Activation of SHIP by NADPH Oxidase-stimulated Lyn Leads to Enhanced Apoptosis in Neutrophils*

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Neutrophils undergo rapid spontaneous apoptosis. Multiple antiapoptotic stimuli can inhibit this process via activation of the Akt pathway. However, despite no such effect singly, combined anti- and proapoptotic stimuli inhibit Akt activity, leaving the cells susceptible to accelerated apoptosis. The blockade of Akt activation depended on reduced phosphoinositide 3,4,5-trisphosphate levels but not decreased phosphatidylinositol 3-kinase activity, thus implicating the involvement of an inositol phosphatase. Evidence for SHIP involvement was provided by SHIP localization to membrane receptors and subsequent activation along with the observed inability of SHIP 1/− neutrophils to exhibit enhanced apoptosis with the stimulus combination. Activation of SHIP was found to depend on Lyn activation, and this, in turn, required NADPH oxidase. Neutrophils from chronic granulomatous disease patients and Lyn 1/− mice no longer responded to the combined stimuli. Thus, we propose a role for oxidants and Lyn in SHIP regulation and suggest a novel mechanism for regulating neutrophil apoptosis.

Once they have been released from the bone marrow, neutrophils have a remarkably short life span of only a few hours, with 80% undergoing apoptosis within 16–20 h of in vitro culture (1). This rapid induction of apoptosis is thought to be the result of a preprogrammed, or spontaneous, apoptotic sequence that, in the absence of additional signals, leads to surface changes on the cell and clearance from the circulation (2, 3). We have recently suggested that repetitive engagement of cell surface β2-integrins within the vasculature, including transvascular emigration of the cells during an inflammatory reaction, may serve as one protective stimulus to prolong the cells' life span (4, 5). G-protein-linked serpine receptors, such as the receptors for fMLP, cytokines, and chemotactic factors, have also been shown to block the endogenous apoptotic pathways (4, 6–8). However, within the inflammatory lesion, neutrophils encounter a complex milieu of pro- and antiapoptotic stimuli whose combined effect will determine the life span of the cells. Protective stimuli include integrin ligation, a wide variety of chemokines and other chemotactants, GM-CSF,1 and even bacterial lipopolysaccharide (9, 10). In contrast, proapoptotic stimuli commonly encountered within an inflammatory lesion would include TNFα, Fas ligand, and a variety of oxidants (11–13) sometimes referred to as “stress” stimuli. The combination of proapoptotic stimuli with integrin activation appears to initiate a synergistic induction of apoptosis (4), a process that may be critical for surface exposure of phosphatidylserine and eventual clearance from the tissue by local phagocytes. This clearance, in turn, results in the production of anti-inflammatory molecules and the subsequent resolution of the inflammatory reaction (3).

Apoptotic processes in neutrophils can be regulated to extend the cells' life span through major pathways including PI 3-kinases and Akt, mitogen-activated protein kinases of the extracellular signal-regulated kinase subgroup (15), and activation of NF-κB (16). These processes not only block spontaneous apoptosis but have also been shown to block apoptosis induced by UV irradiation, TNFα, or ligation of Fas (17–19). In the combined presence of integrin engagement and proapoptotic stress stimuli, however, we have provided evidence for downregulation or bypass of these antiapoptotic effects with induction of rapid neutrophil apoptosis (4).

The serine/threonine kinase Akt has been shown to inhibit both spontaneous and stress-induced apoptosis and appears to be responsible for regulating growth factor-mediated cell survival (20). Akt is activated by a variety of growth factors via PI 3-kinase-generated phosphatidylinositols (20). PI 3-kinase phosphorylates phosphoinositides (PI) on the 3-position of the inositol ring to generate PtdIns 3-monophosphate, PtdIns 3,4-bisphosphate, and PtdIns 3,4,5-trisphosphate (21). Generation of PtdIns 3,4,5-trisphosphate allows for recruitment of Akt to the plasma membrane via its PH domain (22). Once at the plasma membrane, PDK1 and an as yet unknown serine/threonine kinase phosphorylate Akt, whereby inducing its activation (22). Active Akt is thought to inhibit apoptosis in a variety of ways both upstream and downstream of mitochondrial perturbation. It can inhibit caspase 9 activity, phosphorylate proapoptotic Bel-2 family members such as Bad, or regulate activation of transcription factors such as CREB, NF-κB, and members of the Forkhead family (20, 22, 23). This makes Akt a prime candidate for regulation in the circumstance of paradoxical anti- and proapoptotic signal combinations. Prevention of

1 The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; PI 3-kinase, phosphatidylinositol 3-kinase; TNF, tumor necrosis factor; DHR, dihydrorhodamine-123; LPS, lipopolysaccharide; DPI, diphenyleneiodonium chloride; PtdIns, phosphatidylinositol; PIPα, phosphatidylinositol 3,4,5-trisphosphate; PIPβ, phosphatidylinositol 4,5-bisphosphate; CGD, chronic granulomatous disease.
Akt activation in this circumstance could occur by the inhibition of PI 3-kinase or the activation of a phosphatidylinositol phosphatase, each leading to reduced Akt activation and translocation.

