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Role of Rac1 in myocardial TNF-alpha expression in sepsis

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Physiology

Role of Rac1 in myocardial tumor necrosis factor-alpha expression and cardiac dysfunction during endotoxemia

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Abstract

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine and its high levels of expression in the heart leads to cardiac dysfunction in sepsis. However, the underlying molecular mechanisms of regulating myocardial TNF-α expression are not fully understood. The aim of this thesis was to investigate the role of Rac1 in myocardial TNF-α expression and cardiac dysfunction during sepsis. Studies were performed using cultured neonatal cardiomyocytes and a mouse model of endotoxemia.

I showed that lipopolysaccharides (LPS) activated Rac1 in the myocardium. To detect the mechanisms of Rac1 activation, phosphoinositide-3 kinase (PI3K) activity was measured. LPS activated PI3K, which was responsible for LPS-induced Rac1 activation in cardiomyocytes. PI3K-mediated Rac1 activation increased NADPH oxidase activity, O_2^- generation, and ERK1/2 phosphorylation, leading to increased TNF-α mRNA expression in the myocardium. Moreover, the LPS-activated PI3K/Rac1/NADPH oxidase pathway inhibited myocardial Na/K-ATPase activity. The suppression of Na/K-ATPase activity enhanced TNF-α protein levels without any measurable effect on TNF-α mRNA expression or stability. Furthermore, inhibition of Na/K-ATPase activity resulted in activation of mammalian target of rapamycin (mTOR) in cardiomyocytes via Ca^{2+}/ calmodulin-dependent protein kinases (CaMKs). mTOR activity increased LPS-induced TNF-α protein levels without any apparent effect on TNF-α mRNA expression. Most importantly, cardiomyocyte-specific Rac1 deletion significantly decreased myocardial TNF-α expression and improved cardiac function during endotoxemia *in vivo*.

I also demonstrated that Rac1 mediated LPS-induced p21-activated kinase (PAK) 1 activation, which increased p38 and JNK1 phosphorylation and TNF-α expression in

cardiomyocytes. On the other hand, LPS increased mitogen-activated protein kinase phosphatase-1 (MKP-1) expression in the myocardium *in vivo* and in cultured neonatal cardiomyocytes *in vitro*. Inhibition of Rac1, PAK1 and JNK1 decreased LPS-induced myocardial MKP-1 expression. LPS-induced ERK1/2 and p38 phosphorylation was prolonged in MKP-$1^{-/-}$ myocardium. Additionally, myocardial TNF-α mRNA and protein levels were enhanced in MKP-$1^{-/-}$ mice compared to WT mice in endotoxemia, which was associated with a further decrease in cardiac function.

In conclusion, PI3K-mediated Rac1 activation is required for induction of TNF-α mRNA and protein expression in cardiomyocytes and cardiac dysfunction during endotoxemia. Rac1 promotes TNF-α mRNA expression via NADPH oxidase/ERK1/2 and PAK1/p38 pathways. Rac1-mediated NADPH oxidase activation enhances TNF-α protein production via Na/K-ATPase inhibition and Ca^{2+}/CaMK-dependent mTOR activation. On the other hand, Rac1/PAK1 pathway induces myocardial MKP-1 expression via JNK1. MKP-1 attenuates ERK1/2 and p38 activation, thus limiting myocardial TNF-α expression and improving cardiac function in endotoxemia. These findings provide novel insight into the signal transduction mechanisms that regulate myocardial TNF-α expression, and may have therapeutic implications in the treatment of sepsis.

Key words: sepsis, phosphoinositide-3 kinase, Rac1 GTPase, NADPH oxidase, Na/K-ATPase, mammalian target of rapamycin, p21-activated kinase 1, mitogen-activated protein kinase phosphatase-1, tumor necrosis factor-alpha, cardiac function

Statement of co-Authorship

These studies outlined in chapters 2-4 were performed by Ting Zhang in the laboratory of Dr. Qingping Feng, with the assistance if co-authors as listed below.

Dr. Qingping Feng contributed to the experimental design, data interpretation, and manuscript preparation for all the studies in this thesis. My advisory committee consisting of Dr. Frank Beier, Dr. Peter Chidiac and Dr. Douglas Jones also provided intellectual guidance to my studies. In addition, Dr. Xiangru Lu provided training in experimental techniques.

Chapter 2: Dr. Frank Beier provided $Rac1^{f/f}$ mice. Dr. Xiangru Lu performed the *ex vivo* measurements of cardiac function procedures.

Chapter 3: Dr. Peter Chidiac provided technical support on [^{3}H]-leucine incorporation. Dr. Stephen M. Sims and Dr. Houxiang Hu assisted with Ca^{2+} transient measurements.

Chapter 4: Dr. Xiangru Lu performed *ex vivo* cardiac function measurements. Mr. Paul Arnold measured the MKP-1 mRNA, TNF-α mRNA and protein expression in WT and MKP-$1^{-/-}$ myocardium (Figure 4.2B and 4.3). Dr. Yusen Liu provided MKP-$1^{-/-}$ mice.

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List of Abbreviations

AID	autoinhibitory domain
AMPK	AMP-activated protein kinase
Ang II	angiotensin II
AP-1	activator protein 1
ATG13	autophagy-related gene 13
CaMKK-β	calmodulin-dependent protein kinase kinase-β
CCL2	CC-chemokine ligand 2
CH	calponin homology
COX	cyclooxygenase
Deptor	DEP-domain-containing mTOR-interacting protein
4E-BPs	eIF4E-binding proteins
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
DAG	diacylglycerol
DH	Dbl homology
DLC1	dynein light chain 1
DPI	diphenyleneiodonium
DUSP2	dual specific phosphatase 2
eEF2	eukaryotic elongation factor 2
eEF2K	eukaryotic elongation factor 2 kinase
EGFR	epidermal growth factor receptor
eIF4E	eukaryotic translation initiation factor 4E

FBS	fetal bovine serum
FIP200	focal adhesion kinase family-interacting protein of 200 kDa
FKBP12	FK-binding protein 12
FoxO1	forkhead box protein O1
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GEF	guanine nucleotide exchange factor
GIT1	G protein-coupled receptor kinase-interactor 1
HVH3	human VH1-like PTPase-3
ICAM	intercellular adhesion molecule
IFN-γ	interferon-gamma
IL	interleukin
iNOS	inducible nitric oxide synthase
IQGAPs	IQ motif containing GTPase activating proteins
IRAKs	interleulin-1 receptor-associated protein kinases
LBP	lipopolysaccharide-binding protein
LIMK	LIM domain kinase
LPS	lipopolysaccharide
m7G	7 methyl guanosine cap
MAPK	mitogen-activated protein kinase
MHC	myosin heavy-chain
MKK	MAPK-kinase
MKKK	MKK-kinase

MKPs	MAPK phosphatases
MLCK	myosin light-chain kinase
mLST8	mammalian lethal with Sec13 protein 8
MOI	multiplicity of infection
mSIN1	mammalian stress-activated protein kinase interacting protein
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
MyBP	myosin binding protein
MyD88	myeloid differentiation factor 88
NCX	Na^{+}/Ca^{2+} exchanger
O_2^-	superoxide
p70S6K	protein 70 S6 kinase
PAKs	p21-activated kinases
PBD	p21-binding domain
PDK1	phosphoinositide-dependent kinase 1
PG	prostaglandins
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
PIX	PAK-interacting exchange factor
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PP2A	protein phosphatase 2A

PP2B	protein phosphatases 2B
PPARγ	peroxisome proliferator-activated receptor-γ
PRAS40	proline rich Akt substrate 40 kDa
Protor-1	protein observed with Rictor-1
PtdIns(4,5)P2	phosphatidylinositol (4,5)-diphosphate
Raptor	regulatory-associated protein of mTOR
$Rac1^{CKO}$	cardiomyocyte-specific Rac1 knockout mice
$Rac1^{f/f}$	Rac1 floxed mice
Rho	Ras-homologous
Rictor	rapamycin-insensitive companion of mTOR
ROS	reactive oxygen species
RT-PCR	reverse-transcriptase polymerase chain reaction
RyRs,	ryanodine receptors
SERCA	sarcoplasmic reticulum Ca^{2+}-ATPase
siRNAs	small interfering RNAs
SIRS	systemic inflammatory response syndrome
SR	sarcoplasmic reticulum
TGF-β	transforming growth factor-β
TIRAP	interleukin-1 receptor domain-containing adaptor protein
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
TSC	tuberous sclerosis complex
ULK1	Unc-51-like kinase 1

VCAM	vascular cell adhesion molecule
WAVE	Wiskott–Aldrich syndrome protein with a V-domain
WT	wild-type

*and † were used for denoting significance.

μ was used for micro, α was used for alpha

Chapter 1. Introduction

Literature Review

Portions of this chapter appear in

Zhang T, Feng Q. (2010) Nitric oxide and calcium signaling regulate myocardial tumor necrosis factor-alpha expression and cardiac function in sepsis. *Can J Physiol Pharmacol* 88:92-104. **Used with permission**.

1.1 Sepsis and myocardial dysfunction

1.1.1 Overview of sepsis

Sepsis is induced by a systemic immune response to infection. Sepsis, severe sepsis and septic shock represent increasingly grave stages. Sepsis is clinically identified when a patient has met two or more of the stated criteria for systemic inflammatory response syndrome (SIRS) and there is an evidence of infection. These criteria include body temperature greater than 38.5°C or less than 35.0°C, heart rate greater than 90 beats/min, respiratory rate greater than 20 breaths per minute or arterial CO_2 tension less than 32 mm Hg, white blood cell count greater than 12,000/mm^3 or less than 4,000/mm^3 or greater than 10% immature forms. Severe sepsis is defined as sepsis associated with organ dysfunction, hypoperfusion or hypotension (Merx & Weber 2007). Septic shock is defined as severe sepsis with hypotension, despite adequate fluid resuscitation**,** along with the presence of abnormalities in organ perfusion (Levy et al. 2003).

The reported mortality rate in the United States is 20-30% in sepsis and 40-80% in septic shock (Angus & Wax 2001). In Canada, over 30% of hospitalized patients die with sepsis, compared to 18% of stroke patients and 9.1% for heart attack patients (CIHI 2007; CIHI 2009). The incidence of sepsis and sepsis-related deaths is increasing by 1.5% per year due to an aging population, the use of more invasive medical procedures and

widespread antibiotic resistance (Angus et al. 2001). This increasing incidence continuously puts a substantial burden on health care. A recent study showed that the total annual hospital cost of severe sepsis in the United States was approximately $16.7 billion on the basis of 751,000 cases per year with 215,000 associated deaths (Angus et al. 2001).

1.1.2 Myocardial dysfunction during sepsis

Septic patients normally have significant cardiac morbidity. Studies have shown that 40% to 50% of patients with prolonged septic shock develop myocardial depression, characterized by decreased contractility and impaired myocardial compliance (Rudiger & Singer 2007). Myocardial dysfunction during sepsis causes a high risk of developing multi-organ failure, which is associated with a high mortality (Court et al. 2002).

Clinically, septic shock is divided into two phases: an early hyperdynamic phase followed by a hypodynamic phase. At the hyperdynamic phase of septic shock, vasodilation from histamine, bradykinins, serotonin, and endorphins released during the early stages of sepsis significantly decreases total peripheral vascular resistance (Court et al. 2002). After adequate volume resuscitation, the cardiac output is elevated and tissue perfusion is increased. However, besides vasodilation, these vasoactive mediators also induce a marked capillary permeability and the so called “third space” fluid loss (ascites and pleural effusions), leading to hypovolemia. The rise in cardiac output is limited by hypovolemia and a fall in preload due to low cardiac filling pressures. The hypodynamic phase of septic shock is characterized by decreased cardiac output, a sign of cardiac dysfunction, and increased peripheral vascular resistance. The hypodynamic phase occurs between 4 and 7 hours after the injection of endotoxin into mice (Ullrich et al. 2000).

Rodents enter the hypodynamic phase 20 hours after cecal ligation and puncture (Yang et al. 2002).

An early hypothesis was that global myocardial ischemia results in cardiac dysfunction in sepsis (Merx & Weber 2007). However, later findings dismissed this theory. It has been reported that septic patients show a decreased oxygen difference between the coronary artery and coronary sinus, and increased coronary blood flow (Cunnion et al. 1986). Levy et al. found that septic mice exhibited reduced cardiac performance, higher deposits of glycogen and myocardial glucose uptake while arterial oxygen tension and myocardial perfusion are well preserved (Levy et al. 2005), suggesting that cardiac dysfunction is not due to myocardial ischemia during sepsis.

The expression of adhesion molecules, including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, is enhanced in coronary endothelium and cardiomyocytes after lipopolysaccharide (LPS) stimulation (Merx & Weber 2007). These molecules increase myocardial neutrophil accumulation. Antibody blockade of ICAM-1 and VCAM-1 inhibits LPS-induced cardiac dysfunction. However, neutrophil depletion in sepsis does not offer any protection of cardiac function, suggesting that the effect of these adhesion molecules on myocardial dysfunction is independent of neutrophil accumulation (Raeburn et al. 2002). In addition, the activities of proapopotic caspases are increased in the myocardium in endotoxemia, but the rate of myocardial apoptosis is too low and disproportionate to the severity of cardiac dysfunction (Carlson et al. 2005).

A number of factors have been identified to contribute to myocardial depression in sepsis. These factors include prostanoids, endothelin-1, nitric oxide and pro-inflammatory

cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor-alpha (TNF-α), (Kumar et al. 2000). Importantly, TNF-α treatment directly depresses cardiac contractility, implying that TNF-α plays a key role in myocardial dysfunction during sepsis (Walley et al. 1994).

1.2 TNF-α in sepsis

1.2.1 Overview

TNF-α is a 17 kDa protein that was initially discovered as the molecule associated with necrosis in mouse tumors. It is an important pro-inflammatory factor that contributes to a wide range of pathologies (Meldrum 1998; Tracey & Cerami 1993). Systemic administration of TNF-α results in manifestations that are very similar to sepsis including hypotension, metabolic acidosis, diffuse pulmonary infiltration, pulmonary and gastrointestinal hemorrhage, tubular necrosis and death (Tracey et al. 1986). Notably, TNF-α impairs contractile function in cultured cardiomyocytes and isolated hearts as well as cardiac function in intact animals (Bozkurt et al. 1998; Grandel et al. 2000; Oral et al. 1997). The administration of TNF-α antiserum or TNF-α binding proteins improves cardiac function in endotoxemic mice (Peng et al. 2003b) and rats (Meng et al. 1998), and increases survival (Peng et al. 2003b). These inhibitory effects on the heart are mediated by the autocrine, paracrine and endocrine actions of TNF-α during sepsis. To this end, macrophages and monocytes produce TNF-α and release it to the circulation (Schlag et al. 1991). Circulating TNF-α acts on the heart and promotes cytokine expression in cardiomyocytes. Additionally, cardiomyocytes themselves are the predominant local source of TNF-α in the myocardium during sepsis (Grandel et al. 2000; Kapadia et al.

1995; Peng et al. 2003a). Significant induction of TNF-α in the myocardium in septic rodent models supports the notion that local TNF-α is a major contributor to cardiac dysfunction (Grandel et al. 2000).

1.2.2 LPS signaling in TNF-α expression

Endotoxins, such as LPS, are the major pathogens responsible for myocardial dysfunction during sepsis (Natanson et al. 1989; Suffredini et al. 1989). Cardiovascular changes mediated by LPS simulate sepsis in both animal models (Natanson et al. 1989) and human volunteers (Kumar et al. 2005; Suffredini et al. 1989). LPS binds to LPS-binding protein (LBP), which presents LPS to CD-14, a membrane glycoprotein with a glycosylinositol tail. With the assistance from MD-2, the LPS-LBP-CD-14 complex activates toll-like receptor (TLR)-4, the LPS receptor. Activation of TLR-4 results in the recruitment of myeloid differentiation factor (MyD) 88 and Toll/interleukin-1 receptor domain-containing adaptor protein (TIRAP). Binding of MyD88 promotes association with the interleulin-1 receptor-associated protein kinases (IRAKs), IRAK4 and IRAK1. IRAK4 phosphorylates IRAK-1, triggering IRAK-1 kinase activity, which in turn activates downstream signaling pathways, leading to the activation of transcription factors such as NF-κB and production of TNF-α (Monick & Hunninghake 2003). Studies have demonstrated that NADPH oxidase, mitogen-activated protein kinase (MAPK) signaling and intracellular Ca^{2+} all contribute to LPS-induced TNF-α expression in cardiomyocytes (Zhang & Feng 2010).

1.2.3 Cardiac effects of TNF-α

A major mechanism of TNF-α-induced myocardial dysfunction involves disturbances 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 the induction of cardiomyocyte apoptosis (Song et al. 2000; Zhao et al. 2006). Studies have shown that myocardial dysfunction induced by TNF-α has two distinct phases. The early phase occurs within minutes after TNF-α exposure. During this phase, sphingosine production is increased and disturbs intercellular Ca^{2+} homeostasis. The late phase occurs hours after TNF-α exposure. During this phase, inducible nitric oxide synthase (iNOS) expression and cadiomyocyte apoptosis are increased. Also pyruvate dehydrogenase activity and mitochondrial function are inhibited (Meldrum 1998). The purpose of this thesis is to elucidate and understand the signal transduction mechanisms that regulate myocardial TNF-α expression and cardiac dysfunction in endotoxemia.

1.3 Rac GTPases

1.3.1 Overview

Rac GTPases are low molecular weight (20-30 kDa) monomeric GTP-binding proteins, which are a subfamily of the Ras-homologous (Rho) GTPase family (Bustelo et al. 2007; Fritz & Kaina 2006). Rac family contains 4 members, Rac1, Rac2, Rac3 and RhoG (Haataja et al. 1997). Rac proteins play an important role in various cellular events, including actin cytoskeletal reorganization, transformation, proliferation, apoptosis, gene expression, superoxide production and migration (Bustelo et al. 2007; Fritz & Kaina 2006). Rac1, Rac2 and Rac3 have similar genetic sequence with different expression

patterns. Rac1 is ubiquitously expressed, whereas Rac2 expression is mostly restricted to cells of hematopoietic origin and Rac3 is expressed in the brain, liver, lung and pancreas. RhoG has the lowest sequence similarity to Rac1 and is ubiquitously expressed (Heasman & Ridley 2008).

1.3.2 Activation of Rac GTPases

Rac GTPases act as molecular switches, cycling between active (Rac-GTP) and inactive (Rac-GDP) forms. GTP binding and hydrolysis are facilitated by 2 major classes of proteins, guanine nucleotide exchange factors (GEFs) and the GTPase activating proteins (GAPs), respectively. Under basal conditions, inactive Rac localizes in the cytosol and generally binds to a GDP dissociation inhibitor (GDI), such as ARHGDIA, ARHGDIB and ARHGDIG. Binding with GDI maintains the Rac in a GDP-bound state in the cytosol and also promotes Rac dissociation from the membrane. Upon stimulation by various factors such as cytokines, Rac-GDP dissociates from its GDI. GEFs, such as P-Rex, SWAP-70, Tiam, Pix, Sos and Vav, become activated and open the GTPase's nucleotide-binding site allowing the exchange of GDP for GTP on Rac proteins. Inactivation of Rac proteins occurs through the hydrolysis of GTP to GDP by their intrinsic GTPase activity, which is promoted by GAPs, such ARHGAP6, ARHGAP10 and CHN (Schmidt & Hall 2002) (Figure 1.1).

Phosphoinositide 3-kinase (PI3K) is one of the activators of Rac-GEFs. PI3K phosphorylates the phosphoinositide at the 3-OH position of the inositol ring. When the type 1 PI3Ks are activated, they use phosphatidylinositol (4,5)-diphosphate (PtdIns(4,5)P2) as a substrate and produce the second messenger phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)P3). PtdIns(3,4,5)P3 can bind to the pleckstrin

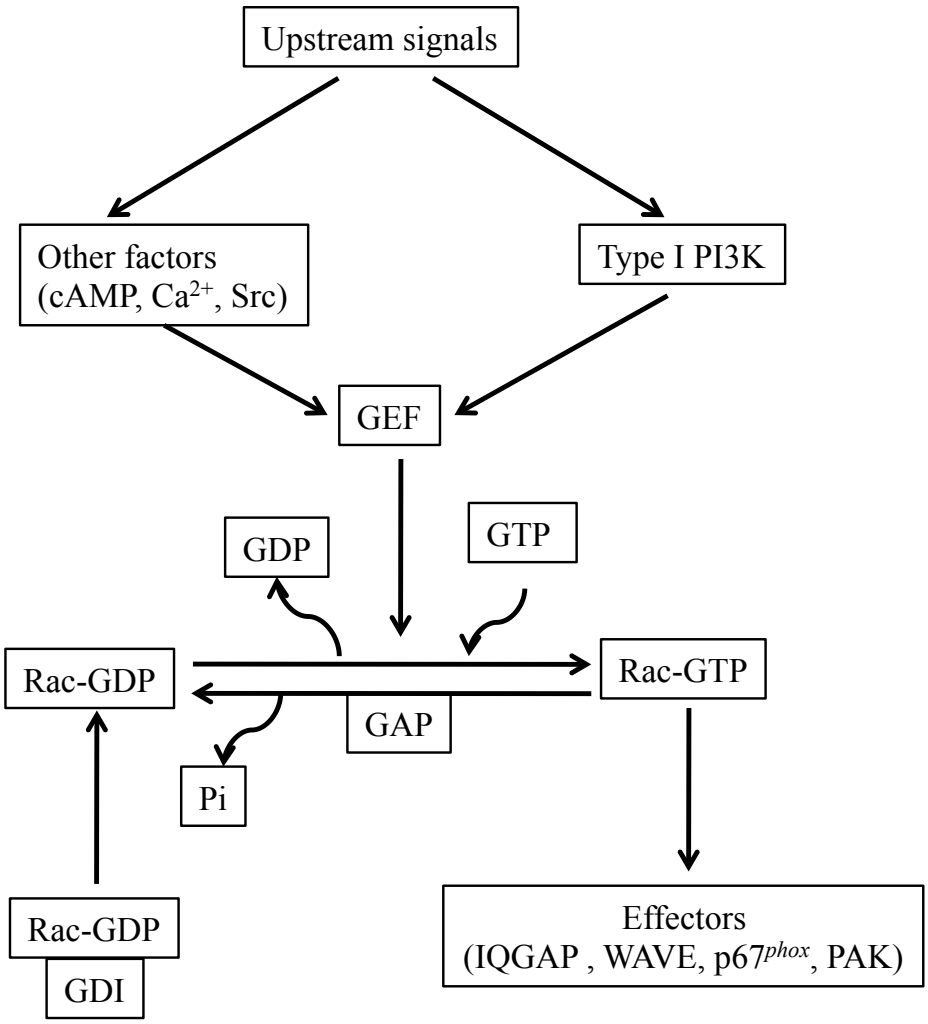

Figure 1.1. Schematic of Rac signaling pathways. In resting cells, Rac-GDP is generally bound to a GDI. Upon stimulation by various factors, Rac-GDP dissociates from GDI and attaches to the membrane. GEFs become activated in PI3K-dependent or –independent ways and allow the exchange of GDP for GTP on Rac. Inactivation of Rac proteins occurs through the hydrolysis of GTP to GDP by their intrinsic GTPase activity, which can be greatly accelerated by GAPs. Activated Rac GTPases interact with specific effectors (IQGAP, WAVE, $p67^{phox}$ and PAK) which influence diverse physiological outcomes.

homology (PH) domain of target proteins and then induce their membrane translocation and/or conformational changes (Welch et al. 2003). Rac-GEFs contain tandem Dbl homology (DH)/PH domains. Several Rac-GEFs, such as P-Rex, SWAP-70, Tiam, Pix, Sos and Vav have been reported to be activated by PI3K (Burridge & Wennerberg 2004; Welch et al. 2003).

Besides PI3Ks, Rac-GEFs can also be activated by PI3K-independent mechanisms. For example, Vav consists of three regions, an N-terminal autoinhibitor region, a central catalytic region with a DH/PH domain and a C-terminal recruitment region. The N-terminal autoinhibitory region has a calponin homology (CH) domain and an acidic region with conserved tyrosines. Lck- and Src-mediated Tyr174 phosphorylation disrupts the interaction between the acidic region and the DH domain, thereby liberating the catalytic site. In addition, some GEFs are activated by common second messengers, such as cyclic adenosine monophosphate (cAMP), Ca^{2+}, and diacylglycerol (DAG) (Bos et al. 2007).

1.3.3 Effectors of Rac GTPases

Activated Rac GTPases interact with specific effectors, which coordinate activation of a multitude of signaling cascades and influence diverse physiological outcomes. These effectors include IQ motif containing GTPase activating proteins (IQGAPs), Wiskott–Aldrich syndrome protein with a V-domain (WAVE), p21-activated kinases (PAKs), and $p67^{phox}$ (Burridge & Wennerberg 2004; Ellenbroek & Collard 2007). IQGAP, WAVE and PAK are cytoskeletal remodeling proteins and known to regulate the formation of lamellipodia, filopodia and microtubule which are necessary for cell migration, adhesion and wound healing. Interaction of Rac-GTP with $p67^{phox}$ results in

activation of NADPH oxidase, which is a major source of reactive oxygen species (ROS) (Moldovan et al. 2006). Since PAKs and NADPH oxidase are key regulators of cardiac function in physiological and pathophysiological conditions (Bendall et al. 2002; Li et al. 2002; Mohazzab et al. 1997; Sheehan et al. 2007; Xiao et al. 2002a), it is important to understand the molecular structure, expression, functions of NADPH oxidase and PAKs in the myocardium.

1.3.4 Rac GTPases in the heart

Rac1 and RhoG are expressed in cardiomyocytes, whereas Rac2 and Rac3 are undetectable in the heart (Satoh et al. 2006). Numerous studies have demonstrated that RhoG acts either upstream of or in parallel to Rac1 to mediate neurite outgrowth, cell motility, endocytosis and immune responses (Katoh et al. 2006; Katoh & Negishi 2003; Vigorito et al. 2004). However, the role of RhoG in regulating cardiac function has not been elucidated.

Rac1 knockout mice are embryonic lethal due to defects in germ-layer formation (Sugihara et al. 1998), and thus tissue specific knockouts have been widely used to study Rac1 function (Heasman & Ridley 2008). A cardiac-specific Rac1 deletion mouse model is now available and shows decreased NADPH oxidase activity and superoxide anion production. Angiotensin II (AngII)-induced cardiac hypertrophy is diminished in these mice (Satoh et al. 2006). Rac1 transgenic mice that express constitutively activated Rac1 specifically in the myocardium have been created. These transgenic mice develop cardiac hypertrophy within weeks after birth (Sussman et al. 2000). In addition, Rac1 enhances hyperglycemia-induced apoptosis in cardiomyocytes. The effect of Rac1 is mediated through NADPH oxidase activation and is associated with mitochondrial ROS generation

(Shen et al. 2009). A recent study demonstrated that LPS induces Rac1 activation, which contributes to NADPH oxidase activity and phosphorylation of ERK1/2 and p38 MAPK, leading to TNF-α expression in the heart (Zhu et al. 2009). The aims of my project were to investigate the molecular mechanisms of myocardial Rac1 activation as well as downstream effectors of Rac1 regulating TNF-α mRNA expression, protein synthesis and cardiac dysfunction during endotoxemia.

1.4 NADPH oxidase

1.4.1 Overview

NADPH oxidase is a multi-component enzyme system that catalyzes the NADPH dependent reduction of O_2 to O_2^- (Babior 1999). The prototypic NADPH oxidase is composed of membrane bound flavocytochrome b558, which consists of $gp91^{phox}$ (Nox2) and $p22^{phox}$ ("phox" represents phagocyte oxidase), cytosolic regulatory proteins ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) and Rac GTPase. The catalytic core of NADPH oxidase is the membrane-integrated flavocytochrome b558. Under basal conditions, the cytosolic complex is separate from the membrane-bound flavocytochrome b558. Upon stimulation, NADPH oxidase activation is triggered by the phosphorylation of the cytosolic *phox* proteins and their translocation to the plasma membrane. Concomitantly, Rac is activated and interacts with flavocytochrome b558 to form a binding partner for $p67^{phox}$. The complete assembly of NADPH oxidase components is crucial for subsequent O_2^- production (Figure 1.2). Increased O_2^- production is known to modulate cardiomyocyte apoptosis, inflammatory response, contractility and cardiac hypertrophy (Babior 1999; Griendling et al. 2000).

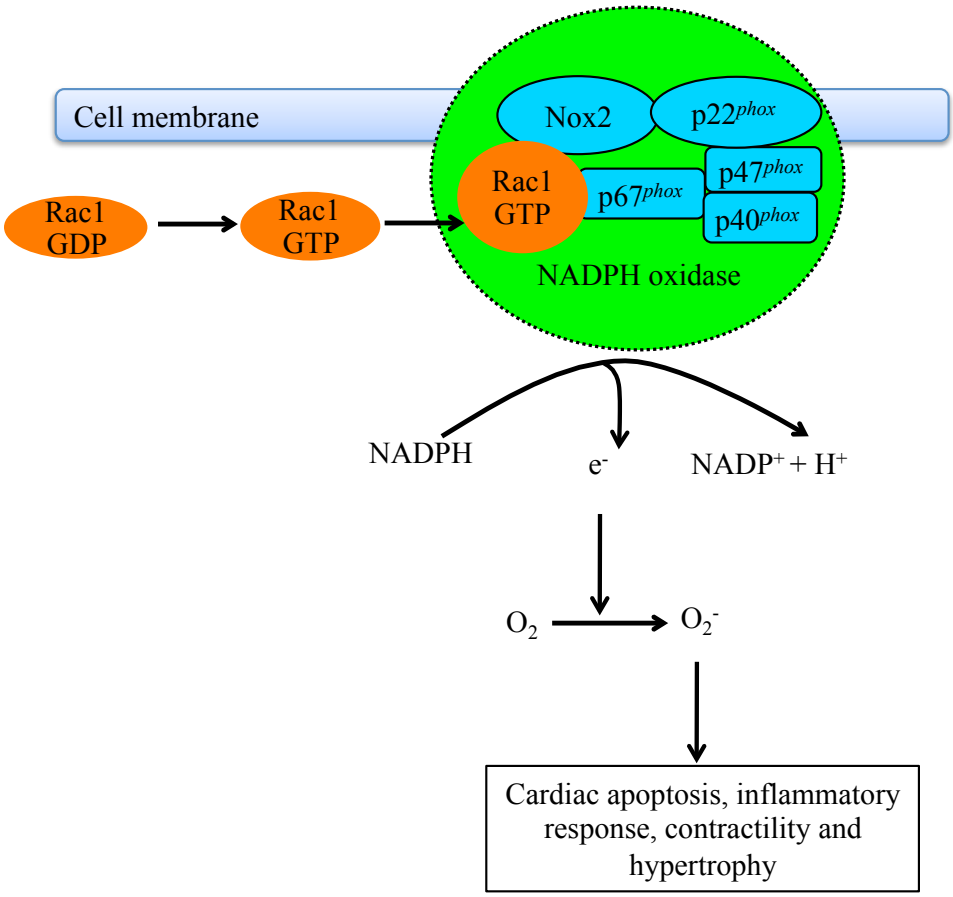

Figure 1.2. Schematic of NADPH oxidase signaling pathways. NADPH oxidase is composed of membrane bound flavocytochrome b558, which consists of gp91^{phox} (Nox2) and p22^{phox}, cytosolic regulatory proteins (p40^{phox}, p47^{phox} and p67^{phox}) and Rac GTPase. Upon stimulation, NADPH oxidase activation is triggered by the phosphorylation of cytosolic *phox* proteins and their translocation to the plasma membrane. Concomitantly, Rac is activated and interacts with flavocytochrome b558 to form a binding partner for p67^{phox}.

There are at least six homologues of gp91^{phox} (Nox2): Nox1, Nox3, Nox4, Nox5 and Duox1/2. Both Nox1 and Nox4 contain 6 trans-membrane domains with NADPH-binding domain in their C-terminal regions and two hemes. They form a complex with p22^{phox}. Nox5, Duox 1 and Duox 2 have a Ca^{2+}-binding site, whereas Duox1 and Duox2 have an additional transmembrane and a peroxidase-like domain (Lambeth et al. 2000; Leto & Geiszt 2006). Nox2 and Nox4 are expressed in cardiomyocytes. They exhibit different subcellular localization and play an important role in mediating oxidative stress. Nox2 is primarily localized on the plasma membrane, whereas Nox4 is found primarily on intracellular membranes, mitochondria, the endoplasmic reticulum or the nucleus (Maejima et al. 2011). Activation of Nox2 requires assembly of NADPH oxidase components and participates in Ang II-induced cardiac hypertrophy. NOX4 produces O_2^- and H_2O_2, which do not require the association with cytosolic regulatory proteins and Rac. NOX4 also is required for pressure overload-induced cardiac hypertrophy and heart failure (Byrne et al. 2003).

1.4.2 NADPH oxidase and myocardial TNF-α expression during endotoxemia

NADPH oxidase is a major source of O_2^- in the myocardium under pathophysiological conditions. The generation of O_2^- cascades into multiple cardiac effects including hypertrophy, hypertension, fibrosis, apoptosis, proliferation and differentiation of ES cells into cardiomyogenic cell lineage (Bendall et al. 2002; Li et al. 2002; Mohazzab et al. 1997; Xiao et al. 2002a). LPS stimulation markedly increases NADPH oxidase activity and O_2^- production in cardiomyocytes. NADPH oxidase inhibitors, such as diphenyleneiodonium (DPI) and apocynin, decrease LPS-induced TNF-α expression (Peng et al. 2005b). Additionally, both O_2^- production and TNF-α

production during LPS stimulation are blunted in $Nox2^{-/-}$ cardiomyocytes. Furthermore, deficiency of Nox2 also improves myocardial function in endotoxemia (Peng et al. 2005b). These studies demonstrate a key role of Nox2-containing NADPH oxidase in myocardial TNF-α expression and cardiac dysfunction in sepsis. MAPKs are downstream factors of Nox2-containing NADPH oxidase signaling as pharmacological inhibition of NADPH oxidase blocked ERK1/2, p38 and JNK phosphorylation induced by LPS stimulation (Peng et al. 2005a; b).

Interestingly, NADPH 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 and PGI_2 (Grandel et al. 2000; Hocherl et al. 2002; Liu et al. 1996; Peng et al. 2005a). COX has two isoforms, the constitutive COX-1 and an inducible COX-2. The full expression of COX-2 requires NF-κB (Rhee & 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, it is conceivable that upregulation of COX-2 enhances myocardial TNF-α expression and decreases cardiac function during sepsis. Our group demonstrated that an NADPH oxidase/MAPK/NF-κB-dependent pathway leads to COX-2 expression in LPS-stimulated cardiomyocytes (Peng et al. 2005a). Indeed, inhibition of either NADPH oxidase or MAPK activity prevents both NF-κB activation and COX-2/PGE_2 production induced by LPS (Peng et al. 2005a). Thus, O_2^- produced from NADPH oxidase promotes LPS-induced TNF-α expression via a MAPK/NF-κB/COX-2 signaling pathway in cardiomyocytes.

1.5 PAKs

1.5.1 Overview

PAKs are serine-threonine protein kinases (Bosco et al. 2009) and each consists of a kinase domain at the C-terminas and a regulatory domain at N-terminus containing a p21-binding domain (PBD). The family consists of two groups. Group I PAKs, including PAK1, PAK2 and PAK3, are activated by GTPases such as Cdc42 and Rac. These PAKs have an autoinhibitory domain (AID) which overlaps with the PBD. The AID interacts with the catalytic domain forming an intramolecular inhibitory conformation. This inhibition is released when activated Cdc42 or Rac binds with the PBD and straightens out the protein. After this conformational change, PAK autophosphorylates Thr423 (for PAK1) in the activation loop of the kinase. Phosphorylation of this site prevents refolding and consequent inhibition, even in the absence of GTPases. All Group I PAKs contain a threonine at the position corresponding to Thr423 in PAK1. PAK1, PAK2 and PAK3 have different expression patterns (Bagrodia & Cerione 1999). PAK1 is mainly expressed in the brain, muscle, heart, and spleen. PAK2 is ubiquitously expressed. PAK3 is expressed in the brain and heart. The group II PAKs, including PAK4, PAK5 and PAK6, have no AID and can also bind to activated Rho-GTPases. However, binding with GTPases leads to the translocation of group II PAKs but has no apparent effect on kinase activity (Bagrodia & Cerione 1999; Molli et al. 2009).

