Activation of PI3-kinase/PKB contributes to delay in neutrophil apoptosis after thermal injury

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Neutrophil apoptosis is delayed under trauma and/or sepsis injury conditions. The molecular mechanism for the delay in apoptosis has not been well defined. We investigated whether activation of phosphatidylinositol 3-kinase (PI3-kinase)/PKB signaling pathway contributes to the delay in neutrophil apoptosis with thermal injury. Rats were subjected to burns (30% total body surface area, 98°C for 10 s), and euthanized 24 h later. Blood neutrophils were isolated with the use of Ficoll gradient centrifugation and cultured for the indicated time periods. Apoptosis was determined using annexin V and PI labeling and flow cytometry. NF-κB activation was examined using gel mobility shift assay and confocal microscopy. Expression levels of inhibitory apoptosis proteins (IAPs), including cellular IAP1 (cIAP1), cIAP2, X-linked IAP (XIAP), and survivin, and Bcl-2 family members such as Bcl-xl and Bad, were determined by Western blot analysis and/or RT-PCR, real-time PCR. The results showed that in culture, the decrease in apoptosis of neutrophils from thermally injured rats was prevented in the presence of PI3-kinase inhibitors wortmannin and LY-294002. There was upregulation of PKB and Bad phosphorylation and NF-κB activation in N-formyl-L-methionyl-L-leucyl-L-phenylalanine-stimulated neutrophils from thermally injured rats compared with the sham injured group. Increased Bad phosphorylation and NF-κB activation were also attenuated by wortmannin. Bcl-xl expression in neutrophils was upregulated with thermal injury and inhibited in the presence of wortmannin. However, the expression of IAP family members was neither affected by thermal injury nor inhibited by wortmannin. These data suggest that the delay in neutrophil apoptosis with thermal injury is partly caused by activation of PI3-kinase/PKB signaling and NF-κB, which appeared to be related to the increased Bcl-xl expression and phosphorylation of Bad, but not IAP expression.

Activation of PI3-kinase/PKB contributes to delay in neutrophil apoptosis after thermal injury

While spontaneous neutrophil apoptosis has been shown to be delayed in the presence of granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage CSF, tumor necrosis factor-α (TNF-α) is known to both promote and delay PMN apoptosis (2, 35, 36). The release of such mediators is known to occur in burn injury and can cause delay in neutrophil apoptosis leading to exacerbation of inflammation after burns (10). Our previous study (15) has shown that a delay in neutrophil apoptosis after burn injury is accompanied by suppression of mitochondrial apoptotic mechanisms. To date, no studies have provided any significant information as to neutrophil signaling mechanisms and their effects on anti-/proapoptotic targets that might be relevant to the delay in apoptosis with burns.

Phosphatidylinositol-3 kinase (PI3-kinase)/protein kinase B (PKB) has been shown to be a key signal transducer for survival factors such as growth factors, cytokines, and integrins in various cell types (14, 38). PKB is a major mediator of survival signals downstream of PI3-kinase (19). Its activity is positively regulated by phosphorylation on residues Thr308 and Ser473 downstream of PI3-kinase. PKB phosphorylates Bad at the Ser136 residue that is critical for sequestration to 14-3-3 (a cytosolic molecular chaperone) (41). After apoptotic stimuli, Bad is released from 14-3-3 and subsequently dimerizes with the anti-apoptotic protein Bcxl. Because Bcxl resides constitutively bound to Bax, Bad displaces and releases Bax from Bcxl. Thereafter, Bax translocates to the mitochondria and promotes release of cytochrome c, formation of the apoptosome, and activation of the caspase cascade (22).

