs (\( \delta \) and \( \epsilon \)) but had no effect on atypical PKCs (\( \zeta \) and \( \lambda \)) (Fig. 4C). Incubation of chelerythrine (5 \mu M) with these isozymes immunoprecipitated from cell lysates did not significantly change their kinase activities as compared with solvent control (Fig. 4D). Based on these and the results described above, we conclude that chelerythrine-induced JNK1 and p38 activation does not require the inhibition of PKC.

Chelerythrine-induced JNK1 and p38 Activation Is Inhibited by Antioxidants—Lack of effect of chelerythrine on PKC raises the question as to the initial signal that led to the activation of MEKK1 \( \rightarrow \) MKK4 \( \rightarrow \) JNK module. The iminium bond of chelerythrine is known to be reactive to cellular thiols including GSH and sulfhydryl group of protein (47). As a result, treatment with chelerythrine may cause depletion of intracellular GSH pool and/or protein thiol. Since GSH is a primary antioxidant responsible for the removal of extra peroxides and the maintenance of cellular redox status, depletion of GSH by chelerythrine will cause accumulation of peroxides and thereby generate oxidative stress. To test this hypothesis, we measured the peroxide levels in chelerythrine-treated cells using a peroxide-sensitive fluorescent dye, H\(_2\)DCFDA. This chemical is oxidized to the peroxide-sensitive fluorescent dye, H\(_2\)DCF. FACScan analysis revealed that chelerythrine...
were treated with chelerythrine (CHE), 5-chloromethylmesidin. H2O2 (1 mM) was included as a positive control (Fig. 5A). Addition of aminotriazole, a catalase inhibitor, slightly enhanced the chelerythrine-induced accumulation of peroxides (Fig. 5A). H2O2 (1 mM) was included as positive control for 30 min. Intracellular peroxides were analyzed by FACScan as described under “Materials and Methods.” PKC activity was assayed with histone H1 as substrate. B, PKC was first isolated from the untreated HeLa cells and then incubated with different concentrations of chelerythrine (C), GF-109203X (G), or Go 6983 (G) for 30 min at 30 °C. PKC activity was assayed as in A. C, HeLa cells were either untreated or treated with PMA (100 nM) for 36 h. PKC isozymes were detected with the specific antibodies by Western blotting. D, PKC isozymes were immunoprecipitated from untreated HeLa cells with the specific antibodies and incubated with chelerythrine (5 μM) or solvent (0.1% Me2SO) for 30 min at 30 °C. PKC activity was assayed with histone H1 as substrate. The activity of each PKC isozyme detected in solvent control was used as 100%. The results shown are averages of three separate experiments.

After demonstrating the oxidative stress induced by chelerythrine, we next studied the effects of pretreatment of cells with antioxidants, NAC, DTT, and GSH prior to chelerythrine stimulation. Activation of JNK1 by chelerythrine was inhibited by NAC, DTT, and GSH pretreatment (Fig. 5B). Similarly, p38 activation was blocked in the presence of these antioxidants (Fig. 5C). Treatment with the antioxidants alone did not affect the JNK1 and p38 activity over the time period of experiments (data not shown). These results suggest that induction of oxidative stress plays a crucial role in chelerythrine-induced JNK1 and p38 activation.

Chelerythrine Induces Apoptosis in HeLa cells, Which Is Prevented by Antioxidants—Previous studies show that chelerythrine induced apoptosis in leukemia and lymphoma cells (28, 33). To address the biological significance of chelerythrine-induced JNK1 and p38 activation, we examined their roles in apoptosis. As shown in Fig. 6A (bottom panel), HeLa cells after treatment with chelerythrine (5 μM) for 24 h showed extensive nuclear condensation or fragmentation when stained with a fluorescent DNA-binding agent, DAPI, indicative of apoptotic cell death. In contrast, control cells (0.1% Me2SO-treated cells) showed normal nuclear morphology (Fig. 6A, top panel). Chelerythrine-induced apoptosis was further confirmed by DNA laddering, which showed a dose-dependent effect (Fig. 6B).

To provide primary evidence for the role of JNK1 or p38 in the induction of apoptosis by chelerythrine, we examined the effects of antioxidants, NAC, DTT, and GSH, which have been shown above to inhibit chelerythrine-induced JNK and p38 activation. Pretreatment with these antioxidants significantly inhibited chelerythrine-induced apoptosis as determined by DAPI staining (Fig. 6C). Cotreatments of antioxidants with solvent (0.1% Me2SO) had little effect on cell viability.

