Cardiac Myocytes Activated by Septic Plasma Promote Neutrophil Transendothelial Migration
Role of Platelet-Activating Factor and the Chemokines LIX and KC

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Abstract—Cardiac myocytes isolated from rats with peritonitis (cecal ligation and perforation; CLP) promote PMN transendothelial migration. Herein, we assessed (1) the mechanisms involved in cardiac myocyte activation during peritonitis and (2) the means by which these activated myocytes promote PMN transendothelial migration. Plasma obtained from mice subjected to CLP (septic plasma) activated isolated cardiac myocytes as evidenced by (1) increased nuclear levels of nuclear factor-κB (NF-κB) and (2) their ability to promote PMN migration across endothelial cell monolayers. Pretreatment of septic plasma with an antibody against tumor necrosis factor-α (TNF-α), but not interleukin-1β (IL-1β), blunted the ability of septic plasma to activate the myocytes. However, septic plasma obtained from TNF-α-deficient mice could still activate the myocytes; an effect attenuated by an antibody against IL-1β. If the myocytes were pretreated with a proteasome inhibitor (MG 132) to prevent NF-κB activation, the myocyte-induced PMN transendothelial migration was compromised. The activated myocytes released platelet-activating factor (PAF), and myocyte-induced PMN migration was abrogated by a PAF receptor antagonist (WEB 2086). These myocytes also released the CXC chemokines LIX and KC; an event prevented by MG 132. Antibodies against LIX and KC abrogated the myocyte-induced PMN migration. However, LIX and KC, but not PAF, could promote PMN migration when used at concentrations produced by activated myocytes. These observations indicate that TNF-α and IL-1β are, in part, responsible for the ability of septic plasma to activate cardiac myocytes. The activated myocytes promote PMN transendothelial migration, an effect attributable to LIX and KC, and possibly, PAF. (Circ Res. 2004;94:944-951.)

Key Words: nuclear factor-κB • interleukin-1β • tumor necrosis factor-α • mice

Sepsis is a generalized systemic inflammatory response to a local severe infection resulting in remote organ injury and dysfunction.¹ The infectious insult results in the activation of myeloid cells, which subsequently produce cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and release them into the systemic circulation. These cytokines can activate circulating neutrophils (PMN) and the endothelial cells lining the blood vessels of various organs, converting both to a proadhesive phenotype.¹ This facilitates PMN accumulation within these organs (lung, liver, heart, etc), where they eventually extravasate and contribute to organ dysfunction.²⁻⁵ This inflammatory response is further amplified by the ability of circulating cytokines to activate the nuclear transcription factor nuclear factor-κB (NF-κB) in myeloid and endothelial cells.⁶⁻⁸ Activation of NF-κB results in the transactivation of various proinflammatory genes (eg, those encoding for cytokines and chemokines).⁹⁻¹¹ Thus, once the systemic inflammatory response is initiated the cascade of circulating inflammatory mediators continues to escalate, and if unabated, can result in multiple organ dysfunction and ultimately, death.¹²

One of the vital organs adversely affected during the clinical course of sepsis is the heart.¹³⁻¹⁵ Ventricular contractile function is compromised in septic patients; an event attributed to the cytokines TNF-α and IL-1β.¹⁴ Animal models of sepsis have provided some additional insights into the cardiac inflammation and dysfunction incurred in this pathology. For example, induction of peritonitis in rats results in PMN accumulation in the heart (increased tissue myeloperoxidase activity).¹²⁻¹⁵ The sepsis-induced PMN infiltration of the heart is associated with an impaired cardiac contractile activity.² Furthermore, cardiac myocytes isolated from these animals exhibit a proinflammatory phenotype. These myocytes (1) have elevated nuclear levels of NF-κB, (2) activate endothelial cells to express adhesion molecules, (3) promote PMN transendothelial migration, and (4) are proadhesive for PMN.²⁻¹⁶ With respect to the latter phenomenon, others have shown that cytokine-activated cardiac...
myocytes are proadhesive for PMN\textsuperscript{2,17–22} and that these adherent PMN induce an increase in myocyte oxidant stress and impair myocyte contractile function.\textsuperscript{18–20} Taken together, the in vivo and in vitro animal studies indicate that during the course of a systemic inflammatory response cardiac myocytes can become activated, promote PMN extravasation into the cardiac interstitium, and facilitate PMN adhesion to myocytes that ultimately results in PMN-mediated myocyte dysfunction.

