3-23-2009

KATP Channel Action in Vascular Tone Regulation During Septic Shock: Beyond Physiology

Weiwei Shi

K_{ATP} CHANNEL ACTION IN VASCULAR TONE REGULATION DURING SEPTIC SHOCK: BEYOND PHYSIOLOGY

by

WEIWEI SHI

Under the Direction of Chun Jiang

ABSTRACT

Septic shock is a major cause of deaths resulting from uncontrolled inflammation and circulatory failure. Recent studies suggest that the vascular isoform of ATP-sensitive K^+ (K_{ATP}) channels is an important contributor to septic susceptibility. To understand the molecular mechanisms for channel regulation during sepsis, we performed studies in isolated endothelium-denuded mesenteric rings. Lipopolysaccharides (LPS) induced vascular relaxation and hyporeactivity to phenylephrine. The LPS-treated aortic smooth muscle cells displayed hyperpolarization and augmentation of K_{ATP} channel activity. Both were due to an up-regulation of Kir6.1 and SUR2B surface expression. The up-regulation relied on transcriptional and translational mechanisms, in which nuclear factor-κB (NF-κB) and Protein kinase A (PKA) played a critical role.

Oxidative stress occurs during sepsis and may act as another regulatory mechanism affecting K_{ATP} channel activity and vascular contractility. We found that micromolar concentrations of H_2O_2 impaired the pinacidil-induced vasodilation. The effect attributed to the suppression of K_{ATP} channel activity, which can be fully produced by reactivity oxidants. Unlike

the Kir6.1/SUR2B channel, the Kir6.2/SUR2B channel was insensitive to 1mM H_2O_2, indicating that the modulation sites are located in Kir6.1. Site-directed mutational analysis showed that three cysteine residues located in N-terminus and the core region of Kir6.1 were likely to mediate the redox-dependent channel modulation.

Arginine vasopressin (AVP) is a vasoconstrictor that is successfully applied to manage sepsis. However, the downstream target of AVP is uncertain. Our studies show that AVP-induced vasoconstriction depended on V1a receptor, Protein kinase C (PKC) and K_{ATP} channel. Additionally, AVP decreased Kir6.1/SUR2B channel activity through V1a receptor. The inhibitory effect was caused by a suppression of the channel open state probability. The channel inhibition was mediated by phosphorylation of the channel protein by PKC.

The widespread involvement of the vascular K_{ATP} channel in vascular responses to endotoxemia strongly suggests that the temporospatial control of channel activity may constitute an important intervention to vascular tone, blood pressure and organ-tissue perfusion in septic shock. Such a control appears feasible by targeting several modulatory mechanisms of intracellular signaling, Kir6.1/SUR2B expression, redox state and channel protein phosphorylation as demonstrated in this dissertation.

INDEX WORDS: Kir6.1, SUR2B, ATP-sensitive K^+ channels, Vascular tone, Sepsis, Lipopolysaccharides, Nuclear factor-kappa B, Protein kinase A, Arginine vasopressin

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WEIWEI SHI

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2009

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by

WEIWEI SHI

Committee Chair: Chun Jiang

Committee: Delon Barfuss
Deborah Baro
Walter William Walthall

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
May 2009

ACKNOWLEDGEMENTS

I thank my advisor Dr. Chun Jiang, for his guidance that showed me a stairway to the heaven of research, and his encouragement that motivates me to explore an unknown scientific kingdom. The author also appreciates my committee members, Dr. Delon Barfuss, Dr. Deborah Baro, and Dr. Walter William Walthall, for their valuable discussions and generous assistance in experimental techniques and English communication. The author was supported by a Molecular Basis of Disease (MBD) Fellowship from Georgia State University. The author would like to acknowledge the members from Dr. Chun Jiang's lab for technical assistances. Special thanks to Drs. Ningren Cui, Yun Shi, and Runping Wang for their helps in experimental techniques. I appreciate Ms Xiaoli Zhang, Dr. Asheebo Rojas, Mr. Yang Yang, and Ms. Zhongyin Wu for their assistances. The author would like to thank Dr. Dalin Zhun and Ms. Shuang Zhang in Haerbin Medical University. Our collaboration made the project run smoothly.

I also give special acknowledgements to Dr. Yun Liu for her aid with the protein experiments, Mrs. Ping Liang-Jiang for her help with DNA sequencing, and Mrs. Debby Walthall for her instruction in real time PCR and spectrophotometry. My appreciation is also paid to Ms. Hongmei Zhang, Mr. Xueliang Gao, Mr. Binh Ha, Ms. Xiaojing Chen, and all other members of the Department of Biology who provided their assistance and share research experiences with me during my Ph. D. education.

The author would like to thank his family, especially his wife, Rong Chen and his daughter, Jiali Shi, for their continuous encouragement and support. Finally, the author would like to express his appreciation to his parents: Mr. Shiren Shen and Mrs. Juan Meng, for supporting me to be a medical doctor.

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ABBREVIATIONS

ABC	ATP binding cassette
AC	adenylyl cyclase
ADP	adenosine diphosphate
Ang II	angiotensin II
ATP	adenosine triphosphate
AVP	arginine vasopressin
BSA	bovine serum albumin
C	closed state
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CD14	cluster of differentiation 14
cDNA	complementary DNA
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene-related peptide
CNG	cyclic nucleotide gated ion channel
CO_2	carbon dioxide
CRE	cAMP-responsive element
CREB	cAMP response element binding protein
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle medium
DMFR	dimethyl fumarate
DMSO	dimethyl sulfoxide
DTNB	5, 5′-dithiobis-2-nitrobenzoic acid
DTT	dithiothreitol
EC_{50}	half maximal effective concentration
ECG	electrocardiogram
ED	endothelium-denuded
EDHF	endothelium-derived hyperpolarizing factor

EET	epoxyeicosatrienoic acid
EGTA	ethylene glycol-bis-β-aminoethylether-N,N,N',N'-tetraacetic acid
EI	endothelium-intact
ET-1	endothelin-1
eNOS	endothelial nitric oxide synthase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
Epac	exchanging protein directly activated by cAMP
FBS	fetal bovine serum
GPCR	G protein coupled receptors
H	Hill coefficient
H_2O_2	hydrogen peroxide
H_2S	hydrogen sulfide
HEK	human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGB1	high mobility group B-1
HO·	hydroxyl radical
IC_{50}	the concentration of a substance providing 50% inhibition
ICL	intracellular linker
ICU	intensive care unit
IκB	inhibitor of NF-κB
IKK	IκB kinase
IL-1	interleukin-1
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
IP_3	inositol-1, 4, 5-triphosphate
IRAK	IL-1 receptor-associated kinase
K_{ATP}	ATP-sensitive K^+ channel
K_{Ca}	Ca^{2+}-activated K^+ channel
KCO	K_{ATP} channel opener
Kir	inward rectifier K^+ channel

Kir6.x	members in the sixth subfamily of inward rectifier K^+ channels
Kir6.2ΔC36	inward rectifier K^+ channel 6.2 with 36 amino acids truncated
K_{NDP}	nucleotide diphosphate activated K^+ channel
KO	gene Knockout
Kv	voltage-gated K^+ channel
LBP	LPS-binding protein
LPS	lipopolysaccharides
mCD14	membrane bound CD14
MD2	Myeloid differentiation protein-2
Mg^{2+}	Magnesium
MIF	migration inhibitory factor
MODS	multiple organ dysfunction syndrome
MyD88	myeloid differentiation primary-response protein 88
NBD	nucleotide bonding domain
NDP	nucleotide diphosphate
NEMO	NF-κB essential modifier, also known as IKKγ
NF-κB	nuclear factor kappa B
NO	nitric oxide
NOS	nitric oxide synthase
O	open state
O_2^-	superoxide
P_aCO_2	arterial partial pressure of carbon dioxide
PAMP	pathogen-associated molecular patterns
pCMPS	*p*-chloromercuriphenylsulphonate
PCR	polymerase chain reaction
4α-PDD	4α-phorbol 12,13-didecanoate
2-PDS	2,2′-dithiodipyridine
PDTC	pyrrolidine dithiocarbamate
PE	phenylephrine
PH	pleckstrin homology
PHHI	persistent hyperinsulinemic hypoglycemia of infancy

PI_3K	phosphatidyl inositol 3-OH kinase
PIP_2	phosphatidylinositol bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKCi	PKC inhibitor peptide 19-31
PKG	protein kinase G
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
P_o	open-state probability
PO_2	pressure of oxygen
PVDF	polyvinylidene fluoride
RIP1	receptor interacting protein -1
ROMK	renal outer medullary K^+ channel
ROS	reactive oxygen species
Rp-cAMP	Rp-adenosine 3′,5′-cyclic monophosphorothioate
RQ	relative quantitation
RT-PCR	reverse transcriptase polymerase chain reaction
sCD14	soluble CD14
S.E.	standard error
SIRS	systemic inflammatory response syndrome
SMC	smooth muscle cell
SOD	superoxide dismutase
SUR	sulphonylurea receptor
TAK1	Transforming growth factor-β-activated kinase-1
TBST	Tris-buffered saline Tween-20
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll-like receptor
TM	transmembrane segment
TM1	the first transmembrane segment in Kir
TM2	the second transmembrane segment in Kir
TMD	transmembrane domain

TNF-α	tumor necrosis factor-α
TRAF6	TNF-receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing IFN-β
TRP	transient potential channels
V1a receptor	Vasopressin 1a receptor
VDCC	voltage-dependent Ca^{2+} channel
VIP	vasoactive intestinal polypeptide
VSM	vascular smooth muscle
VSMC	vascular smooth muscle cell
W_A	Walker A motif (a conserved motif for nucleotide binding in all ABC proteins)
W_B	Warker B motif (a conserved motif for nucleotide binding in all ABC proteins)
wt	wild type
YM-AVP	[Deamino-Pen1, Tyr(Me)2, Arg8]-vasopressin

1. Specific Aims and Hypotheses

Sepsis is a severe medical condition caused by several septic pathogens, especially bacterial lipopolysaccharides (LPS), leading to lethal cardiovascular dysfunction and death (Hotchkiss and Karl, 2003; Merx et al., 2007). Although systemic infection is a problem, most people die of circulation failure or septic shock. It is known that cardiovascular responses to sepsis are rather diverse and the prognosis can be very different between individuals. However, the underlying mechanisms for the sepsis susceptibility are unclear (Hotchkiss and Karl, 2003). Recent studies indicate that functional integrity of vascular ATP-sensitive K^+ (K_{ATP}) channels is a crucial factor for sepsis susceptibility (Kane et al., 2006; Croker et al., 2007). The vascular K_{ATP} channels are members of the inward rectifier K^+ channel family. The channels consist of 4 pore-forming Kir6.x subunits and 4 sulfonylurea receptor (SUR) subunits. The major isoform in vascular smooth muscles (VSM) consists of Kir6.1 and SUR2B, although the Kir6.2/SUR2B may also exist in the tissue. The vascular K_{ATP} channel is a common target of several circulating hormones, neurotransmitters, and cellular metabolites. Channel modulation by these extracellular signaling molecules allows the channel to play an important role in regulating vascular tone. Genetic knockout of either Kir6.1 or SUR2B subunit leads to spontaneous coronary vasospasm and sudden death (Chutkow et al., 2002; Miki et al., 2002). The vascular K_{ATP} channel participates in septic responses. Genome-wide mutagenesis studies have led to the discovery of four strains of mice that are highly susceptible to various septic pathogens (Croker et al., 2007). All of these mice carry a null mutation in the locus of the Kir6.1 gene (*Kcnj8*). Consistently, mice with a *Kcnj8* knockout exhibit cardiovascular abnormalities with a high mortality when exposed to a normally sub-lethal dose of LPS (Kane et al., 2006). Although these recent studies indicate that the Kir6.1/SUR2B channel is an important player in systemic responses to sepsis,

how the channel is affected by LPS remains unclear. Is the channel activated by LPS in VSM leading to vasodilation? Does the channel activation result from changes in channel protein expression or functional activity? Is the channel a direct target of LPS? How is the channel affected by oxidative stress occurring in sepsis? Can the resultant changes in K^+ currents during septic shock be controlled by circulating hormones and neurotransmitters? What are the crucial intracellular signaling pathways involved in the channel modulation by LPS and circulating hormones? The understanding of these regulatory mechanisms will provide information for novel therapeutic approaches to manage septic shock, and improve the survival rate of the disease. Therefore, these are the questions I addressed in my dissertation work. More specially, I addressed the following hypotheses:

A. The vascular K_{ATP} channel is activated with LPS exposure via activation of distinct intracellular signaling systems for Kir6.1/SUR2B gene expression.

B. With the development of systemic oxidative stress, the channel activity is suppressed by reactive oxygen species through direct interaction with the channel protein.

C. The vasodilation and vascular hyporeactivity to vasoconstrictors can be improved with AVP through phosphorylation modulation of the channel protein.

2. General Introduction: Vascular K_{ATP} Channels in Septic Shock

2.1. Septic shock

Septic shock is a medical emergency due to uncontrolled systemic infection and circulation failure. Septic shock is caused by several septic pathogens following systemic inflammatory response syndrome (SIRS) (Bone et al., 1992. See Table 2-1 for detailed nomenclatures of sepsis-related diseases). Approximately 750,000 cases of sepsis occur every year in the United States, and 215,000 deaths are caused by septic shock (Parrillo, 2008). The incidence of the disease has been increasing over the past decade owing to the increase in aging populations and antibiotic-resistant bacteria (Martin et al., 2003). Although septic shock has been intensively studied for decades, it remains the major cause of death in intensive care units, with the mortality rate between 30-70% (Riedemann et al., 2003a). A number of infectious microorganisms and tissue derivatives can cause sepsis, while LPS are the most important septic pathogenic toxins. LPS are the major component of the outer cell wall in Gram-negative bacteria, and can remain active even after the bacteria are lysed (Baron, 1996). Currently, there is no effective antidote against LPS for therapeutical purposes. In addition, the outcome of septic shock is determined by the host inflammatory responses, especially the cardiovascular response (Hotchkiss and Karl, 2003).

Table 2-1. Nomenclatures of sepsis-related diseases (Riedemann et al., 2003b)

SIRS	Body temperature >38.3°C or <36°C Heart rate >90 beats/min Respiratory rate >20 breaths/min or $PaCO_2$ <32 mmHg White blood cell count >12 $\times 10^9$/l or <4 $\times 10^9$/l, or >10% immature band forms
Sepsis	Systemic response to infection, with two or more of the conditions described in SIRS (SIRS $^+$ infectious evidence)
Severe sepsis	Sepsis together with organ dysfunction, hypoperfusion, or hypotension including lactic acidosis, oliguria, or acute alteration in mental state
Septic shock	Sepsis-induced hypotension (e.g., systolic blood pressure <90 mmHg or a reduction of >40 mmHg from base line) in spite of adequate fluid resuscitation, along with the presence of perfusion abnormalities that may contain lactic acidosis, oliguria, or an acute change in mental state. Vasopressor- or inotropic-treated patients may not be hypotensive at the time of measurement
MODS	The appearance of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention

P_aCO_2, arterial partial pressure of carbon dioxide; MODS, multiple organ dysfunction syndrome.

2.1.1. Pathological characteristics of septic shock

Excessive systemic inflammation, microcirculation dysfunction and myocardial depression are three major pathological characteristics of septic shock. Understanding these characteristics is helpful to develop more specific therpaeutic strategies to manage sepsis.

2.1.1.1. Excessive systemic inflammation

Innate immunity is activated in the early stage of infection, recruiting immune cells to build a defense wall. Mononuclear phagocytes are the central players in this process, releasing classic pro-inflammatory cytokines,e.g., interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) (Cohen, 2002). In later 1980s, TNF-α was found to produce similar septic symptoms as LPS administration (Michie et al., 1988). Since then, more pro-inflammatory cytokines, e.g., IL-1, macrophage migration inhibitory factor (MIF), and high mobility group B-1

(HMGB1) that are secreted from neutrophils, macrophages, and other immune cells have been identified to mediate systemic inflammation (Bozza et al., 1999; Dinarello, 1997; Wang et al., 1999). It is now generally accepted that host responses to systemic inflammation instead of the infection itself has a major impact on the outcome of sepsis (Cohen, 2002; Nystrom, 1998). The magnitude and duration of host responses influence the process of tissue damage, hypotension, multiple organ failure, and death (Tracey et al., 1986; Wang et al., 1999).

2.1.1.2. Microcirculation dysfunction

Microcirculation impairment was noticed in septic patients as early as in 1951. Some patients with severe infection are clammy, pale and hypotensive with low volume pulses (Waisbren, 1951). These symptoms are caused by a decreased peripheral vascular resistance and a re-distribution of blood flow to protect more important organs (heart, brain, etc.). The excessive low perfusion in microcirculation finally leads to shock, a lethal condition which is characterized by low blood pressure and insufficient blood perfusion to organs and tissues (Spronk et al., 2004). The hemodynamic changes are caused by peripheral vasodilation accompanied by high cardiac output. The consequential reduction in vascular resistance is thought to be a major cause of death in septic shock (Groeneveld et al., 1986; Parrillo, 1985). The cardiovascular dysfunction is partially due to over-production of nitric oxide (NO). Inhibition of nitirc oxide synthase (NOS) paritially reverses the contractile responses to norepinephrine (Julou-Schaeffer et al., 1990). The restoration can occur even after the endothelium is denuded, suggesting inducible NOS (iNOS) within vascular smooth muscle cells (VSMCs) is an important player. In addition, iNOS-null mice have been found to be resistant to LPS induced-vascular hypocontractility (Gunnett et al., 1998). Therefore, NO generated by iNOS is an important mediator that suppresses vascular contractility in sepsis. In comparison,

endothelial NOS (eNOS)-derived NO seems to serve for the protection of endothelial function (Cerwinka et al., 2002; Wiel et al., 2000), Chronic overexpression of eNOS in the mouse endothelium results in an increased resistance to LPS-induced hypotension and death (Yamashita et al., 2000), suggesting eNOS-derived NO has a beneficial effect on survival of sepsis. Although the use of NOS inhibitors was believed to be a novel therapeutic strategy to manage septic shock (Petros et al., 1991), several clinical trials in the middle and late 1990s that used non-selective NOS inhibitors were unsuccessful, due to serious and unpredicted complications (Cobb, 1999; Landry and Oliver, 2001; Petros et al., 1994). In addition, all NOS knockouts (eNOS, iNOS and neuronal NOS) failed to restore the impaired capillary blood flow in septic mice (Tyml et al., 2008). Therefore, how NO acts in the disease remains uncertain although its impact on septic shock cannot be ignored.

A remarkable finding in septic patients is that vascular contractile responses to vasoconstrictors are impaired during sepsis. Adrenergic stimulants fail to induce vasoconstriction despite increased levels of catecholamine (Chernow et al., 1982). Further studies show that the contractile response to angiotensin II and serotonin is also decreased (Umans et al., 1993). As an exception, AVP, a nanopeptide released from the posterior pituitary gland, produces effective vasoconstriction during sepsis and has been successfully used in therapy together with catecholamines recently (Dellinger et al., 2004). The molecular mechanisms for the vasoconstriction effect of AVP were investigated in this study and will be discussed below.

2.1.1.3. Myocardial depression

Myocardial depression is another important characteristic of sepsis. Both stroke volume and ejection fraction are compromised in septic patients despite normal or even higher cardiac output (Abel, 1989; Friedman et al., 1998; Parker et al., 1984). Septic patients with

cardiovascular dysfunction have significantly higher mortality by 70%- 90% in comparison to ~20% in patients without cardiovascular disorders (Parrillo et al., 1990). The release of myocardial depressant substances, such as TNF-α (Sharma et al., 1997), IL-1 (Fisher et al., 1994), IL-6 (Damas et al., 1992), prostanoids (Reines et al., 1982), endothelin-1 (ET-1) (Konrad et al., 2004), NO (Ullrich et al., 2000), contribute to the process of myocardial depression. A previous hypothesis that sepsis induced global myocardial ischemia is demonstrated to be incorrect at least in the early stage of endotoxemia. Instead, septic patients show a higher coronary blood flow and lower coronary artery– coronary sinus PO_2 difference (Cunnion et al., 1986). In addition, the levels of high energy phosphates in myocardium are sustained during septic shock, suggesting the myocardium depression is not mediated by metabolic stress (Hotchkiss and Karl, 1992; Solomon et al., 1994).

2.1.2. TLR4 receptor and downstream transcriptional factors

As mentioned above, septic process is initiated by several pathogens, especially LPS. These molecules are recognized by the innate immune system, and have been named pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 1998). LPS, a typical PAMP, are components of the outer cell wall from Gram-negative bacteria. In contrast, the septic pathogens in Gram-positive bacteria are peptidoglycan and lipoteichoic acid instead of LPS. All these PAMPs bind to cell-surface receptors, through which they activate downstream intracellular signaling cascades and produce cellular responses (Modlin et al., 1999; Wang et al., 2000).

Wright et al. found that activation of host cells required a LPS-binding protein (LBP) and an opsonic receptor CD14 (Wright et al., 1990). There are two isoforms of CD14. Besides a soluble CD14 (sCD14) that exists in circulation with a dynamic level according to the process of

sepsis, another CD14 (mCD14) is attached on the outer surface of cellular membranes (Landmann et al., 1995). Since mCD14 does not have an intracellular tail, the protein itself unlikely induces downstream signaling events after LPS stimulation. In other words, other proteins must exist to transduce the signal. Exploratory research soon led to the discovery of a Toll protein in *Drosophila*, which controls dorsal–ventral patterning in embryos and activates the transcription factor Dorsal upon binding to its ligand Spatzle (Anderson et al., 1985; Morisato and Anderson, 1994). Later studies indicate that the Toll/Dorsal signaling pathway contributes to an anti-fungal immune response in adult *Drosophila* (Lemaitre et al., 1996). Interestingly, the Toll/Dorsal signaling is highly homologous to the mammalian IL-1 signaling pathway. This observation later led to the identification of human Toll-like receptors (TLRs) and their functions in immune system (Medzhitov et al., 1997; Poltorak et al., 1998). Today, more than thirteen TLRs have been determined to be targets of various PAMPs from bacteria, fungi and host endogenous tissues (Kawai and Akira, 2007). Specially, TLR4 is the receptor of LPS. Upon ligand binding to the TLRs, intracellular signaling is provoked leading to activation of several transcriptional factors, especially nuclear factor kappa B (NF-κB).

2.1.2.1. NF-κB

NF-κB is a homo- or heterodimer of Rel family protein subunits p50, p52, p65 (RelA), c-Rel, and RelB (Ghosh et al., 1998). The most prominent form of NF-κB is the p50/p65 heterodimer (De Martin et al., 2000). The inactivated NF-κB is held in the cytoplasm with its activity suppressed by the inhibitors of NF-κB (IκBs). Upon ligand (e.g. LPS) binding to TLR4, a serial of intracellular signals is initiated causing degradation of IκBs (Figure 2-1). The active NF-κB is then translocated to the nucleus where it promotes transcription of several proinflammatory genes (Baeuerle and Baltimore, 1996).

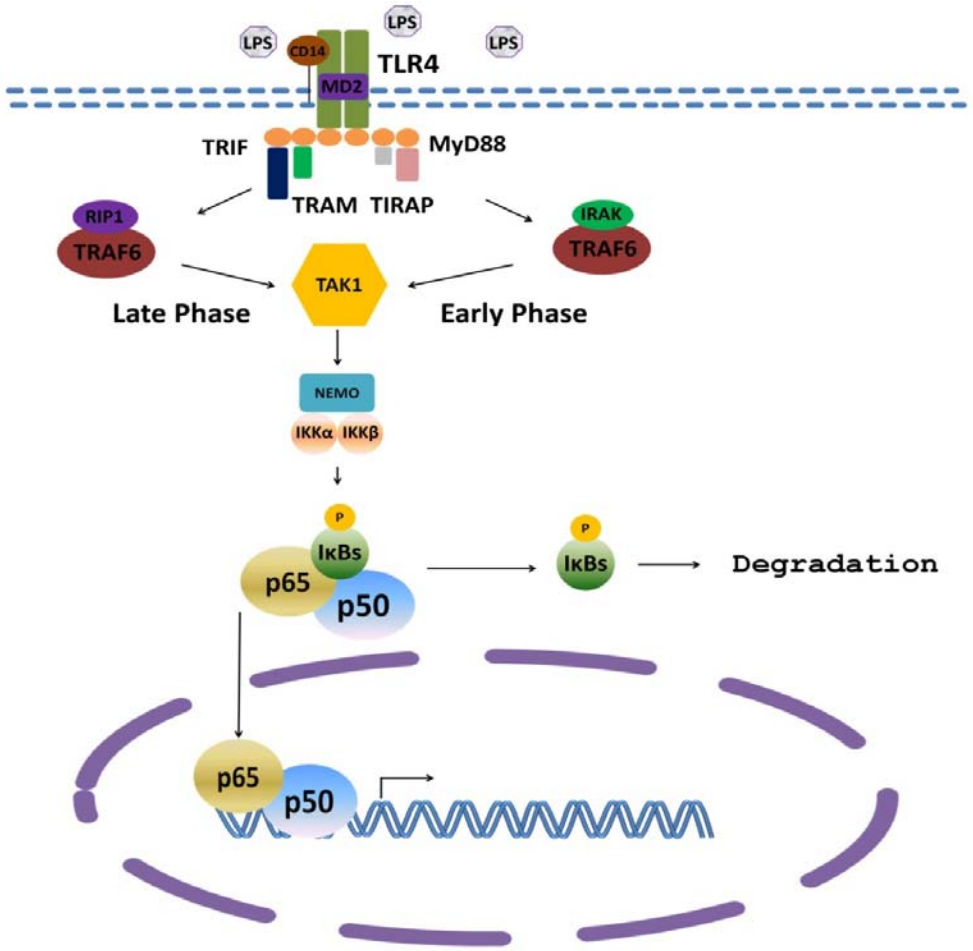


Figure 2-1. TLR4 signaling pathway.

After stimulation by ligands (e.g. LPS), TLR4 dimerizes and undergoes conformational changes to recruit downstream signaling proteins. The TLR4 dimer launches MyD88- and TRIF-dependent pathways through TIRAP and TRAM respectively. MyD88 mediates early phase activation of NF-κB. It phosphorylates IRAKs, which consequently interact with TRAF6, leading to activation of a TAK1 complex. The activated TAK1 complex then acts on IKK complex consisting of IKKα, IKKβ and NEMO, which later phosphorylate IκBs. Without stimulation, NF-κB (p65–p50 heterodimer) is located in cytoplasm and inhibited by IκBs. When IκBs are phosphorylated and degraded in a proteasome, NF-κB becomes active and is then translocated into the nucleus. In addition to the MyD88-dependent pathway, NF-κB is alternatively activated through TRIF-dependent pathway in the late phase. TRIF interact with TRAF6 and RIP1. TRAF6 then activates TAK1 in a manner similar to that in the MyD88-dependent pathway.

2.1.2.2. PKA and CREB

In addition to NF-κB, the cAMP-dependent protein kinase A (PKA)-dependent cAMP response element-binding protein (CREB) is another potentially important signaling pathway that has been noticed recently to contribute to sepsis. It is well known that PKA plays a role in vasodilating hormone-induced vasodilation. Upon stimulation by vasodilators, the Gs-coupled receptors are activated, followed by activation of adenylyl cyclase (AC) and production of adenosine 3', 5' cyclic monophosphate (cAMP) (Figure 2-2). Besides cyclic nucleotide gated ion channels (CNG channels) and guanine exchanging factors Epac (exchanging protein directly activated by cAMP), the major cAMP receptor is PKA (Skalhegg and Tasken, 2000; Walsh et al., 1968). An elevated cAMP activates PKA, which subsequently phosphorylates serine /threonine residue(s) of targeted proteins. In addition to vasodilation, PKA contributes to regulation of different kinds of cellular processes, such as gene transcription, cell differentiation, ion channel permeability, etc. (Skalhegg and Tasken, 2000). Acumulating evidence also suggests that the cAMP/ PKA signaling is up-regulated in several cell types during sepsis. For example, LPS up-regulate forskolin-induced adenylyl cyclase activity in a concentration- and time-dependent manner in murine macrophages (Osawa et al., 2006). LPS elevates PKA activity in human monocytes (Mandrekar et al., 2007). Furthermore, a unique C subunit of PKA (cPKA) has been found to form a ternary complex with NF-κB and IκB instead of R subunit. It is activated when IκB is degraded in response to LPS stimulation and subsequently enhances p65 transcriptional activity through Ser276 phosphorylation in p65 (Zhong et al., 1997).

