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ARTICLES

An Oxidative Stress Mechanism Mediates Chelerythrine-Induced Heparin-Binding EGF-Like Growth Factor Ectodomain Shedding

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Abstract Regulated shedding of cell surface proteins is a mechanism for rapid activation of autocrine and paracrine signaling. Here we report that chelerythrine, a protein kinase C (PKC) inhibitor that possesses a variety of biological functions, is a potent inducer of heparin-binding epidermal growth factor-like growth factor (HB-EGF) shedding from the cell surface. Chelerythrine induced a time- and dose-dependent shedding of an HB-EGF–alkaline phosphatase (HB-EGF-AP) fusion protein expressed in MC2 rat prostate epithelial cells. The soluble form of HB-EGF-AP bound to heparin and exhibited potent biological activity as measured by DNA synthesis assay. Chelerythrine-induced HB-EGF shedding was metalloproteinase-(MMP-) mediated because specific MMP antagonists inhibited shedding by $\geq$60%. Chelerythrine stimulated production of reactive oxygen species, and antioxidants prevented chelerythrine-induced HB-EGF shedding, suggesting that the production of intracellular peroxides is necessary for this event. Consistent with this possibility, antioxidant- and MMP-inhibitable shedding was also demonstrated when hydrogen peroxide was used as an inducer. Although JNK/SAPK and p38 MAPK pathways were activated by chelerythine, these signaling mechanisms were not required to mediate the shedding event. However, JNK signaling was involved in chelerythrine-stimulated apoptosis. Our results suggest that HB-EGF shedding induced by chelerythrine is mediated predominantly via the production of reactive oxygen species. J. Cell. Biochem. 94: 39–49, 2005.

Key words: HB-EGF; shedding; metalloproteinase; chelerythrine; reactive oxygen species

Abbreviations used: HB-EGF, heparin-binding epidermal growth factor-like growth factor; EGFR, epidermal growth factor receptor; GPCR, G protein-coupled receptor; TPA, tetradecanoyl-phorbol 13-acetate; H_2O_2, hydrogen peroxide; 2′,7′-DCFH-DA, 2′,7′-dichlorofluorescein diacetate; GSH, glutathione; DTT, dithiothreitol; NAC, *N*-acetyl-L-cysteine; Ab, antibody; AP, alkaline phosphatase; MAPK, mitogen activated protein kinase; SAPK/JNK, stress activated protein kinase/c-Jun N-terminal kinase; MMP, metalloproteinase; Hsp, heat shock protein; ROS, reactive oxygen species.The Children's Hospital/Harvard Urological Diseases Research Center is an NIDDK-sponsored George M. O'Brien Urology Research Center.

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Ectodomain shedding is an important mechanism for the regulation of biological activities of membrane proteins such as growth factors, growth factor receptors and cytokines. This irreversible post-translational modification is finely modulated and is of much physiologic interest because shedding of membrane proteins changes their structure, location, mode of action, and potentially their biological functions.

Heparin-binding EGF-like growth factor (HB-EGF) is a direct activating ligand for the EGF receptor (EGFR/ErbB1) and the related tyrosine kinase, ErbB4 (reviewed in [Raab and Klagsbrun, 1997]). As with other EGFR ligands, HB-EGF is initially expressed as a transmembrane precursor (proHB-EGF) from which the mature, secreted form is derived by proteolytic

processing [Goishi et al., 1995; Suzuki et al., 1997; Dethlefsen et al., 1998]. Membrane-bound proHB-EGF is capable of activating its cognate receptors on adjacent cells, a process termed juxtacrine signaling [Goishi et al., 1995; Takemura et al., 1997]. Once mobilized from the cell surface, the soluble form of HB-EGF possesses autocrine and paracrine activity. HB-EGF cleavage also appears to be a necessary element of the EGFR transactivation mechanism that occurs in response to molecules that are not direct EGFR ligands, such as ligands for G-protein coupled receptors (GPCRs) [Prenzel et al., 1999; Umata et al., 2001]. These observations suggest an important regulatory function for shedding of the HB-EGF protein.

HB-EGF ectodomain shedding can be triggered by a variety of inducers, such as 12-O-tetradecanoylphorbol 13-acetate (TPA or PMA), a potent activator of protein kinase C (PKC) [Goishi et al., 1995; Gechtman et al., 1999] and lysophosphatidic acid (LPA) [Umata et al., 2001]. Signaling effectors such as PKCδ, MDC9 (ADAM9/Meltrinγ) [Izumi et al., 1998], and the Ras/Raf/MEK signaling pathway [Gechtman et al., 1999; Umata et al., 2001] have been implicated in the regulated cleavage of HB-EGF. Calcium influx also strongly stimulates HB-EGF processing independently of PKC [Dethlefsen et al., 1998]. HB-EGF gene expression and protein synthesis are upregulated in response to a variety of environmental stimuli such as oxidant stress [Sakai et al., 2001], high glucose and hyperosmolarity [Asakawa et al., 1996], and mechanical stretch [Nguyen et al., 1999; Park et al., 1999]. HB-EGF up-regulation has been demonstrated in the neointima following balloon injury to rat carotid arteries [Igura et al., 1996], in foamy macrophages within human atherosclerotic plaques [Miyagawa et al., 1995], and in kidney epithelium and bladder smooth muscle in response to urinary tract obstruction [Borer et al., 1999; Nguyen et al., 2000]. Based on these findings, it is reasonable to postulate that aberrant regulation of shedding may also play a role in situations where HB-EGF protein levels are increased as a result of pathologic processes.

