Signaling mechanisms of elevated neutrophil O₂⁻ generation after burn injury

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SABEH, Farideh, Philip Hockberger, and Mohammed M. Sayeed. Signaling mechanisms of elevated neutrophil O₂⁻ generation after burn injury. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R476–R485, 1998.—A full skin thickness burn injury was produced in anesthetized rats by exposing 25% of total body surface area to 98°C water for 10 s. Sham (exposed to 37°C water) and burn rats were killed 1, 3, 7, or 10 days later. The role of Ca²⁺ signaling and Ca²⁺-related protein kinase C (PKC) activation in neutrophil O₂⁻ generation was ascertained by evaluating the effect of treatment of the rats with the Ca²⁺ entry blocker, diltiazem. There was an overt enhancement of O₂⁻ generation by polymorphonuclear leukocytes from burn rats on days 1, 3, and 7 postburn, with the peak release occurring on day 3 postburn. O₂⁻ generation comparable to the sham was noted on day 10 after the burn. O₂⁻ releases on days 1, 3, and 7 postburn were accompanied by marked elevation of Ca²⁺ and PKC responses. Like the O₂⁻ release, intracellular Ca²⁺ concentration ([Ca²⁺]) response on day 10 after burn was suppressed to levels found in the sham group. The treatment of burn rats with diltiazem prevented the upregulation of both [Ca²⁺] and PKC responses as well as O₂⁻ generation in neutrophils in rats on days 1, 3, and 7 after the burn. Because previous studies have shown that increases in [Ca²⁺] precede O₂⁻ generation and degranulation, our results suggest that neutrophil O₂⁻ release enhancement in the early stages after burn injury (e.g., days 1-7 postburn) results from an overactivation of the Ca²⁺ and PKC signaling pathways. The heightened O₂⁻ generation during the early burn injury phase might play a role in tissue damage in one or more of host organs.

thermal injury; rat; intracellular calcium signaling; protein kinase C activation; reduced nicotinamide adenine dinucleotide phosphate oxidase; intracellular calcium antagonist

BURN INJURY AND TRAUMA can lead to remote tissue damage and multiple organ failure (2, 16, 26, 43, 59). Polymorphonuclear leukocytes (PMNs) presumably play a role in mediating these responses by adhering to endothelial cells in target tissue or organ and releasing reactive oxygen species (ROS) and hydrolytic enzymes (27, 50, 54, 55). The release of the ROS begins with the activation of NADPH oxidase, an intramembrane electron transport chain that reduces oxygen to superoxide anion, O₂⁻ (3–5). NADPH oxidase activity is triggered by a variety of soluble and insoluble agonists such as N-formylmethionyl-leucyl-phenylalanine (fMLP), phospholipid, and opsonized particles (44, 48). Further reduction of O₂⁻ leads to the formation of H₂O₂ and its interaction with other compounds, resulting in the formation of ·OH, HClO⁻, and other toxic metabolites that can play a role in not only bacterial killing but also host tissue damage. There are numerous reports on the reactivity and toxicity of the ROS formed from the superoxide anion (O₂⁻) during the course of inflammatory conditions, including burns (1, 14, 18, 20, 33, 47, 65). Nonetheless, the intracellular signaling mechanisms responsible for PMN activation leading to the release of this free radical in the inflammatory conditions are not known.

There are reports indicating the involvement of burn injury-related inflammatory mediators that may lead to PMN priming and augmented responses such as PMN aggregation, degranulation, and O₂⁻ generation (23, 51, 53, 58). Such augmented PMN responses could cause endothelial damage, capillary leak, and ultimately remote organ injury (9, 12, 56). The peptide fMLP, which may be released from the invading bacteria and/or degenerating euukaryotic mitochondria at sites of initial tissue injury, can trigger the PMN responses (11, 37). An increase in intracellular Ca²⁺ concentration ([Ca²⁺]) precedes PMN O₂⁻ generation and degranulation (59, 52, 63). Stimulation of PMNs with fMLP results in the activation of phospholipase C through a specific receptor-linked G protein with the formation of inositol trisphosphate (IP₃) and diacylglycerol (DAG) (42). IP₃ induces the release of Ca²⁺ from the intracellular stores, leading to an elevation in [Ca²⁺], while DAG remains associated with the membrane and participates in protein kinase C (PKC) activation (19, 35). A portion of the fMLP-induced elevation in [Ca²⁺] is due to Ca²⁺ influx into the PMNs (61, 62). The fMLP-induced PMN activation can also proceed via a tyrosine kinase activation (24, 57). The respiratory burst response induced by direct activation of PKC, by phorbol esters or DAG, is known to be slower in onset than the fMLP-mediated response (15, 34, 45).

