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Lyn- and ERK-mediated vs. Ca^{2+}-mediated neutrophil O_2^- responses with thermal injury

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Fazal, Nadeem, Walid M. Al-Ghoul, Megan J. Schmidt, Mashkoor A. Choudhry, and Mohammed M. Sayeed. Lyn- and ERK-mediated vs. Ca^{2+}-mediated neutrophil O_2^- responses with thermal injury. *Am J Physiol Cell Physiol* 283: C1469–C1479, 2002. First published June 20, 2002; 10.1152/ajpcell.00114.2002.—We evaluated the dependency of neutrophil O_2^- production on PTK-Lyn and MAPK-ERK1/2 in rats after thermal injury. Activation of PTK-Lyn was assessed by immunoprecipitation. Phosphorylation of ERK1/2 was assessed by Western blot analysis. O_2^- production was measured by isoluminol-enhanced luminometry. Imaging technique was employed to measure neutrophil $[Ca^{2+}]_i$ in individual cells. Thermal injury caused marked upregulation of Lyn and ERK1/2 accompanying enhanced neutrophil O_2^- production. Treatment of rats with PTK blocker (AG556) or MAPK blocker (AG1478) before burn injury caused complete inhibition of the respective kinase activation. Both AG556 and AG1478 produced an ~66% inhibition in O_2^- production. Treatment with diltiazem (DZ) produced an ~37% inhibition of O_2^- production without affecting Lyn or ERK1/2 activation with burn injury. Ca^{2+} mobilization was upregulated with burn injury but not affected by treatment of burn rats with AG556. Unlike the partial inhibition of burn-induced O_2^- production by AG556, AG1478, or DZ, platelet-activating factor antagonist (PAFa) treatment of burn rats produced near complete inhibition of O_2^- production. PAFa treatment also blocked activation of Lyn. The findings suggest that the near complete inhibition of O_2^- production by PAFa was a result of blockade of PTK as well as Ca^{2+} signaling. Overall, our studies show that enhanced neutrophil O_2^- production after thermal injury is a result of potentiation of Ca^{2+}-linked and -independent signaling triggered by inflammatory agents such as PAF.

burn; rat; polymorphonuclear neutrophil; protein kinase C signaling; platelet-activating factor blockade; Lyn blockade; extracellular signal-regulated kinase 1/2 blockade

POLYMORPHONUCLEAR NEUTROPHILS (PMNs) play a frontline defensive role following inflammation and injury (38). Several previous studies have shown that blood neutrophils harvested from burn/trauma-injured hosts, in the early stages of the host injury, generate effector responses of higher magnitude than those produced by neutrophils from uninjured hosts. The excessive effector responses in the injured host may emanate from an injury-induced priming of the neutrophils in vivo. Such priming of neutrophils with burn injury could be due to one or more of the known mediators generated during the course of burn injury. These mediators bind to their receptors to initiate a series of G protein-mediated intramembrane signaling events followed by activation of Ca^{2+}-dependent and -independent signaling pathways. Whereas activation of the Ca^{2+}-dependent pathway involves the βγ-subunit of G_i protein (10), the Ca^{2+}-independent pathway is presumably mediated by G_i protein's α-subunit-linked stimulation of protein tyrosine kinases such as Lyn (3). Although the activation of these Ca^{2+}-dependent and -independent signaling pathways stimulates neutrophils to produce various effector responses (43), a prior priming of the neutrophils by inflammatory agents such as TNF-α or granulocyte macrophage colony-stimulating factor (GM-CSF) through the activation of G_i protein-independent pathways is known to result in an effector response of a much greater magnitude (17). Inflammatory mediators activating G_i protein-linked pathways may also mediate prior priming of neutrophils (40, 43).

Neutrophil activation by a number of agonists involves both Ca^{2+} signaling and sequential tyrosine phosphorylation of proteins including members of the Src family protein tyrosine kinase (PTK), such as Lyn, and mitogen-activated protein kinase (MAPK), such as ERK1/2 (13). The ERK p42/44 MAPK is a member of distinct serine/threonine kinase pathways in PMNs that relay signals from surface receptors to elicit cellular responses such as transcription, chemotaxis, and phagocytosis (4, 16, 27). Both platelet-activating factor (PAF) and formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) cause activation of p42/44 MAPK in PMNs and have been implicated in regulating multiple PMN functions, including NADPH oxidase activity and chemotaxis (16). The importance of PTK and MAPK pathways has been substantiated by the results of recent in vitro studies demonstrating that both tyrosine kinase and MAPK inhibitors abrogate several neutrophil re-

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sponses, including oxidative burst (1, 9, 23). These studies support the hypothesis that tyrosine phosphorylation via PTK and MAPK plays an active functional role in the regulation of neutrophil activation under inflammatory conditions such as trauma, burn, and sepsis.

This study followed three major lines of investigations. First, to obtain information on the molecular identity of the kinases participating in burn-induced neutrophil activation, we focused on the Src family of tyrosine kinase, Lyn, and the MAPK, ERK 1/2, which could particularly affect neutrophil oxidant production after burns (25, 41). Second, we compared the involvement of the above signaling pathways in the burn-induced modulations in O_2^- production by neutrophils. Third, we investigated whether or not Lyn and ERK1/2 signaling depended on intracellular Ca^{2+} mobilization during the burn-induced inflammatory condition. Changes in Ca^{2+} mobilization have been implicated in regulation of protein phosphorylation in PMNs, especially for the receptor-linked priming and activation of the PMN oxidase by fMLP (15, 22). To ascertain the link between the signaling pathways and oxidant production function modulation with burn injury, we treated the injured animals with specific signaling pathway inhibitors so as to prevent burn-related PMN signaling changes in vivo. In our study, we abrogated Lyn, ERK1/2, and PAF receptor activation in vivo by employing, respectively, the PTK inhibitor AG556 (6, 19, 26, 28, 36), the MAPK inhibitor AG1478 (7, 34, 44), and the PAF-16 antagonist (11, 29, 42), which previously have been shown to be efficacious through in vivo studies. We ascertained that AG556, AG1478, and PAF-16 antagonist could be administered to control animals without any gross untoward effects.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats weighing 250–275g were obtained from Harlan (Indianapolis, IN). The rats were acclimatized in the animal quarters for 3 days before their use. The care of animals was in accordance with the guidelines set forth by Loyola University Chicago Medical Center Animal Care and Use Committee.

Thermal injury protocol. The animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (45 mg/kg body wt). The hair on the animals' backs was clipped. The animals were then placed in a supine position in a plastic template that exposed 30% of the total body surface area. In the sham group, the exposed backs were immersed for 10 s in a room temperature water bath. In the burn group, full-thickness scald burns were inflicted by immersing the back of the animal in 95°C water for 10 s. Rats were quickly dried after the exposure to hot water to avoid additional injury. The animals in each group were resuscitated with a 10-ml intraperitoneal injection of normal saline. The animals were housed in cages after thermal injury and killed at 24 h postinjury after being anesthetized with pentobarbital sodium (45 mg/kg) and exsanguinated through a cardiac puncture.