Dominant-negative Mutants of MEKK1, MKK4, JNK1, and p38 Block Chelerythrine-induced Apoptosis—To obtain additional evidence for the role of JNK and p38 pathways in chelerythrine-induced apoptosis, we performed transient transfection death assay with dominant-negative mutants, MEKK1(KR), MKK4(ala), JNK1(APF), and p38(AGF). HeLa cells were transfected with β-galactosidase expression vector and stained with X-gal and DAPI to detect the number of blue and green fluorescent cells, respectively. Cotreatment of dominant-negative mutants with chelerythrine significantly blocked chelerythrine-induced apoptosis (Fig. 6D).

FIG. 6. Cytotoxic effects of chelerythrine on HeLa cells, which is prevented by antioxidants. A, cultured HeLa cells were either treated with chelerythrine (CHE, 2.5 μM), or NAC (20 mM), DTT (5 mM), or GSH (5 mM) for 1 h. B, cultured HeLa cells were either treated with chelerythrine (CHE, 2.5 μM) or solvent (0.1% Me2SO) for 24 h. C, cultured HeLa cells were either treated with NAC (20 mM), DTT (5 mM), or GSH (5 mM) for 1 h. D, cultured HeLa cells were transfected with MEKK1(KR), MKK4(ala), JNK1(APF), and p38(AGF) expression vector, and transfected with β-galactosidase expression vector and stained with X-gal and DAPI to detect blue and green fluorescent cells, respectively.
Role of MAPKs in Chelerythrine-induced Apoptosis

FIG. 6. Effect of antioxidants on chelerythrine-induced apoptosis. A and B, induction of apoptosis by chelerythrine. HeLa cells were treated with 2.5 or 5 μM chelerythrine for 24 h or left untreated as control. Apoptotic cells were detected by DAPI staining (A) or DNA laddering (B) as described under “Materials and Methods.” C, inhibition of chelerythrine-induced apoptosis by antioxidants. HeLa cells were pretreated with catalase (CAT, 3,000 units/ml), NAC (20 mM), DTT (5 mM), or GSH (5 mM) for 1 h and then challenged with chelerythrine (CHE, 5 μM) or solvent (0.1% Me2SO) for 24 h in the continuing presence of the antioxidants. Apoptotic cells were quantitated with DAPI staining.

and the empty vector or the vectors encoding kinase mutants. After transfection, cells were either treated with chelerythrine or with solvent (0.1% Me2SO) as control. To ensure that the majority of cells were attached to the plates after chelerythrine treatment, a suitable treatment time period was determined from preliminary experiments. Following treatment with chelerythrine (5 μM for 12 h), the cells were stained with X-gal to examine β-galactosidase-expressing cells (blue in color) (Fig. 7A). Cells transfected with empty vector and treated with solvent had normal appearance (a in Fig. 7A). When treated with chelerythrine, most of cells including the transfected (blue color) and the untransfected (light color) assumed a characteristic apoptotic appearance (round shape shown in b of Fig. 7A). However, the majority of cells transfected with MEKK1(KR) (c in Fig. 7A), MKK4(ala) (d in Fig. 7A), JNK1(APF) (e in Fig. 7A), or p38(AGF) (f in Fig. 7A) showed normal shape in the presence of chelerythrine, whereas most of untransfected cells (light color) in these plates showed apoptotic morphology.

To quantitate apoptosis, the number of blue cells and their morphology were determined. The results were expressed as the percentage of round blue cells (apoptotic cells) as factor of total blue cells (22). As shown in Fig. 7B, expression of dominant-negative mutants of MEKK1 and MKK4 substantially inhibited chelerythrine-induced apoptosis. Although expression of a dominant-negative mutant of JNK or p38 alone showed less inhibition, transfection with both mutants together provided a pronounced protective effect against chelerythrine-induced cell death and even more effective than a dominant-negative mutant of MEKK1 or MKK4. This result suggests that activation of both JNK and p38 is required for the full induction of apoptosis by chelerythrine.