SHIP (SH2-containing inositol 5-phosphatase) is a 145-kDa protein highly expressed in hematopoietic cells (24). It specifically hydrolyzes the 5'-phosphate from PtdIns 1,3,4,5-tetraphosphate and PtdIns 3,4,5-trisphosphate, the predominant product of PI 3-kinase (25). SHIP overexpression has been shown to inhibit Akt activity, whereas SHIP null cells exhibit sustained Akt activity (26). SHIP has also been shown to down-regulate prosurvival and proliferative signals in vivo as reviewed in Ref. 27. Stimulation of B cells by the antigen receptor activates PI 3-kinase and its downstream target Akt and results in proliferation (28). In contrast, co-clustering with the plasma membrane. Once at the plasma membrane, SHIP appears to associate with the ITIM domain of Fc receptor and activate PI 3-kinase and its downstream target Akt and reactivation of these stimuli leads to the complete abrogation of Akt activity via a decrease in PIP3 levels independent of alterations in PI 3-kinase activity. The blockade of Akt appears to be due to NADPH oxidase-derived oxidants and their ability to activate Lyn, which can aid in the recruitment of SHIP to the plasma membrane. Once at the plasma membrane, SHIP appears to associate with the β2-integrin as well as other anti-apoptotic signaling receptors, and once there it can modulate PIP3 levels and subsequently apoptosis.

MATERIALS AND METHODS

Antibodies and Reagents—The following antibodies were obtained from commercial sources and were confirmed to bind to human PMN by flow cytometry. Anti-HLA-ABC W6/32 was obtained from DAKO (Carpinteria, CA), anti–anti-VIM12, anti–CD11b/CD18, and anti-CD11c (Caltag, Burlingame, CA). Anti-Akt, anti-SHIP, and anti-Lyn antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-Akt and anti-phospho-tyrosine (4G10) monoclonal antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Unless specified in the figure legends, mAbs were used at a final concentration of 1 μg/ml. Human and murine TNFα were obtained from R & D Systems (Minneapolis, MN) and used at a final concentration of 1000 units/ml. Human anti-Fas IgM clone CH11 was obtained from Upstate Biotechnology and used at a concentration of 1 μg/ml to induce apoptosis. Murine anti-Fas clone J02 was obtained from Pharmingen (San Diego, CA), was used at 1 μg/ml, and was cross-linked with an anti-Armenian hamster secondary antibody obtained from Jackson Immunochemicals (Bar Harbor, ME) at a 1:1 ratio. The PI 3-kinase inhibitor (LY294002; 2′-(4-morpholinyl)-8-phenyl-1H-1-benzopyran-4-one) was obtained from Calbiochem and used at 100 μM. The NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI) and catalase were obtained from Sigma (St. Louis, MO). PtdIns 4,5-bisphosphate and PtdIns 4-monophosphate were obtained from Sigma. Neutrophil Isolation—Human neutrophils were purified from whole blood as described previously (33). Neutrophil purity was >95% with typically ≤2% eosinophils.

Assessment of Apoptosis in Suspension Neutrophils—Following isolation, neutrophils were suspended at 2 × 106 cells/ml in RPMI supplemented with 1% LPS-free bovine serum albumin (Sigma; A-9393) and incubated in conical polypropylene tubes (Sarstedt, as control; Sparks, NV). Neutrophils, with or without β2-integrin reagent VIM12 (1 μg/ml) or anti-HLA (1 μg/ml), were allowed to apoptose for 4 h in the presence or absence of anti-Fas IgM or TNFα (1000 units/ml). Apoptosis was assessed as by Whitlock et al. (4). Akt Immunoprecipitation and Kinase Assay—Akt activity was measured using an enzyme activity kit (Upstate Biotechnology) with some modification of the provided protocol. Cells were resuspended at 7.5 × 10⁶/ml and stimulated with either 10 ng of GM-CSF (R & D Systems) or 5 μg/ml LPS (List Biological) for 5 min or 1 μg/ml VIM12 or anti-HLA for 15 min in the presence or absence of 1 μg/ml anti-Fas IgM or 1000 units/ml TNFα. Cells were lysed in ice-cold radioimmunoprecipitation assay buffer supplemented with 15 μg/ml leupeptin and aprotonin, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mM sodium orthovanadate, and Akt activity was measured as by Whitlock et al. (4). Western blots were carried out the same way, except immunoprecipitates were boiled in Lammeli buffer, run on a 10% SDS-PAGE gel, and probed with a 1:1000 sheep anti-phospho-Akt antibody (Upstate Biotechnology). Blots were stripped as by Frasch et al. (8) and reprobed with an anti-Akt antibody for equal loading.

Lyn and SHIP Immunoprecipitation and Western Blots—Lyn samples were stimulated for 10 min with the indicated stimuli and then lysed in Lyn Tris-HCl buffer (10 mM Tris-HCl, pH 7.5, 50 mM sodium pyrophosphate, 50 mM NaCl, 1 mM Na3VO4, 1% Nonidet P-40 with 1 mM phenylmethylsulfonyl fluoride and 15 μg/ml leupeptin and aprotinin) for 10 min on ice. Lysates were then cleared by centrifugation, and supernatants were transferred to 25 μg of protein A-Sepharose (Zymed Laboratories Inc.) beads containing 1 μg of rabbit anti-Lyn antibody (Santa Cruz Biotechnology) for 4 h with rotation. Immunoprecipitates were then boiled, run on a 10% gel, transferred, and then probed with 1:1000 anti-phosphotyrosine 4G10 (Upstate Biotechnology) overnight. SHIP samples were lysed according to Liu et al. (27). SHIP was immunoprecipitated, run on a 7% gel, and probed with anti-CD11b clone 44 (Sigma) or anti-phospho-tyrosine 4G10 overnight. Immunoblots were visualized with ECL detection reagents (Amersham Biosciences). All immunoblots were stripped as by Frasch et al. (8) and then reprobed with either anti-Lyn or anti-SHIP to confirm equal protein levels.