Neutrophil signaling blockers. Tyrphostins (also known as AG compounds) inhibit PTKs by binding to the substrate binding site. The dose and route of administration for each of the inhibitors of neutrophil signaling were used as determined by previous studies. PTK inhibitor AG556 has been successfully used in vivo by other researchers (6, 19, 28, 36); the dose of 5 mg/kg was originally determined by Sevransky et al. (36). They also determined the half-life of AG556 (9.2 ± 4.5 min immediately and 7.4 ± 4.6 min after 6 h) in their multiorgan failure study in a canine *Escherichia coli* peritonitis model. Similarly, Brenner et al. (6) used the same dose of AG556 once a day by intraperitoneal injection in mice. Jarrar et al. (19) used the same dose of AG556 intraperitoneally in a trauma-hemorrhage model of rats. Rice et al. (34) and Busse et al. (7) used AG1478 in vivo in a rat model of pulmonary fibrosis and in retinoblastoma tumors in mice, respectively. Diltiazem at micromolar concentrations, such as are expected to prevail in the circulation of rats administered with 2 mg/kg diltiazem, can inhibit a plasma membrane receptor-gated Ca^{2+} channel (36) and thus decrease Ca^{2+} influx. Moreover, other investigators have found PAF-16 antagonist to be efficacious in in vivo studies (11, 29, 42). Tokumura et al. (42) calculated that the IC_{50} for PAF-16 antagonist was 40 nM. Our rat is ~230 g, and the volume of distribution is about 161 ml (70%). We used 10 times the IC_{50}, i.e., 400 nM; to achieve this concentration, we injected PAF-16 antagonist at the dose of 90 μg/rat.

Administration of neutrophil signaling blockers in vivo. Tyrphostins AG556 and AG1478 were purchased from Calbiochem (San Diego, CA). AG556 was originally dissolved in dimethyl sulfoxide (DMSO; 10,000 μM: 5 mg/1.49 ml) diluted with Hanks' balanced salt solution (HBSS) and given subcutaneously (5 mg/kg body wt) in a volume of 0.100 ml. AG1478 was dissolved in DMSO and injected intraperitoneally (5 mg/kg body wt) in a final volume of 1.0 ml. PAF-16 antagonist (0.35 mg/kg body wt; Calbiochem, La Jolla, CA) was dissolved in 1.0 ml of normal saline per animal and injected intravenously in a single bolus. As described in previous studies (35), the Ca^{2+} channel blocker diltiazem hydrochloride (Sigma, St. Louis, MO) was administered intravenously as a single bolus of 1.0 ml to rats at 2 mg/kg body wt. AG556, AG1478, diltiazem, or PAF-16 antagonist were administered to the animals 1 h before subjecting them to burn injury.

Preparation of blood neutrophils. At euthanization, rats were anesthetized with pentobarbital sodium (40–50 mg/kg ip) and blood (10–12 ml) was collected by cardiac puncture to heparinized syringes. Neutrophils were isolated from the heparinized blood by using the standard Ficoll-paque (Pharmacia) cell separation technique followed by dextran sedimentation and hypotonic red blood cell lysis. Neutrophils were then washed and resuspended in HBSS buffer. Neutrophil preparations routinely contained ≥95% neutrophils, as identified by the Giemsa stain, and were found to be ~98% viable by the trypan blue exclusion technique.

Stimulation of PMN with fMLP and lysate preparation. PMN obtained from sham and thermally injured rats were stimulated with fMLP (1 μM) for 180 s at 37°C. The stimulation was stopped by cell solubilization in a phosphorylation lysis buffer (PLB: 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 100 mM NaF, 1 mM $MgCl_2$, 10 mM $Na_4P_2O_7$, 200 μM Na_3VO_4, 0.5% Triton X-100, and 10% glycerol) on ice for 45–50 min. Lysates were centrifuged at 10,000 rpm for 5 min at 4°C.

Immunoprecipitation. Lysates were incubated with monoclonal antibodies to Lyn p56/p53 protein (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h, and then the mixture was incubated with protein G-Sepharose beads for another 2 h (18). These incubations were carried out at 4°C. The precipitates were washed three times in PLB without added glycerol.

In vitro kinase assay. Kinase assays were performed by using previously described methods (18). After the final wash, immune complexes were collected and washed twice with in vitro kinase buffer (50 mM Tris·HCl, pH 7.4, 10 mM $MnCl_2$, and 0.1% Triton X-100). After these washes, kinase assays were performed by incubating immune complexes first with 5 μg/ml acid-treated enolase and then for 30 min with 10 μCi [^{32}P]ATP. This incubation was carried out at room temperature (28°C). Samples were analyzed on SDS-PAGE (9%), and the proteins were transferred to the Immobilon membrane. Phosphoproteins were analyzed by autoradiography, and the intensity of the bands was assessed using densitometry.

Immunoblot analysis. Immunoblot detection of ERK1/2 was done with phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) purchased from Cell Signaling Technology (Beverley, MA). The amount of protein in each sample was quantitated with the Bio-Rad protein assay system using BSA standards. Cell lysates (5×10^6 cell equivalent/lane) were analyzed by electrophoresis on 10% polyacrylamide gels (12). The resolved proteins were electrophoretically transferred to Immobilon-P. After transfer, the membranes were first incubated at room temperature for at least 1 h in the blocking buffer consisting of 10% BSA in Tris-buffered saline (TBS; pH 7.5). Membranes were then incubated in ERK1/2 antibody at 1:200 dilution in 10 ml of primary antibody dilution buffer with gentle agitation overnight at 4°C. After the membrane had been rinsed five times with wash buffer containing 0.05% Tween 20 in TBS (pH 8), the membrane was incubated with horseradish peroxidase-conjugated (HRP) secondary antibody (1:2,000) and HRP-conjugated anti-biotin antibody (1:1,000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 h at room temperature. After five rinses with wash buffer, the transfers were color developed for 1 min by enhanced chemiluminescence (ECL; Amersham) Western blotting reagents. The relative intensity/content of an individual protein band was quantitated by densitometry (Hewlett-Packard, HP ScanJet Scanner). In each batch of experiments, blot intensities were measured in terms of densitometric units in sham and burn group rat neutrophils. Statistical analyses were carried out on values in densitometric units.

Isoluminol-enhanced luminometry. Luminometry was used to measure O_2^- production by MRX Microplate Reader (Dynex Technologies, Chantilly, VA). In each assay, 100 μl of freshly prepared 10^{-4} M isoluminol (Sigma) in HBSS was added to individual wells of white polystyrene microtiter 96-well plates and allowed to equilibrate at 37°C for 30 min. Fifty microliters of neutrophils at 5×10^6 ml^{-1} were then added, and the plate was incubated at 37°C. After a 15-min equilibration period, background readings were recorded on an MLX analyzer every 5 min for 20 min until a steady value was obtained. The cells were then stimulated with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml). The kinetics of O_2^- production were recorded every 2 min over a 60-min period using MLX software. The results were expressed in relative luminescence units (RLU). Each experiment was performed in triplicate, and control wells with isoluminol alone and isoluminol with cells were included in each experiment.

