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Ph.D. Dissertation

黃芩有效成分—黃芩素 (baicalein) 及漢黃芩素 (wogonin) 心臟保護作用的機轉研究

Mechanisms of cardioprotective effects of baicalein and wogonin, two active components from *Scutellaria baicalensis* Georgi

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中文摘要

黃芩是傳統中藥常用的藥物，可清熱燥濕、瀉火解毒、止血、安胎。Baicalein (黃芩素) 及 wogonin (漢黃芩素) 是黃芩的二種主要黃酮素 (flavones)，被證實具有抗氧化及抗發炎作用。本論文的研究目的：於急性發炎動物模式下，探討 wogonin 及 baicalein 是否可產生活體心臟的保護作用；(1) 大鼠麻醉後進行開胸手術，將冠狀動脈結紮四十五分鐘，再循環二小時期，觀察 wogonin (5, 10, 20 mg/kg, ip) 是否可以降低心臟損傷，並探討可能機轉；(2) 將清醒動物靜脈注射 lipopolysaccharide (LPS) 10 mg/kg 以誘發敗血症，注射 LPS 半小時後再給 baicalein 10 mg/kg，於 LPS 注射六小時後，觀察 baicalein 是否可以改善敗血症引起的心臟功能不良，並探討可能機轉。於心肌缺血-再循環模式下，預先給予 wogonin 10 mg/kg 可以明顯延後心肌缺血造成的心室早期收縮 (ventricular premature contractions; VPC) 及心室性心搏過速 (ventricular tachycardia; VT) 發生，可明顯抑制 VT 及心室性纖維顫動 (ventricular fibrillation; VF) 之發生率，可明顯降低心肌缺血造成的死亡率，亦可明顯降低心律不整分數 (Arrhythmia Scores)。但是低劑量及高劑量 wogonin (5 & 20 mg/kg) 未能明顯改善心律不整。再循環期間，wogonin (10 mg/kg) 可以明顯降低心肌組織釋放超氧游離基 (superoxide anion)，及血漿中組織壞死因子 (tissue necrosis factor-α) 含量，再循環二小時後，缺血區內心肌組織內單核球趨化蛋白-1 (monocyte chemoattractant protein-1; MCP-1)、磷酸化 p38 mitogen-activated protein kinase (p38 MAPK)、磷酸化 p65、磷酸化 IκBα 以及活化態

caspase-3 蛋白質表現量明顯增加，而 wogonin (10 mg/kg) 可以明顯降低上述蛋白質表現量。另外，於敗血症模式下，LPS 投予六小時後血壓心跳明顯下降，離體心臟收縮功能明顯降低，給予 baicalein 可以維持血壓免於休克，心跳明顯增加，心臟收縮功能亦明顯改善；LPS 投予六小時後心室肌內血基質氧化酶-1 (heme oxygenase-1; HO-1) 蛋白明顯低於 sham 組，baicalein 可以明顯增加 HO-1 表現量，並且降低心室組織超氧游離基含量；LPS 投予六小時後心室組織內 inducible nitric oxide synthase、MCP-1、磷酸化 p65、磷酸化 IκBα 蛋白質表現量及 caspase-3 活性明顯增加，給予 baicalein 後可以明顯降低上述蛋白表現量以及 caspase-3 活性。結論：wogonin 於心肌缺血時具有抗心律不整作用，可降低死亡率，並且改善缺血-再循環引起之發炎反應及心肌傷害 (壞死及凋亡)，wogonin 可能藉由其抗氧化作用及抑制細胞內 NF-κB 及 p38 MAPK 訊息傳遞路徑之活化而達到此保護作用； baicalein 改善敗血症引起之心臟收縮功能不良，此與 baicalein 可降低氧化壓力、抗發炎作用及減少細胞凋亡有關。由此活體實驗結果可知： wogonin 及 baicalein 可以保護心臟免於急性發炎的傷害。

關鍵字：漢黃芩素、心肌缺血/再循環損傷、心律不整、單核球趨化蛋白-1、血基質氧化酶-1、Nuclear factor-κB、p38 Mitogen-activated protein kinase、黃芩素、敗血症、心肌功能不良、細胞凋亡、發炎、氧化壓力

Abstract

Wogonin and baicalein are flavonoids isolated from *Scutellaria baicalensis* Georgi, a traditional Chinese medicine, and possesses antioxidant and anti-inflammatory effects. The aims of this study are (1) to investigate the *in vivo* effect of wogonin on myocardial ischemia/reperfusion injury in an open-chest anesthetized rat model, which was induced by 45-min left coronary artery occlusion and 2-h reperfusion; (2) to evaluate the protective effect of baicalein on myocardial dysfunction caused by endotoxemia in rats and to explore the possible mechanisms. Rats were treated with wogonin (5, 10, and 20 mg/kg, i.p.) 40 min prior to ischemia or treatment with wogonin 10 mg/kg 15 min after occlusion. Pretreatment with wogonin 10 mg/kg significantly delayed the occurrence of ventricular premature contractions and tachycardia, and suppressed the incidence of ventricular tachycardia and ventricular fibrillation, and mortality elicited by ischemia when compared with the control group, accompanied with reducing the arrhythmia scores. After 2-h reperfusion, pre- and post-treatment with wogonin 10 mg/kg significantly reduced the infarct size, and plasma levels of creatine kinase-muscle-brain fraction and lactate dehydrogenase. Wogonin also significantly reduced the elevation of plasma tissue necrosis factor-α and superoxide anion production in myocardium with ischemia/reperfusion. The expression of monocyte chemoattractant protein-1 (MCP-1), phosphorylated p38 mitogen-activated protein kinase (MAPK), p65 and IκBα, and active caspase-3 in ischemic myocardium pronouncedly increased in the control group, those were significantly attenuated by treatment with wogonin. On the other hand, baicalein (10 mg/kg, i.v.) was administered to conscious Wistar rats 30 min after lipopolysaccharide (LPS; 10 mg/kg,

intravenous) challenge. Six hours after LPS administration, the contractile function of the isolated heart was examined using the Langendorff technique. Post-treatment with baicalein significantly attenuated the LPS-induced hypotension with accompanying tachycardia. The contractile function of isolated heart was significantly preserved 6 h after LPS administration, following treatment with baicalein. Furthermore, baicalein induced the expression of heme oxygenase-1 protein and reduced superoxide anion formation in the myocardium of LPS-treated rats. Cardiac levels of inducible nitric oxide synthase, monocyte chemoattractant protein-1, phospho-IκBα and phospho-p65 protein and caspase-3 activity significantly increased 6 h after LPS challenge but baicalein significantly attenuated these LPS-induced changes. In conclusion, wogonin demonstrated *in vivo* cardioprotective effects by attenuation of the severity of ischemia-induced arrhythmias and irreversible ischemia/reperfusion injury, which is associated with its antioxidant capacity, and anti-inflammatory effects. Suppression of nuclear factor-κB and p38 MAPK activation, and inhibition of monocyte chemoattractant protein-1 expression contribute to the beneficial effects of wogonin. Baicalein improves myocardial contractility in LPS-induced sepsis, which may be related to reductions in oxidative stress, myocardial inflammatory responses and apoptosis.

Key Words:

Wogonin, Myocardial ischemia/reperfusion injury; Arrhythmias; Monocyte chemoattractant protein-1; Heme oxygenase-1; Nuclear factor-κB; p38 Mitogen-activated protein kinase; Baicalein, Sepsis, Myocardial dysfunction, Apoptosis, Inflammation, Oxidative stress

Abbreviations

VPC	Ventricular premature contractions
VT	Ventricular tachycardia
VF	Ventricular fibrillation
NF-κB	Nuclear factor-κB
TNF-α	Tissue necrosis factor-α
MCP-1	Monocyte chemoattractant protein-1
p38 MAPK	p38 mitogen-activated protein kinase
LPS	Lipopolysaccharide
ROS	Reactive oxygen species
LVDP	Left ventricular developed pressure
iNOS	Inducible nitirc oxide synthase
HO	Heme oxygenase
ICAM-1	Intercellular adhesion molecule-1
VCAM-1	Vascular cell adhesion molecule-1

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Chapter 1 Introduction

Inflammation has emerged as a critical biological process contributing to nearly all aspects of cardiovascular diseases including myocardial infarction (Frangogiannis et al., 2002), myocardial ischemia/reperfusion injury (Yellon & Hausenloy, 2007), atherosclerosis (Shalhoub et al., 2011), atrial fibrillation (Issac et al., 2007), heart failure (Picano et al., 2010) and septic shock (Merx and Weber, 2007). It is believed that inflammation is part of the nonspecific immune response that occurs in reaction to any type of bodily injury and that the cardinal signs of inflammation can be explained by increased blood flow, elevated cellular metabolism, vasodilatation, release of soluble mediators, extravasation of fluids and cellular influx. Inflammation has very specific characteristics, whether acute or chronic, and the innate immune system plays a pivotal role, as it mediates the first response. Infiltration of innate immune system cells, specifically neutrophils and macrophages, characterizes the acute inflammation, while infiltration of T lymphocytes and plasma cells are features of chronic inflammation. Monocytes/macrophages play a central role in both, contributing to the final consequence of chronic inflammation which is represented by the loss of tissue function due to fibrosis (Ferrero-Miliani et al., 2007).

The heart is as a pump of circulatory system in human body. It has a specialized muscle that contracts regularly and continuously, pumping blood to the body and the lungs. The pumping action is caused by a flow of electricity through the heart that repeats itself in a cycle. If this electrical activity is disrupted - for example by a disturbance in the heart's rhythm known as an 'arrhythmia' - it can affect the heart's ability to pump properly, which may cause reduction in blood pressure and sudden death.

1. Myocardial Ischemia/Reperfusion Injury

Ischemic heart disease is the leading cause of death in the industrialized world. Coronary vessels and the human heart are frequently subjected to ischemia reperfusion during acute coronary syndromes by balloon angioplasty and open-heart surgery. In its most severe form following cardiac transplantion, primary organ dysfunction frequently occurs, which remains a significant clinical problem and an important cause of perioperative morbidity and mortality. The treatment of acute ischemic heart disease has entered a new era in which mortality can be approximately halved by procedures that allow the rapid return of blood flow to the ischemic zone of the myocardium, i.e., reperfusion therapy. Early reperfusion following acute myocardial infarction saves heart muscle and lives, especially when achieved by coronary angioplasty and stenting of ruptured plaques coupled with adjunctive therapies (e.g. aspirin, clopi dogrel, glycoprotein IIb/IIIa inhibitors or heparin) to maintain vessel patency (Schoming et al., 2000; Montalescot et al., 2001). Reperfusion, however, may lead to further complications such as diminished cardiac contractile function (stunning) and arrhythmia. Moreover, there is experimental evidence that irreversible cell injury leading to necrosis and apoptosis may be precipitated by reperfusion. Therefore, development of cardioprotective agents to improve myocardial function, decrease the incidence of arrhythmias, delay the onset of necrosis, and limit the total extent of infarction during ischemia/reperfusion is of great clinical importance. Earlier pharmacological approaches to attenuate the consequences of ischemia/reperfusion injury have been of limited experimental efficacy or have failed to translate into useful clinical treatments (Ferdinandy et al., 2007).