The main objective of the present study was to assess the mechanisms involved in cardiac myocyte activation during peritonitis and the means by which these activated myocytes promote PMN transendothelial migration. To this end, we subjected mice to cecal ligation and perforation (CLP) to induce peritonitis and plasma obtained from these animals was used in an in vitro model of the vascular-interstitial interface. Using this model, we tested various aspects of the working hypothesis presented in Figure 1.

**Materials and Methods**

**Cells**

Neonatal cardiac myocytes and adult cardiac endothelial cells were isolated from mice (wild-type; C57BL6; Jackson Laboratories) and cultured as described previously.\textsuperscript{23} PMN were isolated from the bone marrow of the long leg bones as previously described.\textsuperscript{23} All animal protocols were reviewed and approved by the University of Western Ontario Committee on Animal Care.

**CLP Model**

Adult C57BL6 mice, anesthetized with ketamine (150 mg/kg BW) and xylazine (7.5 mg/kg BW) subcutaneously, were subjected to cecal ligation and perforation (CLP) to induce peritonitis and plasma obtained from these animals was used in an in vitro model of the vascular-interstitial interface. Using this model, we tested various aspects of the working hypothesis presented in Figure 1.

**Experimental Protocols and Assays**

Confluent myocyte cultures were treated with plasma from CLP (septic plasma) or sham-operated mice diluted in supplemented M199 (M199 with 10% FCS, 100 U/mL penicillin G, and 100 μg/mL streptomycin) for 4 hours and various assays performed.

**Role of TNF-α and IL-1β**

Myocytes conditioned with septic plasma (plasma from CLP mice) were washed with PBS, incubated for 60 minutes with M199, and the supernatants collected and used to assess PMN transendothelial migration as previously described.\textsuperscript{23,25}

Two general approaches were used to assess the role of TNF-α and IL-1β. In some experiments, affinity-purified polyclonal anti-

bodies directed to TNF-α and IL-1β (1 μg/mL; ID Labs) were added to septic plasma before incubation with the myocytes. In other experiments, septic plasma was obtained from TNF-α–deficient mice (C57BL6 background; Jackson Laboratories).

**Role of NF-κB**

NF-κB in nuclear extracts obtained from the myocytes was assessed using an electrophoretic mobility shift assay (EMSA) as previously described.\textsuperscript{2} In addition, to prevent NF-κB activation, myocytes were pretreated with 2.5 μg/mL of the proteasome inhibitor MG-132 for 30 minutes before experimental interventions. These experimental maneuvers included (1) PMN transendothelial migration and (2) myocyte generation of chemokines.

**Role of Platelet-Activating Factor (PAF) and Chemokines (LIX and KC)**

PAF concentrations in the supernatants from myocytes conditioned with SHAM or septic plasma were measured using a scintillation proximity assay (Amersham) according to the manufacturer’s instructions. This assay measures both the C16 and the C18 forms of PAF, which represent 70% of the PAF extracted from the bovine heart. LIX and KC concentrations within cardiac myocytes and in supernatants bathing the myocytes were quantified using an ELISA (ABC peroxidase system) as previously described.\textsuperscript{23} In another set of experiments, a PAF receptor antagonist (WEB 2086, 20 μg/mL; Boehringer-Ingelheim) or affinity-purified polyclonal antibodies directed to LIX and KC (1 μg/mL; ID Labs) was added to the supernatants obtained from myocytes activated by septic plasma prior to their use in the migration assays. In addition, PAF (Sigma) or the chemokines LIX plus KC (ID Labs Inc), were used in the migration assay.

**Statistical Analysis**

All values are expressed as mean±SEM. Statistical analysis was performed using an ANOVA and a Student’s t test with Bonferroni corrections for multiple comparisons. A value of \(P<0.05\) was considered to be significant.

**Results**

**Characterization of the In Vitro Model**

To determine an effective dilution of septic plasma to be used in our in vitro model, different dilutions of septic plasma were added to the myocyte monolayers and their effect on myocyte beating rate evaluated 4 hours later. As shown in Figure 2A, septic plasma reduced the beating rate of myocytes at dilutions of 1:50 and 1:20 in M199. A dilution of 1:20 represents an underestimate of the plasma dilution that is present in myocardial interstitial fluid.\textsuperscript{26} The decrease in myocyte beating rate was transient, ie, recovery to control
It has previously been shown that circulating levels of TNF-α and IL-1β are elevated in mice subjected to CLP.27–30 Thus, the contribution of these two cytokines to the ability of septic plasma to activate cardiac myocytes was assessed. Antibodies, directed to murine TNF-α and IL-1β, were added to septic plasma for 1 hour before exposing the myocytes to the plasma. As shown in Figure 3A, the combination of these two antibodies was effective in reducing the ability of septic plasma to activate cardiac myocytes with respect to their ability to promote PMN transendothelial migration (57% reduction). To evaluate the relative contribution of these two cytokines, we assessed the effects of the antibodies individually. As shown in Figure 3B, the antibody against TNF-α reduced the ability of septic plasma to induce a proinflammatory state in myocytes, ie, a 40% reduction in PMN transendothelial migration. By contrast, the anti–IL-1β antibody was without effect (Figure 3B).