Fura 2 Ca^{2+} imaging. Neutrophils suspended in HBSS were loaded with 10 μM fura 2-AM (Molecular Probes, Eugene, OR) for 1 h at room temperature. A drop of neutrophil suspension (100 μl) was placed on a 1-μm-thick coverslip and examined with the ×40 oil-immersion objective of an inverted Nikon Microscope. Computerized fura 2 ratio imaging was then performed with the aid of MetaFluor software (Series 4.5; Universal Imaging, West Chester, PA) and the associated hardware, including a SenSys charge-coupled device camera (Photometrics) and a Metaltek shutter. With the use of this setup, real-time fluorescence images were generated by exposing cells to alternating 340- and 380-nm excitation wavelengths and automatically collecting associated fura 2 emissions through a 505-nm band-pass filter (24). The following steps were followed for each cell sample. First, cells were focused for optimal fluorescence fura 2 signal and background correction was set up based on an adjacent blank part of the coverslip. Second, a field of cells was chosen, and its 340-nm, 380-nm, and ratio images were optimized. Third, a computer-controlled series of 30 subsequent images was acquired at 20-s intervals. Of the 30-point image series, the first 5 images were kept as baseline images, because cellular stimulation with 1 μM fMLP was administered in vitro after the 5th point (100 s). Fourth, acquired data were then used to generate 340/380 ratio images and curves for analysis and presentation.

Statistical analysis. All statistical analyses were carried out using the Statistical Package, Social Sciences Software Program (SigmaStat version 2.0; SPSS, Chicago, IL). To determine inter- and intragroup differences between variables, a one-way repeated-measures ANOVA, followed by a pairwise multiple comparison procedure (Tukey's post hoc test), was performed. The statistical analysis of the different experimental groups included the comparison of sham-burned, *day 1* postburn, and inhibitor-treated burned rats in *n* animals. Statistical significance was assigned at *P* values <0.05.

RESULTS

Burn-induced activation of PTK-Lyn. In an initial series of experiments, we examined whether PTK-Lyn is modulated by burn-induced PMN activation. Figure 1 shows immunoblot and blot densitometric analyses of

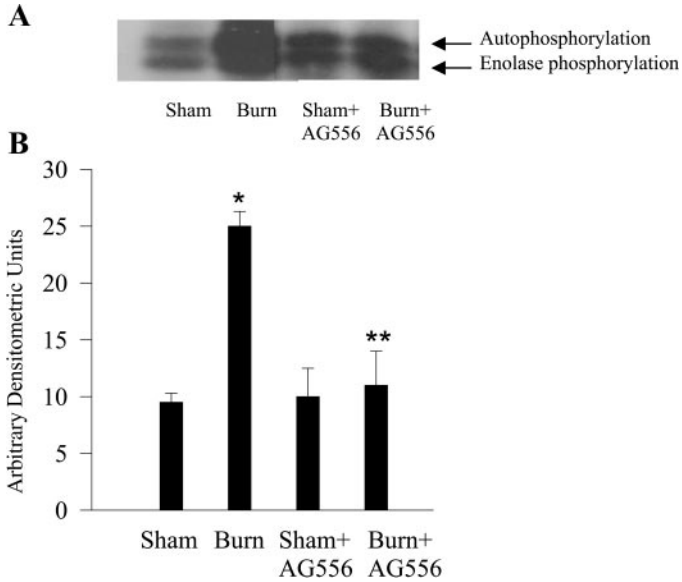

Fig. 1. *A*: representative blot showing formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP; 1 μM)-stimulated PTK-Lyn autophosphorylation and enolase phosphorylation. *Lane 1*, sham rats; *lane 2*, burn rats; *lane 3*, sham rats treated with PTK inhibitor AG556; *lane 4*, burn rats treated with AG556. *B*: densitometric analyses of PTK-Lyn blots from 3 experiments. Values represents means ± SE ($n = 9$ animals) in arbitrary densitometric units. *$P < 0.05$, sham vs. burn. **$P < 0.5$, burn vs. burn + AG556.

Lyn autophosphorylation of neutrophils as well as the ability of Lyn to phosphorylate other proteins (kinase activity), evaluated by examining phosphorylation of enolase. There was no detectable Lyn autophosphorylation or its kinase activity in unstimulated neutrophils from sham or burn animal groups (data not shown). Stimulation of neutrophils with fMLP (1 μM) from *day 1* postburn or sham rats with fMLP caused significant elevations in both Lyn autophosphorylation and the kinase activity. The fMLP-caused autophosphorylation and kinase activity as determined by densitometric analysis were 2.5 times higher in the burn animals than in the sham group (Fig. 1).

We examined the ability of the tyrosine kinase inhibitor (AG556) to inhibit the burn-related activation of Lyn autophosphorylation and Lyn kinase activity in PMNs. The pretreatment of rats with PTK blocker AG556 significantly downregulated Lyn activity in the burn animal neutrophils (Fig. 1).

Effect of PTK inhibitor AG556 on PMN O_2^- production. O_2^- production was measured in neutrophils before and after their stimulation with PMA (100 ng/ml) by isoluminol-enhanced luminometry. Figure 2 shows O_2^- production as a function of time in neutrophils from sham and burn (*day 1* postburn) rats and the effect of the addition of exogenous superoxide dismutase (SOD) on burn and sham rat neutrophils. In neutrophils from the burn rat group, PMA caused an approximately four- to fivefold increase in the initial rate of O_2^- production compared with the sham group. The rate increases were such that a peak value of O_2^- production was observed within 9–12 min, after which time O_2^- production declined and returned to the prestimulation level by 60 min (Fig. 2*A*). Peak O_2^- production in the burn group (2.3 ± 0.5 RLU) was significantly higher ($P < 0.05$) than in the sham group (0.5 ± 0.2 RLU). SOD completely eliminated extracellular release of O_2^- in both the sham and burn rat neutrophils as determined by isoluminol chemiluminescence (Fig. 2*B*). This experiment ascertained the release of O_2^- occurring into the extracellular milieu.

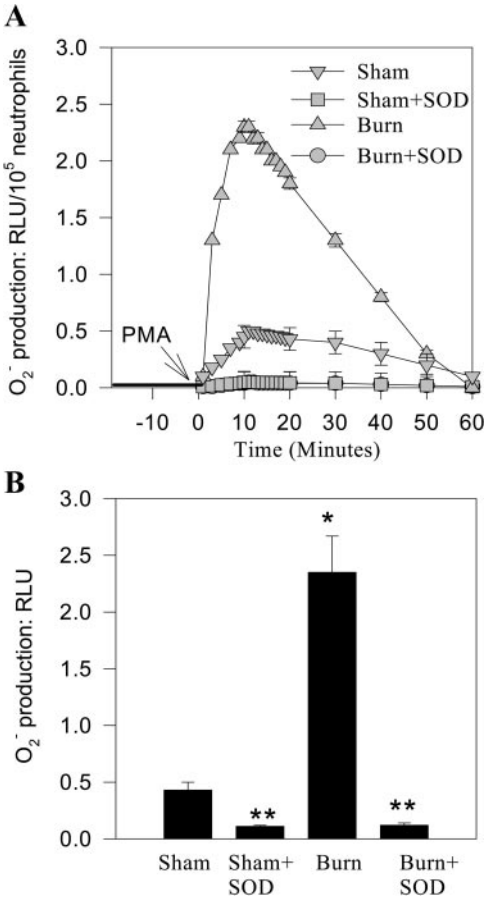

Fig. 2. *A*: chemiluminescence of O_2^- produced by blood polymorphonuclear neutrophils (PMNs) following PMA (100 ng/ml) stimulation (arrow) at *time 0* in sham and burn rats with and without superoxide dismutase (SOD). *B*: bar graph showing means ± SE, in relative luminescence units (RLU), from >12 sham or burn rats. *$P < 0.05$, sham vs. burn. **$P < 0.05$, burn vs. burn + SOD.