Metabolic Labeling and Lipid Extraction—Neutrophils were suspended at 3 × 10⁶/ml in buffer A (30 mM Heps, pH 7.2, 110 mM NaCl, 10 mM KCl, 1 mM MgCl2, 10 mM glucose) and 1 μCi/ml [32P]orthophosphate (PerkinElmer Life Sciences). Cells were incubated at 37°C for 30 min, at which point they were washed three times in buffer A. Labeled cells were then treated as in the apoptosis studies for 10 min. Reactions were stopped with the addition of 3 ml of chloroform/methanol (1:2, v/v) followed by 4 ml of chloroform, 2.4 M HCl (1:1, v/v). The resultant organic phase was removed, and the aqueous phase was washed three times with chloroform/acetone/acetic acid/water (80:30:28:24:14, v/v/v/v/v). Radiolabeled phospholipids were detected by autoradiography. Standard PtdIns/PtdIns-4-P/PtdIns-3,4-P2 were co-chromatographed with Standard PtdIns/PtdIns-4-P/PtdIns-3,4-P2 were co-chromatographed and visualized by I2 vapor.

PI 3-Kinase Assays—Neutrophils were resuspended at 5 × 10⁶/ml and treated as above for 10 min, at which point they were lysed in 500 μl of lysis buffer containing 1% Nonidet P-40, 20 μg/ml Tris-HCl, pH 7.5, 137 mM NaCl, 10% glycerol, 1 mM MgCl2, 1 mM CaCl2, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 15 μg/ml leupeptin, and aprotinin at 4°C for 20 min. Lysates were transferred to protein A-Sepharose (Zymed Laboratories Inc., San Francisco, CA) beads containing 1 μg of anti-PI 3-kinase antibody (Upstate Biotechnology clone UB93–3) for 2 h, at which point they were spun down and washed three times with kinase buffer (50 mM HEPES (pH 7.25), 0.5 mM EDTA, and 5 mM MgCl2). Reaction were then performed as described previously (24). Briefly, immunoprecipitates were resuspended in 40 μl of the wash buffer containing 25 μM ATP and 20 μCi of [32P]orthophosphate (PerkinElmer Life Sciences). Phosphorylated reactions were resuspended with 10 μl of PtdIns 4,5-bisphosphate in 20 mM HEPES buffer (pH 7.4). Reactions were allowed to go for 10 min at 37°C and then stopped with 20 μl of 6 N HCl and 160 μl of chloroform/methanol (1:1, v/v). Samples were allowed to sit for 10 min and then spun for 10 min to separate the organic and aqueous phase. The lower organic phase was applied to silica 60 TLC plates (Whatman) impregnated with 1% potassium oxalate. Plates were developed in chloroform/acetone/methanol/acetic acid/water (80:30:28:24:14, v/v/v/v/v). Radiolabeled phospholipids were detected by autoradiography. Standard PtdIns/PtdIns-4-P/PtdIns-3,4-P2 were co-chromatographed.
SHIP Activity Assay—Preparation of \[^{32}P\]PtdIns 3,4,5-trisphosphate and SHIP assays were carried out as described before (24, 32). Briefly, samples were stimulated as in the Akt activity assay for 10 min, at which time samples were lysed and immunoprecipitated with anti-SHIP for 2 h at 4°C. Samples were then incubated with 25 \(\mu\)M \[^{32}P\]PIP3 or 50 \(\mu\)M 3H-labeled PtdIns 1,3,4,5-P4 in reaction buffer for 20 min. Reactions were stopped with 100 \(\mu\)l of chloroform/methanol (1:1) and 100 \(\mu\)l of 2 M KCl. Dried phospholipids were dissolved in 30 \(\mu\)l of chloroform/methanol (1:1) and separated on TLC as in the metabolic labeling. \[^{32}P\] samples were visualized by autoradiograph, with the position of \[^{32}P\]PtdIns 3,4,5-trisphosphate and \[^{32}P\]PtdIns 3,4-bisphosphate determined by the mobility of \[^{32}P\] in vitro labeled phosphatidylinositol and PIP\(_3\) standards. 3H samples were washed, dried, and re-suspended and run as above; positions were confirmed with \(I_x\) visualization of commercial standards as well as the position of \[^{32}P\]PtdIns 3,4,5-trisphosphate and \[^{32}P\]PtdIns 3,4-bisphosphate prepared standards. Inositol 1,3,4,5-tetraphosphate was excised from TLC plates solubilized in HCl/methanol (1:1) for 1 h and then counted in a scintillation counter as described by Siddiqi et al. (35).

Preparation of Murine Peritoneal Neutrophils and Assessment of Apoptosis—Mouse peritoneal exudate neutrophils were isolated following the methods of Savige et al. (36). Cytospun samples revealed a highly enriched neutrophil population (>90%) with occasional macrophages. Typical yields were \(2 \times 10^6\) peritoneal neutrophils/mouse for SHIP and Lyn +/- and \(3 \times 10^6\) peritoneal neutrophils/mouse for SHIP and Lyn +/− mice. Neutrophils were resuspended at \(2 \times 10^6\) cells/ml and incubated with 1 \(\mu\)g/ml of the murine activating anti-CD18 antibody, clone M18/2 (37), from Pharmingen (San Diego, CA), in the presence or absence of murine TNF\(_{\alpha}\), anti-Fas (Jo2), which was cross-linked with secondary antibody. Cells were incubated for 4 h before apoptosis was assessed with the human neutrophils.

Intracellular Oxidant Measurement—Dihydrosortheimine-123 (DHR; Molecular Probes, Inc., Eugene, OR) was performed as described by Vowells and colleagues (38). Briefly, neutrophils were preloaded with 1.0 \(\times\) 10\(^{-5}\) \(\mu\)M dihydrorhodamine-123 for 5 min in a 37 °C shaking water bath prior to the addition of stimuli. Following a 2-h incubation at 37 °C, cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry (FL-2, FACScalibur; Becton Dickinson).