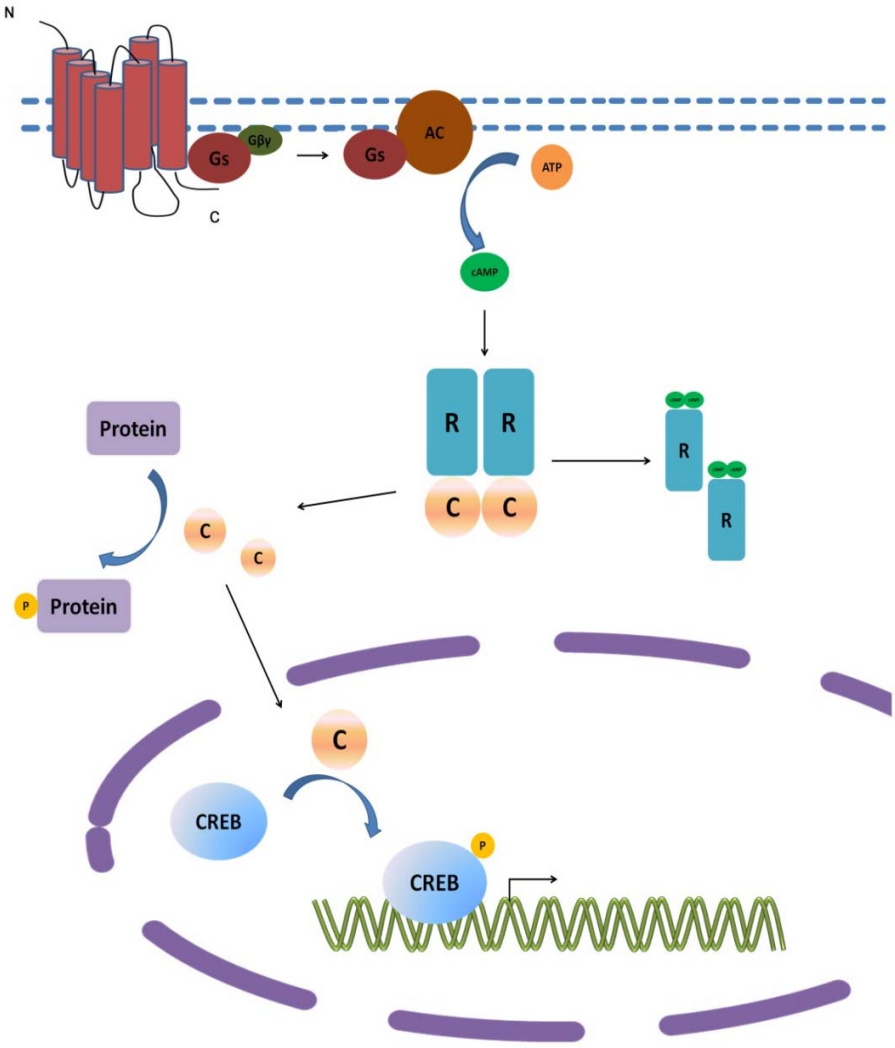


Figure 2-2. PKA-CREB pathway.

Following hormones and neurotransmitters bind to Gs protein-coupled receptors, adenylyl cyclase (AC) is activated and in turn converts ATP to cAMP. In the absence of cAMP, PKA is an inactive tetramer composing of two catalytic subunits (C) and two regulatory subunits (R). When intracellular cAMP level is elevated, the R subunit forms a dimer with four molecules of cAMP is dissociated from two C subunits, which become active and consequently phosphorylate serine and threonine residues of specific proteins. The catalytic subunit of PKA is translocated to nucleus, and activates CREB through phosphorylation at Ser133. The phosphorylated CREB then induces downstream transcriptional events.

PKA modulates gene transcription through cAMP response element binding protein (CREB) (Gonzalez and Montminy, 1989), which forms a homodimer and binds to a conserved cAMP-responsive element (CRE), TGACGTCA, in targeted genes. Phosphorylation at Ser133

promotes recruitment of the transcriptional co-activator CREB-binding protein (CBP) or p300, resulting in activation of CREB (Mayr and Montminy, 2001). CREB activity is enhanced in multiple cell and tissue types during LPS exposure, such as rat aortic smooth muscle cells (Yang et al., 2002), mouse macrophages (Kang et al., 2008) , rat lung tissue (Ye and Liu, 2001), and human bladder epithelial cells (Song et al., 2007). Therefore, CREB is another important transcriptional factor involved in LPS signaling.

2.1.2.3. Interaction between NF-κB and CREB

How are the NF-κB and CREB pathways related to each other? CBP, or its homolog p300 that was originally found as a co-activator of CREB, is required for transcriptional activation of many transcriptional factors, including p65 (Sheppard et al., 1999). Because the total amount of CBP is limited in the nucleus, the competition between NF-κB and CREB to bind CBP becomes an important mechanism contributing to the transcriptional activity regulation related to these factors (Figure 2-3, A) (Ollivier et al., 1996; Parry and Mackman, 1997; Zhong et al., 1998). Inhibition of glycogen synthase kinase 3, a housekeeping kinase which is located to the downstream of the phosphatidyl inositol 3-OH kinase (PI_3K) pathway that is activated upon LPS stimulation, increases the binding of CREB to CBP and decreases the CBP binding to p65 (Martin et al., 2005). In an *in vivo* study, interactions between CBP and p65 or CREB are enhanced in a time-dependent manner (Shenkar et al., 2001). A significant binding of p65 to CBP is found only at 30 min after LPS exposure, while the CREB-CBP interaction increases as early as 15 min after endotoxemia, and returns to baseline in 60 min. NF-κB seems to interact with CREB in the promoter regions of several genes, such as IL-6 (Figure 2-3, B). Mutation in p65-binding residue attenuates the effects of CREB. In contrast, CRE mutation decreases the stimulatory effects of p65. Therefore, both p65 and CREB are required for the maximal

stimulation of angiotensin II (Ang II)-induced IL-6 expression (Sahar et al., 2007). In addition, NF-κB contributes to the inhibitory effect of TNF-α on renin transcription via binding to a CRE in the mouse renin promoter (Figure 2-3, C) (Todorov et al., 2004).

NF-κB also can elevate PKA or CREB expression in some types of cells (Figure 2-3, D). Knockdown of neuronal NF-κB suppresses PKA α subunit (a catalytic subunit) expression in the hippocampus, down-regulating PKA-dependent CREB phosphorylation (Kaltschmidt et al., 2006). Moreover, NF-κB increases CREB promoter activity in Sertoli and NIH 3T3 cells (Delfino and Walker, 1999). The interaction between NF-κB and CREB is complex, depending on the various stimulators and different cell types.

2.1.3. K_{ATP} channel plays a role in sepsis

The involvement of vascular K_{ATP} channels in sepsis has been shown by several groups using different animal models. Administration of K_{ATP} channel blocker, such as glibenclamide, attenuates LPS-induced hypotension through elevation of systemic resistance in dog, pig and sheep (Landry and Oliver, 1992; Lange et al., 2007; Vanelli et al., 1995). Because glibenclamide does not alter hemodynamic of vehicle-treated animals (Landry and Oliver, 1992; Vanelli et al., 1995), it is likely K_{ATP} channel activity is enhanced during sepsis. Dexamethasone, a member of corticosteroid family, improves vascular reactivity that is suppressed by LPS *in vivo* possibly through inhibition of the K_{ATP} channel expression (d'Emmanuele di Villa Bianca et al., 2003). The participation of K_{ATP} channels in sepsis is further demonstrated by recent studies from transgenic mouse models. Kane et al. reported that Kir6.1-null mice have a high susceptibility to sepsis (Kane et al., 2006). Another group screened thousands of mice with random mutations induced by N-ethyl-N-nitrosourea, and found four strains of animals that are hypersensitive to

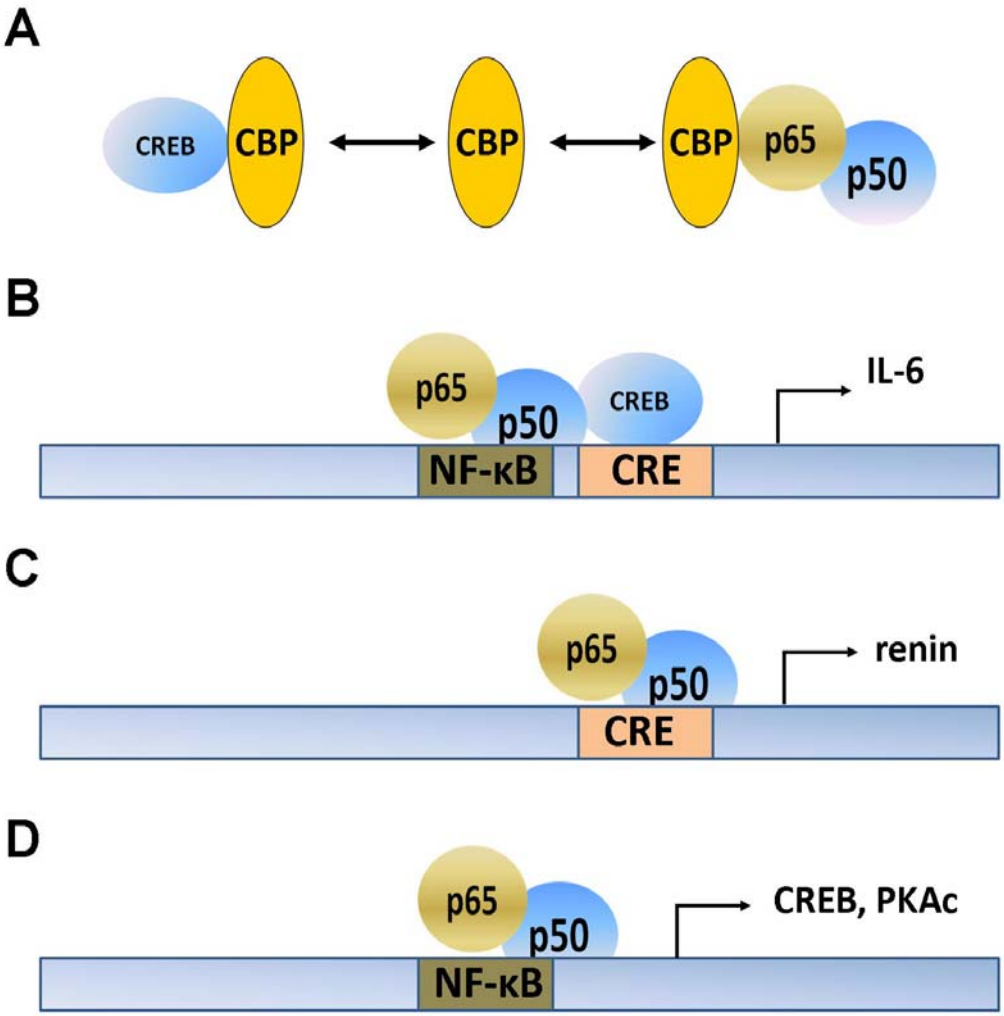


Figure 2-3. Linkages between NF-κB and CREB.

A. NF-κB and CREB competes with each other to bind CBP (Ollivier et al., 1996; Parry and Mackman, 1997); B. NF-κB acts on CREB in promoter region of IL-6 (Sahar et al., 2007) ; C. NF-κB binds to a CRE in the mouse renin promoter (Todorov et al., 2004); D. NF-κB increases CREB or PKAc promoter activity (Delfino and Walker, 1999; Kaltschmidt et al., 2006).

sub-lethal dose of LPS. All of these mice carry a null mutation of Kir6.1 gene (*Kcnj8*), which is caused by a deletion of exon 1, part of exon2, together with an inversion of intron 2 (Croker et al., 2007). Since some gene mutations (TNF-α, IFN-β, TRIF, MyD88, etc.) increase the survival ability from LPS exposure, Croker et al. also performed experiments in animals carrying

mutations in either of these genes together with Kir6.1 disruption. Surprisingly, the compound homozygosity renders a phenotype that is still highly sensitive to LPS. Therefore, the functional integrity of K_{ATP} channels is crucial for survival in sepsis.

2.2. Molecular aspects of K_{ATP} channels

The K_{ATP} channels refer to a group of K^+ channels whose activity is inhibited by intracellular ATP at physiological concentrations. Such a channel inhibition is independent of the high energy phosphate as non-hydrolysable ATP analogs such as AMP-PNP inhibit these channels as potently as ATP. ADP activates the K_{ATP} channels, especially the vascular isoform.

2.2.1. History

In 1983, Noma recorded an ATP-sensitive K^+ current in cardiac muscle, the characteristic of which is quite distinct from other inward rectifier K^+ channels, since the current is inhibited when intracellular ATP concentration is greater than 1 mM (Noma, 1983). This is the first report to show the existence of K_{ATP} channel. Since then, the expression of K_{ATP} channels has been identified in skeletal muscle (Spruce et al., 1985) and pancreatic β cells (Trube et al., 1986). Because some chemicals (e.g., diazoxide), had been known to have vasodilating effect long time before (Andersson, 1973; Rhodes and Sutter, 1971), later studies demonstrated that they activate K_{ATP} channels (Escande et al., 1988; Trube et al., 1986).

In 1989, Standen found a novel K_{ATP} current in rabbit mesenteric arterial smooth muscle cells. The current is activated by vasoactive intestinal polypeptide (VIP) and K_{ATP} channel opener cromakalim, and inhibited by glibenclamide, displaying the similar features as the K_{ATP} channel identified previously (Standen et al., 1989). Subsequently, K_{ATP} channels were found to be distributed ubiquitously in vasculatures (Nelson et al., 1990; Winquist et al., 1989). Because

the molecular basis of the channels was unknown, the studies relied heavily on pharmacological tools. Later, the understanding of channel biophysical properties and physiological functions were spurred after the successful cloning of Kir6.x and SUR subunits of K_{ATP} channels in the mid-1990s (Aguilar-Bryan et al., 1995; Inagaki et al., 1995; Isomoto et al., 1996), and the introduction of K_{ATP} channel transgenic mice in 2000s (Chutkow et al., 2002; Chutkow et al., 2001; Kakkar et al., 2006; Malester et al., 2007; Miki et al., 2002).

2.2.2. Molecular structures of vascular K_{ATP} channels

K_{ATP} channels are an octameric complexes consisting of 4 pore-forming Kir6 subunits (inward rectifier K^+ channel 6.x or Kir6.x) and 4 accessory sulfonylurea receptor (SUR) subunits (Figure 2-4). To date, two Kir6.x genes (*KCNJ8* for Kir6.1, and *KCNJ11* for Kir6.2) and two SUR genes (*ABCC8* for SUR1 and *ABCC9* for SUR2) have been identified. The Kir6.x shares 40-50% homology in amino acid sequence with previously cloned Kir members. Further studies indicated it has 2 transmembrane helixes (M1 and M2), cytoplasmic N- and C-termini and a pore-forming loop containing the glycine-phenylalanine-glycine motif necessary for K^+ selectivity in the H5 region. In symmetrical 140 mM K^+ recording conditions, the unitary conductance of Kir6.1-containing channels is ~35 pS (Kir6.1/SUR2B) (Yamada et al., 1997), whereas Kir6.2-containing channels is ~80 pS (Kir6.2/SUR2B) (Isomoto et al., 1996).

Functional expression of Kir6 requires SUR subunit (Ammala et al., 1996), which belongs to ATP-binding cassette transporter family. SUR1 is mainly expressed in pancreatic β cells. SUR2 has two variants: SUR2A and SUR2B. The only difference between them is the last 42 amino acids in the C terminus, which is caused by alternative splicing of exon 38 in *ABCC9* (Chutkow et al., 1996; Isomoto et al., 1996). SUR2A is predominantly expressed in the heart and skeletal muscles (Inagaki et al., 1996; Isomoto et al., 1996), whereas SUR2B is generally

distributed in smooth muscle cells. SUR contains 2 transmembrane domains (TMD1 and TMD2) with each containing 6 transmembrane segments, plus an N-terminal transmembrane domain containing 5 transmembrane segments (TMD0). There are two large intracellular loops between the adjacent TMDs containing nucleotide binding domains (NBD1 and NBD2). Within the NBDs, three motifs are critical for nucleotide binding: a Walker A motif (W_A), a Walker B motif (W_B), and a linker region (Campbell et al., 2003).

In a SUR2B model that was reported by our group, NBD1 and NBD2 form a dimer with two nucleotide-binding pockets at their interface. The TMD1 acts on NBDs through short intracellular linker 1 and 2 (ICL1 and ICL2, Figure 2-4) (Shi et al., 2008a). ICL1 interacts with both NBDs crossing the first nucleotide-binding region, whereas ICL2 is close to the center of NBD2. Therefore, TMD1 mainly interacts with NBD2. A similar interaction also exists in TMD2-NBD1. PKA phosphorylation changes the NBD2 interface, leading to movement of ICL2 and enhances the interaction between these two protein domains (Shi et al., 2008a).

2.2.3. Pharmacology of K_{ATP} channels

Both activators and inhibitors of K_{ATP} channel have been used in clinical therapy for a long time. Some compounds, such as sulfonylureas, had been applied to treat type II diabetes even before they were known as blockers of K_{ATP} channel. Understanding the pharmacological properties is very important for us to study the channel's function.

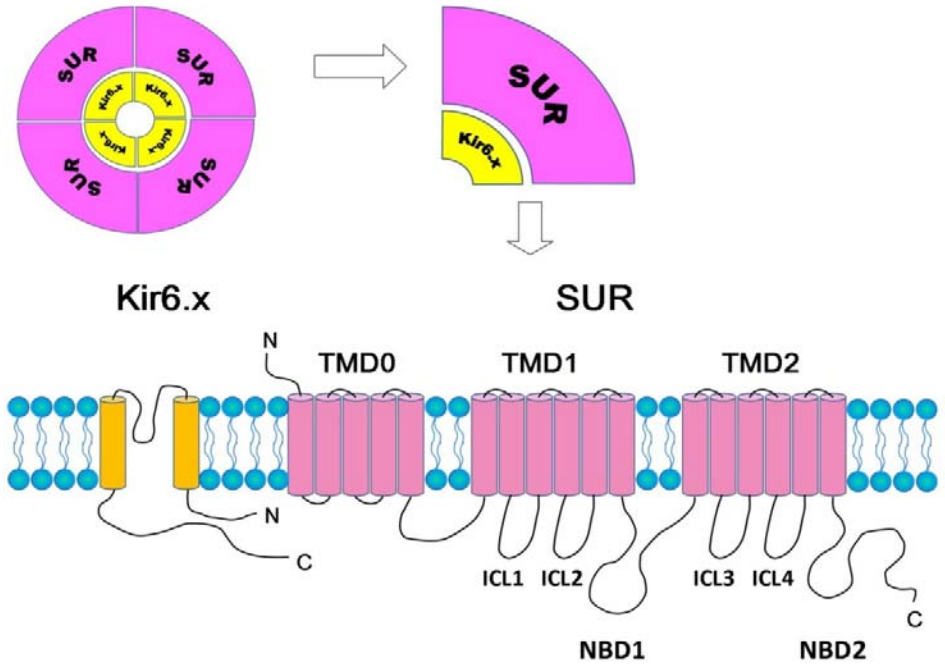


Figure 2-4. Schematic representation of K_{ATP} channel.

K_{ATP} channels are octameric complex containing 4 pore-forming Kir6.x subunits and 4 accessory sulfonylurea receptor (SUR) subunits. Kir6.x has 2 transmembrane domains, cytoplasmic N- and C-termini and a pore-forming loop. SUR contains 2 transmembrane domains with 6 transmembrane segments respectively (TMD1 and TMD2), plus an N-terminal transmembrane domain with 5 transmembrane segments (TMD0). The nucleotide binding domain-1 (NBD1) is located between TMD1 and TMD2, and NBD2 is located C-terminal to TMD2. The intracellular linkers (ICLs) between transmembrane helices are illuminated.

2.2.3.1. K_{ATP} channel inhibitors

Sulfonylureas, such as tolbutamide and glibenclamide (glyburide), have been widely used in anti-type II diabetes since 1950s (Patlak, 2002). The therapeutic effect is through stimulating insulin release by suppression of pancreatic K_{ATP} channels. Sulfonylurea binds to TMD2 of SUR subunit, especially the region between transmembrane segment (TM) 15 and 16 (Ashfield et al., 1999; Babenko et al., 1999). A Ser1237, which is located in the intracellular loop between TM15 and TM16, is a critical site for tolbutamide to display high-affinity inhibition. Glibenclamide has both sulfonylurea and benzamido groups. It binds to SUR1 at two regions: a sulfonylurea

binding site that is the same as tolbutamide, and a bezoamido binding site (Ashcroft and Gribble, 2000b). SUR2 lacks the sulfonylurea binding site; however, tolbutamide still inhibits SUR2B-containing channels (Isomoto et al., 1996), suggesting the C-terminal 42 amino acids of SUR2B comprise additional sulfonylurea binding regions. Since sulfonylurea has some nonspecific effects (Cocks et al., 1990), another type of inhibitor blocking the pore region of Kir6 subunit is developed. One example is PNU-37883A, which displays more potent inhibitory effect on Kir6.1/SUR2B than Kir6.2/SUR2B (Kovalev et al., 2004).

2.2.3.2. K_{ATP} channel activators

K_{ATP} channel openers (KCOs), such as diazoxide, cromakalim, pinacidil, and nicorandil, are a group of chemicals with diverse structures, and activate K_{ATP} channels via binding to SUR subunit (Ashcroft and Gribble, 2000a). Nicorandil has been used as an antianginal drug for several years, and significantly achieves a better outcome of stable angina by decreasing major coronary events (IONA Study Group, 2002). Different SUR subunits display distinct sensitivities to KCOs. For example, diazoxide stimulates all K_{ATP} channel containing SUR1, SUR2A (in the presence of MgADP), and SUR2B. However, Kir6.2/SUR2A channels are only weakly activated by diazoxide in the absence of ADP (D'Hahan et al., 1999). Pinacidil and cromakalim activate SUR2A and SUR2B instead of SUR1 (Seino and Miki, 2003). Recent studies show that KCOs such as pinacidil stimulate ATP hydrolysis at NBD2 and increase channel opening by stabilizing channels in a Mg-nucleotide bound state (Bienengraeber et al., 2000; Zingman et al., 2001). This phenomenon becomes more significant in Kir6.1/SUR2B channel because pinacidil barely activates the channel without nucleotide (Satoh et al., 1998). Therefore, the stimulatory effect of KCOs depends on the interaction with SUR subunit as well as the cellular nucleotide level.

2.2.4. Evidence of vascular K_{ATP} channel function from transgenic mouse models

Transgenic mouse models provide new strategies to understand the roles of K_{ATP} channels in vasculature. A detailed comparison among these transgenic mice is listed in Table 2-2. Generally, both Kir6.1 and SUR2-null mice exhibit frequently spontaneous ST segment elevation due to coronary arterial spasm and sudden early death, suggesting K_{ATP} channel is critical for vascular tone regulation (Chutkow et al., 2002; Miki et al., 2002). In addition, Kir6.1 knockout mice display a high mortality during sepsis, indicating K_{ATP} channel has a protective role against sepsis or septic shock (Kane et al., 2006). Since K_{ATP} channel is expressed in vascular endothelium as well as smooth muscle cells, both of which contribute to vascular tone regulation, the function of endothelial K_{ATP} channel has been noticed recently. In a transgenic animal model, SUR2B expression is selectively restored in smooth muscle cells of SUR2-null mice. The new strain shows a similar coronary vasospasm as Kir6.1-null mice (Kakkar et al., 2006). Another type of transgenic animals which express dominant negative Kir6.1 subunits in endothelium exhibit an elevated coronary perfusion pressure due to an increase in ET-1 release (Malester et al., 2007). All these observations suggest that endothelial K_{ATP} channel is important for coronary circulation.

2.3. Distribution and regulation of vascular K_{ATP} channels

2.3.1. Functional expression of vascular K_{ATP} channels

The evidences cumulated in the last 20 years demonstrate that K_{ATP} channels universally exist in vasculatures and play an important role in regulation of vascular tone. The functions of the vascular K_{ATP} channels in major parts of circulatory system are described blow.

Table 2-2. Summary of vascular K_{ATP} channel transgenic mouse models

Transgenic model	Kir6.1 KO	SUR2 KO	SM22-SUR2B	Tg[Tek-Kir6.1-AAA]
Genotype	Kir6.1 $^{-/-}$	SUR2 $^{-/-}$	Rescued SUR2B in VSMC of SUR2 $^{-/-}$ mice	Dominant negative Kir6.1 in endothelium
Mortality	50% in 5-6 weeks	65% male KO, and 35% female KO in 30 weeks	N/A	N/A
Blood Pressure under anesthesia	–	↑	N/A	N/A
Heart rate	–	–	–	N/A
Frequency of spontaneous elevation of ST segment	↑	↑	↑	–
Coronary artery	N/A	stenoses	Vasospasm	–
Coronary perfusion pressure	↑	N/A	↑	↑
LPS susceptibility	↑	N/A	N/A	N/A
Others		insulin responsiveness of skeletal muscle ↑		ET-1 release ↑
References	(Croker et al., 2007; Kane et al., 2006; Miki et al., 2002)	(Chutkow et al., 2002; Chutkow et al., 2001)	(Kakkar et al., 2006)	(Malester et al., 2007)

ECG, Electrocardiogram. ↑, increase. –, no change. N/A, not reported.

2.3.1.1. Coronary circulation

Although coronary blood flow only accounts for less than 1% of cardiac stroke volume (Duncker and Bache, 2008), the coronary circulation is extremely important because coronary artery disease which is characterized as impairments of coronary circulation is responsible for 52% death in cardiovascular diseases (American Heart Association, 2008). K_{ATP} channel plays a substantial role in coronary arterial tone regulation. Endothelium-denuded porcine and human

coronary arteries are relaxed by pinacidil, suggesting K_{ATP} channel are expressed in coronary arterial smooth muscle (Gollasch et al., 1995). *In situ* hybridization shows that both Kir6.1 and SUR2B mRNAs are present in coronary arteries (Li et al., 2003). An immunohistochemical study indicates both Kir6.1 and SUR2 proteins are distributed in the smooth muscle and endothelial cells of coronary resistance vessels. In addition, Kir6.1 protein is detected in endothelial capillaries, whereas Kir6.2 protein expression is found in endothelial cells instead of (Morrissey et al., 2005). Mice with genetic disruptions of either Kir6.1 or SUR2 subunit develop spontaneous coronary vascular spasm and sudden death (Chutkow et al., 2002; Miki et al., 2002), strongly suggesting that a functional K_{ATP} channel is critical for coronary circulation. In another transgenic mouse model using SUR2-null mice, SUR2 expression is selectively rescued in smooth muscle cells; however, the new transgenic animals still exhibits frequent episodes of spontaneous ST segment elevation, implying that coronary endothelial K_{ATP} channel is also important (Kakkar et al., 2006). In fact, Kir6.1, Kir6.2, and SUR2B are detected in human coronary endothelium by using Western blotting and immunoprecipitation assays, and these subunits form a heteromeric complex based on confocal microscopy images (Yoshida et al., 2004).

2.3.1.2. Cerebral circulation

Patch clamp studies suggest the existence of K_{ATP} channels in cerebrovascular smooth muscle (Kleppisch and Nelson, 1995b) and endothelium (Janigro et al., 1993). The channel expression in rat basilar and middle cerebral artery is likely composed of SUR2B together with Kir6.1 or Kir6.2 by a reverse transcriptase PCR (RT-PCR) study (Jansen-Olesen et al., 2005). K_{ATP} channels in cerebral circulation are subjected to the regulation by endogenous vasoconstrictors (e.g. serotonin and histamine) and vasodilators (e.g. calcitonin gene-related

peptide (CGRP), adenosine) (Kleppisch and Nelson, 1995b). Additionally, either hypotension or hypoxia stimulates K_{ATP} channels through releases of prostaglandins and cAMP, or NO and opioids, respectively, leading to pial artery relaxation (Armstead, 1998, 1999).

2.3.1.3. Pulmonary circulation

The expression of Kir6.1 and SUR2B in human pulmonary arterial smooth muscle cells is confirmed by RT-PCR and patch clamp since a 28-29 pS channel is activated by levcromakalim (Cui et al., 2002). In addition, ET-1, an important vasoconstrictor in pulmonary circulation, reduces the K_{ATP} current in a concentration-dependent manner, and the inhibitory effect is more potent in rabbit pulmonary arterial smooth muscle cells (SMCs) than in coronary arterial SMCs (Park et al., 2005). On the contrary, a low level expression of Kir6.2 (both mRNA and protein) instead of Kir6.1 is detected in rat pulmonary microvascular endothelial cells. Kir6.2 expression and an inwardly rectified membrane current are increased significantly when the endothelial cells are adapted to flow or shear stress. Flow termination induces cellular membrane depolarization, which is inhibited by a K_{ATP} channel opener cromakalim. These studies suggest that the expression of K_{ATP} channels pulmonary vasculture is dynamic and underwent regulation by shear stress (Chatterjee et al., 2003). In a microarray study, Kir6.2 expression is enhanced in lung tissues from patients diagnosed with primary pulmonary hypertension, a rare lung disease caused by progressive pulmonary arterial constriction for unknown reasons, leading to pulmonary hypertension and right heart failure (Geraci et al., 2001). Besides pulmonary arteries, K_{ATP} channels also participate in vascular tone regulation of pulmonary veins, because levcromakalim induces an endothelium-dependent and glibenclamide-sensitive pulmonary vein relaxation (Roh et al., 2006). All these observations suggest that K_{ATP} channel plays an important role in pulmonary vasculature, and contributes to regulation of pulmonary vascular resistance.

2.3.1.4. Fetoplacental circulation

Kir6.1 expression in placental vasculature is verified by RT-PCR and Western blot (Wareing et al., 2006a). Inhibiting K_{ATP} channels by glibenclamide attenuates CGRP-induced relaxation of chorionic artery (Dong et al., 2004) and increases perfusion pressure of placental cotyledons to a plateau level (Bisseling et al., 2005). Furthermore, pinacidil induces a significant relaxation of chorionic plate arteries and veins precontracted by U-46619, a stable thromboxane A_2 analog (Wareing et al., 2006a). All this evidence indicates that K_{ATP} channels participate in vascular tone regulation in placental vessels.

2.3.1.5. Other major vasculatures

The expression of K_{ATP} channels is identified in aorta, mesenteric artery, renal artery, retinal arterioles and vasculatures in skeletal muscle (Bryan and Marshall, 1999; Hein et al., 2006; Li et al., 2003). Both Kir6.1 and SUR2B are detected in aorta by RT-PCR, microarray or Western blot (Ren et al., 2003; Sampson et al., 2004; Tivesten et al., 2004). Glibenclamide induces a 24-mV depolarization in rat aortic SMCs (Mishra and Aaronson, 1999), and suppresses adenosine-induced NO release from aorta, suggesting K_{ATP} channel is functionally expressed in both aortic smooth muscle and endothelial cells (Ray and Marshall, 2006). The expression of K_{ATP} channel subunits could be altered in certain diseases. For example, aortic SMCs dissociated from diabetic rats displays a decreased SUR2B mRNA rather than Kir6.1 and Kir6.2 (Ren et al., 2003).

