REVIEW / SYNTHÈSE

Nitric oxide and calcium signaling regulate myocardial tumor necrosis factor-α expression and cardiac function in sepsis[1]

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Abstract: Myocardial tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, is a critical inducer of myocardial dysfunction in sepsis. The purpose of this review is to summarize the mechanisms through which TNF-α production is regulated in cardiomyocytes in response to lipopolysaccharide (LPS), a key pathogen-associated molecular pattern (PAMP) in sepsis. These mechanisms include Nox2-containing NAD(P)H oxidase, phospholipase C (PLC)γ1, and Ca^{2+} signaling pathways. Activation of these pathways increases TNF-α expression via activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK). Conversely, activation of c-Jun NH_2-terminal kinase 1 (JNK1) negatively regulates TNF-α production through inhibition of ERK1/2 and p38 MAPK activity. Interestingly, endothelial nitric oxide synthase (eNOS) promotes TNF-α expression by enhancing p38 MAPK activation, whereas neuronal NOS (nNOS) inhibits TNF-α production by reducing Ca^{2+}-dependent ERK1/2 activity. Therefore, the JNK1 and nNOS inhibitory pathways represent a "brake" that limits myocardial TNF-α expression in sepsis. Further understanding of these signal transduction mechanisms may lead to novel pharmacological therapies in sepsis.

Key words: sepsis, cardiac dysfunction, tumor necrosis factor-α, nitric oxide, calcium, signal transduction.

Résumé : Le facteur de nécrose tumorale α (TNF-α) myocardique, une cytokine proinflammatoire, est un inducteur important de dysfonction myocardique au cours de la sepsie. La présente synthèse a pour but de résumer les mécanismes de régulation de la production de TNF-α dans les cardiomyocytes en réponse au lipopolysaccharide (LPS), une structure moléculaire clé associée aux pathogènes (PAMP) durant la sepsie. Ces mécanismes comprennent les voies de signalisation de la NAD(P)H oxydase Nox2, de la phospholipase C (PLC)γ1 et du Ca^2. L'activation de ces voies augmente l'expression de TNF-α par l'activation de ERK1/2 et de p38 MAPK. Inversement, l'activation de la protéine JNK1 cause une régulation négative de la production de TNF-α par l'inhibition de l'activité de ERK1/2 et de p38 MAPK. Fait intéressant, la monoxyde d'azote synthase endothéliale (eNOS) favorise l'expression de TNF-α en stimulant l'action de p38 MAPK, alors que la NOS neuronale (nNOS) l'inhibe en réduisant l'activité de ERK1/2 dépendante du Ca^{2+}. Ainsi, les voies inhibitrices de JNK1 et de nNOS représentent une « pause » qui limite l'expression du TNF-α myocardique durant la sepsie. Une meilleure compréhension de ces mécanismes de transduction des signaux pourrait conduire à de nouveaux traitements pharmacologiques durant la sepsie.

Mots-clés : sepsie, dysfonction cardiaque, facteur de nécrose α, monoxyde d'azote, calcium, transduction du signal.

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Introduction

Sepsis, a systemic inflammatory response syndrome (SIRS) to infection, is a common clinical problem that occurs in 2%–11% of all hospital or intensive care unit admissions (Angus and Wax 2001). Sepsis, severe sepsis, and septic shock represent increasingly grave stages. Septic patients normally have significant cardiac morbidity. Studies have shown that 40%–50% of patients with prolonged septic shock develop myocardial depression, as defined by a reduced ejection fraction. Myocardial dysfunction renders septic patients at high risk of developing multiorgan failure, which is associated with a high mortality (Court et al. 2002). The reported mortality rate is 20%–30% in sepsis and 40%–80% in septic shock (Angus and Wax 2001).

Endotoxins or lipopolysaccharide (LPS) are the major pathogen-associated molecular patterns (PAMP) responsible for myocardial depression during sepsis (Natanson et al. 1989; Suffredini et al. 1989). Cardiovascular changes in-

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duced by LPS were virtually identical to the changes that occur during sepsis in both research animals (Natanson et al. 1989) and human volunteers (Kumar et al. 2005; Suffredini et al. 1989). The inhibitory effect of LPS on cardiac function is mediated through the production of proinflammatory cytokines, such as interleukin-1β (IL-1β), IL-4, and tumor necrosis factor-α (TNF-α) (Parrillo et al. 1985). Among these cytokines, TNF-α has been proposed as one of the major factors (Natanson et al. 1989; Peng et al. 2003*a*). Indeed, systemic administration of TNF-α results in manifestations that are very similar to those seen in sepsis, including hypotension, metabolic acidosis, diffuse pulmonary infiltration, pulmonary and gastrointestinal hemorrhage, tubular necrosis, and death (Tracey et al. 1986). More importantly, TNF-α impairs cardiac contractile function in intact animals, isolated hearts, and cardiomyocytes (Bozkurt et al. 1998; Grandel et al. 2000; Oral et al. 1997). LPS-induced myocardial depression is completely abrogated in the presence of TNF-α antiserum (Grandel et al. 2000). Furthermore, administration of TNF-α-binding proteins preserves myocardial function in endotoxemic mice (Peng et al. 2003*b*) and rats (Meng et al. 1998) and increases survival (Peng et al. 2003*b*). A major mechanism by which TNF-α induces myocardial dysfunction is through alteration of Ca^{2+} homeostasis. Specifically, TNF-α disrupts Ca^{2+} influx through L-type Ca^{2+} channels and Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (Krown et al. 1995). In addition to Ca^{2+} dysregulation, TNF-α also causes direct cytotoxicity, oxidative stress, disruption of excitation–contraction coupling, upregulation of other cardiac suppressing cytokines (e.g., IL-1β), and induction of cardiomyocyte apoptosis (Song et al. 2000; Zhao et al. 2006).

Studies have shown that TNF-α-induced myocardial dysfunction has 2 distinct phases. The early phase, which occurs within minutes after TNF-α exposure, is related to sphingosine production leading to disturbances of intracellular Ca^{2+} homeostasis (Krown et al. 1995; Yokoyama et al. 1993). The late phase, which occurs hours after TNF-α exposure, is mediated by inducible nitric oxide synthase (iNOS) expression, apoptosis (Song et al. 2000; Stein et al. 1996), and inhibition of both pyruvate dehydrogenase activity and mitochondrial function (Li et al. 2001; Zell et al. 1997).

