

## NEUTROPHIL SIGNALING ALTERATION:

## AN ADVERSE INFLAMMATORY RESPONSE AFTER BURN SHOCK

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**Abstract** The inflammatory response syndrome in shock-like states might frequently be accompanied by an oxidative cell/tissue damage in one or more organ-systems in the body. The inflammatory response related hyperactivation of neutrophils can contribute to oxidative cell/tissue damage. Studies discussed in this review examined the role of cell signaling pathways in the hyperactivation of neutrophils in an early stage of burn injury shock. The studies were carried out in peripheral blood neutrophils isolated from rats with a 25% body surface area scald burn. Neutrophil cell signaling responses were evaluated by measuring cytosolic  $[Ca^{2+}]$  and protein kinase C activity, and were correlated with neutrophil superoxide production. The cytosolic  $[Ca^{2+}]$  and protein kinase C responses were highly upregulated along with enhanced superoxide production in the early phase of burn injury. The treatment of burn-injured rats with the calcium antagonist diltiazem abrogated enhanced  $Ca^{2+}$  and protein kinase C signaling and superoxide generation. The signaling upregulation in neutrophils could result from potentiation of actions of burn-injury induced chemotactic mediators on the leukocytes. The neutrophil signaling upregulation leading to increased superoxide generation could thus be responsible for the oxidative cell/tissue damage. The organ-system dysfunction/failure accompanying burn shock may be initiated with the oxidative cell/tissue damage.

**Resumen** *Transducción de señales leucocitarias: respuesta inflamatoria adversa en el shock por quemaduras.* El síndrome de la respuesta inflamatoria en estados de shock está frecuentemente acompañado por daño tisular debido a mecanismos oxidativos que afectan uno o más órganos. La hiperactivación de polimorfonucleares neutrófilos asociada a la respuesta inflamatoria puede contribuir al daño oxidativo que se observa en los tejidos. En esta revisión se discutirá el papel de distintas señales en la hiperactivación de neutrófilos en estados tempranos del shock térmico provocado por quemaduras (*burn shock*). Los estudios realizados fueron llevados a cabo con neutrófilos aislados de sangre periférica de ratas que tienen un 25% de la superficie corporal escaldada. La medición del calcio citosólico y la actividad de la proteína quinasa C fueron evaluados como medida de las señales recibidas por los neutrófilos y fueron correlacionadas con la producción de superóxido. La concentración de calcio citosólico y la respuesta de la proteína quinasa C, así como la producción de superóxido, estaban muy aumentadas en las fases tempranas de la injuria térmica. El tratamiento de estas ratas con antagonistas del calcio como el diltiazem elimina el aumento de los parámetros evaluados. El aumento de la capacidad de respuesta de los neutrófilos podría deberse a la generación de mediadores quimiotácticos por los leucocitos de animales que han sufrido el shock térmico. La capacidad aumentada de la respuesta oxidativa en los neutrófilos puede ser la causa inicial que conduce al daño orgánico múltiple en el shock por quemaduras.

**Key words:** neutrophil signaling, burn shock, inflammation, oxidative cell damage

## I. Shock: The outcome of an inflammatory response

The phenomenon of shock after trauma injuries with or without severe blood loss was well recognized in the early to mid 1800s<sup>1</sup>. Subsequent observations in injured humans as well as in animal models of shock have documented disturbances in individual organ systems within the body. Starting with the initial observations im-

plicating shock-induced derangements in the nervous system<sup>2</sup>, studies in the field of shock have identified adverse effects in the heart and vascular system followed, but not necessarily in a set order, by disturbances in the pulmonary, renal, and hepatic functions<sup>3</sup>. More recent observations have given credence to shock-related damage in the gastrointestinal absorptive and mucosal barrier functions<sup>4</sup>. The concepts of shock culminating in multiple organ system dysfunction or failure was promulgated by Professor Arthur Baue in the early 1980s<sup>5</sup>. Figure 1 illustrates the emergence of the sepsis syndrome, also known as SIRS (systemic inflammatory response syndrome), after acute injuries such as