G-CSF is known to prolong neutrophil survival by inhibiting activation of the caspase cascade. The effect of G-CSF could be abolished by cycloheximide, suggesting that protein synthesis is required for the cytokine’s antiapoptotic effect. Previous studies (23, 37) have also shown that Bcxl and inhibitory apoptosis protein (IAP) family proteins are direct targets for transcriptional regulation by NF-κB. Bcxl promotes cell survival via formation of regulatory mitochondrial inner membrane channels that inhibit the release of cytochrome c from mitochondria to cytosol (27, 40). The IAP family includes cellular IAP1 (cIAP1), cIAP2, X-linked IAP (XIAP), and survivin. TNF upregulates cIAP2 through activation of NF-κB in Jurkat cells (5). The expression of survivin is increased by stimulation with G-CSF and granulocyte-macrophage-CSF (3, 12).

In this study, we evaluated the role of PI3-kinase in the delay of apoptosis of neutrophils from burn-injured rats by assessing PKB phosphorylation. Specifically, we ascertained the downstream events of PI3-kinase/PKB activation in neutrophils during early vs. later culture periods.

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The recruitment of many hyperactive polymorphonuclear neutrophils (PMNs) at an inflammatory site may cause secondary tissue injury and contribute to neutrophil-mediated diseases, such as acute respiratory distress syndrome and chronic obstructive pulmonary disease (25, 39). PMNs undergo spontaneous apoptosis in vitro and in vivo, and this process has been recognized as a crucial mechanism for promoting resolution of inflammation (30).

While spontaneous neutrophil apoptosis has been shown to be delayed in the presence of granulocyte colony-stimulating protein (G-CSF); nuclear factor-κB; Bcxl; Bad; inhibitory apoptosis protein; burn injury

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MATERIALS AND METHODS

Rat burn injury model. Adult male Sprague-Dawley rats (~250 g) were purchased from Harlan (Indianapolis, IN). The rats were acclimatized in the animal quarters for 3 days before experiments were initiated. The care of animals was in accordance with the guidelines set forth by Loyola University Medical Center Animal Care and Use Committee. The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt). The hair was shaved off the rats’ backs. The rats were then placed in a supine position in a plastic template that exposed 30% of the total body surface area. In the burn group, full-thickness skin scars were inflicted by immersing the backs of the rats in 95°C water for 10 s. In the sham group, the procedure was performed using a room temperature water bath. Rats were quickly dried and resuscitated with 10 ml of normal saline administered intraperitoneally. The rats were euthanized at 24 h postinjury by exsanguination through cardiac puncture.

Neutrophil isolation and culturing. Blood (10–12 ml) was collected through cardiac puncture into heparinized syringes. Neutrophils were isolated from heparinized blood via Ficoll-Paque (Pharmacia, Peapack, NJ) gradient centrifugation. The erythrocyte/granulocyte pellet was diluted 1:1 with normal saline. Erythrocytes were sedimented by 3% dextran (Sigma, St. Louis, MO) in 0.9% saline and incubated for 1 h. Supernatants were collected and centrifuged for 10 min at 4°C. Remaining red blood cells were lysed in distilled water. The freshly isolated neutrophils were resuspended in modified RPMI 1640 (Cellgro Mediatech, Herndon, VA) containing 10% heat-inactivated fetal calf serum (Cellgro Mediatech), 100 U/ml penicillin, 100 µg/ml streptomycin, and 300 µg/ml glutamine (GIBCO-BRL, Grand Island, NY). The neutrophil preparation routinely contained ≥95% neutrophils as identified by the Giemsa stain and were found to be ≥98% viable by the Trypan blue exclusion technique. Five milliliters of cell suspension were put into each well of 6-well plates (Fisher Scientific, Pittsburgh, PA), which were then incubated at 37°C with or without 100 nM wortmannin (Calbiochem), 10 µM LY-294002 (Calbiochem), and cycloheximide (CHX) (Sigma) (1, 5, and 10 µg/ml) for the indicated periods in a humidified incubator containing 95% atmosphere and 5% CO2. To evaluate the effect of injury-related agonists modulating apoptosis in vivo, neutrophils were stimulated with 100 nM N-formyl-l-methionyl-l-leucyl-l-phenylalanine (fMLP) (Sigma) before being assessed by phosphorylation of PKB and Bad and for determining translocation of NF-κB in vitro.