2.3.2. Metabolites

2.3.2.1. ATP/ADP

It is not strange that vascular K_{ATP} channels are regulated by ATP; however, the inhibitory effects of ATP on these channels are variable in different reports (Quayle et al., 1997). Since intracellular ATP concentration in physiological condition is relatively high (1-11.7 mM) (Randak and Welsh, 2005), vascular K_{ATP} channels usually show a low activity without other stimuli. In comparison, intracellular ADP concentration ranges between 0.1 and 3 mM (Randak and Welsh, 2005), and exhibits stimulating effect on K_{ATP} channel. According to this characteristic, vascular K_{ATP} channel was also named as K_{NDP} channel in early stage (Beech et al., 1993; Cole et al., 2000; Zhang and Bolton, 1996).

2.3.2.2. pH

pH controls regional blood flow in circulation, because increase in arterial carbon dioxide level (hypercapnia) and decrease in the extracellular pH result in acidosis, and sequentially relax blood vessels, especially cerebral arterioles (Kontos et al., 1977; Tian et al., 1995). Hypercapnic acidosis induces vasodilation through activation of K_{ATP} channels in vascular smooth muscles, with maximal effect at pH 6.5 to 6.8 (Wang et al., 2003). Blockade of K_{ATP} channels attenuated the vasodilation, which is observed in cerebral arterioles, basil artery, coronary artery, mesenteric artery or internal mammary artery (Ishizaka and Kuo, 1996; Kinoshita et al., 1997; Rohra et al., 2005; Santa et al., 2003; Wang et al., 2003; Wei and Kontos, 1999).

2.3.2.3. Reactive oxygen species (ROS)

Increased production of ROS, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO\cdot$), and impaired antioxidant defenses are two important features of

oxidative stress, which contributes to the development and progression of many types of diseases, such as diabetes, atherosclerosis and sepsis (A. C. Maritim, 2003; Cosentino et al., 1997; Giugliano et al., 1996) . The effect of ROS on K_{ATP} channel has been noticed recently. Cerebral arterioles treated with superoxide show a less vasodilation to cromakalim (Ross and Armstead, 2003). Pinacidil-induced vasodilation in cerebral arterioles is impaired in insulin-resistant rats fed with fructose, but is completely reversed by treatment with superoxide dismutase (SOD) and catalase (Erdos et al., 2004). Aprikalim-induced vasodilation in coronary arterioles is also attenuated in diabetic patients (Miura et al., 2003). Therefore, the ROS released in oxidative stress reduces vascular K_{ATP} activity.

2.3.2.4. NO

The effect of NO on vascular K_{ATP} channels is controversial. NO hyperpolarizes smooth muscle cells in rabbit mesenteric arteries through increasing cGMP and activating K_{ATP} channels (Murphy and Brayden, 1995). In addition, NO released from skeletal muscle vasculatures during excise may activate vascular K_{ATP} channels, and antagonizes sympathetic vasoconstriction, providing a delicate mechanism to regulate blood flow in exercising skeletal muscles (Thomas and Victor, 1998). Lactate, an important metabolic product in retina, relaxes retinal arterioles through activation of NOS and guanylyl cyclase, and K_{ATP} channel opening (Hein et al., 2006). However, the opposite observations show that NO donor sodium nitroprusside (SNP) fails to activate K_{ATP} currents isolated from rabbit mesenteric arterial smooth muscle cells and pig coronary arterial SMCs (Quayle et al., 1994; Wellman et al., 1998). Therefore, the exact role of NO in regulating vascular K_{ATP} channels is still unclear.

2.3.2.5. Eicosanoids

Epoxyeicosatrienoic acids (EETs) are cytochrome *P*-450 metabolites of arachidonic acid synthesized in endothelial cells (Rosolowsky and Campbell, 1996). Since EETs are participated in vasodilation by hyperpolarizing cell membrane, some groups classified them in endothelium-derived hyperpolarizing factors (EDHFs) (Archer et al., 2003; Campbell and Harder, 1999). Both 11, 12-EET and 14, 15-EET induce dose-dependent vasodilation in isolated small mesenteric arteries through activation of K_{ATP} channels (Bolon et al., 2007; Brueggemann et al., 2005), but the underlying mechanisms were different: 11, 12-EET extracellularly activates mesenteric SMC K_{ATP} channels through PKA (Bolon et al., 2007); whereas the stimulation of 14, 15-EET depends on ADP-ribosylation of G_s (Bolon et al., 2007).

2.3.2.6. H_2S

Hydrogen sulfide (H_2S) is a product from L-cysteine metabolism catalyzed by cystathionine-γ-lyase and cystathionine-β-synthase in mammalian tissues (Zhao and Wang, 2002; Zhao et al., 2001). Endogenous H_2S has been detected in various vascular tissues (e.g. aorta, tail, and mesenteric arteries) (Cheng et al., 2004). H_2S in physiological concentrations (nearly 45 μM) induces vasodilation in rat aorta and transient reduction of blood pressure through activation of K_{ATP} channels (Zhao and Wang, 2002; Zhao et al., 2001). Another study shows that exogenous H_2S activates K_{ATP} channels and hyperpolarizes cell membrane in rat mesenteric arterial SMC in a way independent of PKG (Tang et al., 2005).

Recently, a slow-releasing hydrophilic H_2S compound GYY4137 has been demonstrated to display vasorelaxing effect in rat endothelium-intact aortic rings and perfused rat renal vasculature through stimulation of vascular K_{ATP} channels (Li et al., 2008). Because GYY4137

reduces blood pressure in hypertensive rats without changing heart rate or contracting force *in vitro*, it could be a promising drug for anti-hypertension therapy in future.

2.3.3. Hormones and neurotransmitters

The effects of hormones and neurotransmitters on vascular K_{ATP} channels recently have been well studied (Table 2-3). An excellent review could be referred to the dissertation of Dr. Yun Shi from our group. Since their regulatory mechanisms are so significant, it is still worthy to provide a short summary.

Hormones or neurotransmitters are classified into two groups based on vasoactive functions: vasoconstrictors and vasodilators. The receptors of these substances are coupled to G_q and G_s respectively (Sperelakis, 1998). G_q activation stimulates phospholipase C (PLC), which catalyzes membrane phospholipids to produce diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG in turn activates protein kinase C (PKC). G_s activation increases the activity of adenylyl cyclase, which converts ATP to cAMP. The elevated cAMP level results in the release of the catalytic subunits of PKA. Vascular K_{ATP} channels are substrates of both PKC and PKA, but the phosphorylation mechanisms are quite different: PKC targets distal C-terminus of Kir6.1 subunit leading to channel inhibition (Shi et al., 2008b), whereas PKA acts on NBD2 of SUR2B subunit resulting in channel activation (Shi et al., 2008a; Shi et al., 2007b).

Table 2-3. Summary of hormones and neurotransmitters targeting vascular K_{ATP} channels

Hormone/Neurotransmitter		Receptor	Tissues	References
Vasoconstrictors	noradrenaline	α_2	rat tail artery	(Tan et al., 2007)
	ET-1	N/A	rabbit coronary and pulmonary arteries	(Park et al., 2005)
	Ang II	N/A	rat mesenteric artery	(Kubo et al., 1997)
	AVP	V_{1a}	rat mesenteric artery	(Shi et al., 2007a)
	neuropeptide Y	NPY	rabbit mesenteric artery	(Bonev and Nelson, 1996)
	serotonin	5-HT_2	rabbit mesenteric artery	(Bonev and Nelson, 1996)
	histamine	H_1	rabbit mesenteric artery	(Bonev and Nelson, 1996)
Vasodilators	adenosine	A_2	rat mesenteric artery, guinea pig coronary artery	(Kleppisch and Nelson, 1995a; Mutafova-Yambolieva and Keef, 1997)
	VIP	VPAC1	rat mesenteric artery	(Yang et al., 2008)
	CGRP	N/A	rabbit mesenteric artery, pig coronary artery	(Quayle et al., 1994; Wellman et al., 1998)

N/A, Not reported.

3. Significance

Despite the wide use of antibiotics, the incidence of sepsis has continued to increase in the past few decades. Indeed, sepsis is still a leading cause of death in the intensive care unit (ICU) and accounts for over $15 billion of medicare expenses annually in the United States (Martin et al., 2003). However, effective therapeutics are still unavailable, and the prognosis remains largely unpredictable. There are even controversies in antibiotics usage, as bacterial lysis tends to release more septic pathogens and worsen septic shock. Although the cytokine-directed immune response to sepsis has been intensively studied, accumulative evidence suggests that the cytokine response is an unsatisfactory index for the prognosis of sepsis. A similar stage of infection may be manageable in one individual but becomes deadly in another. The variable responses and vulnerabilities attribute to the genetic composition of individuals, in which the vascular K_{ATP} channel plays a role. K_{ATP} channels regulate resting membrane potentials and thus the activity of voltage-gated ion channels (Nichols, 2006). The voltage-gated Ca^{2+} channels are the chief mechanism for Ca^{2+} entry in VSMs, affecting vascular tones, blood pressure and local blood perfusion (Cribbs, 2006).

Although the K_{ATP} channel plays an important role in systemic responses to sepsis, it is unclear how the channel changes its activity or expression during endotoxemia: What intracellular signaling systems are activated and determine the vascular SMC response to endotoxemia? Do LPS have direct effect on the channel activity? How does the channel activity change with oxidative stress, an important pathophysiologic event during sepsis? Can the channel activity be modulated by vasoconstrictors known to be useful in sepsis management? This dissertation is thus aimed at addressing these questions with an objective to understand the regulatory mechanisms of vascular K_{ATP} channel by LPS. A combination of electrophysiological,

molecular biological and pharmacological approaches was adopted. Our data from these studies demonstrated that the expression of vascular K_{ATP} channels was up-regulated by LPS through NF-κB and CREB dependent pathways, and contributed to LPS-induced vascular hyporeactivity. Anti-septic vasopressor AVP elevated vascular tone by targeting PKC and K_{ATP} channels. H_2O_2, one of ROS which are produced during septic oxidative stress, impaired vasodilation through inhibiting Kir6.1/SUR2B channel. The mechanisms of the channel regulation elucidated in this dissertation provide information on the function of K_{ATP} channel during sepsis, and perhaps the development of new anti-septic therapeutics.

4. Material and Methods

4.1. Chemicals and cDNAs

Chemicals used in my studies were purchased from Sigma (St. Louis, MO) unless otherwise stated. All chemicals were prepared as high concentration stocks in double distilled H_2O or dimethyl sulfoxide (DMSO), and were diluted in the recording solution to experimental concentrations immediately before usage. In cases where DMSO was used, its concentration was maintained at less than 0.1% in the experimental solutions. AVP, glibenclamide and pinacidil were applied to cells using a perfusion system. To avoid ATP degradation, all ATP containing solutions were made immediately before experiments and used no longer than 4hrs.

Rat Kir6.1 (GenBank accession # D42145) and mouse SUR2B (GenBank accession # D86038) were cloned in a eukaryotic expression vector, pcNDA3.1, and used for mammalian cell expression. Human TLR4 (GenBank accession # NM_138554) and CD14 were cloned in pcDNA3 (GenBank accession # NM_000591) by Dr. Golenbock at University of Massachusetts. Human MD-2 (GenBank accession # NM_015364) was cloned in a mammalian expression vector pEFBOS by Dr. Sachiko Akashi-Takamura at the University of Tokyo. Human AVP receptor 1A with NH_2-terminal 3XHA tag (AVPR1A, GenBank accession no. AY322550) in pcNDA3.1 was purchased from http://www.cDNA.org (Rolla, MO).Wild-type V1a receptor was prepared by removing 3XHA-tag with PCR and was cloned into pcNDA3.1. Dynamin 2 wt -GFP, and dynamin 2/K44A-GFP were kindly donated from Dr. Mark McNiven (Mayo Clinic, Rochester, MN).

4.2. Cell culture

All types of cells were grown at 37 °C in a humidified atmosphere of 95% air and 5%CO_2, and were routinely split when cell density reached 90-100% confluence. Rat aortic SMCs (A10, CRL-1476, ATCC, Manassas, VA) were cultured as a monolayer in the DMEM with 10% fetal bovine serum. Human embryonic kidney cells (HEK293, CRL-1573, ATCC, Manassas, VA) were grown in the DMEM-F12 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin.

4.3. Transfection

The HEK-293 cells were used to express the K_{ATP} channels. Transfection was performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in which 0.7 μg Kir6.1, 2.1 μg SUR2B, 1.0 μg TLR4, 0.2μg MD2 and 0.2 μg CD14 were added to a 35-mm petri dish. To facilitate the identification of positively transfected cells, 0.4 μg pEGFP-N2 cDNA (Clontech, Palo Alto, CA) was added to the cDNA mixture. In AVP project, 1.8 μg vasopressin 1a (V1a) receptor cDNA was mixed with 0.7μg Kir6.1 and 2.1μg SUR2B per 35mm petri dish. Cells were dissociated from the monolayer using 0.25% trypsin ~24 h after transfection. A few drops of the cell suspension were added on to 5 x 5-mm coverslips in a 35-mm petri dish. The cells were then incubated in DMEM-F12 for 24–48 h before experiments.

4.4. Molecular biology

Site-specific mutations were performed using PCR. Pfu Ultra polymerase was purchased from Stratagene (La Jolla, CA). PCR cocktail preparation was shown in Table 9-1. Briefly, SUR2B cDNA (0.1μg) was denatured at 95 °C for 5 min followed by 18 cycles of 30 s at 95 °C, 30 s at 55°C, 25 mins at 68 °C, and a final elongation for 10 mins at 70 °C. For Kir6.1

mutagenesis, the extension step was changed to 16 mins at 70 °C. XL-blue competent cells were transformed with the PCR products. Plasmid DNA was purified using mini prep kit (Qiagen, Valencia, CA). The correct mutants were confirmed by DNA sequencing.

Table 4-1. PCR cocktail preparation

PCR cocktail Components	Volume (µl)	Concentration
Forward Primer	0.5	50 µM
Reverse Primer	0.5	50 µM
Plasmid DNA	1	0.1 µg/µl
Pfu Ultra	2	
10X Pfu buffer	5	
DMSO	2.5	
dNTPs	1	10 mM
H_2O	37.5	
Total	50	

4.5. Mesenteric arterial rings

Mesenteric arterial rings were obtained from Sprague-Dawley rats (250–350 g) in accordance with the guidelines for the care and use of laboratory animals by Georgia State University and Harbin Medical University. The rats were anesthetized by inhaling saturated halothane vapor followed by cervical dislocation. The mesenteric arteries were dissected free and transferred to ice-cold Krebs solution containing (in mM): 118.0 NaCl, 25.0 $NaHCO_3$, 3.6 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 11.0 glucose, and 2.5 $CaCl_2$. The arteries were cut into 6–8 endothelium-intact rings of 2 mm in length and stored in Krebs solution. Endothelium-denuded rings were also used in which the endothelium was removed by a rough plastic tube and tested by the loss of response to acetylcholine. During the experiment, a ring was mounted on a force-electricity transducer (Model FT-302, iWorx/CBSciences, Dover, NH) in a tissue bath. With a 0.5 g preload, the ring was allowed to equilibrate in the tissue bath for 30min when the tension was reduced to ~0.3 g. The tissue bath was filled with Krebs solution and perfused with 5% CO_2

at 36°C. Arterial tone was measured as changes in isometric force. Only rings that showed a clear vasoconstriction response to 1.0 μM phenylephrine were used in the study.

4.6. Acute dissociation of vascular smooth myocytes

All animal experiments complied with the Institutional Animal Care and Use Committee approval of the Georgia State University. Mice (15–20 g) were anesthetized by inhalation of saturated halothane vapor followed by cervical dislocation. The aorta was dissected free, cut into small segments (1 mm), and placed in 5-ml solution containing (in mM): 140 NaCl, 5.4 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 10 HEPES, and 10 D-glucose at room temperature for 10 min. The tissues were then placed in 1-ml solution with 20 units of papain (Worthington) and 1.25 mg DTT. After digestion for 15 min at 35°C, the tissue was washed once and then transferred to 1-ml solution containing 440 units of collagenase (CLS II; Worthington) and 1.25 mg trypsin inhibitor (Sigma) for 5-10 min. After being thoroughly washed, the tissue was moved to 1-ml solution containing 20% fetal bovine serum and triturated with a fire-polished Pasteur pipette to obtain single smooth muscle cells. The cells were stored on ice and used within 8 h. A drop of cells was put in a 35-mm tissue culture dish and the cells were allowed to attach to the surface in 15 min. The cells that had clear smooth muscle morphology and did not show evident swelling or shrinkage were used for patch studies.

4.7. Patch clamping

Patch-clamp experiments were performed at room temperature as described previously. In brief, fire-polished patch pipettes with resistance of 40-50 MΩ were made with 1.2-mm borosilicate glass capillaries. Whole cell recording was performed in single-cell voltage clamp. Current records were low-pass filtered (2 kHz, Bessel 4-pole filter, –3 dB), digitized (20 kHz, 16-bit resolution), and stored on a computer hard drive for later analysis using the Clampfit 9

software (Axon Instruments). The bath solution contained (in mM): 10.0 KCl, 135.0 potassium gluconate, 5.0 EGTA, 5.0 glucose, and 10.0 HEPES (pH = 7.4). The pipette solution contained (in mM): 10.0 KCl, 133.0 potassium gluconate, 5.0 EGTA, 5.0 glucose, 1 K_2ATP, 0.5 NaADP, and 10.0 HEPES (pH = 7.4), in which the free Mg^{2+} concentration was adjusted to 1 mM using a $[Ca^{++}]/[Mg^{++}]$ calculation software. For membrane potential measurement from aortic smooth muscle cells, bath solution contained (in mM): 3.0 KCl, 140.0 NaCl, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 10.0 glucose, and 10.0 HEPES (pH = 7.4 with NaOH). Pipette solution is the same as that used in whole cell patch clamping.

Single-channel conductance was measured with slope command potentials from 100 to –100 mV. The open-state probability (P_o) was calculated by first measuring the time t_j spent at current levels corresponding to $j = 0, 1, 2, \ldots N$ channels open, based on all obvious openings during the entire period of recording. P_o was then obtained as

$$P_o = \left(\sum_{j=1}^{N} t_j j \right) / T \cdot N,$$

where N is the number of channels active in the patch, and T is the duration of recordings. P_o values were calculated from one to three stretches of data of 20 s each acquired with Clampex 8 (Axon Instruments). In this study, we used NP_o instead of P_o to express overall channel activity in which the number of openings was not counted.

4.8. Reverse transcription PCR

Total RNA was extracted from mouse aortic tissues with an RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. cDNA was reversely transcribed from total RNA in a 20-µl reaction containing 200 U Superscript II Reverse Transcriptase (Invitrogen), 0.5 µg oligo$(dT)_{12}$-

$_{18}$ primers, 0.5 mM dNTPs, 40 U RNaseOut and 10 mM DTT. The RT product was treated with 5 U RNase H for 20 mins.

For PCR analysis of K_{ATP} channel subunits, we designed primers targeting the mRNA sequence of K_{ATP} channel subunits (Table 4-2). PCR was performed in a Perkin Elmer GeneAmp 2400 in a final volume of 50 μl including 1μl of the RT product, 1.25 units of GoTaq DNA polymerase (Promega, Madison, WI, USA), 250 μM dNTP, 2.5 μl DMSO and 0.5μM primers. RT product from aortic tissues was denatured at 95 °C for 5 min followed by 30 cycles of 45 s at 95 °C, 45 s at 52 °C, 75 s at 72 °C, and a final elongation for 10 min at 72 °C. 5 μl of PCR products were separated by electrophoresis on a 2% agarose gel and visualized with ethidium bromide under UV fluorescence.

Table 4-2. RT-PCR primers

Target Gene	Primers	Accession No.	Size (bp)
Kir6.1	Fw: TGGCTGCTCTTCGCTATC Re: GGGCTACGCTTGTCAATC	NM_008428	578
Kir6.2	Fw: AGGGCATTATCCCTGAGG Re: GCGTTGATCATCAGCCC	NM_010602	569
SUR2B	Fw: GAAGTCCTCCTTATCCCTGG Re: ACGGACAAACGAGGCAAAC	NM_011511	592
GAPDH	Fw: TGCTGAGTATGTCGTGGAG Re: ACCAGGAAATGAGCTTGAC	NM_008084	668

m, mouse; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

4.9. Real time PCR

Real time PCR was performed with an Applied Biosystems 7500 Fast Real-Time PCR system. Primers were specifically designed by using Applied Biosystems Primer Express 3.0 and listed in Table 4-3. The specificity of the primers was confirmed with a BLAST program. Each 20-μl reaction contained 1x Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 0.2 μM

forward and reverse primers, 0.04 µl ROX reference dye, and 0.4 µl of cDNA. Thermal cycling conditions included an initial UNG incubation at 50°C for 2 min, Platinum *Taq* DNA polymerase activation at 95°C for 2 min, 40 cycles of denaturing at 95°C for 3 s, and annealing and extension at 60°C for 30 s, followed by routine melting curve analysis. Relative quantitation (RQ) of target gene expression was calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The first step in the RQ analysis is to normalize target gene expression level to GAPDH (ΔC_t). The second step is to compare the difference between the normalized target gene expression in LPS-treated and untreated samples ($\Delta\Delta C_t$). Each experiment was repeated 3-4 times in four samples.

Table 4-3. Real time PCR primers

Target Gene	Primers	Accession No.	Size (bp)
Kir6.1	Fw: CGCAAACCCGAGTCTTCTAGGA Re: CCTGGCCAACATCTTCCTTTCAC	NM_008428	101
Kir6.2	Fw: GCCCTGCGTCACAAGCA Re: GGACCTCGATGGAGAAAAGGA	NM_010602	39
SUR2B	Fw: CCATAGCTCATCGGGTTCACA Re: CGGACAAACGAGGCAAACAC	NM_011511	133
GAPDH	Fw: CCAGCCTCGTCCCGTAGA Re: TGCCGTGAGTGGAGTCATACTG	NM_008084	179

4.10. Nuclear protein extraction and Western blotting

A10 cells in 90-100% confluence were rendered quiescent in DMEM with 0.5% FBS for 6 h before experiments. Nuclear proteins were extracted using Nuclear Extract kit (Active motif, Carlsbad, CA). Protein concentration was determined by BCA assay (Pierce, Rockford, IL). The nuclear proteins in Laemmli sample buffer were boiled in 95-100°C 5 minutes. The samples (50

μg) were loaded onto each well, separated on 10% SDS-PAGE and transferred onto PVDF membranes (Bio-RAD, Hercules, CA). Nonspecific binding sites were blocked by 1-h incubation of the membranes in TBST/5% nonfat milk, followed by blotting with primary antibodies diluted in TBST/5% BSA overnight. Most primary antibodies were purchased from Cell Signaling Technology (Boston, MA). Anti-p65 IgG was provided by Santa Cruz Biotechnology (Santa Cruz, CA). The membrane was then incubated in horseradish peroxidase (HRP)-conjugated secondary antibody ($1:10^4$, Jackson immunoresearch), and detected by SuperSignal ECL substrate (Thermo scientific, Rockford, IL) according to the manufacturer's instruction.

4.11. PKC phosphorylation site prediction

PKC phosphorylation sites were predicted using two online programs *Kinasephos*, (http://kinasephos.mbc.nctu.edu.tw/) (Huang et al., 2005) and *NetPhosK* (http://www.cbs.dtu.dk/services/NetPhosK) (Blom et al., 2004). A serine or threonine was considered for further studies as a putative PKC site if there were basic amino acids at $^{+}2$ or $^{+}3$ position.

4.12. Data analysis

Data are presented as the mean ± S.E. (Standard error) of each group. Differences in means were tested with single factor ANOVA or Student's *t* test, and were accepted as significant if $P \leq 0.05$.

5. Result 1: Lipopolysaccharides Upregulate Kir6.1/SUR2B Channel Expression and Enhance Vascular K_{ATP} Channel Activity via NF-κB and PKA Dependent Signaling Pathway

Note that Ms. Shuang Zhang and Dr. Ningren Cui performed vascular ring studies. Dr. Ningren Cui, Mr. Yang Yang and Ms. Zhongying Wu conducted the patch clamping study. The rest of the work (estimated to be ~80%) was done by Weiwei Shi. Ms. Honyu Gai provided some technical assistance.

5.1. Abstract

Sepsis is a major cause of deaths worldwide. Recent studies indicate that the septic susceptibility is attributable to the vascular K_{ATP} channel. However, the mechanisms underlying the channel modulation during sepsis are still unclear. Here we show how septic pathogen LPS elevate the K_{ATP} channel activity and expression. In isolated mesenteric arterial rings, phenylephrine (PE) induced concentration-dependent vasoconstriction that was relaxed by pinacidil, a selective K_{ATP} channel opener. The PE response was mostly eliminated after a pretreatment with LPS. In acutely dissociated aortic smooth myocytes the LPS treatment augmented K_{ATP} channel activity. Quantitative PCR analysis showed that LPS raised Kir6.1 and SUR2B transcripts in a concentration-dependent manner, which was suppressed by a transcriptional inhibitor actinomycin D and translational inhibitor actidione. The LPS effects were abolished with an exposure to either NF-κB inhibitors or PKA blockers. PKA was needed for the maintenance of basal phosphorylation of K_{ATP} channels and CREB, while the CREB expression was NF-κB-dependent. LPS had no direct effect on the Kir6.1/SUR2B channel transiently expressed in HEK-293 cells. Thus, the effect of LPS on vasodilation involves upregulation of K_{ATP} channel expression, in which a NF-κB and PKA dependent signaling plays a central role.

5.2. Introduction

Sepsis is a severe medical condition caused by several septic pathogens including LPS, leading to lethal cardiovascular dysfunction and death (Hotchkiss and Karl, 2003; Merx and Weber, 2007). Accumulating evidence indicates that cardiovascular responses to septic pathogens are rather diverse and the prognosis can be very different between individuals. However, the underlying mechanisms for the sepsis susceptibility are unclear (Hotchkiss and Karl, 2003). Recent studies indicate that functional integrity of vascular K_{ATP} channels is a crucial factor for the sepsis susceptibility (Croker et al., 2007; Kane et al., 2006).

Vascular K_{ATP} channels are members of inward rectifier K^+ channel (Kir) family. The Kir6.1/SUR2B is the major isoform in vascular smooth muscles (VSM). The VSM K_{ATP} channel is modulated by several vasoactive hormones and neurotransmitters, such as α and β adrenergic receptor agonists, anginotension II, arginine vasopressin, adenosine, calcitonin gene-related peptide, vasoactive intestinal polypeptide, etc. (Bonev and Nelson, 1996; Nelson et al., 1990; Shi et al., 2007a; Shi et al., 2008a; Yang et al., 2008). In addition, metabolites pH, ATP, ADP, EETs, H_2S are important K_{ATP} channel regulators (Kamouchi and Kitamura, 1994; Tang et al., 2005; Wang et al., 2003; Ye et al., 2005). Genetic knockout of either subunit of the vascular K_{ATP} channel leads to spontaneous coronary vasospasm and sudden death, consistent with their function in vascular tone regulation (Chutkow et al., 2002; Miki et al., 2002).

The vascular K_{ATP} channel plays a role in septic susceptibility. Studies with genome-wide chemical mutations have led to an identification of four strains of mice that are highly vulnerable to various septic pathogens (Croker et al., 2007). All of these mice carry a null mutation of Kir6.1 gene (*Kcnj8*), which is caused by a deletion of exon 1 and part of exon2, and an inversion of intron 2. Consistently, mice with Kir6.1-knockout exhibit cardiovascular abnormalities with a

high mortality when exposed to a sub-lethal dose of LPS (Kane et al., 2006). Although these recent studies indicate that the vascular K_{ATP} channel is an important player in systemic responses to sepsis, how the channel is affected by LPS remains unclear. Does the channel activation result from changes in channel protein expression or functional activity? Is the channel a direct target of LPS? What are the intracellular signaling pathways critical for the channel activation by LPS? To address these questions, we performed this study.

5.3. Results

5.3.1 K_{ATP} channels in the LPS-induced vascular hyporeactivity to vasoconstrictor

Vascular responses to LPS exposure (from *Escherichia coli* 0127:B8) were studied in isolated perfused rings from mesenteric arteries. Endothelium was mechanically removed immediately before mounting. The endothelium elimination was confirmed as the ring failed to respond to 1μM acetylcholine (Yang et al. 2008). The ring was mounted on a force-electricity transducer with a 0.3g preload and allowed to equilibrate for a half of an hour before the experiment. Isometric contraction was produced by exposures to the adrenergic α-receptor agonist phenylephrine (PE). PE produced concentration-dependent constrictions of the rings with the maximum effect reached at ~10μM (Figure 5-1, A). At the peak constriction, pinacidil, a K_{ATP} channel opener, relaxed the vasoconstriction almost completely, suggesting that the K_{ATP} channel was involved (Figure 5-1, A). The PE-induced contraction was significantly suppressed following an exposure to LPS. The LPS effect had a clear concentration-dependent pattern. The vascular reactivity to 1μM PE was reduced ~60% with 0.1μg/ml LPS, and almost completely lost with an exposure to 1μg/ml LPS (Figure 5-1, B). These concentrations of LPS have been previously shown to be relavent to sepsis *in vivo* (Connelly et al., 2005; Fujita et al., 2006).

5.3.2. Activation of K_{ATP} channels with LPS exposure

Membrane potentials of SMCs freshly dissociated from the mouse aorta were examined in whole-cell current clamp. With physiological concentration of K^+ in the bath and pipette solutions, the SMCs had a resting membrane potential -56.4±3.8 mV (n=10). The effect of LPS on membrane potentials was studied in two groups of cells with one group treated with 1μg/ml LPS and the other with the solvent vehicle (Figure 5-1, C). Although no significant changes in membrane potentials were seen between these groups at 0, 2 and 6 hrs of exposures (P>0.05, n=9 to 11 for each time point), the membrane potential at 16 hrs was more hyperpolaried in the LPS-treated group than in the vehicle-treated (-60.1±3.5 mV, n=6, vs. -46.5±4.9 mV, n=6; P<0.05).