Myocardial ischemia develops when coronary blood supply to myocardium is reduced, either in terms of absolute flow rate (low-flow or no-flow ischemia) or relative to increased tissue demand (demand ischemia). A pivotal feature of ischemia is that oxygen supply to the mitochondria is inadequate to support oxidative phosphorylation (Opie, 1990; Hearse, 1996; Ganz & Braunwald, 1997). In experimental models and in clinical situations, ischemia may be followed by reperfusion, that is, the re-admission of oxygen and metabolic substrates with washout of ischemic metabolites. The process of reperfusion is associated with further biochemical, structural, and functional changes in myocardium and may determine cell survival and cell death.

1.1. Arrhythmias Induced by Ischemia

During ischemia, arrhythmias may develop, ranging in severity from isolated ventricular premature beats, through runs of ventricular tachycardia, to ventricular fibrillation (Tennant and Wiggers, 1935; Curtis et al., 1987; Carmeliet, 1999). Early arrhythmias (phase I arrhythmias) after coronary artery occlusion may contribute to sudden cardiac death following coronary occlusion (Janse and Wit, 1989). In experimental models of coronary occlusion, the incidence and duration of arrhythmias has been used as an injury index although it is important to note that arrhythmias develop before the onset of irreversible tissue injury. Reperfusion of myocardium after relatively brief periods of ischemia may also precipitate a pattern of arrhythmia ranging in severity (Manning and Hearse, 1984; Carmeliet, 1999). Clinically reperfusion-induced arrhythmia may be observed during thrombolysis (Goldberg et al., 1983) and after percutaneous coronary intervention (Holdright et al., 1996).

The major changes associated with ischemic injury include: (1) intracellular acidosis, loss of intracellular K^+, and accumulation of metabolites; (2) intracellular Ca^{2+} overload, loss of gap junction expression/function, and irreversible cellular injury; and (3) elevated levels of oxidative stress, progressive accumulation of reactive oxygen species (ROS), and mitochondrial dysfunction. Ischemia is electrocardiographically characterized by marked QT-interval shortening and ST-segment elevation (Surawicz, 1986). The morphology of the ischemic action potential exhibits a significantly depolarized resting membrane potential, a slowed rate of rise of the action potential upstroke, and reduced action potential amplitude and duration (Di Diego & Antzelevitch 2003). Ischemia causes a rapid (within the first 2 minutes) depolarization of the resting membrane potential. This is thought to be largely caused by a rapid redistribution of K^+ ions from the intracellular to the extracellular space (Fozzard & Makielski, 1985). The mechanisms underlying ischemia-induced loss of intracellular K^+ ions likely involve the opening or the outward adenosine triphosphate-sensitive K (K_{ATP}) current, the inhibition of the Na^+/K^+ ATPase activity (Weiss et al., 1992), and the intracellular loss of K^+ to anaerobic glycolysis and intracellular acidification (Weiss et al., 1989). Finally, since at rest, resting membrane potential is determined primarily by the ratio of extracellular to intracellular K^+, hyperkalemia is expected to cause an elevation of membrane potential to more depolarized values, having major consequences on the gating of the voltage-sensitive Na^+ current.

Reduced intracellular pH caused by the accumulation of metabolic by-products is a hallmark of ischemic injury, which is thought to stimulate the Na^+-H^+ exchange pathway in an attempt to extrude H^+ from the cell (Pierce &

Meng, 1992). This will lead to accelerated Ca^{2+} entry via reverse mode Na^{+}-Ca^{2+} exchanger (NCX) activity, which attempts to restore intracellular Na^{+} levels and prevent their accumulation (Allen & Orchard, 1983; Orchard et al., 1985). This NCX-mediated transient inward (I_{ti}) current can then result in intracellular Ca^{2+} overload, spontaneous rises in membrane potential that manifest as delayed afterdepolarizations (DADs) (Bers et al, 2002). Therefore, at the cellular level, Ca^{2+} overload secondary to Na^{+} overload can increase the likelihood of spontaneous calcium release events (Ca^{2+} spark), which can increase the probability that DAD-generating Ca^{2+} waves will be induced. Whether these calcium-dependent triggers can reach the threshold for propagation in the intact myocardium and their exact involvement in the mechanism of arrhythmias remain to be elucidated. Although membrane excitability is generally depressed in ischemia and may hinder the propagation of DAD-induced premature beats, reduced gap junction function as a consequence of altered expression, distribution, and/or phosphorylation of Cx43 may paradoxically promote the successful propagation of premature beats by reducing the passive loss of electrotonic current to neighboring myocytes and thereby preserving it for membrane potential depolarization (Rohr et al., 1997; Rudy, 1998). Furthermore, oxidative stress is involved in the pathogenesis of clinical arrhythmias by predisposing to the development of electrical remodeling and endothelial dysfunction. Rapid atrial rates lead to the development of electrical remodeling and the formation of peroxynitrite, a marker of high oxidative burden. This ultimately leads to the development of sustained atrial fibrillation (Carnes et al., 2001). Experimental atrial fibrillation is associated with increased left atrial NAD(P)H and xanthine oxidase activity,

thereby causing an increase in the formation of superoxide (Dudley et al., 2005).

1.2. Lethal Reperfusion Injury

The term 'lethal reperfusion injury' specifically refers to cell death associated with transient ischemia that can be prevented by interventions applied at the time of reperfusion (Piper et al., 1998). Therefore, it is the component of cell death occurring as a consequence of reperfusion.

Animal models of myocardial ischemia show that cessation of blood flow rapidly depletes cardiomyocytes of high-energy phosphates, with an immediate shift to anaerobic glycolysis and a decrease in cytosolic pH (Jennings et al. 1990). Reduction in ATP and creatine phosphate reduces contractility and impairs the activity of ATP-dependent ion pumps within cell membranes, resulting in intracellular calcium accumulation. With sustained ischemia, membrane integrity further deteriorates with disruption of cellular organelles, and accumulation of water and electrolytes. The cell death that ensues initiates an inflammatory response within the infarct and border zone area, and in some cases within myocardium remote from the infarct area (Neri Serneri et al., 2003; Abbate et al., 2004; Frangogiannis et al., 2002).

Early reperfusion by partial spontaneous fibrinolysis of the plaque-bound thrombus, by collaterals or by early therapeutic intervention, can allow recovery of myocardial function, occasionally with little biochemical evidence of myonecrosis. With longer duration of ischemia, however, reperfusion can result in a cascade of events leading to cardiomyocyte death, directly or by initiation of apoptosis. Within minutes of reperfusion, ROS are generated by

reoxygenated tissues—possibly from xanthine oxidase, the mitochondrial electron transport chain, or NADPH oxidase, among other sources. This action leads to damage of the endothelium, release of chemotactic cytokines, and expression of cell adhesion molecules on the endothelial surface (Semenza, 2000). Activated platelets and neutrophils, followed by lymphocytes, monocytes and macrophages, mast cells and eosinophils, attach to the damaged endothelium of the microcirculation and infiltrate adjacent myocardium. Complement activation recruits larger numbers of inflammatory cells that occlude the microvasculature of reperfused territories, compromising blood flow—the so called no-reflow phenomenon (Rezkalla & Kloner 2002). Accumulation of the terminal components of complement (the membrane attack complex C5b-9) causes direct cellular injury. Endothelial injury, with loss of endothelium-derived relaxant factors such as nitric oxide (NO), promotes microvascular constriction and further reduction in myocardial perfusion. Ischemia and reperfusion also activate cell-signaling cascades leading to apoptosis (Olivetti et al., 1996; Zhao et al., 2000; Prasad et al., 2009) (Appendix Fig. 1).

1.3. Potential Mediators of Lethal Reperfusion Injury

1.3.1. Oxygen Paradox

Experimental studies have established that the reperfusion of ischemic myocardium generates oxidative stress, which itself can mediate myocardial injury (Zweier, 1988). Oxidative stress is part of the oxygen paradox, in which the reoxygenation of ischemic myocardium generates a degree of myocardial injury that greatly exceeds the injury induced by ischemia alone. During

myocardial reperfusion, ROS are generated by xanthine oxidase (mainly from endothelial cells) and the re-energized electron transport chain in the cardiomyocyte mitochondria. Several hours later, a further source of ROS is NADPH oxidase (mainly from neutrophils). ROS mediate myocardial injury by inducing mitochondrial PTP opening, acting as neutrophil chemoattractants, mediating dysfunction of the sarcoplasmic reticulum and contributing to intracellular Ca^{2+} overload, damaging the cell membrane by lipid peroxidation, inducing enzyme denaturation, and causing direct oxidative damage to DNA. The role of oxidative stress in lethal reperfusion injury is clouded by the inconclusive results of animal and clinical studies of cardioprotection by antioxidant reperfusion therapy (Yellon & Hausenloy, 2007). Oxidative stress during myocardial reperfusion also reduces the bioavailability of the intracellular signaling molecule, NO, thereby removing its cardioprotective effects. These effects include the inhibition of neutrophil accumulation, inactivation of superoxide radicals, and improvement of coronary blood flow (Zweier & Talukder, 2006).

1.3.2. Calcium Paradox

At the time of myocardial reperfusion, there is an abrupt increase in intracellular Ca^{2+}, which is secondary to sarcolemmal-membrane damage and oxidative stress–induced dysfunction of the sarcoplasmic reticulum. These two forms of injury overwhelm the normal mechanisms that regulate Ca^{2+} in the cardiomyocyte; this phenomenon is termed the calcium paradox (Piper et al., 1998). The result is intracellular and mitochondrial Ca^{2+} overload, and this excess of Ca^{2+} induces cardiomyocyte death by causing hypercontracture of the

heart cells and mitochondrial PTP opening (Piper et al., 1998). Attenuating intracellular Ca^{2+} overload with pharmacologic antagonists of the sarcolemmal Ca^{2+} ion channel, the mitochondrial Ca^{2+} uniporter, or the sodium-hydrogen exchanger decreases myocardial infarct size by up to 50% in experimental studies (Klein et al., 1989; Gumina et al., 1999). However, the results of the corresponding clinical studies have been negative (Boden et al., 2000; Zeymer et al., 2001).

1.3.3. Inflammation

An inflammatory response starts upon reperfusion. The inflammatory response may be triggered by constituents of the damaged cells (cell debris) or by the disrupted tissue matrix (eg, by activation of the complement cascade or of macrophages, endothelial, dendritic, and other cells via binding of heat shock and nuclear proteins or heparan sulfate to Toll-like receptors). The inflammatory response, however, may also start from cells, especially endothelial cells and macrophages, which are intrinsically activated by the sequence of anoxia and reoxygenation, for instance, owing to an anoxic increase in cytosolic Ca^{2+}. ROS may be generated by activated macrophages, neutrophils, endothelial cells, and platelets and may also be released by injured or even dead cells (cell debris). Mediators such as cytokines and chemokines may be formed by macrophages, lymphocytes, neutrophils, and endothelial cells. Decreased/NO formation, increased formation of endothelin-1, and blood coagulation may result in disturbances of microvascular perfusion. Several of the compounds released during the inflammatory response, such as ROS, the cytokine TNF-α, and high concentrations of NO may produce cytotoxicity and

thus induce additional cell injury. Because of the microvascular dysregulation, even anoxic cell injury may still occur in the reperfusion phase. Cell injury occurring during the reperfusion period additionally perpetuates the inflammatory response. Cell injury in the reperfusion phase, however, also results from the inflammatory response (de Groot & Rauen, 2007).