Annexin V analysis of neutrophil apoptosis. Neutrophil apoptosis was measured by flow cytometry with the annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay kit (Pharmingen/BD Biosciences, San Diego, CA). The experiment was performed using the manufacturer’s instructions with minor changes. Briefly, after isolation or incubation, neutrophils were washed twice with ice-cold PBS and then resuspended in binding buffer. Neutrophils were analyzed by flow cytometry within 1 h of annexin V-PI labeling. Viable neutrophils were defined as positive for annexin V-FITC but negative for PI staining; necrotic annexin V-PI labeling. Viable neutrophils were defined as negative for nuclei and Rel A was performed. After isolation or incubation, the experiment was measured by flow cytometry with the annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay kit and for determining translocation of NF-κB in vitro.

Confocal laser scanning microscopy. Immunohistochemical staining for nuclei and Rel A was performed. After isolation or incubation, 106 neutrophils were washed once in ice-cold PBS and were fixed with 2% (wt/vol) paraformaldehyde in PBS for 15 min at room temperature, washed twice in PBS, and permeabilized in buffer containing 1% Triton X-100 (wt/vol) and 1% (wt/vol) bovine serum albumin (Sigma) in PBS. Neutrophils were incubated with antibody against Rel A (Cell Signaling, Beverly, MA) or resuspended in buffer without antibody (as negative control of staining). After incubation, neutrophils were washed twice and resuspended in secondary antibody (Alexa 488-conjugated goat-anti-rabbit IgG; Molecular Probes, Eugene, OR) at a final concentration of 5.0 µg/ml. Incubation with the secondary antibody was for 30 min. After three washes in PBS, neutrophils were RNase treated and counterstained with PI to label DNA for nuclear localization. Cytospins were prepared on glass slides, air dried, and mounted in a mounting medium (Baxter Healthcare, Deerfield, IL). The slides were analyzed with a confocal laser-scanning microscope.

RNA extraction, RT-PCR, and real-time PCR. Total RNA was isolated from neutrophils with RNase Mini Kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). Neutrophils were washed twice with ice-cold PBS and then lysed in Buffer RLT. The lysates were pipetted directly onto a QiaShredder spin column placed in a 2-ml collection tube and centrifuged for 2 min at maximum speed. After centrifugation, 600 µl of 70% ethanol were added to the homogenized lysates and mixed well by pipetting. Further DNA removal was performed with Qiagen RNase-free DNase set. RNase-free water (30 µl) was pipetted onto the RNeasy silica membrane, and the resulting RNA was in the elution.

To generate cDNA, 2 µg of total RNA were used for each reaction. The reaction mixtures (20 µl) contained RNA, oligo(dT) primer, dNTP mixture, and Omniscript reverse transcriptase (Qiagen). The reaction mixtures were incubated for 60 min at 37°C. One-tenth of the synthesized cDNA was then amplified. PCR reaction mixtures (25 µl) contained cDNA, dNTP mixture, MgCl2 (1.5 mM), Taq DNA polymerase (2.5 U/µl), and forward and reverse primers. The following primer sets were used: rat eIAP (forward: 5’-ACATTTCCCACGT-GCCCATTC-3’, reverse: 5’-CTCTGTGCTCGTCTCTCTT-3’); rat cIAP2 (forward: 5’-CCACCTGCTCCCTAACAATCCT-3’, reverse: 5’-GGGTCACTCGGTTTCTCCCAAC-3’); rat XIAP (forward: 5’-CCGAGCCGGGGTTTCTCCATAC-3’, reverse: 5’-ACAGG-ACGGT CACAGGGTT-3’); rat survivin (forward: 5’-CAACCTGGGACCTGATGACAT-3’, reverse: 5’-CCACCCATAGATCTGTCGG-3’); and rat GAPDH (forward: 5’-CATCTGACCATTTGGGTTG-3’, reverse: 5’-CTCTGTACCCACCTTCTTGT-3’). The samples were denatured at 95°C for 15 min and amplified for 32 cycles (RGAPDH) or 32 cycles (cIAP1, cIAP2, XIAP, and survivin), with the last cycle extended at 72°C for 10 min. Samples were resolved on a 2% agarose gel and visualized with ethidium bromide.

