Oxidants Inhibit ERK/MAPK and Prevent Its Ability to Delay Neutrophil Apoptosis Downstream of Mitochondrial Changes and at the Level of XIAP*

Normal spontaneous apoptosis in neutrophils is enhanced by “stress” stimuli such as tumor necrosis factor-α, Fas ligand, and oxidants, and this effect is inhibited by anti-apoptotic stimuli including granulocyte-macrophage colony-stimulating factor, lipopolysaccharide, and formyl-methionine-leucine-phenylalanine. In this report we demonstrate that anti-apoptotic stimuli protect neutrophils from stress-induced apoptosis via activation of the ERK/MAPK pathway. The protection occurs downstream of mitochondrial alterations assessed as a decrease in membrane potential concomitant with enhanced cytochrome c release. ERK activation was shown to inhibit apoptosis by maintaining levels of XIAP, which is normally decreased in the presence of the pro-apoptotic/stress stimuli. This report also demonstrates that potent intra- and extracellular oxidants inhibit the protective effect of ERK. Oxidant-dependent inhibition of ERK was because of activation of p38 MAPK and activation of the protein phosphatases PP1 and PP2A. Our data suggest that ERK suppresses stress-induced apoptosis downstream of mitochondrial alterations by maintaining XIAP levels and that oxidants block this effect through activation of p38 and protein phosphatases.

Apoptosis is an essential process for development, normal growth, and tissue homeostasis. Neutrophils are unique in their ability to undergo rapid spontaneous apoptosis once released from the bone marrow, resulting in their clearance from the circulation within a few hours. During in vitro culture, 80% will undergo apoptosis within 16–20 h (1). Recently, we have suggested that repetitive engagement of cell surface β2 integrins within the vasculature, as well as during transvascular emigration of the cells during inflammation, may serve as a protective stimulus to prolong the life span of the cell (2, 3). Cytokines, LPS, and chemotactic factors also block the spontaneous apoptotic process in the neutrophil (2). TNFα, Fas ligand, and a variety of oxidants, which are encountered within an inflammatory lesion (4–6), are sometimes referred to as “stress” stimuli and can accelerate neutrophil apoptosis. Enhanced apoptosis during an inflammatory reaction may help prevent tissue damage as it promotes the surface exposure of phosphatidylserine and ultimately results in the removal of neutrophils from the blood or tissue by local phagocytes.

The apoptotic process in neutrophils is delayed by at least three major pathways involving 1) PI3K/Akt, 2) the mitogen-activated protein kinase (MAPK) of the extracellular signal-regulated kinase (ERK) subgroup (7), and 3) activation of NF-κB (8). These pathways not only block spontaneous apoptosis but also prevent apoptosis induced by UV irradiation, TNFα, or Fas ligation (5, 9). Our previous investigations have determined that the combined presence of integrin activation (but not integrin clustering) and pro-apoptotic stress stimuli down-regulate or bypass these anti-apoptotic pathways resulting in markedly enhanced neutrophil apoptosis (2).

The three mitogen-activated protein kinases MAPK/ERK, c-Jun NH2-terminal kinase/stress-activated protein kinase, and p38 have been implicated in controlling neutrophil apoptosis through the regulation of downstream kinases and activation of transcription factors. ERK activation is by a variety of necessary growth factors including GM-CSF, LPS, and fMLP and is important for their ability to inhibit apoptosis (7). ERK activation also inhibits apoptosis induced by stress stimuli such as oxidants, TNFα, UV light, as well as growth factor withdrawal (7). In this report we demonstrate that ERK blocks spontaneous and stress-induced neutrophil apoptosis downstream of mitochondrial injury and release of cytochrome c, a process also noted in other cell types (10).

The inhibitor of apoptosis (IAP) family is a likely target for ERK-mediated protection. These proteins were initially identified in baculovirus and are highly conserved across species. They act directly on caspsases, distal to mitochondrial perturbation (11). The IAP family consists of cIAP-1, cIAP-2, XIAP, neuronal apoptosis inhibitory protein, and survivin. Their baculoviral IAP repeat domains recruit and inhibit caspase activity, regulate cell cycle progression, and modulate receptor-mediated signal transduction (11, 12). XIAP is a potent inhibitor of apoptosis that directly binds to and inhibits caspase 3, 7, and 9 and has recently been implicated in protecting T-cells, epithelial cells, and bone marrow macrophages from a variety of pro-apoptotic/stress stimuli including dexamethasone, cisplatin, etoposide, and Fas ligation (13–15). XIAP contains a ring finger domain that becomes highly ubiquitinated upon the induction of apoptosis, in part due to auto-ubiquitination resulting in rapid degradation of the protein (16). Ring mutants

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† To whom correspondence should be addressed: National Jewish Medical and Research Center, Denver, Colorado 80206, the §Department of Pathology, University of Colorado Health Sciences Center, Denver, Colorado 80262, and the ¶Department of Biology, West Virginia Wesleyan College, Buckhannon, West Virginia 26201.
of XIAP display higher protein levels after exposure to apoptotic stimuli and in fact delay dexamethasone-induced apoptosis in T-cells (13). XIAP activity is also regulated by the SMAC/DIABLO protein, which binds the baculoviral IAP repeat 3 domain and disassociates XIAP from the caspases (17).