1.3.4. ROS

ROS are highly unstable oxygen molecules with unpaired electrons. They are capable of oxidizing many biological molecules, such as proteins, lipids, and DNA (Hess & Manson, 1984). ROS are known to stimulate various protein kinase cascades that underlie inflammatory gene expression. Indeed, much influence of redox imbalance on inflammatory disease is through the manipulation of transcription factors and the subsequent effect on inflammatory gene expression. Nuclear factor-κB (NF-κB) and Activating Protein-1 (AP-1) are two such transcription factors whose activation state and transactivation ability appears to depend highly on the redox state of the cells. In addition, there is clear evidence for the involvement of NF-kB in the initiation and progression of inflammatory vascular disease that is associated with a significant oxidant environment. Activated NF-κB has been demonstrated in the myocytes of canine hearts both during ischemia and following reperfusion (Fan et al., 2002) and in rat hearts following ischemia–reperfusion (Chandrasekar et al., 2001). ROS have been widely accepted as universal second messengers of NF-κB activation (Schreck et al., 1991). Although the molecular mechanisms conferring redox sensitivity of NF-κB activation in certain cells remain to be fully elucidated, a critical role of amino terminal tyrosine residue 42 and the

carboxy terminal pest domain of Inhibitor-κB (IκBα) in oxidant-mediated and antioxidant-sensitive activation of NF-κB is emerging (Imbert et al., 1996; Schoonbroodt et al., 2000). The NF-κB signaling pathways are shown in the Appendix Fig. 2 (Hayden & Ghosh, 2008).

1.3.5. p38 Mitogen-activated protein kinase (MAPK) pathways

There is a wealth of recent information regarding activation of the MAPK pathways by ROS species and cytokines in many cellular systems including vascular endothelial, smooth muscle cells and myocytes. Because of the recently uncovered multifaceted role of the MAPK systems in cellular regulation, an increasing body of evidence suggests that the different subfamilies of the MAPK cascade systems might fulfil the role in the determination of balance between survival and apoptosis (Marczin et al., 2003). Various intracellular signalling pathways are thought to play a critical role in the myocardial response to ischaemia and consequent pathological remodelling. MAPK are activated during ischaemia and may contribute to the structural and functional changes. MAPK are highly conserved serine/threonine kinases that are activated by a dual phosphorylation of a Thr-X-Tyr motif, in response to wide a variety of stimuli such as cytokines, osmotic and other environmental stresses and consequently play a role in numerous cell functions including growth and proliferation (Clark et al., 2007). Three of the five major MAPK cascades have been extensively studied in the heart: extracellular signal-regulated kinase (ERK1 and ERK2), c-Jun N-terminal kinases (JNK1 and JNK2) and p38 kinases. It has been shown that JNK and p38 contribute to, whereas ERK/ERK2 protect against, apoptotic cell death. Although the

mechanisms by which p38 and JNK induce apoptosis may be cell and stimulus specific, there is overwhelming evidence that the activation of p38 MAPK (or p38) that occurs during prolonged ischaemia accelerates injury since its inhibition by pharmacological or genetic means slows the rate of infarction/death (Saurin et al., 2000; Martin et al., 2001).

p38 MAPK are activated by a wide range of extracellular influences, including radiation, ultraviolet light, heat shock, osmotic stress, proinflammatory cytokines such as IL-1 and TNF-α, and certain mitogens (Sugden & Clerk, 1998) in addition to myocardial ischaemia (Bogoyevitch et al., 1996; Saurin et al., 2000; Luss et al., 2000; Ping & Murphy, 2000). Furthermore, the consequent activation of p38 MAPK is intimately involved in multiple cellular responses, including growth, proliferation, differentiation, and death (English et al., 1999; Ono & Han, 2000).

It was fist demonstrated as early as 1996 that p38α and β are activated in response to ischaemia and reperfusion in the heart (Bogoyevitch et al., 1996). There is increasing evidence from preclinical investigations that inhibition of p38 during prolonged ischaemia slows the rate of infarction/death and inhibits the production of inflammatory cytokines, such as TNF-α, IL-1 and IL-8, which aggravate ischaemic injury (Young et al., 1997). p38s phosphorylate a number of known transcription factors to alter their transactivating potential influencing gene expression. However, the immediate downstream targets of p38 that aggravate myocardial injury are still largely unknown. Interestingly, TNF-α also activates p38 and thus p38 has been considered as the keystone in an autoamplifying cytokine cascade by most investigators and an attractive target for antiinflammatory drug development (Lee et al., 2000; Kuma et al., 2005).

A proapoptotic role for p38α and/or p38β during myocardial ischaemia is suggested by protection of cardiac myocytes from ischaemic damage using a selective p38α/p38β isoform inhibitor, SB203580 (Wang et al., 1998). Inhibition of p38α activation during prolonged ischaemia, but not β, resulted in an increase in cell viability (Saurin et al., 2000). It has been suggested that p38α activation in cardiac myocytes is sufficient to cause apoptosis whereas activation of the β isoforms leads to protection and hypertrophy (Wang et al., 1998).

1.3.6. Monocyte chemoattractant protein-1 (MCP-1)

In a canine model, induction of MCP-1 mRNA occurred in previously ischemic area within the first hour of reperfusion, peaked at 3 hours, and persisted throughout the first 2 days of reperfusion (Kumar et al., 1997). MCP-1, a potent monocyte attractant, is a member of the CC chemokine subfamily. Chemokines include a superfamily of small, secreted proteins that play a central role in many homeostatic and pathological processes in human body. Though initial research identified these molecules as regulators of leukocyte trafficking (Schall & Bacon, 1994), subsequent research has pointed to its involvement in other aspects of the inflammatory process, such as fibrosis, tissue remodelling and angiogenesis (Lukacs, 2001). Chemokines control the migration of neutrophils, lymphocytes, antigen-presenting cells, including dendritic cells and cells of monocyte/macrophage lineage (Deshmane et al., 2009). In response to an inflammatory insult, chemokines coordinate the recruitment, activation and homing of leukocytes during the different phases of both innate and adaptive inflammatory responses (Rot & von Andrian, 2004).

Chemokines are a family of small molecules with a molecular weight of 8-14 kDa. To date approximately 50 human chemokines and 20 G-protein-coupled chemokine receptors have been identified. Most chemokines also have at least four cysteines in highly conserved positions and three distinct domains. Based on their genetic organization and the position of two highly conserved cysteine residues at the N-terminus, chemokines can be divided into four subgroups, the CC, CXC, C and CX3C families (Moser & Loetscher, 2001). Chemokines of the CC family have adjacent cysteines close to the N terminus (Zlotnik et al., 2006). As a general rule, members of the CC family are primarily targeting monocytes and T-cells, whereas CXC chemokines affect mainly neutrophils. Five members of the family of monocyte chemoattractant proteins have been identified so far. MCP-1, MCP-2, MCP-3, MCP-4 and MCP-5 constitute a subfamily within the CC chemokines (Proost et al., 1996).

The MCP-1 expression can be induced by a variety of mediators including platelet-derived growth factor, interleukins IL-1 and IL-4, tissue necrosis factor α, vascular endothelial growth factor, bacterial lipopolysaccharide, and interferon γ (Sheikine & Hansson, 2004). MCP-1 is produced by many cell types, including epithelial, endothelial, smooth muscle, fibroblasts, astrocytes, monocytes and microglial cells and recruits monocytes, memory T-cells, and dendritic cells to sites of tissue injury and infection (Yadav et al., 2010). These cells are important for anti-viral immune responses in the peripheral circulation and in tissues. However, major source of MCP-1 is monocytes and macrophages (Yoshimura et al., 1989a, 1989b) and their activity is controlled by IFN-γ, IL-4, IL-10, and IL-13 (Fiorentino et al., 1989).

MCP-1 exerts its effects through binding to G-protein-coupled receptors on

the surface of leukocytes targeted for activation and migration. These receptors, once activated, trigger a set of cellular reactions that result in inositol triphosphate formation, intracellular calcium release, and PKC activation (Melgarejo et al., 2009). The classic MCP-1 receptors (CCR2) belong to the family of heptahelical, pertussis-sensitive, G protein-coupled receptors (Myers et al., 1995) (Appendix Fig. 3). The MAPK ERK1 and ERK2, Janus kinase JAK2, the stress activated kinases JNK1 and p38, phospholipase C and two isoforms of PI3-kinase (p85/p110 and C2α) have all been implicated in MCP-1 signal transduction (Yadav et al., 2010). MCP-1 is known to trigger the firm arrest of rolling monocytes on endothelial monolayers expressing E-selectin (Gerszten et al., 1999) and may have a role in spreading and shape change of monocytes attached to the endothelium (Weber et al., 1999). Two nuclear factor-B-binding sites located approximately 2.6 kb from the transcription initiation site appear to function as the critical elements in MCP-1 induction in response to IL-1β and TNF-α (Melgarejo et al., 2009). Cytokine-activated B-binding complexes p65/p65 and p65/c-Rel bind to these sites and result in an enhancement of MCP-1 gene transcription.

1.4. Cell Death: Necrosis & Apoptosis

Historically, IR-induced cardiac myocyte death has been broadly classified as occurring by either necrosis or apoptosis. A) Necrosis (Greek for: death, causing to die) is a degenerative process in which cellular integrity is lost and the release of cytosolic contents provokes an inflammatory response (Searle et al, 1982). The necrosis is the most common pathway of cell death during reperfusion is also demonstrated by the facts that enzyme release occurring

during initial reperfusion accurately predicts final infarct size and that reperfused infarcts are mainly composed of areas of contraction band necrosis as shown by quantitative histology (Barrabes et al., 1996); and B) Apoptosis (from Greek: falling off, figurative for the falling of leaves) is a highly regulated, genetically determined mechanism that does not provoke an inflammatory response (Saraste, 1999). Moreover, apoptosis requires energy in form of ATP for its successful completion. Apoptosis plays a role in pathophysiological conditions but is also essential in normal tissue homeostasis, allowing the organ or tissue to rid itself of cells which are dysfunctional or no longer needed. Apoptotic cell death is characterized by cell shrinkage, membrane blebbing, and nuclear condensation and degradation. The cell is eventually broken into small membrane-enclosed pieces (apoptotic bodies), which *in vivo* are removed by macrophages, or taken up by neighboring cells. This prevents the release of cellular compounds and thus ensures that an inflammatory response is not provoked.

Two major apoptotic pathways are active in mammalian cells, including the cardiac muscle cell. Mitochondria play a key role in the ‘‘intrinsic’’ pathway. The mitochondrial death pathway is mediated by intracellular and extracellular death-signals that impinge upon mitochondria leading to the disruption of normal mitochondrial physiology, leading to opening of the mitochondrial permeability transition pore (MPTP). The MPTP is a nonspecific pore comprised of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT) and cyclophilin D as well as other proteins. The MPTP permits the movement of small molecules (<1.5 kDa) between the cytosol and the mitochondrial matrix. Upon permeabilization of the

mitochondrion, several intermembrane proteins are released into the cytosol, including cytochrome c, Smac/DIABLO, endonuclease G (Endo G), Omim/Htr and apoptosis-inducing factor (AIF). Specifically, cytochrome c binds to the cytosolic protein apaf1 facilitating formation of the ‘‘apoptosome’’ complex, which results in caspase-9 activation that in turn provokes caspase-3 activation. Secondly, the ‘‘extrinsic’’ death-receptor pathway is triggered by binding of members of the death-receptor superfamily, such as Fas/CD95 and TNF-α to their cognate receptors, which induces receptor clustering. Activation of the cognate receptors triggers recruitment of the adaptor molecule, FADD. This results in the generation of a death-inducing signaling complex (DISC), which is capable to activate caspase-8 and caspase-3. The ‘‘extrinsic’’ and ‘‘intrinsic’’ pathways converge upon the effector caspases, resulting in the cleavage of substrates and cell death. Connections between the ‘‘intrinsic’’ and ‘‘extrinsic’’ pathways exist through caspase-8 mediated cleavage of Bid, resulting in its translocation to mitochondria, where it activates Bak and promotes cytochrome c release. Caspases can be inhibited by inhibitory of apoptosis (IAP)-proteins, which in turn can be inhibited by Smac/DIABLO (van Empel et al., 2005) (Appendix Fig. 4).

