Effects of burn injury on myocardial signaling and cytokine secretion: possible role of PKC

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Tan J, Maass DL, White DJ, Horton JW. Effects of burn injury on myocardial signaling and cytokine secretion: possible role of PKC. Am J Physiol Regul Integr Comp Physiol 292: R887–R896, 2007. First published September 21, 2006; doi:10.1152/ajpregu.00555.2006.—This study examined the effects of major burn injury on the cellular distribution of several PKC isoforms in adult rat hearts and examined the hypothesis that PKC plays a regulatory role in cardiomyocyte cytokine secretion. Burn trauma was given over 40% total body surface area in Sprague-Dawley rats. An in vitro model of burn injury included addition of burn serum, 10% by volume, to primary cardiomyocyte cultures (collagen perfusion). In vivo burn injury produced redistribution of PKCε, PKCe, and PKCα from the cytosol (soluble) to the membrane (particulate) component of the myocardium. This activation of the PKC isoforms was evident 2 h after burn injury and progressively increased over 24 h postburn. Addition of burn serum to isolated myocytes produced similar PKC isoform redistribution from the soluble to the particulate compartment, promoted myocyte Ca2+ and Na+ loading, and promoted robust myocyte secretion of inflammatory cytokines similar to that reported after in vivo burn injury. Pretreating cardiomyocytes with either calphostin or PKCe inhibitory peptide, a potent inhibitor of PKCe, prevented burn serum-related redistribution of the PKCe isoform and prevented burn serum-related cardiomyocyte secretion of TNF-α, IL-1β, IL-6, and IL-10. These data suggest that the PKCe isoform plays a pivotal role in myocardial inflammatory response to injury, altering cardiac function by modulating cardiomyocyte inflammatory cytokine response to injury.

cardiomyocytes; protein kinase C isoforms; protein kinase C inhibition; burn serum challenge; calphostin; protein kinase C epsilon inhibitory peptide

MYOCARDIAL DYSFUNCTION is a major determinant of multiple organ failure and mortality in hemorrhagic shock, major burn injury, or sepsis (1, 18, 20, 25, 33, 34, 42). One cellular response that has been shown to be a common feature of these diverse insults/injuries is cardiomyocyte secretion of inflammatory cytokines (5, 19, 25, 27). Furthermore, cardiomyocyte secretion of TNF-α, IL-1β, and IL-6 after burn injury or sepsis has been shown to produce compartmental or myocardial tissue levels of cytokines that are higher than cytokine levels measured in the systemic circulation (18, 42). Although the specific mechanisms by which inflammatory cytokines after myocardial contraction and relaxation remain unclear, intracellular signaling events regulated by inflammatory cytokines have been proposed to include activation of numerous kinases including PKC, p38 MAPK, JNK, and tyrosine kinase (2, 30). PKC activation includes translocation of the enzyme from the cytosol to membrane, which, in turn, regulates several aspects of myocardial function including myofilament structure and function, activation of the Na+/H+ exchanger, phosphorylation of troponin T and I subunits, phosphorylation of phospholamban, and cytosolic Ca2+ homeostasis (3, 16, 31, 35, 37, 40). Recent attention has focused on the role of PKC inflammatory responses to injury and disease, and PKC inhibition has been shown to attenuate inflammatory cytokine responses by several cell types including peritoneal and alveolar macrophages and vascular smooth muscle cells (9, 14, 29).

The PKC family of enzymes includes several isoforms; both the Ca2+-dependent isoforms (PKCα and PKCβ) and the Ca2+-independent isoforms (PKCδ and PKCe) are present in neonatal myocardium, whereas PKCδ, PKCe, and PKCα isoforms predominate in adult myocardium (9, 32, 39). Although the specific role of specific PKC isoforms in injury and disease remains unclear, a role for PKC activation in the myocardial depression that occurs in injury and disease has been supported by the finding that PKC inhibition or absence of PKC (knock-out mice) improves contractile function in congestive heart failure and ischemia reperfusion injury (7, 11, 12, 22).

The purpose of the present study was to examine the role of several PKC isoforms that have been shown to predominate in the adult myocardium (32, 39) and to play a pivotal role in myocardial responses to injury and disease. Specifically, myocardial PKCδ, PKCe, and PKCα were examined after in vivo burn injury or after in vitro challenge of myocytes with burn serum. This study was directed to further define the role of PKC activation in the cardiomyocyte inflammatory cytokine responses that have been shown to occur after burn injury. The use of primary cardiomyocyte cultures allowed us to eliminate the confounding effects of administering PKC inhibitors in vivo and inhibition of PKC on multiple cell types in numerous organs.