Real-time PCR analysis of IAPs was performed on a GeneAmp 5700 sequence detection system (Applied Biosystems). Platinum QPCR Supermix-UDG (Life Technologies, Grand Island, NY), 12.5 µM Rox reference dye (Life Technologies), SYBR Green DNA binding dye diluted 1:2,000, forward and reverse primers (2.5 µM in 10 mM Tris), RNase- and DNase-free H2O, and appropriate sample cDNA were added to each sample. Negative controls included samples without reverse transcriptase or RNA or cDNA. Controls, standards, and samples were run in triplicate (20 µl volume) in a 96-well optical reaction plate and capped with optical caps (Applied Biosystems). The real-time PCR thermal cycler profile was run as follows: one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, 40 cycles of denaturing at 95°C for 15 s, and annealing and elongation at 60°C for 1 min, followed by a dissociation protocol run to test the melting temperature of the product. The data were analyzed using GeneAmp 5700 SDS software (Applied Biosystems). In each experiment, GAPDH PCR products were used as an endogenous reference to calculate the relative amount of IAP mRNA.

Western blot analysis. The whole cell lysates were obtained as follows. After being washed with cold PBS, the cell pellets were resuspended in lysis buffer, which contained 50 µl piperazine-N,N’-bis(2-ethanesulfonic acid)/KOH, pH 6.5, 2 mM EDTA, 0.1% 3-(cholamidopropyl)dimethylammonio)-1-propanesulfonate, 5 mM DTT, 20 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml sodium orthovanadate, and 2 mM PMSF (all purchased from Sigma). The cells were subjected to three freeze/thaw cycles. The lysates were centrifuged at 4°C, and the supernatant fraction was drawn off. Protein concentrations were determined by the Bradford method.
Fig. 1. The effect of phosphatidylinositol 3-kinase (PI3-kinase) blockers, wortmannin, and LY-294002 on polymorphonuclear neutrophil (PMN) apoptosis. Neutrophils isolated from sham or burn-injured rats were cultured for 8 h with or without N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (or wortmannin or LY-294002). PMNs were stained with annexin V and PI and analyzed with FACScan. Cells stained positive with annexin V and negative for propidium iodide (PI) were counted as apoptotic cells. The percentage of apoptotic neutrophils expressed as apoptotic cells in the counted cell population. Values represent means ± SE of 8 separate experiments. *P < 0.05.

Fig. 2. Increased phosphorylation of PKB and Bad in neutrophils from burn-injured rats was attenuated by wortmannin. Neutrophils were isolated from sham- or burn-injured animal samples without stimulation. Western blot analysis was performed with phosphorylated Bad and PKB antibodies. Densitometric analyses were shown. 