The present study examined the role of [Ca²⁺] and PKC signaling pathways in O₂⁻ production by PMNs from rats with a full skin thickness burn comprising 25% of the total body surface area (TBSA). Additionally, a specific objective of our study was to determine whether or not potential burn-related PMN [Ca²⁺] and PKC signaling alterations could be therapeutically modulated to prevent any inappropriate PMN O₂⁻ generation in burn rats. In preliminary studies, we found an overt augmentation of O₂⁻ generation accompanied by an upregulation of [Ca²⁺] and PKC signaling in PMNs from rats on days 1-3 postburn (49). We hypothesized that the PMN [Ca²⁺] and PKC upregulation could be prevented by treatment of rats with calcium channel blockers, e.g., diltiazem, which have been shown to attenuate the entry of Ca²⁺ into cells (32) as...
well as its release from intracellular stores in PMNs and other cell types in vitro (46). These actions of the Ca²⁺ entry blocker could be exerted on a plasma membrane receptor-gated Ca²⁺ channel (30, 32) and/or an intracellular store Ca²⁺ release channel (46, 66). We evaluated the efficacy of treatment of burned rats with the Ca²⁺ entry blocker diltiazem on O₂ release, and PKC responses in the circulating PMNs.

**METHODS**

Rat burn model. Male Sprague-Dawley rats (250–300 g) were divided into two groups: sham and burn. The animals were intraaperitoneally anesthetized with pentobarbital sodium, 40–50 mg/kg, the hair on their backs was shaved off, and they were placed in a polypropylene cradle with a rectangular opening to allow exposure of the shaved skin area. The cradle was then lowered into a water bath (95–97°C) for 10 s, causing a full-thickness third-degree burn comprising 25% of TBSA (28). This type of injury destroys pain perception. The burn rats were quickly dried off to prevent any additional heat injury and resuscitated with lactate-Ringer solution (3 ml·kg⁻¹·%TBSA⁻¹). Sham rats were immersed in 37°C water. While the animals were recovering from anesthesia, they were kept warm under a heat lamp and observed frequently for a period of 4–6 h. The animals had access to food and water. The sham and burn rats were killed 1, 3, 7, or 10 days later. There was no mortality in the sham or burn animals. Some of the sham and burn rats were killed 1, 3, 7, or 10 days later. Other groups of sham and burn rats received diltiazem (2 mg/kg) at 2 and 24 h postresuscitation and were killed 24 h later. Other groups of sham and burn rats were killed 1, 3, 7, or 10 days later. Other groups of sham and burn rats were killed 24 h later. Other groups of sham and burn rats received diltiazem (2 mg/kg) at 2 and 24 h postresuscitation and were killed on day 3 or at 2, 24, 48, and 72 h later and were killed on day 7.

Preparation of peripheral neutrophils. Rats were killed under general anesthesia with pentobarbital sodium, 40–50 mg/kg ip, and the blood (10–12 ml) was collected into heparinized syringes by cardipuncture. PMNs were isolated from the heparinized blood using the standard Ficoll–paque (Pharmacia) cell separation technique followed by dextran sedimentation and hypotonic red blood cell lysis (10). PMNs were then washed and resuspended in Hanks’ balanced salt solution (HBSS) buffer. PMN preparations routinely contained >95% PMN as identified by the Giemsa stain and were found to be >98% viable by the trypan blue exclusion technique.

**Superoxide anion measurement.** Superoxide anion production in whole cells was determined by the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c (31). The reaction mixture (2 ml) contained 500 × 10⁴ neutrophils, 75 µM cytochrome c and phosphate buffer (in mM: 138 NaCl, 2.7 KCl, 1.0 MgCl₂, 0.6 CaCl₂, 5 glucose, and 10 NaH₂PO₄/ Na₂HPO₄, pH 7.4). Some assays contained an additional 50 µg/ml SOD. Superoxide production was initiated by the addition of 1 µM fMLP. The absorbance of reduced cytochrome c was recorded continuously at 550 nm at room temperature until a maximal signal was obtained. The maximum rate of superoxide formation was calculated from the slope of the response in nanomoles per minute per 10⁶ cells, using the specific absorbance of reduced cytochrome c of 21.1 mM/cm. All of the organic and inorganic reagents were purchased from Sigma, St. Louis, MO, unless reported otherwise.