TNF-α causes cardiac dysfunction through autocrine and paracrine mechanisms in sepsis. Macrophages and monocytes are the predominant source of circulating TNF-α in response to LPS stimulation (Schlag et al. 1991). In the myocardium, however, cardiomyocytes themselves are the major local source of TNF-α (Grandel et al. 2000; Kapadia et al. 1995; Peng et al. 2003*a*). Thus, myocardial TNF-α production plays a key role in cardiac dysfunction during sepsis (Peng et al. 2003*b*). It has been well established that LPS binds to LPS-binding protein (LBP), which presents LPS to CD14, a membrane glycoprotein with a glycosylinositol tail. With the assistance of MD-2, the LPS–LBP–CD14 complex activates Toll-like receptor (TLR)4, which is the LPS receptor. Activation of TLR4 leads to the downstream activation of signaling pathways and causes the production of TNF-α (Monick and Hunninghake 2003). This review focuses on the molecular mechanisms by which LPS induces TNF-α expression in cardiomyocytes and on the role of NO and calcium signaling in the regulation of myocardial TNF-α expression and cardiac function in sepsis.

Effect of mitogen-activated protein kinases on cardiac TNF-α expression

Mitogen-activated protein kinases (MAPKs) are a group of serine/threonine protein kinases that are highly conserved across eukaryotic species. There are 3 well-characterized subfamilies: p38, extracellular signal-regulated kinases 1 and 2 (ERK1/2), and c-Jun NH_2-terminal kinases (JNKs). MAPK pathways are activated by a dual-specificity serine/threonine MAPK kinase (MKK) that, in turn, is phosphorylated and activated by an upstream MAPK kinase kinase (MKKK) at 2 serine residues. The phosphorylation of the threonine and tyrosine residues on MAPKs results in a conformational change of the protein that increases substrate accessibility and catalysis (Cobb and Goldsmith 2000). Activated MAPKs can phosphorylate and activate a wide range of downstream targets, such as protein kinases and transcription factors, which lead to changes in gene transcription (Davis 1993; Hazzalin and Mahadevan 2002). In addition to transcriptional regulation, MAPKs can also modulate the expression of targeted genes by altering mRNA stability, transport, and translation (Dong et al. 2002). These signaling cascades are not only involved in normal cellular process, but have also been implicated in pathology of many diseases, such as cancer, atherosclerosis, diabetes, arthritis, and septic shock (Liu et al. 2007).

p38 MAPK

To date, 5 p38 MAPK isoforms have been identified: p38α, p38β, p38β2, p38γ, and p38δ. The expression of p38α and p38β is ubiquitous, whereas the expression of p38γ and p38δ is tissue-specific. p38γ is synthesized only in skeletal muscle and p38δ is expressed in lung, kidney, testis, pancreas, and small intestine (Ono and Han 2000). The p38 MAPK pathway is known to be responsible for a multitude of cellular events, such as cell growth, proliferation, differentiation, inflammation, and death (Herlaar and Brown 1999). Recently, p38 MAPK has been recognized as an important regulator of heart function in various animal models. For example, transgenic mice with targeted activation of p38 MAPK in cardiomyocytes have impaired systolic and diastolic functions of the heart (Liao et al. 2001). In this study, p38 MAPK was activated in the heart during myocardial ischemia and reperfusion. Furthermore, deletion of one allele of the p38α MAPK gene significantly decreased infarct size after myocardial ischemia and reperfusion (Otsu et al. 2003). In spontaneously hypertensive stroke-prone rats receiving a high-salt and high-fat diet, myocardial p38 MAPK was activated during the development of cardiac hypertrophy, and this increased activity of p38 resulted in hypertensive end-organ damage and premature mortality (Behr et al. 2001). Moreover, accumulating evidence suggests a key role for p38 MAPK activation in cardiac TNF-α expression and myocardial dysfunction in sepsis. For instance, LPS has been shown to increase p38 MAPK phosphorylation in cultured neonatal cardiomyocytes (Peng et al. 2003*a*, 2003*b*, 2005*a*, 2005*b*). Pharmacological inhibition of p38 MAPK or specific downregulation of p38α significantly sup-

pressed both TNF-α mRNA and protein levels in cardiomyocytes (Peng et al. 2003*b*). Consistent with these in vitro studies, p38 MAPK has also been shown to be activated during sepsis and to induce downstream myocardial TNF-α production. Furthermore, inhibition of p38 MAPK activity significantly reversed LPS-induced left ventricular depression and decreased mortality to 65% in endotoxemia (Peng et al. 2003*b*). Thus, p38 MAPK activation represents an important mechanism leading to increased myocardial TNF-α production and cardiac dysfunction during endotoxemia.

ERK1/2

Phosphorylation of ERK1 and ERK2 MAPKs (p44 and p42 MAPK, respectively) are catalyzed by MEK1/2, which in turn are activated by Raf-1. The ERKs can phosphorylate transcription factors, such as Elk-1, SAP1, Ets, and nuclear factor-κB (NF-κB), thereby regulating expression of proteins required for a given response. Activation of ERK1/2 results in the induction of TNF-α expression during sepsis in several cell types, including cardiomyocytes (Peng et al. 2005*a*, 2005*b*; Rosengart et al. 2000). Indeed, ERK1/2 is phosphorylated transiently but dramatically by LPS stimulation in cardiomyocytes. Inhibition of ERK1/2 by ERK short interfering RNA (siRNA) or pharmacological inhibitors such as PD98059 and U0126 abrogates TNF-α production in response to LPS (Geoghegan-Morphet et al. 2007; Peng et al. 2005*b*), suggesting an important role for ERK1/2 in LPS-induced TNF-α expression. Furthermore, inhibition of ERK1/2 by intraperitoneal treatment of U0126 decreases TNF-α production and protects the lung from LPS-induced injury in mice (Schuh and Pahl 2009). However, the in vivo effects of ERK inhibition on myocardial TNF-α expression and cardiac function in sepsis remain to be investigated.