Oxidants, intra- and extracellular, have long been associated with the induction of apoptosis, due in part to their ability to modify protein activity and alter intracellular signaling (18, 19). Oxidants may also activate the ubiquitination pathway, in particular ubiquitin-protein isopeptide ligase (20), resulting in the degradation of important anti-apoptotic molecules such as XIAP. Oxidants are also known activators of the MAPK family, particularly p38 and c-Jun NH2-terminal kinase (21). The effect of p38 on apoptosis still remains undetermined; there are reports demonstrating that activation of p38 enhances apoptosis and that inhibiting p38 results in enhanced survival

Fig. 1. ERK protection of stress-induced apoptosis occurs downstream of mitochondrial changes. A, addition of the ERK activator fMLP inhibited Fas IgM-induced apoptosis without preventing mitochondrial damage. Cells were stimulated with fMLP (1 × 10⁻⁷ M) in the presence or absence of the MEK-1 inhibitor PD-98059 (25 μM) and IgM anti-Fas (1 μg/ml). Apoptosis was assessed by morphology, and bars represent percent enhancement of apoptosis compared with untreated control (n = 3 ± S.D.). B, neutrophils were incubated with JC-1 (75 nM) after 4 h, and mitochondrial damage was measured as decreased red fluorescence by flow cytometry. Histogram shows decreased red fluorescence and is representative of three independent experiments. C, ERK activation does not prevent Fas-induced release of cytochrome c. Cells were stimulated as in A for 2 h at which point Mitotracker red (red), was added for 10 min and cells were cytospun, fixed, and stained for cytochrome c (green). Nuclei are shown in blue. White arrows indicate apoptotic nuclei, and the scale bar is equal to 5 μm.
(22); however, other studies indicate that p38 may be involved in inhibiting apoptosis (23). In neutrophils p38 is involved in UV-induced apoptosis and has also been linked to promoting spontaneous apoptosis (5, 24). The effect of oxidant-stimulated p38 activity on neutrophil apoptosis has not yet been explored, but its ability to suppress ERK activity in epithelial cells and macrophages suggests a possible mechanism for a pro-apoptotic effect (25–27).

In this report we demonstrate that stress-induced apoptosis is associated with mitochondrial damage and degradation, or decreased synthesis, of the potent anti-apoptotic molecule XIAP. ERK activation inhibits stress-induced apoptosis by maintaining XIAP levels but does not prevent the mitochondrial changes including decreased mitochondrial membrane potential and release of cytochrome c. Addition of oxidants blocked ERK activation through the stimulation of p38 and subsequent activation of protein phosphatases (PP1 and PP2A).

**Materials and Methods**

Reagents and Antibodies—The following monoclonal antibodies were obtained from commercial sources and were confirmed to bind to human neutrophils by flow cytometry. Anti-αM-β2LPM19c was obtained from Dako (Carpinteria, CA) and anti-αM-Vim12 from Caltag Laboratories (Burlingame, CA). Unless specified otherwise, monoclonal antibodies were used at a final concentration of 2 μg/ml. All antibodies were tested as diazyl preparations to rule out nonspecific effects of commercial diluents. Formyl-methionine-leucine-phenylalanine (fMLP), was used at 10−7 M; t-butyl hydroperoxide was used at a final concentration of 100 μM; (−)-2-oxo-4-thiazolidine-carboxylic acid (OTC) was used at 10 μM, and all were obtained from Sigma. TNFα was obtained from R&D Systems (Minneapolis, MN) and was used at a final concentration of 1,000 units/ml. IgM anti-Fas clone CH11 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY), and used at a concentration of 1 μg/ml for cell apoptosis. The MEK-1 inhibitor PD-98059 was used at 25 μM; NADPH oxidase inhibitor diphenyleniodonium chloride (DPI) was used at 10 μM; p38 inhibitor SB-208035 was used at 10 μM, and the protein phosphatase inhibitors calyculin and okadaic acid were used at 20 nM and 20 μg/ml, respectively. All inhibitors were obtained from Calbiochem. Anti-phospho-MEK and total MEK-2 were obtained from Cell Signaling (Beverly, MA) and were used at 1:1000 in 5% BSA; anti-cytochrome c was obtained from Pharmingen and used at 1 μg/ml.

**Neutrophil Isolation**—Human neutrophils were purified from whole blood by using a Percoll gradient as described previously (28). Neutrophil purity was 95% with typically less than 2% eosinophils and cell viability greater than 98%.

**Assessment of Apoptosis in Suspension Neutrophils**—After isolation, neutrophils were suspended at 5 × 106 cells/ml in RPMI supplemented with 1% LPS-free BSA (Sigma, A-1923) and incubated in conical polycarbonate tubes (Sarstedt, Newton, NC). Neutrophils were allowed to apoptose spontaneously or were exposed to IgM anti-Fas (1 μg/ml), t-butyl hydroperoxide (100 μM), hydrogen peroxide (100 μM), or UV irradiation (10 min) in the presence or absence of fMLP (1 × 10−7 M). Experiments carried out with the MEK inhibitor PD-98059 (25 μM), p38 inhibitor SB-208035 (10 μM), anti-oxidants DPI or OTC (10 μM), or protein phosphatase inhibitors, calyculin (50 μM) and okadaic acid (2 μg/ml), were used a 30-min preincubation before addition of stimuli. Apoptosis was assessed after 2 h for UV irradiation and 4 h for all other stimuli. 2 × 105 cells were diluted and cytospun at 600 rpm for 3 min onto glass slides and were stained with a modified Wright-Giemsa stain. Cells were counted as apoptotic if their nuclei lost segmentation and had nuclear condensation.