To evaluate the potential contribution of the Lyn activity to the O_2^- production response in PMNs, animals were treated with PTK blocker (AG556) before they were subjected to burn injury. O_2^- production was then measured *day 1* postburn in neutrophils before and after their stimulation with PMA (100 ng/ml). In sham rat neutrophils, O_2^- production following PMA stimulation remained relatively stable over a period of 10–12 min at the level of 0.5 ± 0.2 RLU. The O_2^- production by burn (*day 1* postburn) rat neutrophil increased to 2.7 ± 0.3 RLU over the 12-min period following PMA addition. AG556 treatment of burn animals downregulated the O_2^- production response to a significantly lower level (1.1 ± 0.2 RLU). The inhibition of O_2^- production implicates a role of Lyn activation in burn-induced superoxide generation (Fig. 3*A*).

Effect of PTK inhibitor AG556 on PMN intracellular Ca^{2+} mobilization. Figure 4*A* shows digitized Ca^{2+} images from a representative sham, burn, and burn-treated rat, and Fig. 4*B* demonstrates digitized fluorescence ratios (F_{340}/F_{380}) representing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) obtained in neutrophils. The frequency of cells showing pseudocolors of blue and green, representing unstimulated resting cells, seemed to be comparable in the sham, burn, and AG556-treated burn animals. Upon stimulation with fMLP, a significant number of sham rat neutrophils transformed into cells with pseudocolors in the yellow and red range, corresponding to an image ratio around 1.3. The fMLP stimulation of burn rat neutrophils caused a transformation into the pseudocolor red and white range, corresponding to image ratios >2. Imaging of individual neutrophils confirmed that fMLP-induced elevations in the burn group were markedly higher than for cells in the sham group. Figure 4*B* shows the elevation in the digitized fluorescence ratios as a function of time following fMLP stimulation of neutrophils from sham and burn rats with and without AG556 treatment. The Ca^{2+} image analyses did not show a measurable effect of treatment with AG556 on the basal $[Ca^{2+}]_i$ or fMLP-mediated $[Ca^{2+}]_i$ elevation in

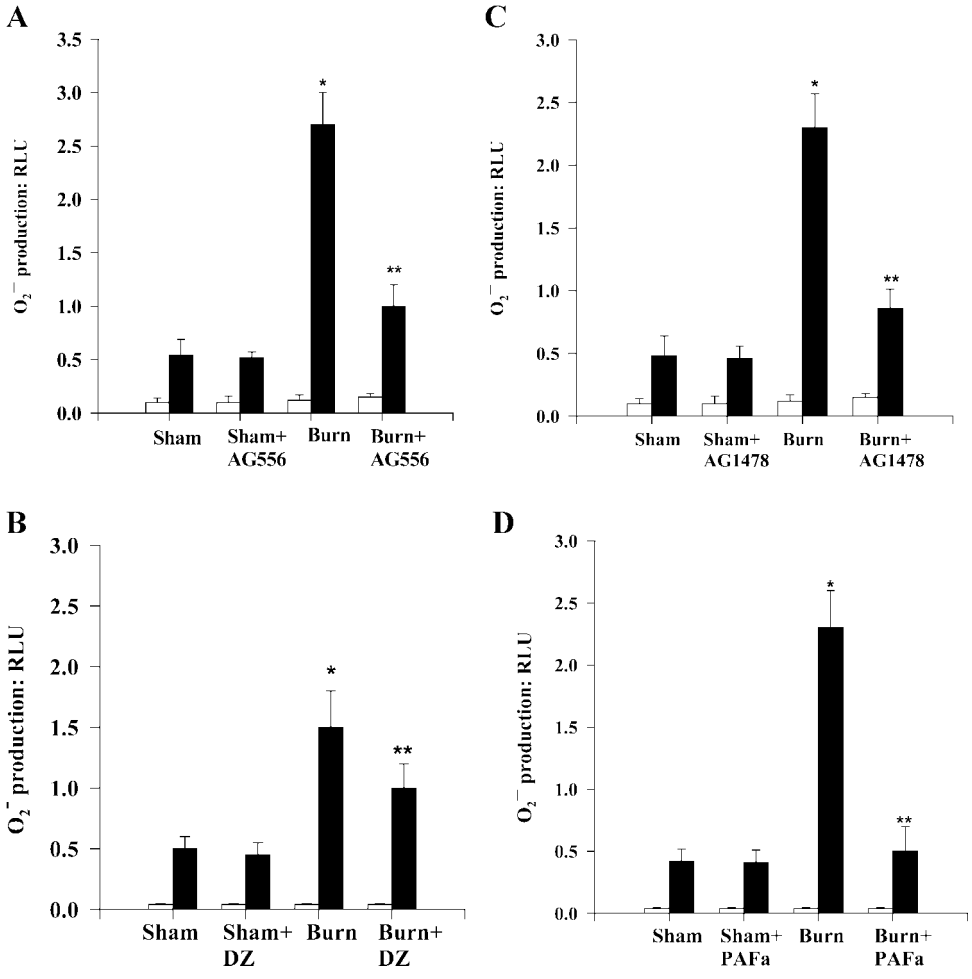

Fig. 3. O_2^- production by PMA-stimulated PMNs (filled bars) isolated from sham or burn rats with or without treatment with PTK inhibitor AG556 (*A*), diltiazem (DZ) (*B*), AG1478 (*C*), or PAF-16 antagonist (PAFa) (*D*). Values represent means ± SE (n = 12 animals). Unstimulated PMNs (open bars) produced insignificant levels of O_2^-. *$P < 0.05$, sham vs. burn. **$P < 0.05$, burn vs. burn + treatment.

the sham animals. These analyses in burn rat neutrophils also did not show any effect of AG556 treatment on fMLP-mediated $[Ca^{2+}]_i$ responses.

Effect of Ca^{2+} channel blocker on burn-induced Lyn activation. To determine the contributions of intracellular Ca^{2+} mobilization in burn-induced upregulation of Lyn, we treated the animals in vivo with the Ca^{2+} channel blocker diltiazem and determined Lyn activation in PMNs. The dose of diltiazem was chosen as reported previously to block Ca^{2+} mobilization in the neutrophil (35). The results indicated that Lyn autophosphorylation as well as enolase phosphorylation by Lyn in neutrophils from diltiazem-pretreated burn rats (*day 1* postburn) was unaffected (Fig. 5).

Role of Ca^{2+} channel blocker in burn-induced PMN O_2^- production. To determine the role of Ca^{2+} mobilization in burn-induced enhanced PMN O_2^- production, we treated the animals with diltiazem before they were subjected to burn injury (Fig. 3*B*). O_2^- production was measured in neutrophils before and after their stimulation with PMA (100 ng/ml). The sham neutrophil O_2^- production (0.5 ± 0.1 RLU) following PMA stimulation was not significantly inhibited by diltiazem treatment. O_2^- production in the untreated burn animal groups was significantly higher (1.5 ± 0.3 RLU) than in the sham group. There was a significant reduction ($P <$ 0.05) of O_2^- production in diltiazem-treated burn animal PMNs (1.0 ± 0.2 RLU) compared with the untreated burn group. This partial inhibition of O_2^- production by diltiazem treatment of rats implicated a Ca^{2+}-independent pathway contributing to the maximal O_2^- production response obtained by stimulation of cells with PMA.