MATERIALS AND METHODS

Experimental model. Adult Sprague-Dawley male rats (320–350 g) obtained from Harlan Laboratories (Houston, TX) were conditioned in-house for 5–6 days after arrival with commercial rat chow and tap water available at will. All experiments performed in this study were reviewed and approved by The University of Texas Southwestern Medical Center’s Institutional Review Board for the care and handling of laboratory animals and conformed to all guidelines for animal care as outlined by the American Physiological Society and the National Institutes of Health.

Burn procedure. Animals were deeply anesthetized (isoflurane) and secured in a constructed template device, and the surface of the skin exposed through the aperture in the template was immersed in 100°C water for 10 s on the back and upper sides. Use of the template produced well-circumscribed full-thickness dermal burns over 40% of

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total body surface area (TBSA). Exposure to this water temperature in adult rats destroys all underlying nerves and avoids injury to underlying organs. Sham burn rats were subjected to identical preparation, except that they were immersed in room temperature water to serve as controls. Immediately after immersion, rats were dried, and lactated Ringer solution (4 ml/kg per %burn) was given intraperitoneally. One-half of the calculated volume was given immediately postburn, and the remaining volume was given 8 h postburn. The total volume of Ringer given over the first 24 h postburn was 50–56 ml. Buprenorphine (0.05–0.1 mg/kg) was given every 12 h during the postburn period. Burned rats did not display discomfort or pain, moved freely about the cage, and consumed food and water within 15 min after recovering from isoflurane anesthesia. Rats with sham burns also received identical regimens of analgesics (buprenorphine) throughout the study period.

Cardiomyocyte isolation. For preparation of primary cardiomyocyte cultures, adult control Sprague-Dawley rats were heparinized and decapitated; hearts were harvested and placed in a petri dish containing ice-cold (4°C) heart medium [113 mM NaCl, 4.7 mM KCl, 0.6 mM KH2PO4, 1.2 mM NaHCO3, 10 mM NaHCO3, 20 mM D-glucose, 0.5X minimum essential medium (MEM) amino acids (50X, Gibco-BRL 11130-051), 10 mM HEPES, 30 mM taurine, 2.0 mM carnitine, and 2.0 mM creatine]. Hearts were cannulated via the aorta and perfused with heart medium at a rate of 12 ml/min for a total of 5 min in a nonrecirculating mode. Perfusion was then changed to a recirculating mode, and enzymatic digestion was accomplished with a digestion solution that contained 34.5 ml of heart medium plus 50 mg of collagenase II ( Worthington 4177, lot no. MOB3771), 50 mg of bovine serum albumin (BSA) fraction V (Gibco-BRL 11018-025), 0.5 ml of trypsin (2.5%, 10X, Gibco-BRL 15090-046), and 15 μM CaCl2. This solution was recirculated through the heart at a flow rate of 12 ml/min for 20 min. All solutions perfusing the heart were maintained at a constant temperature of 37°C. At the end of the enzymatic digestion, the ventricles were removed and mechanically disassociated in 6 ml of enzymatic digestion solution containing a 6-ml aliquot of 2× BSA solution (2 mg of BSA fraction V to 100 ml of heart medium). After mechanical disassociation with fine forceps, the tissue homogenate was filtered through a mesh filter into a conical tube. The cells adhering to the filter were collected by washing with an additional 10-ml aliquot of 1× BSA solution (100 ml of heart medium and 1 g of BSA fraction V). Cells were then allowed to pellet in the conical tube for 10 min. The supernatant was removed, and the pellet was resuspended in 10 ml of 1× BSA. The cells were washed and pelleted further in BSA buffer with increasing increments of Ca2+ (100, 200, and 500 μM, to a final concentration of 1,000 μM). After the final pelleting step, the supernatant was removed, and the pellet was resuspended in MEM [prepared by adding 10.8 g of 2X BSA (Sigma M-1018), 11.9 mM NaHCO3, 10 mM HEPES, and 10 ml of penicillin/streptomycin (100X; Gibco-BRL 1540-122) with 950 ml of MilliQ water]; total volume was adjusted to 1 liter. At the time of MEM preparation, the medium was bubbled with 95% O2:5% CO2 for 15 min and the pH was adjusted to 7.1 with 1 M NaOH. The solution was then filter sterilized and stored at 4°C until use. At the final concentration of Ca2+2, the cardiomyocyte cell number was calculated and myocyte viability was determined (19, 27).