**Mitochondria Staining**—After stimulation, neutrophils were incubated with 10 μM JC-1 (Molecular Probes, Eugene, OR) at 37 °C for 10 min, centrifuged, resuspended in cold phosphate-buffered saline, and analyzed by flow cytometry. Increases in mean green fluorescence intensity (FL-1) correspond to a loss of fluorescent JC-1 aggregates in the mitochondria that occurs upon decreased mitochondrial membrane potential (29). The increase in JC-1 green fluorescence corresponds to a decrease in JC-1 red fluorescence indicating the relative levels of JC aggregates in the mitochondria.

**Cytochrome c Immunostaining**—Human neutrophils were pretreated for 20 min with the MEK-1 inhibitor PD-98059 at which point DML (1 × 10−7 M) and IgM anti-Fas (1 μg/ml) were added for 2 h. After 2 h 75 nm Mitotracker Red CMXRos (Molecular Probes) was added to the sample for 15 min at 37 °C after which cells were cytopspun. Cytochrome c staining was performed as described in Kennedy et al. (30) with the anti-cytochrome c antibody (1 μg/ml) (Pharmingen) and a fluorescein isothiocyanate-conjugated secondary antibody. After fixation and cytochrome c staining, cells were washed (twice) and mounted. Images were collected with Slidebook (31) on a Leica DMRXA microscope at ×62 magnification.

**Intracellular Oxidant Staining Using Dihydrorhodamine-123**—Cells were resuspended at 5 × 106/ml, and dihydorhodamine-123 (Molecular Probes) was used as described by Vowells et al. (31). Briefly, neutrophils were preloaded with 1.0 × 10−5 M dihydorhodamine-123 for 5 min in a 37 °C shaking water bath prior to addition of stimuli. 2 μg/ml of either the monoclonal antibody Vim12 or 2LPM19c, 1 × 10−7 M fMLP or 1000 units/ml of TNFα. Following a 1-h incubation at 37 °C, cells were put on ice and analyzed by flow cytometry (FL-2, FACSCalibur, BD Biosciences). Neutrophils were washed (twice) and cytopspun (FACSCalibur, BD Biosciences).

**XIAP Western Blots**—Cells were resuspended at 2 × 106 cells/ml and samples were stimulated for 4 h with stress stimuli IgM anti-Fas (1 μg/ml), t-butyl hydroperoxide (100 μM), or TNFα (1000 units/ml) in the presence or absence of fMLP (1 × 10−7 M) or the proteosome inhibitor MG-132 (Calbiochem) (30 μM). fMLP-treated cells were also preincubated for 30 min with the MEK-1 inhibitor PD-98059 (25 μM) before stimulation with fMLP. After 4 h cells were lysed in 100 μl of lysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM Na3VO4, 1% Nonidet P-40 with 1 mM phenylmethylsulfonyl fluoride, and 15 μg/ml leupeptin and aprotinin) for 10 min; centrifuged, resuspended in cold phosphate-buffered saline, and analyzed by flow cytometry (FL-1, FACSCalibur, BD Biosciences). XIAP protein levels in total cell lysates were assessed by Western blotting using anti-XIAP antibody (1:1000 in 5% BSA; Cell Signaling).
Oxidant-dependent Inhibition of ERK

**RESULTS**

ERK Inhibits Stress-induced Apoptosis Downstream of Mitochondrial Changes—Recent studies have established the importance of the ERK/MAPK pathway in protecting cells from apoptosis. Previously, we demonstrated that clustering of the β2 integrin inhibits stress-induced neutrophil apoptosis via the ERK pathway, apparently acting downstream of mitochondrial perturbation (2, 32). To pursue the apparent divergence between ERK inhibition of apoptosis but not mitochondrial damage, we established a system by using fMLP, a stimulus known to enhance of apoptosis compared with untreated control (\( n = 3 \pm S.D. \)). The significance of inhibition of enhanced apoptotic by fMLP alone and fMLP plus 2LPM19c was determined by single mean comparisons to control percent inhibition using the Tukey-Kramer test (*, \( p < 0.05 \), compared with control apoptosis).

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**Measurement of Phospho-p38 and ERK—**Neutrophils were resuspended at 5 × 10^6 cells/ml in RPMI supplemented with 1% LPS-free BSA and transferred to sterile Eppendorf tubes. Stimuli were added, and neutrophils were washed three times with 0.75% phosphoric acid, and analyzed by liquid scintillation counting.

**Data Analysis—**Averages and S.D. values were calculated from at least three experiments. Statistical analysis was carried out using the JMP statistical program (SAS Institute, Cary, NC). The Tukey-Kramer and Dunnett’s parametric tests were used for single and multiple comparisons, respectively.