In this study, we found that chelerythrine, a PKC inhibitor, and hydrogen peroxide (H_2O_2) are both potent inducers of HB-EGF cell surface processing. Our findings suggest that reactive oxygen species (ROS) are mediators in the regulated shedding of HB-EGF.

EXPERIMENTAL PROCEDURES

Materials

Chelerythrine was purchased from ALEXIS Inc. (San Diego, CA). Hydrogen peroxide (H_2O_2), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), *N*-acetyl-L-cysteine (NAC), glutathione (GSH), dithiothreitol (DTT), and superoxide dismutase (SOD) were from Sigma Chemical Co. (St. Louis, MO). BB3489 was kindly provided by British Biotech (Oxford, UK). BB2156, genistein, wortmannin, PD098059, SB203580, rapamycin, catalase, and SP600125 were purchased from Calbiochem Inc. (San Diego, CA). The following monoclonal (mAb) and polyclonal antibodies (pAbs) were used: goat anti-human HB-EGF pAb (R&D System, Inc., Minneapolis, MN), anti-AP mAb (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phosphorylated JNK/SAPK (Thr183/Tyr185) pAb, anti-phosphorylated p-p38/MAPK (Thr180/Tyr182) pAb, anti-phosphorylated p-Erk/MAPK (Thr202/Tyr204) pAb (Cell Signaling, Beverly, MA); anti-β-actin (Sigma Chemical Co.).

Cultured Cells and Transfection

The proHB-EGF-AP fusion construct (pRc/CMV-proHB-EGF-AP) has been described [Dethlefsen et al., 1998]. NbMC2 (MC2) rat prostate carcinoma cells [Freeman et al., 1994] expressing high level of alkaline phosphatase (AP)-tagged HB-EGF (MC2-HB-AP cells) or vector only (MC2-vector cells) were established and cultured as previously described [Dethlefsen et al., 1998]. The kinase-negative JNK1 in pcDNA, DN-JNK1, was kindly provided by Dr. Roger J. Davis at the University of Massachusetts Medical School. Transient transfection into subconfluent cells was performed with Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Indirect Immunofluorescence Cell Staining

Low density MC2-HB-AP and MC2-vector cells were fixed with ice cold methanol, and incubated with anti-AP antibody in 2% BSA solution, followed by secondary antibody conjugated to Texas-Red. After mounting and incubation with DAPI-containing mounting agent, cells were analyzed by fluorescence microscopy.

HB-EGF Secretion

Secretion of HB-EGF was measured by determining levels of AP activity in the medium using cells expressing proHB-EGF-AP fusion proteins as described previously [Dethlefsen et al., 1998]. Briefly, 1×10^6 cells/well were seeded in 6-cm dishes for 24 h. Cells were first starved in serum free medium for the indicated times followed by treatment with the various agents. Conditioned medium was collected at the indicated times and AP activity was measured spectrophotometrically.

Membrane Fractionation

Total cell membranes were prepared by resuspending cells in hypotonic buffer [50 mM HEPES pH 7.4; 10 mM NaCl; 5 mM $MgCl_2$; 0.1 mM EDTA; 1 mM PMSF; and COMPLETE protease inhibitor cocktail tablet (Roche, Mannheim, Germany)] followed by mechanical disruption (12 strokes with a Dounce homogenizer) and centrifugation (9,000*g* for 10 min).

Immunoblotting

Cells were solubilized with lysis buffer [1% Nonidet P-40; 50 mM Tris pH 7.4; 10 mM NaCl; 1 mM NaF; 5 mM $MgCl_2$; 0.1 mM EDTA; 1 mM PMSF; and COMPLETE protease inhibitor cocktail tablet (Roche)] and 20 μg protein was subjected to SDS–PAGE and electroblotted onto nitrocellulose (NC). Blots were stained with Ponceau S to verify uniform transfer and equal protein loading (where appropriate), and subjected to immunoblotting with the various antibodies.

Heparin Chromatography and Determination of Mitogenic Activity

Conditioned media were collected after stimulation with chelerythrine and fractionated by heparin-affinity FPLC, followed by determination of mitogenic activity in the eluted fractions, as previously described [Dethlefsen et al., 1998].

Reactive Oxygen Species (ROS) Measurement

Subconfluent MC2-HB-AP cells were incubated at the indicated conditions with 10 μM 2′,7′-DCFH-DA at 37°C for 20 min. Cells were then washed in PBS and fluorescence measured at 488 nm excitation and 525 nm emission by flow cytometry [Hong et al., 2002].

Determination of Apoptosis

Apoptosis was determined by the genomic DNA fragmentation assay, MTT assay and FACS analysis. To detect DNA laddering MC-2-HB-AP cells (8.5×10^5) seeded in 6-cm dishes were subjected to chelerythrine treatment for 0–4 h. Cellular DNA was extracted, precipitated, and separated by electrophoresis in 1.8% agarose gels [Lin et al., 1999]. For MTT assay, MC2-HB-AP cells seeded in 96-well plates were incubated in chelerythrine solution containing the indicated inhibitors for 4 h. To measure apoptosis by FACS, MC2-HB-AP cells incubated under the same conditions as above were fixed, followed by staining with propidium iodide.