1.5.2 Biological effects of PAKs

PAKs are important in cell motility, survival, proliferation and gene expression. Primarily, PAKs are considered as regulators of the actin cytoskeletal organization and cell motility. They phosphorylate several cytoskeleton regulators, such as LIM domain

kinase (LIMK), myosin light-chain kinase (MLCK), paxillin and filamin A, thereby modulating actin dynamics, cytoskeleton remodeling and focal adhesion dynamics (Molli et al. 2009). LIMK is an actin-binding kinase which phosphorylates cofilin and prevents the depolymerization of filament. PAK activates LIMK by phosphorylation and regulates cell motility. MLCK phosphorylates myosin light chain (MLC) and enables the myosin to bind to the actin filament. PAK blocks MLCK activity by phosphorylation and results in decreased stress fibers. Paxillin localizes to focal adhesions and functions as an adaptor protein. PAK phosphorylates paxillin which promotes the binding between paxillin and G protein-coupled receptor kinase-interactor 1 (GIT1). GIT1 forms a complex with PAK-interacting exchange factor (PIX) and PAK, and promotes cell migration, protrusion and adhesion turnover. Filamin A is an actin-binding protein and necessary for building of the cytoskeleton that gives structure to cells and allows them to change shape and move. PAK1 phosphorylates filamin A and regulates actin dynamics at the leading edge of motile cells (Molli et al. 2009).

PAKs have both pro- and anti-apoptotic functions. PAK1 improves Bcl2 activity by phosphorylating Bad and blocking the interaction between Bcl2 and Bad (Schurmann et al. 2000). Furthermore, PAK1 is capable of activating dynein light chain 1 (DLC1) and subsequently prevents inactivation of Bcl-2 (Vadlamudi et al. 2004). Activated PAK4 increases cell survival through decreasing caspase activation and promoting Bad phosphorylation (Gnesutta et al. 2001). Specifically, PAK2 shows a dual effect in regulating cell survival and death. DNA damage and serum starvation activate caspases which cleaves PAK-2 proteolytically, producing a constitutively active fragment, PAK-2p34. Activation of PAK2 as a full-length enzyme or as a proteolytic fragment leads to

different effects. Activation of full-length PAK2 by Rac or Cdc42 inhibits the interaction between Bad and Bcl-2 and promotes cell survival. In contrast, activation of PAK-2p34 correlates with the induction of apoptosis (Jakobi et al. 2001).

1.5.3 PAKs in the heart

PAK1, PAK2 and PAK3 are all expressed in the myocardium. Activated PAK1 localizes to sarcomeric Z-disks, interacts with protein phosphatase 2A (PP2A), and reduces phosphorylation of cardiac troponin I and myosin binding protein (MyBP)-C. These posttranslational modifications influence contractility by altering Ca^{2+} sensitivity (Sheehan et al. 2007). Recent studies showed that PAK1 colocalizes with both the $\alpha 1_C$-subunit of the L-type Ca^{2+} channel and PP2A. Expressing constitutively active PAK1 in adult rat ventricular myocytes decreases the time to peak shortening, rate of $[Ca^{2+}]_i$ decay and time of relengthening, suggesting that PAK1 modifies Ca^{2+} flux (Sheehan et al. 2009). Thus, PAK1 regulates cardiomyocyte contractility by facilitating the dephosphorylation of proteins that control myofilament activity and intracellular Ca^{2+} flux. Buscemi et al. reported that activated PAK3 increases fiber bundle Ca^{2+} sensitivity via phosphorylation of myofilament proteins (desmin, troponin I, troponin T) (Buscemi et al. 2002). In addition, PAK has been implicated in Rac1-induced cardiac hypertrophy in transgenic mice (Sussman et al. 2000). There is evidence that PAK regulates IL-1 expression in macrophages through activation of MAPKs (Hsu et al. 2001). An objective of this thesis was to investigate if PAK1 plays a role in myocardial TNF-α expression and myocardial depression in endotoxemia (Chapter 4).

1.6 Na/K-ATPase

1.6.1 Overview

Na/K-ATPase is located in the plasma membrane and contains a catalytic α subunit, a β subunit and a regulatory subunit, FXYD. The α-subunit has 10 transmembrane segments and contains binding sites for Na^+, K^+, ATP and proteins, such as Src. The β subunit is a glycoprotein, necessary for the transfer of the entire enzyme molecule from the endoplasmic reticulum, the site of synthesis, to the plasma membrane. There are presently four isoforms of the α subunit (α_1, α_2, α_3, α_4) and three isoforms of the β subunit (β_1, β_2, β_3). Mouse hearts express α1, α2, β1 and β2, with α1 and β1 as the major isoforms. The FXYD protein family is a family of small membrane proteins sharing a conserved Pro-Phe-X-Tyr-Asp motif in the extracellular N-terminal domain. In mammals, the FXYD family contains seven members: FXYD1- FXYD7 (Geering 2006).

1.6.2 Regulation of Na/K-ATPase activity

Na/K-ATPase activity is regulated by a variety of factors that affect its activation and expression. The simplest and most straightforward determinant of this pump activity is the concentrations of substrates. Since intracellular Na^+ concentration is a critical factor for determining the Na/K transport rate, any change in intracellular Na^+ will have an impact on its activation. Besides, this pump is also sensitive to the changes in the intracellular ATP and extracellular K^+ concentrations.

Peptide hormones and neurotransmitters regulate Na/K-ATPase expression, translocation or endocytosis via the protein kinases (protein kinase A (PKA), PKC and protein kinase G (PKG)), phospholipases, and phosphatases (protein phosphatases 2B, PP2B) in a tissue specific manner (Therien & Blostein 2000). For example, in guinea-pig

ventricular myocytes, an isoprenaline-induced increase in Na/K-ATPase current is mediated by cAMP, which activates PKA (Gao et al. 1994). Noradrenaline analogously increases Na/K-ATPase current via PKC (Wang et al. 1998). In addition, steroid hormones regulate Na/K-ATPase subunit expression. For example, aldosterone stimulates α and β subunit gene transcription and leads to an increased pump number in the cell membrane (Therien & Blostein 2000). Recently, a novel mechanism of Na/K-ATPase activation has been reported. Ang II inhibits myocardial Na/K-ATPase via activating NADPH oxidase and glutathionylating the β subunit (Figtree et al. 2009).

FXYD proteins do not affect Na/K-ATPase subunit expression but change the transport properties in a tissue-specific way (Geering 2006). FXYD1, known also as phospholemman, is expressed in the heart and liver, and has multiple phosphorylation sites at its cytosolic carboxyl terminus. In resting cardiomyocytes, FXYD1 binds with the α subunit and inhibits Na/K-ATPase activity by reducing its affinity to intracellular Na^+. Upon stimulation, FXYD1 is phosphorylated by PKC and dissociates from Na/K-ATPase, leading to increased Na^+ affinity (Bers & Despa 2006). FXYD2 and FXYD4 are mainly expressed in kidney. FXYD2 decreases Na^+ affinity of Na/K-ATPase, which favors an efficient reabsorption of Na^+ at a high cellular Na^+ load. However, FXYD4 increases affinity for Na^+ which allows Na^+ reabsorption at low intracellular Na^+ concentrations (Beguin et al. 2001). FXYD3 is detectable in the skin, colon, stomach and uterus and apparently decreases both Na^+ and K^+ affinity (Crambert et al. 2005). FXYD7 is exclusively expressed in the brain and produces low K^+ affinity (Beguin et al. 2002).

1.6.3 Biological effects of Na/K-ATPase

The primary function of Na/K-ATPase is to catalyze the chemical hydrolysis of

ATP to mediate active extrusion of Na^+ and intracellular accumulation of K^+. In resting cells, the ion binding sites of Na/K-ATPase are open to the inside of the cell. Once three Na^+ ions bind to the sites, the pump is phosphorylated by ATP. The phosphorylation causes a conformational change and exposes the Na^+ ions to the outside of the cells. Since the phosphorylated form of the pump has a low affinity for Na^+ ions, Na^+ ions are released. At the same time, two K^+ ions bind to the binding sites which promote the dephosphorylation of the pump. The removal of the phosphate causes another conformational change that result in the return of the ion binding sites to the inside of the cell. Consequently, the K^+ ions are released inside the cell. Na/K-ATPase is the main ion pump for Na^+ and K^+ transmembrane distribution, and therefore it plays an essential role in the maintenance of the Na^+ and K^+ gradients. These gradients are needed to facilitate transport, regulate cellular volume, and maintain the resting membrane potential.

In addition to pumping ions, Na/K-ATPase also acts as a signal transducer. Binding of ouabain, a specific Na/K-ATPase inhibitor, with Na/K-ATPase results in the interaction with the cytoplasmic tyrosine kinase Src directly. The Na/K-ATPase/Src complex acts as a "binary receptor" and activates multiple downstream protein kinases including epidermal growth factor receptor (EGFR), MAPKs and PKC (Aydemir-Koksoy et al. 2001; Haas et al. 2000; Haas et al. 2002). These kinases affect various cellular functions, such as gene expression, the production of ROS, the formation of tight junctions, cell proliferation and attachment, the modification of immune responses, the induction of polarity, ion fluxes and protein trafficking (Aydemir-Koksoy et al. 2001; Contreras et al. 1999; Xie & Cai 2003).

1.6.4 Na/K-ATPase in the heart

Although Na/K-ATPase is present in all cells, it also contributes to specialized tissue functions. In cardiomyocytes, Na/K-ATPase activity is tightly coupled to the activation of the Na^+/Ca^{2+} exchanger (NCX), which transports one Ca^{2+} ion in exchange for three Na^+ ions, therefore regulating cardiac contractility (Barwe et al. 2009; Clausen 2003; James et al. 1999). Under physiological conditions, NCX is driven by the high Ca^{2+} transient and works in the Ca^{2+} extrusion mode. When Na/K-ATPase is inhibited, $[Na^+]_i$ is elevated and subsequently drives NCX in a reverse mode. Ca^{2+} enters the cells through NCX, resulting in increased cellular and sarcoplasmic reticulum (SR) Ca^{2+} concentrations. The higher intracellular Ca^{2+} concentrations lead to greater Ca^{2+} transients and enhanced contractility.

Na/K-ATPase also participates in cardiomyocyte apoptosis and cardiac hypertrophy (Huang et al. 1997; Sapia et al. 2010). Ouabain induces cardiomyocyte apoptosis. This effect of ouabain is mediated by reverse mode NCX-dependent activation of calmodulin-dependent protein kinase (CaMK) II, and is counterbalanced by the simultaneous activation of PI3K/AKT signaling (Sapia et al. 2010). In addition, ouabain increases hypertrophic gene expression in cultured cardiomyocytes via Ca^{2+}/CaMKs and PKC (Huang et al. 1997).

Na/K-ATPase activity is decreased in multiple organs during sepsis (Guzman et al. 1995; Koksel et al. 2006; Ohmori et al. 1991). LPS markedly decreases Na/K-ATPase activity in the lung (Guzman et al. 1995; Koksel et al. 2006) and kidney (Guzman et al. 1995; Koksel et al. 2006). Inhibition of Na/K-ATPase potentiates LPS-induced cytokine mRNA expression, including TNF-α mRNA, in macrophages (Ohmori et al. 1991).

However, changes in Na/K-ATPase activity in the heart during sepsis are not known. An objective of this thesis was to determine myocardial Na/K-ATPase activity and to investigate if Na/K-ATPase regulates myocardial TNF-α expression during endotoxemia (Chapter 3).

1.7 Calcium signaling and TNF-α expression during endotoxemia

Calcium is an important diffusible second messenger and 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+} makes its entrance into the cytoplasm either from outside the cell through the cell membrane via Ca^{2+} channels, or from internal Ca^{2+} storages such as the sarcoplasmic reticulum, endoplasmic reticulum and mitochondria. Intracellular Ca^{2+} is removed from the cell by transport proteins, such as plasma membrane Ca^{2+}-ATPase and the 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 are increased in LPS-stimulated macrophages and Kupffer cells leading to 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 mediated by PLCγ (Zhou et al. 2006). In addition, a correlation between Ca^{2+}/calmodulin, ERK activity and TNF-α expression is established in mononuclear cells (Mendez-Samperio et al. 2006; Rosengart et al. 2000b). Indeed, either the chelation of releasable intracellular stores of

Ca^{2+} or the inhibition of calmodulin decreased ERK1/2 phosphorylation and TNF-α levels induced by *Mycobacterium bovis* infection (Mendez-Samperio et al. 2006). Similarly, inhibition of Ca^{2+} /CaMK II prevented ERK1/2 activation and the TNF-α expression in response to LPS stimulation (Rosengart et al. 2000b).

Intracellular Ca^{2+} levels in cardiomyocytes are mainly regulated throught L-type Ca^{2+} channels and sarcoplasmic reticulum Ca^{2+} release channels (or ryanodine receptors, RyRs) during depolarization, which initiates contraction. L-type Ca^{2+} channels are located primarily on the sarcolemma in close proximity with the sarcoplasmic reticulum, and generate an inward Ca^{2+} current during activation. Ca^{2+} entering through L-type Ca^{2+} channels locally increases Ca^{2+} concentration near RyRs and triggers Ca^{2+} release from the sarcoplasmic reticulum (via Ca^{2+} induced Ca^{2+} release). When Ca^{2+} binds to troponin C, it switches on the myofilaments and causes contraction. During relaxation and diastolic filling, Ca^{2+} dissociates from troponin C, thereby turning off the contractile machinery. Ca^{2+} is removed from the cytosol mainly via the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), which takes Ca^{2+} back into the sarcoplasmic reticulum, and the NCX, and to a much less extent via sarcolemmal Ca^{2+}-ATPase and the mitochondrial Ca^{2+} uniporter (Bers 2000). Apart from an essential role in cardiac excitation-contraction coupling, Ca^{2+} has been shown to be important in numerous physiological and pathophysiological processes in the heart, including cardiac hypertrophy, apoptosis, arrhythmia and heart failure as well as gene expression (Erickson & Anderson 2008; Frey et al. 2000).

Sepsis is associated with increased myocardial TNF-α expression and cardiac dysfunction, both of which are regulated by cardiomyocyte Ca^{2+} homeostasis. Recent

studies have demonstrated that LPS increases Ca^{2+} transients and ERK1/2 phosphorylation, leading to TNF-α expression in cardiomyocytes and cardiac dysfunction (Geoghegan-Morphet et al. 2007). Treatment with ERK siRNA decreased LPS-induced ERK activity and TNF-α expression, confirming an important role of ERK activation. Furthermore, inhibition of L-type Ca^{2+} channel activity by verapamil abrogated ERK1/2 phosphorylation and TNF-α production during LPS stimulation. These data suggest that Ca^{2+} signaling results in ERK activation and TNF-α expression in cardiomyocytes during LPS stimulation (Geoghegan-Morphet et al. 2007).

1.8 Mammalian target of rapamycin

1.8.1 Overview

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase and contains 2549 amino acids. It forms two multi-protein complexes with distinct functions, mTOR complex (mTORC)1 and mTORC2. mTORC1 consists of 5 components: mTOR, mammalian lethal with Sec13 protein 8 (mLST8), regulatory-associated protein of mTOR (Raptor), DEP-domain-containing mTOR-interacting protein (Deptor) and proline rich Akt substrate 40 kDa (PRAS40). Raptor contributes to the assembly of the complex and substrate recruitment for mTOR. The interaction of mLST8 with mTOR stabilizes mTOR-Raptor association. PRAS40 and Deptor are negative regulators of mTORC1 activity (Laplante & Sabatini 2009). PRAS40 binds to Raptor and inhibits mTOR kinase activity by blocking substrate access to Raptor. Stimuli, such as insulin, cause a release of PRAS40 from Raptor and allow substrate presentation to mTORC1 (Wang et al. 2007).

mTORC2 is comprised of six different proteins: mTOR; rapamycin-insensitive

companion of mTOR (Rictor), protein observed with Rictor-1 (Protor-1), mammalian stress-activated protein kinase interacting protein (mSIN1), Deptor and mLST8. Rictor and mSIN1 build the structural foundation of mTORC2. mLST8 is necessary for mTORC2 stability and activity. Deptor decreases mTORC2 activity (Laplante & Sabatini 2009).

1.8.2 Regulation of mTOR signalling

mTORC1 activation is regulated by the PI3K/Akt pathway, AMP-activated protein kinase (AMPK), the tuberous sclerosis complex (TSC)1/TSC2 complex and Ca^{2+} (Averous & Proud 2006; Hay & Sonenberg 2004; Hoyer-Hansen et al. 2007; Lenz & Avruch 2005). Studies have demonstrated that growth factors activate PI3K/Akt, ERK1/2 or p90 ribosmal S6 kinase 1 pathways which lead to the inhibition of TSC1/2 complex activation. The TSC1/2 complex comprises TSC1 and TSC2 and functions as a GAP for Rheb, a small Ras-related GTPase. Since activated Rheb directly activates mTORC1, TSC1/2, due to its GAP activity, negatively regulates mTORC1 activity (Hay & Sonenberg 2004; Laplante & Sabatini 2009). In energy depletion and hypoxic conditions, AMPK is activated upon the decline of the intracellular ATP levels. Activated AMPK phosphorylates TSC2, leading to a reduction in mTORC1 activity (Hay & Sonenberg 2004). In primary mouse neurons, glutamatergic stimulation activates mTOR in a Ca^{2+}/CaMK II dependent fashion (Lenz & Avruch 2005) (Figure 1.3).

On the other hand, intracellular amino acids regulate mTORC1 via TSC1/2-independent mechanisms. Rag proteins are required for mTORC1 activation induced by amino acids. Rag binds with Raptor and promotes the intracellular localization of mTOR to a compartment that contains its activator Rheb, resulting in the activation of mTORC1

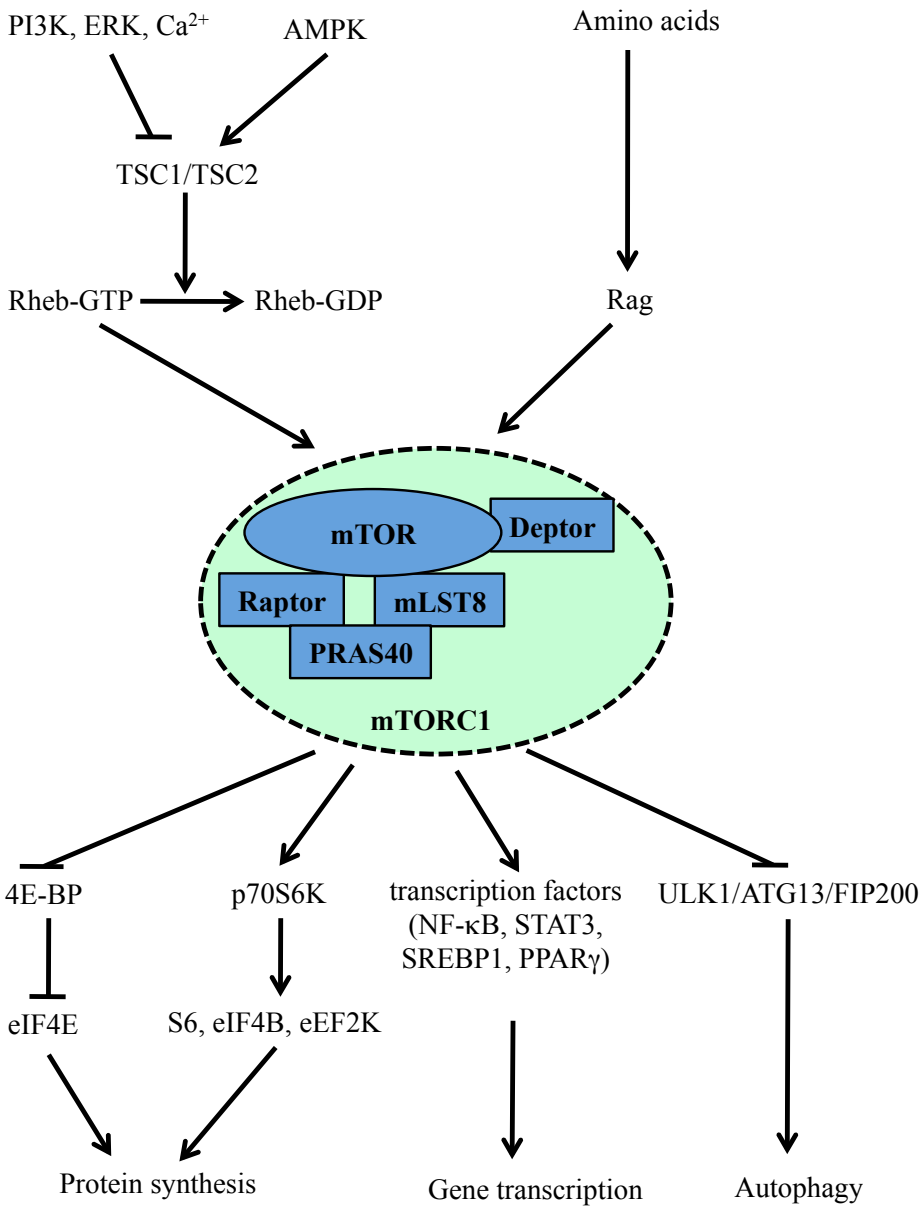

Figure 1.3. Schematic of mTORC1 signaling pathways. mTORC1 consists of 5 components: mTOR, mLST8, Raptor, Deptor and PRAS40. PI3K, AMPK and calcium activate mTORC1 by inhibiting TSC1/TSC2 and activating Rheb. Amino acides activate mTORC1 through Rag. mTORC1 plays a role in regulating protein synthesis, gene expression and autophagy via 4E-BP, p70S6K, transcription factors (NF-κB, STAT3, SREBP1 and PPARγ) and the ULK1/ATG13/FIP200 complex.

(Kim et al. 2008; Sancak et al. 2008) (Figure 1.3).

The mechanisms that activate mTORC2 are not well understood. Akt, a downstream factor of mTORC2, is phosphorylated by phosphoinositi-dedependent kinase (PDK) 1 which recruites to the membrane and binds with PIP3 at PH domain. Since mSIN1, the component of mTORC2, contains a C-terminal PH domain, it is possible that mSIN1 mediates the translocation of mTORC2 to the membrane and promotes phosphorylation of Akt (Laplante & Sabatini 2009). Further studies are required to verify this hypothesis.

1.8.3 Biological effects of mTORC1 signalling

mTORC1 acts as a central regulator of cell survival, proliferation and metabolism by controlling biosynthesis of proteins, lipids and organelles and autophagy (Laplante & Sabatini 2009) (Figure 1.3). It primarily controls protein synthesis by regulating translation. The 5' end of all nuclear-transcribed mRNAs possess a 7 methyl guanosine cap (m7G) which is essential in cap-dependent protein translation initiation and is recognized by eukaryotic translation initiation factor 4E (eIF4E). eIF4E forms a complex with eIF4G and the RNA helicase. This complex binds the 5' cap and unwinds the mRNA 5'-proximal secondary structure to facilitate the binding of the 40S ribosomal subunit. The interaction between eIF4E and eIF4G is regulated by the eIF4E-binding proteins (4E-BPs), a family of translational repressor proteins. The 4E-BPs compete with eIF4G proteins for an overlapping binding site on eIF4E. This process is prevented by 4E-BP phosphorylation. mTORC1 promotes protein synthesis by directly phosphorylating 4E-BPs which enables eIF4E to bind with eIF4G to initiate translation (Hay & Sonenberg 2004).

Besides 4E-BPs, activated mTORC1 can also phosphorylate protein 70 S6 kinase (p70S6K). p70S6K promotes translation by activating the ribosomal protein S6, eIF4B, and eukaryotic elongation factor 2 kinase (eEF2K). Activated ribosomal protein S6 is necessary for recruitment of mRNA to ribosomes and the formation of translation initiation complexes. eIF4B is a protein involved in unraveling the inhibitory secondary structure in the 5'-UTRs of certain mRNAs. p70S6K phosphorylates eIF4B and promotes its association with translation initiation complexes. Eukaryotic elongation factor 2 (eEF2) mediates the translocation step of translation elongation and is inactivated by eEF2K. p70S6K negatively regulates eEF2K and thereby activates eEF2 (Laplante & Sabatini 2009).

mTORC1 also controls the transcription of certain genes via transcription factors such as NF-κB, STAT3, peroxisome proliferator-activated receptor-γ (PPARγ) and regulatory element binding protein 1 (SREBP1). SREBP1 and PPARγ modulate gene expression involved in lipid and cholesterol homeostasis (Kim & Chen 2004). mTORC1 regulates lipid synthesis through SREBP1 and PPARγ (Huffman et al. 2002; Kim & Chen 2004; Porstmann et al. 2008). In macrophages, mTORC1 promotes LPS-induced pro-inflammatory factor expression via NF-κB but inhibits IL-10 expression via STAT3 (Weichhart et al. 2008).

Autophagy is the process of self-digestion by a cell through the action of enzymes originating within the same cell. This process is necessary for maintaining a balance between the synthesis, degradation, and subsequent recycling of cellular products. mTORC1 inhibits autophagy through a protein complex containing focal adhesion kinase family-interacting protein of 200 kDa (FIP200), autophagy-related gene 13 (ATG13) and

unc-51-like kinase 1 (ULK1). ULK1 is a mammalian serine/threonine protein kinase and plays a key role in the initial stages of autophagy. FIP200 and ATG13 are critical for the correct localization of ULK1 to the pre-autophagosome and the stability of ULK1 protein. mTORC1 phosphorylates and thereby represses ULK1 and ATG13, leading to inhibition of autophagy (Ganley et al. 2009; Jung et al. 2009).

1.8.4 Biological effects of mTORC2 signaling

mTORC2 participates in cell survival, metabolism, proliferation and cytoskeleton organization (Laplante & Sabatini 2009). Akt is a well known factor contributing to cell survival, metabolism and proliferation. Activation of Akt requires phosphorylation at Ser308 and Ser473 of the protein (Manning 2004). Deletion of mTORC2 components inhibits the phosphorylation of Akt at Ser473 and activity of transcription factors (forkhead box protein O1, FoxO1 and FoxO3α). These two transcription factors control the expression of genes involved in cell survival, proliferation and metabolism (Guertin et al. 2006). mTORC2 controls the actin cytoskeleton by paxillin phosphorylation, relocalization of paxillin to focal adhesions, and the activation of Rho-GTPases (Jacinto et al. 2004).

1.8.5 mTOR in the heart

mTOR is now considered to be an essential regulator of cardiac function, contributing to the development of cardiac hypertrophy in physiological or pathological conditions (Balasubramanian et al. 2009; McMullen et al. 2004; Shioi et al. 2003). In the pressure-overloaded myocardium, both mTORC1 and mTORC2 are activated. Rapamycin, an inhibitor of mTORC1, promotes the regression of compensated hypertrophy as well as decompensated hypertrophy, via a p70 ribosomal S6 kinase 1–dependent mechanism

(McMullen et al. 2004; Shioi et al. 2003). Akt, the downstream mediator of mTORC2, promotes cardiomyocyte growth and survival, which are critical events in hypertrophic heart, supporting a role of mTORC2 signaling in hypertrophy (Balasubramanian et al. 2009). Moreover, under hypoxic conditions, inhibition of mTORC1 by everolimus decreases infarct size and left ventricular remodeling after myocardial infarction through enhanced autophagy in the border region of the infarct zone (Buss et al. 2009).

Recent studies have reported that LPS activates mTOR in phagocytes, bone marrow cells (Chen et al. 2010) and HL-1 cells (Weichhart et al. 2008). However, the effect of mTOR on pro-inflammatory factor expression is still controversial. mTOR promotes pro-inflammatory factors (TNF-α, IL-12) in monocyte-derived dendritic cells (Haidinger et al. 2010). On the contrary, mTOR is a negative regulator of TNF-α production in monocytes (Weichhart et al. 2008), macrophages (Baker et al. 2009) and HL-1 cells (Song et al. 2010). However, the role of mTOR in the heart during sepsis has not been elucidated. An objective of this thesis was to investigate if mTOR regulates myocardial TNF-α production during endotoxemia (Chapter 3).

1.9 Regulation of TNF-α expression by MAPKs during endotoxemia

MAPKs are a family of serine/threonine protein kinases. There are three well-characterized subfamilies: p38, ERK1/2 and JNK. MAPK pathways are activated by a dual-specificity serine-threonine MAPK-kinase (MKK) that, in turn, is phosphorylated and activated by an upstream MKK-kinase (MKKK) at two serine residues. The phosphorylation of MAPKs leads to increased substrate accessibility and catalysis (Cobb & Goldsmith 2000). Activated MAPKs can activate a wide range of downstream targets

and regulate gene transcription, mRNA stability, transport and translation (Davis 1993; Dong et al. 2002; Hazzalin & Mahadevan 2002). These signaling cascades are not only involved in normal cellular process, but have also been implicated in the pathology of many diseases, such as cancer, atherosclerosis, diabetes, arthritis and septic shock (Liu et al. 2007).

1.9.1 p38

To date, five p38 isoforms have been identified: p38α, p38β, p38β2, p38γ and p38δ. The p38α and p38β genes are ubiquitously expressed. However, p38γ and p38δ are differentially expressed in different tissues. p38γ is synthesized mostly in skeletal muscle and p38δ is expressed in lung, kidney, testis, pancreas and small intestine (Ono & Han 2000). The p38 pathway is known to be responsible for a multitude of cellular events, such as proliferation, differentiation, apoptosis and inflammation (Herlaar & Brown 1999). Recently, p38 has been recognized as an important regulator of heart function in various animal models. For example, transgenic mice with targeted activation of p38 in cardiomyocytes have impaired systolic as well as diastolic functions of the heart (Liao et al. 2001). p38 is activated in the myocardium during ischemia and reperfusion. Deletion of p38α gene decreases infarct size following myocardial ischemia and reperfusion (Otsu et al. 2003). In spontaneously hypertensive stroke-prone rats receiving a high-salt/high-fat diet, myocardial p38 is activated during the development of cardiac hypertrophy and contributes to hypertensive end-organ damage and premature mortality (Behr et al. 2001). Moreover, evidence suggests a key role of p38 activity in myocardial TNF-α expression and cardiac dysfunction in sepsis. For instance, LPS increases p38 phosphorylation in primary neonatal cardiomyocytes (Peng et al. 2005a; b; Peng et al. 2003a; Peng et al.

2003b). Pharmacological inhibition of p38 or specific down-regulation of p38α significantly suppresses both TNF-α mRNA and protein levels in cardiomyocytes (Peng et al. 2003b). Consistent with these *in vitro* studies, p38 is also activated and mediates myocardial TNF-α expression in sepsis. Inhibition of p38 activity significantly reverses LPS-induced left ventricular depression and decreases mortality to 65% in endotoxemia (Peng et al. 2003b). Therefore, p38 activation plays a critical role in myocardial TNF-α expression and myocardial depression in endotoxemia (Zhang & Feng 2010).

1.9.2 ERK1/2

Phosphorylation of ERK1 and ERK2 (p44 MAPK 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 NF-κB, thereby regulating expression of proteins required for a given response. Activation of ERK1/2 promotes TNF-α expression during sepsis in several types of cells including cardiomyocytes (Peng et al. 2005a; b; Rosengart et al. 2000b). Indeed, ERK1/2 in cardiomyocytes is phosphorylated transiently but dramatically by LPS stimulation. Inhibition of ERK1/2 by ERK siRNA or pharmacological inhibitors (e.g., PD98059 and U0126) abrogates TNF-α production induced by LPS (Geoghegan-Morphet et al. 2007; Peng et al. 2005b), suggesting that ERK1/2 play an important role in TNF-α expression. Furthermore, inhibition of ERK1/2 by U0126 decreases TNF-α production and protects the lung from LPS-induced injury in mice (Schuh & Pahl 2009).

1.9.3 JNKs

JNKs are activated by MKK4 and MKK7. The JNK family contains three members: JNK1, JNK2 and JNK3. JNK1 and JNK2 are expressed in the myocardium (Ip

& Davis 1998). LPS activates JNK and increases TNF-α transcription and translation in macrophages and monocytes (Comalada et al. 2003; Swantek et al. 1997). LPS also increases phosphorylation of JNKs in cultured mouse neonatal cardiomyocytes (Peng et al. 2005a; Peng et al. 2009). Unexpectedly, JNK1 activation decreases TNF-α expression in these cells (Peng et al. 2009). Deficiency of the JNK1 gene further enhances TNF-α expression in cardiomyocytes in response to LPS. In addition, blocking JNK1 signaling pathway also enhances the ROS production stimulated by LPS (Peng et al. 2009), which may be due to the increase of TNF-α levels (Garg & Aggarwal 2002). Increased ROS production and TNF-α expression may lead to myocardial depression. Indeed, cardiac function in $JNK1^{-/-}$ mice is further decreased in endotoxemia, compared with wild-type animals (Peng et al. 2009). These data demonstrate that JNK1 activation inhibits myocardial TNF-α expression and improves cardiac function in endotoxemia. In addition, deficiency in JNK1 enhances LPS-induced p38 and ERK1/2 activity (Peng et al. 2009), suggesting crosstalk among the MAPK subfamily members in cardiomyocytes during sepsis.

1.9.4 Crosstalk among MAPKs

Crosstalk among different MAPK signaling pathways may determine the final biological response upon stimulation and the nature of the crosstalk varies depending on the cell types and stimuli. For example, transforming growth factor-β (TGF-β)-stimulated ERK1/2 activation caused dephosphorylation of LPS-induced p38 in macrophages (Xiao et al. 2002b). Conversely, activation of p38 by adenoviral expression of MKK3b dephosphorylates MEK1/2 via phosphatase 2A in human skin fibroblasts (Junttila et al. 2008; Li et al. 2003). Additionally, sustained JNK activation due to mitogenic factors

inhibits ERK activity in COS-7 cells (Shen et al. 2003).

In cardiac myocytes, activation of different MAPKs shows completely opposite effects in sepsis. In this regard, activation of p38 and ERK1/2 promote TNF-α production, while activation of JNK1 inhibits TNF-α expression during LPS stimulation (Peng et al. 2009). Our group has shown that deficiency in JNK1 enhances LPS-induced p38 and ERK1/2 activity and TNF-α production in myocardium, suggesting a negative feedback of JNK1 on p38 and ERK1/2 activity (Peng et al. 2009). Interestingly, the inhibitory effects of JNK1 are mediated by c-fos, an immediate early response gene involved in a wide spectrum of cellular response including cell proliferation, growth and cytokine production (Herrera & Robertson 1996; Lee et al. 2004). The expression of c-fos is enhanced by LPS stimulation in the myocardium, and disrupted by an adenovirus encoding dominant negative mutant of JNK1 and a JNK pharmacological inhibitor (SP600125), suggesting the c-fos is downstream of JNK1 (Meng et al. 1998; Peng et al. 2009). Furthermore, deficiency of c-fos gene augmented p38 and ERK1/2 activity and cardiac TNF-α expression. Conversely, over-expression of c-fos inhibited TNF-α production associated with reductions in p38 and ERK1/2 activity. More importantly, deficiency in c-fos resulted in a further damage in cardiac function during endotoxemia (Peng et al. 2009). These results demonstrate the inhibitory effects of c-fos on p38 and ERK1/2 activity, and TNF-α expression, leading to improvement in cardiac function during endotoxemia. c-fos can dimerise with c-jun to form the activator protein 1 (AP-1), which upregulates the transcription of a diverse range of genes. However, the effects of c-fos in cardiac myocytes are independent of AP-1 since inhibition the formation of c-fos/c-jun AP-1 complex or treatment with a decoy AP-1 oligodeoxynucleotide did not

alter TNF-α expression (Peng et al. 2009). Therefore, the JNK1 inhibits LPS-induced TNF-α expression through inhibition of ERK1/2 and p38 activation (Zhang & Feng 2010). This negative crosstalk is mediated by c-fos (Figure 1.4).