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to activate ERK and inhibit stress-induced apoptosis (5), and a variety of pro-apoptotic stimuli, IgM anti-Fas and UV, to analyze the effect of ERK on apoptosis and mitochondrial function. Neutrophils were incubated with activating IgM anti-Fas in the presence or absence of fMLP and the MEK-1 inhibitor PD-98059 for 4 h, and apoptosis was assessed by morphology (Fig. 1A). Fig. 1B demonstrates the decrease in mitochondrial membrane potential assessed by JC-1 fluorescence. JC-1 fluoresces green in a monomeric form; however, when it is transported into healthy mitochondria it aggregates and fluoresces red. Cells were analyzed for a decrease in red JC-1 fluorescence, indicating a decrease in mitochondrial membrane potential. IgM anti-Fas treatment resulted in a marked decrease in red JC-1 fluorescence, correlating with the enhancement of apoptosis. Although fMLP treatment inhibited anti-Fas-induced apoptosis (Fig. 1A), it did not prevent the decrease in JC-1 red fluorescence (Fig. 1B). Addition of the MEK-1 inhibitor PD-98059 reversed the protective effect of fMLP on

**FIG. 4.** Oxidant stimulation of p38 inhibited ERK activation. A, cells pretreated with the p38 inhibitor SB-208035 restored the protective effect of fMLP on UV-induced apoptosis in the presence of oxidants. Neutrophils were pretreated with SB-208035 for 30 min prior to addition of the oxidant stimuli, H2O2, t-buty1 hydroperoxide, or the β2 integrin activating antibody Vim12 for 30 min. Cells were then stimulated with fMLP and exposed to UV irradiation for 5 min. Samples were incubated for 2 h, and apoptosis was assessed by morphology. Bars represent percent enhancement of apoptosis compared with untreated control as determined by morphologic analysis (n = 3 ± S.D). B, oxidant treatment results in p38 phosphorylation (P-P38). Neutrophils were treated with t-buty1 hydroperoxide (100 μM) or Vim12 (2 μg/ml) for 20 and 40 min. Cells were lysed, run on SDS-PAGE, and blots probed for anti-phospho-p38. Immunoblot is representative of three experiments. C, anti-oxidant addition, DPI (10 μM) or OTC (10 μg/ml) prevented Vim12 activation of p38. Neutrophils were pretreated with anti-oxidants DPI or OTC for 30 min prior to stimulation with Vim12 for 40 min. Cells were lysed, run on SDS-PAGE, and blots probed with an anti-phospho-p38 antibody. D, the oxidant, t-buty1 hydroperoxide, inhibited fMLP-induced ERK activity. Cells were treated with t-buty1 hydroperoxide for 20 min and then exposed to fMLP for 1, 3, 6, or 12 min. Cells were lysed, run on an SDS-polyacrylamide gel, and probed with an anti-phospho-ERK antibody. E, oxidant inhibition of ERK was reversed with the p38 inhibitor SB-208035. Cells were pretreated with SB-208035 for 30 min prior to stimulation with t-buty1 hydroperoxide for 20 min followed by stimulation with fMLP for 1 or 3 min. Cells were processed as in D.
Fas-induced apoptosis (Fig. 1A) but did not alter JC-1 red fluorescence (Fig. 1B).

To confirm these observations, cytochrome c loss was analyzed. Neutrophils were treated with IgM anti-Fas in the presence or absence of fMLP, and cytospins were performed and cells stained for cytochrome c. As cells apoptose, cytochrome c is released into the cytoplasm resulting in an observable reduction in cytochrome c staining in the mitochondria. Treatment with anti-Fas led to a significant decrease in cytochrome c mitochondrial staining, as well as decreased Mitotracker red staining (Fig. 1C). Although addition of fMLP inhibited apoptosis, loss of cytochrome c and Mitotracker red staining (loss of mitochondria membrane potential) was still observed. Pretreatment with PD-98059 restored anti-Fas-induced apoptosis (Fig. 1A) but did not alter cytochrome c release (Fig. 1C). Similar enhancement of apoptosis and injury to the mitochondria were seen with oxidant and TNFα stimulation as the ERK activator, fMLP or GM-CSF, inhibited apoptosis but not mitochondrial changes (data not shown).

**ERK Inhibits the Decrease in XIAP Caused by Pro-apoptotic Stimuli**—A primary candidate for ERK regulation is XIAP of the IAP family. XIAP blocks apoptosis by binding to caspase 9, 3, and 7 and inhibiting their activity. To pursue the involvement of XIAP in neutrophil apoptosis and the ability of ERK to regulate its activity, the levels of XIAP in neutrophils exposed to various pro-apoptotic/stress stimuli was analyzed. Neutrophils were treated with TNFα, IgM anti-Fas, or the oxidant t-butyl hydroperoxide for 4 h. Cells treated with pro-apoptotic stimuli exhibited a marked decrease in XIAP levels compared with untreated cells (Fig. 2A). To determine whether the reduction of XIAP was because of degradation, the proteosome inhibitor MG-132 (30 μM) was added prior to IgM anti-Fas treatment. Cells treated with the proteosome inhibitor no longer showed a decrease in XIAP (Fig. 2B). These data support the suggestion that pro-apoptotic stimuli promote the degradation of XIAP, and this may contribute to their ability to enhance apoptosis.

Fig. 2C demonstrates that ERK activation maintains XIAP levels in the presence of a stress stimulus. Neutrophils were stimulated with fMLP and exposed to IgM anti-Fas for 4 h. fMLP treatment maintained XIAP levels, whereas pretreatment with the MEK-1 inhibitor PD-98059 returned XIAP levels to those from cells treated with pro-apoptotic stimuli alone.