Statistical Analysis

Data were compared using a paired Student *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

In our previous report, NbMC2 (hereafter MC2) cells expressing HB-EGF fusion proteins were used as a model system to study HB-EGF shedding [Dethlefsen et al., 1998]. We showed that a variety of agents, including TPA, ionomycin, TNFα and others induced proteolytic cleavage and release of soluble HB-EGF into the medium. Among the agents tested, chelerythrine, a PKC inhibitor, was found to be among the most potent in inducing HB-EGF secretion [Dethlefsen et al., 1998]. In the present study, we sought to determine the basis for the potent shedding effect induced by chelerythrine. We used a chimeric protein in which membrane-anchored proHB-EGF was fused to a reporter enzyme, AP, within the mature HB-EGF ectodomain. This construct has been described in detail [Dethlefsen et al., 1998]. The HB-EGF-AP fusion protein strategy provides a sensitive means of quantifying shedding by measuring AP levels in the cell medium.

Membrane localization of proHB-EGF-AP in stably transfected MC2 cells (MC2-HB-AP cells) was confirmed by immunofluorescence staining (Fig. 1A). Measurement of AP activity present in the medium verified that chelerythrine stimulated shedding of AP activity in a time- and dose-dependent manner (Fig. 1B and C). Chelerythrine stimulated a transient increase in membrane HB-EGF-AP levels (Fig. 1D), consistent with reported observations that

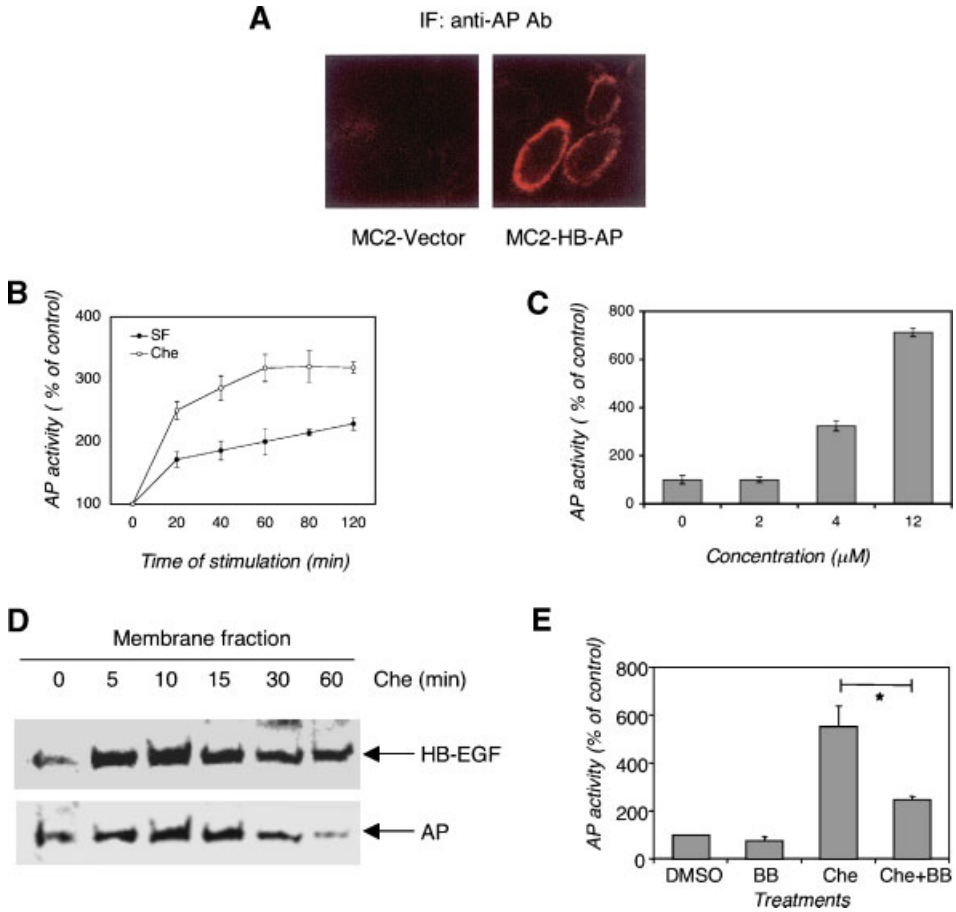

Fig. 1. Chelerythrine stimulates HB-EGF secretion. **A**: Expression of proHB-EGF-AP in MC2 vector-only and MC2-HB-AP cells as demonstrated by immunofluorescence (IF) staining with anti-AP Ab. **B** and **C**: Chelerythrine-induced secretion of HB-EGF-AP increased in a time-and dose-dependent manner MC2-HB-AP cells. Experiments in B were carried out with 4 μM chelerythrine and in C for 1 h under serum free conditions. AP activity in conditioned medium was determined spectrophotometrically as described in Experimental Procedures. **D**: Cell membrane fractions were prepared as described in Experimental Procedures. HB-EGF-AP was detected with Abs against full length HB-EGF or AP by immunoblotting. **E**: Cells were incubated with the MMP inhibitor, BB3489 for 30 min, followed by 1 h chelerythrine treatment. AP activity in conditioned medium was determined as above. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

shedding inducers stimulate transport of proHB-EGF from the cytoplasm to the cell surface [Goishi et al., 1995]. In addition, chelerythrine-induced shedding was inhibited >60% ($P < 0.001$) when cells were pretreated with the matrix metalloproteinase (MMP) inhibitor BB3489, prior to addition of chelerythrine, indicating that the secretion event was MMP-regulated (Fig. 1E). Similar results were obtained when another MMP inhibitor, BB2156, was used prior to addition of chelerythrine (data not shown).