To further probe for a role of TNF-α in the ability of septic plasma to activate myocytes, we used TNF-α–deficient mice. As shown in Figure 4A, plasma obtained from both wild-type and TNF-α–deficient mice subjected to CLP increased the ability of cardiac myocytes to promote PMN transendothelial migration to the same extent. Interestingly, addition of an antibody directed to IL-1β to plasma obtained from TNF-α–deficient mice subjected to CLP reduced the ability of this septic plasma to induce a proinflammatory phenotype in cardiac myocytes, ie, PMN transendothelial migration was reduced by 45% (Figure 4B).

**Role of NF-κB**

Nuclear extracts from myocytes exposed to septic plasma had increased levels of NF-κB as assessed by EMSA (Figure 5A, lane 2). Myocytes pretreated with the proteasome inhibitor MG-132 (to inhibit NF-κB activation) before being exposed to septic plasma had less NF-κB in their nuclei (Figure 5A, lane 3). Pretreatment of myocytes with MG-132 before stimulation with septic plasma reduced their ability to promote PMN transendothelial migration by 50%, indicating that NF-κB activation plays a role in this response (Figure 5B).

**Role of PAF**

PAF (or PAF-like compounds) are generated by a variety of cells in response to NF-κB activation and translocation to the nucleus.31 In the present study, supernatants from myocytes
conditioned with septic plasma had a 3-fold higher concentration of PAF than supernatants from myocytes conditioned with sham plasma (2.3 × 10^{-10} versus 0.7 × 10^{-10} mol/L; means of two experiments). In the present study, addition of the PAF receptor antagonist WEB 2086 to supernatants generated by myocytes conditioned with septic plasma completely blocked PMN transendothelial migration (Figure 6A). Interestingly, when PAF (C16 or C18) rather than supernatants, was added to the basal aspect of the endothelial cell monolayers, they only increased PMN transendothelial migration when used at 10^{-9} mol/L. (Figures 6B and 6C). However, when used at concentrations (10^{-10} mol/L) measured in supernatants from myocytes activated by septic plasma, they failed to do so.

**Role of LIX and KC**

Endotoxemia induces dramatic increases in myocardial LIX and KC message levels and only modest changes in MIP-2 mRNA. Thus, in the present study, we focused on LIX and KC. As shown in Figure 7A, myocytes conditioned with septic plasma had increased intracellular levels of both LIX and KC compared with myocytes stimulated with plasma obtained from sham-operated mice. Pretreatment of myocytes with MG-132 before conditioning with septic plasma significantly reduced the levels of both LIX and KC in the myocytes. Myocytes conditioned with septic plasma released significantly higher amounts of both KC (Figure 7B) and LIX (Figure 7C) into the supernatants compared with mice conditioned with plasma obtained from sham-operated mice. Pretreatment of myocytes with MG-132 significantly reduced the levels of both LIX and KC in the supernatants of myocytes conditioned with septic plasma.

The effects of antibodies directed to LIX and KC on the ability of supernatants from myocytes conditioned with septic plasma to promote PMN transendothelial migration were assessed. When used individually, both antibodies to LIX and KC decreased the ability of these supernatants to promote PMN transendothelial migration (Figure 8A). When used in combination, these antibodies completely prevented the PMN transendothelial migration induced by supernatants from myocytes conditioned with septic plasma. In addition, the combination of LIX and KC at concentrations measured in supernatants (2 and 12 ng/mL M199, respectively; Figure 7) promoted PMN transendothelial migration (Figure 8B).

Because pharmacological inhibition of PAF (Figure 6A) or immunoneutralization of the chemokines (Figure 8A) prevented the PMN transendothelial migration induced by supernatants from activated myocytes, we assessed whether there may be an interaction between these two systems. The PAF receptor antagonist WEB 2086 did not affect the chemokine-induced PMN transendothelial migration (Figure 8B). The WEB compound also did not prevent chemokine production by activated myocytes (data not shown).