Isolation of CGD Neutrophils—CGD blood was obtained with the help of Dr. Dan Ambruso. Blood was drawn from three individuals, each

**Fig. 1. Stimulus induced apoptosis is enhanced by \(\alpha_M\beta_2\) activation.** Activation of the \(\beta_2\)-integrin in combination with the stress stimuli TNF\(_{\alpha}\) or anti-Fas IgM enhances apoptosis due to the abrogation of Akt activity. Neutrophils (2 \(\times\) 10^6/ml) were treated with either VIM12 or anti-HLA (1 \(\mu\)g/ml) in the presence or absence of anti-Fas IgM (1 \(\mu\)g/ml) or TNF\(_{\alpha}\) (100 units/ml) and allowed to incubate for 4 h at 37 °C in polystyrene culture tubes. Apoptosis was assessed by morphology. Bars represent percentage of apoptosis ± S.D. (\(n = 4\)). The significance of enhancement of apoptosis when integrin activation was combined with stress stimuli was determined by single mean comparisons with individual percentages of stress-induced apoptosis using a Tukey-Kramer test (*, \(p < 0.05\), compared with stress induced apoptosis alone). B. Akt activity is inhibited when antiapoptotic and stress stimuli are combined. Cells (5 \(\times\) 10^6/ml) were treated with antiapoptotic stimuli (GM-CSF (1 ng/ml), LPS (100 ng/ml), or VIM12 (1 \(\mu\)g/ml)) in the presence or absence of stress stimuli TNF\(_{\alpha}\) or anti-Fas IgM. GM-CSF and LPS samples were taken at 5 min, and VIM12 and anti-HLA stimulated samples were taken at 20 min. Akt activity was measured in an in vitro kinase assay as the incorporation of \(^{32}P\). Bars represent \(^{32}P\) incorporation above untreated control cells (cpm ± S.D.; \(n = 3\)). Inset, Western gel shows Akt phosphorylation correlating with activity.
displaying different mutations. One patient displayed a gp15 X-linked form, and the other two had an autosomal recessive mutation in either p67 or p22 of NADPH oxidase. All patients were deficient in NADPH oxidase-derived oxidants as tested by superoxide generation by Dr. Ambruso. Neutrophils were isolated as described above.

**Data Analysis**—Averages and SD values were calculated from at least three experiments. Statistical analysis was carried out using the JMP statistical programs (SAS Institute, Cary, NC). Details concerning the statistical tests used for each experiment can be found in the figure legends.

**RESULTS**

Combining Antiapoptotic α5β2-Integrin Activation and Stress Stimuli Enhances Neutrophil Apoptosis—Several cytokines and membrane receptor signals, such as β2-integrin activation, GM-CSF, LPS, and even low doses of TNFα (1 μg/ml) can activate Akt and thus inhibit neutrophil apoptosis (6). However, previous observations have shown that neutrophils incubated simultaneously with the antiapoptotic β2-integrin-activating antibody VIM12 along with the stress stimuli TNFα, anti-Fas IgM, or UV irradiation no longer exhibited inhibition of apoptosis but rather an enhancement of this process (Fig. IA and Ref. 4). Each stress stimulus alone resulted in a marked increase in neutrophil apoptosis compared with control, although we observed that lower concentrations of TNFα had an antiapoptotic effect. On the other hand, combining the opposing stimuli resulted in an increase in apoptosis above each stress stimulus alone.

The Akt/protein kinase B pathway is the common antiapoptotic signaling pathway stimulated by β2-integrin activation, LPS, GM-CSF, and TNFα. Blockade of this pathway upon the combination of antiapoptotic and stress stimuli could result in the enhanced apoptosis seen in our system. Incubation of neutrophils with either 100 nM GM-CSF or 100 ng LPS for 5 min or with 1 μg/ml of the β2-integrin activation antibody VIM12 for 15 min increased Akt activity above base line (Fig. 1B). Stress stimuli alone, either TNFα or anti-Fas IgM, also increased Akt activity. However, the combination of anti-apoptotic and stress stimuli resulted in the complete prevention of Akt activation. This effect on Akt was not seen when cells were treated with a control antibody against HLA, which neither stimulated Akt activity on its own nor attenuated anti-Fas IgM- or TNFα-generated Akt activity. Akt phosphorylation (Fig. 1B, inset) correlated with its activity in that Akt phosphorylation reverted to control levels upon combining VIM12 with anti-Fas IgM or TNFα.

**PtdIns 3,4,5-Trisphosphate Levels, but Not PI 3-kinase Activity Decreases When Antiapoptotic and Stress Stimuli Are Combined**—Since Akt activation requires recruitment of Akt to PIP3 lipids in the plasma membrane via its PH domain (20), the effect of pro- and antiapoptotic stimulus combinations on PIP3 levels was assessed in [32P]orthophosphate domain-labeled neutrophils. Neutrophils were labeled and then incubated with the antiapoptotic β2-integrin activation stimulus in the absence of the stress stimulus. All of the individual stimuli alone enhanced PIP3 production above control; however, upon the combination of antiapoptotic and stress stimuli, the levels of PIP3 significantly decreased back to control levels (Fig. 2A). The combination of anti-HLA and TNFα or anti-Fas IgM showed no reduction in PIP3 levels (data not shown). The reduction in PIP3 suggested either that the combined stimuli inhibited PI 3-kinase activity or that a phosphatidylinositol phosphatase was activated.