Medium conditioned by chelerythrine-treated MC2-HB-AP cells was subjected to heparin-affinity chromatography to determine whether the AP activity detectable in the medium following treatment with the drug represented processed growth factor. Conditioned medium from chelerythrine-treated MC2-HB-AP cells demonstrated a large peak of DNA synthetic activity that eluted from the column in the range 0.7–1.3 M NaCl (Fig. 2B), that was not detectable in vector-only cells (Fig. 2A). An aliquot of this material was refractionated on heparin-affinity FPLC and was demonstrated to contain a peak of AP activity that eluted from the column in a narrow concentration range near 1.0 M NaCl, indicating that the mitogenic peak evident in Figure 2B contained the HB-EGF-AP fusion protein (Fig. 2C and D). Collectively, these data indicate that HB-EGF-AP released into the medium from chelerythrine-treated cells binds to heparin with an affinity comparable to that of the native growth factor, a

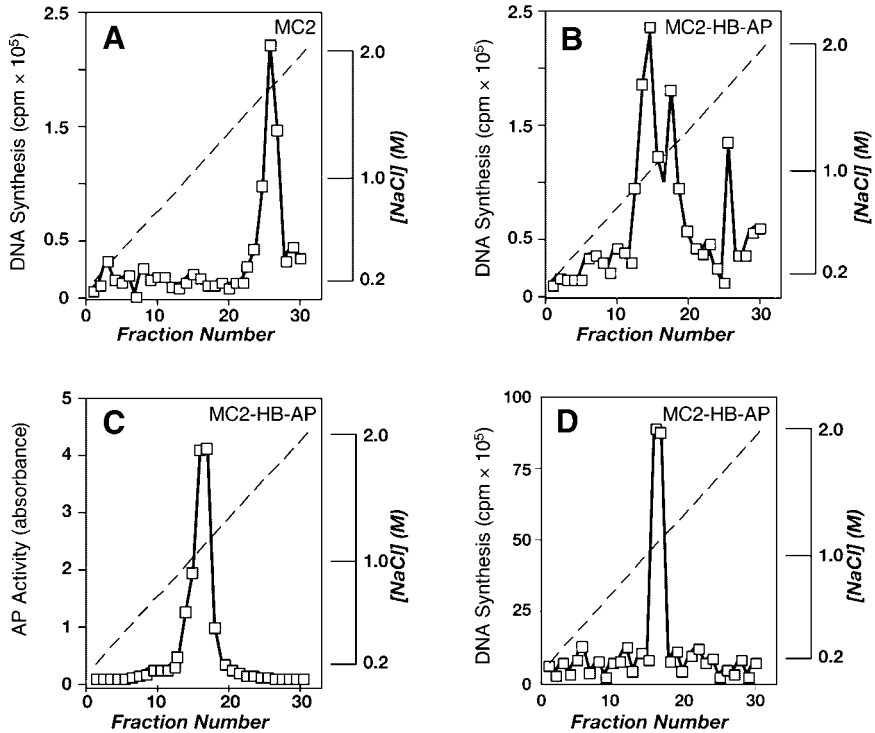

Fig. 2. Secreted HB-EGF-AP exhibits alkaline phosphatase (AP) and mitogenic activity. Vector-transfected MC2 cells and proHB-EGF-AP-expressing cells (MC2-HB-AP cells) were stimulated with 4 μM chelerythrine. Conditioned medium from each of the two cell types was concentrated and fractionated by heparin-affinity FPLC. Fractions were applied to Balb/c3T3 fibroblasts and DNA synthesis rate was measured by tritiated thymidine incorporation. AP activity was measured spectrophotometrically after enzyme assay. **A**: DNA synthesis profile from vector-only cells. **B**: DNA synthesis profile from MC2-HB-AP cells showing a strong growth factor peak between 0.7 and 1.3 M NaCl that is not present in the control cell fractions in panel A. **C** and **D**: Fractions from MC2-HB-AP conditioned medium that eluted from the column in the range of 0.7–1.3 M NaCl were pooled and refractionated by heparin-affinity FPLC. C: AP measurements, indicating that the growth factor activity in the 0.7–1.3 M NaCl fractions obtained from the experiment in panel B contained abundant AP activity. This result demonstrates the presence of the AP moiety originating from the HB-EGF-AP fusion protein released into the medium. D: DNA synthesis profile of the same fractions shown in panel C, verifying that EGF-like motif at the C-terminus of the secreted HB-EGF-AP fusion protein was intact.

result that indicates that the heparin-binding domain (near the processed amino terminus of the protein) is intact. Because the secreted fusion protein retained both AP activity and mitogenic activity, the C-terminal portion of the molecule, which contains the EGF-like motif required for biological activity, was also intact following the shedding event.

Chelerythrine-induced HB-EGF shedding was accompanied by increases in the level of JNK/SAPK and p38 MAPK phosphorylation (Fig. 3A). Consequently, we attempted to evaluate the possible functional contribution of these signal transduction pathways to the shedding mechanism. Notably, detectable increases in phosphorylation of MAPK proteins were first seen 90 min after treatment with the drug, when the induced shedding event is largely concluded, suggesting that these pathways are not shedding effectors. Transient transfection with varying concentrations of a construct encoding a dominant-inhibitory form of JNK1 (DN-JNK1) did not reduce HB-EGF shedding, and actually stimulated shedding at higher DN-JNK1 concentrations (Fig. 3B). Pretreatment with various p38 MAPK inhibitors also did not inhibit shedding (Fig. 3C). Chelerythrine-stimulated JNK/SAPK and p38 MAPK phosphorylation was blocked by transfection with DN-JNK1 and by treatment with pharmacologic p38 inhibitors, respectively (data not shown). These data indicate that JNK/SAPK and p38 MAPK signaling mechanisms are not likely to be shedding mediators, although these kinases are activated concurrently with the shedding process.