**Fluorescent calcium measurements.** The cytosolic free Ca²⁺ concentration, [Ca²⁺], in rat neutrophils was determined by microfluorometry and imaging techniques. PMN (3 × 10⁶ cells) were loaded with 2 µM fura 2-AM (Molecular Probes). For fluorometry, the cells were resuspended in HBSS containing 1 mM CaCl₂ and 1 mM MgCl₂ (GIBCO BRL, Grand Island, NY) and the fluorescence signals of a 0.5-ml stirred PMN suspension were monitored in a F-2000 Hitachi spectrofluorimeter, using 340- and 380-nm excitation wavelengths and 510-nm emission wavelength. The fluorescent ratios [R = fluorescence intensity (F340/F380)] were calculated and converted to [Ca²⁺i using the equation described by Grynkiewicz et al. (25): [Ca²⁺i] = K_d(R - R_{min}/(R_{max} - R)), where R_{max} = F340/F380 (with Ca²⁺i), R_{min} = F340/F380 (no Ca²⁺i), and b = F380 (no Ca²⁺i)/F380 (with Ca²⁺i).

Calcium imaging in single cell. PMNs were loaded with fura 2-AM as described above. The cells were placed on a coverslip and examined with ×100 oil-immersion objective of a Nikon microscope. The cells were then exposed to alternating 340- and 380-nm excitation wavelengths. Fluorescence emitted by fura 2 was collected through a 505-nm band-pass filter. Eight images acquired by a cooled charge-coupled device camera (Photometric) were averaged to reduce the signal-to-noise ratio. The images were digitized, and the data were then analyzed by using Metafluor computer software (Universal Imaging) (38). The imaging system was calibrated using known Ca²⁺-fura concentrations sandwiched between two coverslips ~10 µm apart.

Preparation of subcellular fractions for PKC assays. Isolated neutrophils were stimulated with and without 1 µM fMLP at 37°C in HBSS (without Ca²⁺ and Mg²⁺). The cells were then washed and resuspended in ice-cold extraction buffer containing 0.25 M sucrose, 50 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol. The cells were sonicated twice for 5- to 7-s bursts with a Kontes Micro-Ultrasonic cell dismuter, followed by centrifugation at 100,000 g for 1 h at 4°C to separate the soluble cytosolic fraction. The pellet or membrane fraction was resuspended in the same buffer containing 0.1% Triton X-100 and sonicated again. The total protein concentration in both fractions was determined using bicinchoninic acid protein reagent (Pierce, Rockford, IL). PKC, in both fractions, was assayed according to the method of Yasuda et al. (64) using a synthetic peptide homologous to a sequence of myelin basic protein [MBP4—14; Gln-Lys-Arg-Pro-Ser(8)-Gln-Arg-Ser-Lys-Tyr-Leu]. Aliquots of 25–µl cells fraction were incubated in a 70-µl reaction mixture consisting of 20 mM tris(hydroxymethyl)aminomethane-HCl, pH 7.5, 10 mM magnesium chloride, 25 µM MBP4—14, 0.5 µg phosphatidylserine, 50 ng diolein, 0.1 mM CaCl₂, and 10 µM [γ-³²P]ATP (DuPont-NEN, Boston, MA). After incubation for 12 min at room temperature, the reaction was stopped by addition of 10 µl of 300 mM orthophosphoric acid. Aliquots of the reaction mixture were then spotted onto phosphocellulose paper P-81 (Calbiochem, San Diego, Ca), washed with a sufficient volume of 75 mM phosphoric acid, and counted for ³²P using liquid scintillation spectroscopy. Activity was measured as picomoles of ³²P incorporated per minute per milligram protein.

Statistics. Data are expressed as means ± SE. Comparisons were made using Student’s t-test or analysis of variance as appropriate. The rates of O₂ generation were estimated using linear regression analyses.

**RESULTS**

Measurements of PMN O₂⁻, [Ca²⁺], and PKC activation in diltiazem-treated or untreated rats did not show significant differences on days 1, 3, 7, and 10 after the
PMN superoxide anion release. Figure 1 shows the maximum rate of O$$\textsubscript{2}^-$$ release as well as the total amount of release from PMNs of sham and postburn rats. The maximum rates were determined under conditions of zero-order kinetics with a concentration of the substrate (ferricytochrome c) producing a saturation of the reaction rate. The maximum rates in burn rats on days 1, 3, and 7 postburn were significantly higher than those observed in the sham rats (P < 0.01). On the other hand, the 10-days postburn rates were not statistically different from the sham values (P > 0.05). The O$$\textsubscript{2}^-$$ release, in vitro, continued for ~2 min in all PMN samples studied. As expected, the calculated values of the total amount of O$$\textsubscript{2}^-$$ released by sham and burn rat PMNs varied similarly to the maximum rates. These data suggested an enhanced capacity of superoxide anion production in the early (days 1, 3, and 7 postburn) but not a late stage (day 10 postburn) of burn injury.