Subsequently, K^+ currents were studied in whole-cell voltage clamp. A high concentration of K^+ (145mM) was applied to the bath and pipette solutions, and membrane potential of the cell was held at 0 mV with step hyperpolarizing pulses to -80mV applied to the cell (Shi et al., 2007a). Under this condition, the aortic SMCs exhibited small basal currents upon formation of the whole-cell configuration (72.1±10.5 pA, n=21, Figure 5-2, A, E). Pinacidil (10 μM) augmented the currents by 44% (104.2±16.1 pA, n=21, Figure 5-2, A). The pinacidil-activated currents were strongly inhibited by 10 μM glibenclamide (50.0±9.0 pA, n=18, Figure 5-2, A, C), consistent with the expression of functional K_{ATP} channels in the SMCs. After a treatment of the SMCs with 1μg/ml LPS overnight, the basal currents increased by 43% (103.6±15.2 pA, n=17; Figure 5-2, B, E). The current amplitude was further activated by another45% (150.9± 29.5 pA, n=17; Figure 5-2, B, D) in the presence of pinacidil. These results therefore indicate that the SMC-endogenous K_{ATP} currents are augmented with LPS exposure.

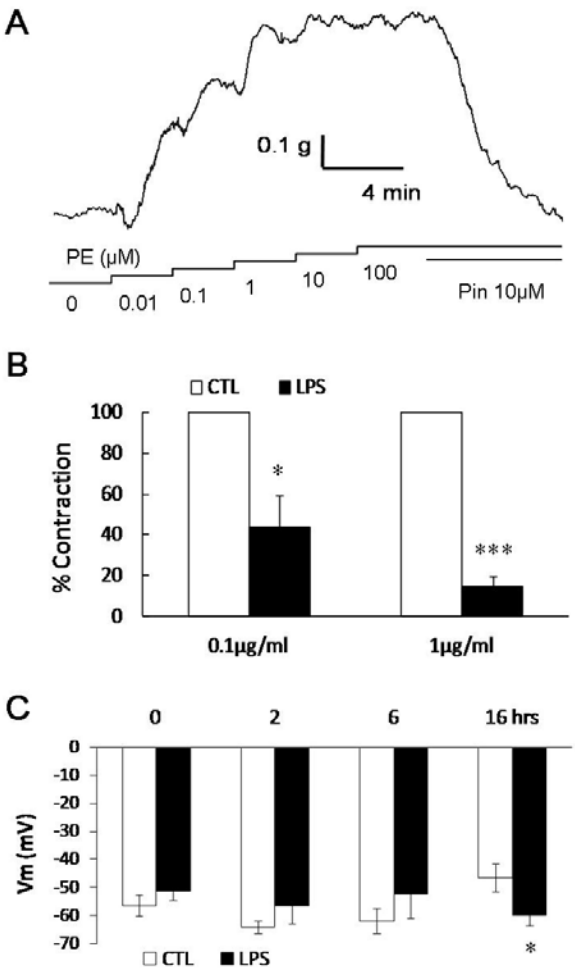


Figure 5-1. K_{ATP} channels play a role in the vascular responses to LPS treatment.

A. An isolated rat mesenteric arterial ring was exposured to graded concentrations of PE with 3 mins for each dose. PE produced a concentration-dependent vasoconstriction. At the peak contraction, the K_{ATP} channel opener pinacidil relaxed the ring almost completely in the presence of 100μM PE within 5 mins. The relationship was described using the Hill equation with EC50 2.2μM. **B.** The PE (10 μM) contractility was studied in endothelium-denuded rings. The contractility was calculated as a percentage by dividing the contractile force in LPS-treated rings to the force in control group. The vascular reactivity was decreased by 56.2% (from 0.16 ± 0.02 g, n=5, to 0.07 ± 0.03 g, n=7, $P<0.05$) after a pretreatment with LPS (0.1μg/ml) for 20 hrs. LPS (1μg/ml) further attenuated the vascular contraction by 85.2% (from 0.17 ± 0.03 g, n=13, to 0.02 ± 0.01 g, n=11, $P<0.001$). Data are presented as means ± S.E. **C.** Membrane potentials (Vm) were studied in vascular smooth muscle cells (SMCs) freshly dissociated from the mouse aorta. The effect of LPS on membrane potentials was studied with different exposure time to 1μg/ml LPS in these SMCs. Significant hyperpolarization occurred with a 16 hr exposure (*, $P<0.05$, n=10).

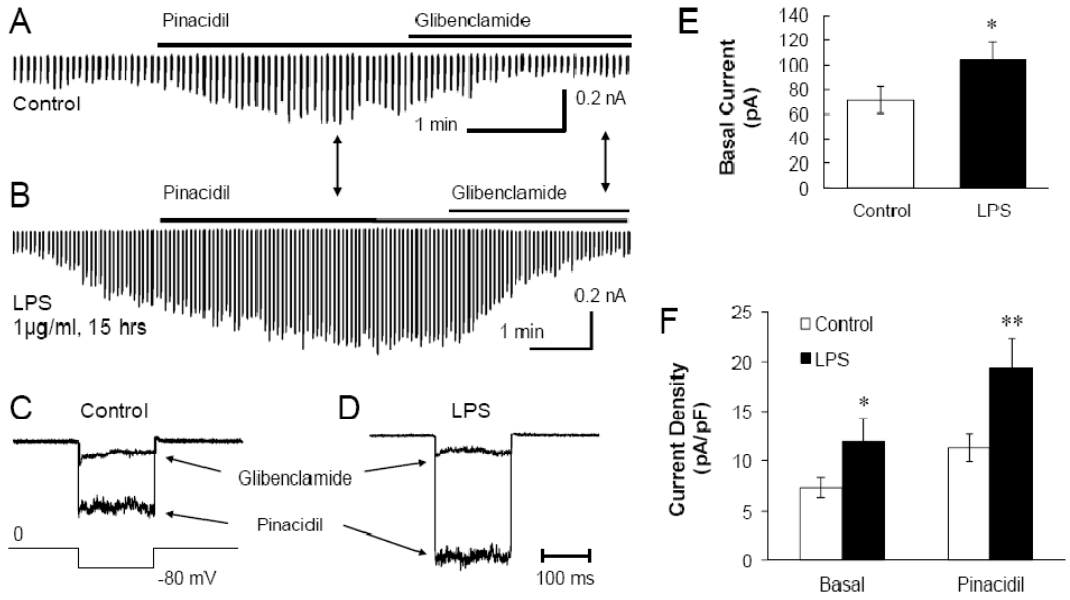


Figure 5-2. Augmentation of K_{ATP} currents with LPS incubation.

Whole-cell voltage clamp was performed in freshly dissociated aortic SMCs. The bath solutions contained 145 mM K^+. The same solution was used in the recording pipette with addition of 1 mM ATP, 0.5 mM ADP, and 1 mM free Mg^{++}. **A.** In a control experiment, small inward currents were seen upon the formation of the whole-cell configuration. The currents were increased by pinacidil (10μM). The maximal activation was reached in 2 min, while glibenclamide (10μM) reduced the currents to a level even below the baseline. **B.** The pinacidil- and glibenclamide-sensitive currents were studied in another SMC that had been treated with LPS (1μg/ml) overnight. The current amplitude increased significantly after the whole-cell patch formation, presumably produced by intracellular dialysis of ADP and Mg^{++}. The currents were further augmented by pinacidil, reaching a peak that doubles that without LPS treatment in A. The pinacidil-activated currents were completely suppressed by glibenclamide. **C. D.** Individual currents produced by a single command pulse were displayed. The currents were taken from the areas indicated by arrows in A and B, respectively. **E.** The effect of LPS on basal currents. After LPS treatment (1μg/ml) overnight, the basal currents were increased from 72.1±10.5 pA to 103.6±15.2 pA (n=21 and 17, respectively. *, $P<0.05$). **F.** Enhancement of current density with LPS treatment. The current density was calculated by dividing the current amplitude by the whole-cell capacitance of each cell. The K_{ATP} currents were isolated by subtracting the currents with glibenclamide (10μM) treatment from the currents with pinacidil (10μM) treatment. After a LPS (1μg/ml) exposure overnight, the density of basal currents increased by 50% (n=21, * $P<0.05$), while the K_{ATP} current density was elevated by 69% (n=17, ** $P<0.01$).

5.3.3. LPS elevated surface expression of vascular K_{ATP} channel

The augmentation of K_{ATP} channel activity may result from an upregulation of the channel expression, post-translational modulation of channel activity (e.g., channel protein phosphorylation by PKA) or both. We therefore undertook experiments to test these possibilities. To show the effect of LPS exposure on the surface expression of K_{ATP} channels, we analyzed the K_{ATP} channel density in dissociated aortic SMCs. In the experiment, the K_{ATP} currents were isolated first by subtracting the currents with the glibenclamide (10μM) treatment from the currents with the pinacidil (10μM) treatment. The isolated currents were then divided by whole-cell capacitance to get the current density. The basal density of the pinacidil and glibenclamide-sensitive currents was 11.4±1.4 pA/pS (n=21, Figure 5-2, F). After a treatment of the cells with LPS (1μg/ml) overnight, the current density was 19.3±3.1 pA/pS (n=17), which was 69% greater than that before LPS exposure (P<0.01; Figure 5-2, F).

5.3.4. Concentration-dependent stimulation of Kir6.1/SUR2B transcription

At the mRNA level, the expression of Kir6.1, Kir6.2 and SUR2B was studied in mouse aorta. RT-PCR with the mRNAs extracted from cultured endothelium-denuded aortic rings showed that the levels of Kir6.1 and SUR2B transcripts were significantly higher in the LPS-treated groups than in the control group (Figure 5-3, A). In contrast, the Kir6.2 mRNA expression did not show any evident change (Figure 5-3, A).

Quantitative PCR analysis showed that LPS (1 μg/ml, 20 hrs) enhanced Kir6.1 transcripts by ~2.9 fold, and SUR2B by 1.5 fold (P<0.001, n=47 and 49, respectively; Figure 5-3, B), whereas Kir6.2 transcripts did not show significant increase (P>0.05, n=32). The effect of LPS on Kir6.1 and SUR2B expressions had a clear concentration-dependence (Figure 5-3, C. D). LPS

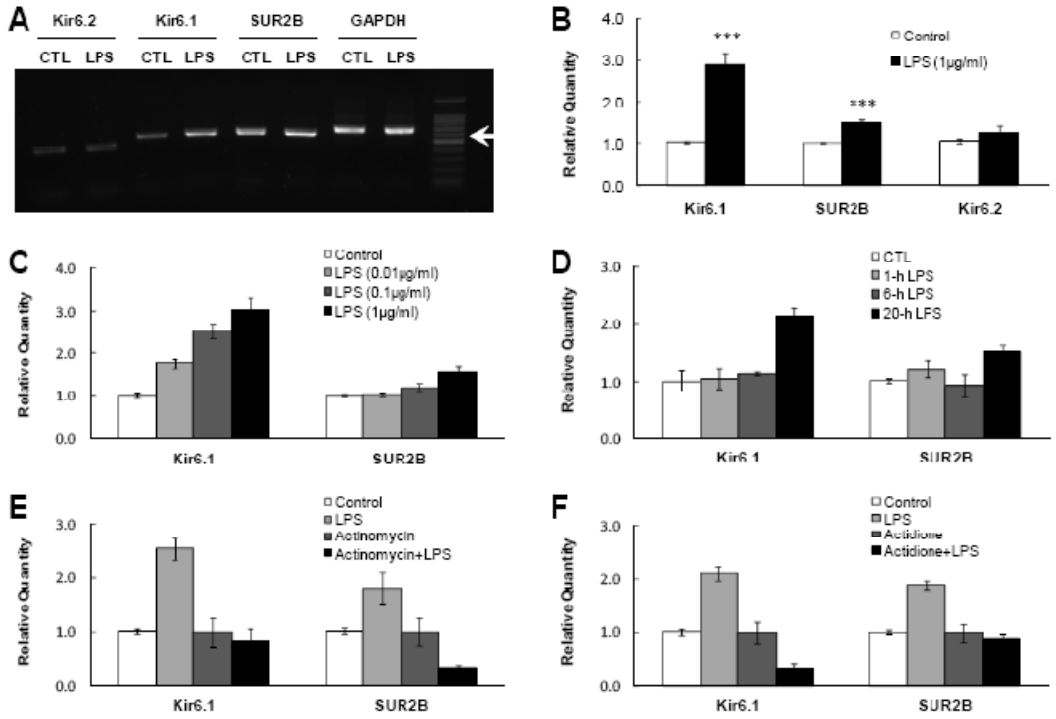


Figure 5-3. Augmentation of K_{ATP} mRNA expression after LPS exposure.

A. Total RNAs were extracted from dissociated SMCs from the mouse aorta after 20h incubation with or without LPS, and were subjected to RT-PCR. In the LPS-treated groups (LPS), transcripts of Kir6.1 and SUR2B were both enhanced in comparison with control (CTL), while the Kir6.2 expression did not change significantly. Note that the arrow indicated a size of 600bp. **B.** Real time PCR was performed to quantify K_{ATP} channel expression. The expression levels of target genes were normalized to the GAPDH mRNA level using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). LPS (1 μg/ml, 20 hrs) increased Kir6.1 transcripts by ~2.9-fold, and SU2B by ~ 1.5-fold (***, $P<0.001$, n=48 and 50, respectively), whereas Kir6.2 did not show a significant increase ($P>0.05$, n=33). Data were obtained from 14 independent experiments with 2-4 samples in each. **C.** LPS stimulated Kir6.1 expression in a concentration-dependent manner. Data were collected from 3 independent experiments with 3-4 samples in each. **D.** Time dependence. A clear upregulation of Kir6.1 and SUR2B mRNA expression was observed with LPS (1 μg/ml) exposure at 20 hrs but not at 1 and 6 hrs. **E.** The expression of K_{ATP} channel in LPS-treated groups was measured and normalized to the vehicle control. After a 20-h treatment with actinomycin D (2 μg/ml, added 1 h before applying 1 μg/ml LPS), the enhancement of Kir6.1 and SUR2B expression was totally eliminated. Data were obtained from 3 independent experiments with 3-4 samples in each. **F.** Similar results were obtained with a 20-h treatment with actidione (2 μg/ml). The data were collected from 2 independent experiments with 4 samples in each.

raised Kir6.1 mRNA expression by 1.8 fold in a concentration as low as 0.01μg/ml, and further stimulated the Kir6.1 expression by 2.5 and 3.0-fold in 0.1 and 1 μg/ml, respectively. In the concentration 0.01 μg/ml, LPS did not exhibit stimulatory effect on SUR2B. A small effect was seen with 0.1 μg/ml LPS, while LPS in 1 μg/ml increased SUR2B mRNA by 1.6-folds (Figure 5-3, C). The upregulation of Kir6.1 and SUR2B expression occurred at ~20 hrs of LPS (1 μg/ml) exposure, while no significant increase in Kir6.1 and SUR2B expression was found at 1 hr and 6 hrs (Figure 5-3, D). Therefore, a 20-h treatment with 1 μg/ml LPS was adopted in further studies.

5.3.5. Involvement of both transcriptional and translational mechanisms

Actinomycin D (2 μg/ml), an RNA polymerase II inhibitor that binds DNA at the transcription initiation complex and blocks RNA elongation (Sobell, 1985), totally eliminated the LPS-induced Kir6.1 and SUR2B expression (Figure 5-3, E). A pretreatment of aortic tissues with actidione (cycloheximide, 2 μg/ml), a protein synthesis inhibitor that binds specifically to the 60S subunit of eukaryotic ribosome (Clotworthy and Traynor, 2006), also blocked the LPS-inducted Kir6.1 and SUR2B expression (Figure 5-3, F). These suggest that both transcriptional and translational mechanisms are required for the LPS effects.

5.3.6. Necessity of NF-κB

Since NF-κB is a critical player in the TLR-4 signaling pathways activated by LPS, it is possible that LPS enhance the K_{ATP} channel expression via the NF-κB pathway. Indeed, we found that p65, a subunit of NF-κB, displayed a strong nuclear accumulation after a 30-min LPS treatment. The nuclear accumulation returned to nearly the basal level after 20 hrs (Figure 5-4, A). Then the NF-κB pathway was further studied using specific NF-κB inhibitors. We tested dimethyl fumarate (DMFR, 100 μM), a NF-κB inhibitor that blocked the nuclear entry of p65 after its release from IκB (Loewe et al., 2002), and pyrrolidine dithiocarbamate (PDTC, 100 μM)

that prevents phosphorylation of IκB (Zhong et al., 1997). One of them was added to aortic tissues 1 h before LPS administration. After overnight incubation with either of the NF-κB inhibitors, the LPS-induced Kir6.1 and SUR2B expressions were strongly suppressed (Figure 5-4, B-D), indicating that the NF-κB signaling is necessary for the K_{ATP} channel upregulation.

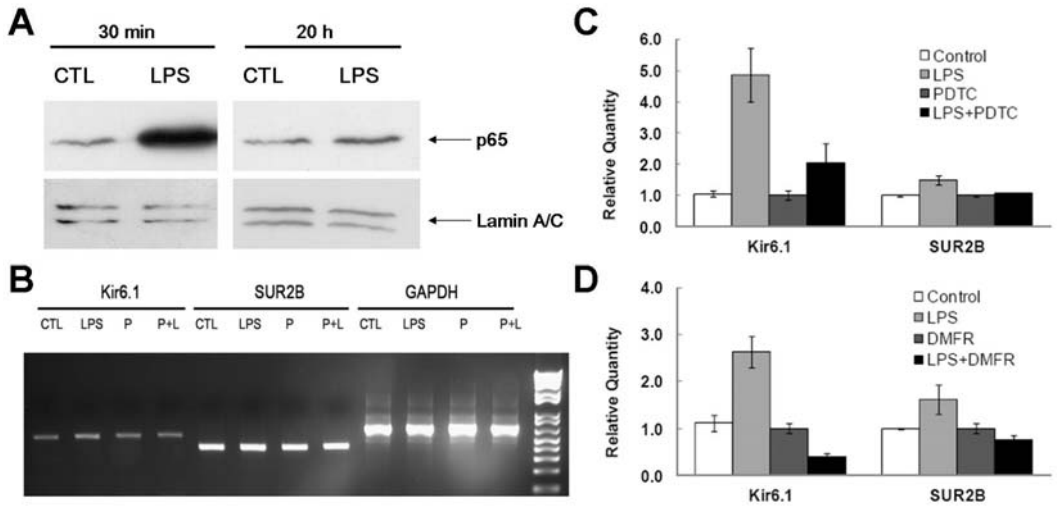


Figure 5-4. Role of NF-κB in the LPS-induced K_{ATP} channel expression.

A. The A10 aortic smooth muscle cells were cultured in the presence or absence of LPS (1μg/ml). Nuclear extracts from the A10 cells were separated on 10% SDS-PAGE and transferred to PVDF membrane. Western blot analysis was performed using anti-p65 antibody. Lamin A/C was used as a loading control. The p65 was accumulated in nucleus with 30-min LPS stimulation. The p65 nuclear accumulation returned to the basal level with a 20 h-LPS treatment. **B.** PDTC attenuated LPS-induced Kir6.1 and SUR2B mRNA expression. A representative RT-PCR was exhibited for the aortic tissues treated with LPS and PDTC. PDTC was applied 1 hour before LPS treatment. The upregulation of Kir6.1 following a 20-h exposure of LPS (L, 1 μg/ml) was blocked by PDTC (P, 0.1 mM). Although SUR2B expression was slightly stimulated by LPS, the upregulation still can be inhibited by PDTC. **C. D.** Total RNAs were subject to real time PCR after 20-h incubation with or without LPS. PDTC (0.1 mM, Figure 5-4, C) or DMFR (0.1 mM, Figure 5-4, D) was applied to the tissue 1 h before LPS administration. The expression of Kir6.1 and SUR2B in the LPS-treated groups was strongly suppressed with either PDTC or DMFR treatment. The data were collected from 3-4 independent experiments with 3 samples in each.

5.3.7. PKA and CREB signaling

The enhanced Kir6.1 and SUR2B expression occurred with 20-h LPS exposure when the nuclear accumulation of NF-κB had declined significantly, suggesting that other intracellular signaling systems may also be involved. Previous studies have shown that LPS exposure augments adenylate cyclase activity in cultured macrophages (Osawa et al., 2006), and PKA plays a role in the LPS-induced suppression of L-type Ca^{++} currents in ventricular myocytes (Zhang et al., 2007). To determine whether PKA activity indeed changes with LPS exposure, we performed PKA activity assay. LPS (1μg/ml) did not increase PKA activity with either 30 min or 20 hr incubation (Figure 5-5, A). However, LPS-induced vascular hyporeactivity was abolished in the presence of a PKA inhibitor Rp-adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMP, 50 μM) (Figure 5-5, B). In addition, blockade of PKA with KT5720 (1 μM) or Rp-cAMP (100 μM) suppressed the LPS effect on the Kir6.1 and SUR2B mRNA upregulation (Figure 5-5, C-E), suggesting the basal PKA activity is extremely important for the effect of LPS.

Three potential mechanisms may allow PKA to enhance Kir6.1/SUR2B channel activity: 1. the channel expression is upregulated by CREB, a well-known PKA-dependent transcriptional factor (Mayr and Montminy, 2001); 2. PKA phosphorylates NF-κB leading to a stronger NF-κB activity (Yoon et al., 2008; Zhong et al., 1997), which may occur during endotoxemia; and 3. Kir6.1/SUR2B channel activity may rise as a result of enhanced channel protein phosphorylation by PKA (Shi et al., 2007b). Experiments were carried out to test each of the possibilities. We found that LPS clearly enhanced the CREB level in the nuclear extracts of A10 aortic SMCs with a 20-hr incubation (Figure 5-6, A). Meanwhile, the level of phospho-CREB was also elevated with LPS exposure (Figure 5-6, A), which is likely due to phosphorylation of the excessive CREB by basal PKA activity. Thus, the enhanced CREB level appeared to be another major

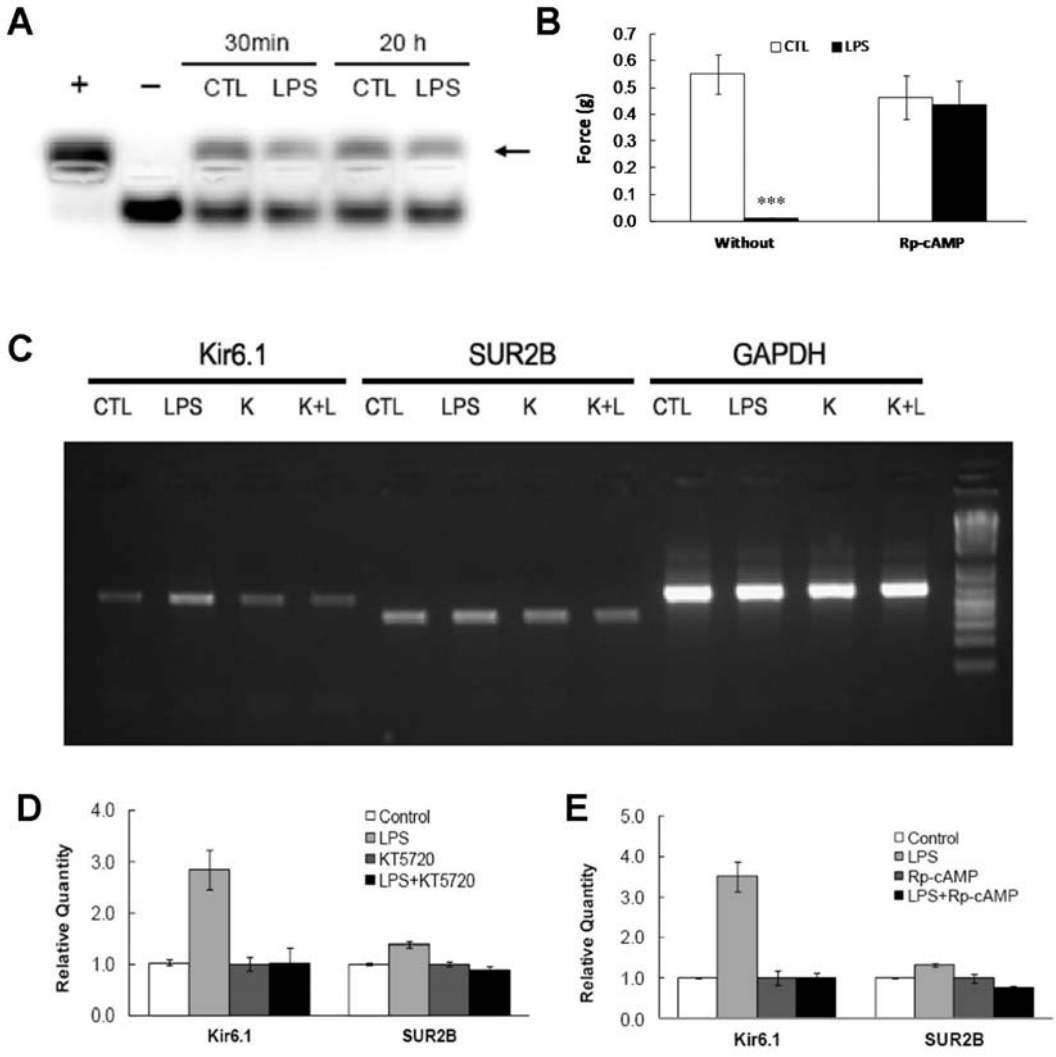


Figure 5-5. Involvement of PKA in the LPS-induced upregulation of K_{ATP} channel expression.

A. PKA assay. Whole cell lysates from A10 cells were collected with and without LPS treatment (1 μg/ml). Protein concentration was determined by the BCA assay. The whole cell extracts (10 μg) were incubated with the Peptag A1 peptide. The phosphorylated peptide migrated toward the anode labeled with an arrow, whereas the non-phosphorylated peptide moved in the opposite direction. PKA catalytic subunit (10 ng) was used as a positive control. LPS exposure (30 min or 20h) did not increase PKA activity in the A10 cells. **B.** Vascular contractility was studied as described in Figure 5-1. Rp-cAMP (50 μM) was added 1 h before applying LPS (1 μg/ml). PE (1μM) increased the contractile force to 0.55±0.07 g (n=10). A 20h LPS exposure strongly suppressed PE (1μM)-induced vasoconstriction (0.01±0.00 g, n=8. *** P<0.001). With the Rp-cAMP treatment, the effect of LPS was almost totally abolished (P>0.05, n=5). C. KT5720 decreased LPS-stimulated Kir6.1 and SUR2B mRNA expression. A representative RT-PCR was exhibited for the aortic tissues treated with LPS and KT5720. KT5720 was applied 1 hour before LPS treatment. The elevated Kir6.1 and SUR2B expression with a 20-h exposure of LPS (L, 1 μg/ml) was blocked by KT5720 (K, 1 μM). D. E. Real time PCR was performed in the total RNAs extracted from dissociated mouse aortic SMCs. Rp-cAMP (100 μM) or KT5720 (1 μM) was used

1 h before LPS administration. The expression of K_{ATP} channel in LPS-treated groups was normalized to the vehicle controls. The LPS + Rp-cAMP or KT5720-treated group was normalized to Rp-cAMP or KT5720-treated group, respectively. The upregulation of Kir6.1 and SUR2B was blocked by either Rp-cAMP or KT5720. Data were collected from 2-4 independent experiments with 3 samples from each.

event in vascular responses to endotoxemia. We also found that the LPS-induced CREB upregulation was blocked by PDTC, indicating that the enhanced CREB level is likely result from the NF-κB signaling (Figure 5-6, B).

Since NF-κB activity can be enhanced by PKA phosphorylation at residue Ser276 of the p65 subunit (Yoon et al., 2008; Zhong et al., 1997), we examined the phosphorylated NF-κB. Our results showed that there was no detectable p65 phosphorylation in the A10 aortic SMCs after LPS treatment, suggesting that the PKA requirement does not seem to be related to NF-κB phosphorylation (Figure 5-6, C).