Cardiomyocyte responses to burn se rum challenge. Myocytes from control (unburned) rats were pipetted into microtiter plates at 5 × 104 cells·mL−1·well−1 (12-well cell culture cluster; Corning, Corning, NY) for 18 h (CO2 incubator at 37°C). Cells were incubated in medium alone (control) or in the presence of burn serum (10% by volume with the serum collected 24 h after a third-degree burn over 40% TBSA in adult rats). Sham burn serum was collected from rats given anesthesia and handled as described for burned rats. Inhibitor studies included pretreating separate aliquots of control myocytes with the PKC inhibitors peptide (Calbiochem, La Jolla, CA; 0.55 mg in 2.5 μl of medium added to 5 × 106 myocytes for 30 min before burn serum challenge) (23, 41). All cells were incubated in medium alone or in burn/sham serum-containing medium in the presence or absence of a PKC inhibitor for 18 h (CO2 incubator at 37°C).

Measurement of myocyte-derived cytokines. Supernatants from cardiomyocytes incubated in the presence or absence of burn serum and/or treated with PKC inhibitor were collected to measure myocyte-secreted pro- and anti-inflammatory mediators TNF-α, IL-1β, IL-6, and IL-10 (rat ELISA; Endogen, Woburn, MA). We previously examined the contribution of contaminating cells (nonmyocytes) in our cardiomyocyte preparations using flow cytometry, cell staining (hematoxyl in and eosin), and light microscopy. We confirmed that <2% of the total cell number in a myocyte preparation were noncardiomyocytes. Since our preparations are 98% cardiomyocytes, we concluded that a majority of the inflammatory cytokines measured in the cardiomyocyte supernatant was indeed cardiomyocyte derived (19, 27).

Preparation of myocardial total protein. Hearts were collected 2, 4, or 24 h postburn, whereas myocytes were collected after in vitro incubation in the presence or absence of the PKC inhibitor calphostin C (50 nM) or PKCε inhibitor peptide; heart tissue or isolated cells were snap-frozen and stored at −80°C until analysis. Whole cell extracts were prepared according to methods described previously (20). Frozen myocardial tissue or frozen cardiomyocytes were homogenized in 200 μl of ice-cold lysis buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, pH 8, 0.1% CHAPS, and protease inhibitors). The homogenized samples were allowed to sit on ice for 20 min and then were centrifuged at 10,000 g for 10 min at 4°C; the supernatant was saved. Total protein concentration was determined on the supernatant by using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Supernatant represented both cytosolic protein and soluble membrane protein and corresponded to the total protein.

Preparation of cytosol and membrane fractions. Cytosol and membrane fractions were prepared according to methods described by Rybin and colleagues (38, 39). Heart tissue was homogenized with a glass-glass homogenizer in 10 ml of ice-cold lysis buffer containing 20 mM Tris·HCl, pH 7.5; 7.6 mM β-mercaptoethanol; 50 mM NaF, 0.1 mM Na3VO4, 2 mM EDTA, 2 mM EGTA, pH 7.4, 1 mM PMSF, and 1 tablet of protease inhibitor cocktail (Complete; Roche Applied Science, Indianapolis, IN). The homogenate was centrifuged at 1,000 g for 15 min to pellet unbroken cells, leaving a membrane-containing supernatant. The resulting supernatant was recentrifuged at 100,000 g for 45 min. The supernatant from the second centrifugation was taken as the cytosolic fraction; the pellet was resuspended in ice-cold lysis buffer containing 1% Triton X-100, homogenized, left on ice for 20 min, and then centrifuged at 100,000 g for 30 min. The supernatant from this step was then used as the solubilized particulate (membrane) fraction (11, 24, 36, 38). Protein concentrations were measured using Bio-Rad protein assay kit (Bio-Rad 500-0006) with BSA as a standard.