PMN [Ca$$^{2+}$$]$$\text{i}$$ responses. The [Ca$$^{2+}$$]$$\text{i}$$ measurements in PMNs from sham and burn rats are shown in Fig. 2. The burn injury caused elevations in both basal [Ca$$^{2+}$$]$$\text{i}$$ and fMLP-mediated [Ca$$^{2+}$$]$$\text{i}$$ responses on days 1, 3, and 7 but not on day 10 postburn. The basal [Ca$$^{2+}$$]$$\text{i}$$ values in PMNs from day 1 (108.6 ± 8.5 nM), day 3 (202.7 ± 20.4 nM), and day 7 (116.7 ± 6.8 nM) postburn groups were significantly higher (P < 0.01) than in the sham group (75.5 ± 5.6 nM). The basal [Ca$$^{2+}$$]$$\text{i}$$ was 40% higher on day 1 and 168% higher on day 3 after the burn than in the sham group; on day 7 the increase was comparable to that on day 1 postburn. The fMLP-induced peak [Ca$$^{2+}$$]$$\text{i}$$ in PMNs was 351.4 ± 21.9 nM on day 1, 336.2 ± 25.3 nM on day 3, and 292.0 ± 11.3 nM on day 7 postburn; each of these values was significantly higher (P < 0.01) than the Ca$$^{2+}$$ response to fMLP in the sham rats (158.7 ± 8.3 nM). On day 10 postburn, the peak fMLP-mediated [Ca$$^{2+}$$]$$\text{i}$$ response was not significantly different from that in the sham rats.

The differences between the basal and the peak fMLP-induced [Ca$$^{2+}$$]$$\text{i}$$ levels (Δ[Ca$$^{2+}$$]$$\text{i}$$, values, Fig. 2) in the burn groups were significantly higher (P < 0.05) compared with the sham value, except in the day 10 postburn group. Although the elevated [Ca$$^{2+}$$]$$\text{i}$$ in the presence of fMLP on day 3 was not significantly different from that on day 1, the Δ value on day 3 was about one-half of the corresponding value on day 1 (P < 0.01). The lower Δ[Ca$$^{2+}$$]$$\text{i}$$ on day 3 was due to the higher basal [Ca$$^{2+}$$]$$\text{i}$$ group. Compared with day 3, the group on day 7 exhibited a lower basal [Ca$$^{2+}$$]$$\text{i}$$, and a higher Δ[Ca$$^{2+}$$]$$\text{i}$$. Both the basal and fMLP-stimulated [Ca$$^{2+}$$]$$\text{i}$$ values in PMNs from the day 10 burn animals were comparable to those in the sham group.

The heightened [Ca$$^{2+}$$]$$\text{i}$$ response with fMLP on day 1 was investigated using the imaging technique. The [Ca$$^{2+}$$]$$\text{i}$$ images of PMNs from sham and day 1 burn rats are shown in Fig. 3. Imaging of individual neutrophils confirmed that fMLP-mediated [Ca$$^{2+}$$]$$\text{i}$$ elevations on day 1 postburn (Fig. 3B) were markedly higher than those in the sham group (Fig. 3A). The Ca$$^{2+}$$ profiles in terms of R340/380 values from burn rat PMNs (Fig. 3B) are markedly different from those in sham rat PMNs (Fig. 3A). In the burn group, the fMLP-mediated sharp rise in Ca$$^{2+}$$ was followed by slowly decreasing levels of Ca$$^{2+}$$ over a period of ~140 s before the return of the Ca$$^{2+}$$ levels to those found before PMN stimulation with fMLP. The slowly decreasing Ca$$^{2+}$$ represented a “shoulder” phase following the Ca$$^{2+}$$ peak. In most cases, the amplitude of the shoulder was much less than the peak Ca$$^{2+}$$ preceding it. In comparison with the burn group, the sham group shoulder phase was much less pronounced, as was the initial rise (peak Ca$$^{2+}$$) in a majority of individual cell Ca$$^{2+}$$ profiles.
Although in some individual cells in the sham group the peak Ca$^{2+}$ reached R340/380 values above 2.75, it remained below 2.00 in most of the cells. The peak Ca$^{2+}$ in the burn group in most cases was well above 5.5, and the shoulder values were above 2.00.