**Endogenous and Exogenous Oxidants Inhibit the Ability of ERK to Prevent Stimulated Apoptosis**—Our previous studies examining the role of β2 integrin engagement in neutrophil apoptosis demonstrated that, in the absence a stress stimulus, both clustering and activation of the β2 integrin were equally anti-apoptotic (2). A significant divergence existed between β2 integrin clustering and clustering accompanied by integrin activation. Clustering activated both the PI3K/Akt and ERK pathways and inhibited stress-induced apoptosis through the ERK pathway. However, β2 integrin activation solely stimulated the PI3K/Akt pathway and paradoxically enhanced stress-induced apoptosis. The major difference between inte-

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**Fig. 5.** ERK activation by fMLP was restored upon the addition of anti-oxidants. *A,* cells pretreated with the anti-oxidants DPI or OTC for 20 min restored fMLP (1 × 10⁻⁷ M) phosphorylation of ERK (P-Erk) in the presence of t-butyl hydroperoxide (100 μM). *B,* oxidants resulting from β2 integrin activation inhibited ERK activity. Cells were incubated with Vim12 (2 μg/ml) in the presence or absence of DPI (10 μM) or the p38 inhibitor SB-208035 (10 μM) for 20 min. Cells were lysed, and ERK activity was assessed in an *in vitro* kinase assay as the incorporation of [³²P]. *Bars* represent [³²P] incorporation above to unstimulated control cells (n = 3 ± S.D.). The significance of enhancement of ERK activity by DPI and SB-208035 was determined by single mean comparisons to Vim12 activity using the Tukey-Kramer test (*, p < 0.05, compared with Vim12).

**Fig. 6.** Oxidants inhibited the upstream activating kinase of ERK, MEK-1. Cells were stimulated with t-butyl hydroperoxide (100 μM) for 20 min followed by fMLP stimulation, and samples were probed for activation of MEK-1 with an anti-phospho-MEK-1 (P-Mek1) antibody.
Oxidant-dependent Inhibition of ERK

Protein phosphatase inhibitors prevent oxidant down-regulation of ERK. A, addition of the protein phosphatase inhibitor okadaic acid prevented t-butyl hydroperoxide (100 μM) blockade of the ability of fMLP to protect cells from UV-induced apoptosis. Bars represent percent enhancement of apoptosis compared with untreated control cells (n = 3 ± S.D.). B, protein phosphatase inhibitors restored ERK activation in the presence of oxidants as determined by SDS-PAGE. Blot is representative of three independent experiments. Cells were pretreated with okadaic acid or calyculin prior to stimulation with t-butyl hydroperoxide followed by fMLP for 5 min. Cells were lysed, run on SDS-PAGE, and probed for anti-phospho-ERK (P-Erk).

Fig. 7. Protein phosphatase inhibitors prevent oxidant down-regulation of ERK. A, addition of the protein phosphatase inhibitor okadaic acid prevented t-butyl hydroperoxide (100 μM) blockade of the ability of fMLP to protect cells from UV-induced apoptosis. Bars represent percent enhancement of apoptosis compared with untreated control cells (n = 3 ± S.D.). B, protein phosphatase inhibitors restored ERK activation in the presence of oxidants as determined by SDS-PAGE. Blot is representative of three independent experiments. Cells were pretreated with okadaic acid or calyculin prior to stimulation with t-butyl hydroperoxide followed by fMLP for 5 min. Cells were lysed, run on SDS-PAGE, and probed for anti-phospho-ERK (P-Erk).

Fig. 8. ERK inhibits apoptosis downstream of the mitochondria through maintained XIAP levels, and oxidant activation of p38 blocks this protective pathway via the stimulation of protein phosphatases. The signaling model shows ERK activity and oxidant inhibition of this. Red and blue bars represent inhibitory pathways. β2 integrin structures are modeled after Stewart and Hogg (41). ability of fMLP to inhibit UV-induced apoptosis in the presence of oxidants (Fig. 4A).

Fig. 4B confirms that the extracellular oxidants and oxidant-generating systems activated p38. Cells treated with the β2 integrin-activating antibody Vim12 demonstrated p38 phosphorylation as early as 20 min, and this continued for 40 min. t-Butyl hydroperoxide stimulation also led to p38 phosphorylation at 20 min with maximal activation occurring at 40 min (Fig. 4B).

To determine whether p38 phosphorylation was oxidant-dependent, neutrophils were pretreated with the NADPH oxidase inhibitor (flavoprotein inhibitor) DPI or OTC (an oxidant scavenger) prior to treatment with the integrin-activating antibody Vim12 or t-butyl hydroperoxide. These agents were able to block p38 phosphorylation, indicating that both Vim12 and t-butyl hydroperoxide activated p38 in an oxidant-dependent manner (Fig. 4C).

The ability of oxidants to reduce (or shorten) fMLP-induced ERK phosphorylation is shown in Fig. 4D. Treatment with t-butyl hydroperoxide for 30 min prior to fMLP stimulation reduced fMLP-induced ERK phosphorylation as early as 3 min and continued to inhibit for up to 12 min. Most importantly, the p38 inhibitor SB-203580 blocked the ability of oxidants to inhibit ERK phosphorylation (Fig. 4E).