Western blot analysis of PKC isoforms. Protein from the total, cytosolic, and particulate cellular (membrane) fractions was subjected to protein immunoblotting as previously described (24, 36, 38). Equal amounts of protein (30 μg) were separated on a 7.5% SDS-polyacrylamide gel and transferred to a polyvinylidenefluoride membrane (Millipore, Bedford, MA). The membrane was blocked by a 1-h incubation at room temperature in a Tris-buffered saline solution (TBS-T; 20 mM Tris, pH 7.6, 135 mM NaCl, and 0.05% Tween) containing 3% BSA and 1% nonfat dry milk. Membranes were probed with antibodies against phosphorylated PKCδ (Tyr-155), PKCe (Ser-729), and PKCα (Ser-657) at 4°C overnight (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Additional membranes were probed with antibodies against nonphosphorylated PKCβ, PKCe, and PKCα at 4°C overnight (1:200 dilution; Santa Cruz Biotechnology). After the primary antibody incubation, the membrane was washed with
three times with TBS-T. The appropriate secondary antibody was then added to the membrane according to the vendor’s recommendation (1:1,000 dilution; Bio-Rad) and incubated for 1 h at room temperature. The membrane was again washed three times with TBS-T and once with TBS. The bound antibodies were detected using SuperSignal Western blotting kits (Pierce, Rockford, IL). Densitometric analysis of Western blots was performed using Multimage (Alpha Innotech, San Leandro, CA).

**Intracellular Ca²⁺ and Na⁺ measurements.** Separate aliquots of cells were loaded with either fura-2 AM for 45 min or sodium-binding benzofurazan isophthalate for 1 h at room temperature in the dark. Myocytes were then suspended in 1.0 mM Ca²⁺-containing MEM and washed to remove extracellular dye; myocytes were placed on a glass slide on the stage of a Nikon inverted microscope. The microscope was interfaced with Groeney optics for epi-illumination, a triocular head, phase optics, a ×30 phase-contrast objective, and a mechanical stage. The excitation illumination source (300-W compact xenon arc illuminator) was equipped with a power supply. In addition, this InCyt Im2 fluorescence imaging system (Intracellular Imaging, Cincinnati, OH) included an imaging workstation and an Intel Pentium Pro200 MHz-based personal computer. The computer-controlled filter changer allowed alternation between the 340- and 380-nm excitation wavelengths. Images were captured by a monochrome charge-coupled device camera equipped with a television relay lens. InCyt Im2 Image software allowed measurement of intracellular Ca²⁺ ([Ca²⁺]i) and Na⁺ concentrations ([Na⁺]) from the ratio of the two fluorescence signals generated from the two excitation wavelengths (340 nm/380 nm); background was removed by the InCyt Im2 software. The calibration procedure included measuring fluorescence ratio with buffers containing different concentrations of either Ca²⁺ or Na⁺. At each wavelength, the fluorescence emissions were collected for 1-min intervals, and the time between data collection was 1–2 min. Since quiescent or noncontracting myocytes were used in these studies, the Ca²⁺ levels measured reflect diastolic levels.

**Statistical analysis.** All values are expressed as means ± SE. Analysis of variance (ANOVA) was used to assess an overall difference among the groups for each of the variables. Levene’s test for equality of variance was used to suggest the multiple comparison procedure to be used. If equality of variance among the four groups was suggested, multiple comparison procedures were performed (Bonferroni); if inequality of variance was suggested by Levene’s test, Tamhane multiple comparisons (which do not assume equal variance) were suggested, multiple comparison procedures were performed. Probability values < 0.05 were considered statistically significant (analysis was performed using SPSS for Windows, version 7.5.1).

**RESULTS**

**Distribution of PKC isoforms after burn injury.** PKC phosphorylation is a recognized mechanism of increasing kinase activity. Upon activation, PKC isoforms are not only phosphorylated but also shift from cell cytosol to cell membrane. Thus we examined distribution of PKC among membrane and cytosolic myocardial fractions, as well as PKC isoform phosphorylation. As shown in Fig. 1, myocardial fractions prepared from control hearts or hearts collected either 2, 4, or 24 h after burn injury were probed on Western blots with phospho-specific antibodies. Actin was used as a loading control. Figure 1 also includes densitometric analysis of several Western blots (n = 5 rats) from cytosolic and membrane fractions prepared at each time period (top band). Although cytosolic PKCα and PKCβ tended to fall 24 h postburn, these changes were not statistically significant (Fig. 1A, bottom). In contrast, densitometric analysis of five to six Western blots prepared from five to six rats at each time period (hearts not pooled) confirmed a significant increase in PKCδ, PKCε, and PKCα in the membrane fraction (Fig. 1B, bottom; P < 0.05). These data confirm that burn injury promoted phosphorylation and translocation of PKCδ, PKCε, and PKCα from cytosol to the membrane fraction.