Chelerythrine was first reported to be a potent PKC inhibitor and, more recently, was found to induce intracellular ROS [Yu et al., 2000]. To determine whether HB-EGF ectodo-

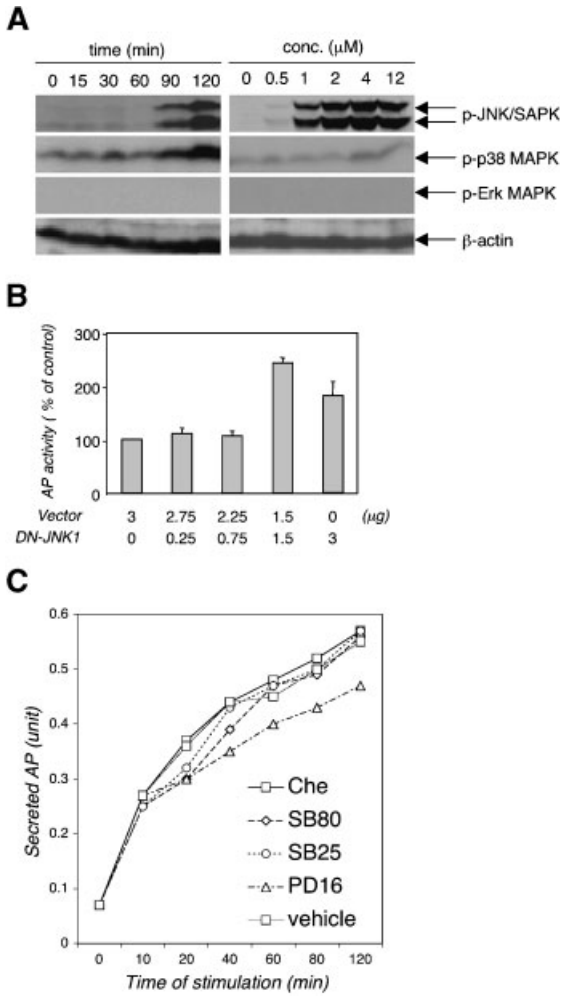

Fig. 3. Chelerythrine-induced HB-EGF secretion is not suppressed by MAPK inhibition. **A**: Chelerythrine treatment activates JNK/SAPK and p38 MAPK, but not Erk/MAPK. MC2-HB-AP cells were treated for the times shown with 4 μM chelerythrine, and with the doses shown for 1 h. Immunoblotting was carried out with Abs against p-JNK/SAPK, p-p38 MAPK, p-Erk/MAPK, and β-actin. **B**: MC2-HB-AP cells were transiently transfected with varying concentrations of DN-JNK1 and vector, with total DNA amount (3 μg) held constant. After chelerythrine treatment (4 μM), as above, AP activity was determined in conditioned medium. **C**: Cells were pretreated with various p38 MAPK inhibitors (10 μM of each inhibitor: SB80, SB203580; SB25, SB220025; PD16, 169316) for 1 h prior to treatment with chelerythrine as above. There was no demonstrable effect of vehicle alone (DMSO) on HB-EGF shedding.

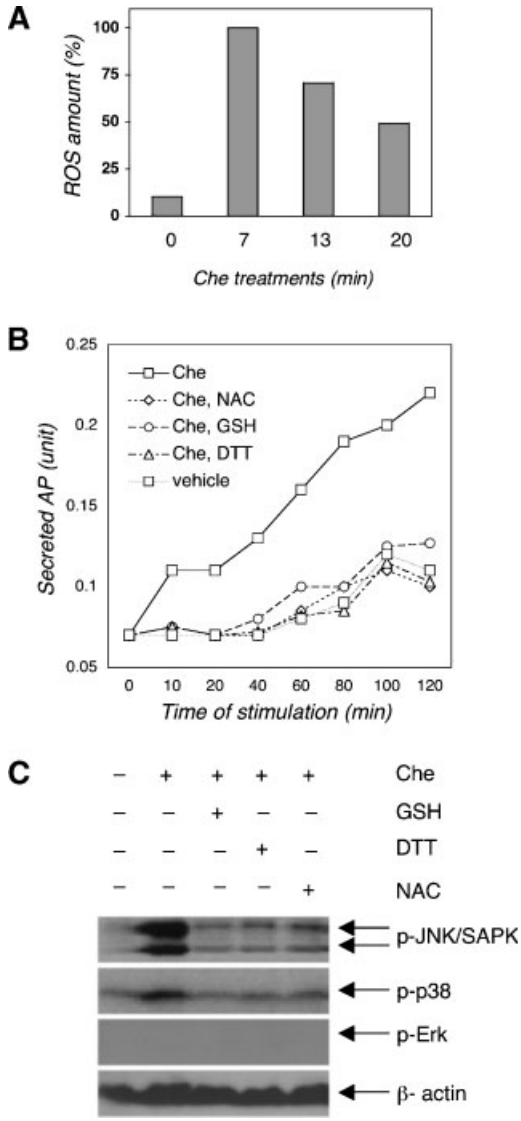

Fig. 4. Effects of ROS inhibitors on chelerythrine-induced HB-EGF processing and MAPK phosphorylation. **A**: MC2-HB-AP cells were incubated with 10 μM 2′,7′-DCFH-DA for 20 min, followed by treatment with 4 μM chelerythrine for the indicated times. ROS levels were measured by FACS. **B**: MC2-HB-AP cells were preincubated with 1 mM GSH, DTT, NAC or vehicle (PBS) for 1 h, followed by stimulation with chelerythrine. AP activity was measured in conditioned medium as above. **C**: Under the same conditions, cell lysates were prepared and immunoblotting was carried out with Abs against p-JNK/SAPK, p-p38 MAPK, p-Erk/MAPK, and β-actin.