To examine whether the reduction in PIP3 levels was due to down-regulated PI 3-kinase, PI 3-kinase activity was measured directly after stimulation with VIM12 in the presence or absence of TNFα or anti-Fas IgM. Control (unstimulated) cells showed moderate PI 3-kinase activity in accordance with reported observations (34). This activity was markedly enhanced in the cells incubated with the antiapoptotic or the stress stimuli alone. Importantly, the stimulation of PI 3-kinase was not inhibited or decreased upon the combination of antiapoptotic β2-integrin activation and stress stimuli (Fig. 2B). However, cells incubated with the PI 3-kinase inhibitor LY294002 (100 nM) showed almost complete inhibition of activity (data not shown).

**Recruitment, Phosphorylation, and Activation of SHIP by the Combination of Stimuli**—Since PI 3-kinase activity was not altered but PIP3 levels were decreased when the two stimulus types were combined, activation of a phosphatidylinositol phosphatase was examined. Candidates included SHIP and PTEN. SHIP recruitment to the plasma membrane was examined due to its predominance in hematopoietic cells and ability to actively down-regulate cytokine-induced Akt activity and regulate integrin signaling (26, 39, 40). Neutrophils incubated with the antiapoptotic integrin-activating antibody VIM12 alone or in the presence or absence of the stress stimuli TNFα or anti-Fas IgM were examined for the...
recruitment of SHIP to the plasma membrane and co-localization with the $\alpha_\text{M}\beta_2$-integrin by immunofluorescence and co-immunoprecipitation (Fig. 3, A and B). Each set of stimuli alone showed no significant SHIP co-localization with the $\alpha_\text{M}\beta_2$-integrin and SHIP, and visualized by confocal microscopy. $\alpha_\text{M}\beta_2$ is shown in green and SHIP in red, whereas yellow represents co-localization of the two. B, the results of confocal microscopy were confirmed by co-immunoprecipitation. Cells were treated with VIM12 in the presence or absence of the stress stimulus TNF$\alpha$ or anti-Fas IgM for 10 min, and SHIP was immunoprecipitated and Western blotted with anti-CD11b. Blots were stripped and reprobed with anti-SHIP to confirm equal loading. One result representative of three independent experiments is shown. C and D, SHIP activity increased when cells were treated with the combination of stimuli. C, neutrophils were treated as in A with the antiapoptotic VIM12 antibody in the presence or absence of the stress stimulus TNF$\alpha$ or anti-Fas IgM for 10 min. Cells were lysed, SHIP was immunoprecipitated, cells were incubated with $[^{32}\text{P}]$PtdIns 3,4,5-trisphosphate for 30 min, and samples were separated on TLC. D, cells were treated as in C except that SHIP lysates were incubated with $[^3\text{H}]$inositol 1,3,4,5-tetraphosphate. Bars represent extracted inositol 1,3,4,5-tetraphosphate ($IP_4$) cpm of $[^3\text{H}]$S.D. ($n = 3$). The position of inositol 1,3,4,5-tetraphosphate was determined with commercial standards visualized by $I_2$ vapor. Lysates treated with a control antibody and resulted in extracted inositol 1,3,4,5-tetraphosphate cpm equal to $3200 \pm 200$ ($n = 3$) (data not shown).

To confirm that the localization of SHIP to the plasma membrane/endothelial $\beta_2$-integrin resulted in increased SHIP activity, neutrophils were incubated as in Fig. 3A for 5 min, cells were lysed, and SHIP was immunoprecipitated. Lysates were then incubated with either a $^{32}\text{P}$-labeled PtdIns 3,4,5-trisphosphate or $^3\text{H}$-labeled inositol 1,3,4,5-tetraphosphate substrate for 20 min. The antiapoptotic and stress stimuli alone induced only a small conversion of $[^{32}\text{P}]\text{PIP}_3$ to PIP$_2$, however, when the two stimuli types were combined, there was a substantial increase in SHIP activity (Fig. 3C). Enhanced SHIP activity was confirmed with an enhanced conversion of $[^3\text{H}]$inositol 1,3,4,5-tetraphosphate to $[^3\text{H}]$inositol 3,4,5-trisphosphate when cells were treated with the stimulus combination (Fig. 3D).

Neutrophils from SHIP $-/-$ Mice Are Not Responsive to the Combination of Antiapoptotic and Stress Stimuli—To further examine the involvement of SHIP in the inhibition of Akt activity, with resultant enhanced neutrophil apoptosis, neutrophil apoptosis from SHIP $-/-$ mice was assessed. Thioglycolate-elicited neutrophils from SHIP $-/-$ and $+/+$ mice were isolated and incubated for 4 h with antiapoptotic mouse $\beta_2$-integrin antibody in the presence or absence of the stress stimuli TNF$\alpha$ or cross-linked anti-Fas (Jo2). In cells from both sets of mice, the antiapoptotic stimulus resulted in decreased apoptosis, whereas the stress stimuli increased their apoptosis (Fig. 4A). When the two types of stimuli were combined in neutrophils from the SHIP $+/+$ mice, apoptosis was enhanced
compared with either the TNFα or cross-linked anti-Fas alone. SHIP −/− neutrophils did not show this enhancement, and in fact β2-integrin ligation continued to inhibit apoptosis in the presence of TNFα or cross-linked anti-Fas.

To confirm that the effects on apoptosis seen in the SHIP −/− mice extended to maintenance of Akt, activity of the enzyme was determined in cells from both sets of mice. β2-integrin activation or TNFα stimulation resulted in measurable Akt activity in neutrophils from both SHIP +/+ and −/− mice (Fig. 4B). As expected, combining the two types of stimuli prevented Akt activation in the SHIP +/+ mice. However, no such decrease in Akt activity was seen in the SHIP −/− mice.