1.10 MAPK phosphatases

1.10.1 Overview

The strength and duration of MAPK signaling are tightly controlled in cells and tissues. Dephosphorylation of MAPKs is the most efficient way for termination of these pathways. Dual-specificity (tyrosine and threonine) MAPK phosphatases (MKPs) have been shown to inactivate MAPK by dephosphorylating phosphothreonine and phosphotyrosine residues (Liu et al. 2007).

In mammalian cells, 11 MKPs have been identified (MKP-1 – MKP-5, MKP-7, MKPX, dual specific phosphatase 2 (DUSP2), human VH1-like PTPase-3 (HVH3), HVH5, DUSP24) (Dickinson & Keyse 2006). According to subcellular localization and patterns of transcriptional regulation, MKPs are divided into two groups. Group 1, including MKP1, MKP2, HVH3 and DUSP2, is primarily localized in the nucleus. Expression of these MKPs can be quickly induced by extracellular stimuli to negatively controls MAPK activity. The MKPs in group 2 are localized in the cytoplasm and/or nuclear compartments. The expression of these genes is induced at a much slower rate compared with the genes that encode the first group of MKPs (Keyse 2000).

1.10.2 MKP-1 and TNF-α expression during endotoxemia

MKP-1, the founding member of this family, is a critical regulator of innate immune

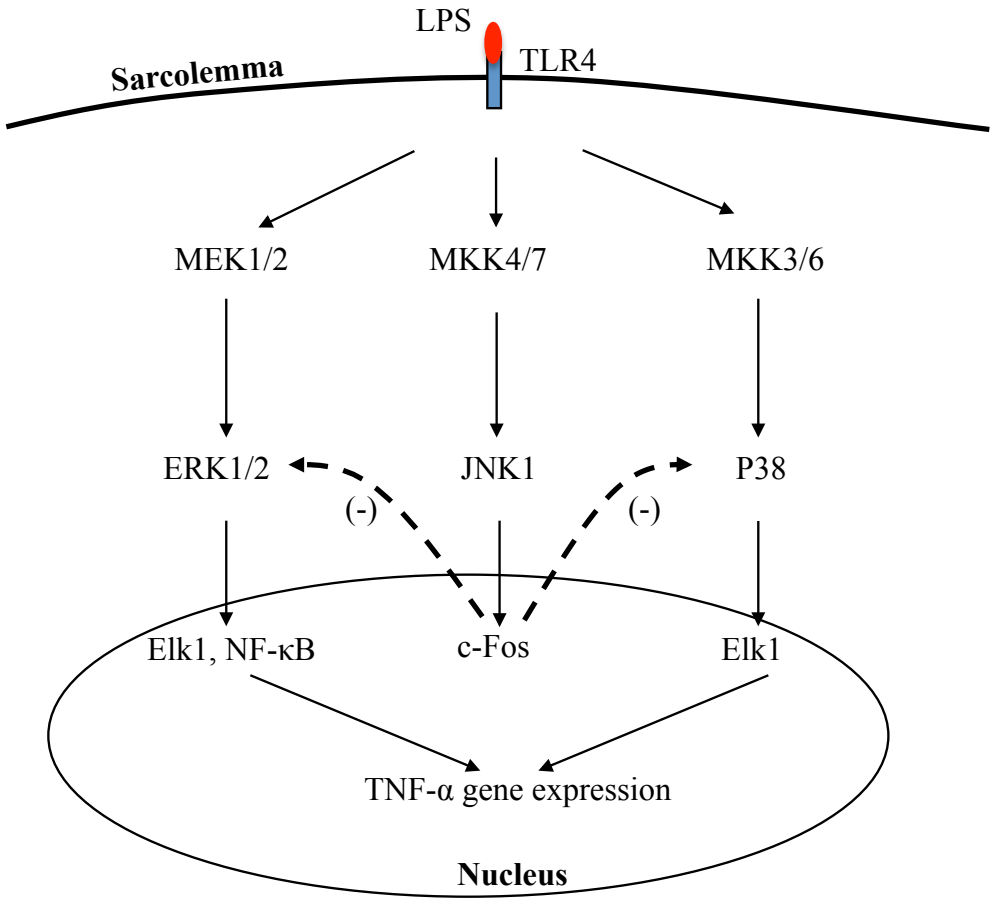

Figure 1.4. Crosstalk among MAPKs on regulating LPS-induced TNF-α expression in cardiomyocytes. ERK1/2 and p38 MAPK activity enhance LPS-induced TNF-α production, while JNK1 activity shows an inhibitory effect. This function of JNK1 is performed by attenuating LPS-induced ERK1/2 and p38 MAPK activity via c-fos. (Adapted from Can. J. Physiol. Pharmacol., Zhang and Feng, 2010).

responses (Liu et al. 2007). Overexpression of MKP-1 accelerates JNK and p38 inactivation and attenuates TNF-α production in RAW264.7 macrophages in response to LPS, indicating that MKP-1 preferentially inactivates p38 and JNK relative to ERK (Chen et al. 2002). Consistently, MKP-1 deficiency leads to sustained activation of p38 and JNK, and enhances pro-inflammatory factor expression in LPS-treated macrophages (Zhao et al. 2006). Furthermore, MKP-$1^{-/-}$ mice exhibit significantly increased serum TNF-α, IL-1β, CC-chemokine ligand 2 (CCL2), interferon-gamma (IFN-γ) and IL-6, and show a higher incidence of multi-organ failure and a greater mortality rate in endotoxemia, compared with wild-type mice (Hammer et al. 2006; Salojin et al. 2006), these findings suggest that MKP1 is an important negative regulator of MAPK and inhibits TNF-α expression during the inflammatory response of the innate immune system (Liu et al. 2007).

1.10.3 Regulation of MKP-1 activity

The phosphatase activity of MKP-1 is regulated by transcriptional induction and protein stability (Liu et al. 2007). MKP-1 expression is inducible. In fibroblasts, MKP-1 induction is dependent on ERK1/2 (Brondello et al. 1997) or JNK activation (Bokemeyer et al. 1996). PKC, Raf-1 and JNK1 are involved in MKP-1 transcriptional induction in macrophages (Sanchez-Tillo et al. 2006; Sanchez-Tillo et al. 2007; Stawowy et al. 2003; Valledor et al. 2000; Valledor et al. 1999). The Ras/Rac pathway is necessary for increasing MKP-1 expression in smooth muscle cells in response to cyclic strain stress (Li et al. 1999).

MKP-1 can be post-translationally modified via phosphorylation, which is not necessary for MKP-1 activity, but leads to stabilization of the protein. Indeed ERK-

induced phosphorylation of MKP1 at Serine 359 and Serine 364 increases MKP1 half-life by two to three folds (Brondello et al. 1999). The reduced degradation results in a greater MKP-1 accumulation and thereby greater MKP-1 activity.

1.10.4 MKP-1 in the heart

MKP-1 has been demonstrated in cardiomyocytes and regulates cardiac hypertrophy and cardiomyocyte apoptosis (Fischer et al. 1998; Kaiser et al. 2004; Palm-Leis et al. 2004). MKP-1 is upregulated in hypertrophic hearts induced by Ang-II (Fischer et al. 1998). Overexpression of MKP-1 in cardiomyocytes significantly inhibits cyclic stretch- and Ang II-induced JNK and p38 activation, and the consequent increase in protein synthesis (Palm-Leis et al. 2004). These results indicate that MKP-1 limits the cardiac hypertrophic response. In addition, transgenic mice overexpressing MKP-1 show significant inhibition of baseline p38 and JNK activation in the heart. Ischemia-induced prominent p38 phosphorylation and cardiomyocyte apoptosis are significantly inhibited in MKP-1 transgenic mice. Consistently, MKP-$1^{-/-}$ mice show greater injury compared with wild-type littermates after ischemia/reperfusion (Kaiser et al. 2004). The evidence supports the notion that MKP-1 decreases cardiomyocyte apoptosis and cardiac injury after ischemia/reperfusion via deactivating p38. Although MKP-1 may protect the heart during hypertrophy and ischemia/reperfusion, the effect of MKP-1 on cardiac dysfunction during sepsis remains unknown. One of the objectives of this study was to elucidate the role of MKP-1 in cardiac TNF-α expression during endotoxemia (see Chapter 4).

1.11 Rationale and hypotheses

The induction of tumor necrosis factor-α (TNF-α) expression in cardiomyocytes leads to myocardial depression during sepsis. However, the underlying molecular mechanisms are not fully understood. Rac1, a small monomeric GTP-binding protein, regulates various cellular functions, such as superoxide (O_2^-) production, microtubule stability, cytoskeletal remodeling and gene transcription (Burridge & Wennerberg 2004; Hordijk 2006). It is an essential regulator of cardiac function under physiological and various pathological conditions (Satoh et al. 2006; Shen et al. 2009; Sussman et al. 2000). A recent study has reported that LPS activates Rac1 in cardiomyocytes. Rac1-GTP activates NADPH oxidase and then leads to TNF-α mRNA expression in cardiomyocytes (Zhu et al. 2009). However the mechanisms of Rac1 activation and the downstream signaling of Rac1 on TNF-α production and myocardial dysfunction during endotoxemia are not fully elucidated. Thus, the **overall hypothesis** of this thesis was that Rac1 promotes TNF-α expression and myocardial dysfunction during endotoxemia (Figure 1.5).

PI3K is an important activator of Rac-GEFs (Welch et al. 2003). PtdIns(3,4,5)P_3, a product of PI3K, activates Rac through PtdIns(3,4,5)P_3-sensitive GEFs in neutrophils and fibroblasts (Burridge & Wennerberg 2004; Welch et al. 2003). On the other hand, Rac can also be activated by PI3K-independent mechanisms, such as cAMP, Ca^{2+}, DAG and Src (Welch et al. 2003). Until now, the mechanisms of myocardial Rac1 activation in endotoxemia are unclear. Thus the first **specific hypothesis** of this thesis (Chapter 2) was that PI3K-mediated Rac1 activation is required for myocardial TNF-α expression and cardiac dysfunction via NADPH oxidase in endotoxemia (Figure 1.5).

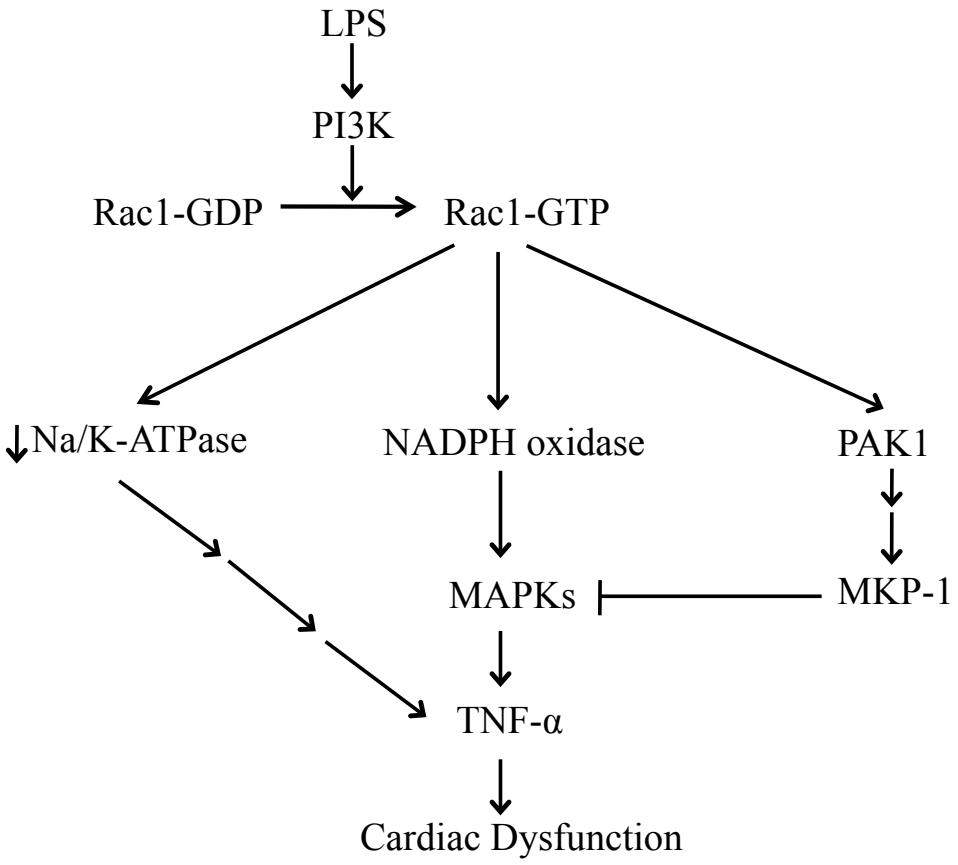

Figure 1.5. Proposed hypotheses of Rac1 in regulating myocardial TNF-α expression and cardiac dysfunction in endotoxemia. Hypothesis 1: PI3K-mediated Rac1 activation is required for myocardial TNF-α expression and cardiac dysfunction via NADPH oxidase in endotoxemia. Hypothesis 2: Rac1 promotes LPS-induced TNF-α production via inhibition of Na/K-ATPase. Hypothesis 3: Rac1/PAK1 induces myocardial MKP-1 expression via JNK1 in response to LPS. It was further hypothesized that increased MKP-1 production limits TNF-α expression and improves heart function in endotoxemia.

Na/K-ATPase catalyzes ATP hydrolysis to actively transport Na^+ out of and K^+ into cells. It is the main pathway for Na^+ extrusion from the cells and therefore plays an essential role in the regulation of intracellular Na^+ concentrations, which, via the Na^+/Ca^{2+} exchanger, control intracellular Ca^{2+}. Our group has reported that LPS increases Ca^{2+} concentration in cardiomyocytes, which participates in TNF-α expression in these cells. In addition, Na/K-ATPase activity is decreased in the lung (Koksel et al. 2006) and kidney (Guzman et al. 1995) and is involved in regulating TNF-α expression in macrophages in response to LPS. However, effects of LPS on Na/K-ATPase activity in the heart are not known and no published study to date has examined the effect of Na/K-ATPase in regulating myocardial TNF-α protein production during endotoxemia. Therefore, the second **specific hypothesis** of this thesis (Chapter 3) was that Rac1 promotes LPS-induced TNF-α production via inhibition of Na/K-ATPase (Figure 1.5).

Although ERK1/2, p38 and JNK1/2 are activated in cardiomyocytes during endotoxemia, they show different effects on TNF-α expression (Peng et al. 2005b; Peng et al. 2003b; Peng et al. 2009; Rosengart et al. 2000a; Thakur et al. 2006). Activated ERK1/2 and p38 promote TNF-α expression (Peng et al. 2005b; Peng et al. 2003b; Rosengart et al. 2000a; Thakur et al. 2006). In contrast, JNK1 decreases TNF-α expression and improves cardiac function during endotoxemia through inactivating ERK1/2 and p38 (Peng et al. 2009). However, the molecular mechanisms of the cross-talk among JNK, ERK1/2 and p38 are not fully understood. MKP-1 is an important negative feedback regulator of innate immune response via its prolonging of p38 and JNK activation and decreasing of the production of pro-inflammatory cytokines (Hammer et al. 2006; Salojin et al. 2006; Zhao et al. 2006). To our knowledge, the effects of MKP-

1 on myocardial TNF-α expression and myocardial dysfunction during endotoxemia have not been investigated. It has been shown that Rac/MAPK is one of the pathways that promote MKP-1 expression (Li et al. 1999). PAK1 is a downstream effector of Rac1 and participates in modulating cardiomyocyte contractility and cardiac hypertrophy (Sheehan et al. 2007; Sussman et al. 2000). There is evidence that PAK regulates proIL-1 expression in macrophages through activation of MAPKs (Hsu et al. 2001). Thus, the third **specific hypothesis of this thesis** (Chapter 4) was that Rac1/PAK1 induces myocardial MKP-1 expression via JNK1 in response to LPS. It was further hypothesized that increased MKP-1 production limits TNF-α expression and improves heart function in endotoxemia (Figure 1.5).

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Chapter 2: PI3K-mediated Rac1 promotes LPS-induced TNF-α expression and cardiac dysfunction via NADPH oxidase

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2.1 Introduction

Sepsis is a major consequence of infectious diseases and one of the leading causes of death in the intensive care unit (Angus & Wax 2001). Myocardial dysfunction induced by endotoxins or lipopolysaccharides (LPS) of Gram-negative bacteria is a common complication of septic shock and renders septic patients at high risk of developing multi-organ failure, which is associated with high mortality (Court et al. 2002). The inhibitory effect of LPS on cardiac function is mediated through the production of pro-inflammatory cytokines (Parrillo et al. 1985). TNF-α is a major cytokine responsible for cardiac dysfunction during sepsis (Bozkurt et al. 1998; Grandel et al. 2000; Meng et al. 1998; Natanson et al. 1989; Oral et al. 1997; Peng et al. 2003; Tracey et al. 1986). However, the molecular mechanisms underlying myocardial TNF-α production during sepsis are not fully understood.

Rac GTPases are a subfamily of Ras-homologous (Rho) GTPases and act as molecular switches, cycling between active GTP-bound and inactive GDP-bound states (Burridge & Wennerberg 2004; Hordijk 2006). The switch is activated when an upstream signal activates a guanine nucleotide exchange factor (GEF) which then acts to facilitate the release of GDP from the Rac GTPase and the subsequent binding of GTP. Rac

activity is terminated by hydrolysis of GTP to GDP, a process which is accelerated by GTPase-activating proteins (GAPs). Rac is an intracellular transducer of signaling and can interact with specific effectors that regulate diverse cellular functions, such as cytoskeletal remodeling, microtubule stability, gene transcription, and superoxide (O_2^-) production (Burridge & Wennerberg 2004; Hordijk 2006).

There are four different Rac proteins: the ubiquitously expressed Rac1, the hematopoietic cell-specific Rac2, Rac3 that is expressed in the brain, liver, lung and pancreas and widespread RhoG (Haataja et al. 1997). Previous studies have shown that LPS increases Rac1 activity in phagocytes, however the effect of Rac1 on TNF-α expression in these cells remains controversial (Monick et al. 2003; Sanlioglu et al. 2001; Thakur et al. 2006). Rac1 is the predominant Rac protein in cardiomyocytes (Pracyk et al. 1998; Satoh et al. 2006). Furthermore, Rac1 is activated during LPS stimulation and contributes to myocardial TNF-α expression (Zhu et al. 2009). However, regulation of Rac1 activation during LPS stimulation is not fully understood. Phosphoinositide-3 kinases (PI3K) are a family of evolutionary conserved signaling molecules that mediate many cellular responses. The production of phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)P_3) from PI3K activates Rac via a PtdIns(3,4,5)P_3-sensitive GEF. However, Rac can also be activated by PI3K-independent mechanisms (Welch et al. 2003). Whether Rac activation in cardiomyocytes during LPS stimulation is mediated by PI3K remains to be determined.

NADPH oxidase is an enzyme system that catalyzes the NADPH-dependent reduction of oxygen to O_2^- and consists of multi-subunits including Nox2 ($gp91^{phox}$), $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac. It has been shown that NADPH oxidase is a

major source of O_2^- in cardiomyocytes under pathophysiological conditions and activation of Rac is essential for NADPH oxidase activation (Moldovan et al. 2006). We have demonstrated that Nox2-containing NADPH oxidase plays a pivotal role in LPS-induced cardiac TNF-α production (Peng et al. 2005). However, the role of Rac1 in cardiac dysfunction during sepsis remains unknown.

In the present study, we hypothesized that Rac1 was necessary for LPS-induced TNF-α expression and myocardial dysfunction via NADPH oxidase activation. To test this hypothesis, a cardiac-specific Rac1-deficient mouse was generated. Our results demonstrated that LPS-induced Rac1 activation in cardiomyocytes is PI3K-dependent. Rac1 deficiency blocked cardiomyocyte TNF-α expression and decreased LPS-induced O_2^- generation. Furthermore, cardiac-specific deficiency of Rac1 improved myocardial function in endotoxemia.

2.2 Materials and methods

2.2.1 Animals and preparation of neonatal mouse cardiomyocytes

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication #85-23, revised 1996) and experimental protocols were approved by Animal Use Subcommittee at the University of Western Ontario. C57BL/6 wild-type (WT) and Rac1 floxed ($Rac1^{f/f}$) mice (Glogauer et al. 2003) were purchased from the Jackson Laboratory (Bar Harbor, Maine). In $Rac1^{f/f}$ mice, LoxP sites were inserted at both sides of exon 1 of the Rac1 gene. Cre transgenic mice ($Cre^{TG/+}$) with over-expression of Cre recombinase under the control of α-myosin heavy-chain (MHC) promoter were provided by Dr. E. Dale Abel

(University of Utah, UT). This Cre recombinase can excise the region between the loxP sites and is specifically expressed in cardiomyocytes since is it under the control of the α-MHC promoter. The generation of cardiomyocyte-specific Rac1 knockout mice ($Rac1^{CKO}$) was achieved by breeding $Rac1^{f/f}$ mice with $Cre^{TG/+}$ mice as we have recently described (Rui et al. 2005).

2.2.2 Isolation and culturing of neonatal mouse cardiomyocytes

The neonatal cardiomyocytes were prepared and cultured according to methods we have previously described (Song et al. 2000). The neonatal cardiomyocyte cultures were prepared from mice born within 24 hours. For each cell culture, 5-10 neonatal mouse hearts were isolated, washed and minced in sodium bicarbonate, Ca^{2+} and Mg^{2+}-free Hanks balanced salt solution (D-Hanks, Sigma, Oakville, Ontario). Cardiomyocytes were dispersed by 10 min of incubation with 22.5 μg/mL liberase 4 (Roche, Laval, Quebec) in D-Hanks at 37 °C with gentle agitation. Cells were collected by centrifugation at 200 g for 5 min and re-suspended in 10% FCS-containing M199 medium (Sigma). Noncardiomyocytes were removed through 1 hour of preplating after which cardiomyocytes were plated in M199 medium containing 10% fetal bovine serum (FBS) on culture plates precoated with 1% gelatin (Sigma). Neonatal cardiomyocytes were cultured in a humidified incubator at 37°C, in the presence of 5% CO_2. A subconfluent spontaneously beating monolayer of cardiomyocytes was formed within 2 days. Cells were treated with LPS (1 μg/ml, Sigma), apocynin (400 μM, Sigma), LY294002 (10 μM, Sigma) and U0126 (10 μM, Sigma) or infected with adenoviruses.

2.2.3 Adenoviral infection of neonatal cardiomyocytes

Cardiomyocytes were infected with adenoviruses carrying a dominant-negative

form of Rac1 (Ad-Rac1N17, Vector Biolabs, Philadelphia, PA), Cre recombinase (Ad-Cre, Vector Biolabs, Philadelphia, PA) or green fluorescence protein (Ad-GFP, a gift from Dr. J. Lipp, Medical University of Vienna, Austria) as a control, at a multiplicity of infection (MOI) of 10 plaque forming units/cell. Adenovirus-mediated gene transfer was implemented as previously described (Peng et al. 2003). Briefly, cells were incubated for 4 hours in the present of minimal volume of M199 containing adenoviruses. Following the incubation, full volume of 10% FBS M199 was applied. All experiments were performed after 48 hours of adenoviral infection.

2.2.4 Rac1 activity assay

Rac1 activity was measured using the EZ-Detect Rac1 activation kit (Pierce, USA) according to the manufacturer's protocol. Briefly, cells or tissues were lysed. Thirty micrograms of protein lysates were taken to detect total Rac1 protein. At the same time, 1 mg of protein lysates were incubated with 20 μg of glutathione S-transferase (GST)-human Pak1-p21 binding domain at 4° C for 1 hour. The beads were washed three times to remove the unbound material and were then boiled in 2x SDS buffer for 5 minutes to elute Rac1-GTP. Rac1-GTP and total Rac1 protein levels were detected by western blot analysis using an anti-Rac1 antibody included in the kit. Rac1 activity was expressed as a ratio of Rac1-GTP to total Rac1 proteins.

2.2.5 PI3K activity assay

PI3K activity in cultured cardiomyocytes or myocardial tissue lysates was determined using a competitive ELISA kit (Echelon Biosciences, Salt Lake City, UT) according to the manufacture's protocol with modifications (Bonnans et al. 2006). Briefly, cell or tissue lysates (25 μg protein) were incubated with phosphatidylinositol

(4,5)-bisphosphate [PI(4,5)P_2] substrate (100 pmol) in 100 μL kinase reaction buffer. The reaction products were incubated with a PI(3,4,5)P_3 detector protein, and then added to the PI(3,4,5)P_3-coated microplate for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric detection at 450 nm was used to detect PI(3,4,5)P_3 detector protein binding to the plate.

2.2.6 Measurement of TNF-α mRNA

Total RNA was extracted from cardiomyocytes using the Trizol Reagent (Gibco-BRL) as per manufacturer's instructions. TNF-α mRNA levels were determined by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) as as previously described (Peng et al. 2003). Total RNA (1 μg) was reverse transcribed using random hexamers. 28S rRNA was used as an internal control. The primers were: TNF-α upstream 5′-CCG ATG GGT TGT ACC TTG TC-3′ and downstream 5′-GGG CTG GGT AGA GAA TGG AT-3′. 28S rRNA upstream 5'-TTG AAA ATC CGG GGG AGA G-3' and downstream 5'-ACA TTG TTC CAA CAT GCC AG-3'. Samples were amplied for 34 cycles using MJ Research Opticon Real-time PCR machine. The levels of TNF-α were compared to that of 28S rRNA, and the relative expression of these genes was obtained.

2.2.7 Measurement of TNF-α protein

TNF-α protein levels were measured using a mouse TNF-α ELISA kit (eBioscience, USA), according to the manufacturer's instructions. Briefly, ELISA plates were coated with coating buffer containing capture antibody for overnight. The wells were washed and blocked with assay diluent. Standards and samples were added to the appropriate well and incubated overnight at 4°C. After wash, wells were incubated with the detection antibody for 1 hour and then avidin-HRP for 30 min at room temperature.

After thorough washing, each well was incubated with substrate solution for 15 min. Reaction was stopped by 1 M H_3PO_4. Plate was read at 450 nm. The concentration of TNF-α was calculated according to the standard curve. The measurements were standardized with cell numbers or expressed as TNF-α levels to total proteins.

2.2.8 Western blot analysis

Thirty micrograms of protein lysates were subjected to separation on a 10% SDS-PAGE gel, followed by electrotransfer to nitrocellulose membranes. Blots were probed with specific antibodies against ERK1/2 (1:500) and phospho-ERK1/2 (1:500), p38 (1:500) and phospho-p38 (1:500; Cell Signaling Technology, Danvers, MA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:3000, Santa Cruz Biotechnology, CA) respectively. Signals were detected by the chemiluminescence detection method and quantified by densitometry.

2.2.9 Lucigenin assay

NADPH-dependent superoxide (O_2^-) generation was measured in cell lysates by lucigenin-enhanced chemiluminescence (40 μg of protein, 100 μM β-NADH, 5 μM lucigenin, sigma). The chemiluminescence was detected by a mutilabel counter (SpectraMax M5, Molecular Devices). Replicates were incubated in the presence of the flavoprotein inhibitor (diphenyleneiodonium, DPI, 10 μM) to ensure O_2^- was generated from NADPH oxidase, as previously described (Peng et al. 2005). The light signal was monitored for 1.5 seconds and counts per second (CPS) were presented as NADPH oxidase activity that was DPI inhibitable.

2.2.10 Isolated mouse heart preparations

Adult $Rac1^{f/f}$ and $Rac1^{CKO}$ (male, 3 months old) mice were treated with LPS

(2mg/kg, i.p.) or saline. After 2 hours, mouse hearts were isolated and perfused in a Langendorff-system with Kreb's-Henseleit buffer at 3 ml/min constant flow. The perfusion buffer was kept at 37 °C and consistently bubbled with a mixture of 95% O_2 and 5% CO_2. Myocardial function was assessed as previously described with modifications (Peng et al. 2005). Briefly, a 6-0 silk suture was passed through the apex of the left ventricle and threaded through a light-weight rigid coupling rod, which was connected to a force-displacement transducer (FT03) to record tension and heart rate. The heart work was calculated by multiplying the force (g) by the heart rate (bpm). Maximal and minimal first derivatives of force ($+dF/dt_{max}$ and $-dF/dt_{min}$) which represent the rate of contraction and relaxation respectively, were analyzed by PowerLab Chart program (AD Instruments).

2.2.11 Statistical analysis

Results are presented as mean ± SEM from at least three independent experiments. Differences between two groups were analyzed by a standard Student t-test. For multigroup comparisons, one or two-way ANOVA followed by Student-Newman-Keuls or Bonferroni post-test was performed. $P < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Myocardial Rac1 activation by LPS

To examine the effect of LPS on Rac1 activity, neonatal cardiomyocytes isolated from C57BL/6 mice were treated with LPS (1 μg/ml) for 5, 15, 30 and 60 minutes. As shown in Figure 2.1A, Rac1 activity in these cells peaked at 15 minutes and declined to

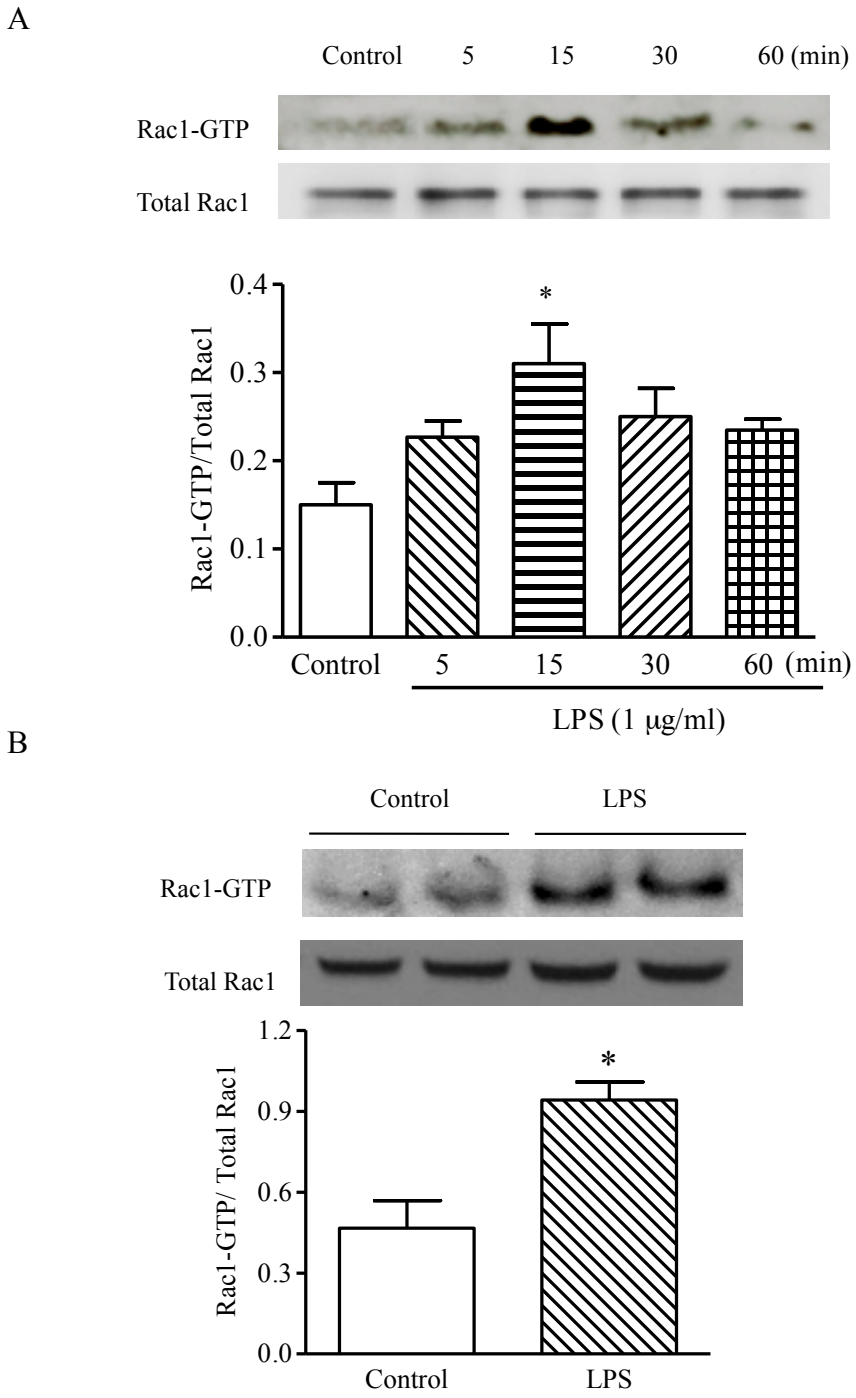

Figure 2.1. LPS activates Rac1 in neonatal cardiomyocytes and in the adult myocardium. A. Cardiomyocytes were isolated from WT mice, cultured for 48 hours, and then treated with vehicle or LPS (1 μg/ml) for 5, 15, 30 and 60 minutes. Rac1 activity was measured using the EZ-Detect Rac1 activation kit. B. Adult male $Rac1^{f/f}$ mice were treated with LPS (2 mg/kg, i.p.) for 30 minutes. Rac1 activity in the left ventricular myocardium was measured as described above. Data are means ± SEM from 3 - 4 mice or independent experiments. **P*<0.05 vs. control.

about control levels by 60 minutes after LPS stimulation. To verify these *in vitro* results, $Rac1^{f/f}$ mice were treated with LPS (2 mg/kg, i.p.) or saline for 30 minutes and myocardial Rac1 activity was measured. In response to LPS, myocardial Rac1 activity was significantly increased ($P<0.05$, Figure 2.1B). These data show that LPS activates Rac1 in cardiomyocytes *in vitro* and in the myocardium *in vivo*.

2.3.2 Rac1 activation and cardiomyocyte TNF-α expression during LPS stimulation

To elucidate the role of Rac1 in LPS-induced TNF-α expression, neonatal cardiomyocytes isolated from WT mice were infected with an adenovirus carrying a dominant negative form of the Rac1 gene (Ad-Rac1N17), which selectively inhibits Rac1 activity. As shown in Figure 2.2A and 2.2B, overexpression of Rac1N17 significantly decreased LPS-induced TNF-α mRNA and protein levels by 60% and 56%, respectively ($P<0.01$). This result was further confirmed using Rac1 deficient cardiomyocytes. Cultured neonatal cardiomyocytes from $Rac1^{f/f}$ mice were infected with Ad-Cre. Expression of Cre recombinase in $Rac1^{f/f}$ cells decreased Rac1 protein levels by 70% (Figure 2.2C). LPS-induced TNF-α mRNA and protein expression were reduced by 67% and 41% in Ad-Cre infected $Rac1^{f/f}$ cells, respectively ($P<0.05$, Figure 2.2D and 2.2E). These data showed that LPS-induced TNF-α expression requires Rac1 activity.