5.3.8. Kir6.1/SUR2B channel was not subject to a direct modification by LPS

Since direct channel modification was another possible mechanism that leads to up-regulated K_{ATP} channel activity, we studied the effect of LPS on Kir6.1/SUR2B transiently expressed in HEK-293 cells. The cell line was chosen for several reasons: 1. the expression of Kir6.1 and SUR2B from plasmids is, to a large degree, independent of genomic regulation; 2. we have shown previously that the Kir6.1/SUR2B channel is phosphorylated by the endogenous PKA in the cells (Shi et al., 2007b; Yang et al., 2008); and 3. the intracellular signaling pathway for LPS is intact although the membrane expression of TLR4, MD2 and CD14 are absent (Chow et al., 1999; Divanovic et al., 2005). Therefore, TLR4, MD2 and CD14 were cotransfected to the cells. To our surprise, the basal current density of the LPS-treated cells did not increase at all in

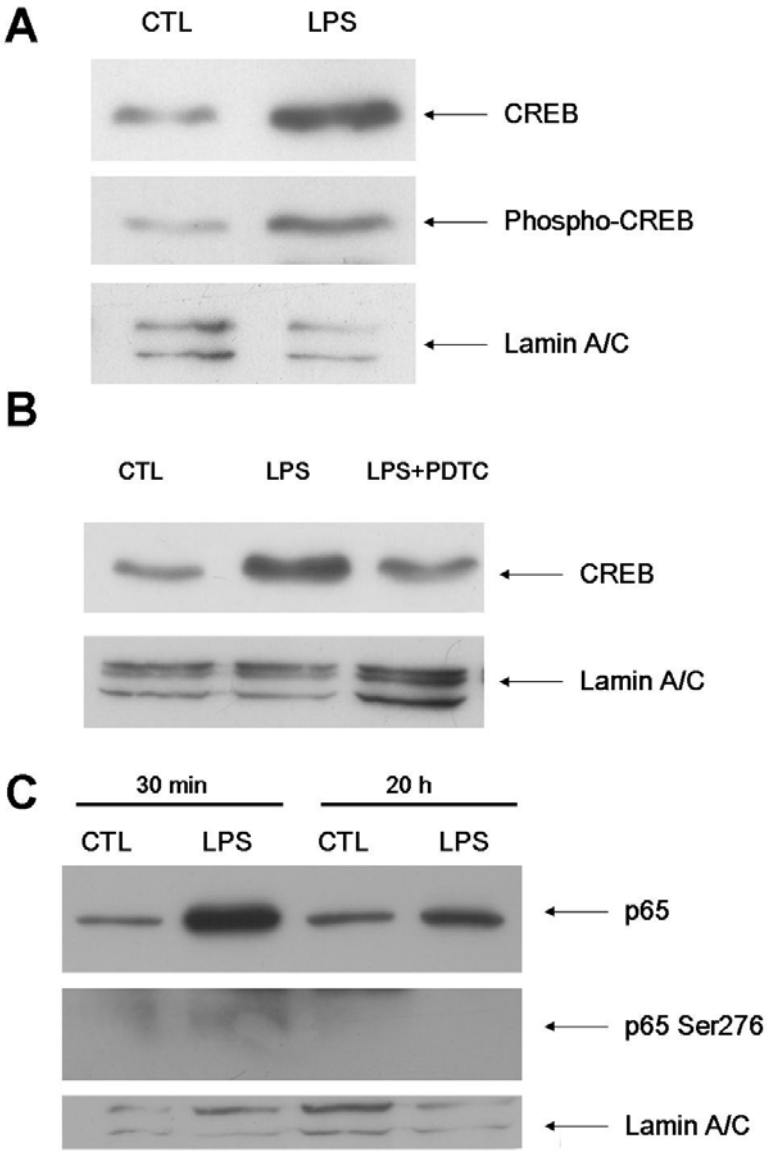


Figure 5-6. The role of PKA in LPS-induced transcriptional regulation.

Western blot was performed using nuclear protein extracted from A10 cells after the cells were incubated with LPS (1μg/ml). **A.** Both CREB and phospho-CREB (CREB Ser133) levels were augmented with a 20-h LPS exposure. **B.** PDTC (0.1 mM) was applied 1 hour before LPS administration. The LPS-induced CREB expression was almost completely abolished in the presence of PDTC. **C.** p65 level was increased with 30-min LPS stimulation, and tended to decline after 20h. The phospho-p65 level (p65 Ser276) remained undetectable with these periods of LPS treatment.

comparison to the vehicle-treated cells (9.8±2.7 pA/pS, n=13, vs. 12.8±3.3 pA/pS, n=15, respectively, *P*>0.05. Figure 5-7, A-C). The pinacidil-induced currents were not different between LPS and vehicle-treated cells either (104.6±29.0 pA/pS, n=13, vs. 138.7±35.8 pA/pS, n=15, respectively, *P*>0.05) (Figure 5-7, A-C). These were not due to a failure of activation of intracellular signaling systems by LPS, as our results indicated that LPS affected the HEK-293 cells, resulting in a clear nuclear accumulation of p65 subunit after a 30 min treatment (Figure 5-7, D). The different responses between native SMCs and HEK-293 cells to LPS suggest that a direct K_{ATP} channel modification does not contribute to the upregulation of the channel activity.

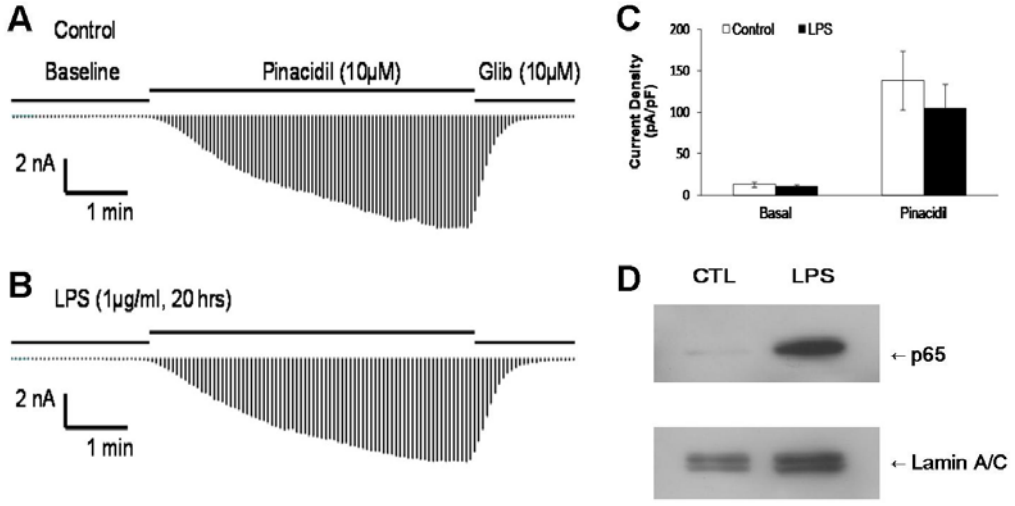


Figure 5-7. LPS failed to raise Kir6.1/SUR2B channel activity in a heterologous expression system. **A.** Kir6.1/SUR2B were co-expressed with TLR4/MD2/CD14 in HEK-293 cells, and whole-cell currents were studied as shown in Figure 5-2. The current amplitude increased markedly in response to pinacidil (10 μM), and was inhibited by glibenclamide (Glib, 10 μM). **B.** Currents were recorded from another cell treated with LPS (1μg/ml) overnight. The currents showed a similar response to pinacidil and glibenclamide. **C.** Comparison of the current density between the control (n=15) and LPS (1μg/ml)-treated cells (n=13). Both basal current density and pinacidil-induced current density were not significantly changed after overnight LPS incubation (*P*>0.05). **D**. Stimulation of NF-κB signaling with LPS exposure in HEK-293 cells. The HEK-293 cells were transfected with human TLR4/MD2/CD14 cDNAs. Two days after transfection, Western blot analysis was performed on the nuclear extracts from the cells. The p65 accumulation was clearly seen 30 min after LPS stimulation.

5.4. Discussion

The outcome of sepsis is determined by not only pathogens but also cardiovascular response (Hotchkiss and Karl, 2003). The major cause of death in sepsis is hypotension and hypoperfusion of several vital organs. Accounting for these are excessive vasodilation and hyporeactivity to vasoconstrictors, in which the vascular K_{ATP} channel plays a role (Kane et al., 2006).

A previous study indicates that administration of glibenclamide recovered blood pressure in dogs with hypotension induced by endotoxemia (Landry and Oliver, 1992). In contrast, glibenclamide did not show any effect in a control group, suggesting K_{ATP} channel's activity is enhanced in LPS-treated animals. The up-regulated channel activity contribute to LPS-induced vascular hyporeactivity (d'Emmanuele di Villa Bianca et al., 2003; O'Brien et al., 2005) and smooth muscle cell hyperpolarization (Wu et al., 2004). Our results are consistent with these findings. By using patch clamp approaches, we demonstrated that whole-cell K_{ATP} currents were elevated after an overnight LPS exposure. The augmentation of whole-cell K_{ATP} currents is due to an increase in surface expression as the channel density increases significantly.

There are a few reports regarding to the transcriptional regulation of K_{ATP} channel with the exposure of LPS. Kir6.1 mRNA is augmented by 4 fold and Kir6.1 protein by 9 fold in rat diaphragm with LPS treatment (24-48h) (Czaika et al., 2000). In experimental colitis, the Kir6.1 mRNA expression in colonic smooth muscle is enhanced by ~22 fold, while SUR2B mRNA decreases by 3 fold (Jin et al., 2004). The increase in the K_{ATP} expression, especially Kir6.1 subunit, is believed to contribute to the dysfunction of visceral smooth muscle contraction during inflammation (Czaika et al., 2000; Jin et al., 2004). In the present study, we have shown, for the first time, that both Kir6.1 and SUR2B mRNA are upregulated after LPS treatment in vascular

SMCs. The effect is related to the newly synthesized mRNAs rather than changes in stability, as a transcriptional inhibitor blocks the mRNA elevation.

Our results also suggest that multiple intracellular signaling systems are involved in the Kir6.1 and SUR2B upregulation during endotoxemia. NF-κB is known to be a key player in the regulation of inflammatory gene expression (Baeuerle and Baltimore, 1996). Activation of NF-κB increases the expression of genes encoding proinflammatory cytokines, such as TNFα, an early mediator of the vasodilatory septic shock (Tracey et al., 1986). In our studies, we have examined the causality between K_{ATP} and NF-κB. The results showed that p65 began to accumulate in the nucleus as soon as 30 min after LPS stimulation. A pretreatment with the NF-κB inhibitor PDTC or DMFR significantly attenuated the LPS-induced K_{ATP} channel expression. Therefore, NF-κB is necessary for the upregulation of the vascular K_{ATP} channel during endotoxemia.

The time course for the LPS-induced cellular membrane hyperpolarization and K_{ATP} channel upregulation suggests that intracellular signaling systems in addition to NF-κB are also important. PKA is one of them. After activation, the catalytic subunit of PKA directly phosphorylates vascular K_{ATP} channel leading to vasodilation (Shi et al., 2007b). In addition, the catalytic subunit is translocated to the nucleus and regulates gene transcription through phosphorylating CREB (Gonzalez and Montminy, 1989). A previous study indicates that LPS (100 μg/ml) activates PKA in primary culture of bovine aortic SMCs (Browner et al., 2004). Another group, however, has shown that LPS (10 μg/ml) inhibits PKA leading to a reduction of electrical coupling in microvascular endothelial cells (Bolon et al., 2007). In the present study, we did not find increased PKA activity with LPS (1μg/ml) exposure in rat aortic SMCs. Moreover, if LPS increased PKA activity, the activity of Kir6.1/SUR2B channel would be

increased by phosphorylation (Shi et al., 2007b). Our patch clamp study showed that the Kir6.1/SUR2B current density in the transfected HEK cells was not changed after LPS exposure, indicating that LPS do not seem to upregulate PKA activity. Therefore, the effect of PKA on LPS-induced upregulated K_{ATP} channel activity appears to be mediated through a mechanism other than enhanced channel phosphorylation.

CREB is a major target of PKA that contributes to gene transcription (Mayr and Montminy, 2001). Our studies suggest that Kir6.1 and SUR2B are likely subject to transcriptional regulation by CREB for the following reasons: 1. Both CREB and phosphor-CREB were elevated 20 hours after LPS exposure; 2. Blocking PKA decreased LPS-induced Kir6.1 and SUR2B mRNA expression. In addition, the activity of NF-κB is important because LPS-induced CREB expression is strongly attenuated by PDTC. It is well accepted that CREB contributes to cardiovascular remodeling process, including VSMC hypertrophy, migration, vascular fibrosis, and possibly angiogenesis (Ichiki, 2006). Our study suggests that CREB also plays an important role in sepsis.

Like CREB, the activity of NF-κB is enhanced by PKA (Yoon et al., 2008; Zhong et al., 1997), which may be a mechanism underlying the NF-κB and PKA dependent K_{ATP} channel upregulation. However, we did not find clear evidence that LPS induce phosphorylation of p65 Ser276, a unique residue that is targeted by PKA. The PKA that phosphorylates p65 upon LPS stimulation could be an atypical isoform,containing only a catalytic subunit which forms a ternary complex with NF-κB and IκB, and is not regulated by cAMP (Zhong et al., 1997). Our results, however, indicate that the LPS effect is blocked by Rp-cAMP, suggesting it is a cAMP-sensitive PKA. Moreover, although a strong PKA stimulation (foskolin, etc.) is able to induce p65 Ser276 phosphorylation in Schwann cells (Yoon et al., 2008), the LPS-treated aortic SMCs

did not display an elevated PKA activity as shown in the present sudy. Thererfore, our data do not agree that NF-κB is a target of PKA.

It is interesting that actidione, a nonselective protein synthesis blocker, suppressed the upregulation of Kir6.1 and SUR2B. The result suggests that some protein synthesis is crucial for the transcriptional regulation of Kir6.1 and SUR2B. Several steps of the intracellular signaling pathways may involve protein synthesis, such as PKA, CREB, NF-κB, protein for nuclear transports, etc. Further studies are needed to identify the proteins that play an important role in the upregulation of Kir6.1 and SUR2B.

In summary, vascular K_{ATP} channel is involved in LPS-induced dysfunction of vasoconstriction. The upregulated vascular K_{ATP} channel activity during LPS exposure is caused by additional expression of Kir6.1 and SUR2B. A transcriptional mechanism which includes NF-κB and CREB signaling contributes to elevation of Kir6.1 and SUR2B mRNA. Although PKA activity is not enhanced during LPS exposure, it plays a crucial role in LPS-induced vascular hyporeactivity and upregulation of Kir6.1 and SUR2B.

6. Result 2: Reactive Oxygen Species Suppressed Kir6.1/SUR2B Channel Activity through Direct Interaction with the Channel Protein

Note that Dr. Ningren Cui performed all vascular ring experiment. Mr. Yang Yang and Ms. Zhongying Wu conducted the study on the identification of intra- vs. extracellular redox sensitivity, while the rest of the work (estimated to be ~80%) was done by Weiwei Shi.

6.1. Abstract

Overproduction of ROS during oxidative stress has an impact on the pathogenesis of several vascular diseases including sepsis, diabetes and atherosclerosis. The impaired vascular K_{ATP} channel function during oxidative stress has been revealed recently, but the underlying mechanism is elusive. Here we reported that H_2O_2 attenuated pinacidil-induced vasodilation in both endothelium-intact and endothelium-denuded mesenteric arterial rings. Patch clamp studies showed that H_2O_2 inhibited pinacidil-activated Kir6.1/SUR2B currents in a dose-dependent manner. Since cysteine residues are the most likely substrates of oxidative modulation, we applied thiol-modifying reagents (2,2′-dithiodipyridine, 2-PDS and 5, 5′-dithiobis-2-nitrobenzoic acid, DTNB) to determine whether thiol oxidation plays a role and identify the critical protein domains and residues that were targeted by oxidation. Kir6.2/SUR2B channel was far less sensitive to H_2O_2 and 2-PDS than the Kir6.1/SUR2B, indicating Kir6.1 is the major player. The membrane permeable 2-PDS suppressed Kir6.1/SUR2B channel in a manner similar to H_2O_2, while the membrane impermeable DTNB had a smaller effect when applied extracellularly, suggesting the major thiol modification occurs in the intracellular domains. Using Kir6.1-Kir6.2 chimeras, two critical protein domains for the thiol modification were identified. Systematic mutational analysis showed that Cys43, Cys120, and Cys176 were critical for the 2-PDS sensitivity. Therefore, these results suggest that H_2O_2 modulates the Kir6.1 channel leading to vascular dysfunction, and such an effect seems to result from thiol oxidation involving Cys43, Cys120, and Cys176 located in the N-terminus and core region of Kir6.1.

6.2. Introduction

Oxidative stress is characterized by an overproduction of reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO\cdot$), and impaired antioxidant defenses, leading to structural damages to proteins, nucleotides, and membrane lipids (Storz and Imlayt, 1999). The ROS production plays an important role in both the normal function of vascular systems and the pathogenesis of vascular diseases, such as diabetes, atherosclerosis, sepsis, Parkinson's disease, Alzheimer's disease, etc. (Aruoma and Halliwell, 1998).

Almost all types of cells distributed in the vascular wall, including SMCs, endothelial cells and fibroblasts, can produce ROS, and are in turn modulated by ROS (Griendling et al., 1994). However, the outcomes of the oxidative stress are controversial. In different studies H_2O_2 is reported to be a vasoconstrictor (Suvorava et al., 2005), vasodilator (Hatoum et al., 2005), or both (Cseko et al., 2004). Since K_{ATP} channels play an important role in vascular tone regulation, the effect of ROS on the K_{ATP} channels may have a profound impact on vascular tone and regional blood flows. Accumulating evidence suggests that the function of vascular K_{ATP} channels is impaired with ROS exposure (Erdos et al., 2004; Miura et al., 2003; Ross and Armstead, 2003). However, the mechanism underlying the K_{ATP} channel modulation by ROS is poorly understood. In contrast to the vascular isoform of K_{ATP} channels, activity of K_{ATP} channels of myocardium and striatal neurons is enhanced by hydroxyl radicals, superoxide and H_2O_2 (Avshalumov and Rice, 2003; Ichinari et al., 1996). Since the disparate responses to ROS may be attributed to the different subunit composition of K_{ATP} channels, detailed studies of these Kir6.1 and Kir6.2 channels may lead to identification of protein domains and amino acid residues critical for the channel modulation by ROS. Therefore, we performed studies to

investigate the molecular basis of the vascular K_{ATP} channel modulation by H_2O_2 and thiol oxidants.

6.3. Results

6.3.1. H_2O_2 exposure resulted in dysfunction of mesenteric arteries, attributable to K_{ATP} channel inhibition

Previous studies have shown that pinacidil exposure leads to relaxation of arterial rings that are pre-contracted by vasoconstrictors including PE and extracellular K^+ (Shi et al., 2007a; Stojnic et al., 2007). In endothelium-intact (EI) mesenteric arterial rings, we also observed the vasorelaxing effect. After a contraction produced by extracellular K^+ (30mM), pinacidil produced a dose-dependent relaxation with EC_{50} 10 μM (Figure 6-1, A, C). Although H_2O_2 produced a rather small vasoconstriction, the exposure of H_2O_2 markedly attenuated the pinacidil-induced vasodilation in a concentration-dependent manner (Figure 6-1, A, C). The effect relied on the concentrations of H_2O_2. The evident effect was seen with 30μM H_2O_2, and a great effect took place with 600μM (Figure 6-1C, D), concentrations that have previously been shown to be produced in vasculatures under different pathophysiological conditions (Colussi et al., 2000; Sandberg and Sayeski, 2004). The attenuation of the pinacidil-induced vasodilation was also observed in endothelium-denuded (ED) rings (Figure 6-1, B, D), suggesting that K_{ATP} channel in smooth muscle cells is inhibited by H_2O_2 exposure.

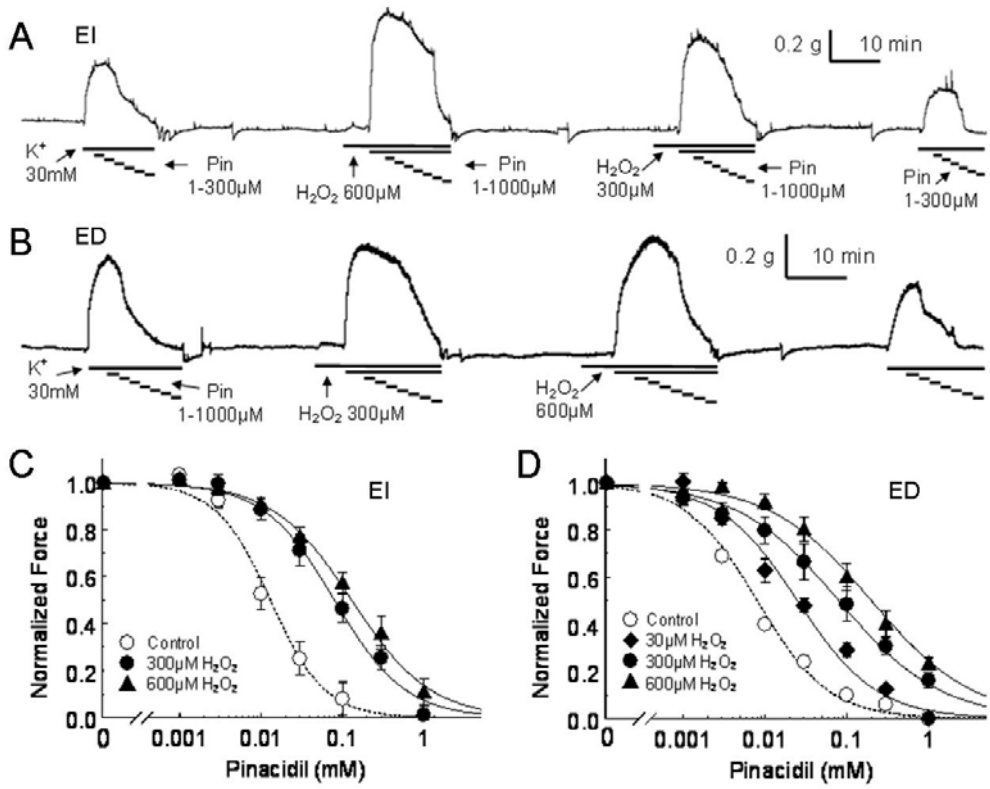


Figure 6-1. Effects of H_2O_2 on the pinacidil-induced vasodilation.

A. Vasoconstriction was studied in an endothelium-intact (EI) mesenteric arterial ring at 36°C in vitro. Isometric contraction was produced by exposures to 30 mM KCl (K^+). The contractile force decreased dose-dependently with an increase in pinacidil (Pin) concentrations. Pre-exposure of H_2O_2 in 300 μM and 600 μM 2 min before KCl impaired the pinacidil-induced vasodilation. **B**. A similar study was performed in an endothelium-denuded (ED) ring. The endothelium elimination was confirmed as the ring failed to respond to 1μM ACh. The pinacidil-induced vasodilation was also compromised with the exposure of H_2O_2. **C**. The contractile force was normalized between the pre-loaded force and the maximal force induced by 30 mM K^+. The vasodilation in EI rings is a function of pinacidil concentrations, and their relationship can be described with the Hill equation (EC_{50} 0.01 mM, h = 1.0). H_2O_2 (300 μM) increased EC_{50} to 0.1 mM. A higher concentration of H_2O_2 (600 μM) only further elevated EC_{50} to 0.2 mM. Data were obtained from 4-5 rings, and shown as means ± SE. **D**. The dose-vasodilation curve was also plotted in ED rings, with EC_{50} 0.008 mM. The exposure of H_2O_2 (30 μM, 300 μM and 600 μM) increased EC_{50} to 0.03, 0.1 and 0.2 mM, respectively. Data were obtained from 4-5 rings, and shown as means ± SE.

6.3.2. H_2O_2 inhibited Kir6.1/SUR2B currents

To examine the molecular mechanism underlying vascular K_{ATP} channel inhibition, we need to know the effect of H_2O_2 on the cloned K_{ATP} channel expressed in HEK293 cells. Indeed, studies in our laboratory showed that the whole-cell Kir6.1/SURB currents were inhibited by extracellular H_2O_2 in a concentration-dependent manner with IC_{50} 1.0 mM (Yang et al. personal communication).

6.3.3. Redox-dependent inhibition of Kir6.1/SUR2B channel by thiol-modifying reagents

It is known that ROS can interact with proteins, especially cysteine residues. Such a thiol oxidation can lead to dysfunction and structural damage of the proteins (Kiley and Storz, 2004). The thiol oxidation may be produced by a number of oxidants including 2-PDS. Thus, we applied 2-PDS, a membrane-permeable thiol modifying reagent, to the cloned K_{ATP} channel. The transfected cells exhibited small baseline currents upon the formation of whole cell configuration (Figure 6-2, A). An exposure to 10 μM pinacidil increased the currents markedly. The pinacidil-activated currents were strongly inhibited by 2-PDS with an IC_{50} 8 μM (Figure 6-2, A, B). To locate the modification sites, we also studied the K_{ATP} currents using DTNB, a membrane-impermeable PDS. At 200 μM, DTNB had very little inhibitory effect. Then, we studied the effects of 2-PDS and DTNB in inside-out patches. Applied to the internal membranes, both reagents showed a similar inhibitory effect on K_{ATP} currents, with IC_{50} ~10 μM (Figure 6-2, C). These studies thus suggest that the redox sensitive site(s) is likely to be located in the intracellular domain of Kir6.1/SUR2B channel.

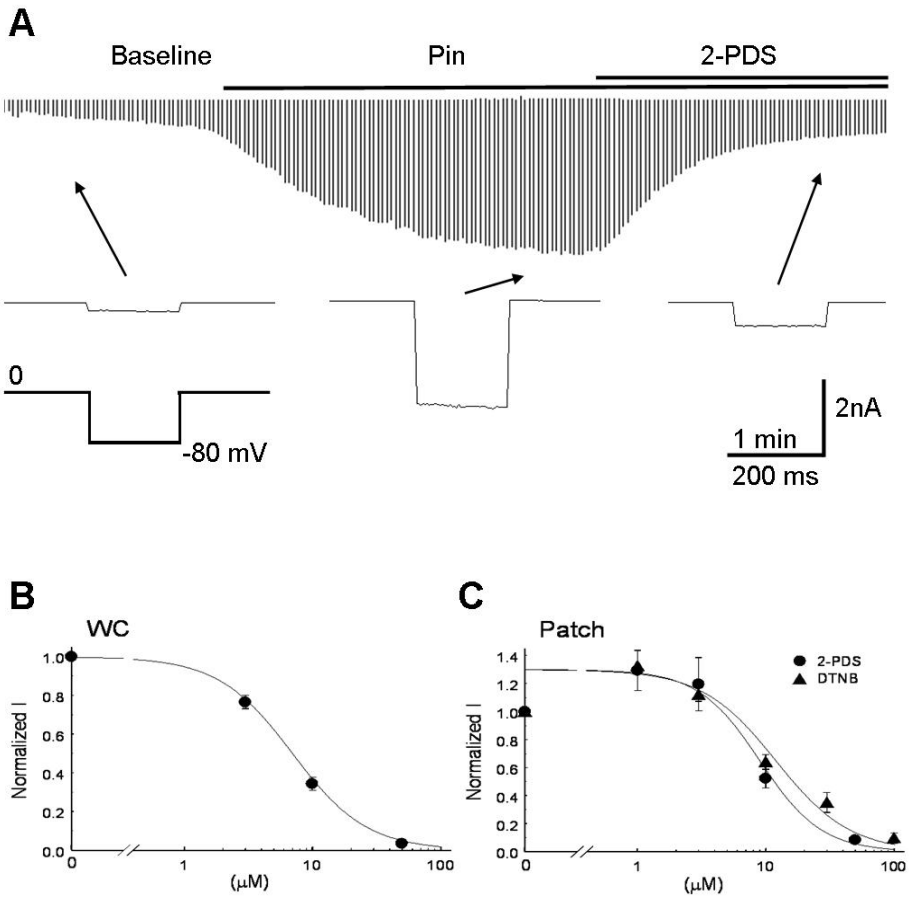


Figure 6-2. Inhibition of Kir6.1/SUR2B currents by thiol modification.

A. Whole cell currents were recorded from a cell transfected with Kir6.1/SUR2B. Currents were strongly activated by pinacidil (Pin; 10 μM), and the maximum activation was reached within 4-5 min of the exposure. The currents were inhibited by 50 μM 2-PDS. *Bottom*. individual currents produced by a single command pulse. **B**. The normalized effect of 2-PDS on whole-cell Kir6.1/SUR2B currents was plotted against the concentration of 2-PDS. The concentration-response relationship was described using the Hill equation with IC_{50} ~8 μM and h = 1.0. **C**. The normalized effects of 2-PDS and DTNB in inside-out patch were plotted against their concentrations, respectively. The dose-response relationship was described using the Hill equation with IC_{50} = 10 μM and h = 1.0. Data were collected from 4-6 cells, and shown as means ± SE.

6.3.4. Kir6.2/SUR2B channel is not sensitive to H_2O_2 and 2-PDS

To elucidate which subunit is targeted, we studied a K_{ATP} channel without the Kir6.1 subunit. The K_{ATP} currents were recorded from HEK cells after a cotransfection of the Kir6.2/SUR2B subunits (Figure 6-3). No clear inhibition was found when the cells were exposed to either H_2O_2 (1 mM) or 2-PDS (50 μM) (-0.01%±0.5%, n=4, and 12.9%±0.5%, n=4,

respectively. Figure 6-3 A and B), suggesting that the SUR2B subunit is not critical for the redox modification, and the different response to H_2O_2 and 2-PDS between these two isoforms of K_{ATP} channels attributes to the Kir6.1 subunit.

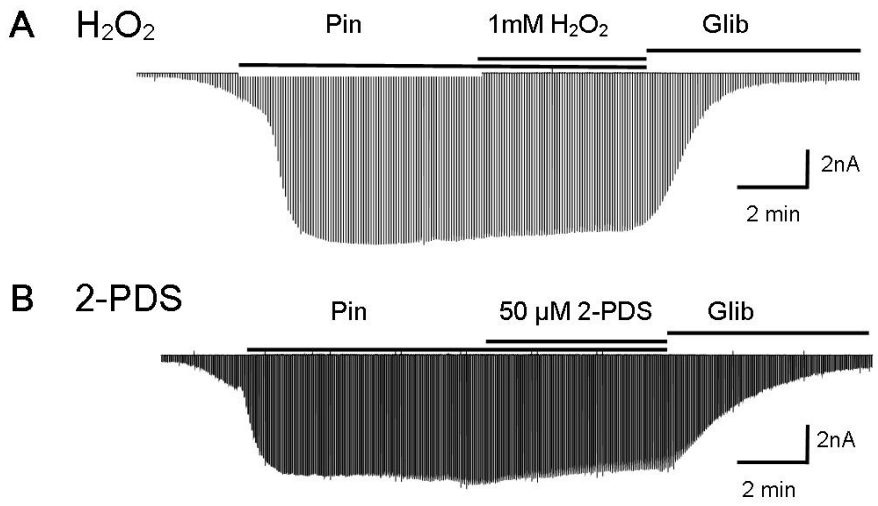


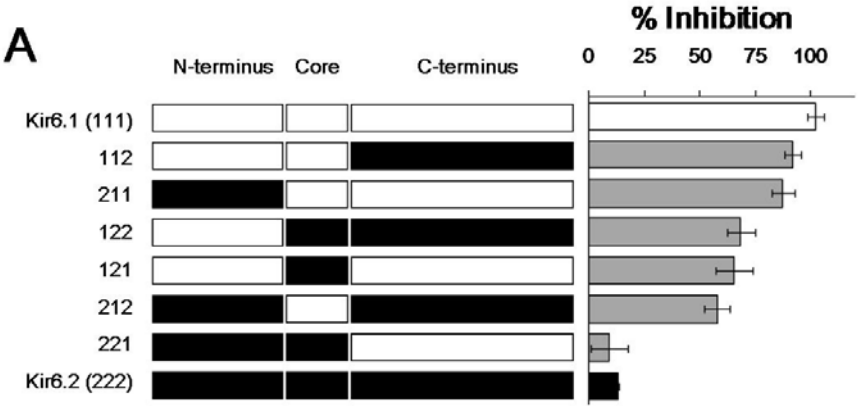
Figure 6-3. Effects of H_2O_2 and 2-PDS on Kir6.2/SUR2B channel expressed in HEK293 cells.
Representative recordings of whole-cell Kir6.1/SUR2B currents were displayed for the cells treated with H_2O_2 and 2-PDS. **A**. shown was the time course for Kir6.2/SUR2B channel modulation by H_2O_2. The whole cell currents were strongly activated by pinacidil (10 μM). The currents were not sensitive to H_2O_2 (50 μM). **B**. In another cell, 2-PDS did not exhibit a clear inhibitory effect on the pinacidil-activated Kir6.2/SUR2B currents.