JNKs

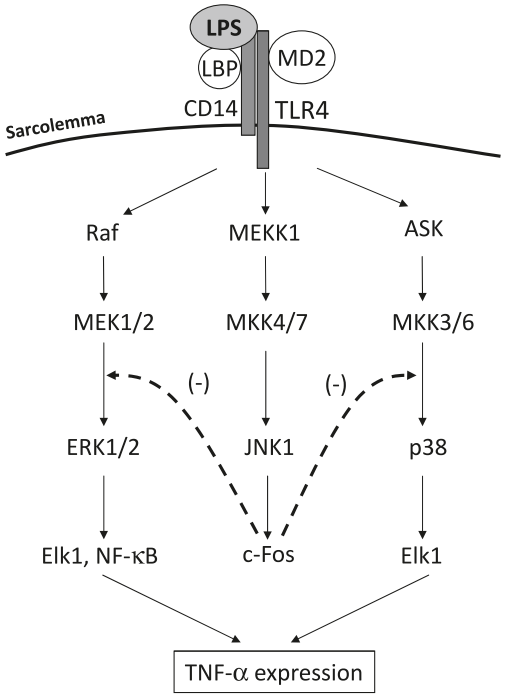
There are 3 JNK isoforms: JNK1, JNK2, and JNK3. JNKs are activated by MKK4 and MKK7. All 3 JNK isoforms are detectable in the heart (Ip and Davis 1998; Strait and Samarel 2000). LPS activates JNK and increases TNF-α mRNA and protein in macrophages and monocytes (Comalada et al. 2003; Swantek et al. 1997). LPS also increases phosphorylation of JNK1 in cultured mouse neonatal cardiomyocytes (Peng et al. 2005*a*, 2009). Unexpectedly, JNK1 activation decreased TNF-α expression in these cells (Peng et al. 2009). Deficiency of *JNK1* gene or inhibition of JNK1 signaling by overexpression of a dominant negative mutant of *JNK1* enhanced LPS-induced TNF-α expression in cardiomyocytes. In addition, blockage of the JNK1 signaling pathway also enhanced the production of reactive oxygen species (ROS) stimulated by LPS (Peng et al. 2009), which may be caused by the upregulation of TNF-α levels (Garg and Aggarwal 2002). Increased ROS production and TNF-α expression may lead to myocardial depression. Indeed, heart work and rate of contraction were further decreased during endotoxemia in *JNK1*$^{-/-}$ mice compared with wild-type animals (Peng et al. 2009). These data demonstrated that JNK1 activation inhibits myocardial TNF-α expression and improves cardiac function in sepsis. The apparent opposing effects between JNK1 and ERK/p38 on TNF-α expression suggest a cross-talk among the MAPK subfamily members in cardiomyocytes during sepsis.

Cross-talk among MAPKs

Cross-talk among different MAPK signaling pathways may determine the final biological response to stimulation, and the nature of the cross-talk varies depending on the cell type and particular stimulus. For example, transforming growth factor-β (TGF-β)-stimulated ERK1/2 activation caused dephosphorylation of LPS-induced p38 MAPK in macrophages (Xiao et al. 2002*b*). Conversely, activation of p38 by adenoviral expression of MKK3β resulted in dephosphorylation of MEK1/2 via phosphatase 2A in human skin fibroblasts (Junttila et al. 2008; Li et al. 2003). Additionally, a negative cross-talk between JNK and ERK1/2 was reported in COS-7 cells in response to activation by mixed lineage kinases (Shen et al. 2003).

In cardiomyocytes, activation of different MAPKs has been shown to result in opposing effects in sepsis. In this regard, activation of ERK1/2 and p38 MAPKs results in TNF-α production, whereas activation of JNK1 MAPK inhibits TNF-α expression during LPS stimulation (Peng et al. 2009). These opposite effects of MAPKs on LPS-induced TNF-α expression prompted us to further study the interactions among the various MAPKs in response to LPS. In fact, we showed that deficiency in JNK1 enhances LPS-induced ERK1/2 and p38 MAPK activity and TNF-α expression in cardiomyocytes, suggesting a JNK1-mediated negative feedback regulation of ERK1/2 and p38 MAPK activity (Peng et al. 2009). Furthermore, the inhibitory effects of JNK1 are mediated by c-*fos*, an immediate-early response gene involved in a wide spectrum of cellular responses, including cell proliferation, growth, and cytokine production (Herrera and Robertson 1996; Lee et al. 2004). The expression of c-*fos* is enhanced by LPS stimulation in cardiomyocytes in vitro and in myocardium in vivo, but it is disrupted by an adenovirus carrying a dominant negative mutant of JNK1 and by a JNK pharmacological inhibitor (SP600125), suggesting c-*fos* is downstream of JNK1 (Meng et al. 1998; Peng et al. 2009). Additionally, overexpression of c-*fos* decreased TNF-α production associated with reductions in ERK1/2 and p38 activity. More importantly, deficiency in c-*fos* resulted in a further decrease in cardiac function during endotoxemia (Peng et al. 2009). These results demonstrated the inhibitory effects of c-*fos* on ERK1/2 and p38 activity and TNF-α expression, leading to improvement in cardiac function during endotoxemia. It is well known that c-Fos can dimerize with c-Jun to form activator protein-1 (AP-1), which upregulates transcription of a diverse range of genes by binding to the AP-1 consensus sites in their promoter regions. However, the effects of c-*fos* in cardiomyocytes are independent of AP-1, as neither overexpression of a dominant negative mutant c-*fos* adenoviral vector that blocks the formation of c-Fos/c-Jun complexes nor treatment with an AP-1 oligodeoxynucleotide decoy were found to alter TNF-α expression (Peng et al. 2009). Therefore, the inhibitory effect of JNK1 on LPS-induced TNF-α expression is due to a c-*fos*-mediated negative cross-talk with ERK1/2 and p38 signaling pathways (Fig. 1). Activation of ERK1/2 and p38 activates downstream transcriptional factors leading to TNF-α expression.

Fig. 1. Cross-talk among MAPKs regulates cardiomyocyte TNF-α expression induced by LPS. LPS through binding to its receptor TLR4 activates JNK1, ERK1/2, and p38 MAPKs. Activation ERK1/2 and p38 enhances LPS-induced TNF-α production, whereas activation of JNK1 shows an inhibitory effect. The negative action of JNK1 is mediated via c-Fos by attenuating the activity of ERK1/2 and p38 MAPK during LPS stimulation. ASK, apoptosis signal-regulating kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; TLR, Toll-like receptor; TNF, tumor necrosis factor.



Nuclear factor-κB and cardiac TNF-α expression

Nuclear factor (NF)-κB is a multisubunit transcription factor composed of members of the Rel/p50 family of polypeptides. The most frequent form of NF-κB is a dimer composed of 2 DNA-binding proteins, namely NF-κB (or p50) and RelA (or p65), although other dimeric combinations also exist (Valen et al. 2001) Under physiological conditions, NF-κB is constitutively located in the cytoplasm in an inactive state bound to an inhibitory protein, IκBα. This inhibitory subunit can be considered as a cytoplasmic anchor, as it prevents the nuclear translocation of NF-κB. Activation of NF-κB involves the degradation of the inhibitory subunit IκBα from the cytoplasmic complex. This releases NF-κB, which then translocates to the nucleus, binds to promoter/enhancer regions of genes containing NF-κB response elements, and recruits proteins such as coactivators and RNA polymerases to promote transcription of target genes (Valen et al. 2001). Activation of NF-κB is associated with numerous pathological conditions, including inflammation and septic shock (Courtois and Gilmore 2006; Karin and Greten 2005).