main shedding by chelerythrine requires ROS induction, we first measured levels of ROS after chelerythrine treatment. ROS levels were transiently increased when MC2-HB-AP cells were incubated with chelerythrine (Fig. 4A). A series of antioxidants (NAC, *N*-acetyl-L-cysteine; GSH, glutathione; DTT, dithiothreitol), used individually, blocked shedding (Fig. 4B), indicating that chelerythrine requires the generation of free radical intermediates to liberate HB-EGF from the cell surface. Similar results were obtained with a fourth antioxidant (catalase; data not shown). Interestingly, treatment with antioxidants also inhibited JNK/SAPK and p38 MAPK phosphorylation induced by chelerythrine (Fig. 4C).

Next, we determined whether shedding was inducible by H_2O_2, another source of ROS. Similar to chelerythrine, 0.01% H_2O_2 potently induced HB-EGF secretion from MC-2-HB-AP cells (Fig. 5A). The H_2O_2-stimulated shedding effect was inhibited completely by the hydroxamic acid compound, BB3489 ($P < 0.001$, Fig. 5B) and another MMP inhibitor, BB2156 (not shown), demonstrating that this effect was the result of MMP-mediated protein cleavage. Although phosphorylation of JNK/SAPK, p38, and Erk MAPKs was stimulated by H_2O_2 (Fig. 5C), HB-EGF shedding by H_2O_2 was not attenuated by inhibitors of p38 MAPK or Erk-MAPK (Fig. 5D). PI3-kinase and $p70^{S6kinase}$ inhibitors were similarly unable to inhibit H_2O_2-stimulated shedding (Fig. 5D). However, shedding was inhibited by pretreatment of cells with the antioxidants NAC, superoxide dismutase (SOD), and catalase (Fig. 5E and F).

Direct observation of MC-2-HB-AP cells treated with chelerythrine suggested that cells were induced to undergo apoptosis in response to the drug. This would be consistent with published