**PKC isoform expression in isolated cardiomyocytes.** To further explore the role of phosphorylated PKC isoforms in myocardial responses to burn injury, we incubated aliquots of primary cardiomyocytes prepared from naive adult Sprague-Dawley rats in medium in the presence or absence of burn serum (10% by volume for 18 h); sham burn serum provided appropriate controls. Burn serum challenge of primary cardiomyocytes produced a significant translocation of PKCδ, PKCε, and PKCα to the membrane fraction (representative Western blots are shown in Fig. 2, top). The densitometric analysis of five to seven Western blots performed on the cytosol fractions prepared from five to six rats per experimental condition is shown in Fig. 2, middle, whereas densitometric analysis of the membrane fraction is shown in Fig. 2, bottom. These results suggest that in vitro burn serum challenge of adult naïve cardiomyocytes produces redistribution of PKC phosphorylated isoforms that closely resembles that confirmed in the myocardium after in vivo burn injury and shown in Fig. 1.

**Inhibition of myocyte PKC expression with calphostin.** We next examined the effects of the PKC inhibitor calphostin on burn serum-related redistribution of PKC isoforms in primary cardiomyocytes. As also shown in Fig. 2, pretreating cardiomyocytes with 50 nM calphostin for 30 min before burn serum challenge prevented the burn serum-related increase in PKCε translocation to the membrane, whereas burn serum-related redistribution of PKCδ and PKCα was not altered by calphostin pretreatment.

**Inhibition of myocyte PKC expression with PKCε inhibitory peptide.** Since calphostin appeared to exert a greater inhibitory effect on PKCε expression after burn serum challenge, we chose to repeat the experiment by pretreating myocytes with PKCε inhibitory peptide, a potent, stable, and specific inhibitor of PKCε. As shown in Fig. 3, burn serum challenge of primary cardiomyocytes produced a significant increase in PKCδ, PKCε, and PKCα translocation to the myocyte membrane fraction, whereas the cytosolic levels of PKC isoforms were not altered significantly. Pretreating aliquots of myocytes with PKCε inhibitory peptide for 30 min before burn serum challenge prevented the burn serum challenge-related increase in PKCε to the myocyte membrane fraction, whereas burn serum-mediated redistribution of PKCδ and PKCα remained significantly increased above control values (Fig. 3).

**Effects of PKC inhibition on cardiomyocyte secretion of cytokines.** The availability of this in vitro burn serum challenge model next allowed us to examine the role of PKC in several aspects of cardiomyocyte cytokine secretion and Ca²⁺ handling. Burn serum challenge in cardiomyocytes (Figs. 4 and 5) produced a significant increase in TNF-α (A), IL-1B (B), IL-6 (C), IL-10 (D), and nitric oxide (NO) secretion (E) compared with cytokine levels secreted by cardiomyocytes incubated in medium alone (control) or in the presence of sham serum (P < 0.05). Pretreating cardiomyocytes with calphostin C, a pharmacological strategy shown in our study to specifically inhibit PKCε activation, prevented burn serum-related increase in cytokine secretion by this cell population (Fig. 4). Addition of
calphostin in the presence of sham burn serum had no significant effect on cardiomyocyte cytokine secretion.

In addition, pretreatment of separate aliquots of primary cardiomyocytes with PKCε inhibitory peptide, an inhibitor of PKCε that has greater stability and potency than other inhibitors, prevented burn serum challenge-related cytokine secretion by myocytes (Fig. 5). Pretreatment of control myocytes in the absence of burn serum challenge (control vehicle) had no effect on cytokine secretion compared with control myocytes treated with vehicle (control + vehicle). These data suggest that PKCε is the isoform that regulates cardiomyocyte secretion of inflammatory cytokines.