RESULTS

PI3-kinase dependence of delayed neutrophil apoptosis with thermal injury. Double labeling of neutrophils with annexin V-FITC and the nuclear stain PI enabled us to distinguish apoptosis (annexin V+, PI−) from cells with damaged cell membranes (annexin V+, PI+). As shown in Fig. 1, the percentage of cell apoptosis was significantly lower in neutrophils from burn-injured rats than in the sham group (P < 0.05) at 8-h culture. In sham rat neutrophils, ~44% of cells displayed apoptosis (annexin V+, PI−) after 8-h culture at 37°C; only 31% of cells were apoptotic in the burn injury group. Previous studies (6) have shown that 100 nM wortmannin and 10 μM LY-294002 produced a near-maximal inhibition of PI3-kinase activity in neutrophils. However, as shown in Fig. 2A, PKB phosphorylation was blocked by PI3-kinase blocker wortmannin. The whole cell lysates were subjected to SDS-PAGE. Western blot analysis was performed with phosphorylated PKB antibody. The same membrane was reprobed with phosphorylated Bad (pPKB) antibody. The same membrane was reprobed with phosphorylated Bad antibody. Densitometric analyses were shown. Results are representative of three independent experiments.

Statistics. The data are expressed as means ± SE. Where applicable, ANOVA analyses were performed to evaluate the significance of differences between control and experimental groups. Statistical significance was defined as P < 0.05.

Molecular Biochemicals). The oligonucleotide used as a probe for the NF-κB consensus 3′-AGTTGAGGG-GACTTTCCCAGG-3′ (Integrated DNA Technologies, Skokie, IL). Probes were labeled with DIG. Binding reaction mixtures (20 μl) containing 6 μg of nuclear extract protein, 1 μg of poly(dl-dc), 0.1 μg of poly-t-lysine, 2 μl of DIG-labeled oligonucleotide (0.4 μg/μl), and 4 μl of binding buffer were incubated for 15 min at room temperature. The protein-DNA complexes were separated on 6% non-denaturing polyacrylamide gels run at 60 V in 0.5× Tris-borate-EDTA buffer. Transfer was performed for 30 min at 400 mA. After cross-linking, the membrane was washed, incubated for 30 min in blocking buffer, and then incubated for 30 min in antibody solution. The DIG-labeled fragments were visualized by an enzyme immunoassay using anti-DIG-AP, Fab fragments, and the chemiluminescent substrate disodium 3-(4-methoxyxyspiro[1,2-dioxyetan-3,2-(5-chloro)tricyclocdecan]-4-yl)phenyl phosphate. The generated chemiluminescent signals were recorded on X-ray film.
with fMLP. This may be because the signaling events triggered to effect neutrophil apoptosis modulation are presumably transient and not detectable without in vitro activation of cells with fMLP.

**PI3-kinase dependence of burn injury on Bad phosphorylation with thermal injury.** To examine the phosphorylation status of Bad in neutrophils from burn-injured rats, Western blot analysis was performed using anti-Bad and selective anti-phospho-Bad antibodies. Figure 2B shows representative immunoblots demonstrating that burn injury causes a marked increase in the phosphorylation of Bad in neutrophils. Phosphorylated Bad is known to play a role in the generation of free Bcl-xl that in turn can cause stabilization of inner mitochondrial membrane and prevention of disruption of its membrane potential for apoptosis (8). As shown in Fig. 2B, the PI3-kinase blocker wortmannin had an inhibitory effect on the phosphorylation of Bad and significantly attenuated the increased levels of phosphorylated Bad in burn-injured rat neutrophils. These data support the hypothesis that PI3-kinase-dependent phosphorylation of Bad might also be an intracellular target causing PMN survival after thermal injury.

**Fig. 3.** Increased NF-κB activation in PMNs from burn-injured rats. After isolation, some neutrophils were stimulated with 100 nM fMLP for 3 min. Nuclear protein was extracted from neutrophils. EMSA was performed with digoxigenin (DIG) Gel Shift Kit. DNA-protein complexes were separated on 6% non-SDS polyacrylamide gels. The densitometric analyses were also performed. The data are expressed as fold changes. The value of nonstimulated sample in sham rats was taken as 1. Results are representative of 3 separate experiments.