1.5. Reperfusion Arrhythmias

During reperfusion, immediate or early arrhythmias occur within a few seconds after reperfusion. They follow ischemia periods of 10–30 min. They start by an automatic stimulus in the reperfused zone and change afterward in a reentry multiple wavelet type of VT or VF. The incidence of arrhythmias depends on the duration of the preceding ischemia, with a frequency maximum

between 10 and 30 min, variable with species. Delayed reperfusion arrhythmias appear as a second period of irregular rhythm when the occlusion period has been longer than 10-20 min. Extra systoles and runs of tachycardia probably originate in surviving Purkinje fibers overlying the ischemic zone. A number of observations suggest early afterdepolarization (EAD) or DAD as possible candidates (Carmeliet 1999). Oxygen radicals could play an important role, and scavengers of radicals act as antiarrhythmics (Bernier & Hearse 1988). There exists a close association between Ca^{2+} overload and ventricular arrhythmias (Brooks et al., 1995). Furthermore, the cellular electrophysiological mechanism for reperfusion arrhythmias appears to include washout of various ions such as lactate and potassium, and toxic metabolic substances that have accumulated in the ischemic zone (Curtis et al., 1993).

2. Sepsis and Heart

Sepsis, defined by consensus conference as "the systemic inflammatory response syndrome (SIRS) that occurs during infection," (Bone et al., 1992) is generally viewed as a disease aggravated by the inappropriate immune response encountered in the affected individual (Hotchkiss & Karl 2003; Riedemann et al., 2003). The current criteria for the establishment of the diagnosis of systemic inflammatory response syndrome, sepsis, and septic shock is shown in the Appendix Table 1 (Bone et al., 1992; Annane et al., 2005). Morbidity and mortality are high, resulting in sepsis and septic shock being the 10th most common cause of death in the United States.5 Morbidity and mortality are high, resulting in sepsis and septic shock being the 10th most common cause of death in the United States (Martin et al., 2003). The incidence of sepsis and

sepsis-related deaths appears to be increasing by 1.5% per year (Angus et al., 2001). In a recent study, the total national hospital cost invoked by severe sepsis in the United States was estimated at approximately $16.7 billion on the basis of an estimated severe sepsis rate of 751 000 cases per year with 215 000 associated deaths annually (Angus et al., 2001). A recent study from Britain documented a 46% in-hospital mortality rate for patients presenting with severe sepsis on admission to the intensive care unit (Padkin et al., 2003).

Sepsis initiates a brisk inflammatory response that directly and indirectly causes widespread tissue injury. Gram-positive and gram-negative bacteria, viruses, and fungi have unique cell-wall molecules called pathogen-associated molecular patterns that bind to pattern-recognition receptors (toll-like receptors;TLRs) on the surface of immune cells. The LPS of gram-negative bacilli binds to LPS-binding protein, CD14 complex. The peptidoglycan of gram-positive bacteria and the LPS of gram-negative bacteria bind to TLR-2 and TLR-4, respectively. Binding of TLR-2 and TLR-4 activates intracellular signal-transduction pathways that lead to the activation of cytosolic NF-κB. Activated NF-κB moves from the cytoplasm to the nucleus, binds to transcription initiation sites, and increases the transcription of cytokines such as TNF-α, IL-1β, and IL-10. TNF-α and IL-1β are proinflammatory cytokines that activate the adaptive immune response but also cause both direct and indirect host injury. IL-10 is an antiinflammatory cytokine that inactivates macrophages and has other antiinflammatory effects. Sepsis increases the activity of iNOS, which increases the synthesis of NO, a potent vasodilator. Cytokines activate endothelial cells by up-regulating adhesion receptors and injure endothelial cells by inducing neutrophils, monocytes, macrophages, and platelets to bind to

endothelial cells. These effector cells release mediators such as proteases, oxidants, prostaglandins, and leukotrienes. Key functions of the endothelium are selective permeability, vasoregulation, and provision of an anticoagulant surface. Proteases, oxidants, prostaglandins, and leukotrienes injure endothelial cells, leading to increased permeability, further vasodilation, and alteration of the procoagulant–anticoagulant balance. Cytokines also activate the coagulation cascade (Russell, 2006).

Calvin et al (1981) were the first to demonstrate myocardial dysfunction in adequately volume-resuscitated septic patients with decreased ejection fraction and increased end-diastolic volume index. Significant reductions in both stroke volume and ejection fraction in septic patients were observed despite normal total cardiac output (Parker et al., 1984). The presence of cardiovascular dysfunction in sepsis is associated with a significantly increased mortality rate of 70% to 90% compared with 20% in septic patients without cardiovascular impairment (Parrillo et al., 1990). The human studies, in conjunction with experimental studies ranging from the cellular level to isolated heart studies and to *in vivo* animal models, have clearly established decreased contractility and impaired myocardial compliance as major factors that cause myocardial dysfunction in sepsis. Thus, myocardial dysfunction in sepsis has been the focus of intense research activity. Although a number of mediators and pathways have been shown to be associated with myocardial depression in sepsis, the precise cause remains unclear (Merx & Weber, 2007).

2.1. Mechanisms Underlying Myocardial Dysfunction in Sepsis

Several circulating factors in septic shock were proposed to be associated with myocardial dysfunction. These include IL-1, IL-8, C3a (Hoffmann et al., 1999) and lysozyme c (Mink et al., 2004), Additional potential candidates for myocardial depressant substance include other cytokines, prostanoids, and NO (Appendix Fig. 5).

(1) Cytokines: TNF-α is an important early mediator of endotoxin-induced shock (Sharma et al., 1997). TNF-α is derived from activated macrophages, but recent studies have shown that TNF-α is also secreted by cardiac myocytes in response to sepsis (Horton et al., 2000). IL-1 is synthesized by monocytes, macrophages, and neutrophils in response to TNF-α and plays a crucial role in the systemic immune response. IL-1 depresses cardiac contractility by stimulating NO synthase (NOS) (Francis et al., 1998). IL-6, another proinflammatory cytokine, has also been implicated in the pathogenesis of sepsis and is considered a more consistent predictor of sepsis than TNF-α because of its prolonged elevation in the circulation (Damas et al., 1992). Although cytokines may very well play a key role in the early decrease in contractility, they cannot explain the prolonged duration of myocardial dysfunction in sepsis, unless they result in the induction or release of additional factors that in turn alter myocardial function, such as prostanoids or NO (Schulz et al., 1992; Finkel et al., 1992).

(2) Prostanoids: Prostanoids are produced by the cyclooxygenase enzyme from arachidonic acid. The expression of cyclooxygenase enzyme-2 is induced, among other stimuli, by LPS and cytokines (cyclooxygenase enzyme-1 is expressed constitutively) (Liu et al., 1996). Elevated levels of prostanoids

such as thromboxane and prostacyclin, which have the potential to alter coronary autoregulation, coronary endothelial function, and intracoronary leukocyte activation, have been demonstrated in septic patients (Reines et al., 1982).

(3) Endothelin-1 (ET-1) upregulation has been demonstrated within 6 hours of LPS-induced septic shock (Shindo et al., 1998). Cardiac overexpression of ET-1 triggers an increase in inflammatory cytokines (among others, TNF-α, IL-1, and IL-6), interstitial inflammatory infiltration, and an inflammatory cardiomyopathy that results in heart failure and death (Yang et al., 2004).

(4) NO: NO has been shown to modulate cardiac function under physiological and a multitude of pathophysiological conditions. In healthy volunteers, low-dose NO increases LV function, whereas inhibition of endogenous NO release by intravenous infusion of the NO synthase inhibitor N^G-monomethyl-L-arginine reduced the stroke volume index (Rassaf et al., 2006). Higher doses of NO have been shown to induce contractile dysfunction by depressing myocardial energy generation (Kelm et al., 1997). Sepsis leads to the expression of iNOS in the myocardium (Preiser et al., 2001; Khadour et al., 2002) followed by high-level NO production, which in turn importantly contributes to myocardial dysfunction, in part through the generation of cytotoxic peroxynitrite, a product of NO and superoxide (Pacher et al., 2007). In iNOS-deficient mice, cardiac function is preserved after endotoxin challenge (Ullrich et al., 2000). Nonspecific NOS inhibition restores cardiac output and stroke volume after LPS injection (Hwang & Yeh, 2003). Strikingly, in septic patients, infusion of methylene blue, a nonspecific NOS inhibitor, improves mean arterial pressure, stroke volume,

and left ventricular stroke work and decreases the requirement for inotropic support but, unfortunately, does not alter outcome (Kirov et al., 2001). An interesting study comparing the inhibition of NO superoxide and peroxynitrite in cytokine-induced myocardial contractile failure found peroxynitrite to indeed be the most promising therapeutic target (Ferdinandy et al., 2000).

(5) Adhesion molecules: Surface-expression upregulation of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) has been demonstrated in murine coronary endothelium and cardiomyocytes after LPS and TNF-α stimulation (Raeburn et al., 2002). After cecal ligation and double puncture, myocardial ICAM-1 expression increases in rats (Neviere et al., 2000). VCAM-1 blockade with antibodies has been shown to prevent myocardial dysfunction and decrease myocardial neutrophil accumulation (Raeburn et al., 2002; Raeburn et al., 2001), whereas both knockout and antibody blockade of ICAM-1 ameliorate myocardial dysfunction in endotoxemia without affecting neutrophil accumulation (Raeburn et al., 2002).

2.2. Production and Role of Free Radicals in Septic Shock

The overt production of superoxide plays a role in the pathological sequelae of septic shock. Firstly superoxide is a proinflammatory mediator. Some of the proinflammatory properties of superoxide pertinent to septic shock include recruitment of neutrophils at sites of inflammation, formation of chemotactic factors, DNA damage, initiation of lipid peroxidation, and release of proinflammatory cytokines such as TNF-α and IL-1β via activation of NF-κB

(Cuzzocrea et al., 2001). The proinflammatory effects of superoxide are then perpetuated by the formation of peroxynitrite, which also deactivates (upon nitration) superoxide dismutase. Peroxynitrite possesses a number of independent proinflammatory/cytotoxic mechanisms including (i) the initiation of lipid peroxidation, (ii) the inactivation of a variety of enzymes, and (iii) depletion of glutathione. Moreover, peroxynitrite can also cause DNA damage resulting in the activation of the nuclear enzyme poly (ADP-ribose) synthetase (PARS), depletion of nicotinamide adenine dinucleotide (NAD), and adenosine triphosphate (ATP), which lead to irreversible cellular damage as evidenced in septic shock (Salvemini & Cuzzocrea, 2002). In addition, ROS have been shown to be involved in NF-κB activation (Schreck et al., 1991; Adcock et al., 1994).