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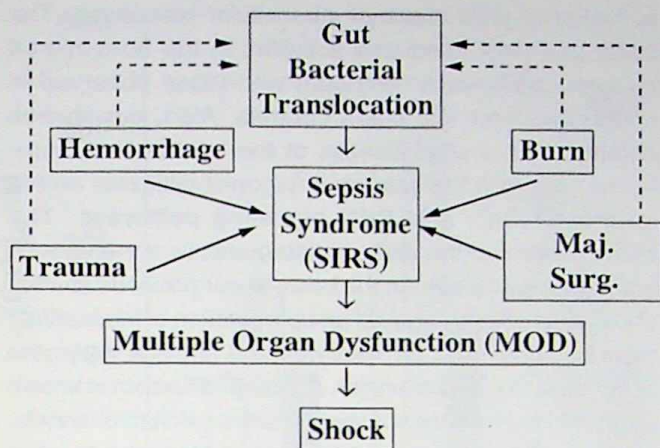


Fig. 1.— Schematic diagram showing emergence of sepsis syndrome after various forms of acute injuries, e.g. burn, trauma etc., and the potential causal relationship between the sepsis syndrome and multiple organ dysfunction. The diagram also emphasizes the injury conditions effecting translocation of bacteria across the gastrointestinal wall to lead to the sepsis syndrome. SIRS = systemic inflammatory response syndrome.

hemorrhage, burn, trauma or major surgical procedures. Also shown in the figure is the role of gut bacterial translocation occurring in the sepsis syndrome and shock. Several studies have shown that trauma and related injuries induce an inflammatory response accompanied by the release of a vast variety of mediators from cells of both the "immune" and "non-immune" systems within the body<sup>6</sup>. The mediators play a role in both an early adaptive activation as well as a late adverse exacerbation of the functions of the various organ systems. Although the recent findings have to a large extent recognized the chemical nature and the cellular sources of these mediators, their induction, expression and release at specific tissue sites, and the mechanisms of their actions at various target cells and tissues remain principal objectives of ongoing investigations. The mediators' actions on their target cells have been designated as either pro- or anti-inflammatory depending on whether they promote or suppress inflammation<sup>7</sup>. The actions of the mediator may be exerted either on cells that themselves produce mediators (autocrine responses), or cells in juxtaposition to the mediator-producing cells (paracrine responses), or cells distant from the mediator-producing cells (endocrine responses).

The research initiated in the author's laboratory in the late 1980s has since focused on intracellular signaling pathways in cells of the "immune" as well as the "non-immune" systems. One research objective has been to determine alterations in signaling pathways that might be responsible for the altered cellular responses contributing to damage or dysfunction in the bodily functions in the inflammatory response syndrome. Our studies were carried out mainly in rat models of sepsis

and burn injury leading to an overt state of inflammatory response and shock.

This review primarily focuses on studies which evaluated the intracellular signalling pathways in the polymorphonuclear neutrophils from rats with a burn injury. The reviewer has previously summarized the findings in the non-immune cell systems (hepatocytes, skeletal myocytes)<sup>8</sup> and in T lymphocytes<sup>9</sup> harvested from animals with a sepsis-induced injury.

## II. Inflammatory response affects signaling in neutrophils

A number of studies have shown polymorphonuclear neutrophils to be activated in an early phase of the inflammatory response syndrome to produce excessive quantities of superoxide free radical along with enhanced chemotactic, chemokinetic and phagocytic responses<sup>10, 11</sup>. Such an activation of neutrophils can evidently provide for their enhanced migration across the microvasculature at tissue sites of infection/inflammation within the body, and for the presumed  $O_2^-$ -related killing of the invading microorganisms. However, the enhanced neutrophil activation can also lead to injury to the host's own cells/tissues. The host cell/tissue injury could be related not only to excessive production of  $O_2^-$  and  $O_2^-$ -derived reactive molecular species (e.g.,  $H_2O_2$ , the hypochlorous and carboxyl anions), which cause oxidative damage to cell/membrane lipids and proteins, but also the hydrolytic enzymes released by neutrophils<sup>12</sup>. The host tissue injury might also be due to attenuation in the activity of scavenger enzyme systems responsible for the clearance of the  $O_2^-$  and related molecular species. The activation of neutrophils in the inflammatory conditions is presumably effected by the mediators, such as the bacteria-derived formyl peptides (e.g., fMLP, formylmethionyl-leucyl-phenylalanine), activated complement (e.g., C5a), and a host of mediators released from leukocytes themselves, such as cytokines, tumor necrosis factor (TNF $\alpha$ ), interleukin-8 (IL-8) or its homologs, granulocyte colony stimulating factor (GCSF), and lipid mediators, platelet activating factor (PAF) and leukotriene B4 (LTB4). All of these mediators have been shown to be released either systemically or regionally in tissues in trauma, septic, and burn injury conditions<sup>13</sup>. Furthermore, there is experimental evidence that not only each of these mediators by itself can activate neutrophils but that there might be a potentiation of the effect of one of these in the presence of one or more of the other mediators. Thus, it is reasonable to assume that there may be an optimum potentiation of the actions of the neutrophil-activating mediators generated after trauma, burn or septic injuries. Figure 2 depicts a flow diagram of trauma/burn/sepsis-related upregulation of the neutrophil-activating agents