2.3.3 Involvement of PI3K in Rac1 activation in cardiomyocytes during LPS stimulation

To investigate the involvement of PI3K in Rac1 activation in cardiomyocytes during LPS stimulation, PI3K activity was determined. In response to LPS (1 μg/ml), PI3K activity was significantly increased in cultured neonatal cardiomyocytes ($P<0.05$, Figure 2.3A). *In vivo* treatment of LPS (2 mg/kg, i.p.) also activated PI3K in the

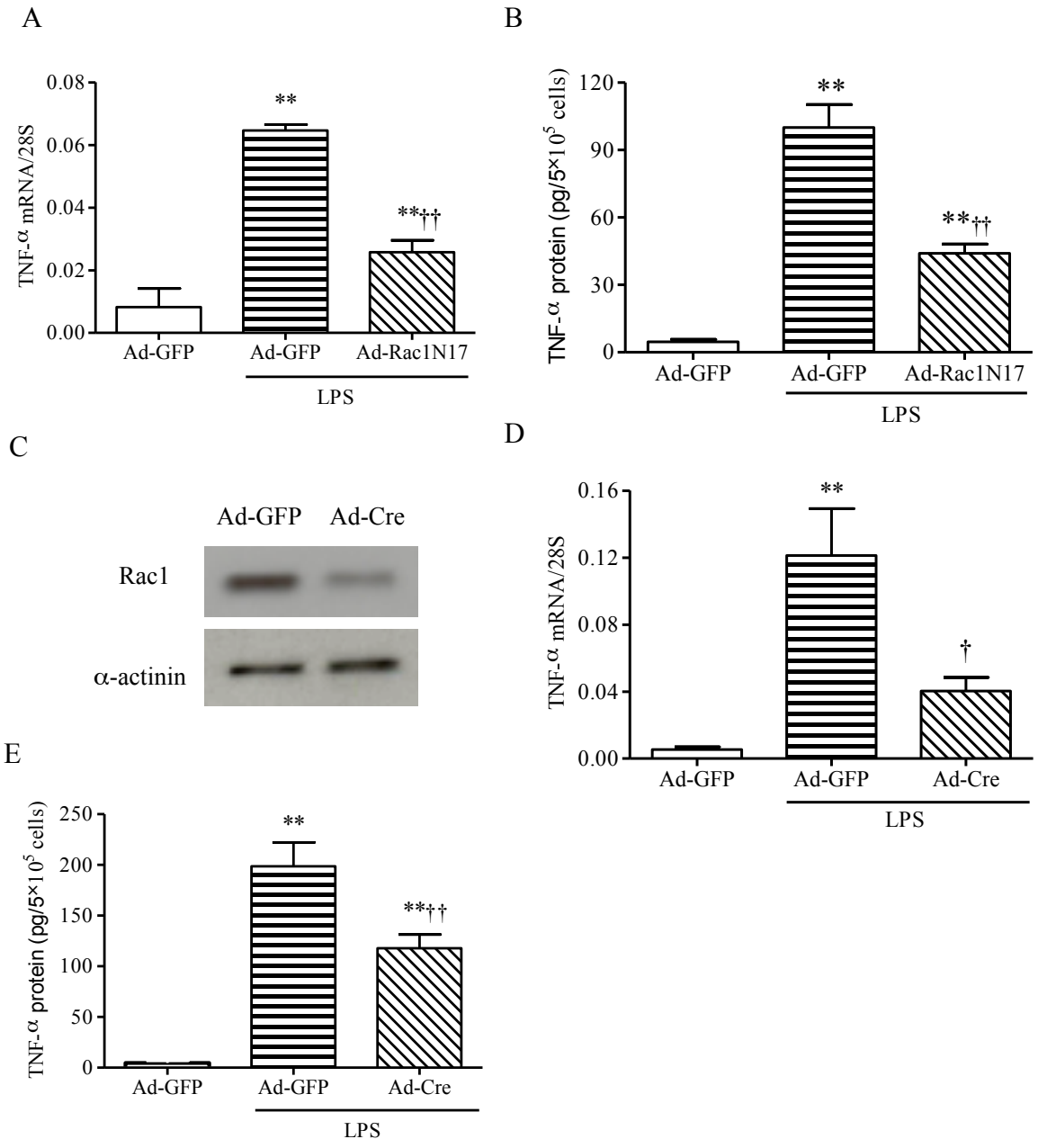

Figure 2.2. Rac1 promotes LPS-induced TNF-α expression in neonatal cardiomyocytes. WT cardiomyocytes were infected with Ad-GFP or Ad-Rac1N17 for 24 hours. Cardiomyocytes were treated with LPS (1 μg/ml) for 3 hours or 5 hours. TNF-α mRNA (A) and TNF-α protein in culture medium (B) were measured by real-time RT-PCR and ELISA, respectively. Neonatal cardiomyocytes from $Rac1^{f/f}$ mice were infected with Ad-GFP and Ad-Cre for 24 hours. Rac1 protein was measured by Western blot analysis (C). $Rac1^{f/f}$ cells, infected with Ad-GFP or Ad-Cre, were treated with LPS for 3 hours or 5 hours. TNF-α mRNA (D) and TNF-α protein in culture medium (E) were measured as described above. Data are means ± SEM from 3 - 7 independent experiments. $^{**}P<0.01$ vs. Ad-GFP; †$P<0.05$, †† $P<0.01$ vs. Ad-GFP+LPS.

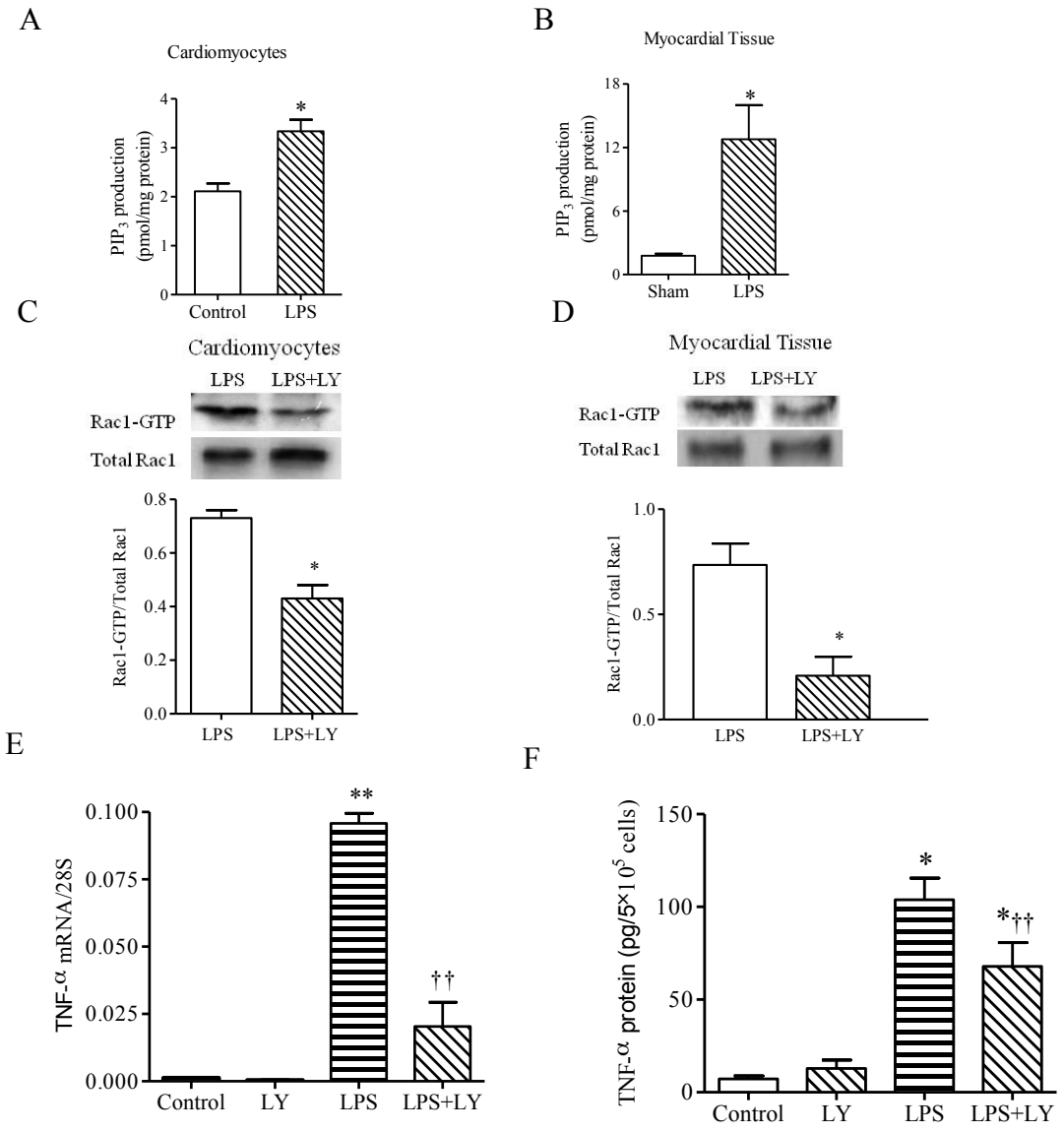

Figure 2.3. PI3K promotes LPS-induced Rac1 activation and TNF-α expression in neonatal cardiomyocytes. Cultured WT neonatal cardiomyocytes was stimulated with LPS at 1 μg/ml for 30 min. WT mice were treated with LPS (2 mg/kg, i.p.) for 30 min. PI3K activities in cardiomyocytes (A) and myocardium (B) were determined by competitive ELISA. Rac1 activities in WT cardiomyocytes (C) and myocardium (D) stimulated with LPS for 30 minutes in the presence or absence of the PI3K inhibitor LY294002 (LY, 10 μM and 7.5 mg/kg, i.p.) were determined by EZ-Detect Rac1 activation kit. WT cardiomyocytes were treated with LPS (1 μg/ml) in the presence or absence of the PI3K inhibitor LY294002 for 3 or 5 hours. TNF-α mRNA (E) and TNF-α protein in culture medium (F) were measured by real-time RT-PCR and ELISA, respectively. Data are means ± SEM from 3 - 5 independent experiments. **P*<0.05, ***P*<0.01 vs. control or sham; †*P*<0.05, ††*P*<0.01 vs. LPS.

myocardium in the adult mice ($P<0.05$, Figure 2.3B). To further study the contribution of PI3K in LPS-induced Rac1 activation, cardiomyocytes were treated with LY294002, a selective inhibitor of PI3K. Our data showed that LY294002 decreased LPS-induced Rac1-GTP by 41% in cardiomyocytes (Figure 2.3C). Similarly, LPS-stimulated Rac1-GTP levels in the myocardium were also blocked by 71% after LY294002 treatment (Figure 2.3D). In addition, LY294002 significantly decreased LPS-induced TNF-α mRNA and protein levels ($P<0.05$, Figure 2.3E and 2.3F). These results indicate that Rac1 activation in cardiomyocytes is mediated by PI3K during LPS stimulation.

2.3.4 Role of PI3K and Rac1 in NADPH oxidase activation during LPS stimulation

Our lab has recently demonstrated that Nox2-containing NADPH oxidase contributes to LPS-induced TNF-α expression in cardiomyocytes (Peng et al. 2005). Consistent with this notion, the present study demonstrated that LPS increased NADPH oxidase-mediated O_2^- generation ($P<0.05$, Figure 2.4A), which was blocked by apocynin, a selective NADPH oxidase inhibitor. Moreover, apocynin, significantly reduced TNF-α mRNA levels by 46% and protein levels by 43% in response to LPS ($P<0.01$, Figure 2.4D and 2.4E). To detect if PI3K activation mediates LPS-induced NADPH oxidase activity, cardiomyocytes were treated with LY294002. Figure 2.4A showed that O_2^- production stimulated by LPS was significantly blocked by LY294002. To determine whether Rac1 is involved in regulating NADPH oxidase activity, cardiomyocytes were treated with Ad-Rac1N17 to specifically block Rac1 activation. Our data showed that LPS-induced O_2^- generation in these cells was significantly inhibited by Ad-Rac1N17 ($P<0.05$, Figure 2.4B). Similarly, O_2^- production was also significantly reduced in Rac1 deficient cardiomyocytes ($P<0.05$, Figure 2.4C). These results suggest that PI3K and

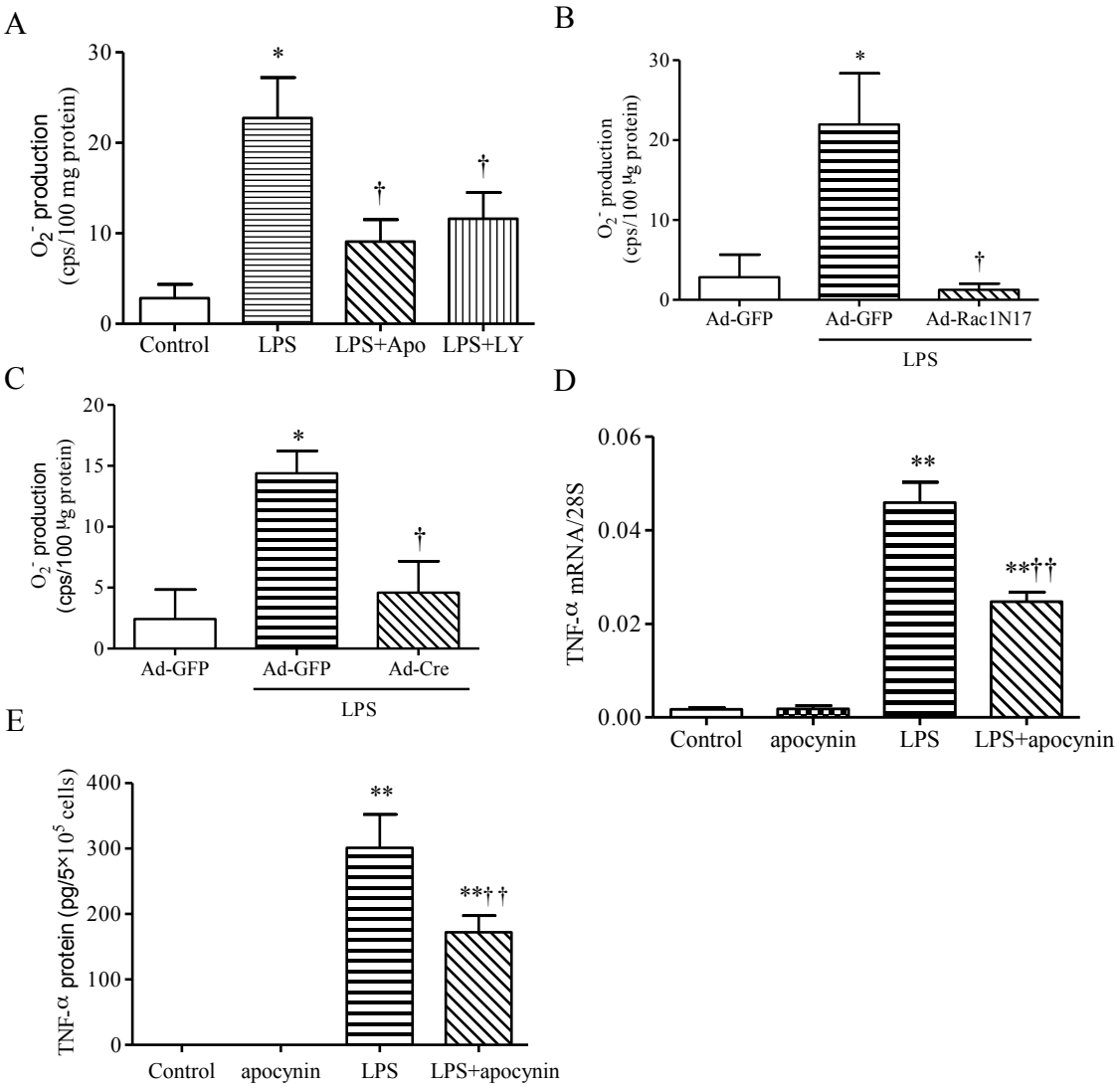

Figure 2.4. PI3K and Rac1 promote LPS-induced superoxide (O_2^-) generation and TNF-α expression in neonatal cardiomyocytes. A. Effects of apocynin and LY294002 on O_2^- production. WT cells were treated with LPS (1 μg/ml) for 2 hours with or without apocynin (400 μM) and LY294002 (LY, 10 μM). O_2^- production was measured by the lucigenin assay. B. Effects of Ad-Rac1N17 on O_2^- production. WT cells were infected with Ad–GFP or Ad-Rac1N17. O_2^- production was measured 2 hours after LPS treatment (1 μg/ml). C. O_2^- production in Rac1 deficient cardiomyocytes. $Rac1^{f/f}$ cardiomyocytes were infected with Ad-GFP or Ad-Cre for 24 hours followed by treatment with LPS for 2 hours. O_2^- production was measured. WT cardiomyocytes were treated with LPS (1 μg/ml) in the presence or absence of 400 μM apocynin for 3 and 5 hours. TNF-α mRNA (D) and TNF-α protein in culture medium (E) were measured by real-time PCR analysis and ELISA, respectively. Data are means ± SEM from 3 - 5 independent experiments. **P*<0.05, ***P* <0.01 vs. control and Ad-GFP; †*P*<0.05, ††*P* <0.01 vs. LPS and Ad-GFP+LPS.

Rac1 are critical for NADPH oxidase activation in cardiomyocytes during LPS stimulation.

2.3.5 Role of PI3K and Rac1 in ERK1/2 activation during LPS stimulation

We have previously shown that activation of ERK1/2 was essential for NADPH oxidase signaling and LPS-induced TNF-α expression in cardiomyocytes (Peng et al. 2005). The effects of LPS on ERK1/2 activation were also determined in the present study. As shown in Figure 2.5A, LPS rapidly increased phosphorylation of ERK1/2 which peaked at 30 minutes and returned to control levels after 2 hours. LPS-induced ERK1/2 phosphorylation was completely blocked by a PI3K inhibitor, LY294002 (Figure 2.5B), suggesting that LPS regulated ERK1/2 activity via PI3K. To examine whether Rac1 activity leads to ERK1/2 phosphorylation, ERK1/2 activation was measured in Ad-Rac1N17 infected cardiomyocytes. Overexpression of Rac1N17 significantly decreased LPS-induced phosphorylation of ERK1/2 compared with Ad-GFP infected group (Figure 2.5C). Furthermore, U0126, a selective ERK1/2 inhibitor, decreased LPS-stimulated TNF-α mRNA and protein levels by 46% and 69%, respectively (Figure 2.5D and 2.5E). Taken together, these results suggest that the effects of PI3K and Rac1 on cardiomyocytes are mediated by ERK1/2.

2.3.6 Role of Rac1 in myocardial dysfunction during endotoxemia

To study the role of Rac1 in myocardial depression during endotoxemia *in vivo*, we generated cardiac-specific Rac1 knockout mice ($Rac1^{CKO}$) using Cre-loxP recombination as described in the methods. Our data showed that the Rac1 protein was

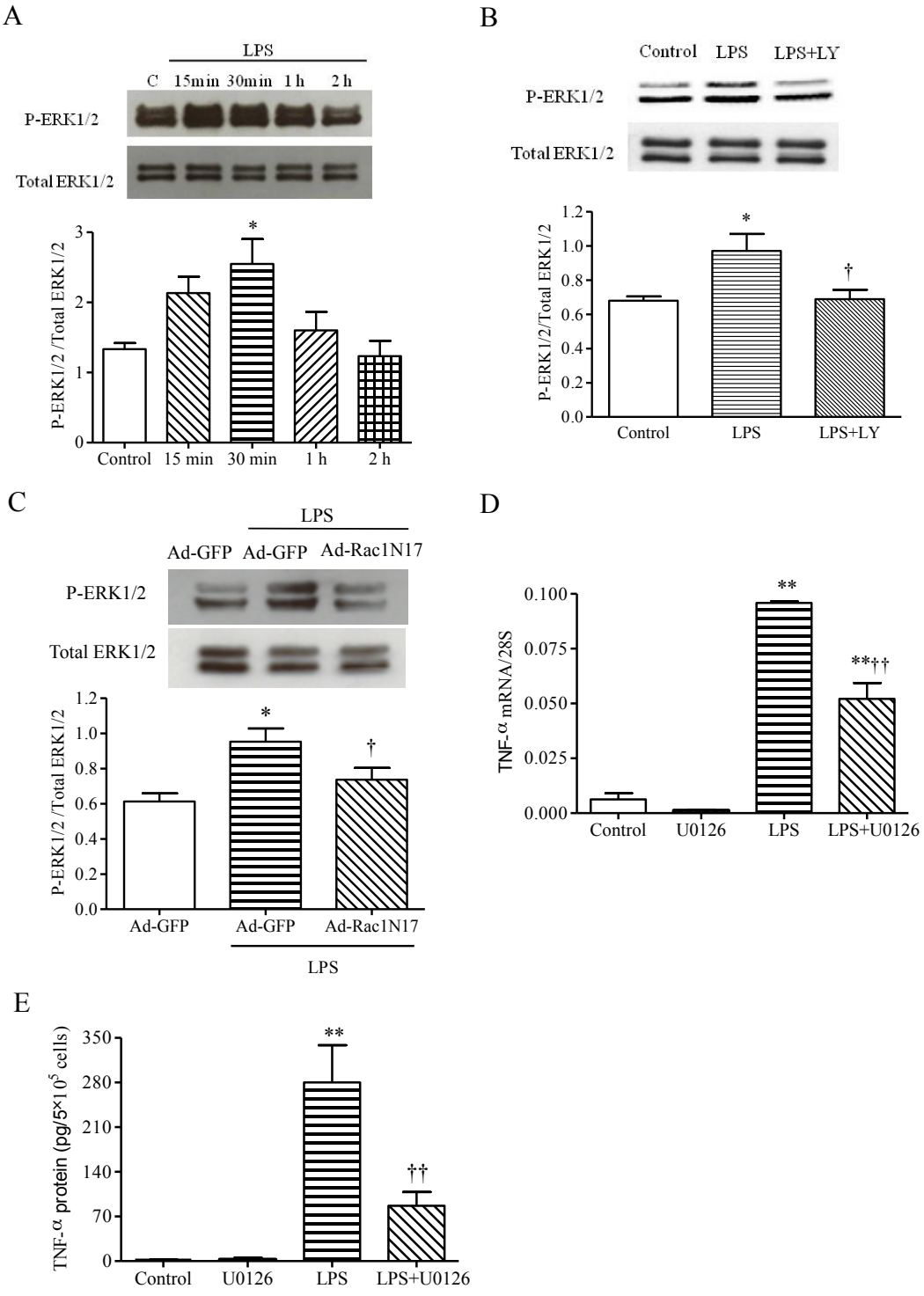
A
LPS
C 15min 30min 1 h 2 h
P-ERK1/2
Total ERK1/2
P-ERK1/2 /Total ERK1/2
Control 15 min 30 min 1 h 2 h
B
Control LPS LPS+LY
P-ERK1/2
Total ERK1/2
P-ERK1/2/Total ERK1/2
Control LPS LPS+LY
C
LPS
Ad-GFP Ad-GFP Ad-Rac1N17
P-ERK1/2
Total ERK1/2
P-ERK1/2 /Total ERK1/2
Ad-GFP Ad-GFP Ad-Rac1N17
LPS
D
TNF-α mRNA/28S
Control U0126 LPS LPS+U0126
E
TNF-α protein (pg/5×10^5 cells)
Control U0126 LPS LPS+U0126

Figure 2.5. PI3K and Rac1 promote LPS-induced ERK1/2 phosphorylation in neonatal cardiomyocytes. A. WT cardiomyocytes were treated with vehicle or LPS (1 μg/ml) for 15 min, 30 min, 1 hour and 2 hours. ERK1/2 phosphorylation in these cells was measured by Western blot analysis. B. WT cardiomyocytes were treated with LPS (1 μg/ml) for 30 min with or without LY294002 (LY, 10 μM). ERK1/2 phosphorylation in these cells was measured. C. Neonatal cardiomyocytes were infected with Ad-GFP or Ad-Rac1N17 for 24 hours. Cardiomyocytes were treated with LPS (1 μg/ml) for 30 min. ERK1/2 phosphorylation was measured as described above. D and E. Cardiomyocytes were treated with LPS with or without the ERK1/2 inhibitor U0126 (10 μM) for 3 and 5 hours. TNF-α mRNA (D) and protein in culture medium (E) were measured by real-time RT-PCR and ELISA, respectively. Data are means ± SEM from 3-5 independent experiments. **P*<0.05, ***P*<0.01 vs. control; †*P*<0.05, ††*P*<0.01 vs. Ad-GFP+LPS and LPS.

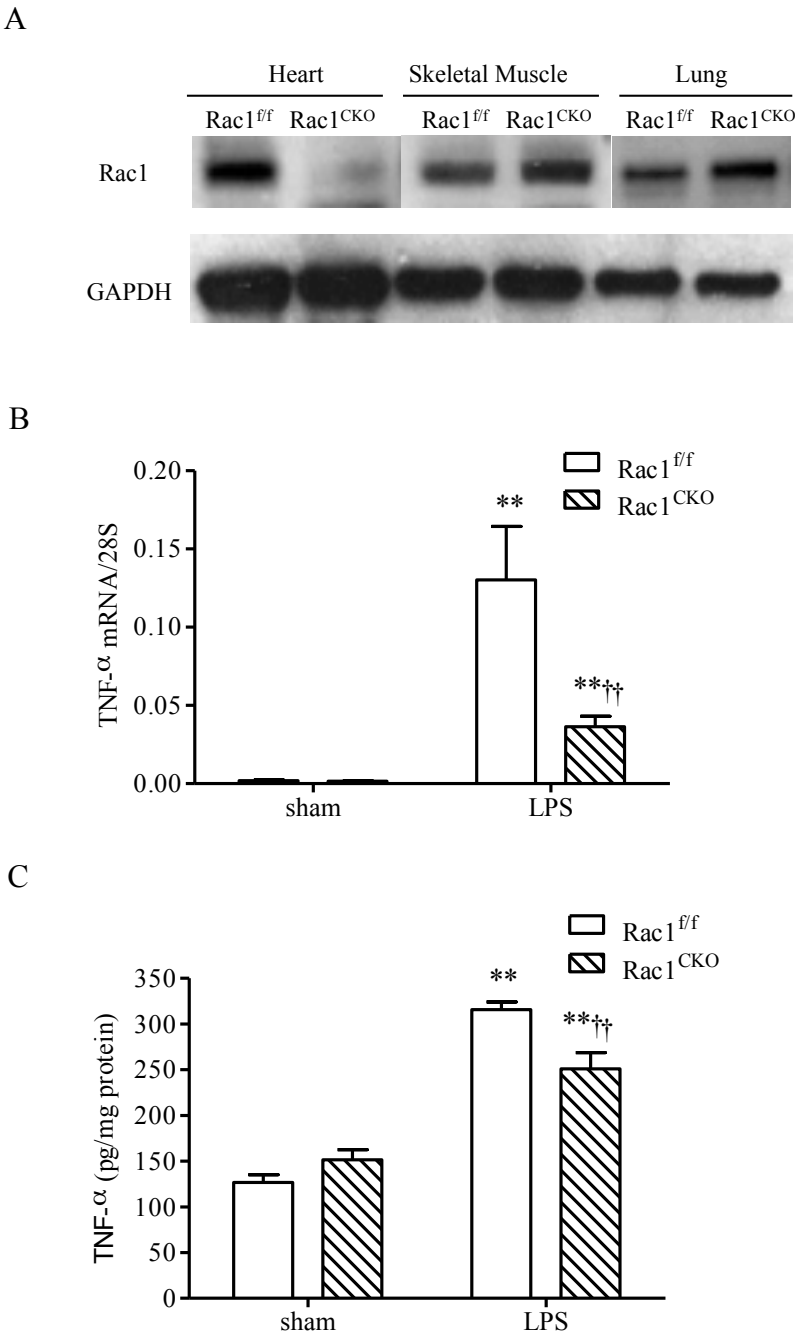

Figure 2.6. TNF-α expression in Rac1$^{f/f}$ and Rac1^{CKO} adult mouse myocardium during endotoxemia. A. Rac1 protein expression in heart, skeletal muscle and lungs in Rac1$^{f/f}$ and Rac1^{CKO} mice as determined by Western blot analysis. TNF-α mRNA (B) and protein (C) levels in Rac1$^{f/f}$ and Rac1^{CKO} heart tissues were measured after 2 and 4 hours of LPS treatment (2 mg/kg, i.p.). Data are means ± SEM, n=3 to 10 per group. ** P<0.01 vs. sham Rac1$^{f/f}$, † P <0.05 vs. LPS Rac1$^{f/f}$.

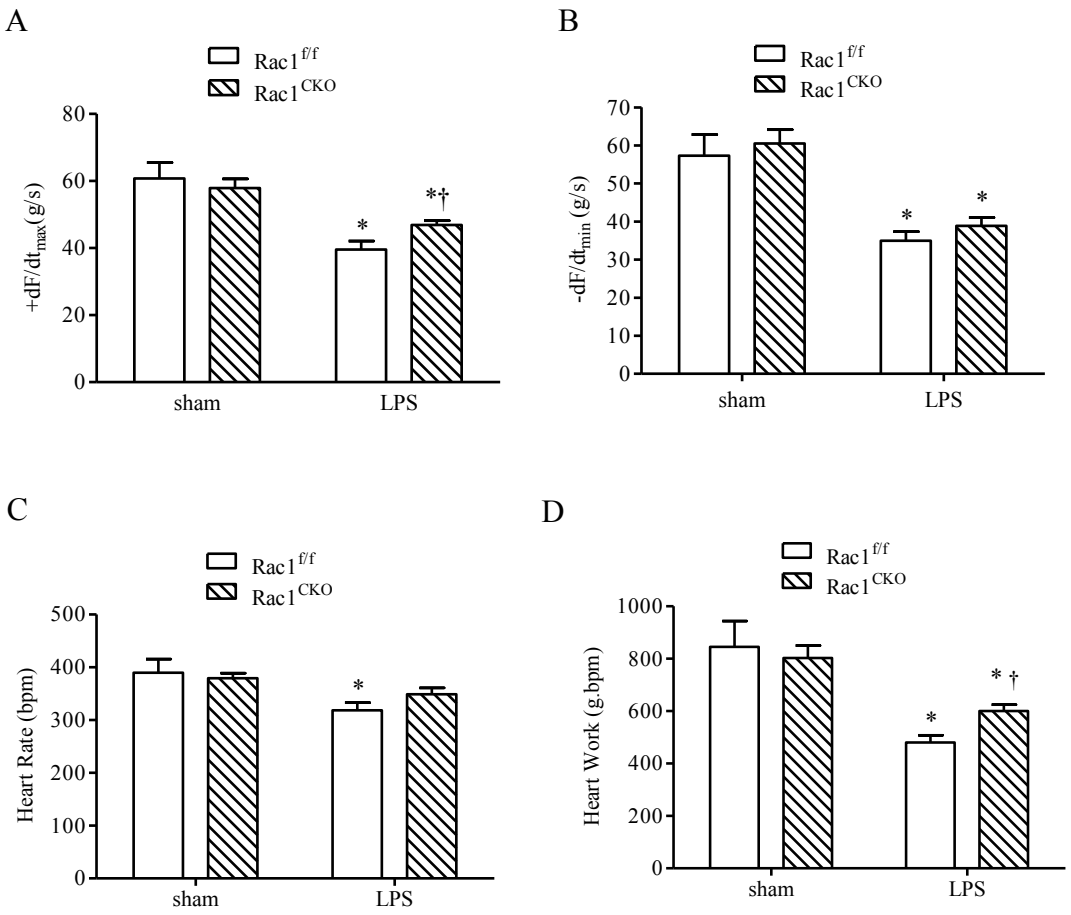

Figure 2.7. Cardiac function in Rac1$^{f/f}$ and Rac1^{CKO} mice after 2 hours of LPS treatment (2 mg/kg, i.p.). Mouse hearts were isolated and perfused using the Langendorff system. Contractile function of heart was determined. Changes in contraction (+d*F*/dt$_{max}$, A), relaxation (-d*F*/dt$_{min}$, B), heart rate (C) and heart work (D) are presented. Data are means ± SEM, n=5 to 7 per group. * *P* <0.05 vs. sham Rac1$^{f/f}$, † *P* <0.05 vs. LPS Rac1$^{f/f}$.

selectively knocked-down in the heart but not in the skeletal muscle and lungs in $Rac1^{CKO}$ mice (Figure 2.6A). $Rac1^{CKO}$ and $Rac1^{f/f}$ mice were treated with vehicle or LPS (2 mg/kg, i.p.). Our data demonstrated that LPS-induced myocardial TNF-α mRNA and protein levels were significantly decreased ($P<0.01$, Figure 2.6B and 6C). After 2 hours of LPS *in vivo* treatment, cardiac function was determined using the Langendorff preparation. The rate of contraction and relaxation, heart rate, and heart work were significantly reduced in both $Rac1^{f/f}$ and $Rac1^{CKO}$ mice after endotoxemia ($P<0.05$, Figure 2.7). However, compared with $Rac1^{f/f}$ mice, heart work and rate of contraction ($+dF/dt_{max}$) were significantly increased in $Rac1^{CKO}$ mice ($P<0.05$, Figure 2.7).

2.4 Discussion

The present study demonstrated for the first time that Rac1-mediated TNF-α expression following LPS stimulation occurs downstream of PI3K signaling in cardiomyocytes. Rac1 activation increased O_2^- generation, and ERK1/2 MAPK phosphorylation leading to increased TNF-α expression in cardiomyocytes. More importantly, our study provided evidence that cardiac-specific Rac1 deficiency improved cardiac function during endotoxemia. PI3K-mediated activation of Rac1 represents a novel signaling pathway by which LPS induces cardiomyocyte TNF-α expression and cardiac dysfunction (Figure 2.8).

Rho GTPases are a large family of proteins that include the Rac proteins (Rac1, 2 and 3) as well as RhoA and Cdc42. The role of Rho GTPases on TNF-α production during LPS stimulation has been studied, but the results differ depending on cell types

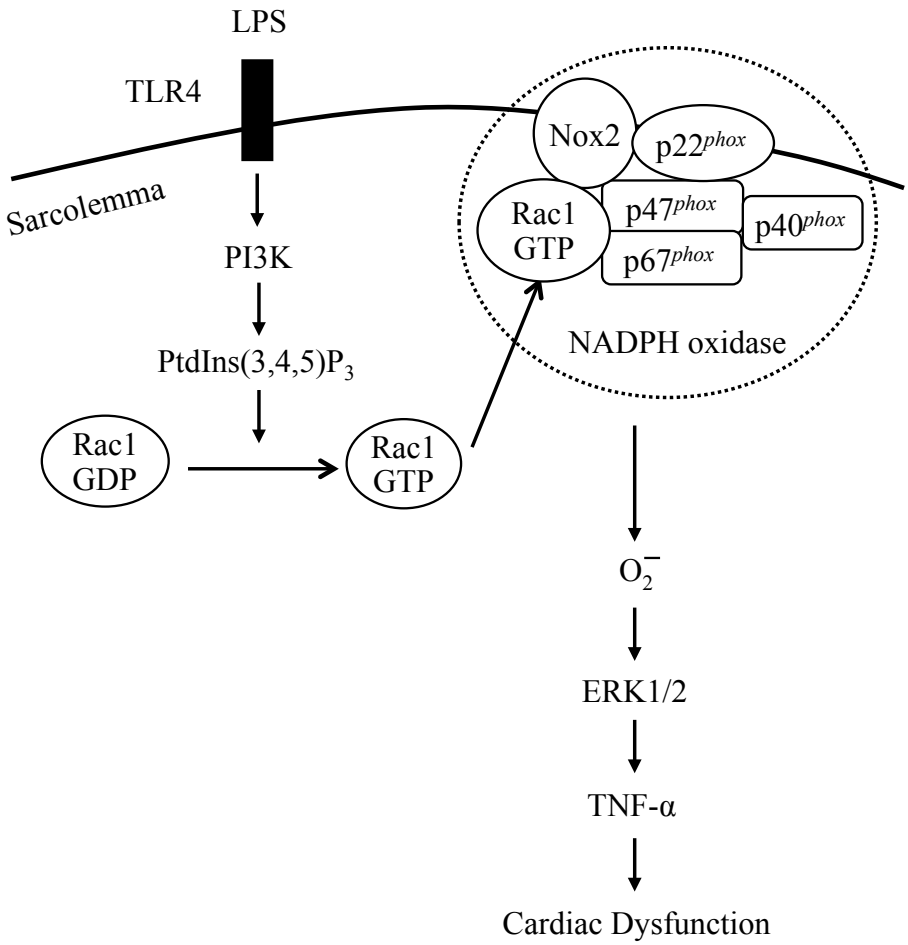

Figure 2.8. Schematic of Rac1 signaling pathway leading to cardiomyocyte TNF-α expression and cardiac dysfunction during LPS stimulation. LPS activates Rac1 via PI3K/PtdIns(3,4,5)P3 signaling. Activation of Rac1 activates NADPH oxidase leading to production of O_2^-, phosphorylation of ERK1/2 MAPK, and expression of TNF-α. Increased myocardial TNF-α production results in cardiac dysfunction during endotoxemia. TLR4, toll-like receptor 4.

and experimental conditions (Fessler et al. 2007; Monick et al. 2003; Sanlioglu et al. 2001; Thakur et al. 2006). For example, toxin B, an inhibitor Rho GTPase family, increased TNF-α production in macrophages in both basal and endotoxemic conditions (Monick et al. 2003). In resting neutrophils, RhoA suppresses TNF-α production by inhibiting NF-κB activity (Fessler et al. 2007). Upon LPS stimulation, RhoA is activated and increases TNF-α expression, suggesting a dual role of RhoA in TNF-α production in human neutrophils (Fessler et al. 2007). Additionally, studies have shown that Rac1 activation promoted LPS-induced TNF-α expression in macrophages and Kupffer cells (Sanlioglu et al. 2001; Thakur et al. 2006). Furthermore, Rac1 mediates myocardial TNF-α expression during LPS stimulation (Zhu et al. 2009). However, the role of Rac1 in cardiac dysfunction during endotoxemia remains unknown. In the present study, we demonstrated that Rac1 is rapidly and transiently activated by LPS in cultured neonatal cardiomyocytes and in the adult myocardium. Rac1 inhibition or deficiency blocked LPS-induced TNF-α production. Furthermore, $Rac1^{CKO}$ mice showed significant improvement in cardiac function during endotoxemia, which was assessed using the Langendorff preparation to avoid the influence of other organs, the systemic circulation and signals from both the central and the autonomic nervous systems. Thus, both *in vitro* and *in vivo* evidence from our study demonstrate that LPS activates Rac1 and promotes cardiomyocyte TNF-α expression leading to cardiac dysfunction.