LPS induces NF-κB activation in cardiomyocytes (Hall et al. 2005; Peng et al. 2005*a*). Selective inhibition of NF-κB activation using dominant negative adenoviral constructs decreases LPS-induced TNF-α expression, demonstrating an important role for NF-κB in this process (Hall et al. 2005). NF-κB activation is downstream of MAPK in the LPS-induced signaling in cardiomyocytes, as inhibition of ERK1/2 and JNK decreases NF-κB activation during LPS stimulation (Peng et al. 2005*a*).

NAD(P)H oxidase and cardiac TNF-α expression

NAD(P)H oxidase is a multicomponent enzyme system that catalyzes the formation of superoxide (O_2^-) from oxygen and NADPH (Babior 1999). The enzyme complex is composed of 2 membrane-bound subunits ($gp91^{phox}$ and $p22^{phox}$, which form flavocytochrome b_{558}, also known as the catalytic core), 3 cytosolic proteins ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$, which form the cytosolic complex), and Rac GTPase. Under basal conditions, the cytosolic complex is separated from the membrane-bound flavocytochrome b_{558}. Upon stimulation, NAD(P)H oxidase activation is triggered by the phosphorylation of the cytosolic phox (phagocyte oxidase) proteins and their translocation with Rac to the membrane-bound flavocytochrome b_{558} to assemble into an active oxidase. NAD(P)H oxidase expression has been demonstrated in cardiomyocytes (Mohazzab et al. 1997; Xiao et al. 2002*a*) and is a major source of O_2^- in the myocardium under pathophysiological conditions (Bendall et al. 2002; Li et al. 2002; Mohazzab et al. 1997; Xiao et al. 2002*a*). For instance, O_2^- production and NAD(P)H oxidase activity were markedly increased in cardiomyocytes in response to LPS stimulation. NAD(P)H oxidase inhibitors, such as diphenyleneiodonium (DPI) and apocynin, decreased LPS-induced TNF-α expression (Peng et al. 2005*b*). Taken together, these data suggest that NAD(P)H oxidase mediates cardiomyocyte TNF-α production induced by LPS.

There are at least 6 homologues of $gp91^{phox}$ (Nox2): Nox1, Nox3, Nox4, Nox5, and Duox1/2 (Lambeth et al. 2000; Leto and Geiszt 2006). Nox2 and Nox4 are expressed in cardiomyocytes in response to angiotensin II and pressure overload, respectively (Byrne et al. 2003). Our recent studies demonstrated that both O_2^- production and TNF-α production were blunted in $Nox2^{-/-}$ cardiomyocytes in response to LPS. Furthermore, deficiency of Nox2 also restored myocardial function at both early (4 h) and late (24 h) stages of endotoxemia (Peng et al. 2005*b*). These studies demonstrated a key role for Nox2-containing NAD(P)H oxidase in myocardial TNF-α expression and cardiac dysfunction during sepsis. A recent study by our laboratory has shown that Nox2-containing NAD(P)H oxidase mediates these effects through regulation of MAPK activity, as pharmacological inhibition of NAD(P)H oxidase blocked phosphorylation of ERK1/2, p38, and JNK MAPK induced by LPS stimulation (Peng et al. 2005*a*, 2005*b*).

NAD(P)H oxidase is also involved in the expression of cyclooxygenase (COX). COX is the rate-limiting enzyme that catalyzes the oxygenation and reduction of arachidonic acid, leading to the formation of cyclic endoperoxides and prostaglandins (PG), including PGE_2, PGI_2, and thromboxane A_2 (Grandel et al. 2000; Höcherl et al. 2002; Liu et al. 1996; Peng et al. 2005*a*). COX has 2 isoforms,

the constitutive COX-1 and an inducible COX-2. The full expression of COX-2 requires NF-κB activity (Rhee and Hwang 2000). Studies have shown that COX-2 is induced during sepsis and facilitates TNF-α expression in the heart (Grandel et al. 2000; Liu et al. 1996). Therefore, upregulation of COX-2 enhances myocardial TNF-α expression and decreases cardiac function during sepsis. Furthermore, we have recently demonstrated that an NAD(P)H oxidase/MAPK/NF-κB-dependent pathway results in induction of COX-2 expression in LPS-stimulated cardiomyocytes (Peng et al. 2005*a*). Indeed, inhibition of either NAD(P)H oxidase or MAPK activity prevented both NF-κB activation and COX-2/PGE_2 production induced by LPS (Peng et al. 2005*a*). Thus, O_2^- produced from NAD(P)H oxidase promotes LPS-induced TNF-α expression via a MAPK/NF-κB/COX-2 signaling pathway in cardiomyocytes.