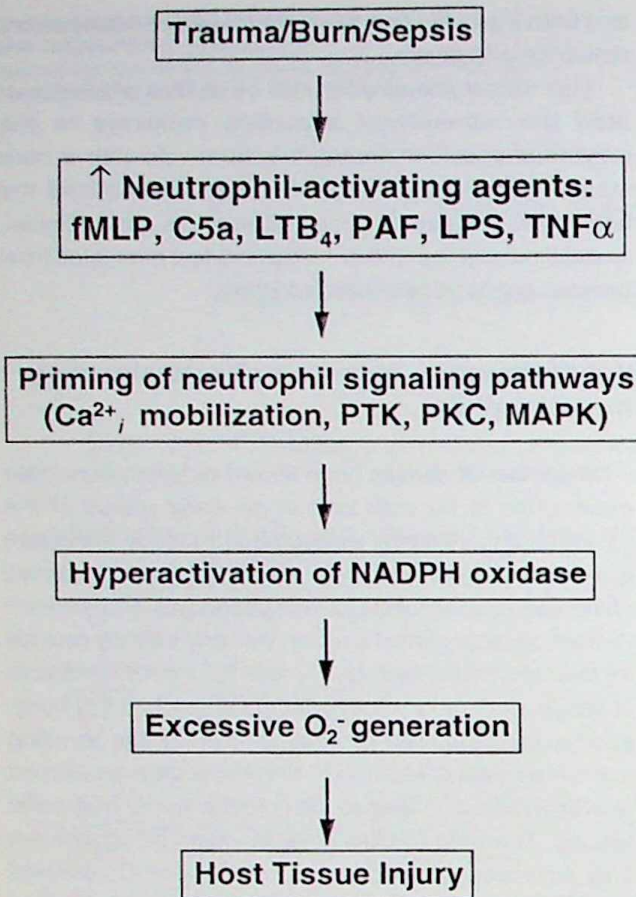


Fig. 2.— Schematic diagram depicting the role of injury-induced inflammatory mediators in the activation of neutrophil signaling pathways responsible for regulating  $O_2^-$ -generating membrane enzyme NADPH oxidase. Also shown in the diagram is the potential injurious effect of excessive  $O_2^-$  generation on host tissues. LPS = lipopolysaccharide, PTK = protein tyrosine kinase, MAPK = mitogen activated protein kinase; see text for other abbreviations in the figure.

and their potential role in modifying the signaling pathways leading to increased activation of the neutrophils'  $O_2^-$ -producing membrane enzyme, NADPH oxidase<sup>11</sup>.

Our experimental approach to assess the potential role of the mediators in the altered regulation of neutrophil intracellular signaling was to isolate circulating neutrophils from burn-injured rats and then assay their signaling activities in the basal state and after their stimulation with fMLP *in vitro*. The neutrophil signaling events assessed were: 1) cytosolic  $Ca^{2+}$  concentration, ( $[Ca^{2+}]_i$ ), and 2) activation of protein kinase C (PKC). In addition we carried out measurements of neutrophil  $O_2^-$  production in the presence of fMLP. It was assumed that injury *per se* had altered the neutrophil cytosolic  $[Ca^{2+}]_i$  and PKC responses which would be reflective in the basal state measurements. The determination of neutrophil activities in the presence of exogenous fMLP presumably evaluated the extent to which injured animal neutrophils could be further