Inhibition of Oxidant Production Restores ERK Activation—Because the inhibition of ERK by t-butyl hydroperoxide was thought to be due to its oxidant properties, cells were pretreated with either DPI or OTC prior to the addition of t-butyl hydroperoxide, and this was followed by fMLP stimulation for 5 min. Samples pretreated with the oxidant inhibitors displayed restored fMLP-induced ERK phosphorylation in the presence of t-butyl hydroperoxide (Fig. 5A).

To address whether oxidants and subsequent p38 activity produced by β2 integrin activation blocked ERK, neutrophils were preincubated with the NADPH oxidase inhibitor DPI or the p38 inhibitor SB-208035 prior to stimulation with the β2 integrin-activating antibody Vim12. Samples treated with the

Oxidant-stimulated p38 Activity Prevents the Activation of ERK—Oxidants are potent modulators of signaling and have been suggested to enhance apoptosis through the activation of p38. To determine whether oxidant inhibition of fMLP/ERK was due to p38, neutrophils were pretreated with the p38 inhibitor SB-203580 prior to oxidant exposure followed by fMLP stimulation and UV irradiation. Samples pretreated with the p38 inhibitor consistently demonstrated a restored...
Vim12 antibody suppressed ERK activity below untreated control values (Fig. 5B). In the presence of both DPI and SB-208035, addition of Vim12 resulted in significant ERK activity. These data indicate that the divergence in signaling between $\beta_2$ integrin clustering and clustering with activation is due to oxidant generation and subsequent p38 stimulation accompanying integrin activation.

Oxidants Inhibit fMLP-induced MEK-1 Activity—To examine where p38 inhibited the ERK pathway, neutrophils were preincubated with the p38 inhibitor SB-208035 prior to addition of the extracellular oxidant t-buty1 hydroperoxide. This was followed by stimulation with fMLP and immunoblotting for phospho-MEK-1. Incubation with t-buty1 hydroperoxide prior to fMLP treatment inhibited MEK-1 phosphorylation at 45 s and 1 and 3 min (Fig. 6). Pretreatment with the p38 inhibitor SB-208035 prior to t-buty1 hydroperoxide stimulation restored fMLP induction of MEK-1 phosphorylation.

Serine/Threonine Phosphatases Mediate Oxidant Inhibition of ERK—Previous reports (32) show that MEK-1 activity can be inhibited by the potent phosphatases PP1 and PP2A. To support a role for these phosphatases, cells were pretreated with the serine/threonine phosphatase inhibitor okadaic acid prior to stimulation with t-buty1 hydroperoxide, and the ability of fMLP to inhibit UV irradiation-induced apoptosis was assessed (Fig. 7A). Pretreatment with the phosphatase inhibitor restored the ability of fMLP to protect neutrophils from UV-induced apoptosis in the presence of oxidants.

The data in Fig. 7B confirm the inhibition of protein phosphatases restored fMLP-induced ERK activity in the presence of potent oxidants. These data indicate that oxidants inhibit MEK-1, and subsequently ERK, via a p38 effect on phosphatases PP1 and/or PP2A. Fig. 8 is a signaling model of ERK-mediated inhibition of neutrophil apoptosis and the negative effect oxidants have on this pathway.

DISCUSSION

Once released from the bone marrow and into the circulation, neutrophils rapidly undergo spontaneous apoptosis. Pro-apoptotic stress stimuli enhance this process, including Fas ligation, TNFα, UV irradiation, or oxidants. Both spontaneous and stimulated apoptosis are inhibited by PI3K/Akt, which appears to block at the level of mitochondrial changes and activation of effector caspases (30), and/or through ERK-dependent processes. This latter pathway appears to act downstream of the mitochondria at the level of caspase inhibition (10, 33). We demonstrate here that stress-induced apoptosis was blocked by a variety of ERK-activating stimuli including growth factors (GM-CSF), ligands for serpentine G-protein-linked receptors (fMLP), or Toll-like receptors (LPS) in circumstances where mitochondrial damage still occurred (Fig. 1).

The most likely candidates for these observations were members of the IAP family. Although we analyzed lysates for several members of the IAP family, including clAP-1 and -2, only XIAP levels were reduced upon addition of an apoptotic stimulus (data not shown). We also focused on XIAP as a likely candidate due to its ability to bind and inhibit the activity of caspases 9, 3, and 7. The importance of XIAP seemed to extend to spontaneous apoptosis, and after 4 h of culture (resulting in about 20% apoptosis), there was a decrease in the levels of XIAP (data not shown). Whether this indicates a similarity between stress-induced and spontaneous apoptosis is not yet clear. It may suggest, however, that during spontaneous apoptosis XIAP is regulated perhaps by degradation but may also be regulated perhaps via pairing with SMAC/DIABLO (17).

As predicted, activation of ERK by fMLP maintained XIAP levels in the presence of stress stimuli, and addition of the MEK-1 inhibitor reversed this protection. These data suggest that XIAP levels are regulated through a complex balance between proteosomal degradation and new synthesis (11). ERK may help maintain XIAP levels in one of two ways, either by inhibiting the ubiquitination pathway, most likely through phosphorylation, or it may induce XIAP synthesis via activation of transcription factors such as Elk1 (34, 35). In fact, inhibition of apoptosis by enhanced XIAP transcription has been associated with ERK activation in acute myeloid leukemia cells and THP-1 cells (34, 36). However, the rapidity with which ERK activation appeared to exert its effect, within 1 min, suggests that much of the regulation is occurring at the level of ubiquitination resulting in reduced degradation.