Lyn Activation by Oxidants Leads to Enhanced Apoptosis When Antiapoptotic and Stress Stimuli Are Combined—It has recently been reported that Lyn, a Src family kinase, can directly phosphorylate SHIP both in vitro and in vivo (32). To determine whether Lyn was activated under the combination of antiapoptotic and stress stimuli, autophosphorylation of Lyn was analyzed. VIM12 alone induced a modest increase in Lyn autophosphorylation and activity compared with each stimulus alone. These results support the idea that Lyn is activated by oxidants when combined with antiapoptotic stimuli.

Oxidant-regulated SHIP Activity

To provide additional support for the suggestion that Lyn activation affected neutrophil function upon the combination of opposing signals, apoptosis was assessed in cells pretreated with the specific Src family kinase inhibitor PP1 (41) and from Lyn −/− mice, and apoptosis was measured (Fig. 5, C and D). Neutrophils treated with PP1 or from Lyn −/− mice no longer showed enhancement of apoptosis when VIM12 was combined with either TNFα or anti-Fas IgM. PP1 treatment alone had no effect on spontaneous or stress-induced apoptosis.

To confirm that the reduced apoptosis observed with the Lyn inhibitor was due to the maintained Akt activity, cells were pretreated for 15 min with PPI before treatment with the combined anti- and proapoptotic stimuli. After 15 min, cells were lysed, and Akt phosphorylation was detected by immunoblot (Fig. 5E). Cells treated with the antiapoptotic β2-integrin-activating antibody, VIM12, showed enhanced phosphorylation that was abrogated upon the combination of stimuli. However, Akt phosphorylation was restored in the cells pretreated with PP1, indicating that the reduced apoptosis was due to the maintained Akt activity.

Lyn Is Involved in the Recruitment of SHIP to the Plasma Membrane and Its Subsequent Activation—To investigate the role of Lyn in SHIP activation, cells were pretreated for 15 min with the Lyn inhibitor PPI prior to being examined for SHIP translocation to the cβ2-integrin. The combination of stimuli showed enhanced recruitment of SHIP to the cβ2-integrin, and this recruitment was inhibited by PP1 (Fig. 6A).

To determine whether the Lyn-dependent recruitment of SHIP to the β2-integrin also affected SHIP activation, a SHIP activity assay was performed on thioglycollate-elicted neutrophils from Lyn −/− mice. Neutrophils from Lyn +/+ mice showed enhanced SHIP activity (i.e. decreased recovery of [3H]inositol 1,3,4,5-tetraphosphate) when treated with the...
The Combination of Antiapoptotic and Stress Stimuli Results in Oxidant Production—Since Lyn activity decreased in the presence of both the NADPH oxidase inhibitor DPI and catalase, the effect of these agents on apoptosis itself was also examined. Cells pretreated with either DPI or catalase no longer showed enhancement of apoptosis when the antiapoptotic β2-integrin-activating antibody, VIM12, was combined with either TNFα or anti-Fas IgM (Fig. 7A). Since the inhibition of oxidants prevented the enhancement of apoptosis, it seemed likely that Akt activity would be restored upon pretreatment with DPI or catalase. Again, VIM12 and the stress stimuli separately resulted in Akt activation, which was absent when the two types of stimuli were combined (Fig. 7B). However, in cells pretreated with DPI or catalase for 10 min, there was complete restoration of Akt activity. Control cells incubated with DPI or catalase alone showed no significant enhancement.

Based upon the ability of NADPH oxidase inhibitors to prevent the enhancement of apoptosis by restoring Akt activity, it seemed reasonable to suggest that the combination of antiapoptotic and stress stimuli led to an increase in oxidant production. To measure this, oxidant production in neutrophils was assessed with DHR, a sensitive probe for the measurement of respiratory burst activity in neutrophils (38). DHR-loaded neutrophils were incubated with either the individual or combined stimuli. The antiapoptotic stimuli VIM12, GM-CSF, and LPS each resulted in an approximately 2-fold increase in mean fluorescence intensity compared with control, with TNFα alone yielding a 5-fold increase (Fig. 7C). However, when the two sets of stimuli were combined, there was a 6–16-fold increase in oxidant production over control with the antiapoptotic stimuli and TNFα combination. The control antibody HLA, when combined with stress stimuli, had no effect.

CGD patients have heritable defects in NADPH oxidase, which results in a limited ability to generate oxidants (42). To support the proposed role for this enzyme in neutrophil apoptosis, neutrophils from three CGD patients were examined. Compared with neutrophils from unaffected individuals, cells from CGD patients no longer showed an increase in apoptosis when treated with the combination of VIM12 and TNFα or anti-Fas IgM (Fig. 7D). Cells treated with each type of stimulus alone showed little difference in apoptosis compared with neutrophils from normal donors.

**DISCUSSION**

The effect on neutrophil apoptosis of a complex cytokine and chemokine milieu, such as that encountered at the site of...
A. inhibition of Lyn prevents SHIP recruitment to the α5β1-integrin. Cells (5 × 10⁶/ml) were pretreated with the Lyn inhibitor PP1 as in B and C and then treated with the combination of stimuli. SHIP co-localization to the α5β1-integrin was performed by SHIP immunoprecipitation and Western blot with anti-CD11b. Blots were stripped and reprobed with anti-SHIP and then anti-SHIP and then anti-CD11b. PP1 was used as the loss of [3H]inositol 1,3,4,5-tetraphosphate. SHIP and SHIP became associated with the plasma membrane (32). In keeping with previous experiments, is shown. B, neutrophils from Lyn −/− mice no longer show enhanced SHIP activity when exposed to the combination of opposing stimuli. Thioglycolate-elicited neutrophils from Lyn +/+ and −/− mice were treated with the mouse integrin-activating antibody in the presence or absence of mouse TNFα, and SHIP activity was measured as the loss of [3H]inositol 1,3,4,5-tetraphosphate. Bars represent extracted cpm of [3H]-labeled inositol 1,3,4,5-tetraphosphate (IP4) ± S.D. (n = 3).