Effect of treatment of rats with diltiazem on PMN [Ca$^{2+}$] responses. Figure 4, A-D, shows the time-related elevation in [Ca$^{2+}$] after fMLP stimulation of PMNs from sham and burn rats with and without diltiazem treatment. Although in the sham animals there was no demonstrable effect of treatment with diltiazem on the basal [Ca$^{2+}$], or fMLP-mediated [Ca$^{2+}$] elevation (Fig. 4A), diltiazem affected [Ca$^{2+}$] in the burn rats. On day 1 after the burn, the treatment did not have an effect on the basal [Ca$^{2+}$] but significantly suppressed the pronounced fMLP-induced [Ca$^{2+}$] elevation seen in the untreated day 1 burn rats (P < 0.025) (Fig. 4B); the fMLP-mediated elevation in the treated rats was comparable to that in the sham group. The Δ[Ca$^{2+}$] in the burn day 1 treated group (77.2 ± 4.5 nM) was significantly lower with diltiazem treatment, compared with the Δ[Ca$^{2+}$] in the untreated burn day 1 PMNs.
The diltiazem treatment led to a decrease of both basal and fMLP-induced [Ca$^{2+}$]$_i$ levels in PMNs from rats on days 3 and 7 postburn. The basal [Ca$^{2+}$]$_i$ (114.5 ± 10.2 nM) and fMLP-induced [Ca$^{2+}$]$_i$ (215.7 ± 6.8 nM) in the diltiazem-treated day 3 postburn group were significantly lower compared with the untreated group's basal (202.7 ± 20.4 nM; $P < 0.01$) and fMLP-induced [Ca$^{2+}$]$_i$ (336.2 ± 25.3 nM; $P < 0.01$), respectively. The $\Delta$[Ca$^{2+}$]$_i$ in the diltiazem-treated burn day 3 rats PMNs (101.0 ± 10.1 nM) was attenuated compared with that in the untreated groups (133.5 ± 13.2, $P < 0.05$). The treatment of day 7 burn rats with diltiazem also significantly affected the basal (116.7 ± 6.8 nM, untreated burn; 50.3 ± 3.8 nM, treated burn; $P < 0.01$), as well as the fMLP-induced [Ca$^{2+}$]$_i$ responses (291.9 ± 11.3 nM, untreated burn; 191.5 ± 11.0 nM, treated burn; $P < 0.025$). The diltiazem treatment attenuated the $\Delta$[Ca$^{2+}$]$_i$, value from 175.5 ± 15.5 nM in the day 7 untreated burn rats to 141.3 ± 14.1 nM ($P < 0.05$) in the treated group. These data suggest that diltiazem therapy led to a substantial downregulation of the burn-induced [Ca$^{2+}$]$_i$ responses.

PMN PKC activation: effect of treatment of rats with diltiazem. Figure 5, A-D, shows the level of activation of PKC activity in PMNs from sham and burn rats with and without their treatment with diltiazem. The measurements of PKC activity in the PMN cytosolic and membrane fractions allowed for the assessment of the translocation of the enzyme into the membrane. The PKC specific activity tended to be higher in the cytosolic (Fig. 5, A and B) than the membrane fractions (Fig. 5, C and D). In the untreated burn rats, the basal cytosolic PKC activity, compared with the sham group value, was ~10% lower ($P < 0.05$) on day 1, ~20% lower ($P < 0.025$) on day 3, and ~10% lower ($P < 0.05$) on day 7 (Fig. 5A). The stimulation of cells with fMLP caused a 10% decrease ($P < 0.05$) in cytosolic PKC activity in the sham group, a 14% decrease ($P < 0.025$) in day 1, a 19% decrease ($P < 0.05$) in day 3, and a 13% decrease ($P < 0.05$) in day 7 groups. The decreases in both basal and post-fMLP cytosolic activities in the sham and burn groups corresponded with increases in the membrane PKC activities. Although the absolute increases in the membrane PKC activities tended to be in the same range as the decreases in the cytosolic activities, the percent changes in the membrane activities were of a greater magnitude. Relative to sham, the increase in basal membrane PKC activity with burn was 88% ($P < 0.01$) in day 1 postburn, 156% ($P < 0.01$) in the day 3 burn, and 86% ($P < 0.05$) in the day 7 burn group. Similarly, the PMN stimulation with fMLP caused higher percentage increases in membrane PKC than the post-fMLP decreases in the cytosolic fractions. The fMLP-mediated membrane PKC was 78% higher ($P < 0.05$) than the basal value in sham animal PMNs, 53% higher ($P < 0.025$) in the day 1 burn group, 47% higher ($P < 0.05$) in the day 3 burn group, and 48% higher ($P < 0.05$) in the day 7 burn group (Fig. 5C). Whereas diltiazem treatment of sham rats did not significantly affect the cytosolic and membrane PKC activities, the treatment of burn (days 1, 3, and 7) rats led to abrogation of both basal and fMLP-related changes in cytosolic (Fig. 5B) as well as membrane (Fig. 5D) PKC activities. These findings suggest that the treatment of burn rats with diltiazem prevented the burn injury-related modulations in basal as well as fMLP-induced translocation of PKC.