2.3. LPS and Cardiac Function

Bacterial LPS are a major trigger of cardiac failure in septic shock. Bacterial LPS induce left ventricular (LV) dysfunction (Annane et al., 2005; Lopez-Bojorquez et al., 2004), characterized by a decrease in both left and right ventricular ejection fractions and increased end-diastolic volumes (Parker et al, 1999, Kumar et al., 2000). LPS-induced cardiac dysfunction may be in part due to produced ROS mediated by inflammatory mediators like TNF-α (Rudiger & Singer, 2007).

2.4. Heme Oxygenase-1 (HO-1) and Cytoprotective Effect in Sepsis

The redox-sensitive gene heme oxygenase-1 (HO-1) can be activated by oxidative stress to induce HO-1 protein expression, resulting in cytoprotective

effects in various diseases (Takahashi et al., 2004). HO has been shown to be important for attenuating the overall production of ROS through its ability to degrade heme and to produce carbon monoxide (CO), biliverdin/bilirubin, and the release of free iron. Excess free heme catalyzes the formation of ROS, which may lead to endothelial cell dysfunction as seen in numerous pathological conditions (Abraham & Kappas, 2005). The current view of HO-dependent protection is that the reaction products of HO activity (i.e., biliverdin, CO, iron), each contribute, alone or in concert, to the restoration of cellular homeostasis under inducing conditions (Appendix Fig. 6) (Ryter et al., 2009).

The transcriptional induction of the gene encoding HO-1 (Hmox1 in mice, HMOX1 in humans) and subsequent synthesis of the corresponding HO-1 protein occurs as a general response to cellular stress (Keyse & Tyrrell, 1989; Applegate et al., 1991). In addition to the substrate heme, a broad spectrum of stimuli can induce HO-1 expression. Such agents include NO, cytokines, heavy metals, hormones, growth factors, thiol-reactive substances, oxidants, extreme oxygen environments, ischemia/reperfusion injury, and ultraviolet-A radiation (Ryter et al., 2006). Since many of these inducing conditions are associated with the stimulation of prooxidant states, HO-1 is considered an inducible defense mechanism against oxidative cellular stress (Keyse & Tyrrell, 1989; Applegate et al., 1991). A subclass of inducing agents includes electrophilic antioxidant compounds, many of which are plant-derived polyphenols, which generally trigger the expression of several detoxification associated genes (including Hmox1, glutathione S-transferase A2 and NADPH: quinone oxidoreductase) through common activation of transcription factor nuclear factor erythroid

2–related factor-2 (Nrf2) (Pickett et al., 2009).

There is accumulating evidence emphasizing the importance of HO-1 in the development of sepsis (Maeda et al., 2008; Tamion et al., 2007; Tracz et al., 2007; Tamion et al., 2006; Moreto et al., 2006; Chang et al., 2006; Poole et al., 2005; Wiesel et al., 2000). It has been suggested that administration of mice with LPS was associated with a marked increase HO-1 gene expression in a site specific organ manner (Suzuki et al., 2000). HO-1-deficient mice develop increased end-organ damage and have increased mortality after LPS administration (Wiesel et al., 2000). In contrast, administration of CO to HO-1-deficient animals attenuates LPS-induced inflammation and end-organ injury (Chung et al., 2008). These studies support the beneficial effects of HO-1 and its by-products such as CO during sepsis. It has been suggested that manipulation of the HO-1 pathway may represent a possible therapeutic strategy to counteract the oxidative stress of endotoxaemia and to limit myocardial deformation (Tamion et al., 2010).

2.5. Chemokines and Sepsis

Chemokines have been shown to participate in the pathogenesis of sepsis (Ramnath et al., 2008). MCP-1, a prototype CC chemokine, is a potent chemoattractant and a regulatory mediator involved in a variety of inflammatory diseases (Luster, 1998). MCP-1 expression is regulated at the transcriptional level by stimulatory agents such as TNF-α, interferon (IFN)-γ, platelet-derived growth factor and stress factors (Melgarejo et al., 2009). Recently, anti-MCP-1 treatment has been proposed to be of potential therapeutic value in the treatment of sepsis and endotoxaemia (Ramnath et al., 2008).

2.6. Apoptosis in Sepsis

There is increasing evidence that apoptosis is also involved in sepsis-induced cardiovascular dysfunction (Ayala et al., 2008; Ward, 2008). Apoptosis is potentially triggered by cytokines, TNF-α, ROS and NO released by infiltrating polymorphonuclear leukocytes or macrophages (Zhao & Vinten-Johansen, 2002). Therapeutic strategies aimed at inhibition of apoptosis have resulted in improved cardiac function in animal models of sepsis (Fauvel et al., 2001; Neviere et al., 2001; Buerke et al., 2008).

3. Scutellaria

3.1. The Properties of Scutellaria and Its Main Active Constituents Wogonin, Baicalein and Baicalin

The dry root of Scutellaria (common name: Huang-Qin in China) (Appendix Fig. 8) is one of the most popular and multi-purpose herb used in China and in several oriental countries. The main Scutellaria species used in the traditional Chinese medicines are Scutellaria baicalensis Georgi, Scutellaria viscidula Bge, Scutellaria amoena C.H., Scutellaria rehderiana Diels, Scutellaria ikonnikovi Juz., Scutellaria likiangensis Diels, and Scutellaria hypericifolia Levl (The grand dictionary of Chinese herbs, 1977). In traditional Chinese medicines, extracts from the Scutellaria radix are widely used for clinical treatment of hyperlipemia, atherosclerosis, hypertension, dysentery, common cold and inflammatory diseases such as atopic dermatitis. Scutellaria has also been recognized as a mild relaxant that affects the neural and muscular-skeletal systems. Apart from above properties, Scutellaria alone, or in combination with

other herbs, has been recently shown to possess cytostatic effect on several cancer cell lines *in vitro* and also *in vivo* in mouse tumor models (Li-Weber, 2009).

The molecular basis of the anti-inflammatory effect of Scutellaria is confirmed to be the bioactive phytochemical flavones. The most frequently described S. baicalensis Georgi is especially famous for its high flavonoids contents (Han et al., 2007; Gao et al., 2008). S. baicalensis Georgi, contains four major flavones: Wogonin (5,7-dihydroxy-8-methoxyflavone), Wogonoside (Wogonin-7-glucuronic acid), Baicalin (7-glucuronic acid, 5,6-dihydroxy-flavone), and Baicalein (5,6,7-trihydroxyfavone) with ratios to the dry material about 1.3%, 3.55%, 5.41%, and 10.11%, respectively (Li-Weber, 2009).

3.2. Therapeutic Aspects of Flavones

Epidemiological studies have shown that dietary intake of flavonoids is significantly associated with a reduced risk of cancer, inflammation and heart disease (Middleton et al., 2000; Havsteen 2002). Indeed, the flavones isolated from the roots of Scutellaria have been shown to exert antioxidant (Gao et al., 1999), anti-viral (Gao et al., 1998; Ma et al., 2002; Huang et al., 2000; Guo et al., 2007), anti-thrombotic (Kimura et al., 1997; Huang et al., 2005), anti-inflammatory (Chi et al., 2003), and anti-cardiovascular illness (Huang et al., 2005; Wang 2007). Some of them also show neuron-protection in vitro (Lee et al., 2003a, 2003b; Son et al., 2004; Piao et al., 2004; Cho & Lee, 2004a), and *in vivo* in a rat ischemic model (Cho & Lee, 2004b). Among these studies, it is noticeable to focus on the anti-inflammatory effects of wogonin and baicalein.

Wogonin possesses the free radical scavenging and antioxidant capacity *in vitro* (Gao et al., 1999) and reduces inducible enzymes expression iNOS and cycloxygenase-2, leading to inhibition of NO and prostaglandin E_2 production respectively, in LPS-activated marcorphages (Kim et al., 1999; Wakabayashi & Yasui, 2000). Interestingly, wogonin inhibits MCP-1 gene expression in human endothelial cells (Chang et al., 2001). Furthermore, wogonin can inhibit IL-1β-induced IL-6 and IL-8 mRNA expression via the suppression of NF-κB binding activities in human retinal pigment epithelial cell line (Nakamura et al., 2003). In *in vivo* studies, wogonin shows the anti-inflammatory effect on TPA-induced skin inflammation (Park et al., 2001) and LPS-induced inflammation in mice (Shen et al., 2002). Similarly, baicalein shows the anti-oxidant (Bochorakova et al., 2003) and anti-inflammatory activities *in vitro* (Wakabayashi, 1999) and *in vivo* (Shen et al., 2003). Recently, we reported that baicalein reduces plasma NO levels *in vivo* in septic rats, leading to improved vasoreactivity, blood pressure and survival rate (Cheng et al., 2007).

4. Aim of the study

This study was designed to observe *in vivo* cardioprotective effects of wogonin and baicalein in two acute inflammatory animal models of reperfusion injury and LPS-induced severe sepsis, respectively. The aim of study (1) to investigate the *in vivo* effect of wogonin on myocardial ischemia/reperfusion injury in an open-chest anesthetized rat model, which was induced by 45-min left coronary artery occlusion and 2-h reperfusion; (2) to evaluate the protective effect of baicalein on myocardial dysfunction caused by endotoxemia in rats.

Chapter 2 Materials and Methods

1. The Rat Model of Myocardial Ischemia/Reperfusion Injury

1.1. Animal Preparations

Male Sprague Dawley rats, weighting 250-280 g, were used for the study. This study was approved by the Institutional Animal Care and Use Committee of National Defense Medical Center, Taiwan. All animals obtained from the National Laboratory Animal Breeding and Research Center of the National Science Council, Taiwan and were handled in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Rats were anesthetized with intraperitoneal (i.p.) pentobarbital sodium (60 mg/kg) and urethane (300 mg/kg). The use of rat preparations as models of myocardial ischemia and infarction induced by left coronary occlusion is common (Kane et al., 1980; Clark et al., 1980; Manning et al., 1984; Curtis et al., 1987). The animal preparations and surgical procedures to induce ischemia and reperfusion were performed as described previously (Chung et al., 2010). Left coronary artery was occluded for 45 min followed by 2 h of reperfusion to induce an irreversible ischemia/reperfusion injury.

1.2. Experimental Groups

The animals were assigned to one of five treatment groups. (1) Control group: rats received the vehicle, dimethyl sulfoxide (i.p., 0.03 ml) 40 min prior to occlusion (n = 30); (2) Pre-Wog 5 group: wogonin (Biotic Chemical, Taiwan) 5 mg/kg was given i.p. 40 min prior to occlusion (n = 15); (3) Pre-Wog 10 group: wogonin 10 mg/kg was given i.p. 40 min prior to occlusion (n = 30); (4) Pre-Wog 20 group: wogonin 20 mg/kg was given i.p. 40 min prior to occlusion (n = 10); (5) Post-Wog 10 group: wogonin 10 mg/kg was administered i.p. 15 min after occlusion (n = 15). The blood pressure, heart rate and electrocardiograms were continuously monitored throughout the experimental period.