6.3.5. Location of potential interaction sites

To identify protein domains necessary for the redox sensitivity, we studied Kir6.1-Kir6.2 chimeras as shown in our previous studies (Shi et al., 2008b). The Kir6.1 and Kir6.2 were separated into three parts: the N terminus, the C terminus, and the core domain containing two transmembrane segments and the pore loop (Figure 6-4, A). All six chimeras displayed functional currents that were sensitive to pinacidil and glibenclamide. When the C terminus was replaced (112), the 2-PDS sensitivity was not significantly changed, suggesting that the C

terminus is not critical. When the N terminus of Kir6.1 was replaced with that of Kir6.2 (named 211), the channel inhibition by 2-PDS was significantly diminished (Figure 6-4, A, B). A similar result was observed for the core domain (121). Consistently, construction of either the N terminus or core domain to the Kir6.2 frame (122 and 212, respectively) was able to retain the 2-PDS sensitivity by ~50%. Therefore, both the N terminus and core domain appear important for 2-PDS-dependent channel inhibition (Figure 6-4, B).

A



B

Constructs	P value	Comments
111, 112	>0.05	C terminal is not important
222, 221	>0.05	C terminal is not important
111, 211	<0.05	N terminal is important
222, 122	<0.05	N terminal is important
111, 121	<0.05	core domain is important
222, 212	<0.05	core domain is important

Figure 6-4. Exploration of critical protein domains contributing to 2-PDS-dependent channel inhibition.

All chimerical Kir6.x subunits were expressed with SUR2B. **A**. shown is a summary of 2-PDS suppression of chimeras. N terminus, core, and C terminus in Kir6.1 refer to amino acid sequences 1–71, 72–186, and 187–424, respectively. N terminus, core, and C terminus in Kir6.2 refer to residues 1–70,

71–176, and 177–390, respectively. **B**. Statistical anaylsis of the response of chimeric channels to 2-PDS. Data were collected from 4-5 cells in each chimera.

There are totally eight cysteine residues in all intracellular domains of human Kir6.1 that were potentially modified by oxidants (Figure 6-5, A). We thus performed systemic mutation screening of all these residues. Mutation of Cys43 or Cys120 to s serine reduced mildly the 2-PDS sensitivity (C43S, 88.0%±4.6%, n=5, * P<0.05, and C120S, 77.6%±5.1%, n=4, * P<0.05, respectively. Figure 6-5, B). Mutation of Cys176 dramatically inhibited the sensitivity (C176A, 26.0%±6.5%, n=6, ** P<0.01, and C176S 67.5%±6.0%, n=5, respectively. *** P<0.001. Figure 6-5, B). Mutation of any other cysteine residues did not significantly change the 2-PDS sensitivity. Consistent with our chimerical study, these cysteine residues are located in the N-terminus and the core domain and seem to be the modulation sites by oxidants.

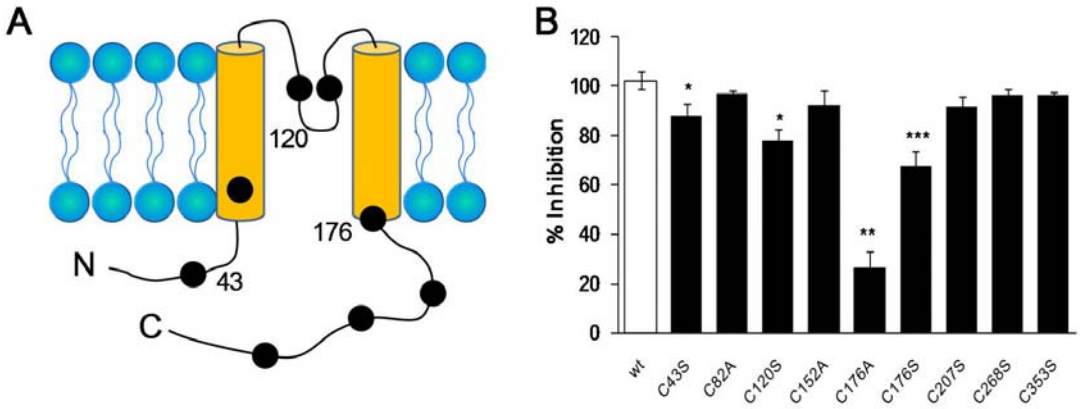


Figure 6-5. Mutagenesis analysis of potential 2-PDS modification of cysteine sites in Kir6.1 subunit.

A. Schematic representation of Kir6.1 subunit. The N and C terminus, as well as eight cysteine residues including Cys43, Cys120 and Cys176, are illustrated. B. Compared with wt, mutations of Cys43, Cys120 and Cys176 attenuated the inhibitory effect of 2-PDS. Data were collected from 4-6 cells for each mutation (*P < 0. 05, **P < 0. 01, ***P < 0. 001).

Based on homologous analysis of Kir6.x amino acid sequences, we noticed that the Cys176 corresponds to Cys166 in Kir6.2, a mutation that decreases ATP and pH sensitivities by disrupting the gating mechanism (Piao et al., 2001; Trapp et al., 1998a; Wang et al., 2007). We found that basal currents recorded from Kir6.1-C176A/SUR2B increased gradually upon a whole-cell patch configuration (Figure 6-6, A). The currents were further stimulated by pinacidil (10 μM), and subsequently inhibited by glibenclamide (10 μM), suggesting that the Cys176 may not be critical for Kir6.1 channel gating. H_2O_2 (3mM and 10mM, note the concentrations are far higher than those used in wild-type channel) failed to produce channel inhibition (0.6%±0.3%, n=5, and 7.6%±3.7%, n=5, respectively. Figure 6-6, B). Consistently, 2-PDS (50 μM) only displayed a weak inhibitory effect on the whole-cell currents (26.0%±6.5%, n=6. Figure 6-6, C).

6.4. Discussion

A major pathological event of inflammation is ROS production. When the cellular antioxidant defense mechanisms are overwhelmed, the excessive amount of ROS tends to react with a large number of macro-molecules including proteins, lipids and nucleotides, and causes damages to the cellular structure and function. In addition to their direct effects, ROS can activate other reactive species such as reactive lipids and reactive carbonyls, propagating oxidative stress within the cell and beyond. Such oxidative stress are seen in vasculatures during sepsis, diabetes, atherosclerosis, etc. (Galley et al., 1996; Macdonald et al., 2003).

Previous studies have suggested that ROS are involved in vascular tone regulation. The pinacidil-induced vasodilation in cerebral arterioles is compromised in insulin-resistant rats fed with fructose, which is completely reversed by treatment with SOD and catalase (Erdos et al., 2004). Superoxide impairs pial arterial dilation induced by K_{ATP} channel opening (Ross and

Armstead, 2003) . Similarly, diabetes reduces human coronary arteriolar dilation in response to K_{ATP} opening, leading to a reduced vasodilation to hypoxia (Miura et al., 2003). Consistent with these previous studies, our results have shown that by targeting at vascular smooth muscles, H_2O_2 induces dysfunction of vasodilation in a K_{ATP} channel dependent manner.

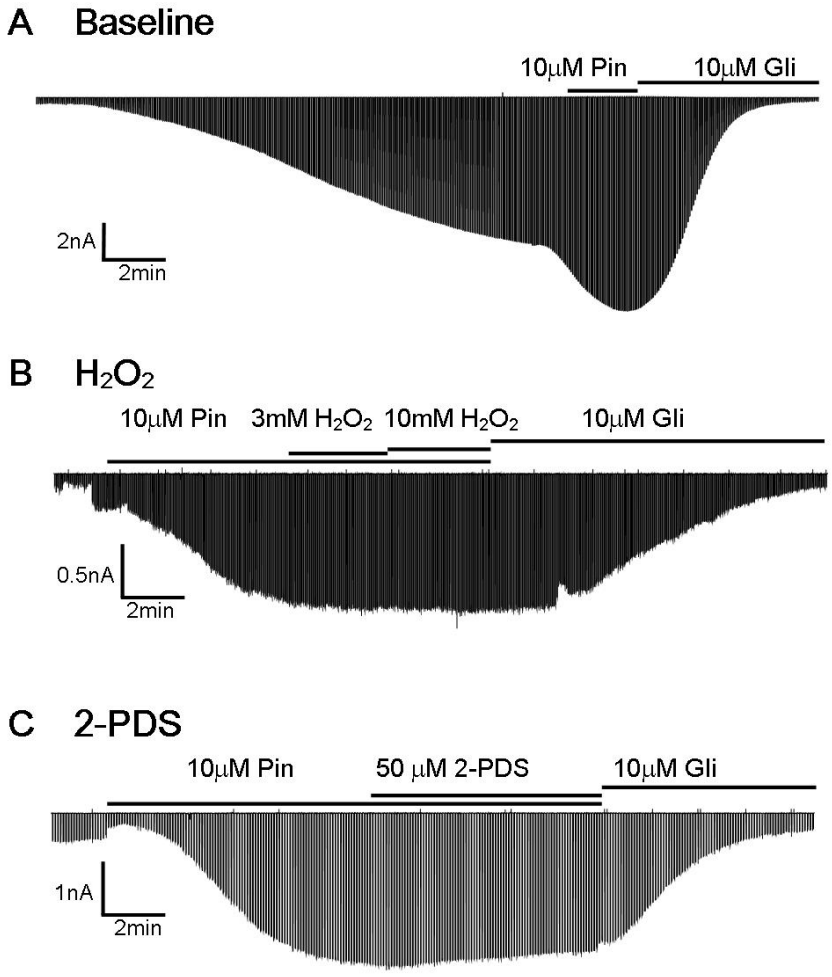


Figure 6-6. Characterization of Cys176 in redox modification.

Whole-cell patch was performed in HEK cells transfected with Kir6.1 C176A/SUR2B. Representative recordings of the whole-cell currents are shown. **A**. The basal current increased spontaneously upon whole cell configuration was formed. The currents were further activated by pinacidil (10μM), and suppressed by 10 μM glibenclamide. **B**. H_2O_2 (3mM and 10 mM) failed to suppress the whole cell currents. **C**. 2-PDS (50 μM) slightly inhibited the whole cell currents.

Since Kir6.1/SUR2B is the major isoform of K_{ATP} channel expressed in smooth muscle cells, we have further examined the effect of H_2O_2 on cloned K_{ATP} channels, and observed that H_2O_2 clearly suppresses the pinacidil-induced currents. Using thiol-modifying reagents with different membrane permeability, the redox sensitive sites are located in intracellular domains. These sites are likely to be in the Kir6.1 but not the SUR2B subunit, because Kir6.1/SUR2B instead of Kir6.2/SUR2B is sensitive to H_2O_2 and 2-PDS. With systematic chimerical construction, the redox sensitive sites are subsequently located in the N-terminus and core region of Kir6.1. In these protein domains, three cysteine residues of Cys176, Cys43 and Cys120 are identified to be most likely targets of the redox regulation.

The Cys176 is an interesting residue. Both C176A and C176S mutations cause a major decrease in the channel response to H_2O_2 and 2-PDS dramatically. Its homologous residue in Kir6.2 (Cys166) contributes to a channel gating mechanism, as mutation of the Cys166 to any amino acids except valine dramatically decreases the ATP and pH sensitivities of the mutant channels, and robustly elevates Po (Trapp et al., 1998a; Wang et al., 2005). This raises a question as to whether the Cys176 in Kir6.1 is a gating site rather than a redox sensor, as its mutation may cause disruption of the channel gating and make the channel constitutively open. This idea however is not supported by our results. We have found that C176A or C176S mutation does not lead to complete opening of the channel, as the basal currents are small and increase spontaneously with intracellular dialysis of ADP. The C176A or C176S mutation does not prevent channel regulation by a K_{ATP} channel opener and inhibitor. At maximal basal channel activity, the channel activity can be further stimulated by pinacidil, and inhibited fully by glibenclamide, indicating that the channel gating is not disrupted. Alternatively, a straightforward interpretation of the decreased response to 2-PDS and H_2O_2 in the C176A or

C176S is that the Cys176 allows the wild-type but not the mutant channel to be modified by H_2O_2 and 2-PDS, leading to channel closure. Although the precise role of the Cys176 in the redox regulation of Kir6.1is still unclear, a differentiation of this site in redox sensing from channel gating helps future studies.

The Cys43 is another redox sensitive site. The corresponding residue in Kir6.2 Cys42 is located in an ATP-binding motif and is subject to thiol modification (Trapp et al., 1998b). A reagent *p*-chloromercuriphenylsulphonate (pCMPS, 50 μM) eliminates Kir6.2/SUR1 currents when applied to an open channel in inside-out patches. The inhibitory effect is reversible since 5mM DTT can partially restore the currents. When the channel is closed by ATP, the Cys42 is not modified by pCMPS, suggesting that it is inaccessible from the bath solution. The C43S in Kir6.1channel is sensitive to thiol modification in the open status as well.

Cys120 in Kir6.1 is close to the pore region, a place that is not accessible from the intracellular side when the channel is in the closed state. A previous study suggested Cys110 in Kir6.2 at a position similar to Cys120 in Kir6.1 may form a disulfide bond with Cys142 (Loussouarn et al., 2001). Our result shows that Kir6.1 C120S channel partially decreased the sensitivity to 2-PDS, consistent with the idea that it is another oxidation site when the channel is open. Kir6.1 Cys152 is a residue corresponding to Kir6.2 Cys142. Mutation of this residue did not change 2-PDS sensitivity, suggesting Cys152 is not important for thiol modification.

In summary, the vascular K_{ATP} channel is inhibited by H_2O_2. The N-terminus and core domain are critical for redox modulation. Three cysteine residues, i.e., Cys43, Cys120 and Cys176, located in these regions are potentially oxidized with H_2O_2 exposure. Oxidation of these

residues appears to cause a suppression of vascular K_{ATP} channel activity during oxidative stress, leading to disruption of vascular responses to vasodilators.

7. Result 3: Arginine Vasopressin Constricts Mesenteric Artery through V1a Receptor, PKC and K_{ATP} Channel

Publication: Weiwei Shi, Ningren Cui, Yun Shi, Xiaoli Zhang, Yang Yang, and Chun Jiang (2007) Arginine vasopressin inhibits Kir6.1/SUR2B channel and constricts the mesenteric artery via V1a receptor and protein kinase C. ***Am J Physiol Regul Integr Comp Physiol.*** 2007; 293:R191-9.

Note: The work was mostly done by Weiwei Shi (>90%). Dr. Ningren Cui performed most vascular ring study. Dr. Yun Shi helped to setup patch clamping. Mrs. Xiaoli Zhang and Mr. Yang Yang did some patch clamp experiments.

7.1. Abstract

AVP is a peptide hormone released from the posterior pituitary gland. With strong vasoconstricting effect it has been used in the treatment of septic shock when α-adrenoceptor agonists are ineffective. To test the hypothesis that the Kir6.1/SUR2B channel is a target molecule of AVP, we performed studies on SMC-endogenous K_{ATP} channel and the cloned Kir6.1/SUR2B channel. In isolated mesenteric artery rings, AVP produced concentration-dependent vasoconstrictions with EC_{50} 6.5 nM. At the maximum effect, pinacidil completely relaxed vasoconstriction in the continuing presence of AVP. The magnitude of the AVP-induced vasoconstriction was significantly reduced after PKC inhibition. In acutely dissociated vascular smooth myocytes, AVP strongly inhibited the cell-endogenous K_{ATP} channel. In the HEK-293 cells transfected with Kir6.1/SUR2B and V1a receptor, AVP produced a concentration-dependent inhibition of the pinacidil-activated currents with IC_{50} 2.0 nM. An exposure to 100 nM PMA, a potent PKC activator, inhibited the pinacidil-activated currents, and abolished the channel inhibition by AVP. Such an effect was not seen with inactive phorbol ester. A pretreatment of the cells with selective PKC blocker significantly diminished the inhibitory effect of AVP. Furthermore, PKC inhibited Kir6.1/SUR2B channel in a way independent of endocytosis. Mutation of potential PKC phosphorylation sites in SUR2B did not affect the channel sensitivity to PKC activators. These results therefore indicate that the Kir6.1/SUR2B channel is a target molecule of AVP, the channel inhibition involves G_q-coupled V1a receptor and PKC, and the PKC-dependent K_{ATP} channel inhibition is mediated on Kir6.1 not SUR2B.

7.2. Introduction

A major challenge in the treatment of septic shock is the control of excessive vasodilation and vascular hyporeactivity to α-adrenergic agonists. The limitation of the α-adrenoceptor agonist usage has led to great efforts to find other alternative vasoconstrictors. AVP is one and has been shown to remain effective in vasoconstriction during sepsis (Sands et al., 1997).

AVP is a nanopeptide synthesized in the hypothalamus and then transported to the posterior pituitary gland where it is released to the systemic circulation. Its release increases with a drop in blood volume or systemic dehydration. AVP performs multiple tasks in blood pressure control, water reabsorption, gluconeogenesis, neurotransmission, and platelet aggregation, depending on cell types and receptor species (Birnbaumer, 2000; Holmes et al., 2003). Three subtypes of AVP receptors have been found: V1a, V2, and V1b. The V1a receptor is mainly expressed in vascular smooth muscle cells, while it is also found in hepatocytes and platelets. In these cells, AVP plays an important role in vasoconstriction, hepatic gluconeogenesis, and platelet aggregation through the V1a receptor (Holmes et al., 2003). The V2 receptor is expressed in the collecting duct principal cells of medullary nephrons, regulating water reabsorption (Bankir, 2001). The V1b receptor is mainly expressed in the pituitary gland (Folny et al., 2003). Acting on the V1a receptor, AVP is a potent vasoconstrictor and has been widely used for therapeutical purposes (Holmes et al., 2001a).

Several Ca^{2+}-permeable channels are activated by AVP, including the T-type Ca^{2+} channels, L-type Ca^{2+} channels, and the receptor-operated cation channels (Brueggemann et al., 2005; Katori et al., 2001; Maruyama et al., 2006) . The opening of these channels contributes to the rise in cytosolic Ca^{2+} and constriction of vascular smooth muscles. Since some of these channels are voltage-dependent, their activation requires depolarization. Thus, the early

depolarization is crucial for the AVP-induced vasoconstriction. It is known that K^+ and Cl^- channels are important regulators of membrane potentials. Indeed, previous studies have suggested that the K_{ATP} channels are inhibited by AVP leading to depolarization of vascular smooth muscle cells (Wakatsuki et al., 1992).

As the major vascular isoform, the Kir6.1/SUR2B channel plays an important role in vascular tone regulation. Kir6.1 knockout mice exhibited a high rate of sudden death associated with spontaneous electrocardiographic ST elevation followed by atrioventricular block, which resembles Prinzmetal angina in humans (Miki et al., 2002). Genetic disruption of the *abcc9* (*SUR2*) gene leads to coronary artery vasospasm and raises resting blood pressures (Chutkow et al., 2002). Vascular K_{ATP} channels are targeted by several vasoactive hormones and neurotransmitters (Bonev and Nelson, 1996). However, the modulation of the vascular K_{ATP} channels by AVP is still controversial (Dumont and Lamontagne, 1995; Wakatsuki et al., 1992). There is evidence that K_{ATP} channels in cardiac myocytes and the insulinoma cell line are also inhibited by AVP, suggesting that Kir6.2/SUR1 and Kir6.2/SUR2A channels are targeted (Martin et al., 1989; Tsuchiya et al., 2002) . Since functional vascular K_{ATP} channels are mainly made of Kir6.1 with SUR2B subunits, the understanding of K_{ATP} channel contribution to vascular tones relies on the demonstration of the precise signal network between neurotransmitters/hormones and K_{ATP} channels. To test the hypothesis that Kir6.1/SUR2B channel is one of the effectors of AVP, we performed these studies. Our results indicated that the Kir6.1/SUR2B channel was inhibited by AVP through the V1a receptor, and the channel inhibition relied on intracellular signal systems involving PKC.

7.3. Results

7.3.1. Constriction of mesenteric artery by activation of V1a receptors

AVP produced concentration-dependent constrictions of the isolated mesenteric artery rings with EC_{50} 6.5 nM (Figure 7-1, A and B). At the maximum effect, pinacidil (Pin, 20 μM) relaxed the vasoconstriction almost completely in the continuing presence of AVP, strongly suggesting that the K_{ATP} channel is involved. A repetitive exposure of AVP in 45 min after the first treatment did not significantly change the reactivity of vascular ring (0.40 ± 0.05 and 0.37 ± 0.05 g, respectively, n = 5 rings from two animals, $P > 0.05$, Figure 7-1, C). This effect did not seem to be mediated through endothelium, as AVP continued producing contractions in endothelium-denuded rings (0.52 ± 0.09 g, n = 3). The effect of AVP was blocked by 30 nM YM-AVP, a selective V1a receptor blocker (Figure 7-1, D and F) (0.260 ± 0.002 and 0.028 ± 0.000 g, respectively, n = 4, $P < 0.05$). The V1a receptor is known to be coupled to G protein $G_{\alpha q}$ activation, which leads to activation of phospholipase C and PKC (Howl and Wheatley, 1995). Therefore, it is possible that AVP-induced vasoconstriction is mediated by activation of the PKC pathway. A pre-exposure to PKC inhibitor, calphostin-C (1 μM), attenuated the AVP-induced vasoconstriction ($P < 0.05$, n = 4; Figure 7-1, E and F). Similar to the effect of AVP, application of PMA (1 μM), a selective PKC activator, produced vasoconstriction (0.41 ± 0.16 g, n = 5) that was relaxed by 10 μM pinacidil (0.05 ± 0.03 g, n = 5 rings from 4 animals).

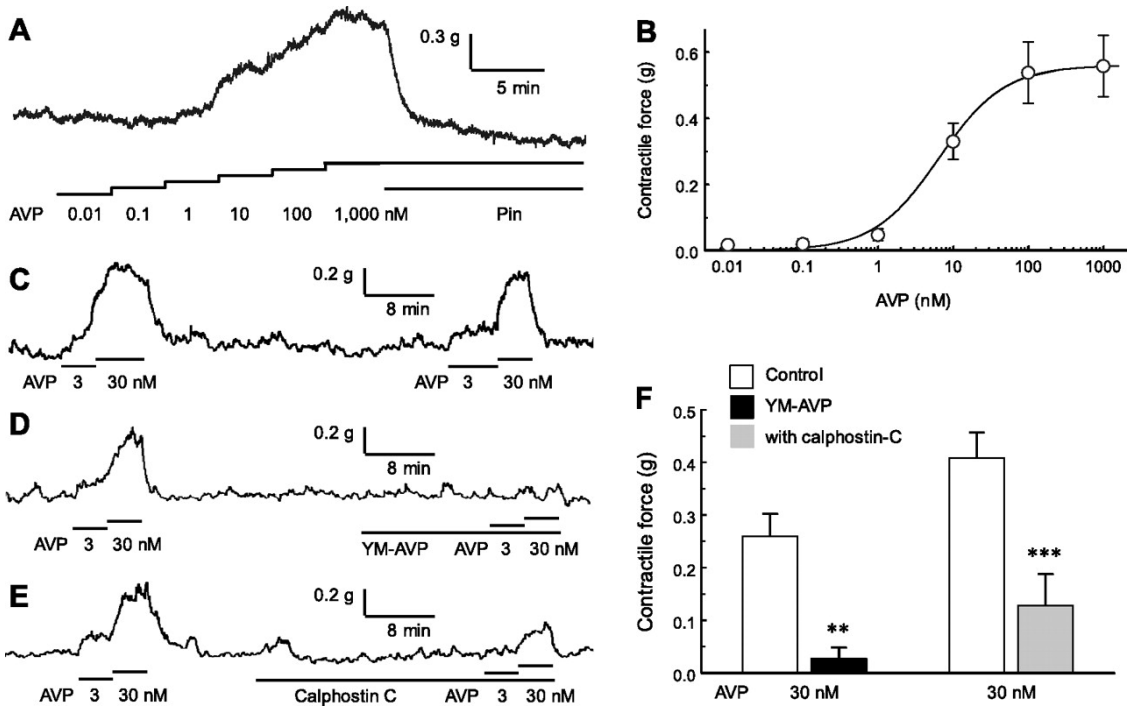


Figure 7-1. Effects of AVP on vascular tone.

Vasoconstriction was studied in endothelium-intact mesenteric arterial rings at 36°C in vitro. **A.** contractile force increased dose-dependently with the increase in AVP concentrations. At the maximum effect, 20 μM pinacidil relaxed the vasoconstriction almost completely in the continuing presence of 1 μM AVP. **B.** contractile force is a function of AVP concentrations, and their relationship can be described with the Hill equation (EC_{50} 6.5 nM, $h = 1.0$, $n = 4$ rings from 2 animals). **C.** contractions were seen with repetitive exposures to AVP. **D** and **E**. in two different rings, the AVP effect was greatly diminished by 30 nM [Deamino-Pen1, Tyr(Me)2, Arg8]-vasopressin (YM-AVP; D) and 1 μM calphostin-C (E). **F**. Summary of the effect of YM-AVP and calphostin-C on the contraction inhibition by AVP. Data are shown as means ± SE ($n = 4$ rings from 3 animals and 5 rings from 4 animals, respectively).

7.3.2. Effects of AVP on endogenous currents of vascular smooth myocytes

Since activation of K_{ATP} channel compromised AVP-induced vasoconstriction, we next investigated if AVP directly regulated vascular K_{ATP} channel. Vascular smooth myocytes (VSMs) were freshly dissociated from the mesenteric arteries (Figure 7-2, A). The bath solutions contained 145 mM K^+ so that the reversal potential of K^+ currents is near 0 mV. The recording pipette was filled with the same solution with the addition of 1 mM ATP, 0.5 mM ADP, and 1 mM free Mg^{2+}. The VSMs showed small baseline currents, and the current amplitude increased

markedly with an exposure to 10 μM pinacidil. For quantitative analysis, we normalized the affected currents between maximum channel inhibition by glibenclamide (Glib, 10 μM) and maximum activation by 10 μM pinacidil. The pinacidil-activated currents were strongly inhibited by 100 nM AVP in the presence of pinacidil (by 63.1 ± 5.3%, n = 6) (Figure 7-2, B and E).

In cell-attached patches, a K^+ current with unitary conductance of ~35pS was observed in the acutely dissociated VSMs (Figure 7-2, C). The current had rather low baseline activity with NP_o 0.004±0.002 (n=4), consistent with a previous report (Nelson et al., 1990). The channel activity increased with an exposure to 10 μM pinacidil and was inhibited with 100 nM AVP in the extracellular solution (Figure 7-2, D, E). The pinacidil-activated current had unitary conductance of 34.8±1.1 pS (n=4), which did not change significantly with AVP (35.3± 0.8 pS, n=4, P>0.05). Application of glibenclamide led to a further inhibition of this current. Therefore, these pharmacological properties of this 35-pS current were consistent with our observations in the whole cell recordings from the VSMs, suggesting that the VSM-endogenous K_{ATP} channel is inhibited by AVP.