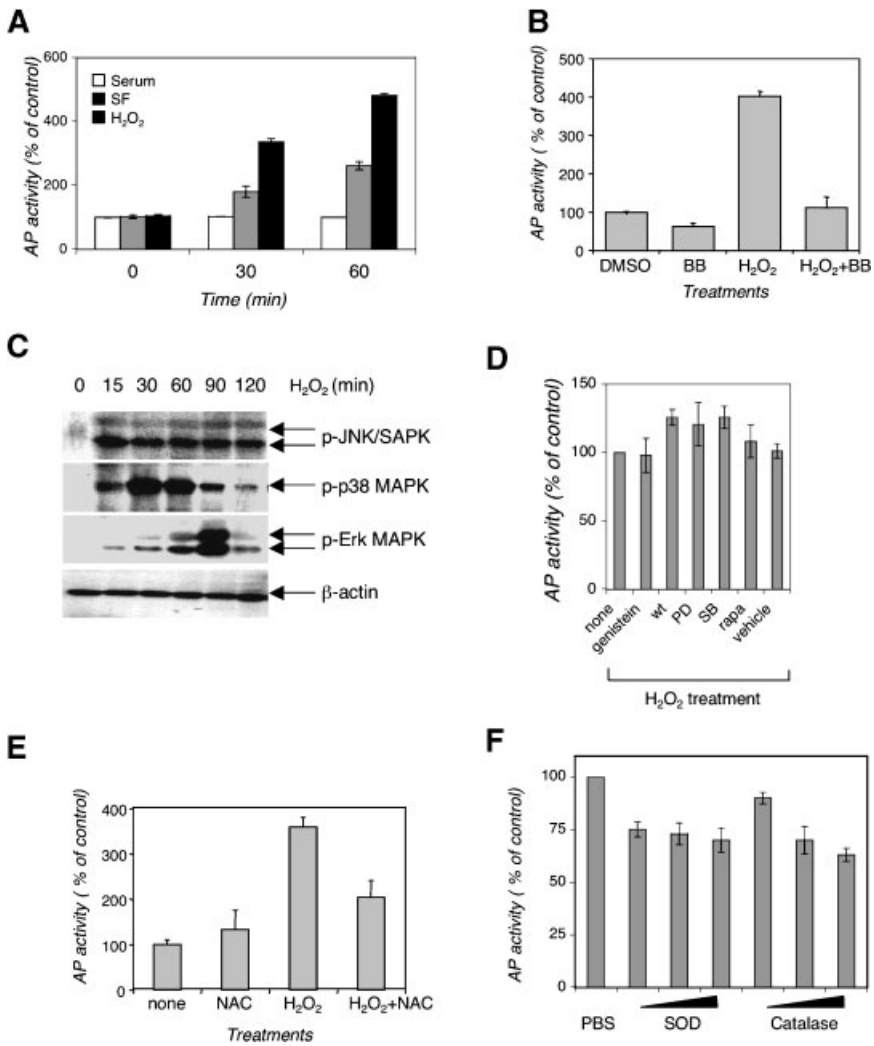

Fig. 5. Effects of H_2O_2 on HB-EGF processing. **A**: MC2-HB-AP cells were incubated with 0.01% H_2O_2 in serum free medium. AP activity was determined in the medium at 0, 30, and 60 min after treatment. **B**: Cells were pretreated with 10 μM BB3489, followed by H_2O_2 treatment under the above conditions. AP activity was then determined in the medium. **C**: Cell lysates were prepared under the same conditions. Phosphorylation of JNK/SAPK, p38 MAPK, and Erk/MAPK was determined by immunoblotting. **D**: Pretreatment of cells with inhibitors or vehicle alone was for 30 min, followed by 0.01% H_2O_2 stimulation (none, no inhibitor; genistein; wt, wortmannin; PD, PD098059; SB, SB203580; rapa, rapamycin). AP activity was then determined in the medium. **E**: Effect of 1 mM NAC on the H_2O_2-induced HB-EGF shedding was determined. AP activity was determined in the medium. **F**: Cells were pretreated with PBS, varying doses of SOD (1, 5, and 10 U) or catalase (1, 5, and 10 U), followed by H_2O_2 treatment for 1 h. AP activity was then determined in the medium.

results showing that chelerythrine is a potent inducer of apoptosis in some cell types [Yu et al., 2000; Yamamoto et al., 2001]. To verify that chelerythrine-induced HB-EGF shedding coincided with physiologic apoptosis, cells were assayed for the presence of low MW DNA fragmentation, a definitive indicator of apoptosis, following treatment with the drug. Chelerythrine induced DNA fragmentation in MC2-HB-AP cells, as shown in Figure 6A. Under conditions of chelerythrine treatment, the JNK inhibitor, SP600125 (Fig. 6B,C), but not the ROS inhibitor, catalase, or the p38 MAPK inhibitor, SB203580 (Fig. 6B), elicited a protective effect. These data suggest that JNK signaling is a primary mediator of apoptosis stimulated by chelerythrine.

DISCUSSION

Growth factors belonging to the EGF-like family are initially expressed as membrane-anchored precursors from which the mature (soluble) proteins are produced by regulated proteolytic cleavage events. This regulatory step facilitates their rapid export into the extracellular space, where they are available to bind cognate receptors in the local environment. Because many or all of the EGF-like growth factors are involved in autocrine and paracrine signaling pathways, this mechanism facilitates rapid activation of these pathways in pathological states, such as wound healing.

In the present study, we describe a ROS-dependent mechanism as the basis for the potent HB-EGF shedding effect of chelerythrine, noted by us in a previous report [Dethlefsen et al., 1998]. The conclusion that chelerythrine stimulates HB-EGF shedding by ROS mobilization is based on: (1) the demonstration that chelerythrine stimulated ROS production; (2) the finding that chelerythrine-induced HB-EGF shedding is inhibitable with several antioxidants; and (3) our ability to induce MMP-dependent HB-EGF shedding with H_2O_2, a source of ROS. We used heparin-affinity FPLC in combination with mitogenesis assays to show that the secretion event induced by chelerythrine results in the release of a mitogenically active protein that binds to heparin with an affinity similar to that of native HB-EGF. Our findings are consistent with previous studies demonstrating that chelerythrine is capable of inducing ROS production, leading to