DISCUSSION

PKC plays an important role in signal transduction (23, 48). In addition to its normal functions in various cellular processes, activation of PKC has also been implicated in the pathogenesis of diseases, especially the role in phorbol ester-promoted tumorigenesis (49). It is therefore of considerable interest to identify or develop PKC inhibitors. By using the PKC isozymes isolated from rat brain, chelerythrine was shown to be a competitive inhibitor with respect to phosphatase acceptor (histone IIc) and a non-competitive inhibitor with respect to ATP. Furthermore, chelerythrine did not inhibit other kinases, such as tyrosine protein kinase, cAMP-dependent protein kinase, and Ca2+-calmodulin-dependent protein kinase with great efficacy (32). Accordingly, chelerythrine has been used as a specific PKC inhibitor in the studies aiming at elucidation of the roles of PKC in various cellular functions. However, by using chelerythrine, a number of studies have obtained the results that would not be anticipated by an inhibitor of PKC (34, 35, 38). Recently, Lee et al. (36) re-evaluated the effects of chelerythrine on PKC activity under the experi-
Role of MAPks in Chelerythrine-induced Apoptosis

mental conditions published previously (32). In contrast to the previous results, they found that chelerythrine did not show a potent inhibitory effect on PKC activity, regardless of the sources of PKC and the presence of different activators. In addition, chelerythrine did not affect phorbol 12,13-dibutyrate binding to the regulatory domain of PKC, and no significant alteration of PKC-α, -β, -γ translocation was observed in human leukemia (HL-60) cells. These results strongly suggest that a mechanism independent of PKC should be considered as responsible for the biological activities of chelerythrine. In the present study, we demonstrated that chelerythrine activated JNK1 and p38, but not ERK2, pathways. The activation of p38 and JNK1 by chelerythrine was independent of PKC inhibition as evidenced by the following: (i) chelerythrine-induced JNK1 and p38 activation was not affected by the pretreatment with GF-109203X and Go6983 that substantially reduced the PKC activity in HeLa cells; (ii) a prolonged treatment with PMA almost completely down-regulated cPKC and nPKC isozymes in HeLa cells but had little effect on chelerythrine-induced JNK1 and p38 activation; (iii) chelerythrine did not inhibit PKC activity present in HeLa cells at the concentrations that stimulated the activities of p38 and JNK1. Thus, activation of JNK1 and p38 pathways as shown in this study represents a novel biochemical property of chelerythrine.

JNK path way is regulated by the upstream MAPKKKs (50, 51). Genetic and biochemical studies have identified a number of such kinases (52), among which MEKK1 has been shown to play a major role in JNK activation by various stimuli such as growth factors (53), microtubule disruption (54), and alkylation agents (55). The stimulated MEKK1 phosphorylates and activates MKK4, which, in turn, activates JNK (56). However, the role of MEKK1 in p38 activation appears to be controversial. Several studies demonstrated that overexpression of MEKK1 induced marked activation of JNK but not p38 (56–58), whereas others showed that overexpressing MEKK1 can phosphorylate and activate p38 (59, 60). Our results suggest that MEKK1 mediated both JNK1 and p38 activation by chelerythrine, because MEKK1 activity was stimulated in chelerythrine-treated cells and blocking this induced MEKK1 activity with its dominant-negative mutant attenuated JNK1 and p38 activation. The role of MEKK1 in chelerythrine-induced JNK1 and p38 activation is further supported by the activation of MKK4 and the inhibitory effect of its dominant-negative mutant on JNK1 and p38 activation. However, it is worthy to note that, when overexpressed at similar levels, the dominant-negative mutants of MEKK1 and MKK4 showed much stronger inhibitory effect on JNK1 activation than that on p38 activation by chelerythrine, suggesting that additional signaling pathways may exist in regulating p38 activity. Indeed, certain upstream MAPKKKs have been identified as components of p38 signaling pathways. These MAPKKKs include TAK1 (61) and ASK1 (62) that directly phosphorylate and activate MKK3 and/or MKK6, leading to the activation of p38. It should be also noted that the dominant-negative mutant of MEKK1 or MKK4 may interact with several partners and hence may block more than one of the parallel MAPK pathways. Thus, whether other related kinases are involved in chelerythrine-induced p38 and JNK activation remains to be studied.