**Discussion**

Studies in humans and animals indicate that during sepsis one of the vital organs adversely affected is the heart. There is a growing body of evidence indicating that during the
course of a systemic inflammatory response, such as sepsis, cardiac myocytes can become activated, promote PMN extravasation into the cardiac interstitium, and facilitate PMN adhesion to myocytes, which ultimately leads to myocyte dysfunction. Herein, we used a reductionist approach using an in vitro model of the cardiac vascular-interstitial interface to identify some of the mediators involved in the sepsis-induced PMN emigration into the cardiac interstitium. We provide evidence that two cytokines, TNF-α and IL-1β, present in plasma from septic (CLP) mice are partially responsible for the ability of this septic plasma to activate cardiac myocytes. The activation of myocytes by septic plasma involves (1) an increase in their nuclear levels of NF-κB and (2) an enhanced ability to promote PMN transendothelial migration. Further, we demonstrate that the activated myocytes release PAF and the two chemokines LIX and KC into the external milieu (supernatants). We also provide strong evidence that the chemokines are responsible for the ability of the activated myocytes to promote PMN transendothelial migration, whereas the role of PAF appears equivocal.

It is generally accepted that the cytokines TNF-α and IL-1β are elevated in the plasma of both septic patients and animals subjected to CLP. Both TNF-α and IL-1β have been shown to produce negative inotropic effects in the heart. Finally, blockage of both TNF-α and IL-1β attenuated the development of cardiac dysfunction in a CLP model of sepsis. In the present study, we show that the ability of septic plasma to activate cardiac myocytes with respect to PMN transendothelial migration can be attributed to TNF-α, but not IL-1β (Figure 3). However, septic plasma from TNF-α deficient (TNF-α−/−) mice was just as efficient in activating the myocytes (Figure 4A). These latter observations are consistent with studies showing that TNF-α−/− mice develop sepsis in response to CLP to a similar degree to that of the wild-type controls. Interestingly, we noted that IL-1β played a role in the ability of septic plasma from TNF-α−/− mice to activate myocytes with respect to their ability to promote PMN transendothelial migration (Figure 4B). These apparently discordant observations may be explained by a "redundancy" in the cytokine system, ie, when a dominant cytokine is removed, another can take its place to achieve the same effect.
resides in the cytoplasm in association with IκB and promote PMN transendothelial migration. role in the ability of septic plasma to induce myocytes to release PAF into the external milieu (supernatants). Further, a PAF receptor antagonist (WEB 2086) completely prevented the production and secretion of both of these chemokines; an effect attenuated by the proteasome inhibitor (Figure 7). Furthermore, a combination of antibodies directed to LIX and KC completely prevented the PMN transendothelial migration induced by activated myocytes (Figure 8A). Finally, the two chemokines, per se, were capable of promoting PMN transendothelial migration when used at concentrations present in supernatants from activated myocytes (Figure 8B). Collectively, these findings strongly indicate that these two chemokines play a critical role in the PMN transendothelial migration noted in our in vitro model.

Mice lack a CXCR1 receptor and, thus, murine CXC chemokines interact with a receptor homologous to the human CXCR2 to promote chemotaxis. In our in vitro system, PMN transendothelial migration occurred in the presence of only two cell types: endothelial cells and PMN. LIX and KC may induce PMN emigration via an action on the endothelial cells and/or PMN. Because murine endothelial cells reportedly do not express the CXCR2 receptor, it seems likely that the two chemokines are interacting with CXCR2 receptors on PMN.

It is tempting to speculate that our findings that the CXC chemokines LIX and KC play a pivotal role in PMN transendothelial migration induced by cardiac myocytes conditioned with septic plasma may be relevant to sepsis in humans. LIX appears closely related to human ENA-78 (epithelial cell-derived neutrophil-activating peptide-78) and GCP-2 (granulocyte chemotactic protein-2), whereas KC appears to be closely related to human GRO-α (growth related oncogene-α). However, the potential targeting of chemokines as a therapeutic regimen for sepsis may not be fruitful for the following reasons. First, it is not entirely clear whether a given CXC chemokine has a specific counterpart in the human chemokine family. Second, the CXC chemokines apparently are differentially expressed in various organs during endotoxemia, ie, MIP and KC predominate in the lung, whereas LIX and KC predominate in the heart. Third, redundancies may exist with respect to the actions of various CXC chemokines. A more fruitful avenue to pursue for the purpose of therapeutic intervention in sepsis in humans may be to target the CXC receptors.