**Fig. 4.** Increased translocation of NF-κB after burn injury was blocked by wortmannin (wort). Freshly isolated PMNs were stimulated with fMLP, and some PMNs were treated with wortmannin. The cells were fixed and permeabilized. Rel A staining was performed using Rel A antibody and a FITC-conjugated secondary antibody. PI was used for nuclear staining. The color green stands for Rel A staining and red represents nuclear staining. Results are representative of 3 independent experiments with at least 3 burn-injured and 3 sham-injured rats. In each experiment, at least 50 neutrophils were counted in at least 5 different fields under oil immersion for one sample.
The nuclear translocation of NF-κB transcription factor was upregulated after burn injury (Fig. 3). Flow cytometry to detect NF-κB activation revealed that activation of the NF-κB that has been implicated in survival signaling and has been shown to increase survival in neutrophils of burn-injured rats. This indicates that the effect of burn injury on neutrophil apoptosis delay at 2-h culture might be partly dependent on protein synthesis but that the decrease of PMN apoptosis in burns at 8-h culture was independent of protein synthesis. Although the absolute values for neutrophil apoptosis given in Fig. 5B in both sham and burn groups were lower than those shown in Fig. 1, the relative change with burn shown in Figs. 5 and 1 was the same. Data in Fig. 6 show that there was significant upregulation of NF-κB activation in freshly isolated burn rat neutrophils compared with sham samples. Increased activation of NF-κB was also apparent in 2-h-cultured PMNs from burn-injured rats. However, at 8-h incubation, there was a decrease in NF-κB activation in the burn group compared with sham. This suggests that NF-κB activation and protein synthesis are prerequisite for the delay in neutrophil apoptosis at 2-h culture but not after a later 8-h culture period.

Effect of burn injury on expression of Bcl-xl in neutrophils. As shown in Fig. 7A, Bcl-xl protein expression was apparently upregulated in neutrophils from burn-injured rats compared with neutrophils from sham rats. This is consistent with our previous study showing that burn injury caused increased expression of Bcl-xl at the mRNA and protein levels (15). To determine whether PI3-kinase signaling pathway was involved in Bcl-xl upregulation in neutrophils after burn injury, wortmannin was used to treat neutrophils at the time of stimulation with fMLP. Figure 7A also shows that fMLP increased the expression of Bcl-xl in neutrophils of both sham- and burn-injured rats, but there was a greater upregulation of Bcl-xl

Role of NF-κB signaling in delayed neutrophil apoptosis after burn injury. A potential downstream target of PI3-kinase is NF-κB that has been implicated in survival signaling and has been shown to be activated with thermal injury (28). An EMSA to detect NF-κB activation revealed that activation of the transcription factor was upregulated after burn injury (Fig. 3). The nuclear translocation of NF-κB was assessed via double labeling of RelA and PI in neutrophils. Figure 4 shows that RelA labeling was much more intense in neutrophils from burn-injured rats than in sham samples. RelA labeling in the sham group, although much less intense than in the burn group samples, was inside the nucleus, and the addition of wortmannin to these samples resulted in an extranuclear distribution of RelA. In the absence of wortmannin, burn rat neutrophils showed the presence of most of RelA inside the nucleus. The addition of wortmannin to the burn rat samples clearly attenuated intranuclear RelA distribution. These data indicate that increased translocation of RelA to nucleus with burn injury is significantly attenuated by wortmannin and that activation of NF-κB occurs downstream from PI3-kinase.

Effect of CHX on neutrophil apoptosis with thermal injury. Treatment of neutrophils with CHX inhibits synthesis of proteins including anti-apoptotic factors and thereby prevents the delay in neutrophil apoptosis (4). As shown in Fig. 5, at 2-h incubation, 5 µg/ml CHX significantly increased PMN apoptosis in both the sham and burn groups (P < 0.05). A similar effect of CHX was seen at a lower (1 µg/ml) and a higher (10 µg/ml) concentration of CHX (data not shown). However, the measured rate of neutrophil apoptosis in the burn group was maintained at a much lower level than it was in the sham group. This suggests that synthesis of anti-apoptotic proteins may be less dependent on protein synthesis in neutrophils from burn group than sham rat PMNs in vitro. At 8-h incubation, CHX significantly increased neutrophil apoptosis in sham group without significantly affecting PMN apoptosis in the burn-injured rats. This indicates that the effect of burn injury on neutrophil apoptosis delay at 2-h culture might be partly dependent on protein synthesis but that the decrease of PMN apoptosis in burns at 8-h culture was independent of protein synthesis.