activated to yield maximal intracellular responses. The basal and fMLP-mediated activities in the burn-injured rat neutrophils were compared with those observed in neutrophils from the sham controls. Also, our studies determined the effectiveness of the treatment of burn-injured rats with the calcium antagonist diltiazem on the neutrophil  $Ca^{2+}$  and PKC signaling pathways. The administration of the calcium antagonist as a therapeutic agent was warranted on the basis of our previous studies showing its efficacy against an upregulation of intracellular calcium signaling in hepatocytes and skeletal myocytes of the septic injured animals<sup>9</sup>. Although diltiazem is known principally to block the voltage-sensitive calcium channels, at relatively higher concentrations it also serves as a blocker of the receptor gated  $Ca^{2+}$  channels in a variety of cell systems<sup>14</sup>. Our previous studies have shown diltiazem's ability to block the receptor-gated calcium channel in the hepatocytes which are known to lack the voltage-sensitive calcium channels<sup>15</sup>.

The basal neutrophil cytosolic  $[Ca^{2+}]_i$  and PKC activity presumably represent the state of one of the neutrophils' well studied signaling networks that is activated by a number of but not all of the neutrophil-activating mediators. For example, whereas fMLP, C5a, LTB<sub>4</sub>, and PAF are known to activate the  $Ca^{2+}$  and PKC linked pathways, and TNF- $\alpha$  and GCSF may act through a  $Ca^{2+}$  independent pathway<sup>16</sup>. Although TNF- $\alpha$  and GCSF by themselves may not activate the calcium linked signaling, they may potentiate the actions of other calcium signal-activating mediators. These considerations lend support to the concept that activation of the intracellular calcium linked pathways may lead to an optimum potentiation of the neutrophil responses in conditions accompanying release of the various inflammatory mediators. The known link between the  $Ca^{2+}$  and PKC signaling components and their combined effect to potentiate the activity of the NADPH oxidase enzyme system in neutrophils<sup>17</sup> would imply that burn/sepsis-related perturbations in the  $Ca^{2+}$  signal would also lead to simultaneous alterations in PKC activation as well as the related effector response, namely  $O_2^-$  generation. In our studies, we quantified  $[Ca^{2+}]_i$  and PKC activation along with assessments of  $O_2^-$  production in neutrophils isolated from sham-operated and burn-injured rats. A scalding burn injury was produced in anesthetized rats by exposing 25% of their total body surface area to 95°C water for a period of 4-5 seconds. This protocol was shown to result in a full thickness skin burn. Whereas rats studied 1-3 days after the burns were designated as those showing early effects of the injury, rats studied 7-10 days after the burn were assumed to be in a late injury phase. Details of the rat burn protocol have been described in earlier reports from our laboratory<sup>19,20</sup>. The methods of isolating neutrophils, and of measurements of neutrophil  $[Ca^{2+}]_i$  and PKC activities have also been described in detail in the earlier

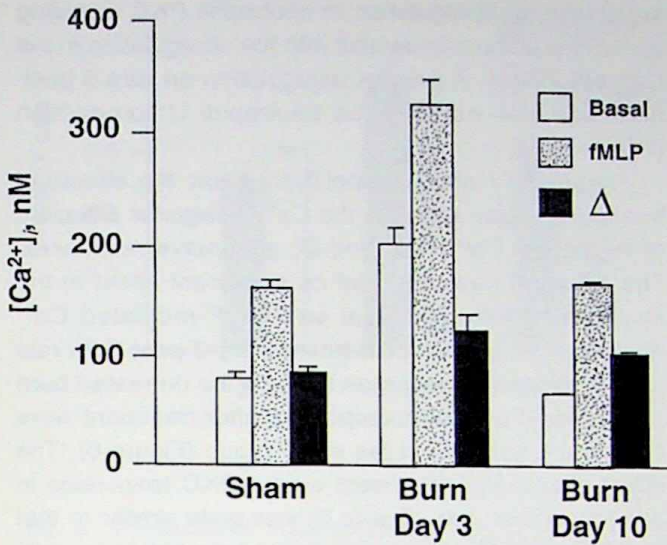


Fig. 3.— Basal and fMLP-elevated [Ca<sup>2+</sup>]<sub>i</sub> in neutrophils from sham and day 3 and day 10 post-burn rats. The differences in basal and fMLP-elevated [Ca<sup>2+</sup>]<sub>i</sub> are shown as Δ values. Bars represent mean ± SE values.