Phosphorylation/activation of p42/44 MAPK in burn-induced PMNs. One of the early events of fMLP signal transduction in neutrophils is the phosphorylation of intracellular proteins, including ERK1/2 MAPK protein. The activation of ERK protein follows dual phosphorylation of discrete threonine and tyrosine residues. We investigated activation of p42/44 MAPK by fMLP via Western blot analyses of whole cell extracts with antibodies to dually phosphorylated (Thr^{202}/Tyr^{204}) p42/44 MAPK (8). PMNs were stimulated with 1 μM fMLP for 5 min, and whole cell lysates were subjected to SDS-PAGE and immunoblotting. Similar aliquots of PMN samples taken from different experimental groups were probed for p42/44 kinase to examine differential phosphorylation patterns. No dual phosphorylation was detected in the PMNs without stimulation with fMLP (data not shown). Phosphorylation of ERK1/2 (Fig. 6*A*) was significantly ($P < 0.05$)

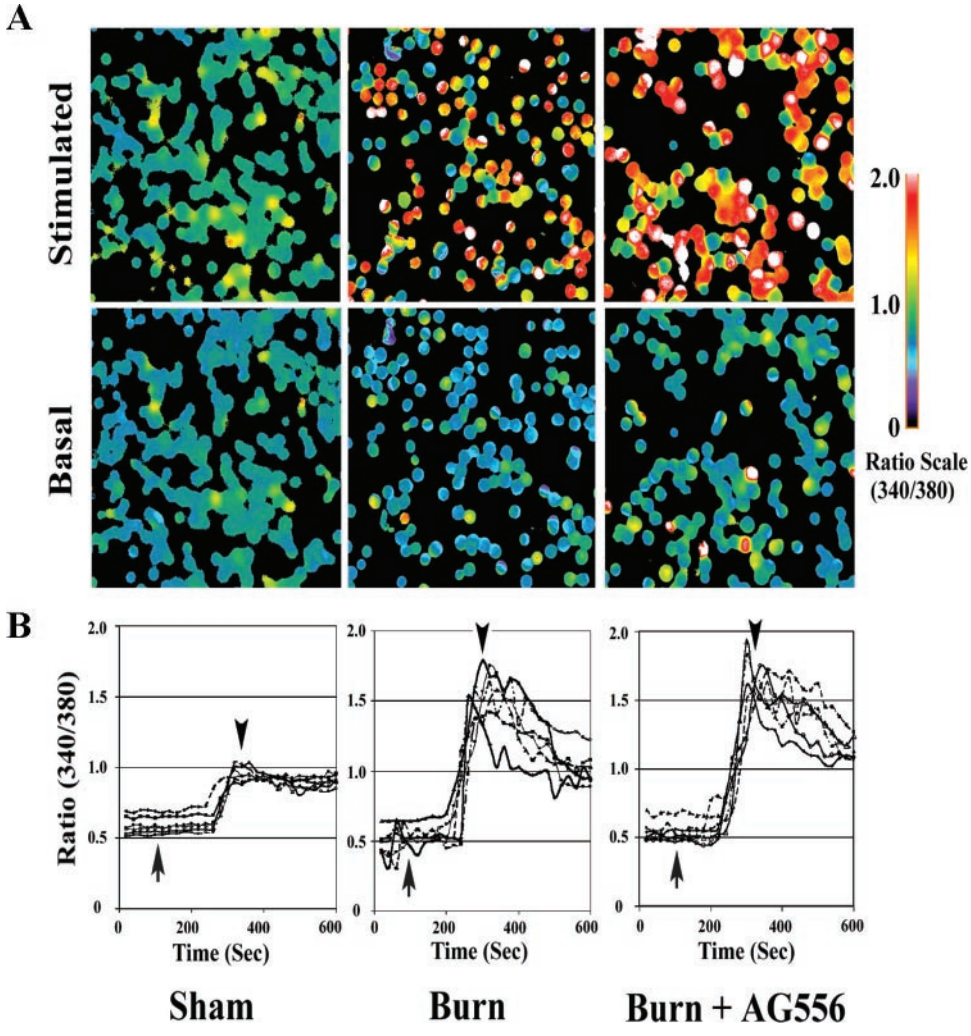

Fig. 4. Comparison of intracellular Ca^{2+} mobilization response in PMNs from sham (*left*), burn (*middle*), and burn + AG556-treated rats (*right*). All intracellular Ca^{2+} responses were determined at basal (unstimulated) level and after stimulation of PMN with fMLP (1 μM). *A*: pseudocolor digitized cellular images of 340/380 fluorescence ratio levels are shown in basal (*bottom*) or stimulated (*top*) conditions. Basal images represent snap shots of neutrophil intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) before fMLP stimulation, and stimulated images depict snapshots of peak neutrophil $[Ca^{2+}]_i$ after fMLP activation. In the basal images, unstimulated neutrophils have low blue-green ratio levels (0.5–0.75 on ratio scale, *far right*). In the stimulated images, stimulated (1 μM fMLP) burn (*middle*) and burn + AG556 neutrophils (*right*) exhibit ratio values in the high red-white ratio range (1.5–2.0 on ratio scale, *far right*); sham neutrophils (*left*) exhibit ratio values in the green-yellow ratio range (0.75–1.0). *B*: ratios (340/380) of fluorescence intensities emitted from fura 2 when excited at 340 (Ca^{2+} bound) and 380 nm (Ca^{2+} free), corresponding to $[Ca^{2+}]_i$ levels, plotted as a function of time. For each experimental condition, traces of 340/380 ratios are shown over a 10-min period for 6 different experiments in which neutrophils responded to fMLP stimulation. Arrows indicate the time point (100 s) when 1 μM fMLP was added. Basal ratio values before fMLP administration were comparable for sham, burn, and burn + AG556 group samples and ranged from 0.5 to 0.75, indicating relatively similar group $[Ca^{2+}]_i$ levels. Notably, however, peak fMLP response (arrowheads) in burn + AG556 group (ratio range 1.5 to 2.0) was comparable to that in burn alone (1.5 to 1.8), which was considerably higher than in sham group (0.75 to 1.0).

elevated on *day 1* postburn neutrophils compared with phosphorylation of this protein in neutrophils from sham animals. Identical aliquots from the same PMNs employed above were probed with a monoclonal antibody to p42/44 MAPK. No differences in the amounts of immunoreactivity were visualized among any of the samples (data not shown).

Attenuation of burn-induced phosphorylation/activation of the p42/44 MAPK by AG1478. To determine whether p42/44 block administered to animals specifically affected the burn-induced activation of the MAPK, we pretreated animals with these agents before performing the sham and burn procedures. PMNs from sham and burn animals with and without MAPK blocker treatments were stimulated with fMLP. The treatment of sham animals with AG1478 (ERK blocker) did not effect ERK1/2 expression in PMNs from sham animals. However, AG1478 treatments of burn animals significantly downregulated ERK1/2 activation in burn animal PMNs (Fig. 6*A*).