Rac GTPases are regulated by GEFs that promote the exchange of GDP for GTP, and GAPs that accelerate the hydrolysis of GTP. Available evidence suggests that Rac activation depends mainly on the activation of GEFs (Welch et al. 2003). In this regard, Rac-activating GEFs such as Vav, Sos, PIX, SWAP-70 and P-Rex, can be activated by

phosphoinositol-3-kinase (PI3K) (Welch et al. 2003). The PI3K family is divided into three major classes: type 1, type II and type III. The type I PI3K comprises a catalytic subunit, p110 and a regulatory adapter subunit p85. Activated type I PI3K is recruited to the plasma membrane, where it can phosphorylate its main substrate PtdIns(4,5)P2 and thereby generate PtdIns(3,4,5)P3. The type II PI3K uses phosphatidylinositol (PI) and PtdIns(4)P as substrates and forms PtdIns(3)P and PtdIns(3,4)P2, respectively. The type III PI3K phosphorylates phoshatidylinositol (PtdIns) to produce phosphoinositol-3-P (PtdIns(3)P). PtdIns(3,4,5)P3, the production of the type I PI3K, can interact with the homology (PH) domain of target proteins, including GEFs, and promote protein activation (Welch et al. 2003). In the present study, type I PI3K activity, as determined by PtdIns(3,4,5)P_3 levels, was significantly increased in cultured cardiomyocytes *in vitro* and in the myocardium *in vivo* during LPS stimulation. Inhibition of PI3K activity by a selective inhibitor, LY294006, significantly decreased Rac1-GTP levels and resulted in a concomitant decrease in TNF-α expression stimulated by LPS. These results indicate that PI3K is required for Rac1 activation in cardiomyocytes during LPS stimulation.

Activated Rac1 interacts with specific effectors that regulate diverse physiological functions. One of these effectors is $p67^{phox}$, a subunit of NADPH oxidase (Bosco et al. 2009). A critical step for NADPH oxidase assembly and activation is the hetero-dimerization of Nox2 with $p67^{phox}$ (Dang et al. 2001). Interestingly, the interaction between $p67^{phox}$ and Rac1 results in increased affinity of $p67^{phox}$ for Nox2 (Nisimoto et al. 1997). In addition, recent studies involving $p67^{phox}$–Rac1 chimeras have reported that Rac1 induced an "activating" conformational change in $p67^{phox}$ (Gorzalczany et al. 2002; Sarfstein et al. 2004). Thus, Rac proteins are required for NADPH oxidase activation and

superoxide production. NADPH oxidase is an important source of reactive oxygen species in the heart and activation of NADPH oxidase has been shown to contribute to the pathogenesis of cardiovascular diseases including: cardiac hypertrophy (Bendall et al. 2002), hypertension (Rajagopalan et al. 1996), atherosclerosis (Warnholtz et al. 1999) and heart failure post myocardial infarction (Gao et al. 2008; Murdoch et al. 2006). We have recently demonstrated that NADPH oxidase is activated in cardiomyocytes which results in myocardial TNF-α expression and cardiac dysfunction during endotoxemia (Peng et al. 2005). In agreement with these data, the present study demonstrated that LPS increases O_2^- generation. Inhibition of NADPH oxidase activity by apocynin significantly decreased LPS-induced TNF-α expression in cardiomyocytes by 50%. This partially inhibitory effect of apocynin on TNF-α expression also indicates the exitance of other parallel signaling pathways, such as eNOS and PLCγ pathways, accounting for the remaining TNF-α expression (Zhang & Feng 2010). Furthermore, we showed that inhibition of PI3K activity decreased LPS-induced O_2^- production. Similarly, deficiency in Rac1 or overexpression of dominant-negative Rac1 significantly suppressed O_2^- generation and TNF-α expression in response to LPS stimulation. These results suggest that PI3K-mediated Rac1 activity promotes neonatal cardiomyocyte TNF-α expression induced by LPS via activation of NADPH oxidase.

MAPKs (p38, ERK1/2 and JNKs) are key signaling molecules involved in the regulation of many biological processes including inflammatory responses and the expression of pro-inflammatory cytokines. Indeed, activation of ERK1/2 and p38 regulates the expression of TNF-α in phagocytes during sepsis (Fessler et al. 2007; Rosengart et al. 2000; Thakur et al. 2006). We have recently demonstrated that ERK1/2

is downstream of NADPH oxidase signaling in LPS-induced TNF-α expression (Peng et al. 2005). In the present study, we showed that LPS increased the phosphorylation of ERK1/2 in cultured neonatal cardiomyocytes. Inhibition of PI3K and Rac1 activity blocked LPS-stimulated ERK1/2 phosphorylation. Moreover, inhibition of ERK1/2 activity decreased LPS-induced TNF-α production. Thus, PI3K-mediated Rac1 activity regulates LPS-induced TNF-α expression in cardiomyocytes via ERK1/2 activation.

In summary, the present study provides strong evidence that Rac1 activation is required for cardiomyocyte TNF-α expression and cardiac dysfunction during endotoxemia. Activation of Rac1 through PI3K increases NADPH oxidase and ERK1/2 activity, leading to increased myocardial TNF-α expression during LPS stimulation (Figure 2.8). Our study suggests that Rac1 may represent a novel therapeutic target for TNF-α expression and myocardial dysfunction in sepsis.

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Chapter 3. Rac1 promotes LPS-induced TNF-α production via Na/K-ATPase

3.1 Introduction

Sepsis is a leading cause of death in hospital intensive care units. Myocardial expression of pro-inflammatory cytokines, especially tumor necrosis factor-alpha (TNF-α) contributes to cardiac dysfunction and high mortality (40-80%) (Dellinger et al. 2008; Merx & Weber 2007; Parrillo et al. 1985). However the underlying molecular mechanisms of cardiac TNF-α production during sepsis remain elusive.

Na/K-ATPase catalyzes ATP hydrolysis to actively transport 3 Na^+ ions out of and 2 K^+ into the cell, and thus maintains trans-membrane gradients of Na^+ and K^+. Changes in intracellular Na^+ regulate intracellular Ca^{2+} via the Na^+/Ca^{2+} exchanger (Bers et al. 2006). In cardiomyocytes, Na/K-ATPase activity is inhibited by phospholemman (Han et al. 2006) and glutathionylation induced by NADPH oxidase (Figtree et al. 2009). In addition, Na/K-ATPase also acts as a signal transducer that regulates protein kinases including Src and ERK (Li & Xie 2009). In the heart, Na/K-ATPase modulates cardiomyocyte apoptosis (Sapia et al. 2010), contractility (Barwe et al. 2009; James et al. 1999) and hypertrophy (Huang et al. 1997) via Ca^{2+}-dependent mechanisms. LPS inhibits Na/K-ATPase activity in the lung (Koksel et al. 2006) and kidney (Guzman et al. 1995). Inhibition of Na/K-ATPase potentiates LPS-induced-cytokine expression including TNF-α in macrophages (Ohmori et al. 1991). Although we have previously shown that LPS increases intracellular Ca^{2+} in cardiomyocytes (Geoghegan-Morphet et al. 2007), changes in Na/K-ATPase activity were not studied. Thus, the role of Na/K-ATPase in myocardial TNF-α expression during endotoxemia remains unknown.

Mammalian target of rapamycin (mTOR), a Ser/Thr protein kinase, acts as a

central regulator of cell growth and metabolism by controlling protein synthesis and other cellular processes (Hay & Sonenberg 2004). It is sensitive to tuberous sclerosis complex (TSC)1/TSC2 activation and intracellular Ca^{2+} concentration (Hay & Sonenberg 2004; Hoyer-Hansen et al. 2007; Lenz & Avruch 2005). Studies have shown that mTOR contributes to cardiac hypertrophy (McMullen et al. 2004; Shioi et al. 2003) and ventricular remodeling after myocardial infarction (Buss et al. 2009). LPS activates mTOR (Baker et al. 2009; Chen et al. 2010; Haidinger et al. 2010; Weichhart et al. 2008), however its role in pro-inflammatory cytokine expression during sepsis remains controversial. For example, mTOR activation by LPS promotes TNF-α and interleukin-12 expression in monocyte-derived dendritic cells (Haidinger et al. 2010). On the contrary, mTOR activation inhibits TNF-α production in monocytes (Weichhart et al. 2008), macrophages (Baker et al. 2009) and HL-1 cells during LPS stimulation (Song et al. 2010). Thus, the effects of mTOR activation are cell type specific. Importantly, the role of mTOR in myocardial TNF-α protein production during endotoxemia remains unknown.

In the present study, we hypothesized that myocardial Na/K-ATPase activity is inhibited during endotoxemia via PI3K/Rac1/NADPH oxidase pathway. We further hypothesized that inhibition of Na/K-ATPase activates Ca^{2+}/CaMK-dependent mTOR, leading to enhanced TNF-α protein production in cardiomyocytes during LPS stimulation. To test these hypotheses, cultured cardiomyocytes and *in vivo* endotoxemia mouse models were employed. Our study demonstrated a novel function of Na/K-ATPase in the regulation of myocardial TNF-α expression during endotoxemia.

3.2. Materials and Methods

3.2.1 Animals and preparation of neonatal mouse cardiomyocytes

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication #85-23, revised 1996) and experimental protocols were approved by Animal Use Subcommittee at the University of Western Ontario. C57BL/6 wild-type (WT) and Rac1 floxed ($Rac1^{f/f}$) mice (Glogauer et al. 2003) were purchased from the Jackson Laboratory (Bar Harbor, Maine). Cre transgenic mice ($Cre^{TG/+}$) which overexpress Cre recombinase under the control of a-myosin heavy-chain (MHC) promoter were provided by Dr. E. Dale Abel (University of Utah, UT). The generation of cardiomyocyte-specific Rac1 knockout mice ($Rac1^{CKO}$) was achieved by breeding $Rac1^{f/f}$ mice with $Cre^{TG/+}$ mice (Rui et al. 2005).

3.2.2 Isolation and culturing of neonatal mouse cardiomyocytes

The neonatal cardiomyocytes were prepared and cultured according to methods we have previously described (Song et al. 2000; Chapter 2). Cells were treated with LPS (Sigma, Oakville, Ontario, Canada), apocynin (Sigma), ouabain (Sigma), KN-93 (Sigma), rapamycin (Cell Signaling Technology, Danvers, MA) or infected with adenoviruses.

3.2.3 Adenoviral infection of neonatal cardiomyocytes

Cardiomyocytes were infected with adenoviruses carrying a dominant-negative form of Rac1 (Ad-Rac1N17, Vector Biolabs, Philadelphia, PA), green fluorescence protein (Ad-GFP, a gift from Dr. J. Lipp, Medical University of Vienna, Austria) and LacZ (Ad-LacZ, Vector Biolabs, Philadelphia, PA) at a multiplicity of infection (MOI) of 10 plaque forming units/cell. Adenovirus-mediated gene transfer was applied as previously described (Peng et al. 2003; Chapter 2).

3.2.4 Na/K-ATPase activity assay

Na/K-ATPase activity in neonatal cardiomyocytes and LV myocardium was determined by a fluorometric method with modifications (Barr et al. 2005; Johansson et al. 2003). Briefly, homogenates were prepared in a buffer containing 250 mM sucrose, 2 mM EDTA, 1.25 mM EGTA, 5 mM NaN_3, and 10 mM Tris (pH 7.4). Homogenates were freeze-thawed four times. 30 μg protein lysates were incubated for 5 min at 37°C in a buffer containing 5 mM $MgCl_2$, 1.25 mM EDTA, 100 mM Tris base (pH 7.4), 1 mM EGTA, and 5 mM NaN_3, with or without 6 mM ouabain. 3-O-methylfluorescein phosphate (3-O-MFP, 160 μM) was added to the lysates followed by incubation at 37°C for 1 min. Activated Na/K-ATPase hydrolyzes 3-O-MFP and forms a fluorescent compound 3-O-MF. To activate Na/K-ATPase, 10 mM KCl was added and fluorescence was recorded. The excitation and emission wavelengths were 470 nm and 515 nm, respectively. The amplitude of the emission was shown to be proportional to the concentration of 3-O-MF, and a standard curve was created using varying concentration of 3-O-MF. K^+-dependent 3-O-MFPase activity was determined by subtracting activity with KCl from the activity without KCl. Na/K-ATPase activity was determined by subtracting K^+-dependent 3-O-MFPase activity without ouabain from the activity with ouabain and expressed as micromoles of liberated phosphate (Pi) per minute per milligram protein.

3.2.5 Measurement of TNF-α mRNA

Total RNA was extracted from cardiomyocytes using the Trizol Reagent (Gibco-BRL) as per manufacturer's instructions. TNF-α mRNA levels were determined by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) as as previously

described (Peng et al. 2003; Chapter 2). TNF-α mRNA stability was assessed in the present of a transcription inhibitor, actinomycin D. Neonatal cardiomyocytes were stimulated with LPS (0.1 μg/ml) with or without ouabain (50 μM) for 3 hours followed by actinomycin D (5 μg/ml) treatment. After 20, 60 and 120 minutes of actinomycin D exposure, total RNA was extracted from cardiomyocytes. TNF-α mRNA levels were measured.

3.2.6 Measurement of TNF-α protein

TNF-α protein levels were measured using a mouse TNF-α ELISA kit (eBioscience, USA), according to the manufacturer's instructions. The measurements were standardized with cell numbers or expressed as TNF-α levels to total proteins.

3.2.7 [^{3}H]-Leucine incorporation

Neonatal cardiomyocytes were seeded into 12-well plates (8×10^5 cells/well). After 48 hours, cells were treated with LPS (1 μg/ml) with or without rapamycin (10 nM) in the present of [^{3}H]-leucine (1 μCi/ml, Amersham, GE Healthcare, Baie d'Urfe, Quebec) for 5 hours. Cells were then washed 3 times with ice-cold PBS. Proteins were precipitated with 5% tricholoroacetic acid (TCA) for 30 minutes on ice. Cell precipitates were washed 2 additional times with TCA and solubilized in 0.2 M NaOH. The radioactivity of [^{3}H]-leucine incorporated into proteins were measured by a liquid scintillation counter (Tri-Carb 2900TR, Perkin-Elmer, Woodbridge, Ontario).

3.2.8 Adult cardiomyocyte isolation

Cardiomyocytes were isolated from the hearts of adult male $Rac1^{f/f}$ and $Rac1^{CKO}$ mice as previously described with modifications (Burger et al. 2009). Hearts were mounted on a Langendorff system and perfused with digestion buffer containing 75

μg/mL of liberase blendzyme IV (Roche, Laval, Quebec). Following digestion, cells were resuspended and exposed to a series of sedimentation and resuspension steps in buffer containing increasing concentrations of Ca^{2+} (12.5 μM-1.5 mM). Healthy, rod-shaped myocytes were used for measurements of Ca^{2+} transients.

3.2.9 Intracellular Ca^{2+} transients

Free intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured in isolated ventricular cardiomyocytes using fura-2-AM as previously described (Burger et al. 2009). Cells were loaded with 1 μM fura-2-AM and then paced at 0.5, 1, 2 and 4 Hz using a 2.5 ms duration pulse. Fluorescence intensity at 510 nm was measured with alternating 345 and 380 nm excitation using a Deltascan monochrometer system (Photon Technology International, London, ON, Canada). $[Ca^{2+}]_i$ was calculated by the methods of Grynkiewicz (Grynkiewicz et al. 1985).

3.2.10 Western blot analysis

Thirty micrograms of protein lysates were subjected to separation on a 10% SDS-PAGE gel, followed by electrotransfer to nitrocellulose membranes. Blots were probed with specific antibodies against total mTOR (1:1000) and phospho-mTOR (1:1000; Cell Signaling Technology, Danvers, MA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:3000, Santa Cruz Biotechnology, CA) respectively. Signals were detected by the chemiluminescence detection method and quantified by densitometry. The relative phosphorylation levels of mTOR under various experimental conditions were estimated by taking the ratio of the densitometric signal acquired with anti-phospho-mTOR to the corresponding signal acquired with anti-mTOR.

3.2.11 Statistical analysis

Results are presented as mean ± SEM. Differences between two groups were analyzed by unpaired Student t-test. For multigroup comparisons, one or two-way ANOVA followed by Student-Newman-Keuls or Bonferroni post-test was performed. A P value less than 0.05 was considered statistically significant.

3.3 Results

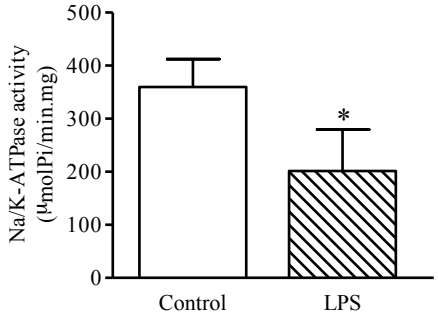
3.3.1 LPS inhibits myocardial Na/K-ATPase activity

To examine the effect of LPS on Na/K-ATPase activity, neonatal cardiomyocytes isolated from C57BL/6 mice were treated with LPS (1 μg/ml) for 2 hours. LPS decreased Na/K-ATPase activity by 44% (Figure 3.1A). To study Na/K-ATPase activity in the myocardium *in vivo*, $Rac1^{f/f}$ mice were treated with LPS (2 mg/kg, i.p.) or saline for 1, 2, 3 or 4 hours, after which hearts were obtained and myocardial Na/K-ATPase activity was measured. After 2 hours of LPS treatment, myocardial Na/K-ATPase activity was decreased by 93%, and returned back to control levels at 4 hours (Figure 3.1B). These data showed that LPS strongly inhibits Na/K-ATPase activity in cardiomyocytes *in vitro* and in myocardium *in vivo*.

3.3.2 Inhibition of Na/K-ATPase promotes TNF-α protein production during LPS stimulation

TNF-α is a major pro-inflammatory cytokine contributing to cardiac dysfunction during endotoxemia. To elucidate the role of Na/K-ATPase on LPS-induced TNF-α expression, neonatal cardiomyocytes were incubated with ouabain, a selective inhibitor of Na/K-ATPase. A concentration-dependent enhancement of LPS-induced TNF-α protein levels was observed following ouabain treatment (Figure 3.2A). Surprisingly, ouabain

A



B

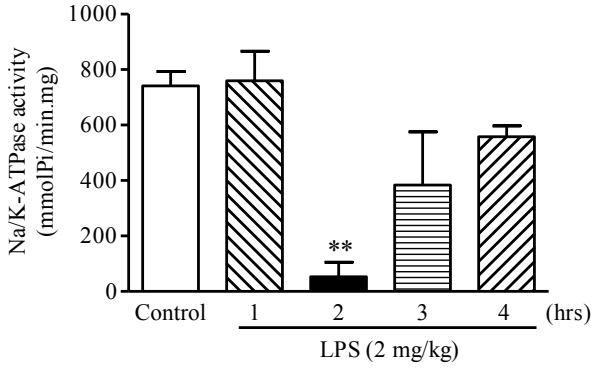


Figure 3.1. LPS inhibits myocardial Na/K-ATPase activity. (A) Neonatal cardiomyocytes from WT mice were treated with vehicle or LPS (1 μg/ml) for 2 hours. Na/K-ATPase activity in these cells was determined using a fluorometric assay. (B) Adult male $Rac1^{f/f}$ mice were treated with LPS (2 mg/kg, i.p. injection) for 1, 2, 3 and 4 hours. Na/K-ATPase activity in the left ventricular myocardium was determined. Data are means ± SEM from 3-5 independent experiments. **P*<0.05, ***P*<0.01 vs. control.

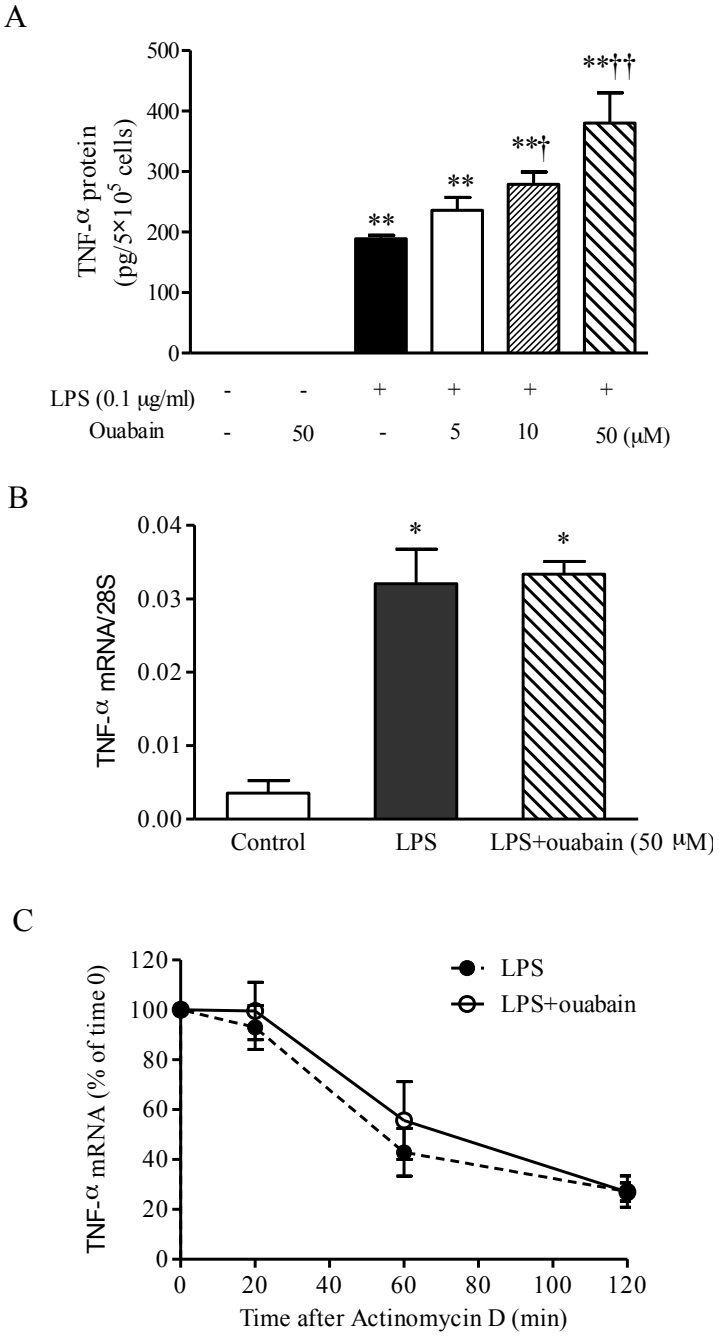


Figure 3.2. Ouabain enhances LPS-induced TNF-α expression. Neonatal cardiomyocytes from WT mice were treated with LPS (0.1 μg/ml) with or without ouabain for 3 or 5 hours. (A) TNF-α protein levels in culture medium were measured by ELISA. (B) TNF-α mRNA levels in cardiomyocytes were determined by real-time RT-PCR. (C) Stability of TNF-α mRNA was assessed in the presence of actinomycin D, an inhibitor of transcription. Data are means ± SEM from 3-4 independent experiments. **P*<0.05 ***P*<0.01 vs. control; †*P*<0.05, ††*P* <0.01 vs. LPS

had no measurable effect on LPS-induced TNF-α mRNA expression (Figure 3.2B). Furthermore, ouabain did not alter TNF-α mRNA stability during LPS stimulation (Figure 3.2C). These data indicate that inhibition of Na/K-ATPase activity enhances LPS-induced TNF-α protein levels without any apparent effects on its mRNA expression or stability in cardiomyocytes.

3.3.3 Inhibition of Na/K-ATPase by LPS is mediated by the PI3K/Rac1/NADPH oxidase pathway

We have recently shown that the PI3K/Rac1/NADPH oxidase pathway is activated and important for cardiac TNF-α production during endotoxemia (Zhang et al. 2011 Chapter 2; Zhu et al. 2009). To determine if PI3K acts as an up-stream regulator of Na/K-ATPase, the PI3K inhibitor LY294002 was employed. LY294002 had no effect on Na/K-ATPase activity under control conditions but completely restored Na/K-ATPase activity after LPS stimulation in neonatal cardiomyocytes (Figure 3.3A). These results were further confirmed *in vivo*. Adult male WT mice were pretreated with LY294002 (7.5 mg/kg, i.p.) for 30 minutes followed by LPS stimulation (2 mg/kg, i.p.). The dose of LY294002 was chosen from our recent study, which showed significant inhibition of PI3K-mediated responses in mice (Wu et al. 2011). As shown in Figure 3.3B, LY294002 blocked myocardial Na/K-ATPase activity suppression by LPS, indicating that LPS inhibits cardiac Na/K-ATPase activity via PI3K.

To determine the effect of Rac1 on Na/K-ATPase activity, neonatal cardiomyocytes isolated from WT mice were infected with an adenovirus encoding a dominant negative form of Rac1 (Ad-Rac1N17), which specifically inhibits Rac1 activity. Overexpression of Rac1N17 restored Na/K-ATPase activity inhibited by LPS

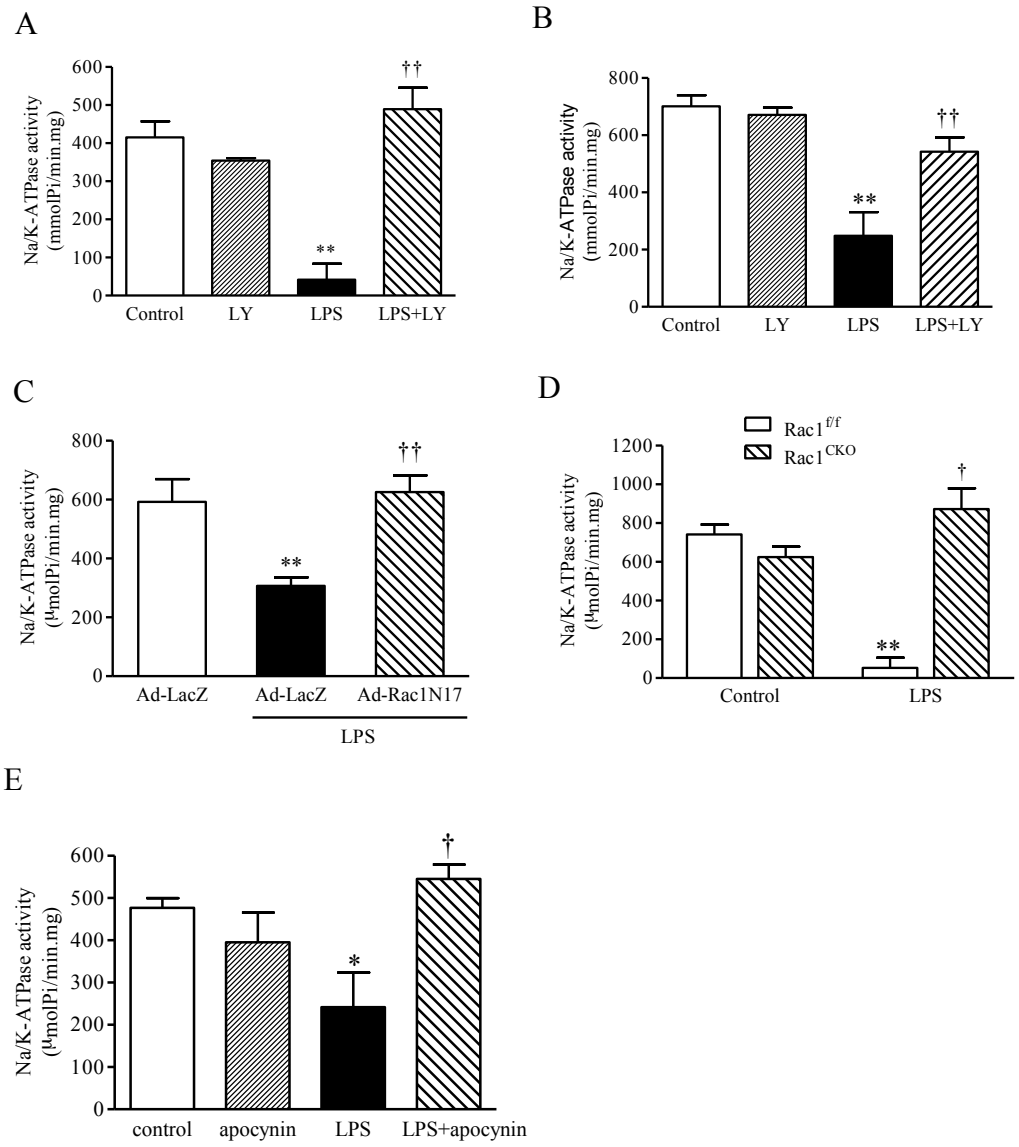
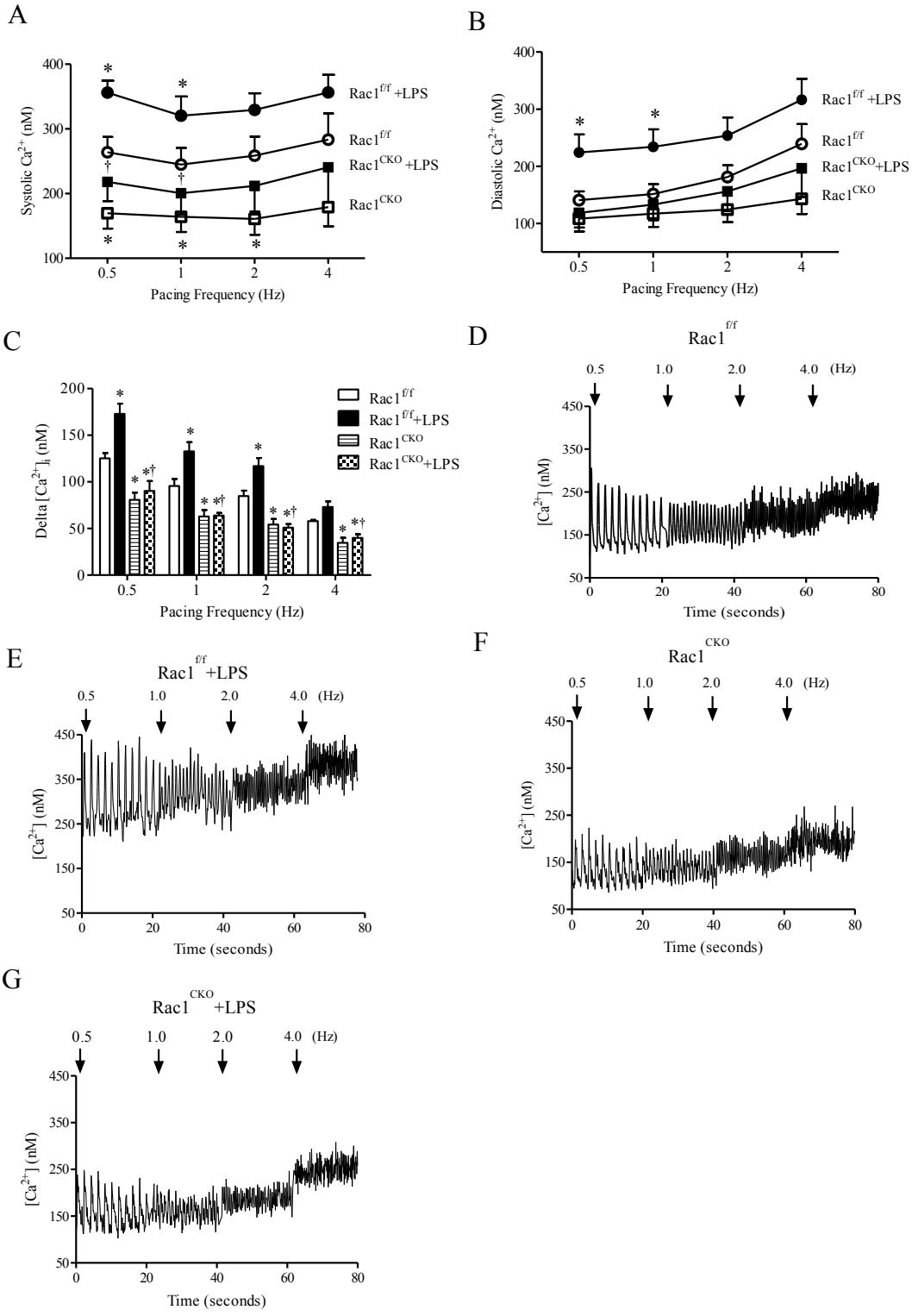


Figure 3.3. LPS-induced PI3K, Rac1 and NADPH oxidase activities suppress Na/K-ATPase. (A) WT neonatal cardiomyocytes were treated with LPS (1 μg/ml) with or without LY294002 (LY, 10 μM) for 2 hours. Na/K-ATPase activity in these cells was measured. (B) Adult male C57BL/6 mice were pretreated with saline (control) or LY294002 (7.5 mg/kg) for 30 minutes followed by treatment with LPS (2 mg/kg, i.p.) for 2 hours. Na/K-ATPase activity in the LV myocardium was determined. (C) WT neonatal cardiomyocytes were infected with Ad-LacZ or Ad-Rac1N17 for 24 hours followed by treatment with LPS (1 μg/ml). Na/K-ATPase activity was measured. (D) Na/K-ATPase activity in $Rac1^{f/f}$ and $Rac1^{CKO}$ myocardium were measured after 2 hours of LPS treatment (2 mg/kg, i.p.). (E) Cells were incubated with LPS with or without apocynin (400 μM) for 2 hours. Na/K-ATPase activity was measured. Data are means ± SEM from 3-5 independent experiments. **P*<0.05, ***P*<0.01 vs. control and Ad-LacZ; †*P*<0.05, ††*P*<0.01 vs. Ad-LacZ+LPS and LPS.