Phospholipase Cγ and cardiac TNF-α expression

Phospholipase C (PLC) is a family of enzymes that cleaves phospholipids and is central to the core machinery for the phosphoinositide signal transduction pathway. Activation of PLCs generates 2 intracellular products: inositol 1,4,5-trisphosphate (IP_3), a universal calcium-mobilizing second messenger, and diacylglycerol (DAG), an activator of protein kinase C. To date, 4 PLC subfamilies, β, γ, δ, and ε, have been identified and are encoded by different genes. In mammals, 4 β, 2 γ, and 4 δ isoforms have been discovered (Rhee 2001). Three PLC isoforms (PLCβ, PLCγ, and PLCδ) are expressed in the heart, with PLCγ1 as the predominant isoform. All 3 isoforms are implicated in cardiac hypertrophy, heart failure, and diabetic cardiomyopathy (Dent et al. 2004; Tappia et al. 1999, 2004). It has been reported that inhibition of PLC decreases TNF-α expression in macrophages and keratinocytes after LPS treatment (Yamamoto et al. 2003; Zhang et al. 2001), suggesting a role for PLC in LPS-stimulated TNF-α expression. To further study the specific role of the PLC isoform involved in LPS-induced TNF-α expression, an integrated pharmacological and genetic approach was employed. We showed that phosphorylation of PLCγ1 increased at 30 min after LPS stimulation in mouse neonatal cardiomyocytes with no significant change in total intracellular PLCγ1 level. Selective inhibition of PLCγ1 by siRNA or a pharmacological inhibitor (U73122) decreased LPS-induced TNF-α synthesis. Furthermore, LPS-induced myocardial dysfunction was significantly improved in *PLCγ1*$^{+/-}$ mice or by U73122 pretreatment (Peng et al. 2008). These results suggest that PLCγ1 signaling induces TNF-α expression and cardiac dysfunction during LPS stimulation. The effect of PLCγ1 activation is mediated by IP_3 and IP_3 receptors (IP_3R), as blockage of either IP_3 or IP_3R in cardiomyocytes abrogated TNF-α production, whereas inhibition or activation of DAG did not alter basal or LPS-stimulated TNF-α expression (Peng et al. 2008). Interestingly, cellular IP_3 can be modulated by overexpression of the ligand-binding domain of its receptors. The N-terminal cytoplasmic region of the human type I IP_3R (residues 1–604) binds IP_3 with a comparable affinity to that of the full-length IP_3R. Removal of residues 1–223 (suppressor region) further increases the affinity for IP_3. Thus, the ligand-binding domain of the human type I IP_3R (residues 224–605) behaves as an effective mobile cytosolic IP_3 buffer, and overexpression of this domain attenuates intracellular Ca^{2+} signaling. To this end, overexpression of the ligand-binding domain of human type I IP_3R inhibited cardiomyocyte TNF-α expression in response to LPS (Peng et al. 2008). The data support the involvement of the PLCγ1/IP_3/IP_3R signaling pathway in the LPS-induced TNF-α expression in cardiomyocytes. However, the exact mechanism downstream of IP_3R is still not fully understood. It is well known that IP_3 binds to IP_3R and elevates intracellular Ca^{2+}. Elevated intracellular Ca^{2+} levels can promote ERK1/2 phosphorylation, which increases myocardial TNF-α expression in sepsis (Geoghegan-Morphet et al. 2007; Peng et al. 2005*b*). Thus, it is possible that ERK1/2 may act downstream of the PLCγ1/IP_3 signaling pathway that mediates the regulation of cardiomyocyte TNF-α expression during LPS stimulation.

Calcium signaling and cardiac TNF-α expression

Calcium is an important diffusible second messenger that controls a wide variety of intracellular processes ranging from secretion and contraction to differentiation and apoptosis (Clapham 2007). Levels of intracellular Ca^{2+} are tightly regulated. Normally, Ca^{2+} enters the cytoplasm either from outside the cell through the Ca^{2+} channels located in the cell membrane, or from some internal Ca^{2+} storage sites such as the sarcoplasmic reticulum, endoplasmic reticulum, and mitochondria. Intracellular Ca^{2+} is removed from the cytosol by transport proteins, such as plasma membrane Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger. Increased intracellular Ca^{2+} concentrations lead to Ca^{2+} binding by regulatory proteins, which control the location, amount, and effect of Ca^{2+} influx and turn the Ca^{2+} signal into a biological response. Calmodulin is considered a major transducer of Ca^{2+} signals (Clapham 2007). Recent studies reported that intracellular Ca^{2+} concentrations were increased in LPS-stimulated macrophages and Kupffer cells, leading to increased TNF-α production (Letari et al. 1991; Seabra et al. 1998; Wheeler et al. 2000; Zhou et al. 2006). The increased Ca^{2+} levels may be due to Ca^{2+} influx and release and are mediated by PLCγ (Zhou et al. 2006). In addition, a correlation between Ca^{2+}/calmodulin, ERK activity, and TNF-α expression has been established in mononuclear cells (Méndez-Samperio et al. 2006; Rosengart et al. 2000). Indeed, either chelation of releasable intracellular stores of Ca^{2+} or inhibition of calmodulin decreased ERK1/2 phosphorylation and TNF-α levels induced by *Mycobacterium bovis* infection (Méndez-Samperio et al. 2006). Similarly, inhibition of Ca^{2+}/calmodulin-dependent protein kinase (CaMK) II prevented ERK1/2 activation and the TNF-α expression in response to LPS stimulation (Rosengart et al. 2000).

Intracellular Ca^{2+} levels in cardiomyocytes are regulated mainly by dihydropyridine receptors, known as the L-type Ca^{2+} channels, and by sarcoplasmic reticulum Ca^{2+} release channels, known as the ryanodine receptors (RyRs). Calcium release from the aforementioned receptors occurs during depolarization and initiates contraction. L-type Ca^{2+} channels, which are located primarily at the sarcolemma in close prox-

imity to the sarcoplasmic reticulum, generate an inward current of Ca^{2+} during activation. Ca^{2+} entering through L-type Ca^{2+} channels increases Ca^{2+} concentration locally near the RyRs and triggers Ca^{2+} release from the sarcoplasmic reticulum through a process known as Ca^{2+}-induced Ca^{2+} release. When Ca^{2+} binds to troponin C, it activates the myofilaments and causes contraction. During relaxation and diastolic filling, Ca^{2+} dissociates from troponin C, thereby switching off the contractile machinery. Ca^{2+} is taken out of the cytosol mainly through the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), which pumps Ca^{2+} back into the sarcoplasmic reticulum. Ca^{2+} is also removed from the cell by the sarcolemmal Na^+/Ca^{2+} exchanger and, to a much lesser extent, via the sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniporter (Bers 2000). Apart from an essential role in cardiac excitation–contraction coupling, Ca^{2+} has been shown to play an important role in numerous physiological and pathophysiological processes in the heart, including cardiac hypertrophy, apoptosis, arrhythmia, and heart failure, as well as regulation of gene expression (Erickson and Anderson 2008; Frey et al. 2000). Sepsis is associated with increased myocardial TNF-α expression and cardiac dysfunction, which are regulated by cardiomyocyte Ca^{2+} homeostasis. Our recent studies have shown that LPS increased the peak level of Ca^{2+} transient, leading to increased ERK1/2 phosphorylation, which consequently increased TNF-α expression in cardiomyocytes and led to cardiac dysfunction (Geoghegan-Morphet et al. 2007). Treatment of neonatal cardiomyocytes with *ERK* siRNA decreased ERK activity and downstream TNF-α expression during LPS stimulation, confirming an important role for ERK. Furthermore, inhibition of L-type Ca^{2+} channel activity by the selective pharmacological inhibitor verapamil abrogated LPS-induced ERK1/2 phosphorylation and TNF-α expression. Taken together, our data suggest that Ca^{2+} signaling results in ERK activation and TNF-α expression in cardiomyocytes during LPS stimulation (Geoghegan-Morphet et al. 2007).