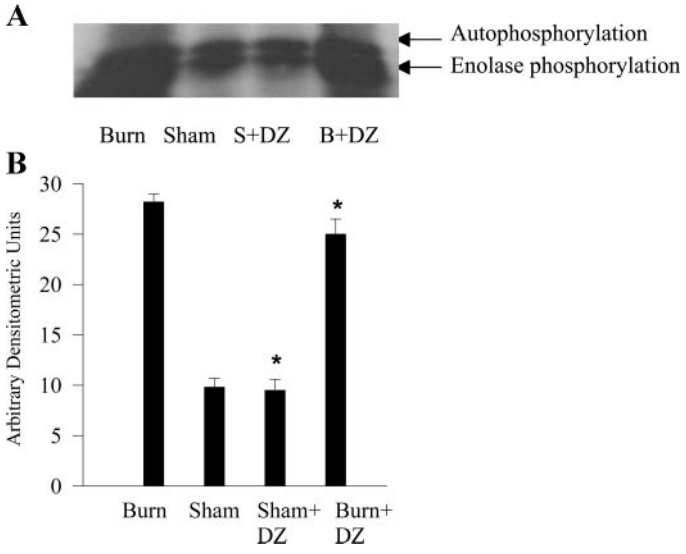

Fig. 5. *A*: representative blot showing fMLP (1 μM)-stimulated PTK-Lyn kinase autophosphorylation (upper band) and enolase phosphorylation by PTK-Lyn (lower band) in neutrophils from sham or burn rats with or without treatment with DZ. *B*: densitometric analyses of PTK-Lyn blots from 2 experiments. Values represents means ± SE (n = 6 animals) in arbitrary densitometric units. *$P > 0.01$, sham vs. sham + DZ or burn vs. burn + DZ.

Effect of PTK inhibitor AG556 on burn-induced phosphorylation/activation of the p42/44 MAPK. To determine whether blockade of a signal upstream to MAPK, i.e., a tyrosine kinase (3), could cause the inhibition of MAPK ERK1/2, we treated the animals with PTK blocker AG556 and assayed PMNs for the expression of ERK1/2 activity. We found that the inhibitor of upstream tyrosine kinase signaling, AG556, blocked burn-induced elevations of ERK1/2 activity (Fig. 6*B*).

Prevention of burn-induced enhancement in PMN O_2^- production with AG1478. We investigated whether ERK1/2 inhibition could lead to modulation of O_2^- production by PMNs from burn-injured rats. Burn and sham animals were treated with the ERK MAPK blocker (AG1478) before they were subjected to burn injury. O_2^- production was measured in neutrophils before and after their stimulation with PMA (100 ng/ml). The sham animal neutrophil O_2^- production (0.5 ± 0.1 RLU) following PMA stimulation was not altered ($P > 0.05$) with treatment of the animal with AG1478 (ERK blocker). O_2^- production response in the untreated burn animal groups was within the range of 2.3 ± 0.2 RLU (Fig. 3*C*). However, there was a significant decrease ($P < 0.05$) in O_2^- production following deactivation of ERK1/2 in the burn group. This substantial blockade of O_2^- production shows a role of the ERK kinase pathway in O_2^- production by PMNs of burn animals.

Effects of Ca^{2+} channel blocker on burn-induced phosphorylation of p42/44 MAPK. To investigate the role of blocking intracellular Ca^{2+} release on burn-induced activation of neutrophil MAPK, we treated the animals with the Ca^{2+} channel blocker diltiazem. Phosphorylation of p42/44 MAPK with fMLP stimulation of PMNs was unaffected by diltiazem pretreatment of the burn animals (Fig. 7). These results suggest that phosphorylation/activation of ERK MAPK was independent of intracellular Ca^{2+} mobilization after burn injury.

Effect of PAF-16 antagonist on Lyn. Figure 8 shows immunoblot and blot densitometric analyses of the Lyn autophosphorylation of neutrophils as well as the ability of Lyn to phosphorylate other proteins (kinase activity) as evaluated by examination of phosphorylation

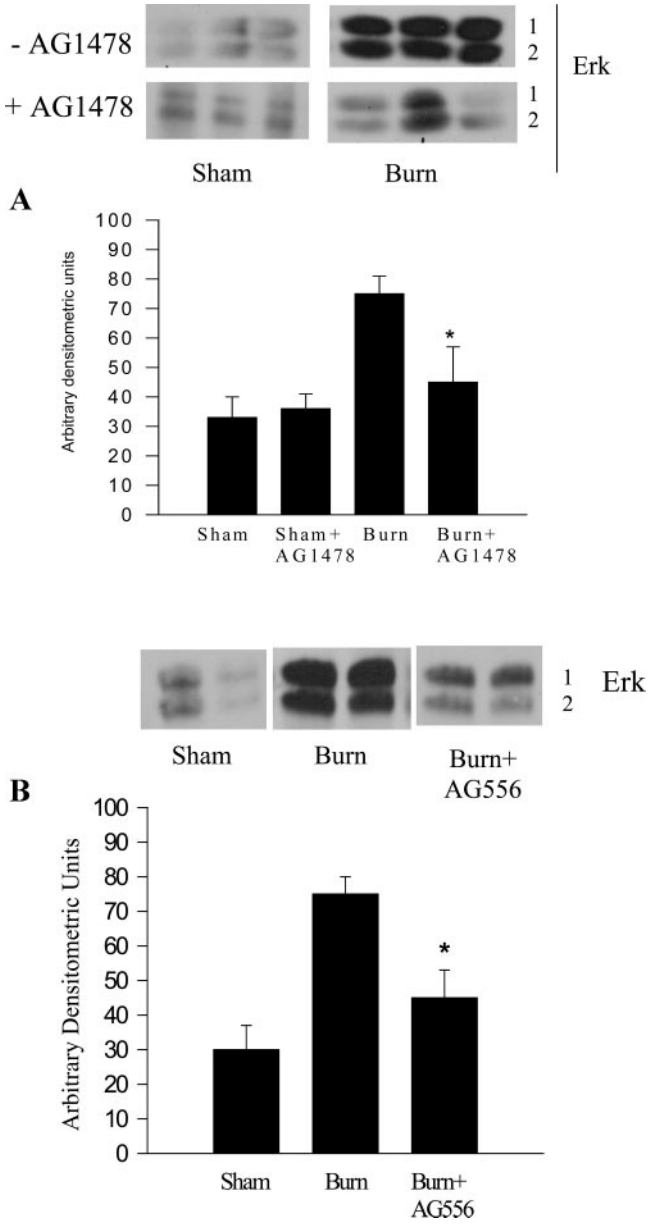

Fig. 6. *A*: representative Western blot shows fMLP (1 μM)-stimulated ERK1/2 activation (phosphorylation) in neutrophils from sham or burn rats treated with MAPK blocker AG1478. Bar graph indicates densitometric analyses of ERK1/2 blot with values shown as means ± SE (n = 5 animals). *$P < 0.05$, burn vs. burn + AG1478. *B*: representative Western blot shows fMLP (1 μM)-stimulated ERK1/2 activation (phosphorylation) in neutrophils from sham or burn rats treated with PTK blocker AG556. Bar graph indicates densitometric analyses of ERK1/2 blot with values shown as means ± SE (n = 4 animals). *$P < 0.05$, burn vs. burn + AG556.