publications. Briefly, [Ca<sup>2+</sup>]<sub>i</sub> was measured by initially labeling neutrophils with the membrane permanent fluorescent Ca<sup>2+</sup> chelating dye Fura 2. Fluorescent signals from the labeled neutrophils, before and after their stimulation with fMLP, were fluorometrically quantified, and the resultant fluorescence intensities converted into Ca<sup>2+</sup> concentration values. PKC activities in the neutrophil cytosolic and membrane fractions were determined after incubating the cell fractions with a synthetic peptide, homologous to a sequence of myelin basic protein, and quantitating the incorporation of <sup>32</sup>P derived from ATP into the peptide. An increase in the <sup>32</sup>P incorporation in the membrane fraction along with its decrease in the cytosolic fraction after neutrophil stimulation with fMLP was taken to represent PKC translocation from the cytosol to membrane and thus its activation. The neutrophil O<sub>2</sub> production was assessed by spectrophotometrically quantifying the reduction of ferricytochrome C.

Neutrophil Ca<sup>2+</sup> signaling responses in the early (day 3 post-burn) to a late (day 10 post-burn) phase after burn injury are shown in Figure 3. The basal [Ca<sup>2+</sup>]<sub>i</sub> on day 3 post-burn was greater than 2x the level in the sham group. However, on day 10 post-burn the basal [Ca<sup>2+</sup>]<sub>i</sub> was either comparable to or less than that found in the sham controls. This suggested that neutrophil Ca<sup>2+</sup> responses were significantly upregulated in the early but not in the later phases after the burn injury. The elevated basal [Ca<sup>2+</sup>]<sub>i</sub> on day 3 post-burn can be attributed to an effect of the inflammatory mediators released into circulation during the early course of injury. Although the identity of the mediators remains unknown, it is reasonable to assume that multiple endogenous mediators contributed to the heightened Ca<sup>2+</sup> response. The effect of neutrophil

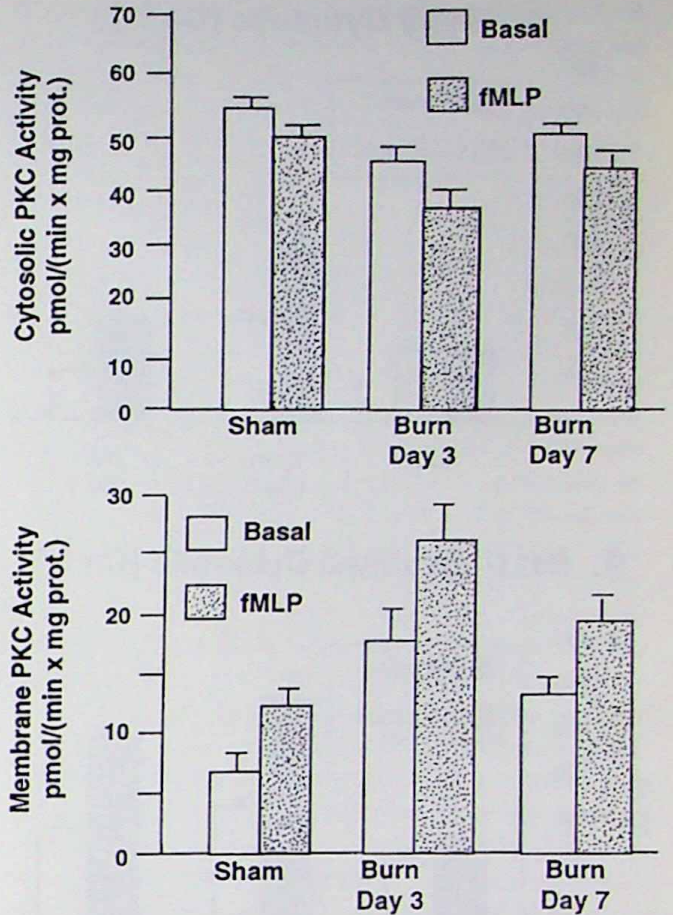


Fig. 4.— Basal and fMLP-mediated PKC activities in the cytosolic and membrane fractions of neutrophils from sham and da6 3 and day 7 post-burn rats. Bars represent mean ± SE values.