(Figure 3.3C). Consistently, LPS decreased Na/K-ATPase activity in the myocardium of $Rac1^{f/f}$ mice but had no effect on $Rac1^{CKO}$ mice (Figure 3.3D). To determine if NADPH oxidase regulates Na/K-ATPase activity in response to LPS, neonatal cardiomyocytes were treated with apocynin, a specific inhibitor of NADPH oxidase. Apocynin prevented the diminution of Na/K-ATPase activity in response to LPS (Figure 3.3E). Taken together, these data implicate the PI3K/Rac1/NADPH oxidase pathway in the inhibition of myocardial Na/K-ATPase activity during LPS stimulation.

3.3.4 LPS-induced intracellular Ca^{2+} is mediated by Rac1

Inhibition of Na/K-ATPase is known to increase intracellular Ca^{2+} (Bers et al. 2006). Since LPS-induced inhibition of Na/K-ATPase is mediated by Rac1 as shown above, the role of Rac1 in intracellular Ca^{2+} during LPS stimulation was studied. To this end, Ca^{2+} transients were recorded using fura-2 in adult cardiomyocytes isolated from $Rac1^{f/f}$ and $Rac1^{CKO}$ mice. These cells were paced over a range of frequencies (0.5 to 4 Hz) (Figure 3.4A-G). $Rac1^{CKO}$ cells showed lower systolic Ca^{2+} compared with $Rac1^{f/f}$ cells, but diastolic Ca^{2+} was similar between these two groups (Figure 3.4A-C). In addition, systolic and diastolic Ca^{2+}, and the difference between systolic and diastolic Ca^{2+} concentrations were all significantly increased by LPS stimulation in $Rac1^{f/f}$ cardiomyocytes, but not in $Rac1^{CKO}$ cells (Figure 3.4A-C). Collectively, these data suggest that Rac1 is not only required for maintaining cardiac Ca^{2+} homeostasis under basal physiological conditions but also contributes to increased intracellular Ca^{2+} in response to LPS.


A
Systolic Ca2+ (nM)
Pacing Frequency (Hz)
Rac1f/f +LPS
Rac1f/f
Rac1CKO +LPS
Rac1CKO
B
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Pacing Frequency (Hz)
Rac1f/f +LPS
Rac1f/f
Rac1CKO+LPS
Rac1CKO
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[Ca2+] (nM)
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Figure 3.4 Rac1 increases LPS-induced intracellular Ca^{2+} transient in cardiomyocytes. Adult cardiomyocytes from $Rac1^{f/f}$ and $Rac1^{CKO}$ hearts treated with vehicle or LPS (5 μg/ml) for 30 minutes were paced at 0.5, 1, 2 and 4 Hz. Intracellular Ca^{2+} transients were recorded. (A) Systolic, (B) diastolic and (C) difference between systolic and diastolic intracellular Ca^{2+} concentrations in response to pacing were determined. (D) and (F) representative tracings from $Rac1^{f/f}$ and $Rac1^{CKO}$, respectively. (E) and (G) representative tracings from $Rac1^{f/f}$ and $Rac1^{CKO}$ stimulated by LPS respectively. Data are mean ± SEM from 4-6 mice per group (8-12 cells). *P<0.05 vs. $Rac1^{f/f}$; †P<0.05 vs. $Rac1^{f/f}$+LPS.

3.3.5 Ca^{2+}/CaMK mediates LPS-induced TNF-α expression

Ca^{2+}/calmodulin-dependent protein kinase (CaMK) activation promotes TNF-α expression in monocytes and macrophages (Liu et al. 2008; Mendez-Samperio et al. 2006; Rosengart et al. 2000). To determine if CaMK regulates TNF-α expression in cardiomyocytes, neonatal cardiomyocytes were pretreated with KN-93, a CaMK inhibitor. KN-93 decreased LPS-induced TNF-α mRNA and protein levels in a dose-dependent manner (Figure 3.5A and B).

3.3.6 mTOR mediates LPS-induced TNF-α protein expression

To gain insight into the molecular mechanisms by which Na/K-ATPase regulates TNF-α protein production, we studied mTOR, a master regulator of protein synthesis, activation during endotoxemia. Our data showed that mTOR phosphorylation was significantly increased in neonatal cardiomyocytes in response to LPS stimulation (Figure 3.6A). Inhibition of mTOR by rapamycin resulted in a significant decrease in LPS-induced TNF-α protein levels in a dose-dependent manner without any effect on TNF-α mRNA expression (Figure 3.6B and C).

To assess the effects of the above treatments on global protein synthesis, [^{3}H]-leucine incorporation was determined in cultured cardiomyocytes. In response to LPS, [^{3}H]-leucine incorporation showed a trend but not significant increase compared to controls (Figure 3.6D). Importantly, treatment with rapamycin did not have a significant effect on [^{3}H]-leucine incorporation during LPS stimulation (Figure 3.6D). Taken together, our data showed that inhibition of mTOR by rapamycin selectively decreases LPS-induced TNF-α protein synthesis.

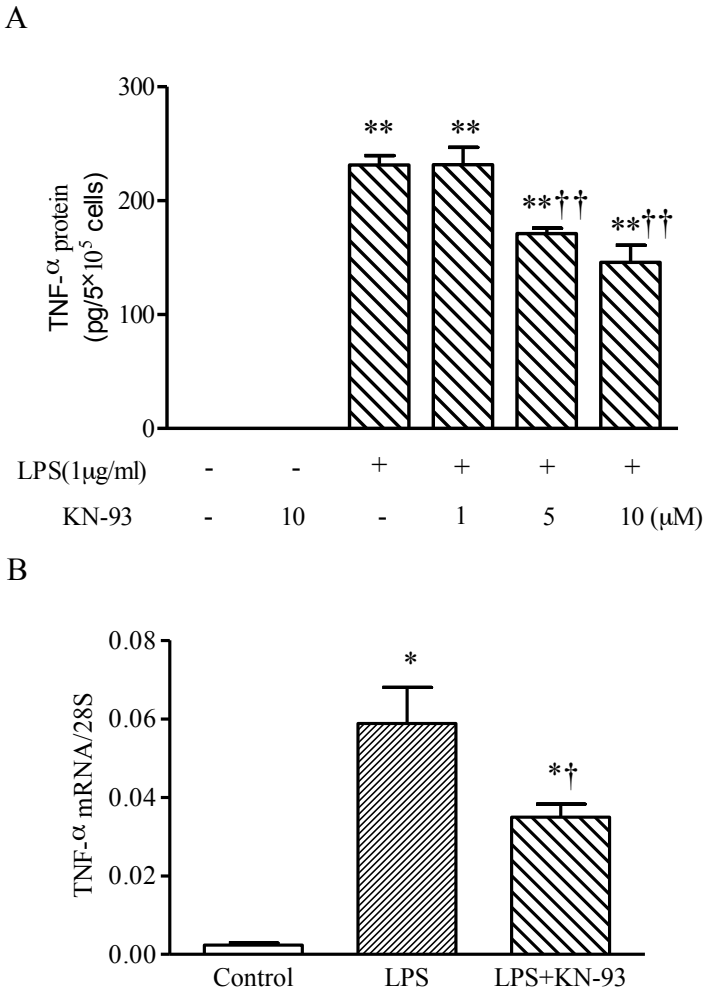

Figure 3.5. Inhibition of CaMK activity diminishes LPS-induced TNF-α production. WT neonatal cardiomyocytes were treated with vehicle or LPS (1 μg/ml) with or without KN-93. TNF-α protein (A) and mRNA (B) levels were measured at 5 and 3 hours after LPS treatment, respectively. Data are mean ± SEM from 3-4 independent experiments. **P* <0.01 vs. control; †*P*<0.01 vs. LPS.

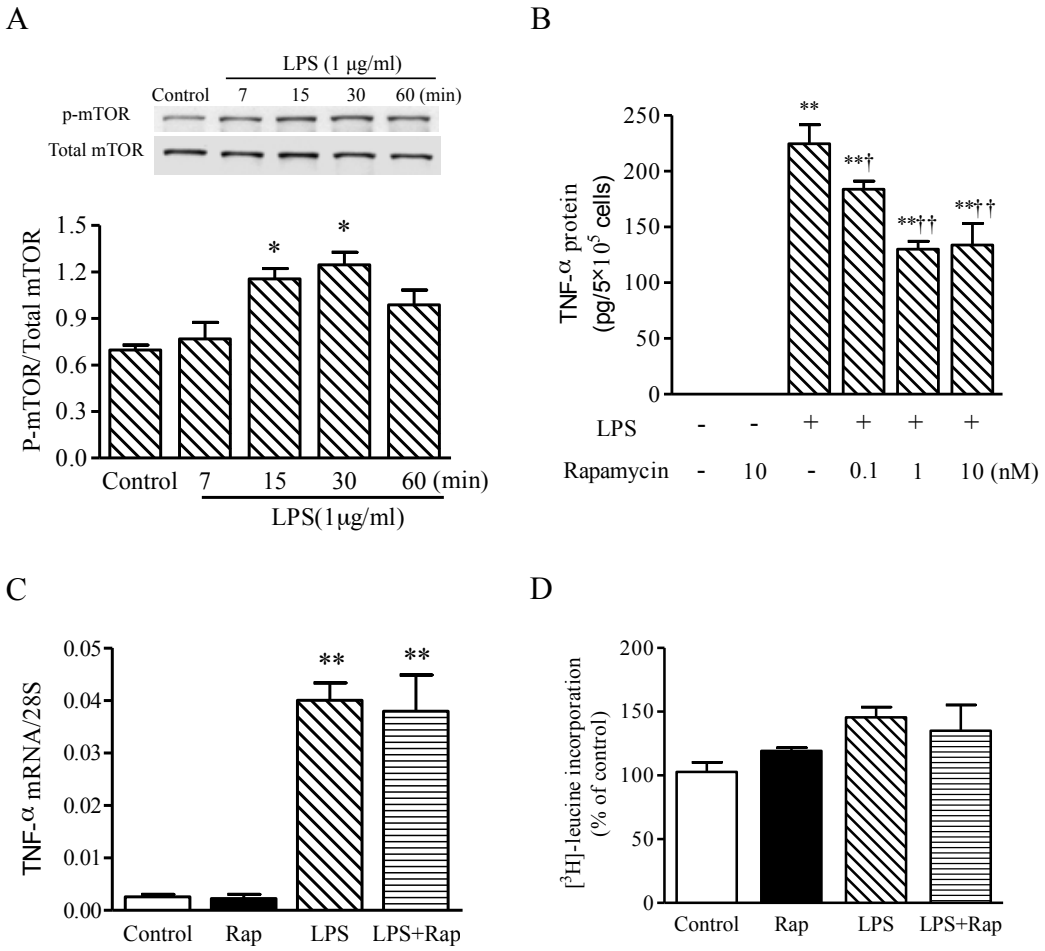

Figure 3.6. mTOR activity promotes LPS-induced TNF-α production in cardiomyocytes. (A) WT neonatal cardiomyocytes were cultured in serum free medium overnight followed by treatment with LPS (1 μg/ml) for 7, 15, 30 and 60 minutes. Phospho-mTOR was determined by western blotting. Cells were treated with vehicle or LPS (1 μg/ml) with or without rapamycin (Rap). TNF-α mRNA (C) and protein (B) levels were measured following 3 and 5 hours of LPS treatment, respectively. (D) Global protein synthesis in cardiomyocytes was assessed by [^{3}H]-leucine incorporation following 5 hours of LPS (1 μg/ml) treatment with or without Rap. Data are mean ± SEM from 3-6 independent experiments. **P* <0.05, ***P* <0.01 vs. control; †*P* <0.01

3.3.7 mTOR activation is mediated by Rac1/ Na/K-ATPase /CaMK signaling

To determine the role of Rac1/ Na/K-ATPase / CaMK signaling on mTOR activity during LPS stimulation, neonatal cardiomyocytes were treated with Ad-Rac1N17, ouabain and KN-93, respectively. Rac1N17 and KN-93 blocked LPS-induced mTOR phosphorylation (Figure 3.7A and C). In contrast, ouabain enhanced mTOR phosphorylation induced by LPS (Figure 3.7B). Taken together, these data suggest that Rac1/ Na/K-ATPase/ CaMK pathway mediates LPS-induced mTOR activation, which promotes TNF-α protein expression.

3.4. Discussion

The present study provides the first evidence that LPS negatively regulates cardiac Na/K-ATPase activity via the PI3K/Rac1/NADPH oxidase pathway. Inhibition of Na/K-ATPase activates mTOR apparently via Ca^{2+}/CaMK, leading to TNF-α protein production in cardiomyocytes. Na/K-ATPase/ Ca^{2+}/CaMK /mTOR signaling represents a novel molecular mechanism by which LPS stimulates cardiac TNF-α protein production (Figure 3.8).

Na/K-ATPase is an important ion transporter and signal transducer located at the cell membrane. It has been demonstrated that Na/K-ATPase activity is decreased during the inflammatory response of the lung, kidney and innate immune system and contributes to pro-inflammatory factor mRNA expression (Guzman et al. 1995; Koksel et al. 2006; Ohmori et al. 1991). Here, we provided both *in vivo* and *in vitro* evidence to show that LPS inhibits Na/K-ATPase activity in the heart. It is interesting to note that Na/K-ATPase inhibition enhances LPS-induced TNF-α protein production without any

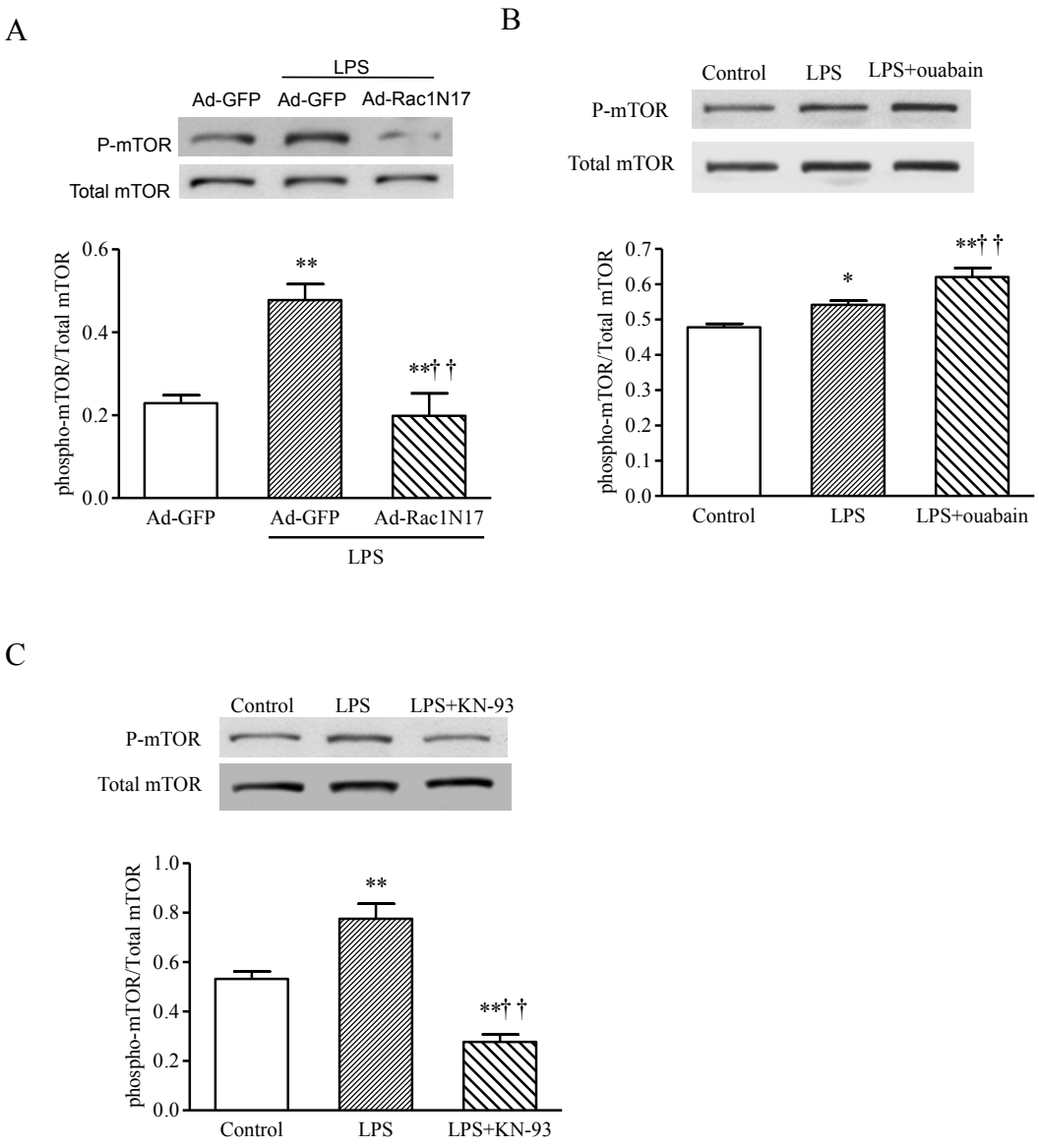

Figure 3.7. Role of Rac1, Na/K-ATPase and CaMK in LPS-induced mTOR activation in cardiomyocytes. (A) WT neonatal cardiomyocytes cultured in serum free medium were infected with Ad-Rac1N17 followed by LPS treatment (1 μg/ml) for 15 min. (B and C) Cultured cardiomyocytes were treated with LPS (1 μg/ml) with ouabain (50 μM, B) or KN-93 (10 μM, C) for 15 minutes. Phospho-mTOR was detected by western blotting. Data are mean ± SEM from 3-4 independent experiments. **P* <0.05, ***P* <0.01 vs. control; †*P* <0.01 vs. LPS.

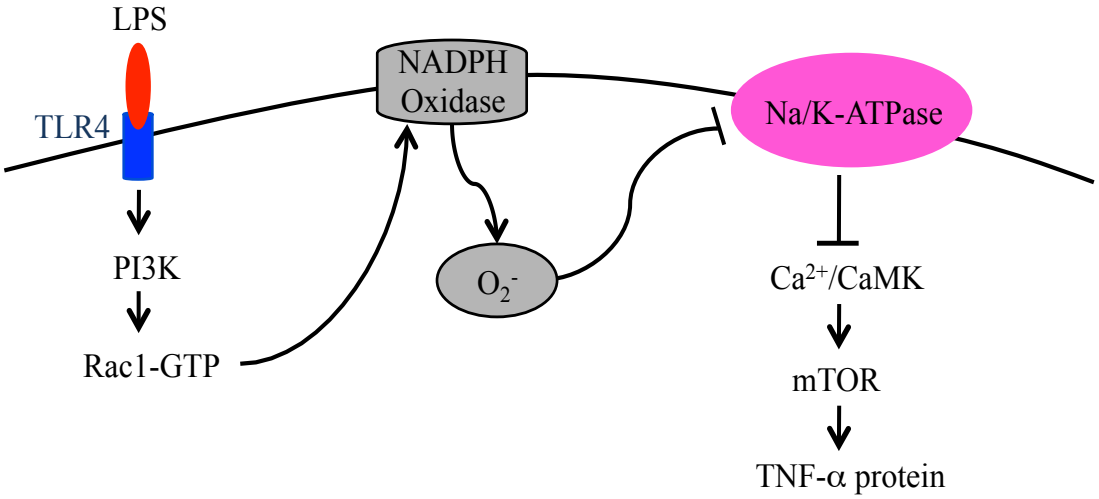

Figure 3.8. Schematic diagram illustrates the involvement of Na/K-ATPase/mTOR signaling pathways leading to cardiomyocyte TNF-α protein production during LPS stimulation. LPS suppresses Na/K-ATPase activity via the PI3K/Rac1/NADPH oxidase pathway in the myocardium. Inhibition of Na/K-ATPase activates mTOR via Ca^{2+}/CaMK. Activated mTOR promotes TNF-α protein production. TLR4, toll-like receptor 4.

apparent effects on TNF-α mRNA expression in cardiomyocytes. Since TNF-α mRNA stability is not affected, these results suggest that inhibition of Na/K-ATPase may promote TNF-α protein translation or inhibit its degradation in cardiomyocytes during LPS stimulation.

Studies have shown that activation of NADPH oxidase inhibits Na/K-ATPase by glutathionylation of its β subunit (Figtree et al. 2009). We have demonstrated previously that LPS activates NADPH oxidase via PI3K/Rac1 signaling in cardiomyocytes (Zhang et al. 2011 Chapter2). In agreement with this finding, the present study showed that the reduction of myocardial Na/K-ATPase activity during LPS stimulation was prevented by inhibition of PI3K, Rac1 or NADPH oxidase, indicating that activation of PI3K/Rac1/NADPH oxidase pathway inhibits myocardial Na/K-ATPase activity during endotoxemia.

It is well established that inhibition of Na/K-ATPase activity increases intracellular Ca^{2+} via the Na^{+}/Ca^{2+} exchange (Bers et al. 2006). We have previously shown that intracellular Ca^{2+} levels are increased by LPS in cardiomyocytes and contribute to TNF-α expression (Geoghegan-Morphet et al. 2007). To study the role of Rac1, a negative regulator of Na/K-ATPase in Ca^{2+} homeostasis, we measured Ca^{2+} transients in cardiomyocytes and found that Rac1 deficient cardiomyocytes exhibited significantly lower intracellular Ca^{2+} levels compared with $Rac1^{f/f}$ cells during basal conditions and in response to LPS, suggesting that activation of Rac1 increases intracellular Ca^{2+} via inhibition of Na/K-ATPase. CaMK is a downstream target of Ca^{2+} (Mendez-Samperio et al. 2006; Rosengart et al. 2000). Our study further demonstrated that inhibition of CaMK decreased TNF-α expression during LPS stimulation. CaMK

appears to affect both TNF-α protein and mRNA levels, which presumably reflects the multiplicity of cellular effects triggered by this kinase. Taken together, our data suggest that LPS activates Rac1/Ca^{2+}/CaMK signaling leading to TNF-α protein expression.

Activation of mTOR stimulates global protein synthesis by directly phosphorylating eukaryotic initiation factor 4E-binding protein (4E-BP) and in the longer term increases levels of ribosomal proteins(Hay & Sonenberg 2004; Ruvinsky & Meyuhas 2006). LPS activates mTOR in phagocytes (Baker et al. 2009; Haidinger et al. 2010; Weichhart et al. 2008), bone marrow cells (Chen et al. 2010) and HL-1 cells (a cardiomyocyte cell line) (Song et al. 2010), however the role of mTOR in pro-inflammatory cytokine expression is cell type specific. For example, rapamycin, an mTOR inhibitor decreased the production of pro-inflammatory cytokines such as IL-12 and TNF-α in dendritic cells (Haidinger et al. 2010). In contrast, rapamycin enhanced LPS-induced TNF-α mRNA and protein levels in monocytes (Weichhart et al. 2008) and macrophages (Baker et al. 2009). Similarly, overexpression of mTOR in HL-1 cells decreased TNF-α protein levels stimulated by LPS (Song et al. 2010). In the present study, we showed that mTOR was rapidly and transiently activated between 15 to 30 minutes after LPS stimulation in neonatal cardiomyocytes cultured in serum free medium. Our data showed that Na/K-ATPase activity were decreased in the cells cultured in medium contain 10% FBS after 2 hours of LPS stimulation. The mismatch between the time points of Na/K-ATPase and mTOR activation is possibly due to different culture conditions with and without serum. Rapamycin decreased TNF-α protein levels after LPS stimulation but had no apparent effect on TNF-α mRNA expression or global protein synthesis. Our data suggest that mTOR is important in LPS-induced TNF-α protein

synthesis in cardiomyocytes.

mTOR activation is regulated by a number of factors including TSC1/TSC2 and Ca^{2+} (Hay & Sonenberg 2004; Hoyer-Hansen et al. 2007; Lenz & Avruch 2005). The relationship between intracellular Ca^{2+} concentrations and mTOR activity is cell-type specific (Hoyer-Hansen et al. 2007; Lenz & Avruch 2005). In primary mouse neurons, glutamateric stimulation activates mTOR in a Ca^{2+}/CaMK-dependent manner (Lenz & Avruch 2005). On the other hand, mTOR activity is inhibited by an increase in cytosolic Ca^{2+} in MCF-7 breast cancer cells (Hoyer-Hansen et al. 2007). The present study showed that LPS-induced inhibition of Na/K-ATPase activity increases intracellular Ca^{2+} in cardiomyocytes. To study the role of CaMK, a downstream effector of Ca^{2+} in mTOR activation, a selective inhibitor of CaMK was employed. Our data showed that inhibition of CaMK decreased mTOR activity in response to LPS. Furthermore, LPS-induced mTOR activity was blocked by inhibition of Rac1 but enhanced by the inhibition of Na/K-ATPase. Our results suggest that Rac1 inhibits Na/K-ATPase and increases Ca^{2+}/CaMK activity, which subsequently activates mTOR and promotes TNF-α protein translation in cardiomyocytes during LPS stimulation. In addition, our study also showed that inhibition of CaMK blocked LPS-induced TNF-α mRNA and protein expression, indicating that there are other parallel pathways through which Na/K-ATPase regulates mTOR activation.

Interestingly, mTOR also promotes the translation of cellular mRNAs that have a 5' terminal oligopyrimidine (TOP) tract adjacent to the cap site (Ruvinsky & Meyuhas 2006). The 5'TOP tracts, which are found in all mRNAs that encode ribosomal proteins, enhance translation upon mTOR activation (Ruvinsky & Meyuhas 2006). Examination

of the 5' untranslated region of TNF-α mRNA reveals that both the mouse (GenBank: U68414) and human (GenBank: NM_000594) forms contain a 5' CTCCCTC sequence, which conforms to the established consensus for regulatory 5'TOP motifs (Ruvinsky & Meyuhas 2006). The presence of this putative 5'TOP motif further supports our hypothesis that TNF-α is controlled at the translational level by mTOR in cardiomyocytes during LPS stimulation.

In conclusion, our study demonstrated that LPS inhibits Na/K-ATPase activity via PI3K/Rac1/NADPH oxidase pathway in the myocardium. Inhibition of Na/K-ATPase activity promotes TNF-α protein production by Ca^{2+}/CaMK-dependent mTOR activation in cardiomyocytes. This Na/K-ATPase/ Ca^{2+}/CaMK /mTOR pathway provides novel insight into the signal transduction mechanisms that regulate myocardial TNF-α expression, and may have therapeutic implications in the treatment of sepsis (Figure 3.8).

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Chapter 4. Rac1 regulates LPS-induced TNF-α expression and cardiac dysfunction via MKP-1

4.1 Introduction

Sepsis is the 10th most common cause of death in the United States (Dellinger et al. 2008). Cardiac dysfunction frequently accompanies severe sepsis and septic shock, and is associated with a significant increase in mortality rate (70% to 90%) compared with septic patients without cardiovascular impairment (20%) (Merx & Weber 2007). Tumor necrosis factor-alpha (TNF-α) produced by cardiomyocytes is a major contributing factor to cardiac dysfunction (Merx & Weber 2007; Parrillo et al. 1985). However, the underlying molecular mechanisms regulating myocardial TNF-α production during sepsis remain elusive.

Mitogen-activated protein kinases (MAPKs) including p38, ERK1/2, and JNK1/2 are key signaling molecules regulating inflammatory responses and the expression of pro-inflammatory cytokines (Dumitru et al. 2000; Kotlyarov et al. 1999). It has been demonstrated that these MAPKs exhibit different effects on myocardial TNF-α expression during endotoxemia (Peng et al. 2005b; Peng et al. 2003; Peng et al. 2009; Rosengart et al. 2000; Thakur et al. 2006). Indeed, activation of ERK1/2 and p38 promotes TNF-α expression (Peng et al. 2005b; Peng et al. 2003; Rosengart et al. 2000; Thakur et al. 2006). In contrast, we recently demonstrated that JNK1 decreases TNF-α expression and improves cardiac function through inhibition of ERK1/2 and p38 activity (Peng et al. 2009). However, the molecular mechanisms by which JNK inhibits ERK1/2 and p38 are not fully understood.

Inactivation of MAPKs is achieved primarily by MAPK phosphatases (MKPs)

that dephosphorylate phosphothreonine and phosphotyrosine residues of MAPKs (Liu et al. 2007). Studies have shown that MKP-1 negatively regulates the inflammatory response of the innate immune system by accelerating MAPK inactivation and attenuating the production of pro-inflammatory cytokines including TNF-α, interleukin (IL)-1β and IL-6 in macrophages following LPS stimulation (Chen et al. 2002; Zhao et al. 2006). Furthermore, deficiency in MKP-1 results in a significantly higher incidence of mortality during endotoxemia, suggesting a protective role of MKP-1 in sepsis (Hammer et al. 2006; Salojin et al. 2006; Zhao et al. 2006). MKP-1 is also expressed in cardiomyocytes and is involved in cardiomyocyte apoptosis and cardiac hypertrophy (Fischer et al. 1998; Kaiser et al. 2004; Palm-Leis et al. 2004). However, the role of MKP-1 in myocardial TNF-α expression and cardiac dysfunction in endotoxemia remains unknown.

MKP-1 has intrinsic phosphatase activity and is inducible in response to extracellular stimuli. The mechanisms responsible for regulating MKP-1 expression are cell type specific (Bokemeyer et al. 1996; Brondello et al. 1997; Li et al. 1999; Sanchez-Tillo et al. 2007). For example, ERK1/2 (Brondello et al. 1997) and JNK (Bokemeyer et al. 1996) are responsible for MKP-1 induction in fibroblasts while ERK, JNK1, and p38 are required for MKP-1 expression in macrophages (Ananieva et al. 2008; Chen et al. 2002; Kim et al. 2008; Sanchez-Tillo et al. 2007). Rac is necessary for cyclic strain stress-induced MKP-1 expression in smooth muscle cells (Li et al. 1999). We have demonstrated that Rac1 is a critical regulator of TNF-α expression and cardiac dysfunction in endotoxemia (Zhang et al. 2011 Chapter 2). Interestingly, p21-activated kinase (PAK), a serine-threonine protein kinase, acts as a downstream effector of Rac

(Molli et al. 2009). Furthermore, activation of the Rac1/PAK pathway increases pro-inflammatory factor expression in macrophages through JNK and p38 (Hsu et al. 2001). PAK1 is the main PAK isoform in cardiomyocytes (Sheehan et al. 2007). The role of PAK1 in TNF-α expression in cardiomyocytes has not been elucidated.

In the present study, we hypothesized that MKP-1 is induced during endotoxemia via the Rac1/PAK1/JNK pathway in cardiomyocytes, leading to inhibition of TNF-α expression and improvement of cardiac function in endotoxemia. To test this hypothesis, cultured cardiomyocytes and MKP-$1^{-/-}$ mice were employed. Our results demonstrated for the first time that MKP-1 represents an important negative feedback mechanism in limiting pro-inflammatory response in the heart during endotoxemia.

4.2 Materials and methods

4.2.1 Animals and preparation of neonatal mouse cardiomyocytes

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication #85-23, revised 1996) and the experimental protocols were approved by the Animal Use Subcommittee at the University of Western Ontario. C57BL/6 wild-type (WT), Rac1 floxed ($Rac1^{f/f}$) and $JNK1^{-/-}$ mice and were purchased from the Jackson Laboratory (Bar Harbor, Maine). Cre transgenic mice ($Cre^{TG/+}$), which over-express Cre recombinase under the control of α-myosin heavy-chain (MHC) promoter, were provided by Dr. E. Dale Abel (University of Utah, UT). The generation of cardiomyocyte-specific Rac1 knockout mice ($Rac1^{CKO}$) was achieved by breeding $Rac1^{f/f}$ mice with $Cre^{TG/+}$ mice as previously described (Rui et al. 2005). MKP-$1^{-/-}$ mice were kindly provided by Bristol-Myers Squibb Pharmaceutical

Research (Dorfman et al. 1996).

4.2.2 Isolation and culturing of neonatal mouse cardiomyocytes

The neonatal cardiomyocytes were prepared and cultured according to methods we have previously described (Song et al. 2000; Chapter 2). Cells were treated with LPS (Sigma, Oakville, Ontario, Canada), p21-activated kinases inhibitor III (IPA-3, EMD Biosciences, San Diego, CA) and SP600125 (Enzo Life Sciences, Plymouth Meeting, PA), infected with adenoviruses or transfected with small interfering RNAs (siRNAs).

4.2.3 Adenoviral infection of neonatal cardiomyocytes

Cardiomyocytes were infected with adenoviruses carrying a dominant-negative form of Rac1 (Ad-Rac1N17, Vector Biolabs, Philadelphia, PA), or LacZ (Ad-LacZ, Vector Biolabs, Philadelphia, PA) at a multiplicity of infection (MOI) of 10 plaque forming units/cell. Adenovirus-mediated gene transfer was applied as previously described (Peng et al. 2003; Chapter 2). All experiments were performed after 48 hours of adenoviral infection.

4.2.4 siRNA transfection of neonatal cardiomyocytes

Cardiomyocytes were treated with murine PAK1 siRNA to knock down PAK1 expression (Santa Cruz Biotechnology Inc., Santa Cruz, CA). A scrambled siRNA (Santa Cruz Biotechnology Inc.) was used as a control. The transfection was performed with transfection reagent (Santa Cruz Biotechnology Inc.) according to the manufacturer's instructions. After transfection, cells were maintained in normal culture medium for additional 48 hours before LPS treatment.

4.2.5 Measurement of TNF-α and MKP-1 mRNA

Total RNA was extracted from cardiomyocytes using the Trizol Reagent (Gibco-

BRL) as per manufacturer's instructions. TNF-α mRNA levels were determined by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) as as previously described (Peng et al. 2003; Chapter 2). 28S rRNA was used as an internal control. The primers were: TNF-α upstream 5′-CCG ATG GGT TGT ACC TTG TC-3′ and downstream 5′-GGG CTG GGT AGA GAA TGG AT-3′; MKP-1 upstream 5′-GGA GAT CCT GTC CTT CCT GTA-3′ and downstream 5′-CTG ATG TCT GCC TTG TGG TTG-3′; 28S rRNA upstream 5'-TTG AAA ATC CGG GGG AGA G-3' and downstream 5'-ACA TTG TTC CAA CAT GCC AG-3'. Samples were amplied for 34 cycles using MJ Research Opticon Real-time PCR machine. The levels of TNF-α and MKP-1 were compared to that of 28S rRNA, and the relative expression of these genes was obtained.

4.2.6 Measurement of TNF-α protein

TNF-α protein levels were measured using a mouse TNF-α ELISA kit (eBioscience, USA), according to the manufacturer's instructions.

4.2.7 Western blot analysis

Ten to twenty micrograms of protein lysates were subjected to separation on a 10% SDS-PAGE gel, followed by electrotransfer to nitrocellulose membranes. Blots were probed with specific antibodies against ERK1/2 (1:500), phospho-ERK1/2 (1:500), p38 (1:500), phospho-p38 (1:500), JNK1/2 (1:500), phospho-JNK1/2 (1:500), PAK1 (1:1000) and phospho-PAK1 (1:500, Cell Signaling Technology, Danvers, MA), MKP-1 (1:1000, Upstate, Lake Placid, NY), α-actinin (1:2000) and GAPDH (1:2000, Santa Cruz Biotechnology Inc.) followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:3000, Santa Cruz Biotechnology, CA) respectively. Signals were detected by chemiluminescence and quantified by densitometry.

4.2.8 Isolated mouse heart preparations

Adult WT and MKP-1$^{-/-}$ mice (male, 10 weeks old) were treated with LPS (10 mg/kg, i.p.) or saline. After 4 hours, hearts were isolated and perfused in a Langendorff system with Kreb's-Henseleit buffer at 3 ml/min constant flow. The Kreb's-Henseleit buffer was kept at 37 °C and consistently bubbled with a mixture of 95% O_2 and 5% CO_2. Myocardial function was detected as previously described (Zhang et al. 2011 Chapter 2).