1.3. Ventricular Arrhythmias

Diagnosis and quantification of arrhythmias conformed with the guidelines of the Lambeth Conventions (Walker et al., 1988). Ventricular arrhythmias were recorded by the time to onset of first arrhythmia, incidence of ventricular tachycardia and ventricular fibrillation (all types), incidence of sustained ventricular fibrillation, and arrhythmia score (Johnston et al., 1983). Sustained ventricular fibrillation was defined as ventricular fibrillation lasting continuously for more than 120 sec (the incidence of ventricular fibrillation

provides a measure of susceptibility to ventricular fibrillation initiation, and the incidence of sustained ventricular fibrillation provides a measure of ventricular fibrillation maintenance in this model) (Curtis & Hearse, 1989; Tsuchihashi & Curtis, 1991). All arrhythmias were scored on a 0-8 arrhythmia scoring scale for 0-30 min post-ligation period (Johnston et al., 1983). The value 0 was given for 0-50 ventricular premature contractions with no ventricular tachycardia or ventricular fibrillation over the observation period; 1, for 50-500 ventricular premature contractions only; 2, for > 500 ventricular premature contractions, or one episode of spontaneously reversible ventricular tachycardia or ventricular fibrillation; 3, for one or more episodes of spontaneously reversible ventricular tachycardia and/or ventricular fibrillation lasting less than 60 sec; 4, for reversible ventricular tachycardia and/or ventricular fibrillation episodes lasting 60-120 sec; 5, for ventricular tachycardia and/or ventricular fibrillation episodes lasting more than 120 sec; 6, fatal ventricular fibrillation starting at > 15 min after occlusion; 7, fatal ventricular fibrillation starting at between 4 min and 14 min 59 sec after occlusion; 8, fatal ventricular fibrillation within 4 min. The mortality in each group was also evaluated.

1.4. Area at risk and infarct

At the end of 2-h reperfusion, the left coronary artery was re-occluded and 0.3 ml Evens blue (3 %) was injected intravenously to denote the area at risk. The heart was then excised and frozen for 90 min (-20 ^{o}C). The entire ventricular area was sectioned into four 3 mm thick slices from the apex to the base and incubated in 1 % triphenyl tetrazolium chloride (phosphate buffer, pH 7.4) for 20 min (37 ^{o}C). The surviving tissue turns a deep red, while the infarct portion is white. The slice was fixed in 10% formalin overnight. The areas of risk and infarct were taken with digital camera. The areas were then measured and analyzed using Image-Pro plus analysis software. Infarct size is presented as a percentage of area at risk (infarct: area at risk).

1.5. Plasma Creatine Kinase-muscle-brain Fraction, Lactate Dehydrogenase and Tissue Necrosis Factor-α Levels Analysis

Acute ischemia/reperfusion injury was assessed with the measurement of plasma creatine kinase-muscle-brain and lactate dehydrogenase levels 60 min after reperfusion (Moss et al., 2007). Plasma levels of creatine kinase-muscle-brain and lactate dehydrogenase were measured using an analyzer of Fuji DRI-CHEM FDC 3000 (Fuji Photo Film, Japan). The tissue necrosis factor-α level was determined by an enzyme-linked immunoadsorbent

assay (rat TNF-α Immunoassay Kit, R&D Systems, USA) according to the manufacturer's instructions.

1.6. Superoxide Anion Production in Ischemic Myocardium after Reperfusion

Superoxide anion production in ischemic cardiomyocytes after ischemia/reperfusion was measured by modified lucigenin-enhanced chemiluminescence, as described previously (Chen et al., 2006). In brief, myocaridium samples (3 × 3 mm) taken from the ischemic regions 30 min after reperfusion. Scintillation plates containing Krebs-HEPES buffer with lucigenin (1.25 mM) were placed into a microplate luminometer (Hidex, Microplate Luminometer, Finland). Counts were obtained in duplicate at a 15-sec interval. Plates containing all components with the exception of organs were counted as background, and these blank values were subtracted from the chemiluminescence signals obtained from the organ samples. All samples were dried in a 90-^{o}C (16 h) oven for expressing results on a milligram myocardium dry weight basis. These results were expressed as count per second / milligram of myocardium dry weight.

1.7. Western Blot Analysis

To elucidate the effect of wogonin on the protein expression of MCP-1, activation of NF-κB, and p38 MAPK signaling pathway, and apoptosis elicited by ischemia/reperfusion, Western blot analysis was used. After 45-min ischemia and 2-h reperfusion, the ischemic region of myocardium was isolated and immediately frozen in liquid nitrogen, and stored at -80 °C until processed. Primary antibodies probed in this experiment were mouse monoclonal anti-phospho-IκBα antibody (Cell signaling, USA; 1:1000), mouse anti-phospho-p65 antibody (Epitomics, USA; 1:1000), mouse monoclonal anti-phospho-p38 MAPK antibody (Cell Signaling; 1:1000), mouse polyclonal anti-MCP-1 antibody (eBioscience, USA; 1:1000), and rabbit monoclonal anti-caspase-3 (active) antibody (Epitomics, USA; 1:500), respectively. The ratios of phospho-IκBα, phospho-p65, phospho-p38 MAPK, MCP-1 or active caspase-3 to α-actin were calculated for statistical analysis to standardize densitometry measurements between individual samples.

2. The Rat Model of Endotoxemia

2.1. Animal Preparation

Wistar-Kyoto rats (Male, 280–300 g) were purchased from the National Laboratory Animal Breeding and Research Center of the National Science

Council, Taiwan. Handling of the animals was in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised in 1985). This study was approved by the Institutional Animal Care and Use Committee of National Defense Medical Center, Taiwan. All animals were housed at an ambient temperature of 23 ± 18 °C and humidity of 55 ± 5%. Rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (40–50 mg/kg). The left carotid artery was cannulated and exteriorized to the back of the neck and connected to a pressure transducer (P23ID, Statham, Oxnard, CA, USA) to measure phasic blood pressure, mean arterial blood pressure (MBP) and heart rate, which were displayed on a polygraph recorder (ML 785 PowerLab, AD instruments, Castle Hill, Australia). The right jugular vein was cannulated and exteriorized to the back of the neck for the administration of drugs. After the catheters were fixed, rats were fasted overnight for recovery but allowed water *ad libitum*.

2.2. Experimental Groups

The animals were randomly allocated into four groups (n = 6 in each group): (1) sham group (1 mL/kg normal saline given intravenously); (2) sham + Bai group (10 mg/kg baicalein given intravenously); (3) LPS group,

Escherichia coli LPS (10 mg/kg, intravenous infusion over 10 min); (4) LPS + Bai group, *E. coli* LPS 10 mg/kg plus baicalein (10 mg/kg, intravenously). The dose of baicalein used was based on our previous study on sepsis (Cheng et al., 2007). Bacterial LPS (*E. coli* serotype 0127:B8, L3127) and baicalein were obtained from Sigma Chemical Company (St. Louis, MO, USA). The experiments were performed on pairs of conscious rats, a model that is likely to be clinically relevant (Mathiak et al., 2000) and avoids the interference of anaesthetics with cytokine release (Yang et al., 2007). After recording baseline haemodynamic variables, LPS was infused and baicalein or vehicle (0.3 mL dimethyl sulfoxide) infusion was started 30 min after LPS treatment. The changes in blood pressure and heart rate were monitored for 6 h in all animal groups. The state of conscious rats after LPS administration became gradually less active: they moved slowly and appeared immobile after 5–6 h. The blood glucose levels significantly increased at 1 h after LPS administration (Δ 50 ± 7.8 mg/dL) compared with basal levels (102 ± 3.1 mg/dL). Hyperglycaemia was used as an indicator of successful induction of sepsis by LPS challenge. At the end of each experiment, the rats were euthanized by intraperitoneal administration of pentobarbital (60 mg/kg) with 5000 USP units of heparin added as an anti-coagulant.

2.3. Isolated Heart Preparation and Left Ventricular Pressure Recording

Hearts were isolated 6 h after LPS administration and perfused with a modified Krebs–Henseleit solution equilibrated with 95% O_2 and 5% CO_2 at a constant flow of 7–9 mL/min and temperature of 37 °C when being mounted on the Langendorff apparatus. The buffer contained 118.0 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$ and 11.0 mM glucose. A 2 F high-fidelity micro-manometer catheter containing a pressure transducer (SPR-407, Millar Institute, Houston, TX, USA) was inserted into the left ventricle via the left atrium. The heart was paced at 300 beats/min and allowed to equilibrate for 15 min. Left ventricle contractility was continuously evaluated by the left ventricular developed pressure (LVDP) and the rates of contraction and relaxation (+dP/dt and –dP/dt) measured using a PowerLab/8SP analogue-to-digital converter (ADInstruments).

2.4. Western Blot Analysis

Six hours after LPS administration, the left ventricular myocardium was isolated, immediately frozen in liquid nitrogen and stored at –80 °C until processed. Detection of the proteins by Western blotting was performed as described previously (Chen et al., 2006). The primary antibody probes in this experiment were mouse monoclonal anti-inducible nitric oxide synthase (iNOS)

(BD Biosciences, USA; 1:2000), anti-phospho-IκBα (Cell Signaling, USA; 1:1000) and anti-phospho-p65 (Epitomics, USA; 1:1000) and mouse polyclonal anti-MCP-1 (eBioscience, USA; 1:1000) and anti-HO-1 (Santa-Cruz, USA; 1:1000). To standardize densitometry measurements between individual samples, the ratios of iNOS, phospho-IκBα, phospho-p65, MCP-1 or HO-1 to α-actin were calculated.

2.5. Superoxide Anion Production in Myocardium 6 h after LPS Administration

Superoxide anion production in the left ventricular myocardium was measured by modified lucigenin-enhanced chemiluminescence and was performed as described previously (Chen et al., 2006; Shih et al., 2008). Samples of left ventricle (3 × 3 mm) taken 6 h after LPS administration were used. Scintillation plates containing Krebs-HEPES buffer with lucigenin (1.25 mM) were placed into a microplate luminometer (Hidex, Microplate Luminometer, Finland). These results were expressed as counts per second (CPS) per milligram dry weight of myocardium.

2.6. Measurement of Caspase-3 Activity in Cardiac Tissue

Cardiac caspase-3 activity was determined using colorimetric assay kits (Assay Designs, MI, USA) according to the manufacturer's instructions. Results are expressed as units/μg protein.

3. Statistical Analysis

The *Chi* square test with Fisher exact test was used to analyze the differences in the incidence of arrhythmias and mortality between the control and wogonin-treated groups. The time to onset of first arrhythmia was $\log_{10}$ transformed to generate Gaussaian-distributed variable (Tsuchihashi et al., 1991). Data are expressed as group mean ± standard error of mean (SEM). Statistical evaluation was performed with one-factor analysis of variance followed by the Newman-Keuls post-hoc comparison test. A *P* value of less than 0.05 was deemed statistically significant.

Chapter 3 Results

1. The Cardioprotective Effect of Wogonin in Myocardial Ischemia/Reperfusion Injury

1.1. Hemodynamics

The hemodynamic data including mean blood pressure and heart rate was summarized in Table 1. Throughout ischemia/reperfusion experimental period, the measurement of mean blood pressure was not significantly different among groups. However, a significant reduction in heart rate was observed at 40 min after treatment with wogonin 10 mg/kg when compared with the control group. During ischemia/reperfusion, the heart rate of rats in Pre-Wog 10 group was lower than that of control group, which appeared significant differences at 1 min and 5 min after occlusion ($P < 0.05$). In the Post-Wog 10 group, wogonin 10 mg/kg was administered at 15 after occlusion. There was no wogonin-treatment during early 15 min of ischemic period. Therefore, the heart rate of Post-Wog 10 group was significantly higher than the pre-Wog 10 group at 1 min before occlusion, 1 min and 5 min after occlusion ($P < 0.05$).