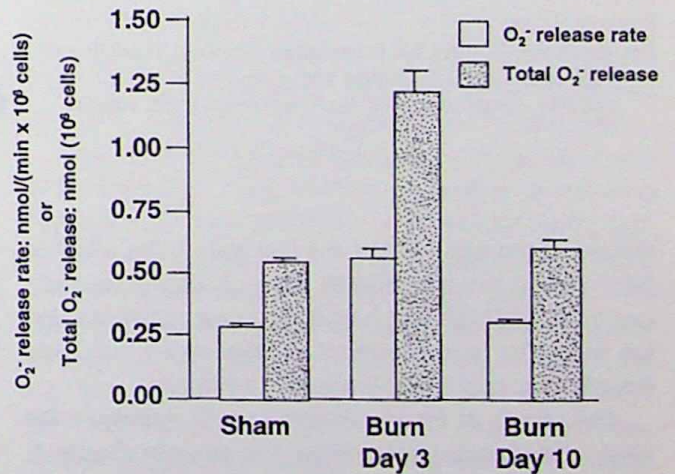


Fig. 5.— O<sub>2</sub><sup>-</sup> release rate and total O<sub>2</sub><sup>-</sup> released from neutrophils of sham and day 3 and day 10 post-burn rats. Bars represent mean ± SE values.

stimulation by fMLP, in vitro, further assessed the enhanced responsiveness of neutrophils harvested from the day 3 burn rats. The enhanced responsiveness to fMLP on day 3 (Figure 3) was apparently due not only to

### A. Basal Cytosolic $[Ca^{2+}]_i$



### B. fMLP-mediated Cytosolic $[Ca^{2+}]_i$



Fig. 6.— Basal (A) and fMLP-mediated (B)  $[Ca^{2+}]_i$  in neutrophils from diltiazem (DZ) treated and untreated sham and day 3 and day 7 post-burn rats. Bars are mean  $\pm$  SE values.

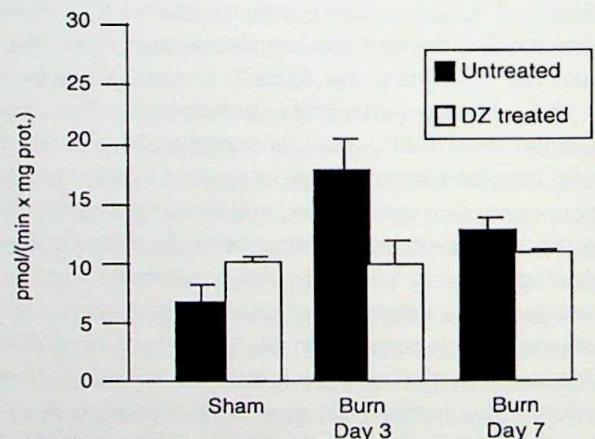
the increased basal  $[Ca^{2+}]_i$ , but due also to the effect of fMLP *per se*; this was evident after calculating the  $\Delta$  values ( $[Ca^{2+}]_i$  fMLP- $[Ca^{2+}]_i$  basal). The neutrophils isolated ten days after burn showed  $Ca^{2+}$  responses which were not different from those in the sham controls.

The results of measurements of PKC activity in the neutrophil membrane fractions are given in Figure 4. There was an apparent enhancement in both the basal and fMLP mediated membrane PKC in neutrophils of rats 3 days after the burn compared to the sham group values. On day 7 after burn, whereas there was an attenuation in both the basal and fMLP-mediated membrane PKC activities compared to the day 3 burn group values, the activities on the day 7 post-burn were still somewhat higher than in the sham control group. These findings

suggested an upregulation in neutrophil PKC signaling on day 3 post-burn in parallel with the upregulation in the  $Ca^{2+}$  responses. A parallel upregulation on day 3 post-burn was also noted in the neutrophil  $O_2^-$  production (Figure 5).