In the present study, septic plasma increased myocyte nuclear levels of the transcription factor, NF-κB (Figure 5A). These results are consistent with our previous studies in which we showed that cardiac myocytes isolated from rats subjected to CLP had elevated levels of NF-κB in their nuclei. Interestingly, both TNF-α and IL-1β are potent activators of NFκB. In quiescent cells, NF-κB usually resides in the cytoplasm in association with IκB, an inhibitory protein that prevents the translocation of NF-κB to the nucleus. On activation of cells by cytokines, IκB is degraded by the proteasome pathway allowing NF-κB to translocate to the nucleus. In the present study, the proteasome inhibitor MG132 largely prevented (1) the increase in cardiac myocyte nuclear NF-κB and (2) the myocyte-induced PMN transendothelial migration (Figure 5). These latter observations indicate that NF-κB plays an important role in the ability of septic plasma to induce myocytes to promote PMN transendothelial migration.

Activation of NF-κB can transactivate the gene encoding for phospholipase A2, the initial enzyme involved in the remodeling pathway of PAF synthesis. In the present study, we show that myocytes conditioned with septic plasma can release PAF into the external milieu (supernatants). Further, a PAF receptor antagonist (WEB 2086) completely prevented the PMN transendothelial migration induced by cardiac myocytes activated by septic plasma (Figure 6A). These observations are in agreement with our previous studies in which a PAF antagonist prevented the PMN transendothelial migration induced by myocytes isolated from the hearts of rats exposed to CLP. However, a disconcerting observation was that PAF, per se, could not promote PMN transendothelial migration when used at concentrations measured in the supernatants from myocytes activated by septic plasma (Figures 6B and 6C). Collectively the data obtained in the present study with respect to the role of PAF appear to be equivocal. Further studies are warranted to more firmly support or refute a role for PAF in the myocyte-induced PMN transendothelial migration.

NF-κB also transactivates genes encoding for the chemokines LIX and KC. In the present study, activation of cardiac myocytes by septic plasma resulted in increased production and secretion of both of these chemokines; an effect attenuated by the proteasome inhibitor (Figure 7). Furthermore, a combination of antibodies directed to LIX and KC completely prevented the PMN transendothelial migration induced by activated myocytes (Figure 8A). Finally, the two chemokines, per se, were capable of promoting PMN transendothelial migration when used at concentrations present in supernatants from activated myocytes (Figure 8B). Collectively, these findings strongly indicate that these two chemokines play a critical role in the PMN transendothelial migration noted in our in vitro model.

Figure 8. A. Addition of antibodies directed against LIX or KC to supernatants obtained from myocytes stimulated with septic plasma significantly reduced PMN transendothelial migration. Addition of both antibodies, concomitantly, to supernatants from myocytes conditioned with septic plasma completely blocked PMN transendothelial migration. FI indicates fold increase, n=6. *P<0.05 compared with SHAM; **P<0.05 compared with CLP.

B. Addition of LIX and KC in M199 (2 and 12 ng/mL, respectively) increased PMN transendothelial migration. PAF antagonist WEB 2086 at 20, 40, and 80 μg/mL did not affect the ability of the chemokines to induce PMN migration; n=4.
The observation that either a PAF antagonist or antibodies to LIX and KC were able to completely block the PMN transendothelial migration induced by the activated myocytes deserves comment. PAF and the chemokines could be acting in parallel. PMN transendothelial migration is dependent on both the activation of the effector cells and the establishment of a chemotactic gradient. PAF is a very potent activator of both PMN and endothelial cells. The chemokines are poor activators of these cells, but stimulate chemotaxis. Thus, it is possible that the PAF receptor antagonist prevents the activation step, whereas the antibodies directed to LIX and KC prevent the establishment of a chemotactic gradient. This explanation for our findings is not without caveats. CXC chemokines are also capable of activating PMN; KC has been shown to increase surface levels of CD11b on PMN. In addition, previous studies indicate that perfusion of the isolated cardiac myocytes with respect to (1) an increase in plasma) was used in our model of the cardiac vascular-interstitial interface. WEB 2086 neither interfered with chemokine production nor their ability to promote PMN transendothelial migration. Alternatively, the WEB compound may be interfering with chemokine interaction with PMN. In short, the exact explanation for the ability of both the PAF antagonist and the chemokine antibodies to completely block PMN transendothelial migration remains speculative.

In summary, using a reductionist approach, we have identified some of the potential mediators involved in the sepsis-induced PMN emigration into the heart. Mice were exposed to CLP to induce sepsis, and the plasma (septic plasma) was used in our model of the cardiac vascular-interstitial interface. We show that this septic plasma can activate isolated cardiac myocytes with respect to (1) an increase in nuclear NF-κB and (2) their ability to promote PMN transendothelial migration. This ability of septic plasma to activate myocytes can be attributed, in part, to TNF-α and IL-1β. The PMN transendothelial migration induced by the activated myocytes is dependent on the production and secretion of the chemokines LIX and KC by the activated myocytes. A role for myocyte-derived PAF remains to be established.

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References


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