Effect of treatment of rats with diltiazem on PMN superoxide anion release. The significance of [Ca$^{2+}$]$_i$ regulation per se, and of potential [Ca$^{2+}$]$_i$-related PKC activation in PMN superoxide anion production during burn injury, was evaluated by assessing the effect of
Diltiazem treatment of burn rats on $O_2^-$ release by fMLP-stimulated PMNs. Figure 6 shows PMN $O_2^-$ production as a function of time over a period of 135 s. The time course of $O_2^-$ release in sham rat PMNs was not significantly different ($P > 0.05$) from PMNs of sham rats treated with diltiazem (Fig. 6A). In the treated day 1 postburn rats, PMN $O_2^-$ release was significantly lower ($P < 0.025$) than that observed in the untreated rats but not significantly different ($P > 0.05$) from that in the sham group (Fig. 6B). Also, we observed a decrease in PMN $O_2^-$ production in diltiazem-treated day 3 ($P < 0.01$) (Fig. 6C) and day 7 postburn ($P > 0.05$) (Fig. 6D) rats compared with the corresponding untreated groups.

Fig. 6. Time course of $O_2^-$ release from PMNs, in response to fMLP, of sham and burn rats with and without treatment with DZ (2 mg/kg). A: PMNs from untreated and DZ-treated sham rats. B: sham and burn day 1, DZ-treated and untreated rats. C: burn day 3 rats with and without DZ treatment compared with sham rats. D: time course of $O_2^-$ release from PMNs of sham and burn day 7 rats with and without treatment with DZ. Data are shown as means ± SE; $n = 20$ for sham, $n = 12$ for burn days 1-7, and $n = 8$ for all DZ-treated rats.
DISCUSSION

The ability of neutrophils to produce superoxide anion after burn injury has been studied frequently in recent years (1, 14, 18, 20, 47, 65). Previous studies showed that the PMN respiratory burst may be impaired in burn patients; this could lead to an increased risk of bacterial invasion and sepsis (1, 18, 47). The PMN O₂⁻ release is the initial step in the formation of toxic reactive oxygen metabolites, which play an essential role in bacterial killing. Beside this beneficial bactericial role, toxic oxygen metabolites could also cause host tissue damage when they are produced in excess quantities during inflammatory conditions. Our present study suggests an enhanced O₂⁻ release by PMNs in the early stages (days 1-7) of burn injury. At day 10 postburn, we did not detect a change in the O₂⁻ generation. These findings are in keeping with some previous studies showing an initial increase in PMN respiratory burst after burn injury to be followed by an attenuation in the later stages of injury (14, 20, 65).

Burn patients often exhibit intense inflammation and sepsis 7-10 days after 15-25% TBSA burns (41). Several previous studies have suggested that neutrophils from burn and trauma patients contribute to the pathogenesis of adult respiratory distress syndrome (ARDS) and multiple organ failure (MOF) (8, 54, 59). ARDS has been reported to develop within 72-96 h postburn (49, 54). ARDS and MOF are associated with massive accumulation of PMNs in the microvasculature, causing microvascular permeability to protein and capillary leak (3-5, 9, 28). Till et al. (54) have shown that the administration to the burn rats (28% TBSA) of catalase and SOD attenuated the burn-induced lung injury. Furthermore, the depletion of neutrophils before burn in experimental animals prevented the lung injury (54). These findings clearly support the role of ROS in organ dysfunction.

The present study has investigated the magnitude of PMN O₂⁻ generation as well as PMN signaling mechanisms responsible for it during the course of burn injury. The activation of NADPH oxidase, the dormant enzyme of the neutrophil plasma membrane, is essential for the production of O₂⁻ in response to neutrophil chemotactic agonists including the N-formylmethionyl peptides, e.g., fMLP (35). Previous studies have amply shown that the chemotactic agonists upregulate NADPH oxidase activity through activation of neutrophil signaling pathways via modulations of both Ca²⁺/Ca²⁺-dependent and Ca²⁺-independent kinase cascades, namely, PKC, tyrosine kinase, and mitogen-activated protein kinase (21, 24, 57). FMLP action on neutrophils can lead to an activation of a soluble tyrosine kinase and eventual NADPH oxidase activation without the elicitation of a Ca²⁺ response (7, 57), and yet under physiological conditions it would cause an elevation of [Ca²⁺], as well as a PKC response before NADPH activation (19, 35, 61). The Ca²⁺-dependent pathways are known to be linked to activation of PKC and a subsequent translocation of certain cytosolic proteins (p47phox and p67phox) to the plasma membrane to form a complex with the dormant NADPH oxidase to cause its activation (17, 36). In this study, the assessments of [Ca²⁺] and PKC signaling behaviors in neutrophils from animals with a burn injury have allowed for an evaluation primarily of the Ca²⁺-dependent pathways. The experimental design of the treatment of animals with the Ca²⁺ entry blocker diltiazem to assess its effect on PMNs from burn animals also provided for an evaluation of potential therapeutic modulations of the PMN’s Ca²⁺-dependent signaling pathways; diltiazem was employed to presumably attenuate [Ca²⁺], elevations either via a decrease in Ca²⁺ influx through the plasma membrane receptor-gated Ca²⁺ channel or through a decrease in the intracellular Ca²⁺ release (32, 46). Furthermore, through the assessment of the effect of diltiazem treatment on PKC activation, we tested the possibility of a Ca²⁺-linked activation of PKC in the burn animal PMNs.