Fig. 5. The effect of cycloheximide (CHX) on neutrophil apoptosis. A: neutrophils were incubated with or without 5 µg/ml cycloheximide for 2 h. Neutrophils were incubated with or without 5 µg/ml cycloheximide for 8 h. After incubation, PMN were stained with annexin V and PI and analyzed by FACSscan. Neutrophil apoptosis was expressed as the percentage of neutrophils with annexin V positive and PI negative in the counted cell population. Values represent means ± SE of four separate experiments. *P < 0.05, compared with no treatment.

Fig. 6. NF-κB activation in neutrophils. Neutrophils were isolated and some were incubated for 2 or 8 h. Six micrograms of nuclear extracts were used in EMSA that was performed with the DIG Gel Shift Kit. DNA protein complexes were separated on 6% non-SDS polyacrylamide gels. S, sham rat PMNs; B, PMNs from burn-injured rats. The densitometric analyses were also performed. The data are expressed as fold changes. The value of nonstimulated PMNs; B, PMNs from burn-injured rats. The densitometric analyses were also performed. The data are expressed as fold changes. The value of nonstimulated PMNs; B, PMNs from burn-injured rats. The densitometric analyses were also performed.
protein expression in neutrophils from the burn group compared with the sham group. The increased expression of Bcl-xl in neutrophils from burn-injured rats was attenuated with wortmannin. As shown in Fig. 8, mRNA or protein expression of IAP family members was not affected by wortmannin.

**DISCUSSION**

The present experiments show that burn injury-caused delay in neutrophil apoptosis is PI3-kinase dependent. Our findings also indicate that PI3-kinase activation with burn injury would result in a downstream activation of NF-κB and Bad phosphorylation in neutrophils. PI3-kinase activation with burns also appears to play a role in downstream upregulation of anti-apoptotic factor Bcl-xl. The decrease in neutrophil apoptosis with thermal injury, however, was not related to modulation in expression of IAPs. Several intracellular signaling pathways have been shown to be involved in cytokine-mediated inhibition of neutrophil apoptosis (1, 6, 11, 21). Our results showing PKB activation and its abrogation with wortmannin, and wortmannin-caused restoration of apoptosis in neutrophils from burn-injured animals, suggest the involvement of a PI3-kinase-PKB pathway in the burn-induced delay in neutrophil apoptosis. Previous studies have shown that cytokine stimulation caused a rapid PI3-kinase-dependent phosphorylation of Bad that prevented apoptosis via the release of Bcl-xl from the Bad-Bcl-xl heterodimer complex. The released Bcl-xl plays a role in the stabilization of mitochondrial membrane and thereby serves as a mechanism for suppression of the mitochondrial pathway of cell apoptosis (40). Our results demonstrate that NF-κB activation with burn injury was also dependent on the PI3-kinase signal and that inhibition of PI3-kinase with wortmannin attenuated both the translocation of NF-κB to nucleus and PMN survival after burn injury. Previous studies (16) have provided important insights into the pathways that lead to NF-κB activation (16). In most cells, Rel/NF-κB subunits that are sequestered in the cytoplasm as inactive homo- or heterodimers bound to one of several IκB proteins. In response to a wide variety of stimuli, IκB is phosphorylated by
IκB kinase complex, leading to a rapid release of NF-κB that translocates to the nucleus (4). IκB kinase complex itself is activated via phosphorylation by PKC, MAPK, and/or phospho-PKB (18, 31, 32). NF-κB induced suppression of cell apoptosis results from transactivation of several cell survival genes, including Bcl-xl (20, 43). Our previous study (15) has provided evidence for a role of the mitochondrial pathway in the delay of PMN apoptosis with burn injury. Recent studies also suggest that neutrophil apoptosis is initiated via the mitochondrial pathway through the mitochondrial permeability transition induced via incorporation of proapoptotic factor Bax into mitochondrial membrane. Thereafter, Bcl-xl mainly functions as an inhibitor of neutrophil apoptosis at the mitochondrial level after burn injury.