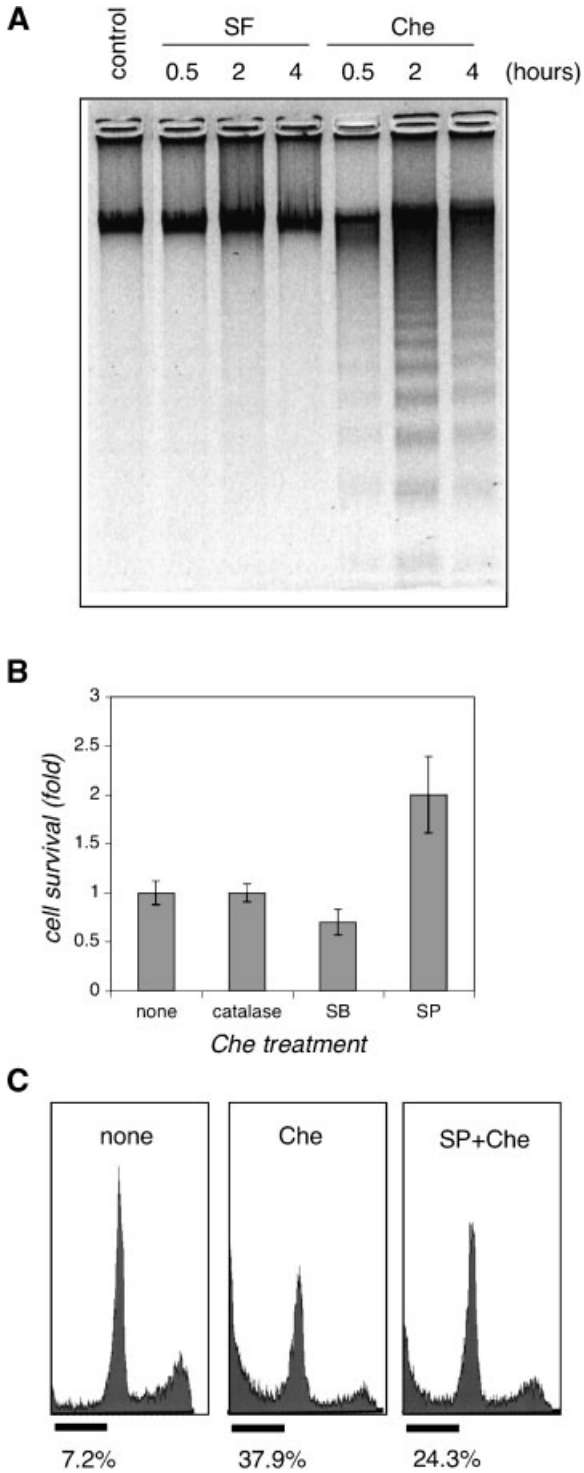

Fig. 6. Chelerythrine induces apoptosis in MC2-HB-AP cells. **A**: MC2-HB-AP cells were treated with 4 μM chelerythrine for the indicated times. Cytoplasmic DNA was collected, subjected to agarose gel electrophoresis, and visualized by ethidium bromide staining. SF = serum-free medium. **B** and **C**: Cells were pretreated with 10 U catalase, 10 μM SB203580 (SB, p38 inhibitor) or SP600125 (SP, JNK inhibitor) for 1 h, followed by chelerythrine treatment for 4 h. MTT assay and FACS analysis were performed to measure the extent of cell apoptosis as described in Experimental Procedures.

apoptosis via a PKC-independent pathway [Yu et al., 2000; Yamamoto et al., 2001]. They are also consistent with previous results implicating ROS in shedding of TNF p75 receptor and I-selectin in response to PMA [Zhang et al., 2001], suggesting the involvement of intracellular peroxides in the regulated cell surface processing of a wide variety of signaling proteins.

We also demonstrate that MAPK pathway activation, which we show occurs in MC2 cells in response to chelerythrine treatment, is not involved in the shedding mechanism. Activation of JNK signaling was, however, involved in the apoptotic effect of chelerythrine. Previous studies have demonstrated that the p38 MAPK pathway can mediate HB-EGF shedding in response to stress signals and inflammatory cytokines [Takenobu et al., 2003]. The p38 pathway has also been implicated in EGFR activation by HB-EGF in response to oxidative and osmotic stress [Fischer et al., 2004]. In contrast to these reports, in our model system the p38 MAPK pathway was found not to be a direct mediator of the shedding event stimulated by chelerythrine and H_2O_2. This finding indicates that a ROS-dependent shedding mechanism can operate independently from alternative signaling mechanisms initiated by stress signals. Interestingly, H_2O_2 was previously demonstrated to induce the expression of HB-EGF mRNA in aortic smooth muscle cells [Che et al., 1997]. Our findings here indicate that a separate, but complementary post-translational mechanism exists that is capable of liberating HB-EGF into the extracellular space. These two mechanisms of HB-EGF regulation (gene expression and proteolytic cleavage/secretion) are likely to operate coordinately in an in vivo setting. Collectively, these data provide additional insight into the mechanism whereby HB-EGF is liberated from cells and may be relevant to pathophysiological processes in which ROS are known to play a role.

A number of HB-EGF shedding agonists have been identified in published studies [Goishi et al., 1995; Dethlefsen et al., 1998; Umata et al., 2001; Takenobu et al., 2003]. Multiple signaling pathways have been implicated in the shedding mechanism, and these in some cases can be independent of each other [Dethlefsen et al., 1998]. It is likely that a variety of stimuli can trigger parallel pathways that converge on cell surface proteases, resulting in the cleavage of the membrane-anchored form of HB-EGF and other cell surface proteins that respond to similar mechanisms. The physiologic role of most shedding processes, and the context in which they operate, remains to be determined. Recently, however, the generation of HB-EGF mutant mice by targeted gene replacement has identified a role for the shedding mechanism in normal cardiovascular development and function of the heart [Yamazaki et al., 2003].

Soluble HB-EGF has been demonstrated in a number of studies to activate cytoprotective intracellular signaling mechanisms [Lin et al., 1999; Michalsky et al., 2001]. The demonstration that chelerythrine triggered a rapid apoptotic response in MC2-HB-AP cells via JNK activation suggests that HB-EGF shedding is a component of a more extensive intracellular mechanism of resistance to apoptotic stimuli. A unique cytoprotective function of membrane-anchored proHB-EGF was recently reported by our laboratory [Lin et al., 2001]. The cytoplasmic domain of proHB-EGF was found to interact with the chaperone protein, BAG-1, and to exist in a complex with the stress-related protein, Hsp70. Upregulation of BAG-1 facilitated HB-EGF shedding induced by etoposide. Our present observations further implicate stress-initiated mechanisms in HB-EGF processing. Stress signals have been demonstrated to upregulate HB-EGF at the mRNA and protein levels in vitro and in vivo [Miyagawa et al., 1995; Asakawa et al., 1996; Igura et al., 1996; Park et al., 1999; Nguyen et al., 2000]. Our findings and those of others suggest that HB-EGF cleavage-secretion is also likely to be upregulated in many of these conditions and that this process may play a physiologic role in the maintenance of tissue homeostasis.

In conclusion, we have demonstrated that the PKC inhibitor, chelerythrine, elicits the production of ROS, and that a reactive oxygen-responsive mechanism is a component of an MMP-dependent mechanism of HB-EGF ectodomain shedding from the cell surface. These findings may be relevant to conditions where rapid mobilization of soluble HB-EGF has an important physiologic role.

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