Figures 6, 7 and 8 respectively show the effects of treatment of burn rats with the  $Ca^{2+}$  antagonist diltiazem (2 mg/kg) on  $Ca^{2+}$ , PKC and  $O_2^-$  generative responses. The diltiazem treatment had no significant effect in the sham rats. Both the basal and fMLP-mediated  $Ca^{2+}$  responses in the diltiazem treated day 3 post-burn rats were significantly lower than those in the untreated burn group; day 3 post-burn responses after treatment were comparable to those in the sham group (Figure 6). The effect of diltiazem treatment on the PKC responses in day 3 post-burn rats (Figure 7) was quite similar to that on  $Ca^{2+}$  responses. The diltiazem treatment apparently prevented upregulation in both  $Ca^{2+}$  and PKC signaling pathways observed on day 3 post-burn. Furthermore,

### A. Basal Membrane PKC Activity



### B. fMLP-mediated Membrane PKC Activity

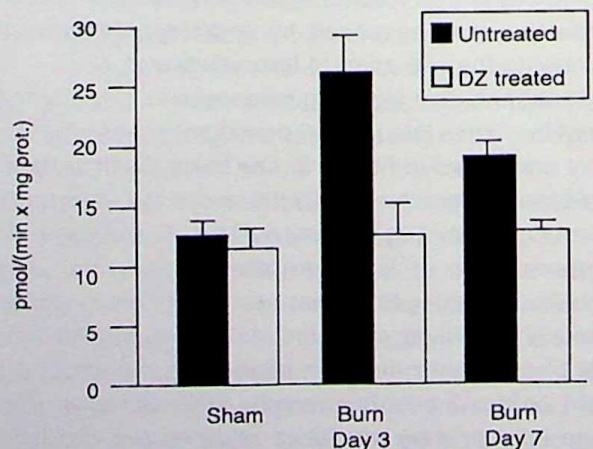


Fig. 7.— Basal (A) and fMLP-mediated (B) membrane PKC activities in neutrophils from diltiazem (DZ) treated and untreated sham and day 3 and day 7 post-burn rats. Bars are mean  $\pm$  SE values.

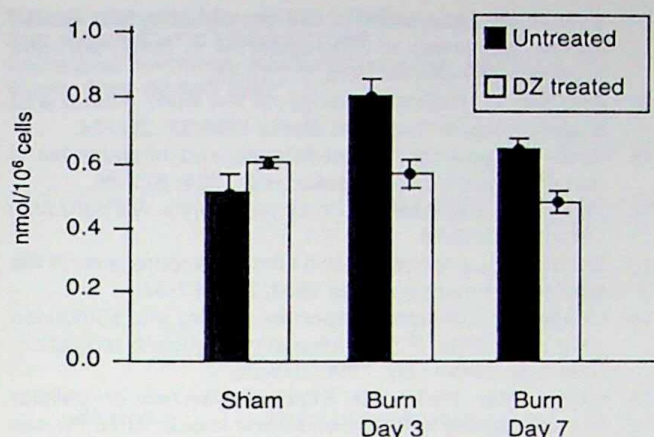


Fig. 8.— O<sub>2</sub><sup>-</sup> release by neutrophils from diltiazem (DZ) treated and untreated sham and da6 3 and da6 10 post-burn rats. Bars are mean ± SE values.

the treatment of burn rats with diltiazem effectively attenuated the burn-induced enhancement in neutrophil O<sub>2</sub><sup>-</sup> production (Figure 8).

### III. Neutrophil signaling alterations contribute to enhanced potential for oxidative cell/tissue injury

The foregoing results of signaling assessments in neutrophils from burn-injured animals clearly demonstrate that Ca<sup>2+</sup> and PKC pathways in these leukocytes are "hyperactive". The latter term is warranted in as much as maximum levels of Ca<sup>2+</sup> and PKC activities in fMLP challenged neutrophils from injured animals were resolutely found to be higher than in neutrophils from control animals. Furthermore, the finding that basal [Ca<sup>2+</sup>]<sub>i</sub> and activated PKC levels in the 3 days post-burn animal neutrophils were higher than in controls supports the concept that development of the "hyperactive" state of neutrophil signaling occurs during an early phase of burn injury. At later stages of injury, namely days 7 and 10 post burn, there was an apparent subsiding of the "hyperactive" state.