**Effects of PKC inhibition on myocyte [Ca$$^{2+}$$] and [Na$$^{+}$$].** Burn serum challenge in cardiomyocytes produced a rise in [Ca$$^{2+}$$], (Figs. 6 and 7, top) and [Na$$^{+}$$], (Figs. 6 and 7, bottom) compared with [Ca$$^{2+}$$] and [Na$$^{+}$$] measured in cardiomyocytes incubated in either medium alone (control) or medium containing 10% sham burn serum. Pretreating the cardiomyocytes with either calphostin (Fig. 6) or PKCε inhibitory peptide (Fig. 7) before burn serum challenge prevented the burn...
serum-related accumulation of Ca$^{2+}$ and Na$^+$ by this cell population. Addition of calphostin or PKCε inhibitory peptide to cardiomyocytes in the absence of burn serum or in the presence of sham burn serum had no effect on either myocyte [Ca$^{2+}]_i$ or [Na$^+$].

**DISCUSSION**

PKC isoforms PKCδ, PKCε, and PKCα have been shown to be expressed in adult cardiomyocytes (9, 32, 36, 39). In the present study, either burn injury in vivo or burn serum challenge of cardiomyocytes in vitro produced a significant redistribution of PKCδ, PKCε, and PKCα, and a significant increase in phosphorylated membrane-bound PKC isoforms. In our study, pretreating isolated cardiomyocytes with calphostin, a nonspecific PKC inhibitor, prevented burn serum-related increase in membrane-associated PKCε, whereas PKCδ and PKCα isoform responses were unaffected by calphostin treatment. Of particular interest in the present study was the finding that calphostin pretreatment of cardiomyocytes and inhibition of PKCε redistribution was associated with reduced cardiomyocyte secretion of inflammatory cytokines. Additional experiments with PKCε inhibitory peptide, a potent, stable, and
Fig. 4. Inflammatory cytokine responses of isolated cardiomyocytes to burn serum challenge in the presence or absence of the PKC inhibitor calphostin. Responses include TNF-α (A), IL-1β (B), IL-6 (C), IL-10 (D), and total nitric oxide (E). All values are means ± SE. *P < 0.05, significant difference from control. †P < 0.05, significant calphostin-related effect compared with the response to burn serum treatment in the absence of PKC inhibition.
Fig. 5. Inflammatory cytokine responses of isolated cardiomyocytes to burn serum challenge in the presence or absence of PKCε inhibitory peptide. Responses include TNF-α (A), IL-1β (B), IL-6 (C), IL-10 (D), and total nitric oxide (E). All values are means ± SE. Each bar on the graph represents 6 aliquots of myocytes collected from 4–5 individual rats for a total of 24–30 cultures of myocytes used for each experimental condition. *P < 0.05, significant difference from control. †P < 0.05, a significant PKCε inhibitory peptide-related effect compared with the response to burn serum treatment in the absence of PKC inhibition.
highly specific inhibitor of the PKCe isoform, confirmed the calphostin data. PKCe inhibition with either calphostin or PKCe inhibitory peptide suppressed TNF-\(\alpha\) secretion by cardiomyocytes to a greater extent than PKCe inhibition reduced IL-1\(\beta\), IL-6, and IL-10. Whether this variable response is related to greater sensitivity of TNF-\(\alpha\) secretory mechanisms to PKCe modulation or to the dose of inhibitory peptide used in our study remains unclear and warrants further study. However, our data suggest that PKCe is the dominant isoform that modulates the postburn inflammatory signaling cascade in cardiomyocytes.

Our use of an in vitro system such as burn serum challenge in isolated cardiomyocytes provides an attractive means of examining cell signaling mechanisms by which a stimulus is transduced to the cell, producing an inflammatory response and secretion of inflammatory cytokines such as TNF-\(\alpha\) and IL-1\(\beta\). Burn serum challenge in cardiomyocytes eliminated the neurohormonal and endocrine responses that occur in the intact animal and allowed us to examine cell-specific responses to insult. Other advantages of our isolated cardiomyocyte model over the use of snap-frozen myocardial tissue include eliminating interactions between cardiomyocytes and nonmyocyte...
cell types that would be expected to confound data interpretation. Our cell preparations were 98% viable cardiomyocytes, and the remaining cells were identified as dead myocytes. A homogenous population of primary cardiomyocytes allowed better definition of signal transduction events and their downstream signaling events. Furthermore, we have shown previously that the primary cardiomyocyte cultures used in the present study exhibit responses to stimuli that are similar to in vivo cardiomyocyte responses (4, 15, 17, 19). Finally, we have shown that concentrations of inflammatory mediators such as TNF-α, IL-1β, IL-6, and NO in myocyte supernatants are severalfold greater than cytokine levels measured in serum after burn injury (4, 15, 17, 19). Thus these data suggest that cytokine levels measured in supernatants after myocyte challenge with burn serum are indeed myocyte-derived cytokines.