The assessments of basal levels of [Ca²⁺] and PKC activity in PMNs from the burn groups revealed alterations in the signaling intermediates in vivo at various stages after the onset of the injury process. On the other hand, the findings of elevations in [Ca²⁺] and PKC activation after stimulation of PMNs from burn animals in vitro with exogenous fMLP presumably determined the maximum capacity of the [Ca²⁺] or PKC signaling in PMNs at various stages of the burn injury process. The data presented here show that on day 1 postburn there was an increase in basal [Ca²⁺], and fMLP-mediated [Ca²⁺] elevation and a resultant increase in Δ[Ca²⁺] corresponding with only a moderate increase in O₂⁻ production. On day 3 there was a large increase in O₂⁻ generation accompanied by a large increase in basal [Ca²⁺], but only a modest increase in Δ[Ca²⁺]. On day 7, the increases in both the basal [Ca²⁺] and fMLP-mediated [Ca²⁺] elevations were comparable to those on day 1, as was the increase in O₂⁻ generation. We interpret these results to indicate that a moderate increase in basal [Ca²⁺] along with a near maximum [Ca²⁺] elevation in response to fMLP (in vitro) found on days 1 and 7 postburn accompanied a moderate increase in O₂⁻ generation. The fMLP-mediated response in vitro presumably shows a burn-induced upregulation of the capacity of the Ca²⁺ response, and basal [Ca²⁺] enhancement indicates a burn injury-related accumulation of PMN Ca²⁺-upregulating signals [namely, the endogenously accumulating Ca²⁺ mobilizing inflammatory mediators such as fMLP itself, C5a, platelet-activating factor (PAF), etc.]. The higher O₂⁻ generation on day 3 may result from not only the increased Ca²⁺ response capacity (fMLP-induced elevation, in vitro) but also by the higher level of increase in basal [Ca²⁺]. The highest basal [Ca²⁺] accompanying the highest level of O₂⁻ generation on day 3 could be due to a greater degree of the accumulation of PMN Ca²⁺-mobilizing inflammatory mediators in vivo. The partial recovery of both the basal Ca²⁺ and O₂⁻ responses on day 7 to the day 1 levels may be reflective of an attenuation of the burn-related inflammatory mediators with a continuing upregulation of the Ca²⁺.
response capacity to a near-maximum level. In the face of the near-maximum [Ca\(^{2+}\)] response (to fMLP) on days 1, 3, and 7 postburn, the ∆[Ca\(^{2+}\)] estimation seemed to be determined primarily by basal [Ca\(^{2+}\)] values in the burn groups. Thus burn injury maximally affected the O\(_2^\cdot\) generation by influencing both the basal [Ca\(^{2+}\)] and the [Ca\(^{2+}\)] response capacity. The changes in ∆[Ca\(^{2+}\)] reflected mainly the effects on the basal [Ca\(^{2+}\)] values. The subsiding of the [Ca\(^{2+}\)] upregulation clearly occurred by day 10 postburn. The burn-related upregulation of Ca\(^{2+}\) signaling could be due to an increase in Ca\(^{2+}\) influx across the plasma membrane and/or an increase in intracellular release of Ca\(^{2+}\) from the cytoplasmic Ca\(^{2+}\) reservoir. Although the mechanism of an augmentation in Ca\(^{2+}\) influx and/or intracellular release remains unknown, these occurrences are borne out by studies showing that PMNs from diltiazem-treated rats exhibit a reversal of these Ca\(^{2+}\) upregulations. Diltiazem could block both the influx of Ca\(^{2+}\) and its intracellular release (32, 46).