The lack of effect of chelerythrine on PKC activity raises the questions as to the initial signals that lead to the activation of JNK1 and p38 pathways. As noted above, chelerythrine-induced JNK1 and p38 activation was suppressed by the antioxidants, NAC, GSH, and DTT, implicating a role of oxidative stress. However, the mechanisms by which chelerythrine-induced oxidative stress activates JNK1 and p38 pathways are not clear. Since iminium bond of chelerythrine is reactive to cellular thiols, including protein sulphydryl groups, treatment with chelerythrine may cause covalent modification of one or multiple upstream components of JNK and p38 pathways, resulting in their activation. Several apical proteins in JNK and p38 pathways have been identified. These proteins include the Rac1 (63), Cdc42 (64), and/or Ras (65). Interestingly, overexpression of a dominant-negative mutant of Rac1, Cdc42, or Ras had no significant effect on chelerythrine induction of JNK and p38 (data not shown). Alternatively, chelerythrine may inhibit a specific phosphatase responsible for down-regulation of one or multiple activated upstream protein kinases. Inhibition of a constitutive dual specificity JNK phosphatase has been recently implicated in the activation of JNK and p38 by arsenite (66), an oxidant that primarily attacks protein sulfhydryls.

Since chelerythrine-stimulated JNK1 and p38 activities require upstream kinases, MEKK1 and MKK4, it is possible that chelerythrine interferes with a phosphatase(s) that is able to regulate MEKK1 activity. In fact, all phosphatases have an active cysteine site that could be the target of oxidants. It is also possible that chelerythrine interacts with glutathione, resulting in its depletion and the increase of reactive oxygen species, which may, in turn, activate JNK and p38 pathways.

Apoptosis, or programmed cell death, is a highly regulated process that involves activation of a cascade of molecular events, leading to cell death that is characterized by plasma membrane blebbing, shrinkage, chromatin condensation, chromosomal DNA fragmentation, and formation of membrane-bound apoptotic bodies (67, 68). A number of studies indicate that activation of JNK or p38 kinase pathway plays a crucial role in apoptosis induced by certain stimuli. For example, in PC-12 neuronal cells, activation of JNK and p38 pathways is required for apoptosis induced by nerve growth factor depletion (21). In U937 and Jurkat cells, interfering with JNK or p38 pathway has been shown to block apoptosis induced by ceramide and UV radiation (22, 69). Activation of JNK and p38 pathways has also been implicated in anticancer drug-induced apoptosis (70, 71). In the present study, we found that chelerythrine induced apoptosis at the concentrations that stimulated JNK and p38 activities. Pretreatment with antioxidants blocked JNK and p38 activation and also attenuated the induction of apoptosis by chelerythrine. Furthermore, specifically blocking JNK and p38 pathways by overexpression of a dominant-negative mutant of MEKK1, MKK4, JNK1, or p38 inhibited chelerythrine-induced apoptosis. Therefore, JNK and p38 pathways are also involved in the regulation of apoptosis induced by chelerythrine.

In summary, we demonstrate that chelerythrine induces JNK and p38 activation without inhibiting PKC activity. Activation of JNK and p38 is mediated by upstream kinases, MEKK1 and MKK4, and regulates apoptosis. Chelerythrine is known to display a variety of biological activities, such as inhibition of taxol-mediated polymerization of rat brain tubulin (47) and histamine release induced by aggregation of the IgE receptors on human basophils (34). Chelerythrine is also shown to stimulate protein phosphorylation in the mitochondrial fraction of rat retina (35). It will be interesting to study whether JNK and p38 play a role in those biological responses induced by chelerythrine.

Acknowledgments—We thank Drs. Anning Lin and Micheal Karin (University of California, San Diego) for providing pcdNA3-4-RA-JNK1 and pGEX-GST-e-Jun-(1–79); Dr. J. Silvio Gutkind (National Institutes of Health, Bethesda) for providing pGEX-GST-ATF2-(1–96); Dr. Zhengbin Yao (Amgen Inc., Boulder, CO) for providing pcdNA3-4-RA-p38; Dr. Roger J. Davis (Howard Hughes Medical Institute, Worcester, MA) for providing pcDNA3-Flag-p38(AGF), pcDNA3-Flag-JNK1(4APF), and pcDNA3-Flag-MKK4(ala); and the members of the Kong laboratory for their critical reading of this manuscript.
Activation of p38 and c-Jun N-terminal Kinase Pathways and Induction of Apoptosis by Chelerythrine Do Not Require Inhibition of Protein Kinase C
Rong Yu, Sandhya Mandlekar, Tse-Hua Tan and A.-N. Tony Kong

J. Biol. Chem. 2000, 275:9612-9619.
doi: 10.1074/jbc.275.13.9612

Access the most updated version of this article at http://www.jbc.org/content/275/13/9612

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 70 references, 36 of which can be accessed free at http://www.jbc.org/content/275/13/9612.full.html#ref-list-1