LPS upregulates myocardial intercellular cell adhesion molecule (ICAM)-1 expression during sepsis (Kukielka et al. 1993; Raeburn et al. 2002). Increased ICAM-1 expression enhances adhesion of polymorphonuclear leukocytes (PMNs) to cardiomyocytes and may contribute to cardiac dysfunction in part through release of ROS. In addition, ICAM-1 also represents an important link between extracellular mechanical stimulation and intracellular signaling. In this regard, binding of ICAM-1 activates cytoskeleton-associated focal adhesion kinase (FAK), increases heterogeneity of intracellular Ca^{2+} release, and decreases cardiomyocyte contractility (Davani et al. 2004).

Nitric oxide and cardiac TNF-α expression

NO is produced from the guanidino group of L-arginine in an NAD(P)H-dependent reaction catalyzed by a family of NOS enzymes (Kelly et al. 1996). Originally identified as a vasodilatory agent, NO is now recognized as an important signaling molecule involved in regulating cardiac function, including the modulation of contractile function, energetics, substrate metabolism, and cell growth and survival (Massion et al. 2003; Sears et al. 2004; Seddon et al. 2007; Shah and MacCarthy 2000). There are 3 distinct isoforms of NOS: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). eNOS and nNOS are Ca^{2+}-dependent enzymes, whereas iNOS is usually induced by cytokines and its enzyme activity is Ca^{2+}-independent. The 3 NOS isoforms are located in distinct subcellular compartments in cardiomyocytes. eNOS is expressed on the sarcolemma, iNOS is located in the cytosol, and nNOS is expressed in the sarcoplasmic reticulum as well as on the sarcolemma (Xu et al. 1999, 2003). The spatial difference of the NOS isoforms has functional implications. For example, eNOS enhances TNF-α biosynthesis in cardiomyocytes in response to LPS, while nNOS exhibits an inhibitory effect via regulating intracellular Ca^{2+} levels (Geoghegan-Morphet et al. 2007; Peng et al. 2003*a*). Activation of eNOS and nNOS produces nanomolar concentrations of NO, while induction of iNOS is associated with micromolar levels of NO production and hypotension in sepsis.

Positive effect of eNOS on cardiac TNF-α expression

Under normal conditions, NO released from eNOS in the heart plays several important physiological roles, including coronary vasodilation, regulation of platelet and neutrophil functions, and tonic inhibition of mitochondrial O_2 consumption (Kelly et al. 1996). Upon stimulation with endotoxins, the levels of phosphorylated eNOS were increased without any accompanying change in total eNOS protein levels (T. Zhang and Q. Feng, unpublished observations). This phosphorylation-mediated activation of eNOS enhances LPS-induced TNF-α production in cardiomyocytes, which can be prevented by treatment with *N*-nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor. Deficiency in eNOS decreased both basal and LPS-stimulated TNF-α mRNA and protein expression. Moreover, the decreased production of TNF-α in *eNOS*$^{-/-}$ cardiomyocytes can be restored to a level similar to that expressed in wild-type cardiomyocytes by either treating with an NO donor, such as diethylenetriamine–NO (DETA-NO), or through overexpression of the *eNOS* gene (Peng et al. 2003*a*). Thus, eNOS plays an important role in regulating cardiomyocyte TNF-α production during LPS stimulation.

The actions of NO are mediated through both soluble guanylate cyclase (sGC)/cGMP-dependent and -independent pathways (Kalra et al. 2000; Peng et al. 2003*a*; Vila-Petroff et al. 1999; Wang et al. 1997). For example, treatment with the exogenous NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) resulted in a time- and dose-dependent increase in myocardial TNF-α mRNA and protein biosynthesis in adult feline hearts and isolated cardiomyocytes (Kalra et al. 2000). The effect of SNAP was completely inhibited by a selective guanylate cyclase inhibitor, 1*H*-(1,2,4)oxadiazolo(4,3-*a*)quinoxalin-1-one (ODQ) (Kalra et al. 2000). Furthermore, deficiency of soluble GCα1 improved cardiac function and survival rate during endotoxemia (Buys et al. 2009). These results suggest that NO signaling and cardiac function during sepsis can be mediated by sGC/cGMP. In addition to the cGMP-dependent mechanism, our studies demonstrated that the effects of NO on cardiomyocyte TNF-α production during LPS stimulation are cGMP-independent (Peng et al. 2003*a*). In this regard, exogenous NO donors increased cGMP levels in mouse neonatal cardiomyocytes. However, inhibition of guanylate cyclase by ODQ had no effect on

TNF-α production induced by LPS. Similarly, 8-Br-cGMP, a cGMP analogue, failed to induce TNF-α production in *eNOS*$^{-/-}$ cardiomyocytes. Deletion of the *eNOS* gene, on the other hand, decreased basal intracellular cAMP levels in neonatal mouse cardiomyocytes, which were restored by NO donor DETA-NO. Inhibition of cAMP production attenuated LPS-induced TNF-α expression (Peng et al. 2003*a*). Whereas a high dose of a cAMP analogue (10 μmol/L 8-Br-cAMP) inhibited TNF-α production in neonatal rat myocytes (Wagner et al. 1998), low doses of the analogue (10 and 100 pmol/L) increased TNF-α production in *eNOS*$^{-/-}$ cardiomyocytes (Peng et al. 2003*a*). Therefore, the effects of eNOS-derived NO on LPS-induced TNF-α production are mediated by adenylate cyclase / cAMP-dependent pathways in mouse cardiomyocytes.

Effects of NO on cAMP production in cardiomyocytes appear to be concentration dependent. Although low levels of NO activate adenylate cyclase activity and increase cAMP production, high levels of NO inhibit adenylate cyclase activity and decrease cAMP production (Vila-Petroff et al. 1999). The biphasic effects of NO are mediated by phosphodiesterases (PDEs), which upon activation break down cAMP to the inactive 5′-AMP. Low levels of NO inhibit PDE3 activity and thus increase cAMP levels, while high levels of NO activate PDE2 and decrease cAMP levels (Lugnier 2006). In addition, LPS induces COX-2 and PGE_2 production in cardiomyocytes (Peng et al. 2005*a*). Induction of COX-2 has also been shown to activate adenylate cyclase and cAMP/protein kinase A (PKA) signaling (Chen et al. 1999). Therefore, eNOS activation releases low levels of NO and induces COX-2 expression, which may contribute to adenylate cyclase activation and cAMP production during LPS stimulation in cardiomyocytes.

cAMP has been shown to activate p38 MAPK via PKA activation in adult mouse cardiomyocytes during β_2-adrenergic receptor stimulation (Zheng et al. 2000). Activation of the cAMP/p38 MAPK signaling pathway has also been demonstrated in mouse neonatal cardiomyocytes in response to LPS (Peng et al. 2003*a*). Addition of an NO donor or a cAMP analogue increased p38 MAPK activity and TNF-α expression induced by LPS in *eNOS*$^{-/-}$ cardiomyocytes. Conversely, phosphorylation of p38 MAPK and release of TNF-α were attenuated by inhibiting NOS and adenylate cyclase activities. Furthermore, inhibition of PKA decreased phosphorylation of p38 MAPK and expression of TNF-α. These data support the notion that the positive effects of eNOS on LPS-stimulated TNF-α expression are mediated via a cAMP/PKA/p38 MAPK pathway (Peng et al. 2003*a*).