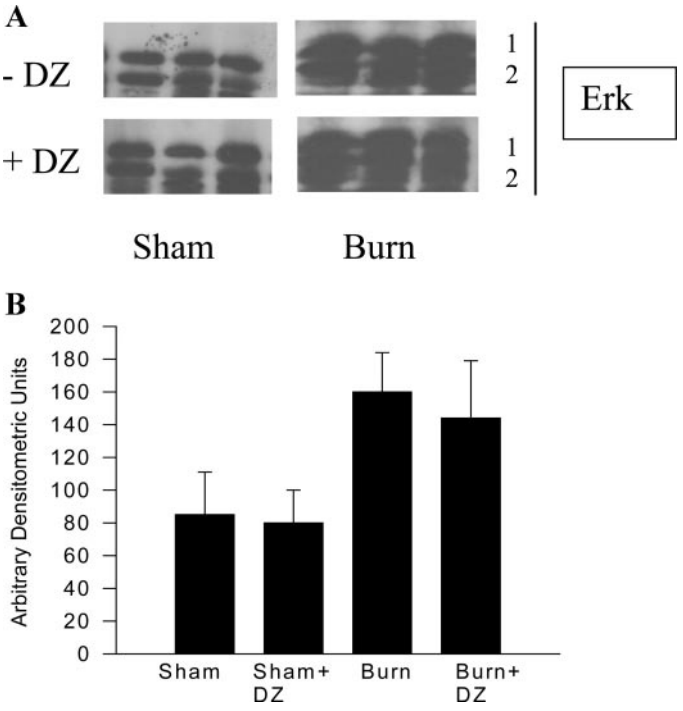

Fig. 7. *A*: representative Western blot shows fMLP (1 μM)-stimulated ERK1/2 activation (phosphorylation) in neutrophils from sham or burn rats treated with Ca^{2+} channel blocker DZ. *B*: densitometric analyses of ERK1/2 blot with values shown as means ± SE (n = 4 animals). *$P > 0.05$, burn vs. burn + DZ.

of enolase. There was no detectable Lyn autophosphorylation or its kinase activity in unstimulated neutrophils from sham or burn animal groups (data not shown). The stimulation of neutrophils from *day 1* postburn or sham rats with fMLP caused significant elevations in both Lyn autophosphorylation and the kinase activity. The fMLP stimulation of both autophosphorylation and kinase activity was five times higher in the burn animals than in the sham group as determined by densitometric analysis (Fig. 8). The pretreatment of rats with PAF-16 antagonist (0.35 mg/kg) had no significant effect on Lyn autophosphorylation by neutrophils in the sham animal group. However, Lyn autophosphorylation as well as enolase phosphorylation activity in neutrophils from PAF-16 antagonist-pretreated burn rats (*day 1* postburn) was significantly ($P < 0.05$) lower than that in the untreated *day 1* postburn rat neutrophils. The autophosphorylation and kinase activities in the PAF-16 antagonist-pretreated *day 1* postburn rats were comparable to those in the neutrophils from sham groups.

Prevention of burn-induced enhancement in PMN O_2^- production with PAF-16 antagonist. We investigated whether PAF inhibition could lead to modulation of O_2^- production by PMNs from burn-injured rats. Burn and sham animals were treated with PAF-16 antagonist before they were subjected to burn injury. O_2^- production was measured in neutrophils before and after their stimulation with PMA (100 ng/ml). The sham animal neutrophil O_2^- production (0.5 ± 0.1 RLU) following PMA stimulation was not altered ($P > 0.05$) with treatment of the animals with PAF-16 antagonist. O_2^- production response in the untreated burn animal groups was within the range of 2.3 ± 0.2 RLU (Fig. 3*D*). However, there was a near complete blockade of burn-induced O_2^- production ($P < 0.05$) following PAF-16 antagonist treatment in the burn group. This blockade of O_2^- production shows a significant role of PAF in O_2^- production by PMNs of burn animals.

DISCUSSION

The present study demonstrates an enhancement in O_2^- production along with a role of Lyn PTK and ERK1/2 MAPK by blood neutrophils isolated from the *day 1* postburn rats. Because blockade of burn-induced PTK-Lyn and MAPK-ERK upregulation also prevented O_2^- production enhancement in the neutrophils, there seems to be a causal relationship between Lyn/ERK signaling and O_2^- production after burn. Our findings also suggest that Lyn and ERK signaling is activated with burn without an involvement of Ca^{2+} signaling. This study also confirms an independent role of Ca^{2+} signaling in the PMN oxidant response with burn, as was shown previously (11, 35). Overall, it appears that the burn-induced oxidant response in PMNs is a result of activation of a Ca^{2+}-independent Lyn PTK and ERK1/2 MAPK pathway as well as a "Ca^{2+}-linked" signaling mechanism. The upregulation of the Ca^{2+}-independent PTK/ERK pathway with burn may be involved in the priming of neutrophils during the course of thermal injury. Previous studies have supported a priming role of the PTK/ERK pathway in the oxidant production response of PMNs (20).

A contribution of our study is that PTK and ERK1/2 blockers, which have hitherto been utilized primarily

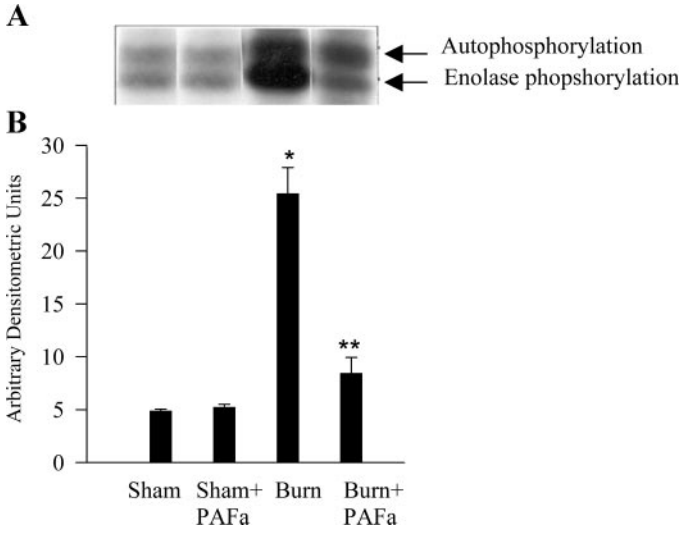

Fig. 8. *A*: representative blot showing fMLP (1 μM)-stimulated PTK-Lyn kinase autophosphorylation and enolase phosphorylation by PTK-Lyn in neutrophils from sham or burn rats with or without treatment with PAFa. *Lane 1*, sham rats; *lane 2*, sham rats treated with PAFa; *lane 3*, burn rats; *lane 4*, burn rats treated with PAFa. *B*: densitometric analyses of PTK-Lyn blots with values shown as means ± SE (n = 8 animals) in arbitrary densitometric units. *$P <$ 0.05, sham vs. burn. **$P < 0.05$, burn vs. burn + PAFa.

as agents efficient in blocking signaling pathways in cells under in vitro conditions, can be administered to animals to produce selective signaling blockade in vivo. Such administrations of blockers to produce cell signaling modulations in vivo could modulate various, and perhaps all, cell systems in the body. Although this possibility cannot be ruled out, it is reasonable to postulate that blockers would exert inhibitory effects only on cell systems in which the targeted signaling pathways would be selectively activated under inflammatory conditions such as prevailing after burns. There is evidence that PMNs are among the cell systems in which MAPKs (ERK and p38) are activated during early stages after burn injury (2).