4.2.9 Statistical analysis

Results are presented as mean ± SEM from at least three independent experiments. Differences between two groups were analyzed by a standard Student t-test. For multigroup comparisons, one or two-way ANOVA followed by Student-Newman-Keuls or Bonferroni post-test was performed. $P < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 LPS increases myocardial MKP-1 expression

To examine whether LPS regulates cardiac MKP-1 expression in cardiomyocytes, we first measured MKP-1 protein levels in neonatal cardiomyocytes and found that MKP1 protein was increased after 1 hour of LPS stimulation (Figure 4.1A). To verify these *in vitro* results, WT mice were treated with LPS or saline for 1 and 1.5 hours. Hearts were then harvested and myocardial MKP-1 mRNA and protein levels were detected. Myocardial MKP-1 mRNA and protein levels were markedly increased after LPS stimulation (Figure 4.1B & 4.1C). These data show that LPS promotes MKP-1

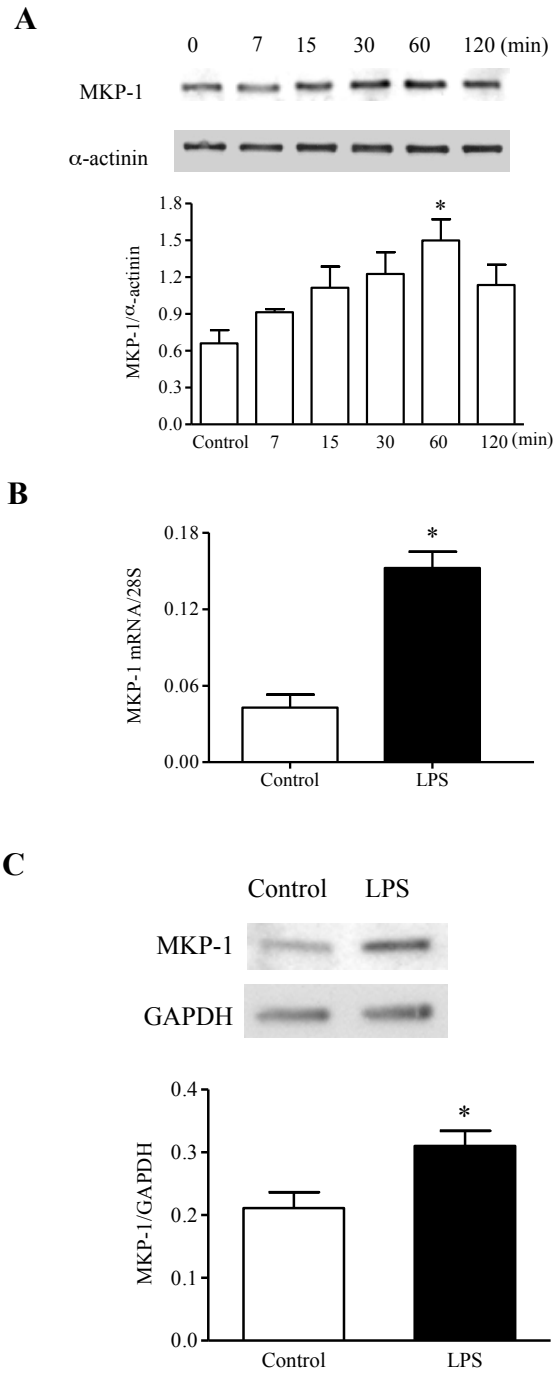


Figure 4.1. LPS induces MKP-1 expression in neonatal cardiomyocytes and in the adult myocardium. (A) WT cardiomyocytes were cultured in serum free medium for 24 hours and then treated with vehicle or LPS (1 μg/ml) for 7, 15, 30, 60 and 120 minutes. MKP-1 protein was detected by western blotting. Adult male WT mice were treated with LPS (4 mg/kg, i.p.) for 1 or 1.5 hours. MKP-1 mRNA (B) and protein (C) levels in the left ventricular myocardium were measured. Data are means ± SEM from 3 - 4 mice or independent experiments. **P*<0.05 vs. control.

expression in cardiomyocytes *in vitro* and in myocardium *in vivo*.

4.3.2 MKP-1 inhibits LPS-induced myocardial ERK1/2 and p38 activation

MKP-1 inactivates MAPKs by dephosphorylating phosphothreonine and phosphotyrosine residues. To determine the role of MKP-1 in cardiac MAPK inactivation after LPS stimulation, we examined myocardial ERK1/2 and p38 phosphorylation in WT and MKP-$1^{-/-}$ mice. In response to LPS, ERK1/2 and p38 phosphorylation was induced in both WT and MKP-$1^{-/-}$ myocardium. Phosphorylation of ERK1/2 was diminished in the WT myocardium between 2 and 3 hours after LPS stimulation, but it was sustained in the MKP-$1^{-/-}$ myocardium. Similarly, p38 phosphorylation was increased in the WT myocardium between 1.5 and 3 hours after LPS stimulation. Importantly, p38 phosphorylation was significantly higher in MKP-$1^{-/-}$ than the WT myocardium (Figure 4.2). Thus, MKP-1 deficiency enhanced ERK1/2 and p38 phosphorylation, demonstrating an important role for MKP-1 in reducing the levels of myocardial ERK1/2 and p38 activation after LPS stimulation.

4.3.3 MKP-1 attenuates myocardial TNF-α expression in endotoxemia

To investigate the role of MKP-1 in myocardial TNF-α expression and heart function during endotoxemia *in vivo*, WT and MKP-$1^{-/-}$ mice were treated with vehicle or LPS. Our data showed that LPS increased myocardial TNF-α expression in both WT and MKP-$1^{-/-}$ mice. Compared with the WT group, MKP-$1^{-/-}$ mice exhibited significantly higher TNF-α mRNAand protein levels (Figure 4.3). These results suggest that MKP-1 limits myocardial TNF-α expression in response to LPS.

4.3.4 MKP-1 improves cardiac function during endotoxemia

Cardiac function was determined using the Langendorff preparation after 4 hours

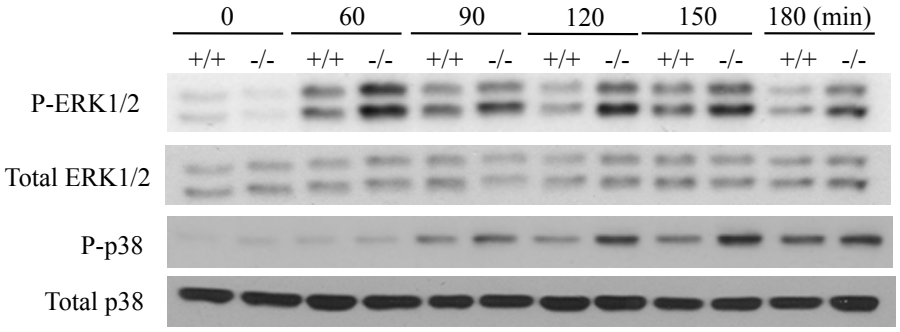


Figure 4.2. MKP-1 inhibits LPS-induced myocardial ERK1/2 and p38 activation. Adult male WT and MKP1$^{-/-}$ mice were treated with LPS (4 mg/kg, i.p.) for 60, 90, 120, 150 and 180 minutes. ERK1/2 and p38 phosphorylation in myocardium were detected.

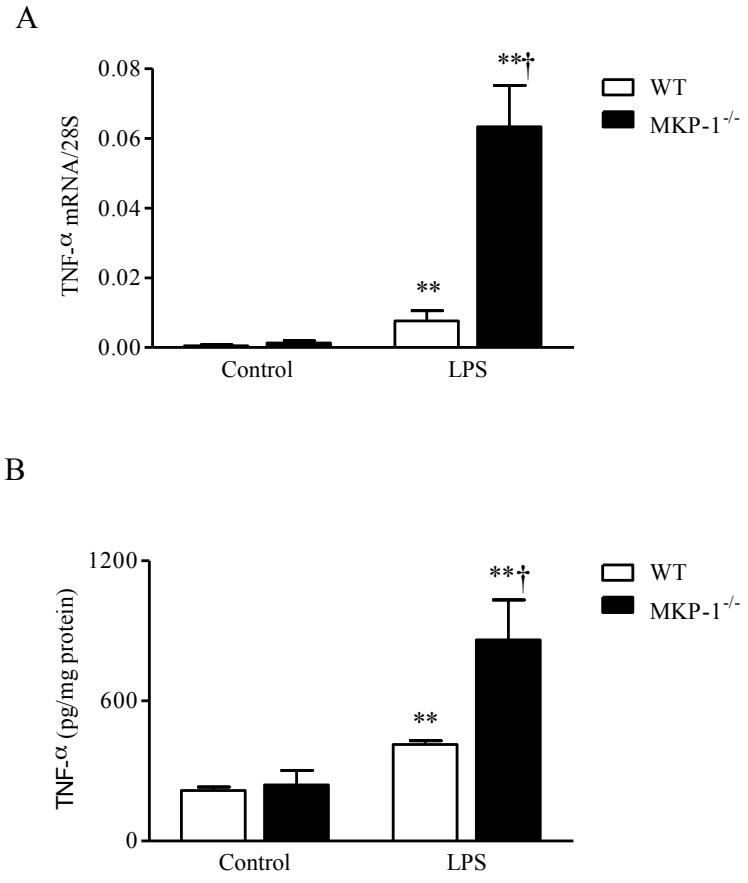


Figure 4.3. TNF-α expression in WT and MKP-1$^{-/-}$ myocardium during endotoxemia. TNF-α mRNA (A) and protein (B) levels in WT and MKP-1$^{-/-}$ heart tissues were measured after 1 and 2 hours of LPS treatment (4 mg/kg, i.p.). Data are means ± SEM, n=3 to 5 per group. ** P <0.01 vs. control, † P <0.05 vs. WT+LPS.

of LPS *in vivo* treatment. The rates of contraction ($+dF/dt_{max}$) and relaxation ($-dF/dt_{min}$), and heart work in LPS-treated hearts were reduced in both WT and MKP-$1^{-/-}$ mice relative to vehicle-treated mice. Furthermore, compared with WT mice, rate of contraction and relaxation and heart work were significantly lower in MKP-$1^{-/-}$ mice (Figure 4.4), indicating that MKP-1 expression improves cardiac function during endotoxemia.

4.3.5 LPS activates PAK1 in cardiomyocytes

Rac contributes to the induction of MKP-1 expression in smooth muscel cells (Li et al. 1999). PAK is a downstream effector of Rac (Molli et al. 2009). Activation of PAK promotes pro-inflammatory cytokine expression (Hsu et al. 2001). To assess the role of PAK1 in cardiac MKP-1 and TNF-α expression during endotoxemia, we first examined PAK1 activity after LPS stimulation. Neonatal cardiomyocytes isolated from WT mice were treated with LPS (1 μg/ml) for 7, 15, 30 and 60 min. PAK1 phosphorylation in these cells peaked at 15 min and then returned to control levels at 60 min (Figure 4.5A), indicating that LPS activates PAK1 in cardiomyocytes.

4.3.6 PAK1 increases LPS-induced TNF-α expression

To elucidate the role of PAK1 in LPS-induced TNF-α expression, neonatal cardiomyocytes were treated with IPA-3, an inhibitor of PAKs. IPA-3 (1–10 μM) decreased LPS-induced TNF-α mRNA expression and TNF-α protein levels in a dose-dependent manner (Figure 4.5B & 4.5C). These results were further verified by using PAK1 siRNA. As shown in Figure 4.5D, PAK1 mRNA expression was decreased by 56% after PAK1 siRNA transfection. The inhibition of PAK1 expression was associated

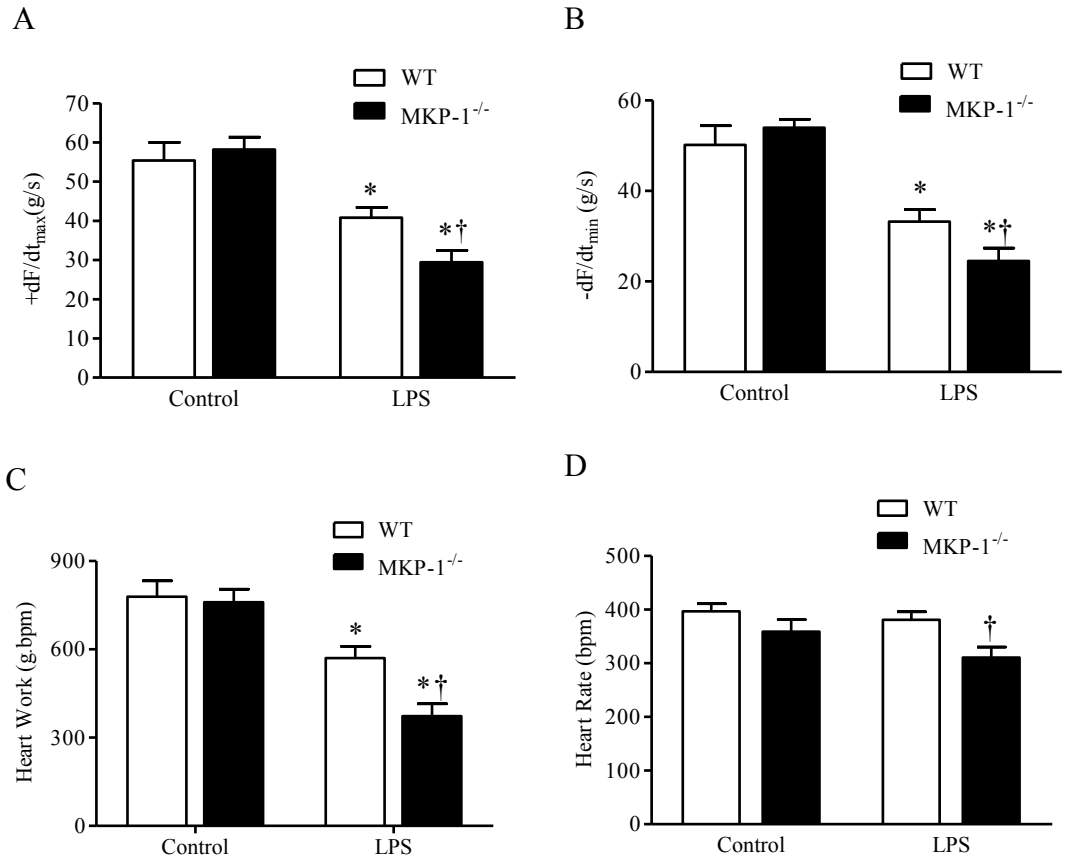


Figure 4.4. Cardiac function in WT and MKP-1$^{-/-}$ mice after 4 hours of LPS treatment (10 mg/kg, i.p.). Mouse hearts were isolated and perfused using the Langendorff system. Contractile function of heart was determined. Changes in contraction (+d*F*/dt$_{max}$, A), relaxation (-d*F*/dt$_{min}$, B) heart work (C) and heart rate (D) are presented. Data are means ± SEM, n=5 to 7 per group. * *P* <0.05 vs. control, † *P* <0.05 vs. WT+LPS.

with decreased TNF-α mRNA and protein levels (Figure 4.5E & 4.5F). These data indicate that activation of PAK1 promotes cardiac TNF-α expression in response to LPS.

4.3.7 PI3K/Rac1 pathway mediates LPS-induced PAK1 activation

It is well known that PAK1 is activated by certain small GTPases such as Rac (Molli et al. 2009). We have demonstrated that the PI3K/Rac1 pathway is activated by LPS and critical for cardiac TNF-α expression during endotoxemia (Zhang et al. 2011 Chapter 2). To determine if PI3K acts as an upstream regulator of PAK1, the PI3K inhibitor, LY294002, was employed. LY294002 significantly decreased PAK1 phosphorylation induced by LPS (Figure 4.6A). To determine the role of Rac1 on PAK1 activity, neonatal cardiomyocytes were infected with an adenovirus encoding a dominant negative form of Rac1 gene (Ad-Rac1N17), which specifically inhibits Rac1 activity. Overexpression of Rac1N17 inhibited PAK1 activity in response to LPS (Figure 4.6B). Taken together, these data imply that LPS activates PAK1 via PI3K/Rac1 in cardiomyocytes.

4.3.8 PAK1 mediates LPS-induced p38 and JNK phosphorylation

To determine whether PAK1 regulates p38, ERK1/2 and JNK phosphorylation, neonatal cardiomyocytes were transfected with PAK1 siRNA. Interestingly, PAK1 siRNA blocked p38 and JNK phosphorylation in response to LPS, but did not show any effect on ERK1/2 phosphorylation (Figure 4.7).

4.3.9 The Rac1/PAK1/JNK pathway mediates LPS-induced MKP-1 expression

To further explore the downstream effectors of the Rac1/PAK1/JNK pathway, MKP-1 expression was studied. Overexpression of Rac1N17 in neonatal cardiomyocytes significantly decreased MKP-1 protein levels (Figure 4.8A). This result was further

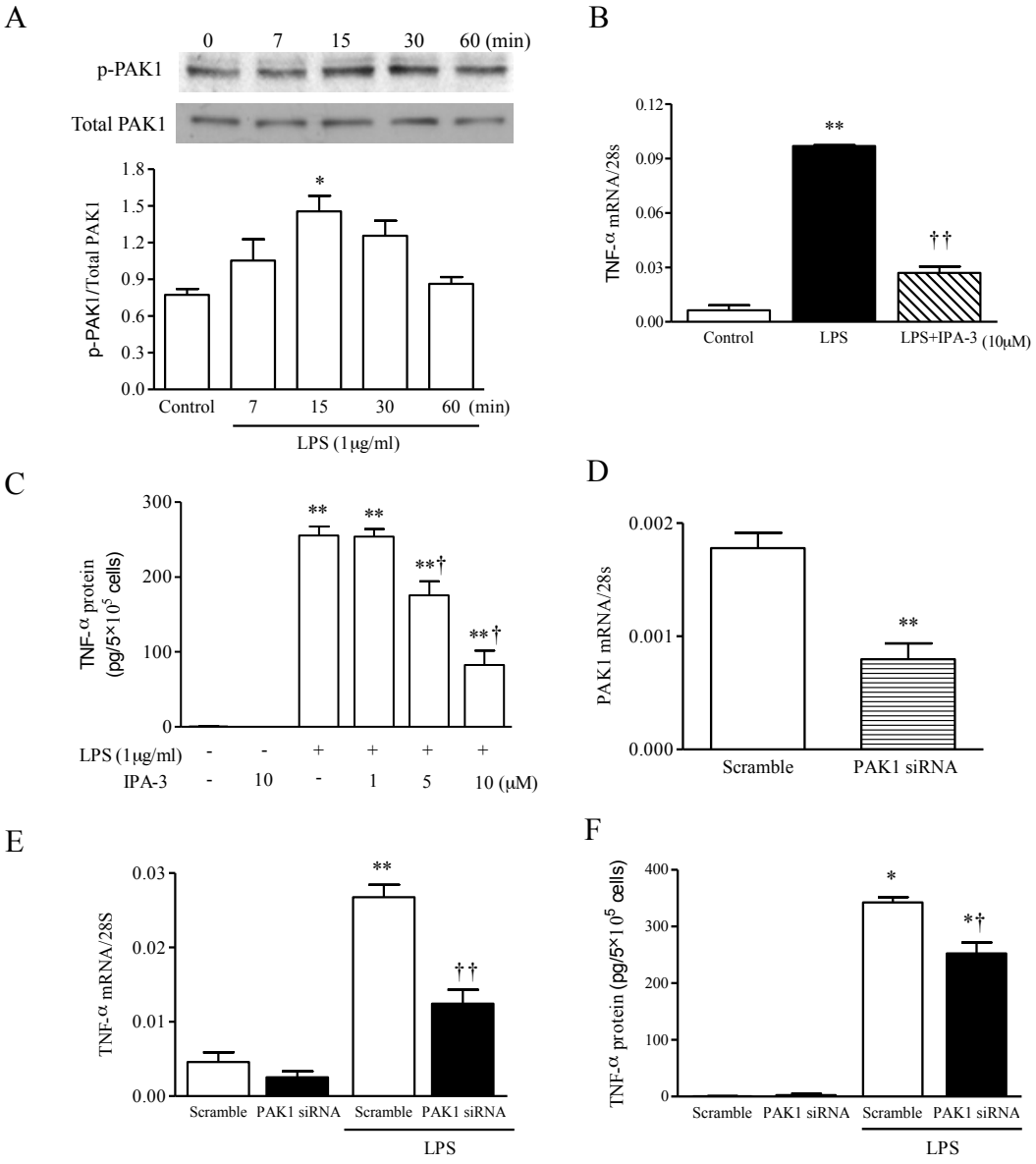


Figure 4.5. PAK1 activity promotes LPS-induced TNF-α expression in neonatal cardiomyocytes. (A) Cardiomyocytes isolated from WT mice were treated with vehicle or LPS (1 μg/ml) for 7, 15, 30 and 60 minutes. PAK1 phosphorylation was measured by western blotting. Cells were treated with IPA-3 with or without LPS (1μg/ml) for 3 or 5 hours. TNF-α mRNA (B) and TNF-α protein in culture medium (C) were measured by real-time RT-PCR and ELISA, respectively. (D) WT cardiomyocytes were transfected with scrambled or PAK1 siRNA. PAK1 mRNA expression in these cells were detected. Cells, transfected with scrambled and PAK1 siRNA, were treated with LPS (1μg/ml) for 3 or 5 hours. TNF-α mRNA (E) and protein levels (F) were measured. Data are means ± SEM from 3 - 4 independent experiments. **P*<0.05, ***P*<0.01 vs. control; †*P*<0.05, †† *P*<0.01 vs. LPS and Scramble+LPS.

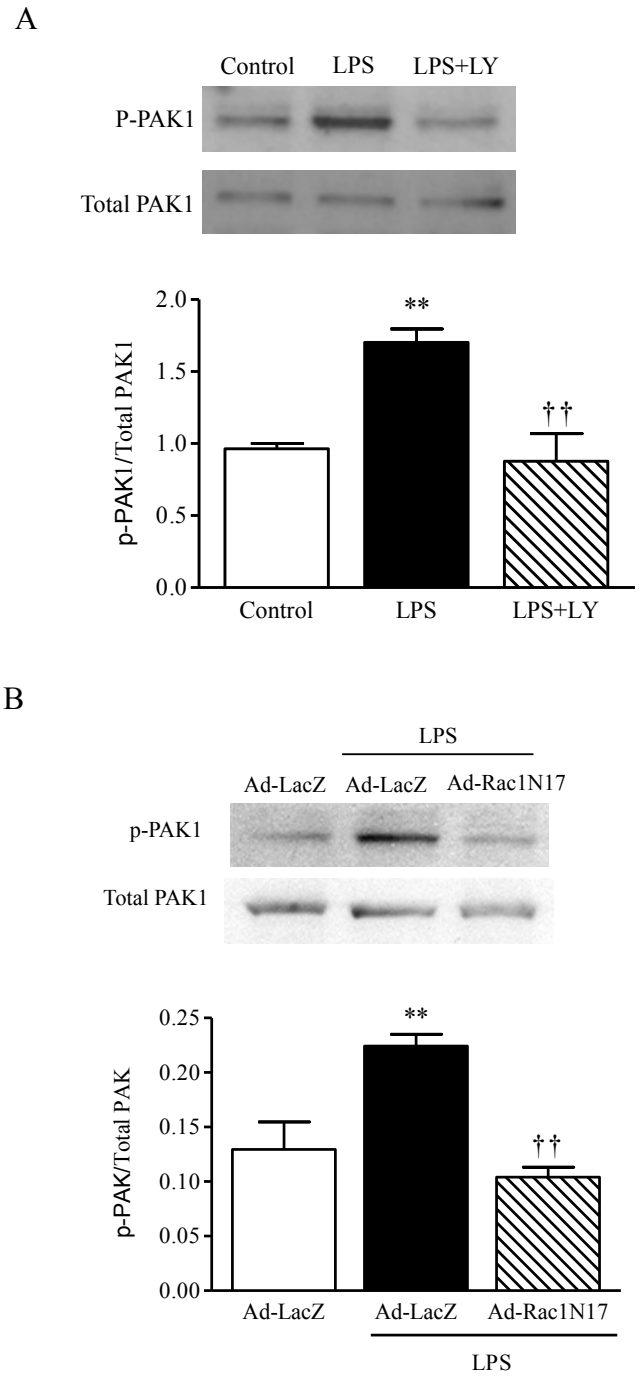


Figure 4.6. PI3K and Rac1 activity increases LPS-induced PAK1 activation. (A) WT neonatal cardiomyocytes were incubated with LPS (1 μg/ml, 15 min) with or without LY294002 (LY). (B) Cells were infected with Ad-Rac1N17 followed by LPS treatment (1 μg/ml) for 15 minutes. PAK1 phosphorylation in these cells was detected by western blotting. Data are mean ± SEM from 3 independent experiments. ***P* <0.01 vs. control; ††*P* <0.01 vs. LPS or Ad-LacZ+LPS.

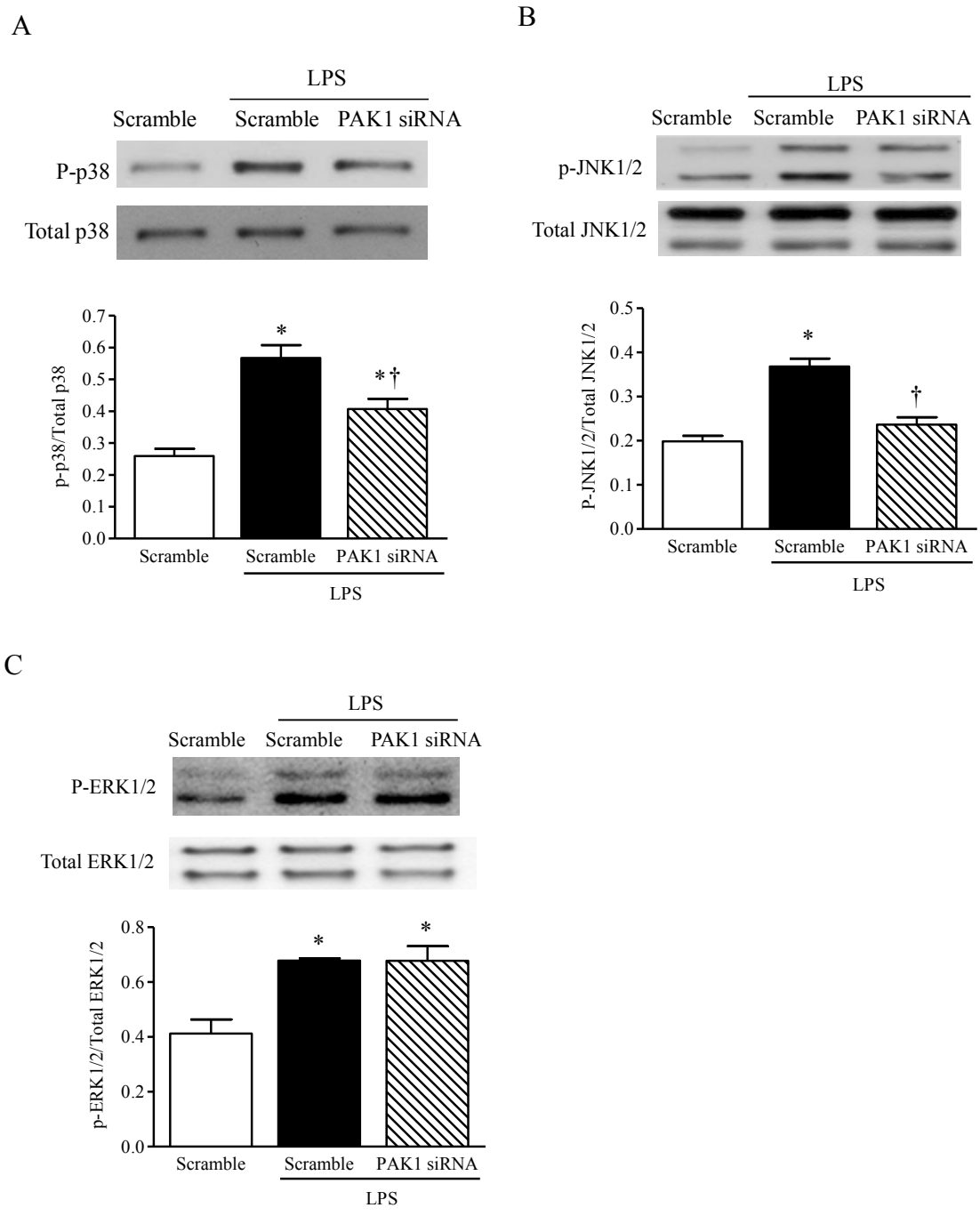
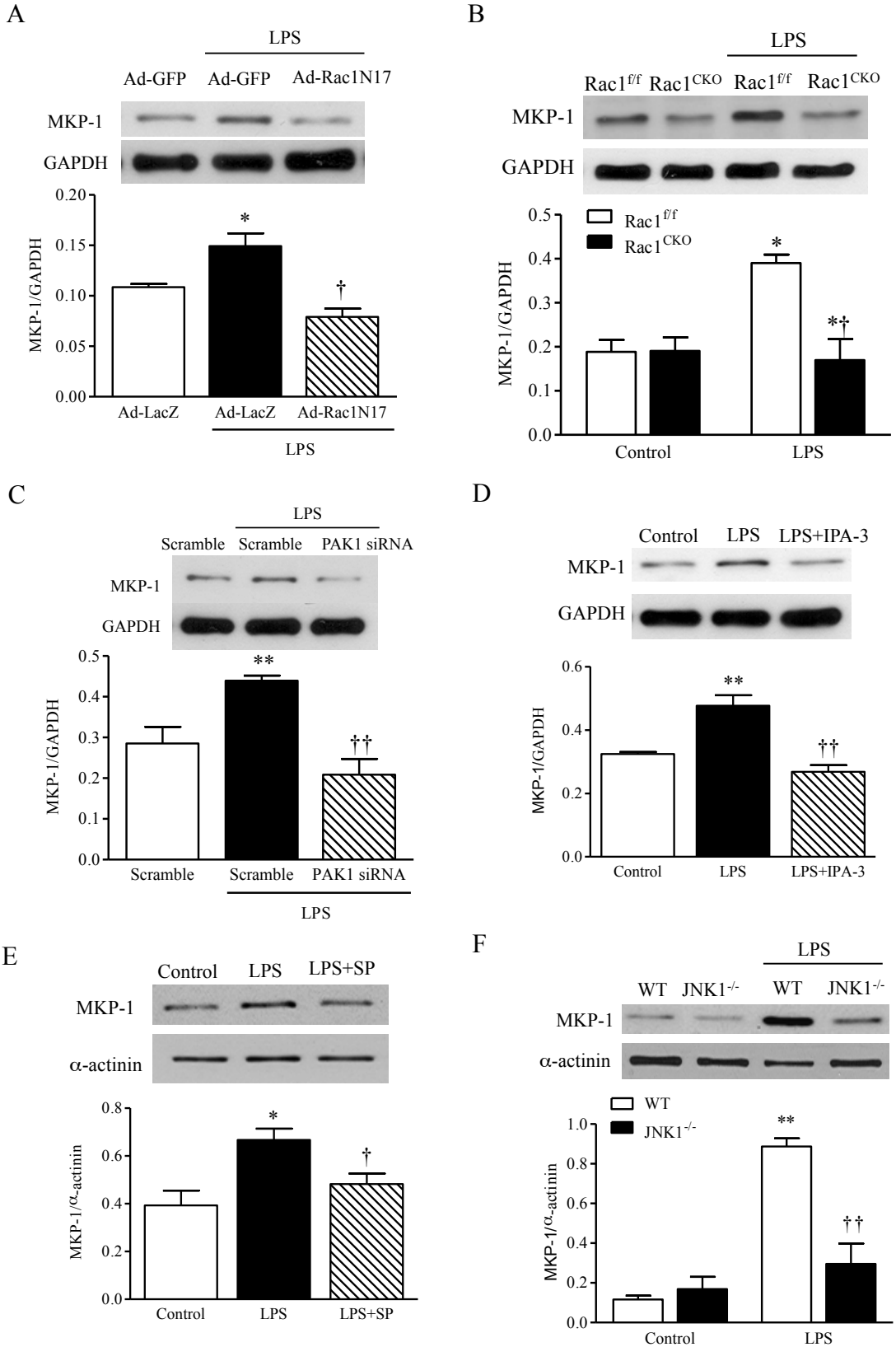


Figure 4.7. Effect of PAK1 on LPS-induced p38, JNK1/2 and ERK1/2 activation in neonatal cardiomyocytes. WT cardiomyocytes, transfected with scrambled and PAK1 siRNA, were treated with LPS (1 μg/ml) for 1 hour. P38 (A), JNK1/2 (B) and ERK1/2 (C) phosphorylation and were detected by western blotting. Data are mean ± SEM from 3 independent experiments. **P* <0.05 vs. control; †*P* <0.05 vs. Scramble+LPS.


A
LPS
Ad-GFP
Ad-GFP
Ad-Rac1N17
MKP-1
GAPDH
MKP-1/GAPDH
0.20
0.15
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0.00
*
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Ad-LacZ
Ad-LacZ
Ad-Rac1N17
LPS
B
LPS
Rac1f/f
Rac1CKO
Rac1f/f
Rac1CKO
MKP-1
GAPDH
MKP-1/GAPDH
0.5
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MKP-1
α-actinin
MKP-1/α-actinin
WT
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Control
LPS

Figure 4.8. Effect of Rac1, PAK1 and JNK on LPS-induced MKP-1 expression. (A) WT neonatal cardiomyocytes were infected with Ad-Rac1N17 followed by LPS treatment (1 μg/ml) for 1 hour. MKP-1 protein levels were detected by western blotting. (B) Adult male $Rac1^{f/f}$ and $Rac1^{CKO}$ mice were treated with LPS (4 mg/kg, i.p. injection) for 1.5 hours. MKP-1 protein levels in the left ventricular myocardium were determined. (C) WT cardiomyocytes were transfected with PAK1 siRNA followed by LPS treatment for 1 hour. Cells were incubated with LPS for 1 hour with or without IPA-3 (10μM, D) and SP600125 (SP, 10μM, E). MKP-1 protein levels in these cells were detected. (F) Adult male WT and $JNK1^{-/-}$ mice were treated with LPS (4 mg/kg, i.p. injection) for 1.5 hours. MKP-1 protein levels in the left ventricular myocardium were determined. Data are mean ± SEM from 3 - 4 independent experiments. **P* <0.05, ***P* <0.01 vs. control; †*P* <0.05, ††*P* <0.01 vs. LPS, Ad-LaZ +LPS, Scramble+LPS.

confirmed *in vivo*. As shown in Figure 4.8B, LPS enhanced MKP-1 protein levels in $Rac1^{f/f}$ but not in the $Rac1^{CKO}$ myocardium. To examine whether PAK1 mediates MKP-1 expression, cardiomyocytes were treated with IPA-3 or transfected with PAK1 siRNA. LPS-induced MKP-1 protein expression was blocked by both IPA-3 and PAK1 siRNA (Figure 4.8C & 4.8D). To determine if JNK is involved in regulating MKP-1 expression, cardiomyocytes were treated with SP600125, an inhibitor of JNK. LPS-induced MKP-1 protein expression was significantly reduced by SP600125 (Figure 4.8E). To verify this result *in vivo*, WT and $JNK1^{-/-}$ mice were treated with LPS (4 mg/kg, i.p.) or saline for 1.5 hours. LPS significantly increased myocardial MKP-1 protein levels in WT mice but had no apparent effect in $JNK1^{-/-}$ mice ($P<0.05$, Figure 4.8F). Taken together, these data indicate that the Rac1/PAK1/JNK pathway mediates LPS-induced MKP-1 expression.