7.3.3. Expression of Kir6.1/SUR2B channels in HEK-293 cells

We further identify the signaling pathway between AVP and K_{ATP} channel using mammalian expression system. Expression of AVP receptors in HEK-293 cells has been successfully used to identify signal pathways of AVP (Innamorati et al., 1998; Terrillon et al., 2004). The Kir6.1/SUR2B channel was transiently expressed in HEK-293 cells. Whole cell patch clamp was performed on GFP-positive cells. Whole cell currents were recorded from these cells under the same condition as for the VSMs. The transfected cells exhibited small baseline currents

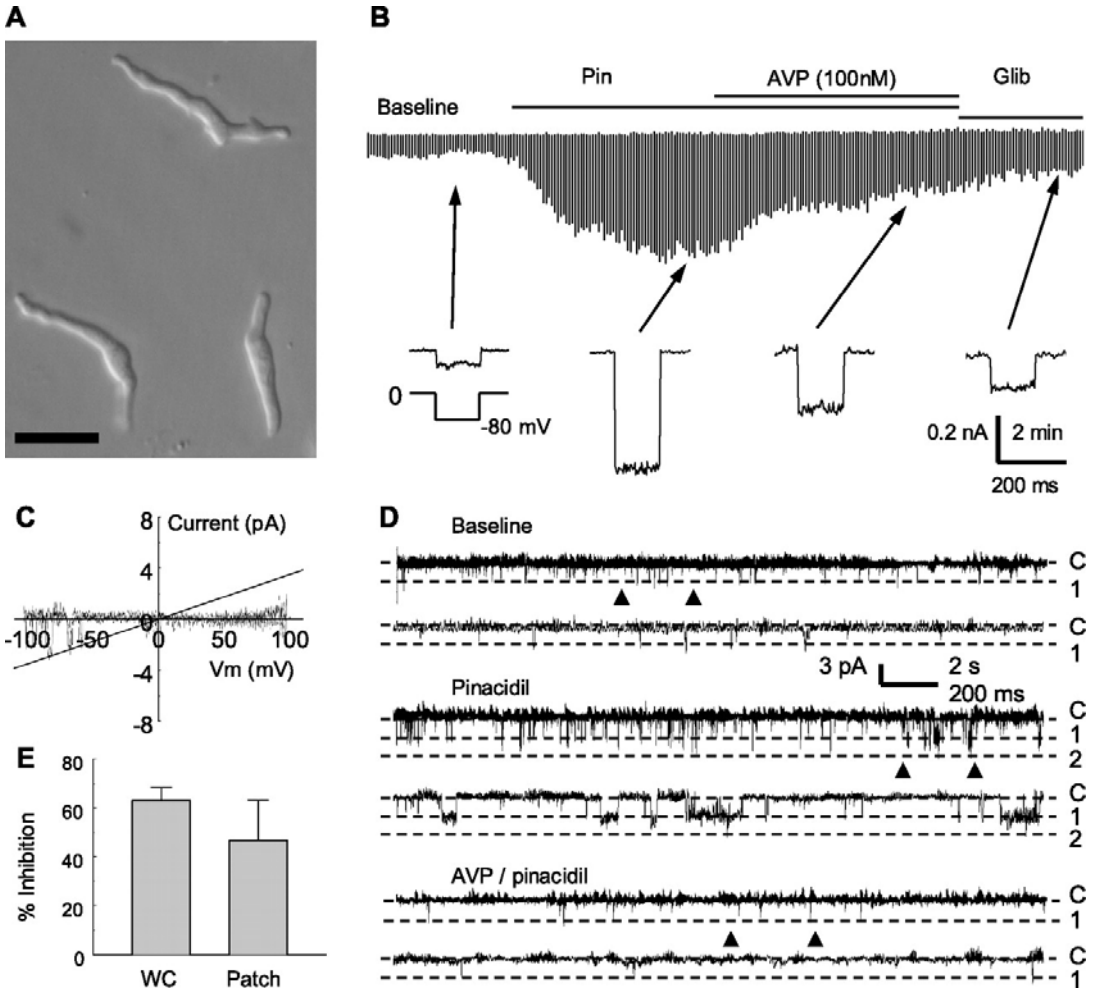


Figure 7-2. Effects of AVP on inward K^+ currents in VSMCs.

A. cells were acutely dissociated from the rat mesenteric artery and photographed before patch-clamp experiments. Calibration = 30 μm. **B.** The whole cell currents were recorded with a holding potential at 0 mV and command pulses of –80 mV every 3 s. After whole cell configuration was formed, the SMC was perfused with extracellular solution for a 5-min baseline recording (shown partially). The whole cell K^+ currents were strongly activated with 10 μM pinacidil. Application of 100 nM AVP suppressed the currents in the presence of pinacidil. The currents were further inhibited by 10 μM glibenclamide. Note that individual records are shown (*bottom*) with time expansion. **C.** single-channel currents were recorded in a cell-attached patch. Currents showed single-channel conductance of 35 pS with ramp voltages from –100 to 100 mV. **D.** with a membrane potential of –80 mV, the channel activity (C) was low at baseline. The single-channel currents were activated with 10 μM pinacidil. The pinacidil-activated currents were strongly inhibited by 100 nM AVP in the presence of pinacidil. The lower trace in each panel is an expansion from the record of upper trace between arrow heads. **E.** summary of percentage inhibition of channel activity by 100 nM AVP in whole cell (WC; $n = 6$) and cell-attached patch ($n = 4$) configurations. No statistical difference was found ($P>0.05$).

upon the formation of whole cell configuration (Figure 7-3, A and B). An exposure to 10 μM pinacidil increased the currents markedly. The pinacidil-activated currents were strongly inhibited by 10 μM glibenclamide (Figure 7-3). These, as well as single-channel properties (see *Biophysical mechanisms*), were consistent with the characteristics of Kir6.1/SUR2B currents reported previously (Teramoto, 2006; Wang et al., 2003; Yamada et al., 1997).

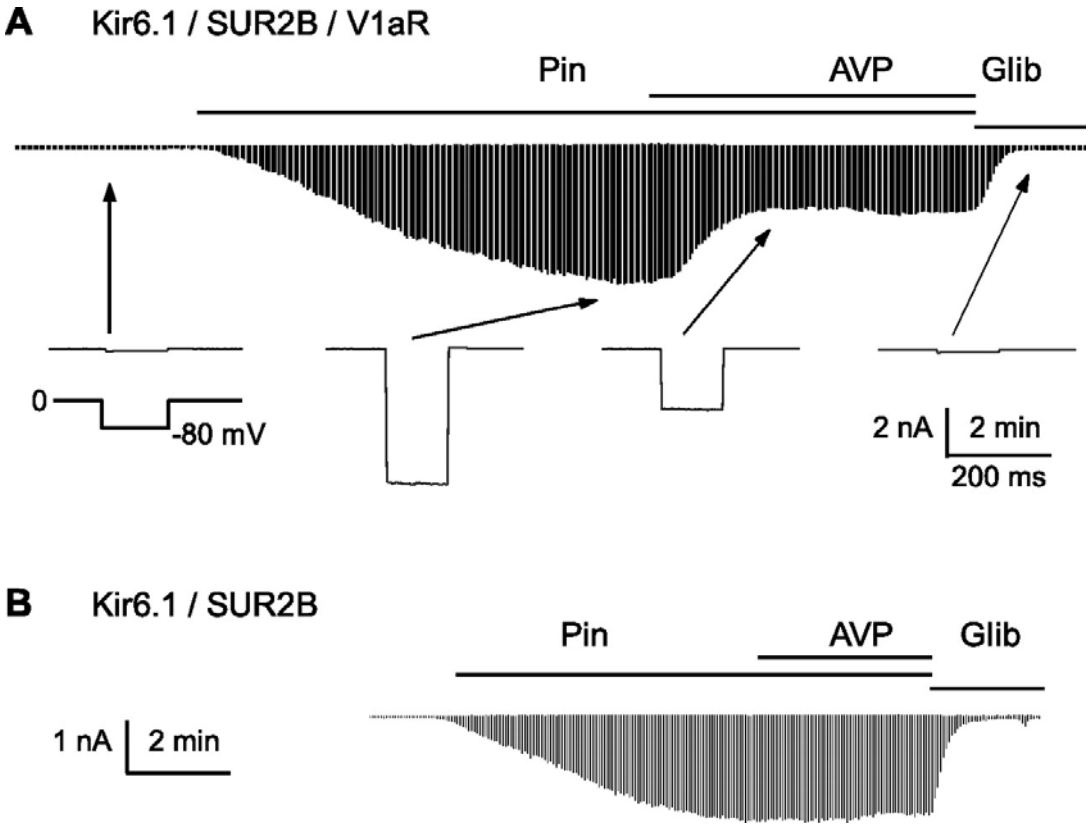


Figure 7-3. Kir6.1/SUR2B channel expressed in HEK-293 cells.

A. whole cell currents were recorded from a HEK-293 cell transfected with Kir6.1/SUR2B/V1a receptor (V1aR) under the same condition as described in Fig. 7-2. Similar to the K_{ATP} channel expressed in SMCs, currents were strongly activated by pinacidil (10 μM), and the maximum activation was reached within 5–6 min of the exposure. The currents were inhibited by 100 nM AVP and further inhibited by glibenclamide (10 μM). *Bottom*: individual currents produced by a single command pulse. **B.** whole cell currents were recorded from a cell transfected with Kir6.1/SUR2B. Currents were strongly activated by pinacidil (10 μM) with the maximum activation reached in 4–5 min of the exposure. Currents did not respond to 100 nM AVP while they were still inhibited by glibenclamide (10 μM).

7.3.4. Inhibition of Kir6.1/SUR2B channels by AVP

When the V1a receptor was cotransfected with Kir6.1/SUR2B in HEK-293 cells, the currents activated by 10 μM pinacidil were strongly inhibited with an exposure to 100 nM AVP plus 10 μM pinacidil (Figure 7-4, A). Evident channel inhibition was seen with 300 pM AVP (16.6 ± 8.1%, n = 8), and stronger inhibition occurred with higher concentrations, with 1 nM (26.4 ± 10.9%, n = 6) and 3 nM (52.2 ± 10.6%, n = 8). The concentration-response relationship can be described by using the Hill equation with IC_{50} 2.0 nM, and (h) 1.0 (Figure 7-4, B). The maximal inhibition was reached with 10 nM AVP (62.9 ± 10.7%, n = 5). Higher concentration showed slightly further inhibitory effect, with 30 nM AVP (64.0 ± 9.6%, n = 11), 100 nM AVP (66.0 ± 5.4%, n = 8), and 300 nM AVP (66.8 ± 8.6%, n = 6). The Kir6.1/SUR2B currents were also studied in cells transfected without V1a receptor in which no evident inhibition of the Kir6.1/SUR2B currents was seen with 100 nM AVP (Figure 7-3, B).

7.3.5. Biophysical mechanisms

In whole cell recordings, the currents activated by pinacidil showed almost a linear conductance with no obvious rectification, which was consistent with previous reports (Figure 7-5, A-D) (Yamada et al., 1997). The currents inhibited by AVP were isolated by subtracting the remaining currents from the maximum currents activated by pinacidil. When the current-voltage relationship of the currents was plotted with the pinacidil-activated currents, they superimposed almost completely (Figure 7-5, E-G), indicating that effect of AVP is not voltage dependent.

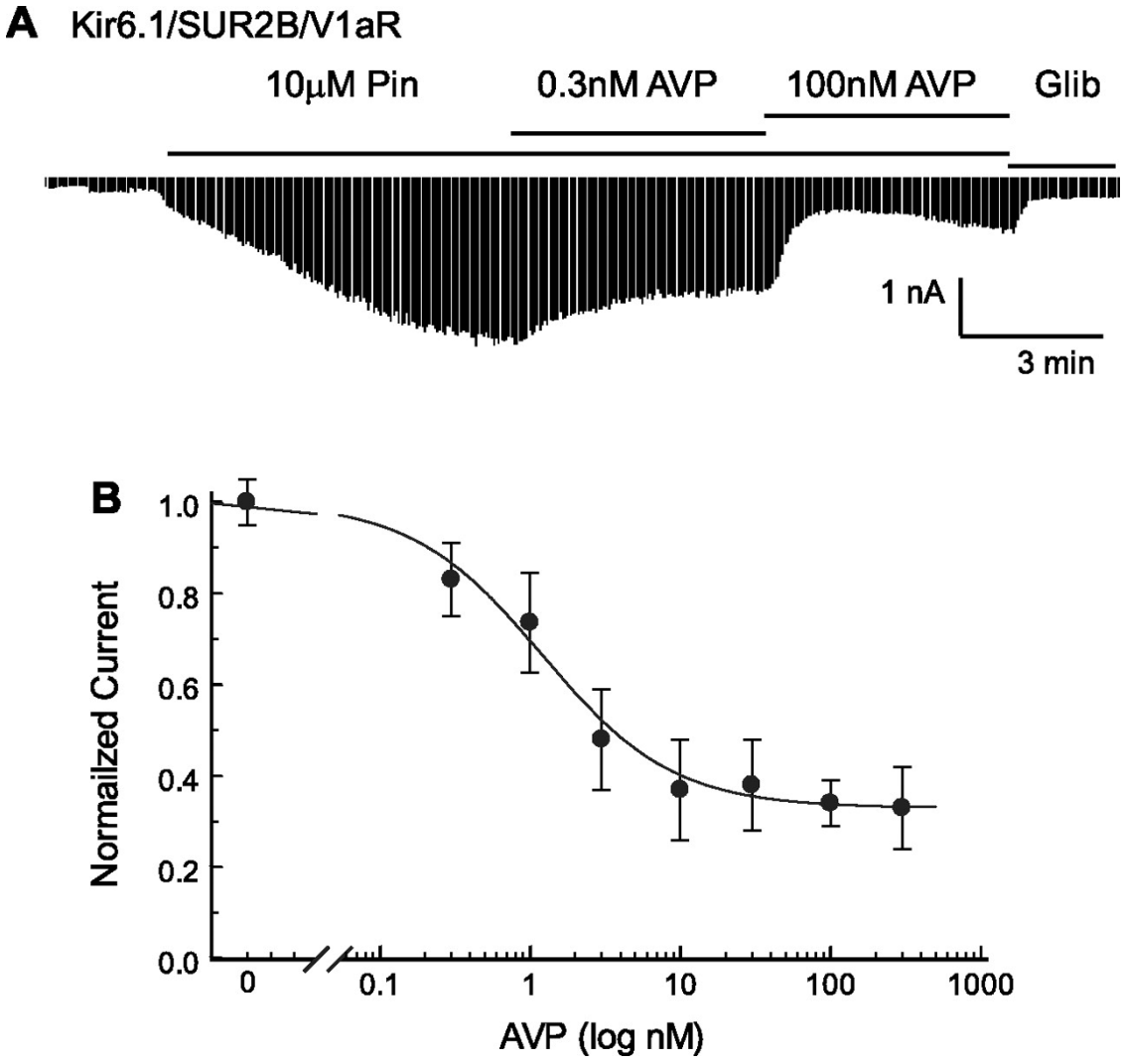


Figure 7-4. Concentration-dependent inhibitions of Kir6.1/SUR2B currents by AVP.

A. clear inhibition of the pinacidil-activated currents occurred with 0.3 nM AVP, and a higher concentration (100 nM) of AVP further decreased the whole cell currents. **B**. effect of AVP was measured and normalized between the maximum channel inhibition by 10 μM glibenclamide and maximum channel activation by 10 μM pinacidil, and was plotted against AVP concentrations. The concentration-response relationship was described using the Hill equation with IC_{50} = 2.0 nM and Hill coefficient (*h*) = 1.0.

In cell-attached patches, currents with single-channel conductance of 39.1 ± 3.3 pS (*n* = 8) were observed. Exposure of the cells to 10 μM pinacidil increased NP_o from 0.021 ± 0.030 to 0.140 ± 0.072 (Figure 7-6). AVP subsequently reduced NP_o to 0.037 ± 0.026 ($P < 0.01$, $n = 5$). In

contrast to NP_o, single-channel conductance did not show any significant change in the presence of AVP (38.0 ± 4.7 pS, *n* = 8) (Figure 7-6). Therefore, these results suggest that the inhibition of whole cell Kir6.1/SUR2B currents by AVP is produced by the suppression of the NP_o without affecting the unitary conductance.

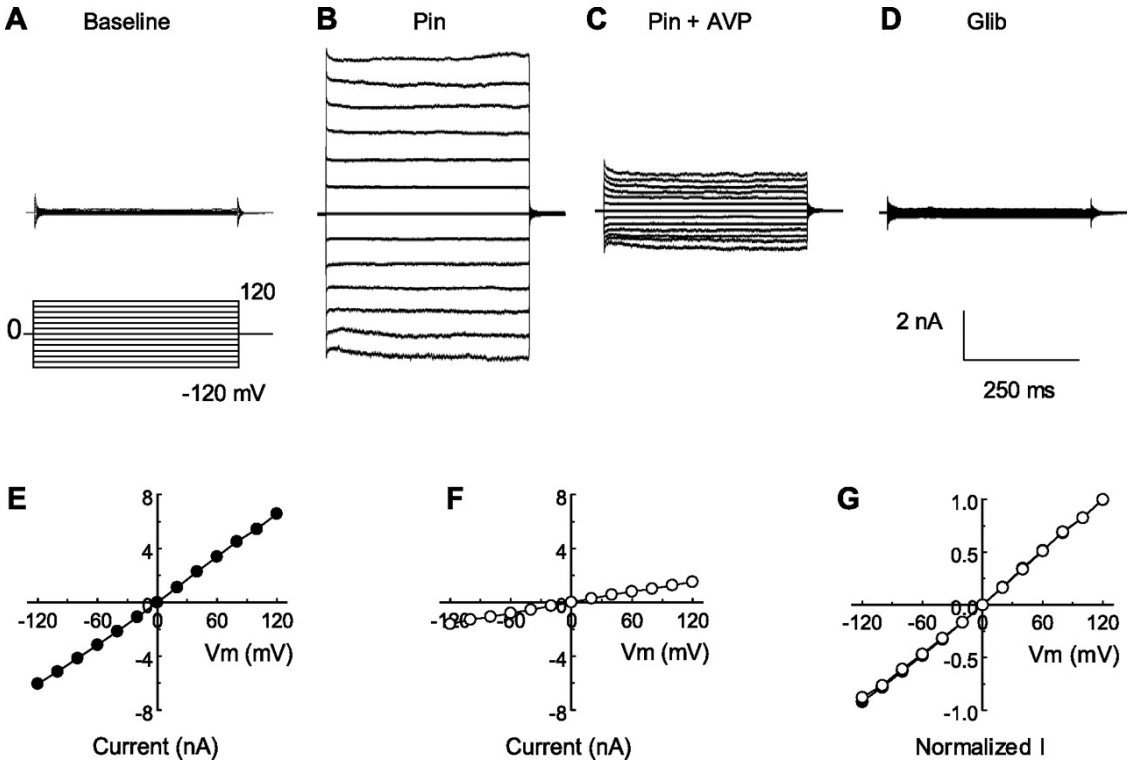


Figure 7-5. Voltage dependence.

A–D. whole cell currents were recorded under the same condition as shown in Fig. 7-2. A series of command pulses were given from –120 mV to 120 mV with 20-mV increments. The voltage protocol did not produce evident currents in the baseline (**A**). The Kir6.1/SUR2B currents were strongly activated with 10 μM pinacidil (**B**). Pinacidil-activated currents were inhibited by 100 nM AVP (**C**) and 10 μM glibenclamide (**D**). When plotted against membrane potential (Vm), the pinacidil-activated currents showed almost a linear conductance (**E**). A similar current-voltage relationship was seen after the AVP exposure (**F**). When the currents inhibited by AVP were isolated with a subtraction of F from E, scaled to the maximum at 120 mV and plotted together with the pinacidil-activated currents, the currents were almost completely superimposed (**G**).

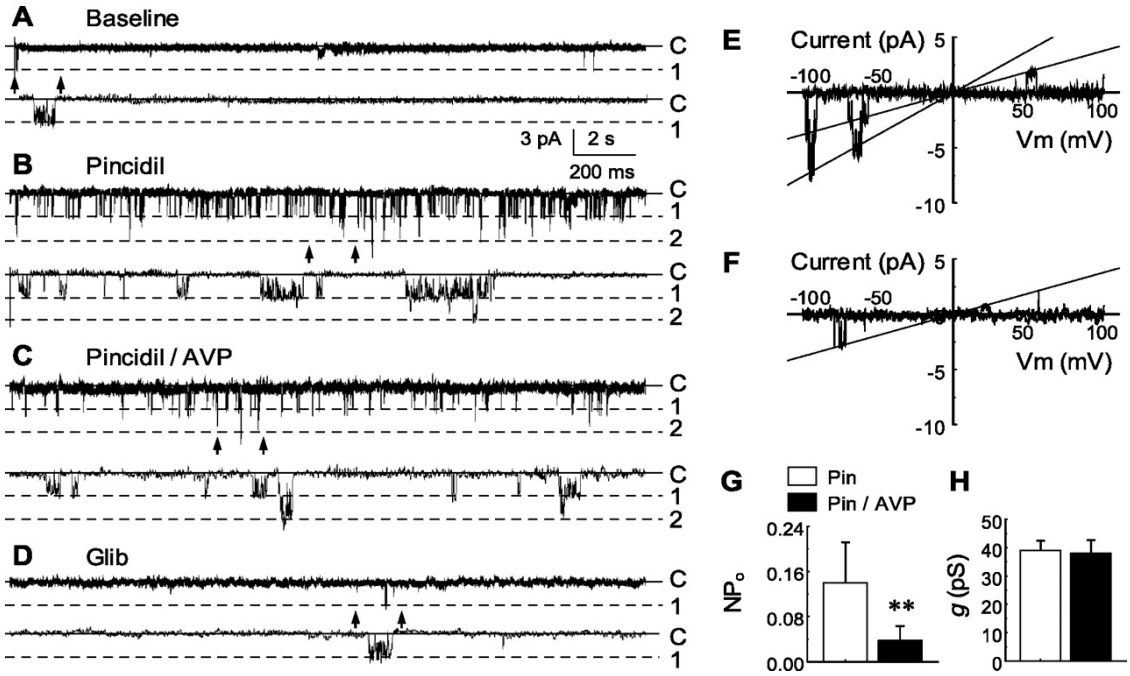


Figure 7-6. Effect of AVP on single-channel currents.

A–D. single-channel currents were recorded in a cell-attached patch. The lower trace in each panel is an expansion from the record of upper trace between arrows. An active channel was seen at baseline (**A**). When the cell was exposed to 10 μM pinacidil, the single-channel current was activated with the appearance of a second active channel (**B**). The currents were strongly inhibited with 100 nM AVP (**C**). Glibenclamide (10 μM) further inhibited the currents (**D**). **E** and **F**. single-channel conductance was measured in the same cell with a ramp voltage from –100 to 100 mV. Two channels were active in **E**. Slope conductance of the currents was 38 pS with pinacidil (**E**) and AVP exposure (**F**). Note that leak currents were manually removed, and the slope conductance was not measured at baseline because of low appearance of the channel. AVP showed significant suppression of overall channel activity in which the number of openings was not counted (NP_o; **G**) but not single-channel conductance (**H**). $^{**}P < 0.01$.

7.3.6. PKC dependence

Previous studies have shown that Kir6.1/SUR2B channel activity is affected by both PKA and PKC (Quinn et al., 2004; Thorneloe et al., 2002), and the V1a receptor is coupled to $G_{\alpha q}$ which links to downstream PKC. Therefore, it is possible that the Kir6.1/SUR2B channel inhibition by AVP is mediated by activation of the PKC pathway. To test this hypothesis, we studied the Kir6.1/SUR2B channel by interference with the PKC signaling system. PMA is a

potent PKC agonist that anchors PKC to the cellular membrane and persistently exposes its catalytic site (Newton, 1995). Administration of 100 nM PMA reduced the pinacidil-activated currents by 84.5 ± 5.9% (n = 6) (Figure 7-7, A). In the presence of PMA, 100 nM AVP had no significant inhibitory effect on the currents (8.4 ± 5.1%, n = 6). In contrast, inactive phorbol ester, 4α-phorbol 12,13-didecanoate (4α-PDD) had little inhibitory effect (13.3 ± 8.5%, n = 4), and the whole currents were further inhibited by 100 nM AVP (63.9 ± 9.9%, n = 4) after the cells were pretreated with 4α-PDD (Figure 7-7, D and E). With pretreatment with 100 nM calphostin-C for 20 min, the inhibitory effect of AVP was significantly diminished (15.4 ± 1.7%, n = 5, $P < 0.01$).

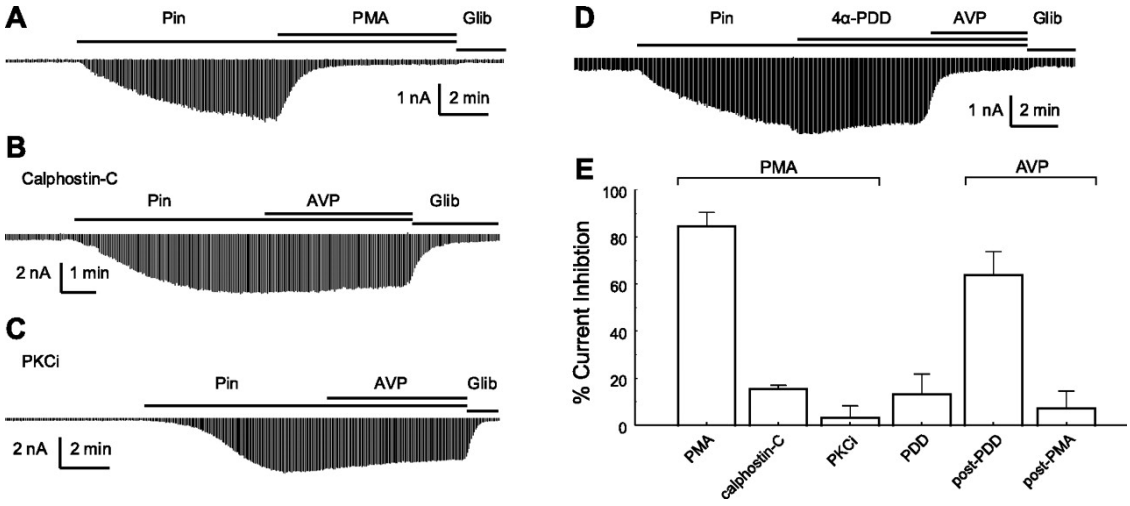


Figure 7-7. PKC dependence.

A. pinacidil-activated currents were markedly inhibited by 100 nM PMA. **B**. inhibitory effect of AVP was diminished with a 20-min pretreatment of 100 nM calphostin-C. **C**. in the presence of 10 μM PKC 19–31 in pipette solution, the current response to AVP was also reduced. **D**. pinacidil-activated currents were not inhibited with a 5-min exposure to 100 nM 4α-phorbol 12,13-didecanoate (4α-PDD), and were inhibited by 100 nM AVP. **E**. Summary of effects of PKC activator on the Kir6.1/SUR2B currents. Data are shown as means ± SE (n = 4 to 8). PDD, 4α-PDD.

When the pipette solution contained PKC inhibitor peptide 19-31 (PKCi, 10 μM) (Figure 7-7, C and E), which acted as a pseudo-substrate by binding to the catalytic site of PKC, 100 nM AVP did not produce any significant channel inhibition (3.2 ± 5.1%, $n = 5$, $P > 0.05$) (Figure 7-7, C and E). Taken together, all of these results suggest that the Kir6.1/SUR2B channel inhibition by AVP is very likely to be mediated through the PKC system.

7.3.7. Endocytosis did not contribute to PKC-dependent inhibitory effect

Channel trafficking is an important mechanism for regulation of channel activity. Upon PKC activation, Kir6.2-containing K_{ATP} channel displayed internalization in a dynamin-dependent way (Hu et al., 2003). We hypothesized that PKC inhibited Kir6.1/SUR2B through a similar trafficking mechanism. Since a dominant-negative dynamin-2 K44A blocked the process of endocytosis, we co-transfected the K44A mutant with Kir6.1 and SUR2B. Wild-type dynamin-2 was used as control. The two groups of cells did not displayed significantly different response to PMA (68.0 ± 3.3%, n=4, and 79.0 ± 9.6%, n=4, respectively, $P>0.05$. Figure 7-8), suggesting that the dynamin-dependent internalization is not critical.

7.3.8. Mutational analysis on SUR2B subunit

If the channel internalization was not a mechanism here, it was likely that PKC inhibited Kir6.1/SUR2B through a direct phosphorylation. Systematic mutational screening was performed on the SUB2B subunit first. Twenty-three PKC consensus residues were found in the intracellular domains of SUR2B. Each site was mutated to alanine or asparagine. All of these mutant SUR2B subunits together with Kir6.1 expressed functional currents similar to the wt

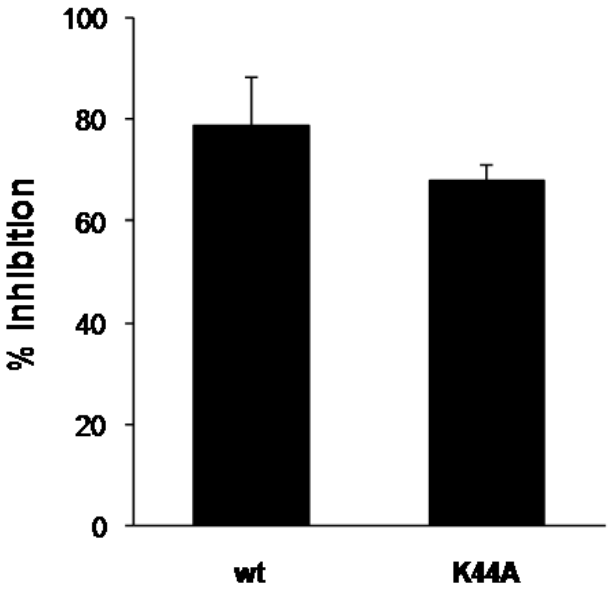


Figure 7-8. PKC inhibited Kir6.1/SUR2B channel independent of endocytosis.

Whole cell patch clamping was performed in HEK-293 cells transfected with Kir6.1, SUR2B and dynamin 2 constructs (wt and K44A). PMA (100 nM) inhibited pinacidil-sensitive currents in wt by 79.0 ± 9.6% (n=4). Expression of dynamin-2 K44A did not significantly disturb the effect of PMA (68.0 ± 3.3%, n=4, $P>0.05$).

channel, i.e., currents that were activated by pinacidil and suppressed by glibenclamide. In wt channel, PMA (100 nM) reduced the pinacidil-activated currents by 80.1 ± 5.4% (n = 10). Among the twenty-three residues, T898N, T972A and T1381A significantly reduced the PKC-dependent channel inhibition (by 41.8±6.6%, n=6, $P<0.001$; 55.6±12.1%, n=7, $P<0.05$ and 48.4±10.1%, n=8, $P<0.001$, respectively).