It can be reasonably assumed that the injury-related release of neutrophil-activating mediators play a role in the "hyperactivation" of neutrophil signaling pathways during the early burn injury phase. Although the precise identity of such mediators affecting the signaling systems remains unknown, the various known chemotactic agents/mediators released after burn injury (e.g. C5a, formyl peptides derived from the invading bacteria IL-8 homologs, TNF $\alpha$ , LTB<sub>4</sub>, PAF, GCSF)<sup>10</sup> are likely responsible for the alterations in signaling pathways. Previous studies have shown that neutrophil Ca<sup>2+</sup> and PKC activities are

potentiated by the actions of various combinations of the aforementioned mediators<sup>16</sup>. Thus the observed "hyperactivation" of Ca<sup>2+</sup> and PKC signaling in burn-injured animal neutrophils could result from neutrophil hypersensitization to the multiple mediators acting in concert or at some time intervals during the burn pathogenesis. Previous studies have referred to such hypersensitization of neutrophils during injury states as a "priming" phenomenon<sup>19</sup>. Primed neutrophils presumably exhibit an enhanced ability to infiltrate into tissues subsequent to their adhesion to endothelium, and migration across the vascular wall and through the tissue interstitium, and eventually their release of O<sub>2</sub><sup>-</sup> and hydrolytic enzymes. Based on our findings, we postulate that under pathophysiologic conditions which lead to recovery from injury, the priming of neutrophils allows them to migrate through the vascular wall to the interstitial sites of injury/infection and then to release O<sub>2</sub><sup>-</sup> and the proteolytic enzymes in such a controlled manner that adverse oxidative/proteolytic effects are exerted on the pathogens but not on host cells/tissues. On the other hand, the postulate would entail that under certain conditions a hyperactivation or priming of neutrophils occurs to such an extent that they release both O<sub>2</sub><sup>-</sup> and proteolytic enzymes as they migrate through the vascular wall and through the interstitium. Such inappropriate releases of O<sub>2</sub><sup>-</sup>/proteolytic enzymes can understandably injure endothelial permeability and interstitial/parenchymal functions in host tissues. The resulting endothelial/microvascular permeability dysfunction and interstitial/parenchymal damage can conceivably contribute to one or more organ/system dysfunction/failure leading to a lethal outcome. The multiple organ/system disturbances emanating from the early burn mediated neutrophil "hyperactivity" could become manifest at later stages of burn injury even though the neutrophil responses themselves may not be upregulated in the later stages.

The findings summarized here show an excessive production of O<sub>2</sub><sup>-</sup> along with the upregulation of signaling pathways (viz. Ca<sup>2+</sup> and PKC) responsible for the O<sub>2</sub><sup>-</sup> production in neutrophils in an early stage of burn injury. Our findings support the linkage between the hypersensitization of the Ca<sup>2+</sup> and PKC pathways and excessive O<sub>2</sub><sup>-</sup> production as both the signaling and the O<sub>2</sub><sup>-</sup> release responses were prevented subsequent to the treatment of the injured animals with the calcium antagonist diltiazem. Diltiazem likely served to control the Ca<sup>2+</sup> response which in turn could modulate PKC activation and O<sub>2</sub><sup>-</sup> generation.

Although our studies do not provide any evidence for host cell/tissue damage associated with the increased neutrophil O<sub>2</sub><sup>-</sup> production, they lend credence to the concept that hypersensitization of neutrophil signaling pathways could primarily be responsible for an uncontrolled O<sub>2</sub><sup>-</sup> production by the neutrophils.

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*Scientists operate within a tribal culture, the rules, mores and idiosyncrasies of which are generally acquired through intellectual osmosis in a mentor-disciple relationship.*

Los científicos operan dentro de una cultura tribal, cuyas reglas, costumbres e idiosincrasias se adquieren en general por ósmosis en una relación mentor-discípulo.

Carl Djerassi

Ethical discourse by science-in-fiction. *Nature* 1998; 393: 511