1.2. Arrhythmias during Ischemic Period

Ventricular arrhythmias commenced within 4-30 min of occlusion, manifesting as ventricular premature contractions, ventricular tachycardia and ventricular fibrillation. All the rats developed arrhythmias during the 30-min post-ligation period. Wogonin 10 mg/kg significantly delayed the occurrence of ventricular premature contractions and ventricular tachycardia (Table 2), and suppressed the incidence of ventricular tachycardia, total ventricular fibrillation and sustained ventricular fibrillation (Table 3), as compared with the control group. The arrhythmia score and mortality of rats in the Pre-Wog 10 group was significantly lower than those of control group ($P < 0.05$). However, pretreatment with 5 and 20 mg/kg of wogonin did not significantly suppress arrhythmia scores and reduce mortality by ischemia when compared with the control group ($P > 0.05$) (Table 3 & Fig. 2). The mortality of Pre-Wog 20 was significantly higher than the Pre-Wog 10 group. In the Post-Wog 10 group, because wogonin was given at 15 min after occlusion, the incidence of ventricular fibrillation (66.7 %) and mortality (33.3 %; 5 rats died, 10 rats survived) were similar to those of control group. In addition, the arrhythmias also occurred in the reperfusion period. Pre- or Post-treatment with wogonin 10 mg/kg did not significantly reduce the counts of ventricular premature contractions when compared with the control group ($P > 0.05$). The count of the

Post-Wog 10 group was significantly more than that of the Pre-Wog 10 group ($P < 0.05$). The incidence of ventricular tachycardia was 15% (3/20) and 20 % (1/5) in the Pre-Wog 10 and Post-Wog 10 groups, respectively, which did not significantly differ from that of the control group (25%, 3/12). The incidence of ventricular fibrillation was 8.3% (1/12) in the control group. There was no occurrence of ventricular fibrillation in the Pre- and Post-Wog 10 groups (Table 4).

1.3. Plasma Levels of Creatine Kinase-muscle-brain and Lactate Dehydrogenase

After 1-h reperfusion, the creatine kinase-muscle-brain level significantly elevated in the control group (5858 ± 436 units/l, n = 10). The creatine kinase-muscle-brain levels of Pre-Wog 5, Pre-Wog 10 and Post-Wog 10 groups are significantly lower than that of control group (Pre-Wog 5: 3610 ± 213, n = 5; Pre-Wog 10: 3500 ± 479, n = 10; Post-Wog 10: 3320 ± 377 units/l; n = 5, $P < 0.05$). Pretreatment with high dose of wogonin (20 mg/kg) did not attenuate the creatine kinase-muscle-brain level when compared with the control group (Pre-Wog 20: 4620 ± 535 units/l, n = 5). There was no significant difference in the creatine kinase-muscle-brain level among wogonin-treated groups (Fig. 3A). The lactate dehydrogenase data of control group was 2562 ± 215 units/l (n=10).

Pretreatment with 5 and 10 mg/kg, and post-treatment with wogonin 10 mg/kg significantly attenuated the levels of lactate dehydrogenase when compared with that of the control group (Pre-Wog 5: 1722 ± 229, n = 5; Pre-Wog 10: 1713 ± 164, n = 10; Post-Wog 10: 1518 ± 220 units/l; n = 5, $P < 0.05$). Pretreatment with high dose of wogonin (20 mg/kg) did not significantly reduce the plasma level of lactate dehydrogenase when compared with that of the control group (Pre-Wog 20: 2268 ± 236 units/l, n = 5). There was no significant difference in the lactate dehydrogenase level among wogonin-treated groups (Fig. 3B).

1.4. Size of infarction after 2 h of reperfusion

No significant differences in the area at risk, expressed as percentage of the total left ventricle, were noted among the groups (Fig. 4). A significant reduction in infarct size, expressed as percentage of the area at risk was noted in groups of pre- and post-treatment with wogonin 10 mg/kg (Pre-Wog 10 and Post-Wog 10 groups), when compared with the control (Pre-Wog 10: 48.2±2.7 %, n = 6, Post-Wog 10: 52.3±2.8 %, n = 5, vs control: 63.1±4.6 %, n = 6) ($P < 0.05$). There is no significant difference between Pre- and Post-Wog 10 groups ($P > 0.05$).

1.5. Superoxide Anion Production in Ischemic Myocardium after Reperfusion

Myocardial superoxide anion production was measured in ischemic regions of the control and wogonin-treated groups after 45-min ischemia/30-min reperfusion (Fig. 5A). Pre- and post-treatment with wogonin significantly inhibited the increase in superoxide anion production in the myocardium after ischemia/reperfusion (Pre-Wog 5 3.5 ± 1.9, n = 5: Pre-Wog 10: 2.6 ± 1.2, n = 6; Post-Wog 10: 5.4 ± 2.3 counts per second/mg tissue weight, n = 5), when compared with that of control group (Control: 15.3 ± 3.5 counts per second/mg tissue weight, n = 6) ($P < 0.05$). There is no significant difference in the superoxide anion level among wogonin-treated groups.

1.6. Plasma Tissue Necrosis Factor-α Levels after Ischemia/Reperfusion

Plasma levels of tissue necrosis factor-α were measured at 1 h after reperfusion. Pretreatment with wogonin 5 and 10 mg/kg significantly decreased the ischemia/reperfusion-induced elevation of plasma tissue necrosis factor-α level as compared with the control group (Control: 75.1 ± 10.1, n=10; Pre-Wog 5: 43.1 ± 4.0, n=5; Pre-Wog 10: 32.5 ± 6.0 ng/ml, n = 10) ($P < 0.05$). The tissue necrosis factor-α levels of Pre-Wog 20 and Post-Wog 10 groups were not

significantly different from that of the control group (Pre-Wog 20 60.1 ± 4.0 ng/ml; n = 5; Post-Wog 10: 48.1 ± 4.4 ng/ml; n = 5) (Fig. 5B).

1.7. Protein Expression after Ischemia/Reperfusion

Western blots on homogenates of ischemic myocardium after 2-h reperfusion was performed to observe the effects of wogonin on ischemia/reperfusion-induced changes of protein expression. Comparing with the control group, both pretreatment and post-treatment with wogonin 10 mg/kg significantly reduced ischemia/reperfusion-induced elevation of MCP-1, phospho-IκBα, phospho-p65, phospho-p38 MAPK, and active caspase-3 protein expression (Fig. 6-10) ($P < 0.05$). There is no significant difference in the expression levels of all observed proteins between the Pre-Wog 10 and Post-Wog 10 groups ($P > 0.05$).

2. The Cardioprotective Effect of Baicalein in Sepsis

2.1. Effects of Baicalein on Haemodynamic Changes in Endotoxaemic Rats

The haemodynamic data including MBP and heart rate are shown in Fig. 12. The basal MBP of rats did not differ significantly between the four groups. In the sham and sham + Bai groups, there was no significant change in MBP during the experimental period. In the LPS group, rats showed a marked fall in

MBP 30 min after LPS administration, which lasted until 1 h after LPS and then progressively increased between 1 and 2 h, followed by an increasing rate of decrease in MBP for 2–6 h after LPS. Post-treatment with baicalein 30 min after LPS administration significantly attenuated the hypotension caused by LPS.

The basal heart rate did not differ significantly between the four groups. In the sham and sham + Bai groups, there was no significant change in heart rate during the experimental period. LPS administration caused a significant increase in heart rate during the first 2–3 h of the experimental period compared with the sham group, and then progressively decreased to the basal level. Post-treatment with baicalein 30 min after LPS administration also resulted in a profound elevation of heart rate, which was maintained at a significantly higher level than in the sham group until the end of the experiment (6 h after LPS).

2.2. Effects of Baicalein on Cardiac Contractile Dysfunction Caused by LPS

The LVDP (Fig. 13A) and average ± dP/dt (Fig. 13B and 13C) evaluated at 6 h after LPS administration were significantly decreased in the hearts of the LPS-treated groups compared with those of the sham group ($P < 0.05$). Post-treatment with baicalein resulted in the recovery of LVDP and ± dP/dt

compared with the LPS groups ($P < 0.05$). Baicalein alone (sham + Bai group) did not significantly affect these parameters of cardiac contractile function.

2.3. Effects of Baicalein on Cardiac iNOS, MCP-1, Phospho-IκBα, Phospho-p65, and HO-1 Protein Expression

Six hours after administration of LPS, the levels of cardiac protein expression of iNOS (Fig. 14A and 14B), MCP-1 (Fig. 15A and 15B), phospho-IκBα (Fig. 16A and 16B) and phospho-p65 (Fig. 17A and 17B) were significantly elevated compared with those in the sham group ($P < 0.05$). Post-treatment with baicalein significantly reduced expression of these pro-inflammatory proteins compared with the LPS group ($P < 0.05$). However, the levels of iNOS and MCP-1 in the LPS + Bai group were significantly higher than those in the sham group ($P < 0.05$). By contrast, the level of HO-1 protein was markedly reduced 6 h after LPS administration compared with that of the sham group ($P < 0.05$), whereas post-treatment with baicalein significantly elevated the induction of HO-1 during endotoxaemia ($P < 0.05$) (Fig. 18A and 18B). Cardiac expression of iNOS, MCP-1, phospho-IκBα, phospho-p65 and HO-1 protein in the sham + Bai group did not differ from those of the sham group.

2.4. Effects of Baicalein on Superoxide Anion Production

The levels of superoxide anion production in left ventricular myocardium 6 h after LPS administration were significantly elevated compared with the sham group. Post-treatment with baicalein significantly inhibited this increase in superoxide anion production compared with that of the LPS group (sham: 23.5 ± 3.9; LPS: 58.6 ± 5.2; LPS + Bai: 35.4 ± 5.1 CPS/mg tissue weight, n = 6) ($P < 0.05$) (Fig. 19). The level of superoxide anion in the sham + Bai group (19.5 ± 2.8 CPS/mg tissue weight, n = 6) did not differ significantly from that of the sham group ($P > 0.05$).

2.5. Effects of Baicalein on Cardiac Caspase-3 Activity

Six hours after administration of LPS, the caspase-3 activity in the LPS group was significantly higher than that of the sham group (sham: 326.3 ± 17.2; LPS: 546.5 ± 16.0 units/μg protein) ($P < 0.05$) (Fig. 20). Post-treatment with baicalein (LPS + Bai: 418.4 ± 23.7 units/μg protein) significantly reduced the induction of caspase-3 activity by LPS ($P < 0.05$), but it remained significantly higher than that of the sham group ($P < 0.05$). The level of caspase-3 activity in the sham + Bai group (345.4 ± 20.6 units/μg protein) did not differ significantly

from that of the sham group. This result indicated that post-treatment with baicalein may attenuate myocardial apoptosis induced by endotoxaemia.

Chapter 4 Discussion

In Myocardial Ischemia/Reperfusion Injury

In the present study, we showed the *in vivo* evidence for the first time that wogonin markedly suppresses ischemia-induced lethal ventricular arrhythmias, contributing to reduce mortality. Besides pretreatment, even given after occurrence of ischemia (post-treatment), wogonin demonstrated myocardial protection against irreversible ischemia/reperfusion injury and apoptosis. Wogonin attenuated ischemia/reperfusion-induced superoxide anion production, and inflammatory responses evidenced by decreases in tissue necrosis factor-α level, and protein expression of chemokine MCP-1, which may be mediated by suppression of activation of NF-κB and p38 MAPK signaling pathways in ischemia/reperfusion myocardium.