In a previous report we demonstrated that clustering of the $\alpha_m$/$\beta_2$ integrins blocked spontaneous and stimulated neutrophil apoptosis through activation of both the Akt and ERK pathways. Most importantly, during integrin activation ERK was no longer activated, and stress-induced apoptosis was enhanced, due in part to oxidant-mediated SHIP activation and negative effects on Akt activation (37). These observations raise the possibility that oxidants generated by integrin activation might also block the ERK pathway, leaving the cells more susceptible to stress-induced apoptosis.

Oxidant down-regulation of ERK activity and the impact this has on neutrophil apoptosis may have important implications in chronic granulomatous disease (CGD) and during inflammatory reactions. CGD patients are unable to generate oxidants through the NADPH pathway, and compared with normal controls, neutrophils from CGD patients have lower levels of spontaneous and stress-induced apoptosis (data not shown and see Ref. 38). This observation may be attributed in part to maintained ERK and XIAP levels. The opposite effect may occur during an inflammatory reaction where oxidant levels are enhanced. These higher oxidant levels may inhibit ERK and lead to the rapid degradation of XIAP and enhanced apoptosis. This may be important for removing the neutrophils before they can degranulate and cause tissue damage.

p38 MAPK is a known downstream target of oxidants (21), and recent reports have suggested that it may have a regulatory effect on ERK (26, 27, 39). As expected, p38 was activated in the presence of exogenous oxidants or when the $\beta_2$ integrin was activated. Furthermore, blockade of p38 with SB-208035 prevented both the oxidant-induced inhibition of ERK and restored its ability to inhibit apoptosis. Further analysis demonstrated that the p38 effect required Src family kinases because addition of the Src family inhibitor, PP1, restored ERK activity in the presence of oxidants (data not known). The oxidants were shown to activate the Src family kinases Hck and Lyn, making them likely candidates for activating pathways leading to phosphorylation of p38 (data not shown).

Blockade of ERK activation by p38 could be a direct effect on the MAPK or on upstream activators. In fact, p38 inhibition was shown to prevent phosphorylation of MEK-1 implicating it as the likely regulatory candidate (27, 33). A number of studies have suggested that regulatory cross-talk between MAPK families is caused by activation of phosphatases and in this case most likely PP1 and/or PP2A (32, 40). In support of this possibility, the phosphatase inhibitors, calyculin and okadaic acid, prevented the oxidant blockade of ERK activation. Because...
they did not alter p38 activation (data not shown), p38-mediated activation of phosphatases is implicated. The data provide potential insight into the complex balance of anti- and pro-apoptotic signals in neutrophils and suggest a potential hierarchy among these stimuli where the presence of pro-apoptotic stress signals such as oxidants may override the precariously balanced anti-apoptotic signals present in the cell. Such stimuli and effects are likely present in inflammatory sites and may be expected to contribute to the effective removal of neutrophils during the resolution of the inflammation.