Negative effect of nNOS on cardiac TNF-α expression

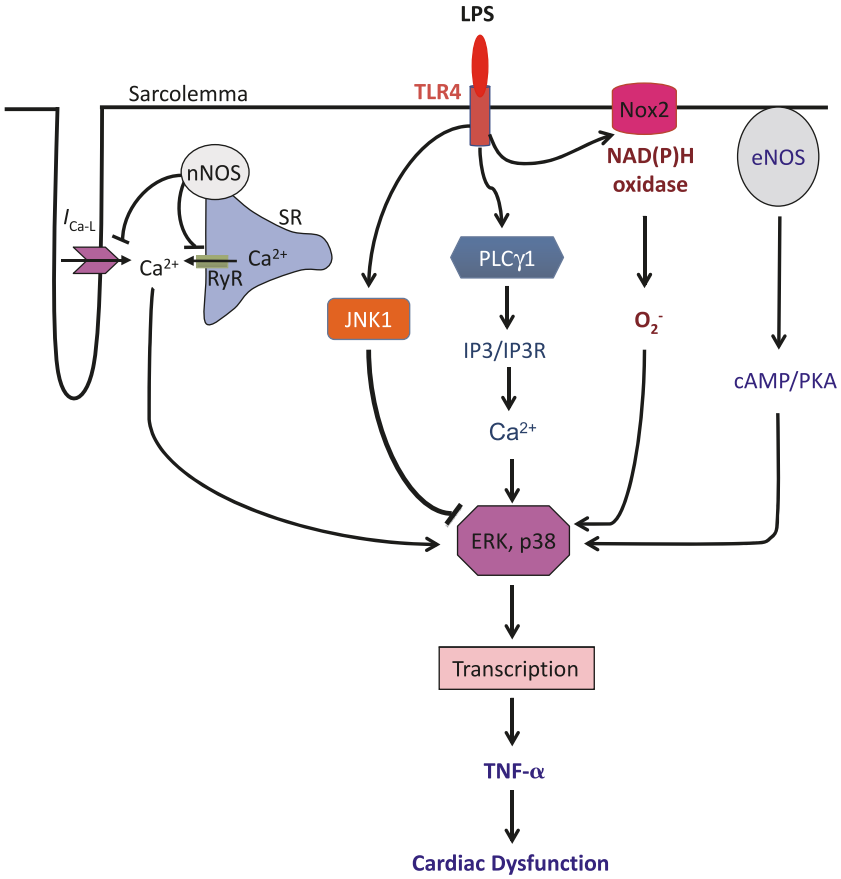
nNOS has been shown to be physically associated with the sarcoplasmic reticulum Ca^{2+} release channel and SERCA2a in cardiomyocytes (Burkard et al. 2007; Damy et al. 2004). When nNOS is overexpressed in cardiomyocytes, it binds to the L-type Ca^{2+} channel (Burkard et al. 2007). Given the close association between nNOS and the proteins that are important in the regulation of Ca^{2+}, it is not surprising that nNOS regulates intracellular Ca^{2+} levels in cardiomyocytes. Studies have shown that nNOS deletion is associated with increased Ca^{2+} transient amplitude and L-type Ca^{2+} currents ($I_{Ca\text{-}L}$), as well as reduced phospholamban phosphorylation and increased cardiomyocyte shortening (Ashley et al. 2002; Heaton et al. 2006; Sears et al. 2003). Additionally, nNOS-derived NO has been shown to inhibit myocardial responsiveness to β-adrenergic stimulation (Ashley et al. 2002; Heaton et al. 2006). Taken together, these observations indicate that nNOS plays an important physiological role in regulating intracellular Ca^{2+} cycling and cardiac function (Casadei 2006). A novel function of nNOS that has recently been identified by our laboratory is its ability to regulate myocardial TNF-α expression during sepsis. The effect of nNOS on TNF-α production is mediated via regulation of intracellular Ca^{2+} levels. We showed that TNF-α expression in response to LPS was enhanced either by disruption of the *nNOS* gene or by an antisense oligonucleotide against nNOS, and that this increase could be abrogated by verapamil, suggesting an important role for intracellular Ca^{2+} and L-type Ca^{2+} channels in this pathway (Geoghegan-Morphet et al. 2007).

ERK1/2 MAPKs can be activated by increases in intracellular Ca^{2+} through the Ca^{2+}-sensitive signaling protein calmodulin (Agell et al. 2002; Maass et al. 2005; Melien et al. 2002; Rosengart et al. 2000). ERK1/2 activation promotes TNF-α expression in cardiomyocytes during LPS stimulation (Peng et al. 2005*b*). Interestingly, ERK1/2 activity in *nNOS*$^{-/-}$ cardiomyocytes was increased, resulting in higher TNF-α expression than that in wild-type cardiomyocytes. The response was attenuated by verapamil (Geoghegan-Morphet et al. 2007). Furthermore, in vivo studies showed that myocardial ERK1/2 activity and TNF-α expression in *nNOS*$^{-/-}$ were increased, leading to decreased cardiac function during endotoxemia compared with wild-type mice (Geoghegan-Morphet et al. 2007). Taken together, nNOS-derived NO inhibits LPS-induced TNF-α expression in cardiomyocytes and improves cardiac function in endotoxemia by reducing L-type Ca^{2+} channel-dependent ERK signaling in cardiomyocytes.