In our experiment, we found that at an early stage of neutrophil culture (2 h), the delay of apoptosis of PMN from burn-injured rats was dependent on upregulated NF-κB activation and increased synthesis of anti-apoptotic factors. However, at a later stage of PMN culture (8 h), the inhibitory effect of thermal injury was not dependent on an increase in synthesis of anti-apoptotic proteins. The phosphorylation of Bad, however, was increased at 8-h neutrophil culture and may be an important factor for PMN survival at this later phase in culture. These data provide information that increased expression of anti-apoptotic Bcl-xl and phosphorylation of Bad, both of which are PI3-kinase-dependent mechanisms for the delay of PMN apoptosis, may operate independently in burn injury. IAP family proteins have been shown to suppress apoptosis induced by a variety of stimuli (13, 34). These proteins directly inhibit activated effector caspases, caspase-3 and caspase-7, and can inhibit the activation of the initiator caspase, caspase-9 (9, 24). Furthermore, it has been reported that IAPs, cIAP1 and cIAP2, bind to TNF receptor-associated factor-1/-2 heterocomplexes and then are recruited to TNF receptors to suppress caspase-8 activation and/or caspase-3 activity (26, 29). IAP family members are inducible by a variety of NF-κB-inducing stimuli, such as TNF, LPS, and G-CSF (7, 33). The findings that proinflammatory stimuli are released into circulation after burn injury could suggest that these stimuli might play roles in inducing IAPs after burn injury. Moreover, cIAP2 expression has been shown to be increased at 1 h after neutrophil culture with G-CSF (13). In our present study, although NF-κB was activated in the freshly isolated and 2-h cultured neutrophils, we failed to demonstrate an effect of burn injury on gene and protein expressions of IAPs in 0- and 1-h cultured neutrophils. These findings suggest that either the IAP-inducing inflammatory stimuli are not expressed at levels required for the activation of IAPs or IAP activation occurs in vivo transiently and then is diminished in freshly isolated PMNs from burn-injured rats. In contrast to the anti-apoptotic activity of NF-κB, it has been shown that NF-κB also promotes apoptosis (17, 42). NF-κB could not only trigger the Fas-death cascade by directly activating the expression of Fas ligand but also regulate gene expression of proapoptotic factors, including Bcl-xs, which antagonizes prosurvival Bcl-2 family members. In our study, we found that at the later stage of neutrophil culture (8 h), NF-κB activity in sham rat PMNs was higher than that in burn-injured rat PMNs and that CHX significantly increased PMN apoptosis in the sham group. This may be related to the proapoptotic effect of NF-κB (such as upregulation of proapoptotic factor expression) on neutrophil apoptosis.

In summary, our results show that delay of neutrophil apoptosis with thermal injury is likely caused by the activation of PI3-kinase-PKB signaling pathway. PI3-kinase-PKB pathway activation in neutrophils after thermal injury occurs upstream of phosphorylation of Bad and NF-κB activation. The anti-apoptotic effect of NF-κB with thermal injury depends on increased expression of Bcl-xl, but not IAPs. Moreover, increased Bcl-xl expression and Bad phosphorylation, which are dependent on PI3-kinase activation, appear to operate independently to cause the delay of PMN apoptosis with burn injury.

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SIGNALING MECHANISM OF DELAYED PMN APOPTOSIS

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