inflammation, is likely to be of critical importance to the resolution of inflammation and thus prevention of further tissue injury (3). Our experiments suggest that stimuli that normally have a protective, antiapoptotic effect paradoxically enhanced neutrophil apoptosis in the presence of stress stimuli. One such antiapoptotic stimulus is the engagement of α5β1-integrins. Activation of this molecule during emigration could have a protective effect, allowing a neutrophil to reach a site of inflammation (5). However, once reaching the inflammatory site, exposure to proapoptotic stimuli such as TNFα, Fas ligand, oxidants, etc., could serve to overcome the antiapoptotic effect and ultimately result in accelerated apoptosis, subsequent cell clearance, and resolution of the inflammatory project. This reversion or enhancement of apoptosis seems to be linked to the down-regulation of Akt activity and subsequent inhibition of antiapoptotic pathways, thus priming the neutrophil for apoptosis.

Recent reports have indicated that blockade of the PI 3-kinase pathway with inhibitors sensitized endothelial and epithelial cells to both Fas- and TNFα-induced death (43, 44), thus setting a precedent for the importance of the PI 3-kinase/Akt pathway in inhibiting stress-induced apoptosis. Our observations support this notion in that neutrophils incubated with the integrin-activating antibody VIM12 and the stress stimulus TNFα or anti-Fas IgM displayed a significant increase in apoptosis, which was shown to be directly linked to loss of Akt activity (Fig. 1B). This inhibitory effect on Akt was seen not only with the combination of integrin activation and stress stimuli but extended to other antiapoptotic stimuli such as GM-CSF and LPS when combined with TNFα or Fas ligation (data not shown).

Akt can be regulated at multiple points, including PI 3-kinase activation, maintenance of PIP3 levels, and activation of PDK1. Therefore, potential alterations in these pathways were examined. Importantly, a striking decrease in PIP3 generation was seen when the antiapoptotic stimulus and stress stimuli were combined. The decrease required both stimuli and suggested that the regulation was occurring at the point of Akt recruitment to the plasma membrane.

PI 3-kinase is activated by growth factors and other antiapoptotic stimuli. Although basal levels of activity were observed in neutrophils from normal donors using an anti-p85 antibody, the various stimuli examined herein were able to enhance this basal activity, and Ly90682 inhibited this activation (data not shown). Most importantly, however, is the fact that there was no decrease in activity when the stress and antiapoptotic stimuli were combined. Although the p85 does not associate with PI 3-kinase γ, we do not feel that this is the predominantly active PI 3-kinase in our system, due to its known activation by glycosphatidylinositol-linked factors such as fMLP and C5a. These data indicate that the regulation of Akt occurred at the PIP3 level but downstream of PI 3-kinase (either α, β, or δ) activation and that other PI 3-kinase-dependent functions would probably be unaffected in our system. This is important, since it has been shown that Fas and TNF can activate PI 3-kinase on their own and that this may actually be important for their ability to induce apoptosis (45).

Based on the reduced PIP3 levels without concomitant alterations in PI 3-kinase activity, activation of an inositol phosphatase was implicated. The prevalence of SHIP in hematopoetic cells as well as its involvement in the negative regulation of BCR signaling (a situation also involving the combination of two signals, BCR and FcγRII) (30, 46) led us to examine the involvement of this inositol phosphatase in neutrophils. Although PTEN is an alternative candidate phosphatase, it has been suggested to predominantly regulate basal levels of Akt, since PTEN null cells have enhanced basal Akt activity, but does not appear to regulate Akt activation following the addition of growth factors (47). On the other hand, SHIP knockout mice show hyperresponsiveness to growth factors, show resistance to Fas-induced death, and display spontaneous pulmonary inflammatory responses (26, 46, 48).

Recently, SHIP activation was shown to be dependent on translocation to the plasma membrane (32). In keeping with this, combined stimulation of neutrophils resulted in enhanced association of SHIP with the α5β1-integrin along with direct evidence of increased SHIP activation. If another antiapoptotic stimulus, GM-CSF, was used in combination with anti-Fas IgM or TNFα, we found that that SHIP associated with the GM-CSFR. However, even with GM-CSF as a stimulus, some of the SHIP became associated with the β2-integrin (data not shown). This observation raises the possibility that neutrophil stimulation might allow for clustering of antiapoptotic and stress receptors in the plasma membrane. Parenthetically, this potential clustering of receptors may initiate the activation of...
NADPH oxidase suggested here to be upstream of SHIP activation.

SHIP activation and its potential involvement in the down-regulation of Akt activity and enhanced apoptosis were pursued in thioglycolate-elicited neutrophils from SHIP−/− mice. As predicted, SHIP−/− neutrophils showed enhanced apoptosis when the two sets of stimuli were combined (Fig. 4A). Consequently, proapoptotic effects can be seen in murine as well as human neutrophils. On the other hand, SHIP−/− cells showed resistance to enhanced apoptosis, if not a slight inhibition. The inability of SHIP−/− mice to respond to the combination of stimuli was shown to be the result of maintained Akt activity (Fig. 4B), thus linking SHIP activation to the down-regulation of Akt activity and enhanced apoptosis seen with the combined stimuli. The data indicate that SHIP activation may be pivotal in regulating neutrophil apoptosis in an inflammatory reaction and preventing tissue damage and subsequent fibrosis.