A role of PKC and subsequent protein phosphorylations in PMN O\(_2^\cdot\) production can be due to either a direct activation of the kinase by DAG or an increase in [Ca\(^{2+}\)] and a subsequent activation of the calcium-dependent α- and β-PKC isoform (36). The calcium-dependent β-PKC isoform has been shown to phosphorylate the cytosolic proteins p47\(^{phox}\) and p67\(^{phox}\), which then are translocated to the membrane before NADPH oxidase activation and O\(_2^\cdot\) generation (17). Recent studies from our laboratory have reported an upregulation of p47\(^{phox}\) and p67\(^{phox}\) in the early phases of burn injury (18–72 h) in a rat burn model with 30% burns (20). Our data on the basal levels of PKC activation in burn rat PMNs support an upregulation in this signaling component somewhat similar to that in the Ca\(^{2+}\) signaling component with the burn injury. A progressive upregulation of the translocation of PKC from the cytosol to the membrane, in vivo, was probably reflective in the basal PKC measurements through day 3 postburn. The lowering of basal cytosolic PKC and the concomitant decrease in basal membrane PKC after burn supported PKC activation in vivo. Although PKC activation on day 7 postburn was of a greater magnitude than the sham group, like the Ca\(^{2+}\) signaling it tended to show a recovery toward the sham level. Unlike the constancy of burn injury-related Ca\(^{2+}\)-signaling maximal capacity as reflected in the response to exogenous fMLP on days 1-7 postburn, the fMLP-induced PKC activation seemed to be upregulated progressively through day 3 postburn and falling off by day 7 postburn. From the apparent constancy of fMLP-induced Ca\(^{2+}\) responses at a maximal level with a concurrent progression of the fMLP-induced PKC activation on days 1-3 postburn, it is reasonable to speculate that the burn-related activation of PKC is an effect of enhanced Ca\(^{2+}\) signaling rather than its occurrence independently of the Ca\(^{2+}\) responses.

The comparison of the time courses of PMN Ca\(^{2+}\) and PKC responses and the O\(_2^\cdot\) generation during the time period from day 1 to day 7 postburn indicates potential relationships between the signaling components and the effector response, viz O\(_2^\cdot\) generation. Apparently, the absolute Ca\(^{2+}\) response to fMLP and the basal [Ca\(^{2+}\)] but not the ∆Ca\(^{2+}\) seemed to increase preceding the increase in fMLP-induced O\(_2^\cdot\) production. The enhancement in the basal [Ca\(^{2+}\)] level in PMNs was plausibly due to their endogenous activation by agonists present in the circulation of injured animals. There seems to be no difference in the duration of fMLP-induced [Ca\(^{2+}\)], as the increase in [Ca\(^{2+}\)]; in both sham and burn rat PMNs dropped back to the basal level ~800 s after addition of fMLP (data not shown). The prevention of the burn-related increase in basal [Ca\(^{2+}\)] or its lowering with the diltiazem treatment on days 1-7 postburn and the prevention of the enhanced [Ca\(^{2+}\)] response to fMLP in PMNs from days 1, 3, and 7 postburn animals demonstrate diltiazem's efficacy in abrogating the burn injury-produced PMN signaling perturbations. The O\(_2^\cdot\) upregulation was clearly accompanied by concomitant enhancements in Ca\(^{2+}\) and PKC signaling at days 1-3 postburn and a tendency of recovery of O\(_2^\cdot\) generation on day 7 along with a recovery in the two signaling components. The importance of Ca\(^{2+}\) signaling upregulation as a potential primary event in triggering the PKC response and the subsequent O\(_2^\cdot\) generation upregulation during burn injury is underscored by studies of the effect of treatment of burn rats with diltiazem. As stated above, diltiazem treatment can suppress either an endogenously occurring increase in PMN Ca\(^{2+}\) influx or a decrease in intracellular Ca\(^{2+}\) release or both during the pathogenesis of burn injury.

Because diltiazem may primarily affect Ca\(^{2+}\) signaling, its effects on the PKC activation and O\(_2^\cdot\) release are likely due to primary modulations in the PMN Ca\(^{2+}\) signaling in the burn animals. Previous studies have shown that human PMNs lack voltage-dependent calcium channels (40); thus the action of diltiazem is presumably not due to its inhibition of the voltage-sensitive Ca\(^{2+}\) channel in the plasma membrane. 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 (30, 32) and thus decrease Ca\(^{2+}\) influx. That diltiazem inhibits the hyper-activation of PMNs by inhibiting the Ca\(^{2+}\) release from intracellular stores of PMNs also remains a possibility (46). The calcium channel blockers have also been suggested to inhibit PKC (6), phospholipase A\(_2\) (13), tumor necrosis factor (TNF)-α release by mononuclear cells (29), and PAF binding to PMNs (22). Increased release of TNF-α and PAF after burn injury and their ability to prime neutrophils for O\(_2^\cdot\) production could also be phenomena that are likely blocked by diltiazem treatment, which would lead to suppression of excessive PMN release of O\(_2^\cdot\) after the burn.

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