Effects of iNOS on cardiac TNF-α expression

iNOS is expressed during sepsis and produces high levels of NO that, while toxic to undesired microbes, may also harm healthy tissues. It has been demonstrated that LPS induces iNOS expression in a number of cell types in the heart, including the endothelium and smooth muscle of the cardiac microvasculature, the endocardium, and cardiomyocytes (Muller et al. 2000; Stein et al. 1996). Myocardial iNOS induction can cause contractile dysfunction in isolated myocytes, perfused working hearts, and in vivo animal preparations. Conversely, NO generated by iNOS also has beneficial effects. For example, NO-derived from iNOS has been shown to suppress viral replication and infection (Lowenstein et al. 1996). In septic shock, iNOS is induced in various organs. The induction of iNOS is mediated by the release of proinflammatory cytokines, including TNF-α, and contributes to delayed vascular hyporeactivity and development of hypotension in septic shock (Feihl et al. 2001). In agreement with these observations, myocardial *iNOS* mRNA expression is significantly increased 18 h after LPS treatment in mice, and inhibition of *iNOS* expression improves cardiac function (Niu et al. 2008). In cultured neonatal cardiomyocytes, iNOS protein expression was detectable only 6 h after LPS treatment, whereas upregulation of TNF-α protein expression occurred in less than 30 min and peaked

Fig. 2. Signal transduction, Ca^{2+}, and NO in the regulation of myocardial TNF-α expression and cardiac function during sepsis. LPS, through binding to its receptor TLR4, activates Nox2-containing NAD(P)H oxidase, PLCγ1, and Ca^{2+} signaling, thus increasing ERK1/2 and p38 MAPK activity leading to TNF-α expression and cardiac dysfunction. LPS also activates JNK1, which displays a negative effect on TNF-α production through inhibition of ERK1/2 and p38 MAPK activity. Interestingly, eNOS promotes TNF-α expression through cAMP/PKA-dependent p38 MAPK mechanisms, whereas nNOS inhibits L-type Ca^{2+} channel ($I_{Ca\text{-}L}$) and RyR activities, decreasing intracellular Ca^{2+} levels and TNF-α production via inhibition of Ca^{2+}-dependent ERK1/2 activity. The balance of positive and negative signaling pathways regulates myocardial TNF-α expression and cardiac function in sepsis. cAMP, cyclic AMP; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; IP_3, inositol 1,4,5-trisphosphate; JNK, c-Jun NH_2-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; nNOS, neuronal NOS; Nox, NAD(P)H oxidase; PKA, protein kinase A; PLC, phospholipase C; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; TLR, Toll-like receptor; TNF, tumor necrosis factor.



around 4–6 h after LPS challenge. Furthermore, TNF-α production was not different between *iNOS*$^{-/-}$ and wild-type cardiomyocytes at 4 h after LPS stimulation, indicating that iNOS is not involved in TNF-α production in cardiomyocytes during early hours of LPS stimulation (Peng et al. 2003*a*).

Other regulators of cardiac TNF-α expression

Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase that was originally identified as a regulator of glucose metabolism. In mammals, GSK3 is encoded by 2 genes, *GSK3α* and *GSK3β*. Recently, GSK3β has been shown to be a key player in many signaling pathways responsible for multiple biological processes including cell proliferation, cell differentiation, embryonic development, cell motility, cell cycle regulation, gene expression, metabolism, and inflammatory responses (Cohen and Frame 2001). However, the role of *GSK3β* in inflammatory responses is controversial (Cuzzocrea et al. 2006; Gao et al. 2008; Martin et al. 2005; Shen et al. 2008; Vines et al. 2006; Whittle et al. 2006). Studies have shown that pharmacological inhibitors of GSK3β attenuated proinflammatory cytokine expression, including that of TNF-α, in the lung (Cuzzocrea et al. 2006) and colon (Whittle et al. 2006) during acute systemic inflammation and in the heart after ischemia and reperfusion (Gao et al. 2008). In addition, administration of a GSK3 inhibitor suppressed the expression of proinflammatory cytokines, including TNF-α, in LPS-stimulated monocytes and mediated protection from endotoxin shock in mice (Martin et al. 2005). In contrast, overexpression of *GSK3β* in mice by recombinant adenoviruses encoding *GSK3β* inhibited TNF-α expression in lung and heart tissues during endotoxemia, suggesting an antiinflammatory role for GSK3β (Vines et al. 2006). Consistent with these findings, inhibition of GSK3β in neonatal cardiomyocytes enhances TNF-α expression in response to LPS. Furthermore, the inhibitory effect of GSK3β on cardiomyocyte TNF-α expression is mediated by the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Shen et al. 2008). Thus, a reduction in Akt activity and

subsequent GSK3β activation induced by LPS may contribute to myocardial dysfunction in sepsis.

Another negative feedback mechanism activated by LPS to prevent runaway inflammation is the expression of suppressor of cytokine signaling (SOCS) proteins (O'Shea and Murray 2008), which are a family of cytokine-induced inhibitors with at least 8 members. The main function of SOCS proteins is to block signal transducers and activators of transcription (STAT) signaling from cytokine receptors. LPS induces SOCS-1 in macrophages. Furthermore, mice deficient in SOCS-1 show enhanced plasma TNF-α production and mortality upon LPS challenge, suggesting an important protective role for SOCS-1 in endotoxemia (Nakagawa et al. 2002). SOCS-1 overexpression has been shown to inhibit TNF-α-induced apoptosis in neonatal rat cardiomyocytes (Yan et al. 2008). However, the role of SOCS-1 in myocardial TNF-α expression and cardiac function during sepsis remains to be determined.

Conclusions

Cardiomyocyte TNF-α production contributes to cardiac dysfunction during endotoxemia. The mechanisms regulating myocardial TNF-α expression are multifactorial and complex. As summarized in Fig. 2, LPS activates TLR4 on the sarcolemma and increases the activities of both Nox2-containing NAD(P)H oxidase and PLCγ1, leading to increased intracellular ROS and Ca^{2+} levels in cardiomyocytes. The increased levels of O_2^- and Ca^{2+} signaling lead to increased ERK1/2 and p38 MAPK phosphorylation and activation of transcription factors, which ultimately lead to increased TNF-α expression. LPS also activates JNK1. Activation of JNK1 increases c-*fos* expression and inhibits ERK1/2 and p38 phosphorylation, however, suggesting a negative cross-talk between JNK1 and p38/ERK1/2 in cardiomyocytes during LPS stimulation. Depending on the NOS isoform, NO has both positive and negative effects on myocardial TNF-α production. eNOS-derived NO enhances LPS-induced TNF-α expression via the cAMP-dependent p38 MAPK pathway. In contrast, NO derived from nNOS decreases intracellular Ca^{2+} levels via inhibition of L-type Ca^{2+} channels and RyRs (Ca^{2+} release channels), inhibits LPS-induced TNF-α expression, and improves cardiac function during endotoxemia. iNOS, however, is not involved in cardiomyocyte TNF-α expression during the first few hours of LPS stimulation. These signaling mechanisms not only provide novel insights into the regulation of myocardial TNF-α expression and cardiac function during LPS stimulation, but may also have therapeutic implications in sepsis.

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