We found that both Lyn autophosphorylation and enolase phosphorylation by Lyn with burn injury was blocked completely by the PTK blocker AG556. This finding indicated a maximal inhibitory effect on this Src kinase pathway activated during burn injury. Because we also found that PMN O_2^- upregulation with burn could be blocked ~66% by that dose of AG556, it would appear that the PTK pathway alone controlled more than half of the burn-induced O_2^- production. Lyn blockade apparently suppressed PKC, because O_2^- production in these experiments was performed after PMN stimulation with PMA, which is known to activate PKC directly (14, 21, 39). PKC is upregulated in the early period after burn (35). PKC activation presumably occurs subsequent to PTK activation in PMNs as has been shown in several previous studies (45). Our previous studies indicated that Ca^{2+} mobilization plays an important role in PKC upregulation in the burn rat neutrophils (35). In this study, we found no effect of PTK blocker AG556 on the Ca^{2+} signal quantified via digital Ca^{2+} image analysis in PMNs. We also tested whether blocking intracellular Ca^{2+} mobilization could affect upregulated PTK-Lyn. The treatment of animals with diltiazem at the maximal effective dose (2 mg/kg) did not show any effect on upregulated Lyn activation. We conclude that Ca^{2+} and PTK-Lyn pathways are activated in parallel and independently of Ca^{2+} signaling to upregulate O_2^- production in PMN in burn injury. An independent role of Ca^{2+} in regulating burn-induced O_2^- production was also evident, because treatment of animals with diltiazem produced a significant inhibitory effect on PMN oxidant response. Whereas AG556 resulted in an ~66% inhibition of the O_2^- response, diltiazem treatment inhibited O_2^- production by ~37%. Thus PMN O_2^- production enhancement with burn injury could be due to a greater contribution of the Ca^{2+}-independent PTK pathway than that of the Ca^{2+}-dependent signaling pathway, suggesting that the overall enhancement of O_2^- production in burn rat PMN is the result of both a Ca^{2+}-independent "priming" of neutrophils and activation of a Ca^{2+}-dependent pathway, both of which upregulate oxidant production by the downstream PKC signaling. Regarding the absence of an effect of diltiazem on the O_2^- response by sham rat neutrophils, we can surmise that in the sham group PMA caused a stimulation of O_2^-, but to a level that was not sensitive to blockade of intracellular Ca^{2+} mobilization. This may be due to a potential Ca^{2+} mobilization-independent PMA action on O_2^- in the sham group. The possibility that Ca^{2+}-independent O_2^- production was predominant in the burn group also was indicated.

Our findings that Lyn protein kinase is essentially involved in fMLP-induced upregulation of O_2^- production after burn are consistent with previous observations in which PTKs, including Lyn and Hck, were shown to upregulate O_2^- and CD11b/CD18 response in human neutrophils (33, 37). Studies have also shown that heterotrimeric G_i protein-coupled receptors activate the Ras-dependent MAPK cascade via activation of both Syk and Src family kinases (22, 32, 33). Lyn-dependent Shc phosphorylation may be one of the mechanisms leading to Ras activation in neutrophils stimulated by fMLP and other chemoattractants (8, 31). However, an alternative tyrosine kinase-independent mechanism has been suggested to mediate fMLP-induced Ras activation in neutrophils (46). Our finding of PTK-Lyn activation with burn could be interpreted to result from either a G_i protein-independent or G_i-linked signal pathway. A G_i protein-independent PTK signaling has been shown to be activated in neutrophils, primed by mediators such as TNF-α and GM-CSF (17). Whether PTK activation occurs in a G_i-dependent or -independent manner, it does seem to proceed via Ca^{2+}-independent signaling pathway.

The upregulation of PMN O_2^- production in burn rats presumably results from actions of mediators released during the early period after burn. Such mediator(s) could cause a priming of neutrophils as well as their subsequent activation. Previous studies support a role of PAF in both priming of neutrophils and the activation of preprimed neutrophils (29, 30). Such actions of PAF during the early course of burn injury could lead to a greater magnitude of PMN O_2^- response than would occur with activation of a population of presumably unprimed neutrophils. Thus PAF may well serve as one of the mediators responsible for the potentiated/enhanced O_2^- production by PMNs of burn-injured rats. Our finding of a near complete abrogation of burn-induced PMN O_2^- production after PAF antagonist treatment supports the suggestion that endogenous PAF release could contribute substantially to the PMN O_2^- response. The blockade of PAF actions on O_2^- production was clearly more pronounced than that obtained by blocking PTK-Lyn/MAPK-ERK or Ca^{2+} signaling pathways. It is likely that burn-induced PAF expression/release contributes to O_2^- response via both priming and activation of preprimed neutrophils. The priming of neutrophils alone could be achieved via Ca^{2+}-independent pathways such as the PTK-Lyn/MAPK-ERK, as shown after PMN priming with TNF-α or GM-CSF (17). Activations of PMNs could be achieved via mediators such as fMLP, C5a, leukotriene B_4, and/or PAF (33) subsequent to priming of TNF-α or GM-CSF. PAF action on PMN proceeding through its seven-transmembrane domain receptor could involve both the turn-on of the Ca^{2+} pathway (5) and the tyrosine phosphorylation of cellular proteins (10) such

as phosphatidylinositol 3-kinase (PI3K), MAPKs, and phospholipases A_2, C, and D, all of which play a role in the O_2^- production response (3). A recent study has also shown that PAF activates PMN MAPK-ERK1/2 through tyrosine kinase and PI3K (15). Precise identity of the tyrosine kinase has, however, remained unknown. Our study suggests that PAF-mediated actions on burn rat neutrophils involve PTK-Lyn. Our previous studies had determined that PAF antagonism in burn rat neutrophils abrogated Ca^{2+} signaling pathway (11). Thus, taken together, our findings support the concept that PAF actions during burn could activate both Ca^{2+}-independent PTK-Lyn and MAPK-ERK pathways and Ca^2-linked pathways. The actions of PAFs may involve both Ca^{2+}-independent and Ca^{2+}-dependent pathways to allow for enhanced O_2^- response.

In summary, our studies demonstrate that Ca^{2+}-independent activations of PTK-Lyn and MAPK-ERK1/2 play an important role in the enhanced O_2^- production by neutrophils following thermal injury. Whereas the activation of Ca^{2+}-linked signaling contributed to approximately one-third of the burn-enhanced neutrophil O_2^- production, the Ca^{2+}-independent Lyn and ERK1/2 activations seem to be responsible for approximately two-thirds of the enhanced O_2^- response. The present study also provides insights into the role of PAF in the activations of the neutrophil signaling pathway and the O_2^- generation response with burn injury in vivo.

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