REFERENCES

1. Savill, J. S., Wylie, A. H., Henson, J. E., Walport, M. J., Henson, P. M., and Haslett, C. (1989) J. Clin. Invest. 83, 865–875
2. Whitlock, B. B., Gardai, S., Fadok, V., Bratton, D., and Henson, P. M. (2000) J. Cell Biol. 151, 1395–1420
3. Watson, R. W. G., Rotstein, O. D., Nathens, A. B., Parodo, J., and Marshall, J. C. (1997) J. Immunol. 158, 945–953
4. Takeda, Y., Watanabe, H., Yonehara, S., Yamashita, T., Saito, S., and Senda, F. (1993) Int. Immunol. 5, 691–694
5. Frasch, S. C., Nick, J. A., Fadok, V. A., Bratton, D. L., Worthen, G. S., and Henson, P. M. (1998) J. Biol. Chem. 273, 8389–8397
6. Liles, W. C., Kiener, P. A., Ledbetter, J. A., Aruffo, A., and Klebanoff, S. J. (1996) J. Exp. Med. 184, 429–440
7. Sweeney, J. F., Nguyen, P. K., Omann, G., and Hinshaw, D. B. (1999) J. Surg. Res. 81, 108–112
8. Ward, C., Chivers, E. R., Lawson, M. F., Prude, J. G., Fujisawa, S., Farraw, S. N., Haslett, C., and Rossi, A. G. (1999) J. Biol. Chem. 274, 4309–4318
9. Tudan, C., Jackson, J. K., Blanis, L., Pelech, S. L., and Burt, H. M. (2000) J. Immunol. 165, 5798–5806
10. Erhardt, P., Schremser, E. J., and Cooper, G. M. (1999) Mol. Cell. Biol. 19, 5308–5315
11. Yang, Y. L., and Li, X. M. (2000) Cell Res. 10, 169–177
12. Roy, N., Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) EMBO J. 16, 6914–6925
13. Yang, Y., and Ashwell, J. D. (1999) Mol. Cell. Biol. 19, 279–288
14. Asselin, E., Mills, G. B., and Tsang, B. K. (2001) Cancer Res. 61, 1862–1868
15. Lin, H., Chen, C., and Chen, B. D. (2001) Biochem. J. 353, 299–306
16. Yang, Y., Fang, S., Jensen, J. P., Weissman, A. M., and Ashwell, J. D. (2000) Science 289, 874–877
17. Liu, Z., Sun, C., Olejniczak, E. T., Meadows, R. P., Betz, S. F., Oost, T., Herrmann, J., Wu, J. C., and Fesik, S. W. (2000) Nature 408, 1004–1008
18. Lieberthal, W., Triaca, V., Koh, J. S., Pagano, P. J., and Levine, J. S. (1998) Am. J. Physiol. 275, F691–F702
19. Chandra, J., Samali, A., and Orrenius, S. (2000) Free Radic. Biol. Med. 29, 323–333
20. Wang, K., Gross, A., Waksman, G., and Korsmeyer, S. J. (1998) Mol. Cell. Biol. 18, 6083–6089
21. Underwood, D. C., Osborn, R. R., Bochnowicz, S., Webb, E. F., Riemann, D. J., Lee, J. C., Romain, A. M., Adams, J. L., Hay, D. W., and Griswold, D. E. (2000) Am. J. Physiol. 278, L895–L902
22. Alvarado-Kristensson, M., Peron-Ares, M. I., Grethe, S., Smith, D., Zheng, L., and Andersson, T. (2002) FEBS J. 16, 129–131
23. Asshai, K., Yasui, S., Hayashi, M., Tamaoki, J., and Nagai, A. (1999) J. Immunol. 162, 1692–1700
24. Haslett, C., Guthrie, L. A., Kopaniak, M. M., Johnston, R. B., Jr., and Henson, P. M. (1998) Am. J. Physiol. 219, 101–110
25. Vowells, S. J., Sekharasri, S., Malek, H. L., Shalit, M., and Fleischer, T. A. (1995) J. Immunol. Methods 187, 89–97
26. Xia, Z., Niessen, L., and Henson, P. M. (1998) J. Exp. Med. 184, 3373–3377
27. Xiao, Y. Q., Malcolm, K., Worthen, G. S., Gardai, S., Schiemann, W. P., Fadok, V. A., Bratton, D. L., and Henson, P. M. (2002) J. Biol. Chem. 277, 14884–14893
28. Haslett, C., Guthrie, L. A., Kopaniak, M. M., Johnston, R. B., Jr., and Henson, P. M. (1998) J. Immunol. 160, 1323–1328
29. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995) Science 273, 108–112
30. Takeda, Y., Watanabe, H., Yonehara, S., Yamashita, T., Saito, S., and Senda, F. (1993) Int. Immunol. 5, 691–694
31. Frasch, S. C., Nick, J. A., Fadok, V. A., Bratton, D. L., Worthen, G. S., and Henson, P. M. (1998) J. Biol. Chem. 273, 8389–8397
32. Lin, H., Chen, C., Li, X., and Chen, B. D. (2002) J. Biol. Chem. 277, 192–199
33. Lin, H., Chen, C., Li, X., and Chen, B. D. (2002) Exp. Cell Res. 272, 192–199
34. Lin, H., Chen, C., Li, X., and Chen, B. D. (2002) Exp. Cell Res. 272, 192–199
35. Chang, L., and Karin, M. (2001) Mol. Cell. Biol. 21, 2373–2383
36. Kasahara, Y., Iwai, K., Yachie, A., Ohta, K., Konno, A., Seki, H., Miyawaki, T., Yanagawa, Y., Hiraga, A., Kanamaru, R., and Tamura, S. (1998) EMBO J. 17, 323–333
37. Milella, M., Kornblau, S. M., Estrov, Z., Carter, B. Z., Lapillonne, H., Harris, D., Konopleva, M., Zhao, S., Estey, E., and Andreeff, M. (2001) J. Clin. Invest. 108, 851–859
38. Kuwano, K., Tsunoda, T., and Andersson, T. (2002) J. Clin. Investig. 109, 851–859
39. Gardai, S., Whitlock, B. B., Helgason, C., Ambruos, D., Fadok, V., Bratton, D., and Henson, P. M. (2002) J. Biol. Chem. 277, 5236–5246
40. Kiossan, Y., Iwai, K., Yachie, A., Ohta, K., Konno, A., Seki, H., Miyawaki, T., and Taniuchi, N. (1997) Blood 89, 1748–1753
41. Zhang, H., Shi, X., Hampong, M., Blanis, L., and Pelech, S. (2001) J. Biol. Chem. 276, 6905–6908
42. Hanada, M., Kobayashi, T., Ohnishi, M., Ikeda, S., Wang, H., Katsura, K., Yanagawa, Y., Hiraga, A., Kanamaru, R., and Tamura, S. (1998) FEBS Lett. 437, 172–176
43. Stewaet, M., and Hogg, N. (1996) J. Cell. Biochem. 61, 554–561
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Shyra J. Gardai, Ben B. Whitlock, Yi Qun Xiao, Donna B. Bratton and Peter M. Henson

J. Biol. Chem. 2004, 279:44695-44703.
doi: 10.1074/jbc.M405313200 originally published online July 30, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405313200

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