Due to the importance of SHIP in the down-regulation of Akt activity, potential upstream activators of SHIP were investigated. Lyn has previously been shown to phosphorylate SHIP, and we found that Lyn autophosphorylation and activity were enhanced upon the combination of opposing stimuli (Fig. 5A). Although we saw a small amount of Lyn phosphorylation with the integrin-activating antibody alone, this correlated with previous reports (49). Lyn phosphorylation was enhanced 2-fold when the stimuli were combined. The importance of Lyn in regulating this response was indicated by the resistance of cells treated with the Lyn inhibitor PP1 or from Lyn−/− mice to enhanced apoptosis upon the combination of antiapoptotic

**FIG. 7.** Oxidant production increases when antiapoptotic and stress stimuli are combined, thus leading to the abrogation of Akt activity. A, inhibition of NADPH oxidase prevents the enhancement of apoptosis upon the combination of stress and antiapoptotic stimuli. Cells (2 × 10^6/ml) were preincubated with either DPI or catalase for 15 min prior to the addition of antiapoptotic VIM12 or stress stimuli TNFα or anti-Fas IgM alone or the combination of both stimuli sets. Samples were incubated for 4 h at 37 °C, and apoptosis was assessed as before. Bars represent percentage of apoptosis ± S.D. (n = 3). The significance of reduced apoptosis upon the DPI or catalase addition was determined by single mean comparisons with individual percentages of apoptosis in nontreated cells using a Tukey-Kramer test (*, p < 0.05, compared with nontreated cell apoptosis alone). B, Akt activity is restored upon NADPH oxidase inactivation. Cells (5 × 10^6/ml) were treated as in A, and an Akt assay was performed as in Fig. 2A. Bars represent 32P incorporation above untreated control cells (cpm ± S.D.; n = 3). C, intracellular oxidant levels increase upon the combination of antiapoptotic and stress stimuli. Cells (5 × 10^6/ml) were loaded with 1 × 10^−5 M DHR and stimulated with antiapoptotic stimuli, TNFα, or the combination as in A. DHR fluorescence was measured by flow cytometry and is expressed as histograms corresponding to FL-2 fluorescence. Data shown are representative of five separate experiments. D, neutrophils from CGD patients no longer respond to the combination of stimuli. Neutrophils were isolated as above from the blood of three separate CGD patients who have a heritable defect in their NADPH oxidase. Cells were incubated at 2 × 10^6/ml with the antiapoptotic VIM12 antibody in the presence or absence of the stress stimuli TNFα or anti-Fas IgM. Samples were incubated for 4 h, and apoptosis was determined by morphology. Bars represent percentage of apoptosis ± S.D. (n = 3).
Fig. 8. The activation of SHIP by combined stimuli is a result of NADPH oxidase-derived oxidant-activated Lyn. The signaling model shows SHIP localization to the β2-integrin upon combined stimulation. Gray bars represent inhibitory pathways. β2-Integrin structures are modeled after Stewart and Hogg (52).

and stress stimuli and correlated with the maintained Akt activity seen upon the combination of opposing stimuli (Fig. 5D).

Although it is known that SHIP must translocate to the plasma membrane for activation, how this recruitment is regulated remains unclear. The inability of SHIP to translocate to the plasma membrane when Lyn was inhibited correlated with SHIP activation as demonstrated with neutrophils from Lyn−/− mice. Based on our data and previous reports in Lyn−/− mice demonstrating abrogated recruitment of SHIP when BCR and FcγRII were co-engaged (50), we propose a role for Lyn in regulating recruitment of SHIP to the plasma membrane. Since Lyn has been shown to phosphorylate SHIP (32), a direct effect is suggested whereby SHIP phosphorylation by Lyn results in SHIP association with membrane receptors and enhanced activity. In particular, SHIP phosphorylation might be necessary for its recruitment from the cytosol to Shc-GRB2 complexes on membrane receptors. Alternatively, Lyn activity might be necessary for receptor phosphorylation, and this in turn may promote SHIP recruitment and activity.

Activation of Lyn in neutrophils has been shown to be a result of oxidant production, in particular H2O2 (51). Our data showing reversal of Lyn phosphorylation in the presence of the NADPH oxidase inhibitor DPI or catalase confirmed these earlier reports. Using dihydrodihydroxyamine-123, we showed that the combination of antiapoptotic and stress stimuli resulted in a significant increase in intracellular oxidant production, and this increase appeared to be linked to NADPH oxidase activity (Fig. 7C). The resultant oxidant production further correlated with enhanced neutrophil apoptosis and decreased Akt activity, since cells pretreated with DPI or catalase no longer demonstrated enhanced apoptosis when exposed to the combination of opposing signals (Fig. 7A). Interestingly, the effect of the exogenous addition of catalase suggests a role for extracellular released H2O2. However, direct measurements of extracellular oxidants did not show (in these suspension conditions) significantly increased oxidant levels (data not shown). Possibly, this reflects an immediate action of these molecules on the cell with minimal free levels for detection. Importantly, an antiapoptotic effect of extracellular catalase on neutrophil apoptosis has been reported previously (14).

Last, a role for NADPH oxidase in the enhancement of apoptosis was confirmed when neutrophils from CGD patients were exposed to the combination of antiapoptotic and stress stimuli. These cells, defective in NADPH oxidase-derived oxidants, no longer showed enhanced apoptosis upon the combination of opposing stimuli as seen with control cells.

In conclusion, it is suggested that the enhancement of apoptosis seen when opposing signals are combined is due to the loss of Akt activity potentiated by oxidant-mediated activation of Lyn and subsequent translocation and activation of SHIP (model in Fig. 8). This potential pathway for the stimulation of neutrophil apoptosis may be critical for the resolution of inflammation and subsequent neutrophil clearance, thus protecting tissue from damage and exacerbation of the inflammatory response.

Acknowledgment—We thank the John Cambier laboratory for access to the SHIP and Lyn −/− mice.

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