The role of PKC activation in inflammatory cytokine responses to burn injury has not, to our knowledge, been examined previously in cardiomyocytes. Our data confirm that a pharmacological intervention that specifically blocked PKCε activity in cardiomyocytes (calphostin or PKCε inhibitory peptide) prevented burn serum-mediated secretion of TNF-α, IL-1β, IL-6, NO, and IL-10. We have shown previously that stress-activated kinases such as mitogen-activated protein kinase (p38 MAPK), a kinase thought to function downstream of PKCε, is activated in burn trauma (2). Those previous studies confirmed that p38 MAPK activation promoted IκB phosphorylation, NF-κB nuclear translocation, and cardiomyocyte secretion of inflammatory cytokines (2, 4, 15). Thus it is likely that PKC may serve as an upstream modulator of the cell signaling cascade that modulates TNF-α, IL-1β, IL-6, and NO secretion by cardiomyocytes.

Cardiomyocytes used in this study had a viability of 98% before burn serum challenge. Exposure of the cardiomyocytes to burn serum produced a modest fall in cell viability (>98% at baseline and 92–95% at 18 h after burn serum challenge). Longer periods of cardiomyocyte exposure to burn serum have been shown to produce a gradual rise in supernatant creatine kinase levels and a progressive decrease in cell viability (15). Therefore, in the present study our incubation period was limited to 18 h. Myocytes retained their rod-shaped morphology, and cardiomyocyte borders and cellular striations were well defined after 18 h of incubation at 37°C. There was no evidence of cellular blebbing, necrosis, or apoptosis in cardiomyocytes used under the experimental conditions described.

That activation of PKCε is deleterious to myocardial function is not a new concept. PKC activation has been shown to reduce myocardial contractility, likely due to impaired mobilization of Ca²⁺ from the sarcoplasmic reticulum, altered Ca²⁺ flux through the slow channel, and altered binding of Ca²⁺ to contractile proteins (43). In the present study, burn serum challenge produced cardiomyocyte accumulation of Ca²⁺, a response that we have shown to be characteristic of in vivo burn injury (18, 42). That burn serum-related alterations in Ca²⁺ homeostasis were PKC dependent was evident from our finding that calphostin or PKCε inhibitory peptide pretreatment of cardiomyocytes prevented burn serum-related accumulation of Ca²⁺ and Na⁺.

Although the mechanisms by which a noninfectious injury such as burn trauma alters myocardial contractile function remain unclear, there is growing evidence that cardiomyocyte secretion of inflammatory cytokines such as TNF-α and IL-1β have profound negative inotropic effects on the heart (8, 10, 25, 28). The pleiotropic nature of these cytokines is well recognized, and TNF-α and IL-1β alter cardiomyocyte responsiveness to both β-adrenergic stimuli as well as non-β-adrenergic agonists (13). In addition, inflammatory cytokines have been shown to activate PKC in several cell types; thus cytokine secretion by cardiomyocytes may activate a positive feedback mechanism, exacerbating PKC activation in the cytokine-secreting cardiomyocytes or acting in a paracrine fashion to promote PKC activation in adjacent cardiomyocytes. The specific mechanisms by which PKCε may alter cardiomyocyte inflammatory cytokine responses remain unclear; however, PKC has been shown to regulate shedding of type I TNF receptors in some cell types, perhaps decreasing the paracrine action of myocyte secreted TNF on adjacent myocytes (26). Alternatively, PKC may regulate NF-κB activation, an essential signaling event in cytokine secretion (6).

The clinical relevance of our study is the fact that myocardial injury and dysfunction occur in several injury and disease states including burn injury, sepsis, ischemia reperfusion, as well as intestinal ischemia reperfusion; cardiac dysfunction has been described as a main determinant of morbidity and mortality in these injury states (1, 5, 18, 19, 20, 25, 27, 33, 34, 42). A better understanding of the cell signaling mechanisms by which an extracellular stimulus such as burn injury triggers a cellular cascade that produces cytokine secretion and organ dysfunction will allow the development of therapeutic strategies that support and maintain cardiac function.

**GRANTS**

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**REFERENCES**