4.4 Discussion

The present study would appear to demonstrate for the first time that myocardial MKP-1 is induced by LPS via the PI3K/Rac1/PAK1/JNK pathway. Enhanced MKP-1 expression attenuates ERK1/2 and p38 phosphorylation, leading to inhibition of myocardial TNF-α expression and improvement of cardiac function during endotoxemia.

MKP-1 is constitutively active, thus its phosphatase actvity depends on its protein expression but does not rely on post-translational modifications such as phosphorylation (Liu et al. 2007). MKP-1 dephosphorylates MAPKs and impedes their cellular functions. Recent studies have shown that MKP-1 expression is up-regulated in macrophages in response to LPS (Chen et al. 2002). Deficiency of MKP-1 enhances p38 and JNK phosphorylation and pro-inflammatory cytokine production including TNF-α, IL-1β and

IL-6 in macrophages (Chen et al. 2002; Zhao et al. 2006). Consistent with this notion, MKP-1$^{-/-}$ mice exhibit significantly higher serum cytokine concentrations and higher mortality rate after LPS stimulation (Hammer et al. 2006; Salojin et al. 2006; Zhao et al. 2006). In the present study, we showed that LPS induced myocardial MKP-1 expression. Furthermore, MKP-1 deficiency enhanced myocardial ERK1/2 and p38 phosphorylation and increased cardiac TNF-α expression. Most importantly, MKP-1 deficiency worsened cardiac function during endotoxemia. Thus, both *in vitro* and *in vivo* evidence demonstrated that LPS induces MKP-1 expression, which inhibits ERK1/2 and p38 activation and myocardial TNF-α expression, and improves cardiac function during endotoxemia.

Rac is one of the factors that are responsible for MKP-1 expression (Li et al. 1999). We recently demonstrated that PI3K-mediated Rac1 activation promotes TNF-α expression and cardiac dysfunction in endotoxemia (Zhang et al. 2011 Chapter 2). PAK proteins are downstream effectors of Rac and regulate many cellular events including cell motility, survival, proliferation and gene expression (Molli et al. 2009). PAK1 is the main isoform of this enzyme in the myocardium and plays an important role in cardiac contractility and hypertrophy (Sheehan et al. 2009; Sussman et al. 2000). Studies have shown that PAK protein is activated and contributes to IL-1 expression through MAPKs upon ligand stimulation in macrophages (Hsu et al. 2001). In the present study, LPS enhanced PAK1 activity in cardiomyocytes. Inhibition of PAK1 activity by the pharmacological inhibitor, IPA-3, or PAK1 expression by PAK1 siRNA decreased LPS-induced TNF-α expression in cardiomyocytes. Furthermore, inhibition of PI3K and Rac1 decreased PAK1 activity in response to LPS in cardiomyocytes. These results indicate

that PI3K/Rac1-mediated PAK1 activation plays an essential role in LPS-induced TNF-α expression in cardiomyocytes.

ERK1/2, p38 and JNK MAPKs are important signaling molecules regulating TNF-α expression in cardiomyocytes (Peng et al. 2005b; Peng et al. 2003; Peng et al. 2009; Rosengart et al. 2000; Thakur et al. 2006). ERK1/2 and p38 are positive regulators of TNF-α expression (Peng et al. 2005b; Peng et al. 2003; Rosengart et al. 2000; Thakur et al. 2006). In contrast, JNK1 inhibits TNF-α expression via inactivating LPS-induced ERK1/2 and p38 MAPKs (Peng et al. 2009). In the present study, inhibition of PAK1 activity blocked LPS-induced p38 and JNK phosphorylation. These results suggest that PAK1 may have both stimulatory and inhibitory effects on LPS-induced TNF-α expression in cardiomyocytes. Its activation on p38 promotes TNF-α expression. On the other hand, PAK1-mediated JNK activation inhibits LPS-induced TNF-α expression.

To address the molecular mechanisms by which JNK inhibits LPS-induced TNF-α expression, we studied the role of MKP-1 in this process. Studies have shown that MAPKs are involved in regulating MKP-1 expression. For example, ERK1/2 and JNK activation are responsible for MKP-1 induction in fibroblasts (Bokemeyer et al. 1996; Brondello et al. 1997). However, the effects of ERK1/2, p38 and JNK on MKP-1 expression in macrophages are controversial. Several studies have showed that ERK1/2 and p38 are required for MKP-1 expression in macrophages (Ananieva et al. 2008; Chen et al. 2002; Kim et al. 2008). On the other hand, Sanchez-Tillo et al. reported that JNK1 is necessary for MKP-1 expression in macrophages which consequently decreases ERK1/2 and p38 phosphorylation levels (Sanchez-Tillo et al. 2007). In the present study we demonstrated that LPS-induced MKP-1 production was blocked by the inhibition of

Rac1, PAK1 and JNK1 in cardiomyocytes, suggesting that the Rac1/PAK1/JNK1 pathway is required for LPS-induced MKP-1 expression in cardiomyocytes. MKP-1 mediates the inhibitory effect of JNK1 on ERK1/2 and p38 activity. Therefore, Rac1/PAK1/JNK1-mediated MKP-1 expression provides a key negative feedback mechanism to limit myocardial TNF-α production and improve cardiac function during endotoxemia.

In conclusion, the current study showed that PAK1 is activated by the PI3K/Rac1 pathway and increases p38 and JNK activity. PAK1 increases TNF-α expression via p38 activation in response to LPS. On the other hand, Rac1/PAK1/JNK1 pathway enhances MKP-1 expression, which inactivates ERK1/2 and p38, limits TNF-α expression and improves cardiac function during endotoxemia (Figure 4.9). Thus, the Rac1/PAK1/JNK1/MKP-1 signaling pathway represents a novel negative feedback mechanism in regulating TNF-α expression and cardiac function in endotoxemia, and may have therapeutic implications in the treatment of sepsis.

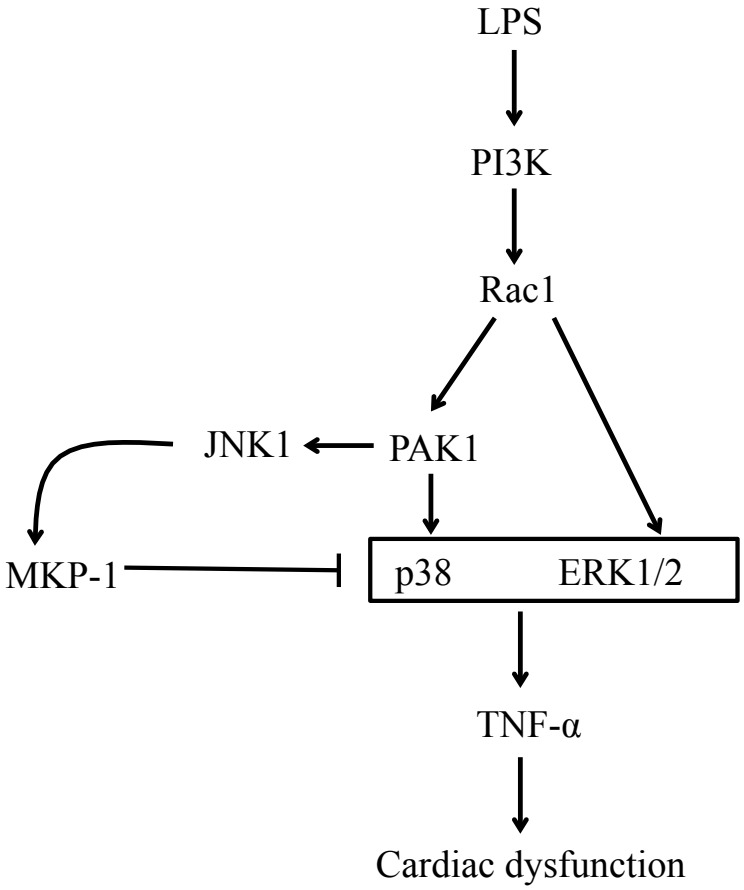

Figure 4.9. Schematic of the MKP-1 signaling pathway regulating TNF-α expression and cardiac function during LPS stimulation. PAK1 is activated by PI3K/Rac1 pathway and increases P38 and JNK activity. PAK1 increases TNF-α expression via p38 activation in response to LPS. On the contrary, Rac1/PAK1/JNK pathway enhances MKP-1 expression, which inactivates ERK1/2 and p38, limits TNF-α expression and improves cardiac function during endotoxemia.

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Chapter 5 Discussion

5.1 Summary and major findings

The overall goal of this thesis was to investigate the role of Rac1 in myocardial TNF-α expression and dysfunction during sepsis. Specifically, the activation of Rac1 and its signaling in the regulation of myocardial TNF-α mRNA expression and protein synthesis in endotoxemia were examined (see Figure 5.1).

In **Chapter 2**, the mechanisms of Rac1 activation in the heart and the role of Rac1 in cardiac dysfunction during endotoxemia were studied. The results showed that LPS activates PI3K in cardiomyocytes both *in vitro* and *in vivo*. Furthermore, activated PI3K is responsible for Rac1 activation. Rac1 activity is required for induction of myocardial TNF-α expression by LPS. This effect of Rac1 on TNF-α expression is mediated by NADPH oxidase and ERK1/2. Finally, using cardiac-specific Rac1 knockout mice, I showed that Rac1 activation leads to myocardial depression during endotoxemia (Figure 5.1).

In **Chapter 3**, I discovered that Rac1 promotes cardiac TNF-α protein synthesis via the Na/K-ATPase/mTOR pathway in endotoxemia. Previous studies have shown that Na/K-ATPase activity is inhibited in macrophages (Ohmori et al. 1991), lung (Koksel et al. 2006) and kidney (Guzman et al. 1995) by LPS. I showed for the first time that Na/K-ATPase activity in the myocardium is decreased during endotoxemia. The suppression of myocardial Na/K-ATPase activity is mediated through PI3K/Rac1/NADPH oxidase pathway. This finding is consistent with a previous recent study showing that NADPH oxidase decreases Na/K-ATPase activity in cardiomyocytes by glutathionylating its β subunit (Figtree et al. 2009). Inhibition of Na/K-ATPase has been shown to potentiate

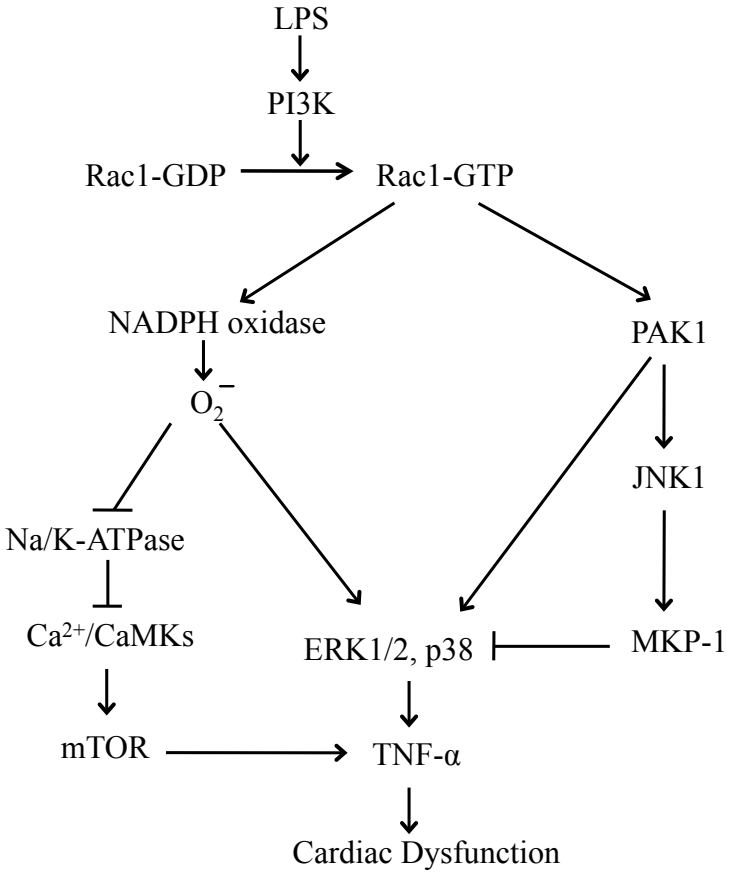

Figure 5.1. Schematic Rac1 signaling pathway regulating TNF-α expression and cardiac function in endotoxemia. LPS activates Rac1 through PI3K. Activation of Rac1 increases NADPH oxidase and ERK1/2 activity, leading to increased myocardial TNF-α mRNA expression. Moreover, Rac1-containing NADPH oxidase inhibits Na/K-ATPase and activates Ca^{2+}/CaMK-dependent mTOR, causing enhanced TNF-α protein synthesis. Another effector of Rac1 is PAK1. PAK1 increases TNF-α expression via p38 MAPK. On the other hand, PAK1 promotes JNK1-dependent MKP-1 expression, which inactivates ERK1/2 and p38 MAPK, reduces TNF-α expression and improves heart function.

LPS-induced cytokine expression in macrophages (Ohmori et al. 1991). A novel finding from my study is that, in cardiomyocytes, reduction of Na/K-ATPase activity leads to enhanced TNF-α protein levels in response to LPS without any measurable effect on TNF-α mRNA expression or stability.

It has been demonstrated that inhibition of Na/K-ATPase activity increases intracellular Ca^{2+} concentrations via the Na/Ca exchanger (Bers et al. 2006). Since Rac1 is a negative regulator of Na/K-ATPase, I measured Ca^{2+} transients in $Rac1^{f/f}$ and $Rac1^{CKO}$ ventricular cardiomyocytes and found that $Rac1^{CKO}$ cardiomyocytes exhibited significantly lower intracellular Ca^{2+} levels compared with $Rac1^{f/f}$ cells in basal conditions and in response to LPS. It appears that Rac1-mediated Na/K-ATPase inhibition increases intracellular Ca^{2+} concentrations during endotoxemia. mTOR is a Ca^{2+} sensitive regulator of protein expression which does so by controlling mRNA expression, translation initiation and elongation (Hay & Sonenberg 2004; Hoyer-Hansen et al. 2007; Lenz & Avruch 2005). Results in **Chapter 3** demonstrated that mTOR is activated by LPS in cardiomyocytes and increases TNF-α protein levels without any significant effect on TNF-α mRNA expression or stability. In addition, LPS-induced mTOR activity was blocked by inhibition of Rac1 and CaMKs but enhanced by inhibition of Na/K-ATPase. Taken together, these results provide the first evidence that inhibition of Na/K-ATPase activates Ca^{2+}/CaMK-dependent mTOR, which increases TNF-α protein production in cardiomyocytes (Figure 5.1).

In the **Chapter 4**, I identified a negative feedback mechanism, which limits myocardial TNF-α expression and improves heart function in endotoxemia. Recent studies have demonstrated that MKP-1 is an important negative regulator of the

inflammatory response of the innate immune system (Chen et al. 2002; Hammer et al. 2006; Salojin et al. 2006; Zhao et al. 2006). In **Chapter 4**, I found that LPS increased MKP-1 expression in the myocardium. Deficiency in MKP-1 prolonged myocardial ERK1/2 and p38 activities and increased cardiac TNF-α expression in response to LPS. MKP-$1^{-/-}$ mice showed a further decrease in cardiac function during endotoxemia, compared to the corresponding WT group. Taken together, these results imply that LPS induces myocardial MKP-1 expression, which inhibits TNF-α expression and improves heart function in endotoxemia.

PAK1 is a downstream effector of Rac1 (Molli et al. 2009) and increases pro-inflammatory factor expression in macrophages through MAPKs (Hsu et al. 2001). My results showed that LPS activated PAK1 through PI3K/Rac1 in cardiomyocytes (Chapter 4), consistent with previous studies (Molli et al. 2009). Activated PAK1 increased p38 MAPK activity and promoted TNF-α expression in cardiomyocytes. Rac1 is necessary for MKP-1 expression in smooth muscle cells (Li et al. 1999). I showed that Rac1/PAK1 activation increased JNK1 activity, which induced MKP-1 expression, leading to decreased TNF-α expression in cardiomyocytes during LPS stimulation. Therefore, I demonstrated for the first time that Rac1/PAK1/JNK1-mediated MKP-1 represents an important negative feedback mechanism in limiting the pro-inflammatory response in the heart during sepsis (Figure 5.1).

Therefore, results from these chapters provide insight into the molecular mechanisms of myocardial Rac1 activation as well as the pathways through which Rac1 regulates TNF-α mRNA expression and protein production in myocardium and cardiac dysfunction during endotoxemia. These studies may increase our understanding of

mechanisms that regulate myocardial TNF-α expression and cardiac function during sepsis.

5.2 Clinical implications

The heart is a major organ that produces TNF-α in sepsis (Grandel et al. 2000; Kapadia et al. 1995; Peng et al. 2003). High levels of TNF-α produced by cardiomyocytes impair cardiac function via direct suppression of cardiac contractility, the induction of myocardial apoptosis, and inflammatory response (Meldrum 1998). Therefore, understanding the underlying mechanisms of myocardial TNF-α production may lead to new therapeutic approaches. A novel finding reported in this thesis is that Rac1 is a critical regulator of myocardial TNF-α production and cardiac dysfunction in sepsis. PI3K-mediated Rac1 activation promotes LPS-induced TNF-α mRNA expression and protein production through the NADPH oxidase/ERK, PAK1/p38 and Na/K-ATPase/mTOR pathways. Cardiomyocyte specific deletion of Rac1 decreased myocardial TNF-α expression and improved cardiac function in endotoxemia. These results suggest that Rac1 may represent a novel therapeutic target for inhibiting TNF-α expression and improving myocardial function in sepsis.

In contrast to its pathogenic effects, Rac1 activation also triggers a negative feedback mechanism to limit myocardial TNF-α production via MKP-1. Myocardial TNF-α mRNA and protein levels in MKP1$^{-/-}$ mice were enhanced compared to WT mice in endotoxemia, which was associated with a further decrease in cardiac function. These results indicate that MKP-1 may protect the heart from sepsis-induced injuries. To this end, future studies may test the hypothesis that cardiomyocyte specific overexpression of

the MKP-1 gene improves cardiac function during sepsis. While cardiac specific overexpression of MKP-1 may benefit the heart during sepsis, a recent study showed that overexpression of MKP-1 in the hippocampus of nonstressed rats produces profound depressive-like responses (Duric et al. 2010). Therefore, tissue specific overexpression of MKP-1 is critical to developing MKP-1 as a treatment for sepsis in humans.

5.3 Study limitations

5.3.1 Use of mouse models to simulate human disease conditions

All of the studies presented in this thesis were done either in primary neonatal cardiomyocytes or adult cardiomyocytes *in vitro* or in an endotoxemic mouse model *in vivo*. Animal models have many advantages in studying signaling pathways. For example, animal models allow for the control of experimental conditions, collection of tissue samples and the manipulation of the genetic systems, which are difficult or not ethical to do in humans. Furthermore, mice have relatively shorter gestation (20 days) and maturation (2 months), which enable experiments to be carried out with sufficient sample size in a reasonably short period of time. In addition, approximately 99% of all mouse genes have a human homologue (Waterston et al. 2002).

This thesis utilized LPS to simulate human sepsis. LPS is an endotoxin produced by Gram-negative bacteria and is effective in inducing a sepsis-like state in animal models. But it does not represent all sepsis-associated infections. It has been demonstrated that 37.6% of the septic cases from 1979 through 2000 were caused by Gram-negative bacterial infection, while the rest resulted from Gram-positive bacterial, polymicrobial and fungi infections (Martin et al. 2003). In addition, clinical studies have

shown that 49% of the infections originated in the respiratory system while 21% were abdominal infection. Patients with abdominal infection are more likely to have septic shock and early renal failure, whereas patients with respiratory infections more commonly exhibit early alterations in neurological function (Volakli et al. 2010). To this end, intraperitoneal injection of LPS has been shown to induce septic shock, renal dysfunction and abnormalities in coagulation (Doi et al. 2009; Levy & Deutschman 2004). Therefore, the endotoxemic model induced by LPS provides an appropriate model for studying many manifestations of sepsis.

5.3.2 Use of pharmacological inhibitors to delineate signaling pathways

In this thesis, the following pharmacological inhibitors were used: LY294002, apocynin, U0126, ouabain, KN-93, rapamycin, IPA-3 and SP600125 to selectively inhibit PI3K, NADPH oxidase, ERK1/2, Na/K-ATPase, CaMKs, mTOR, PAK1 and JNK1, respectively. LY294002 inhibits PI3K activity via competitive inhibition of an ATP binding site on the p85α subunit. At low dose, it specifically abolishes PI3K activity (IC_{50} = 1.40 μM) and does not inhibit other lipid and protein kinases such as PI4K, EGFR, PKA, PKC, MAPKs, ATPase, diacylglycerol kinase or Src (Vlahos et al. 1994). Thus, LY294002 is highly selective for PI3K and represents an excellent tool for studying PI3K-dependent signaling in this thesis.

Apocynin (4'-hydroxy-3'-methoxy-acetophenone) is a cell-permeable, relatively selective inhibitor of NADPH oxidase (IC_{50} value: 10 μM in human neutrophils). Once inside the cell, apocynin reacts with ROS and peroxidase to form an apocynin radical, which prevents the translocation of two essential cytosolic proteins, $p47^{phox}$ and $p67^{phox}$ to the cell membrane and their binding with Nox2, thereby inhibiting the assembly of

NADPH oxidase (Stefanska & Pawliczak 2008). Besides its ability to inhibit NADPH-oxidase, apocynin also inhibits cytochrome P450 (IC_{50} value: 600 μM) (Pietersma et al. 1998), and thromboxane synthase (IC_{50} value: 1 μM- 0.1nM) (Engels et al. 1992). Although, in this thesis, apocynin (400 μM) significantly decreased LPS-induced O_2^- production in cardiomyocytes, the effects of apocynin on thromboxane synthase cannot be excluded.

U0126 is a cell permeable, potent inhibitor of MEK1 and MEK2 (IC_{50} of 72 nM and 58 nM, respectively), both of which activate ERK1/2 (Duncia et al. 1998). U0126 noncompetitively binds with the deltaN3-S218E/S222D site of MEK1/2 and inhibits MEK1/2 activation (Favata et al. 1998). This compound also inhibits other kinases including MKK-3/4/6, p38, PRAK and PKBα at 4-10 times higher IC_{50} concentrations compared with its effect on MEK-1 (Davies et al. 2000; Favata et al. 1998). Although the effect of U0126 on the above protein kinases cannot be completely ruled out, U0126 is still a relatively specific inhibitor of MEK1/2.

Ouabain, a cardiac glycoside, is a specific inhibitor of Na/K-ATPase which dose so by binding to a cavity formed by the transmembrane helices of the α subunit of Na/K-ATPase, thereby blocking ion transportation (Ogawa et al. 2009) or promoting the interaction of Na/K-ATPase with signaling molecules, such as Src and MAPKs (Li & Xie 2009). Ouabain has no effect on the Mg^{2+}-ATPase and the HCO_3^--ATPase (Knauf et al. 1976). Therefore, ouabain selectively inhibits Na/K-ATPase without any measureable effect on other ATPases.

KN-93 is a potent and cell-permeable inhibitor of CaMKs by competitively blocking calmodulin binding to the calmodulin-binding region of CaMK. It inhibits

CaMK I (IC_{50} = 5 μM), CaMK II (IC_{50} = 1 μM) and CaMK IV (IC_{50} = 7 μM) (Means 2008). This compound does not affect the activities of PKA, PKC, MLCK, or Ca^{2+}-phosphodiesterase (Riganti et al. 2009). However, KN-93 shows inhibitory effects on aminopyrine uptake in parietal cells (IC_{50} = 300 nM) (Mamiya et al. 1993) and voltage-gated K^+ channels in smooth muscle cells (IC_{50} = 270 nM) (Ledoux et al. 1999). Interestingly, both Kv4.2 and Kv4.3 channels are substrates of CaMK II in cardiomyocytes (Colinas et al. 2006) and the effects of KN-93 on K^+ channels are a result of CaMK II inhibition, making KN-93 a specific inhibitor of CaMKs in cardiomyocytes.

Rapamycin is a potent allosteric mTORC1 inhibitor by forming a complex with the FK-binding protein 12 (FKBP12). This complex interacts with the multiprotein complex composed of mTOR, mLST8, and raptor, leading to inhibition of mTORC1. A recent study showed that prolonged rapamycin treatment (24 hours) reduces the levels of mTORC2, which phosphorylates and activates Akt (Sarbassov et al. 2006). Rapamycin has no significant inhibitory effect on other kinases (Davies et al. 2000). In the present thesis, cells were treated with rapamycin for 3-5 hours. During this period of time, it seems unlikely that rapamycin would have any nonspecific effect.

IPA-3 is a cell-permeable symmetrical disulfide allosteric inhibitor of group I PAK activation, and 10 μM of IPA-3 inhibits PAK1, PAK2 and PAK3 activity by 90%, 70% and 60%, respectively. IPA-3 binds covalently to the PAK regulatory domain and prevents binding to the upstream activator (Viaud & Peterson 2009). A recent study showed that IPA-3 at 10μM inhibits PKBβ, FGR, GRK4, GSK3, p38α, PLK3 and SGK3 activity to 68%, 51%, 68%, 66%, 70%, 88% and 93% respectively (Deacon et al. 2008). These nonspecific effects of IPA-3 cannot be ruled out in my thesis. To exclude these

nonspecific effects, PAK1 siRNA was also employed to specifically inhibit PAK1 activity.

SP600125 is a potent, cell permeable inhibitor of JNK (IC_{50} = 40 nM for JNK-1 and JNK-2; 90 nM for JNK-3) (Bogoyevitch & Arthur 2008). It competes with ATP for the ATP-binding site of JNK. Unlike ATP, SP600125 cannot be used in the phosphotransfer reaction, thereby inhibiting JNK activity. This agent exhibits greater than 300-fold selectivity for JNK in relation to other MAPKs (ERK and p38) and PKA (Bogoyevitch & Arthur 2008). This limitation of SP600125 cannot be ruled out in the present thesis. To overcome this limitation, $JNK1^{-/-}$ mice were employed to confirm the results of JNK inhibition by SP600125 in this thesis.

5.4 Future directions

Besides regulating TNF-α expression, it is possible that Rac1 contributes to cardiac dysfunction in endotoxemia through other mechanisms that are not explored in this thesis. Studies have shown that LPS increases caspase activation in the heart (Carlson et al. 2005). Inhibition of caspase activity and transgenic overexpression of Bcl-2 improve myocardial function in endotoxemic mice, suggesting that cardiomyocyte apoptosis may play a role in myocardial depression (Fauvel et al. 2001; Lancel et al. 2005). A recent study showed that Rac1 is required for cardiomyocyte apoptosis during hyperglycemia (Shen et al. 2009). Therefore, one of the future studies of interest would be to determine if Rac1 has any effect on cardiomyocyte apoptosis in endotoxemia.

Microcirculatory dysfunction plays an important role in the pathogenesis of sepsis and is characterized by loss of vasomotor reactivity, endothelial cell injury, activation of

coagulation, and disordered leukocyte trafficking (Trzeciak et al. 2008). The endothelium is known to dynamically regulate thrombosis, profibrinolysis, leukocyte adhesion/migration, microvascular tone, permeability, and blood flow in both physiological and pathophysiological conditions (Aird 2004). Numerous studies have demonstrated that LPS enhances adhesion molecule and pro-inflammatory factor expression in endothelial cells and endothelial permeability, leading to leukocyte adhesion, migration and coagulation (Berman et al. 1993; Carlos & Harlan 1994; McCuskey et al. 1996). Rac1 deficiency in endothelial cells prevents endothelial cell migration, tubulogenesis, adhesion, and permeability in response to vascular endothelial growth factor (VEGF) and sphingosine-1-phosphate (S1P) (Tan et al. 2008), indicating a critical role of Rac1 in endothelial cell function. It would be interesting to investigate the role of Rac1 in the microvascular circulation during sepsis.

In addition, in **Chapter 3**, I found that Rac1 deficiency decreased systolic Ca^{2+} concentration in cardiomyocytes through regulating Na/K-ATPase activity. Ca^{2+} has been shown to be important in regulating cardiac contractility, hypertrophy, apoptosis, and arrhythmia as well as gene expression (Erickson & Anderson 2008; Frey et al. 2000). It is possible that Rac1 may regulate cardiac function in other pathophysiological conditions, such as myocardial infarction. In this regarding, it is would be interesting to study the role of Rac1 in cardiomyocyte apoptosis, arrhythmia, infarct healing and left ventricular remodeling after myocardial infarction.

5.5 Conclusions

My studies provide strong evidence that Rac1 is a critical regulator of myocardial

TNF-α expression and cardiac dysfunction in endotoxemia (Figure 5.1). LPS activates Rac1 through PI3K. Activation of Rac1 increases NADPH oxidase and ERK1/2 activity, leading to increased myocardial TNF-α mRNA expression (**Chapter 2**). Furthermore, Rac1-containing NADPH oxidase inhibits Na/K-ATPase and activates Ca^{2+}/CaMK-dependent mTOR, resulting in enhanced TNF-α protein production (**Chapter 3**). These effects of Rac1 on TNF-α expression lead to cardiac dysfunction in endotoxemia. Another downstream effector of Rac1 is PAK1. PAK1 increases TNF-α expression via p38 MAPK. On the other hand, PAK1 promotes JNK1-dependent MKP-1 expression, which inactivates ERK1/2 and p38 MAPK, reduces TNF-α expression and improves cardiac function. Rac1/MKP-1 pathway represents a negative feedback mechanism to limit myocardial TNF-α expression and cardiac dysfunction in endotoxemia (**Chapter 4**). These Rac1 pathways provide novel insights into the signal transduction mechanisms that regulate myocardial TNF-α expression, and may have therapeutic implications in the treatment of sepsis.

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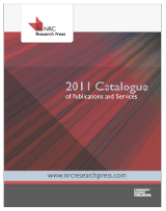

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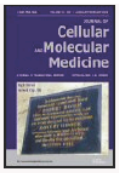

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Appendix 2:

UWO Animal use sub-committee protocol approvals

Western

04.01.2010
***This is the 3rd Renewal of this protocol**
*A Full Protocol submission will be required in **2011**

Dear Dr. **Feng**

Your Animal Use Protocol form entitled:

Modulation of Myocardial Function in Myocardial Infarction, Sepsis and Diabetes

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **04.01.2010 to 03.31.2011**

The protocol number for this project remains as **2007-011**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this *Animal Use Protocol* is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

[redacted]

c.c. T. Carter; W. Lagerwerf

***The* University *of* Western Ontario**
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, ● London, Ontario ● CANADA – N6A 5C1
PH: 519-661-2111 ext. 86770 ● FL 519-661-2028 ● www.uwo.ca / animal

Curriculum Vitae

Ting Zhang

PhD Candidate
Department of Physiology and Pharmacology
M253 Medical Sciences Building
The University of Western Ontario
London, Ontario, Canada, N6G 5C1

EDUCATION

01/2007-present	Ph.D.	Physiology, University of Western Ontario, London, Ontario, Canada Thesis title: Role of Rac1 in myocardial tumor necrosis factor-alpha expression and cardiac dysfunction during endotoxemia
09/2004-08/2006	M.Sc.	Physiology, University of Western Ontario Thesis title: Molecular Mechanisms of Visceral Adiposity in offspring of Maternal Protein Restricted Rats
09/1996-07/2000	B.Sc.	Biochemistry, Central China Normal University, P.R.China

SCHOLARSHIPS AND AWARDS

2011	Graduate Thesis Research Awards Fund
2010-2011	Ontario Graduate Scholarship (OGS)
2010	Passed Ph.D comprehensive exams with Distinction
2009	2nd prize for poster competition, The Department of Physiology and Pharmacology Research Day
2008	1st prize for poster competition, The Department of Physiology and Pharmacology Research Day,
2007-2009	Student Travel Award, Beijing Joint Conference of Physiological Sciences 2008
2007	Schulich Graduate Scholarship
2007-2011	2011Western Graduate Research Scholarships
2006	The Endocrine Society Travel Grant
2006	CIHR Institute of Human Development, Child and Youth Health Travel Award

TEACHING EXPERIENCE

- Lecturer, College of Chemistry and Life Science, South Central University for Nationalities, P.R.China (09/2003 – 06/ 2004)
- Laboratory sections of Cell Biology for 3rd year undergraduate
- Teaching assistant, Medical Science 400, UWO (02/2007 - 03/2007)
- Teaching assistant, Pharmacology 432B, UWO (01/08-05/08; 09/08-12/08)

PUBLICATIONS

Peer-reviewed publications

-Published

- **Zhang T**, Lu X, Beier F and Feng Q, Rac1 activation induces tumor necrosis factor-alpha expression and cardiac dysfunction in endotoxemia, *Journal of Cellular and Molecular Medicine*, 2011,May;15(5):1109-21
- **Zhang T**, Guan H, Yang K, Keratinocyte growth factor promotes preadipocyte proliferation via an autocrine mechanism, *Journal of Cellular Biochemistry*, 2010 Mar 1;109(4):737-46
- **Zhang T**, and Feng Q, Signal transduction and calcium signaling in myocardial tumor necrosis factor-alpha expression and cardiac dysfunction in sepsis: Role of nitric oxide (review), *Canadian Journal of Physiology and Pharmacology*, 2010 Feb;88(2):92-104
- Peng T, **Zhang T**, Lu X and Feng Q, JNK1/c-fos inhibits cardiomyocyte TNF-alpha expression via a negative crosstalk with ERK and p38 MAPK in endotoxemia, *Cardiovascular Research*, 2008, Mar 1;81(4):733-41
- **Zhang T**, Guan H, Arany E et al., Fetal programming of adipogenesis in visceral adiposity in male offspring of maternal protein restricted rats, *Journal of Cellular Biochemistry*, 2007, 101(2): 381-8.
- Zhu D, Luo Q, and **Zhang T**, Thermally-induced effects on normal and tumor of mouse mesentery based on measuring microcirculation parameters and temperature, *Proc. SPIE*, 2005, 5630:44-51
- **Zhang T**, Zhu D, and Luo D, Dynamic measurement of blood flow with optical methods, *Proc. SPIE*, 2003, 4961: 165-173.
- **Zhang T**, Luo Q, Zhu D, Optical monitoring of dynamics of blood flow in locally heated microvessels on intestine mesentery of rats, *Acta Laser Biology Sinica*, 2002, 5: 358-363. (In Chinese)

-In preparation

- **Zhang T**, Lu X, Chidiac P, Sims S and Feng Q, Inhibition of Na/K-ATPase promotes myocardial tumor necrosis factor-alpha protein expression via activation of mTOR in endotoxemia.

- **Zhang T**, Lu X, Arnold P, Liu Y and Feng Q, Mitogen-activated protein kinase phosphatase-1 inhibits myocardial tumor necrosis factor-alpha expression and improves cardiac function during endotoxemia.

Non-peer-reviewed publications

-Meeting abstracts:

- **Zhang T**, Lu X, Sims S, Feng Q, Inhibition of Na/K-ATPase activity enhances myocardial tumor necrosis factor-alpha expression via Ca2+/CaMKII-dependent mTOR activation in endotoxemia, Experimental Biology 2011, poster, April9-13, 2011
- **Zhang T**, Lu X, Feng Q, Rac1 promotes lipopolysaccharide-induced tumor necrosis factor-alpha expression in cardiomyocytes via inhibition of Na/K-ATPase, ISHR2010 KYOTO, poster, May 13-16, 2010
- **Zhang T**, Peng T, Lu X et al., Role of rac1 in lipopolysaccharide-induced tumor necrosis factor-alpha expression and myocardial depression, Beijing Joint Conference of Physiological Sciences 2008, Poster, Oct 19-22, 2008
- **Zhang T**, Guan H, Arany E et al., Maternal protein restriction permanently programs adipocyte growth and development in adult male rat offspring, Annual Meeting of Endocrine Society, *oral presentation*, June 24-27, 2006.
- **Zhang T**, Guan H, and Yang K, KGF is a novel mitogen factor in 3T3-L1 and rat primary preadipocytes, Annual Meeting of Endocrine Society, poster, June 24-27, 2006.