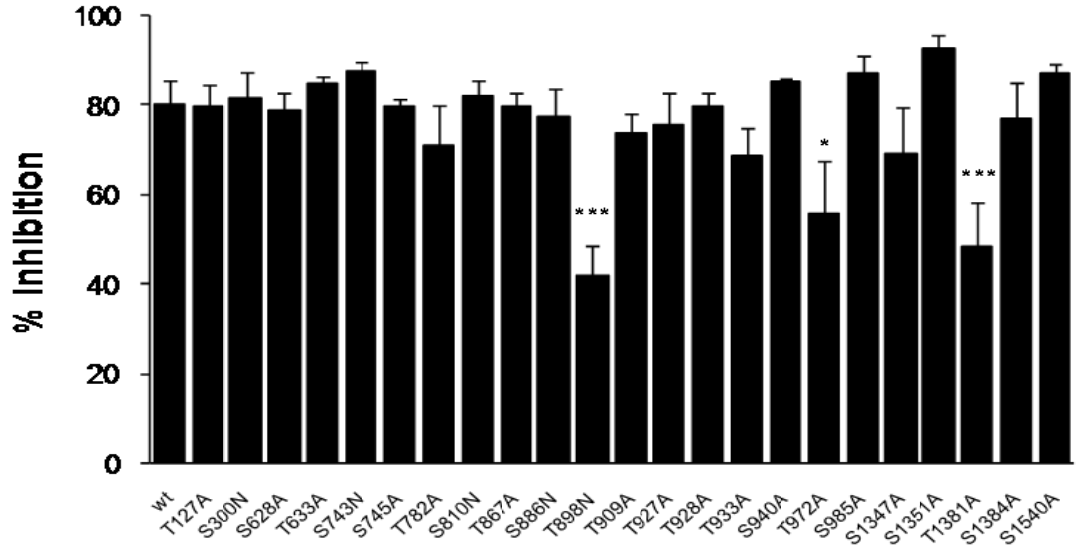


Figure 7-9. Systemic mutation on predicted PKC phosphorylation sites located in SUR2B subunit.

All mutations were constructed on SUR2B. All patch clamping data were collected from 4–8 cells per mutation. T898N, T972A and T1381A displayed a decreased response to PKC activation compared to wt. *P < 0.05. *** P<0.001.

7.3.9. Thr898, Thr972 and Thr1381 were not functional PKC phosphorylation sites

If Thr898, Thr972 and Thr1381 were phosphorylated by PKC, double mutation among these residues should lead to greater inhibition of the PMA effect. To confirm this hypothesis, we constructed SUR2B with T898N/T1381A and T898N/T972A. The T898N/T1381A mutant exhibited a similar sensitivity to PMA (90.9±1.0%, n=4, P>0.05). Since T898N/T972A (2 clones) did not display pinacidil-activated currents, we alternatively transfected SUR2B T972A with Kir6.1 S6N, a predicted PKC consensus residue located in the distal N-terminus of Kir6.1. The channel with this combination was inhibited by 84.7±7.5% (P>0.05) when exposed to PMA.

If these residues were substrates of PKC, the mutant channels should also show a decreased response to AVP. In HEK-293 cells transfected with V1a receptor and Kir6.1/SUR2B,

the pinacidil-induced K_{ATP} currents were strongly inhibited by AVP (76.1±7.1%, n=6). The inhibitory effect was not significantly altered in S898N and T1381A (81.7±5.7%, n=5, P>0.05, and 61.9±9.2%, n=6, *P*>0.05, respectively). Therefore, it is unlikely that Thr898, Thr972 and Thr1381 were phosphorylated by PKC.

Since PKC phosphorylation sites do not seem to be in the SUR2B, the Kir6.1 subunit becomes the only logical target. Indeed, further studies on the Kir6.1 in our laboratory have led to the identification of several PKC sites in the subunit, to which I was a contributor (Shi et al. 2008).

7.4. Discussion

Our results from the present study indicate that the VSM isoform of K_{ATP} channels is one of the major targets of AVP. We have found that AVP strongly inhibits the Kir6.1/SUR2B channel expressed in the HEK-293 cell line. The channel inhibition is mediated by selective suppression of NP_o without effect on the single-channel conductance. Activation of the V1a receptor and PKC system is crucial for the channel inhibition. Similar effects are also observed in acutely dissociated VSMs. AVP constricts mesenteric arteries where Kir6.1/SUR2B channel is expressed (Sampson et al., 2004; Wang et al., 2003), and such a vasoconstriction also depends on the K_{ATP} channel, V1a receptor, and PKC pathway.

As an effective vasoconstrictor, AVP is believed to modulate contractions of vascular smooth muscles through multiple ion channels. At physiological concentrations, AVP stimulates Ca^{2+} spiking in cultured aortic smooth myocytes, which can be abolished by mibefradil at submicromolar concentrations that do not inhibit L-type Ca^{2+} currents, suggesting that the T-type,

or low-threshold voltage-activated Ca^{2+} channels, are activated by AVP (Brueggemann et al., 2005) . Another study showed that the L-type Ca^{2+} channel is also involved in the AVP-induced vasoconstriction (Katori et al., 2001) . In addition to the Ca^{2+} channels, AVP is known to produce Ca^{2+} influx through voltage-dependent and receptor-operated cation channels (Van Renterghem and Lazdunski, 1994) , which was recently demonstrated to be the heteromultimeric TRPC6-TRPC7 channel (Maruyama et al., 2006). Activation of these voltage-independent Ca^{2+} channels and cation channels can raise intracellular Ca^{2+} and initiate contractile filament sliding, when depolarization also occurs simultaneously. The vasoconstriction effect of AVP depends on extracellular Ca^{2+} entry in male rat aorta, whereas female aorta depends more upon intracellular Ca^{2+} released from the sarcoplasmic reticulum. In this regard, gonadal steroid hormones may contribute to aortic vasoconstriction induced by AVP (Eatman et al., 1998). AVP also enhanced sympathetic vasoconstriction at a lower temperature (30°C) by activating V1a receptor (Garcia-Villalon et al., 1999). However, intracellular signal pathways and target molecules of AVP are still not fully understood. Accumulating experimental evidence indicates that K_{ATP} channels are inhibited by AVP leading to depolarization. In guinea pig ventricular myocytes, AVP inhibits K_{ATP} channels through V1a receptors with an IC_{50} of 15 nM (Tsuchiya et al., 2002). In the RINm5F insulin-secreting cell line, AVP inhibits K^+ channels that are closed by tolbutamide and opened by diazoxide (Martin et al., 1989) , suggesting that the Kir6.2/SUR1 channel is involved. In cultured coronary arterial smooth muscle cells, AVP inhibits a K^+ current in both outside-out and cell-attached patches, and this effect can be reversed by a K_{ATP} channel opener nicorandil (Wakatsuki et al., 1992). Our results indicate that the vascular isoform of K_{ATP} channels Kir6.1/SUR2B is indeed targeted by AVP. The effect of AVP is not limited to the heterologous

expression system. We have found that the VSM-endogenous K_{ATP} current is inhibited by AVP to almost the same degree as the Kir6.1/SUR2B channel expressed in HEK-293 cells.

Dumont and Lamontagne (Dumont and Lamontagne, 1995) reported that the AVP-induced vasoconstriction of aortic rings cannot be blocked by glibenclamide and suggested that K_{ATP} channels do not play a role in the vasoconstrictive effect of AVP. Since the effect of the K_{ATP} channel blocker depends on the channel-open state, it may not have significant effect when the channels are closed. When the K_{ATP} channels are opened by lemakalim, the same study indeed showed a significant attenuation of the AVP-induced vasoconstriction, which is consistent with our observations in the present study. The evidence of glibenclamide alone may not be sufficient to overthrow the contribution of these K^+ channels to the AVP effect, as glibenclamide also affects other channels, such as renal outer medullary K^+ channel (ROMK) and cystic fibrosis transmembrane conductance regulator (CFTR) (Ishida-Takahashi et al., 1998; Konstas et al., 2002) , and glibenclamide may act on thromboxane receptors attenuating indirectly the AVP-induced vasoconstriction of placental chorionic plate arteries (Wareing et al., 2006b) . The observations that the K_{ATP} channel openers relax arterial rings in previous reports (Dumont and Lamontagne, 1995; Wakatsuki et al., 1992) and that AVP inhibits the Kir6.1/SUR2B and the tissue-endogenous K_{ATP} channels shown in our current studies strongly suggest that the Kir6.1/SUR2B channel plays, at least in part, a role in the AVP-induced vasoconstriction.

Consistent with the idea that the V1a receptor is the AVP receptor in vascular smooth muscles (Holmes et al., 2003), our studies have shown that YM-AVP, a selective V1 receptor antagonist, blocks the vasoconstriction effect of AVP in mesenteric artery rings. In HEK cells, we have found that Kir6.1/SUR2B currents are inhibited by AVP only when the V1a receptor is coexpressed, indicating that the Kir6.1/SUR2B channel is a downstream effector of the V1a

receptor. The V1a receptor is linked to $G\alpha_q$, which activates the PKC-dependent intracellular signaling system (Birnbaumer, 2000). Several vasoconstrictors, such as angiotensin II, serotonin, and histamine have been shown to inhibit vascular K_{ATP} channels through the PKC signal pathway (Bonev and Nelson, 1996; Kubo et al., 1997). Our results suggest that the inhibition of Kir6.1/SUR2B channels by AVP is also mediated via PKC: *1*) PMA resembles the inhibitory effect of AVP, while 4α-PDD has no obvious effect on whole currents; *2*) Kir6.1/SUR2B channel inhibition by AVP is abolished in the presence of selective PKC antagonists calphostin-C or PKCi; *3*) AVP has no further inhibitory effect when the Kir6.1/SUR2B currents were inhibited by PMA; and *4*) PKC dependence is not limited in the HEK cells, as calphostin-C also blocks the vasoconstriction produce by AVP in mesenteric arteries. Furthermore, we have shown that the inhibition of Kir6.1/SUR2B currents by AVP is mediated via suppression of NP_o instead of unitary conductance, consistent with the studies showing that PKC regulates K_{ATP} channels through gating mechanism (Shi et al., 2008b). In addition to direct phosphorylation of the channel protein, the internalization or endocytosis of the V1a receptor and/or Kir6.1/SUR2B channel may be another mechanism for the decrease in functional channel activity, as shown previously for the Kir6.2 channel (Hu et al., 2003).

The SUR subunit is critical for functional expression of K_{ATP} channel (Zerangue et al., 1999). Both Kir6.1 and Kir6.2 contain short sequence RKR that functions as ER retention and prevents their surface expression (Zerangue et al., 1999). The SUR subunit shields this sequence by coupling with Kir6 subunit and forming an octamer. Coexpression SUR with Kir6 dramatically increases K_{ATP} channel surface expression. Noticeably, Kir6.2Δ36 without RKR sequence displayed inward rectifier K^+ currents that were stimulated by metabolic inhibitor azide but not sensitive to glibenclamide, a SUR binding K_{ATP} channel blocker (Tucker et al., 1997). In

comparison, a similar truncated form of Kir6.1 (Kir6.1Δ45) cannot exhibit currents even with surface expression (Zerangue et al., 1999). Therefore, SUR subunit seems more important for Kir6.1-containing K_{ATP} channels.

The SUR2B as an essential accessory subunit of Kir6.1 in vasculture is subject to various regulations. PKA, a key Ser/Thr kinase that contributes to vasodilation, phosphorylates Ser1387 in SUR2B (Shi et al., 2008a). The SUR subunit is also targeted by most of pharmacological K_{ATP} channel modulators, including sulfonyurea that is applied in anti-diabetic therapy (Seino and Miki, 2003), and nicorandil that is used to treat angina (IONA Study Group, 2002). Although the potential PKC sites may be theoretically located in SUR2B, our studies do not support the idea. Our systematic mutational analysis of all candicate PKC sites failed to prove any in the SUR2B subunit. The negative finding is useful, as it helped our later studies and identified the PKC sites in Kir6.1 (Shi et al. 2008).

Dynamin is a 100-kDa GTPase that is involved in many kinds of endocytosis processes such as clathrin-coated pit endocytosis and caveolar endocytosis (Nichols, 2003). The protein consists of multiple C-terminal SH3 binding proline motifs, a central pleckstrin homology (PH) domain, and an N-terminal GTPase domain. PKC stimulates dynamin by acting on the PH domain (Scaife and Margolis, 1997). Compared to neuronal isoform dynamin 1 and testis isoform dynamin 3, dynamin 2 is widely expressed in different cell types, including vascular SMCs (Kashiwakura et al., 2004). Dynamin contributed to PKC activation-induced K_{ATP} channel internalization in cardiac myocytes (Kir6.2/SUR2A) and CA1 neurons (Kir6.2/SUR1). The internalization prevents extra channel activity which is caused by the direct stimulatory effect of PKC on Kir6.2-containing channels (Zerangue et al., 1999). We did not find that dynamin 2 was involved in PKC regulation on Kir6.1/SUR2B. This could be related to the opposite response of

Kir6.1/SUR2B to PKC. The channel inhibition plus internalization by PKC would lead to excess channel suppression.

In conclusion, AVP is a potent vasoconstrictor that has been known to be useful when systemic circulation loses its reactivity to traditional anti-shock drugs, such as epinephrine and dopamine during septic shock (Friedman et al., 1998). The rationale for the application of AVP is its relative lower plasma concentration (1 pg/ml, 10^{-12} M) and hypersensitivity to its vasoconstrictor effects during such a pathological condition (Mutlu and Factor, 2004). Therapeutic application of AVP can be even more effective if its target molecules and critical intracellular signal pathways are known. In this regard, our current studies appear to constitute a significant step toward the understanding of vascular regulation by AVP and K_{ATP} channels.

8. General Discussion

8.1. K_{ATP} channel in a regulatory matrix

The K_{ATP} channel is critical for vascular tone regulation and a delicate regulatory network was developed during evolution (Figure 8-1). Regulation of K_{ATP} channel actually actually has acute and chronic phases. The fast regulation is through a channel gating mechanism, whereas the slow regulation is operated by a transcriptional mechanism.

8.1.1. Acute regulation

K_{ATP} channels are subject to a direct and fast regulation by intracellular ATP, ADP, pH and phospholipids. Such modulations link the cellular metabolic states to membrane electric activities (Aguilar-Bryan and Bryan, 1999). pH changes in local tissues are very common in heavy exercise, hypoxia, ischemia, and severe diabetes. Previous studies in our group have demonstrated that K_{ATP} channels in pancreatic and cardiac tissues are stimulated by hypercapnia and intracellular acidosis (Li et al., 2005; Piao et al., ; Wu et al., 2002; Xu et al., 2001). The modulations of K_{ATP} channel activity by H^+, ATP and ADP are mediated via directly proton binding to Kir6.x or SUR, leading to alternation in the channel gating mechanism (Dabrowski et al., 2004; Matsuo et al., 2000; Xu et al., 2001). Products during oxidative stress also change vascular K_{ATP} channel activity. H_2O_2 suppresses Kir6.1/SUR2B channel by direct targeting cysteine residues located in the Kir6.1 subunit. In addition to the modulation by the metabolites, K_{ATP} channel activity is regulated by multiple protein kinases (e.g., PKA, PKC). The vasoactive substances bind to their receptors that are coupled to different types of G proteins, leading to activation of PKA and PKC. The phosphorylation sites that are targeted by these two protein kinases are well demonstrated in the vascular K_{ATP} channel (Shi et al., 2008b; Shi et al., 2007b).

The phosphorylation together with ligand-binding (e.g., H^+, ATP and ADP), provide a fast mechanism to regulate vascular tone.

8.1.2. Chronic regulation

Gene transcription is another mechanism that modulates K_{ATP} channel activity in a slow way. The expression of K_{ATP} channel subunits could be altered in some chronic diseases (e.g. diabetes). A declined SUR2B instead of Kir6.1 and Kir6.2 is observed in aortic SMCs dissociated from diabetic rats (Ren et al., 2003). The changes in channel expression change are more significant in sepsis. Both mRNA and protein levels for Kir6.1 are increased in the diaphragm of rats treated with LPS, with the mRNA level augmented by 4-fold in 48h, whereas protein levels enhanced 9-fold after 24h (Czaika et al., 2000). Moreover, Kir6.1 expression in colonic smooth muscle is enhanced by 22-fold, the mRNA level for SUR2B is decreased by 3-fold in experimental colitis (Jin et al., 2004). Flow stress increases the expression of Kir6.2 (both mRNA and protein) in rat pulmonary microvascular endothelial cells. Flow stopping initiates cellular depolarization in a K_{ATP} channel dependent way (Chatterjee et al., 2003). In the present study, we have demonstrated that LPS increase both Kir6.1 and SUR2B mRNA in a NF-κB and PKA dependent fashion. Such an up-regulation increases K_{ATP} channel activity, and may lead to excessive vasodilation during sepsis.

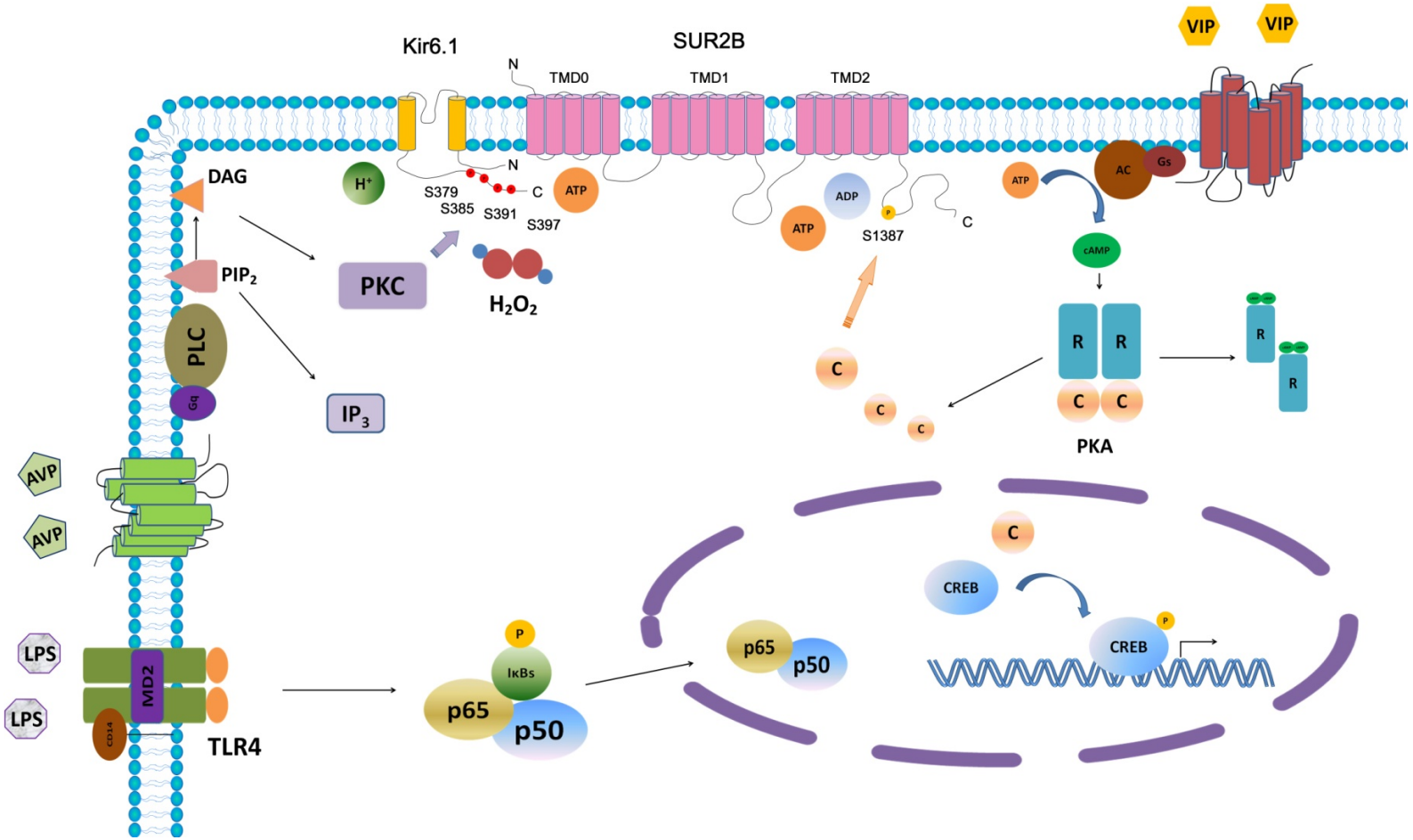

Kir6.1
SUR2B
N
TMD0
TMD1
TMD2
VIP
VIP
DAG
H+
S379
S385
S391
S397
ATP
ADP
S1387
C
AC
Gs
cAMP
PIP2
PKC
H2O2
PLC
Gq
IP3
R
R
C
C
C
C
C
PKA
AVP
AVP
LPS
LPS
MD2
CD14
TLR4
P
IκBs
p65
p50
p65
p50
CREB
CREB
P

Figure 8-1 Schematic representation of vascular K_{ATP} channel in regulatory matrix.

K_{ATP} channels are regulated in multiple levels. Metabolic products (e.g. H^+, ATP, ADP) change channel gating through binding to specific residues. For example, Elevated H^+ (pH 6.8-7.4) stimulates Kir6.2Δ36 channel via binding to His175 (Xu et al., 2001). Both ATP and ADP bind to NBD1 and NBD2 of SUR subunit. In addition, ATP binds to N- and C- termini of Kir6.x subunit (Dabrowski et al., 2004; Matsuo et al., 2000). Vascular K_{ATP} channels are also targeted by vasoconstrictors and vasodilators through protein kinase-dependent ways. Vasopressors, such as AVP, activate PKC, which consequently inhibits the channel through phosphorylating Ser379, Ser385, Ser391, and Ser397 in C-terminus of Kir6.1 (Shi et al., 2007a; Shi et al., 2008b). PKA is stimulated by vasodilators (e.g., VIP), and opens the channel through phosphorylating Ser1387 in SUR2B (Shi et al., 2007b; Yang et al., 2008). In addition, H_2O_2 inhibits vascular K_{ATP} channel activity through a thiol modification mechanism. Furthermore, K_{ATP} channels are subjected to transcriptional regulation. LPS elevate both Kir6.1 and SUR2B expression in a NF-κB and CREB dependent pathways.

8.2. Double-edged vascular K_{ATP} channel during sepsis

Our study, together with the findings from other groups (Czaika et al., 2000; Jin et al., 2004), suggest that the vascular K_{ATP} channel activity is up-regulated during sepsis, leading to severe vasodilation and fatal hypotension. However, Kir6.1-null mice exhibit a high mortality with a challenge of infection, indicating functional K_{ATP} channel is essential for survival from sepsis (Croker et al., 2007; Kane et al., 2006). Therefore, the vascular K_{ATP} channel, with a beneficial side and a dark side, acts as a double-edged sword in the process of sepsis.

8.2.1. A beneficial side of K_{ATP} channel

K_{ATP} channels located in coronary arterioles (<100 μm) become active when coronary pressure is less than 80 mmHg. The activated K_{ATP} channels contribute to coronary vasodilation during reactive hyperemia and hypoxia (Duncker and Bache, 2008). A transgenic mouse model further demonstrates that functional Kir6.1-containing K_{ATP} channels are critical for coronary circulation since the animals exhibit spontaneous coronary vasospasm, a symptom that is similar to Prinzmetal angina in human. An interesting phenomenon is that only one silent mutation in Kir6.1 (C to T, Ile37Ile) is found in patients with coronary spastic angina in studies from 2 individual groups (Emanuele et al., 2003; Tomita et al., 2006). In comparison, dozens of

mutations in the Kir6.2 subunit were discovered in patients with Diabetes mellitus and persistent hyperinsulinemic hypoglycemia of infancy (PHHI) (Gloyn et al., 2006). Such diversity is caused possibly by the lethal outcome of Kir6.1 mutation—the individual carrying Kir6.1 mutation dies early and may be erased during evolution.

Sepsis as an uncontrolled infectious inflammation challenges immune response and changes hemodynamics dramatically. The decreased peripheral resistance requires an adequate cardiac function, but the incidence of myocardial depression during sepsis is so common (nearly 50%) (Maeder et al., 2006) that makes the disease situation even worse. Epidemiological studies show that the cardiovascular impairment during sepsis would elevate the mortality rate from 20% to 90% (Merx and Weber, 2007; Parrillo et al., 1990). Therefore, the LPS-induced up-regulation of K_{ATP} channel doubtlessly plays a protective role for survival from sepsis.

8.2.2. A dark side of K_{ATP} channel

K_{ATP} channels regulate vascular tone in resistant arteries as well as coronary artery. SUR2 is reported to be a susceptibility gene for essential hypertension in humans (Sato et al., 2006). In addition, SUR2-null mice exhibit hypertension (Chutkow et al., 2002). Together with the observations from the major circulatory systems that are described in the Introduction, we can conclude that the K_{ATP} channel plays an important role in the regulation of peripheral circulation. During sepsis, an enhanced channel activity will not only increase coronary arterial perfusion, but also cause severe peripheral vasodilation leading to excess low blood pressure and organ failure. Several therapeutic approaches have already been developed to attenuate septic vasodilation through targeting, at least partially, the K_{ATP} channel.

8.2.2.1 K_{ATP} channel blocker

Glibenclamide as a SUR subunit-binding K_{ATP} channel inhibitor has been tested in several septic animal models. It augments blood pressure through increasing systemic vascular resistance (Landry and Oliver, 1992; Vanelli et al., 1995). However, in a recent double blind clinical trial, glibenclamide neither changed blood pressure, nor decreased requirements of norepinephrine in septic patients (Warrillow et al., 2006). This is due to the complication of using SUR-binding blockers. They will not only inhibit vascular K_{ATP} channels, but also suppress pancreatic K_{ATP} channel activity leading to an increase in insulin secretion. When oral glibenclamide (10 to 30 mg) is provided, a blood glucose concentration will drop before a better hemodynamic is achieved (Morelli et al., 2007). In order to avoid this complication, a new Kir6.x pore blocker PNU-37883A has been developed, and displays better effect to reverse LPS-induced vascular hyporeactivity (O'Brien et al., 2005). Noticeably, Kir6.1/SUR2B channel is more sensitive to PNU-37883A than Kir6.2, suggesting that a proper dose could be found to selectively suppress vascular isoform K_{ATP} channels instead of pancreatic isoform. Hence, developing new blockers specifically targeting Kir6.1 appears to be a promising strategy to manage septic hypotension.

8.2.2.2 AVP

Traditional anti-shock vasopressors, such as norepinephrine, have limitations in management of sepsis, since the high dose of catecholamines may decrease cardiac output and adequate organ perfusion, and increase mortality (Russell et al., 2008). AVP is a special potent vasoconstrictor, because septic patients show insufficiency in AVP secretion and elevated sensitivity to exogenous AVP application (Landry et al., 1997a; Landry et al., 1997b). In a guideline released in 2004, AVP (0.01-0.04 U/min) was recommended for management of sepsis

to avoid usage of high-dose of catecholamines (e.g. norepinephrine, dopamine) (Dellinger et al., 2004). However, a dose higher than 0.03 U/min is not suggested by some groups, since it may induce coronary vasoconstriction and impair cardiac function(Holmes et al., 2001b). Our findings clearly show that vascular K_{ATP} channel is a downstream target of AVP. Therefore, it is not difficult to understand that AVP increases blood pressure through suppression of K_{ATP} channels located in peripheral blood vessels, and decreases coronary perfusion through inhibiting coronary arterial K_{ATP} channels.

8.2.2.3. Transcription interference

Clinical trials suggest that septic patients managed by low dose of corticosteroids displayed a better outcome (Annane et al., 2002). The underlying mechanisms are 1. septic patients exhibit relative adrenal insufficiency (Annane et al., 2000; Rothwell et al., 1991); 2. corticosteroid increases expression of adrenoceptor (Hotchkiss and Karl, 2003), and improves the effect of vasopressors (e.g. norepinephrine, phenylephrine) in septic patients (Annane et al., 2002). K_{ATP} channel is subject to regulation by glucocorticoids. Dexamethasone attenuates LPS-induced K_{ATP} channel activation, and improves vascular reactivity to PE (d'Emmanuele di Villa Bianca et al., 2003). The effect could be caused by down-regulation of K_{ATP} channel expression or synthesis of an intermediate molecule that alters channel expression. Our study suggested that both NF-κB and CREB were required for up-regulation of vascular K_{ATP} channel during sepsis, and blocking either of them attenuated LPS-induced vascular hyporeactivity. Therefore, transcription interference targeting K_{ATP} channels may be a promising therapeutic approach to manage sepsis.

8.3. Summary

The two faces of the K_{ATP} channel set a therapeutic dilemma for administration of sepsis, which means that we need to reasonably control vascular contractility but avoid impairing coronary circulation. All of these depend on an understanding of the molecular mechanism regulating the vascular K_{ATP} channel. The findings in this thesis elucidate the channel regulation at a molecular level, and will shed light on new therapeutic strategies designed to manage sepsis.

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APPENDIX: PUBLICATIONS

1. **Shi, W.**, Cui, N., Shi, Y., Zhang, X., Yang, Y., and Jiang, C. (2007). Arginine vasopressin inhibits Kir6.1/SUR2B channel and constricts the mesenteric artery via V1a receptor and protein kinase C. Am J Physiol Regul Integr Comp Physiol *293*, R191-199.

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5. Shi, Y., Wu, Z., Cui, N., **Shi, W.**, Yang, Y., Zhang, X., Rojas, A., Ha, BT., and Jiang, C. (2007). PKA phosphorylation of SUR2B subunit underscores vascular K_{ATP} channel activation by beta-adrenergic receptors. Am J Physiol Regul Integr Comp Physiol *293*, R1205-1214.

6. Cui, N., Li, L., Wang, X., Shi, Y., **Shi, W.**, and Jiang C. (2006). Elimination of allosteric modulation of myocardial K_{ATP} channels by ATP and protons in two Kir6.2 polymorphisms found in sudden cardiac death. Physiol Genomics *25*, 105-115.

7. Li, L., Shi, Y., Wang, X., **Shi, W.**, and Jiang, C. (2005). Single nucleotide polymorphisms in K_{ATP} channels: muscular impact on type 2 diabetes. Diabetes *54*, 1592-1597.