Ischemia induces decreased intracellular pH caused by the accumulation of metabolic by-products, leading to stimulate the Na^+-H^+ exchange pathway in an attempt to extrude H^+ from the cell, and consequently resulting in accelerated Ca^{2+} entry via reverse mode Na^+-Ca^{2+} exchange activity, which attempts to restore intracellular Na^+ levels and prevent their accumulation. This Na^+-Ca^{2+} exchange-mediated transient inward current can result in intracellular Ca^{2+} overload, spontaneous rises in membrane potential that manifest as delayed

afterdepolarizations (Akar & Akar, 2007). This triggered activity from Ca^{2+} overload may successfully propagate throughout the myocardium and form lethal arrhythmias. In the present study, wogonin showed anti-arrhythmic effect during ischemic insult and reduced mortality. The anti-arrhythmic mechanism of wogonin is still unknown. In previous electrophysiological study, we found that wogonin can suppress L-type Ca^{2+} currents, shorten action potential duration, and reduce Ca^{2+} transient induced electrically in normal rabbit ventricular myocytes (Appendix Fig. 9). Therefore, wogonin may reduce Ca^{2+} overload in ischemic myocardium by restoring the changes in Ca^{2+} handling. Further experiments to explore possible anti-arrhythmic mechanism of wogonin on ischemic or hypoxic cardiomyocytes will be undertaken. On the other hand, oxidative stress is involved in the pathogenesis of arrhythmias. For example, experimental atrial fibrillation is associated with increased left atrial NAD(P)H and xanthine oxidase activity, thereby causing an increase in the formation of superoxide (Dudley et al., 2005). Wogonin has been reported to possess free radical scavenging effects and suppress NADPH-dependent lipid peroxidation (Gao et al., 1999). Meanwhile, in the present study, we showed the inhibitory effect of wogonin on superoxide anion production in myocardium after 45 min-ischemia/30 min-reperfusion (Fig. 2A). Therefore, the antioxidant capacity

of wogonin may participate in the mechanism of antiarrhythmic action. Moreover, elevated heart rate induces conditions, such as increased myocardial oxygen consumption, reduction in time of diastole and myocardial blood supply, which result in the development of myocardial ischemia and arrhythmias in ischemic areas (Lanza et al., 2006). Treatment with wogonin 10 mg/kg gradually reduced heart rate of rats (Table 1), which last to even onset of ischemia and, at least, 5 min after ischemia, suggesting that myocardial ischemia-mediated sympathetic activation can be suppressed by wogonin. Bradycardia produced by wogonin may contribute to antiarrhythmic action during ischemia.

Although pretreatment with wogonin 5 mg/kg did not significantly reduce the arrhythmia score and mortality of rats subjected to ischemia, the anti-reperfusion-injury effect was pronounced (Fig. 2A, 2B), which may be associated with its antioxidant and anti-inflammatory effects (Fig. 3). Unlike wogonin 10 mg/kg, pretreatment wogonin 5 mg/kg did not reduce the heart rate during baseline and early ischemic period (Table 1). This also implies that wogonin 5 mg/kg may not alleviate ischemia-induced Ca^{2+} overloading, and triggered activity, therefore, it did not afford protective effects during ischemic period. Preteatment with high dose of wogonin (20 mg/kg) did not show more

beneficial effects on ischemia-induced arrhythmias, and reperfusion injury, accompanying higher mortality, when compared with the Pre-Wog 10 group. However, wogonin 20 mg/kg did not significantly worsen ischemic insult when compared with the control group. This may be a result that toxic effect of high dose wogonin counteracted its beneficial effects. Moreover, the anti-reperfusion injury effects of post-treatment with wogonin 10 mg/kg did not significantly differ from that of pretreatment indicating wogonin mainly exerted its protective actions during reperfusion period. However, the ventricular premature counts during reperfusion period in the Post-Wog 10 group were significantly more than that of the Pre-Wog 10, suggesting that preceding administration of wogonin into ischemic zone contributed to alleviate reperfusion-induced arrhythmias.

The oxygen free-radical system has been implicated in the pathogenesis of ischemia/reperfusion. Several approaches to protection against free radical damage have been considered to protect myocardium against ischemia/reperfusion injury (Hamilton, 2007). As aforementioned, wogonin is a polyhydroxyflavonoid and has been demonstrated to possess antioxidant, free-radical scavenging, and anti-inflammatory activities (Gao et al., 1999). In the present study, wogonin also suppressed superoxide anion production in

ischemic region after ischemia/reperfusion. Therefore, the antioxidant capacity of wogonin is likely to contribute to reduce ischemia/reperfusion injury. Additionally, increased production of ROS induces changes in the physicochemical properties of the cells and initiates new signal transduction mechanisms, leading to such as the activation of NF-κB transcription factor and MAPK superfamily that result in altered gene expression profile and generally in an activated and proinflammatory cellular phenotype (Marczin et al., 2003). Therefore, wogonin reduced oxidative stress in ischemia/reperfusion is likely further to suppress the activation of nuclear factor-κB transcription factor and p38 MAPK signaling pathway.

Reperfusion injury has to be considered as inflammatory disease (Granger & Kubes, 1994). Accumulating evidence has indicated that ischemia elicits an acute inflammatory response that is greatly augmented by reperfusion. Nuclear factor-κB regulates the expression of numerous inflammatory mediators, including interleukins, cytokines, and cell adhesion molecules (Hall et al., 2006). ROS, cytokines, and shear stress resulting from ischemia/reperfusion injury, stimulate NF-κB via proximal kinase activation. It has been shown that gene transfer of IκBα limits infarct size in a mouse model of myocardial ischemia-reperfusion injury (Squadrito et al., 2003). Specific IKKβ inhibitor

Bay65-1942 can provide both acute and delayed cardioprotection and has been suggested to offer a clinically accessible target for preventing cardiac injury following ischemia/reperfusion (Moss et al., 2007). In the present study, wogonin can reduce the expression of phospho-IκBα and -p65 showing the anti-NF-κB property, which is contributing to reduction of myocardial stress elicited by ischemia/reperfusion.

The pro-inflammatory nuclear factor kappa B transcription factor is a key mediator for MCP-1 expression (Melgarejo et al., 2009). In the canine model, induction of MCP-1 mRNA occurred in previously ischemic area within the first hour of reperfusion, peaked at 3 hours, and persisted throughout the first 2 days of reperfusion (Kumar et al., 1997). Neutralizing antibody to MCP-1 significantly reduce infarct size decreasing adhesion molecule expression and macrophage infiltration in rats (Ono et al., 1999). Enhanced MCP-1 expression in rat kidney during ischemia/reperfusion injury is mediated by oxidative stress and nuclear factor-κB (Sung et al., 2002). In accordance with the results, wogonin inhibits MCP-1 gene expression in human endothelial cells (Chang et al., 2001). In the present study, we also found that wogonin inhibited MCP-1 protein expression in ischemic region after ischemia/reperfusion. The inhibitory effect on MCP-1 may contribute in the beneficial effect of wogonin on

ischemia/reperfusion injury, which is likely mediated by suppression of NF-κB activation and its antioxidant effect.

The p38 MAPK is activated after exposure to many forms of cellular stress, such as endotoxin, proinflammatory cytokins, tissue necrosis factor-α, interleukin-1, osmotic shock, and heat stress (Ravingerova et al., 2003). Activation of p38 MAPK followed by transcription of genes encoding inflammatory molecules indicates an important role of this stress cascade in the cell inflammatory responses. The activation of the p38 MAPK pathway plays essential roles in the production of proinflammatory cytokines (interleukin-1, tissue necrosis factor-α and interleukin-6) (Zarubin & Han, 2005). The production of cytokines further elicited NF-κB activation and apoptosis. The p38 MAPK pathway is a controversial signaling pathway in myocardial responses to ischemic injury. Inhibition of p38 MAPK activation delayed the development of infarcts, increased cell survival, reduced myocardial apoptosis and improved postischemic recovery of cardiac function (Ma et al., 1999; Schneider et al., 2001). In the present study, we showed the cardioprotective effect of wogonin accompanied with suppression of the activation of p38 MAPK, which may be one mechanism of protective action of wogonin.

Ischemia/reperfusion injury results in a variable mixture of apoptotic, necrotic, and normal tissue that depends on both the duration and severity of ischemia. An abundance of evidence indicates that ischemia/reperfusion-induced cardiac cell death occurs from both necrosis and apoptosis (Logue et al., 2005). Cysteine proteases comprising the caspase family have been considered one of the major executioners of programmed cell death or apoptosis (Yaginuma et al., 2001). Apoptosis is potentially triggered by cytokines, tissue necrosis factor-α, ROS, and NO released by infiltrated polymorphonuclear leukocytes or marcophages (Zhao & Vinten-Johansen, 2002). In the present study, we measured the levels of active caspase-3 protein expression to reflect the situation of ischemia/reperfusion-induced apoptosis and found wogonin can attenuate the induction of apoptosis. The antioxidant and anti-inflammatory effect of wogonin likely contribute to this protection.

Although in a previous *in vitro* report, wogonin can not show protection efficacy in a cultured chick cardiomyocyte exposed to ischemia/reperfusion (Chang et al., 2007), it ameliorated ischemia/reperfusion injury *in vivo* in the present study. However, an *in vivo* study is more clinically relevant than that of *in vitro* study of wogonin. Furthermore, the neuroprotective effect of wogonin has been demonstrated *in vivo* in experimental brain injury models (Lee et al.,

2003; Cho & Lee, 2004). These *in vivo* results provide a pharmacological basis for the use of wogonin or *Scutellaria baicalensis* in the treatment or prevention of stroke and acute myocardial infarction.

In Sepsis

In a previous study, we showed that baicalein improves circulatory failure and the survival rate in septic rats (Cheng et al., 2007). Here, we further investigated the cardioprotective effect of baicalein during endotoxaemia, which may directly contribute to prevention of circulatory failure. Baicalein improved cardiac contractile function and prevented occurrence of septic shock 6 h after administration of LPS, accompanied by sustained tachycardia. An anti-inflammatory effect is involved in cardioprotection by baicalein, evidenced by attenuation of cardiac iNOS and MCP-1 protein expression and suppression of cellular NF-κB activation. Induction of cardiac HO-1 production and the anti-apoptotic effects of baicalein may also contribute to prevention of myocardial depression. However, LPS challenge caused complex and serious inflammatory responses. Rats were treated with baicalein 30 min after LPS challenge (i.e., post-treatment) to evaluate the therapeutic effect: the inflammatory responses had been initiated before baicalein treatment. Therefore,

it was difficult to reverse totally the LPS-induced effects, but partial reversal was achieved.

In sepsis, heart rate and cardiac output are increased, seemingly to compensate for a general vasodilatation and to maintain blood pressure (Bradley et al., 1945). In this study, baicalein improved cardiac contractile function and maintained blood pressure at a high level in septic rats, accompanied by a lasting tachycardia. An increase in heart rate can be a compensatory effect to maintain blood pressure and cardiac output to improve perfusion to organs and prevent multiple organ failure at late-stage sepsis. Baicalein alone (sham + Bai group) did not elicit this increase in heart rate. This indicates that baicalein preserved cardiac function, thus maintaining circulatory function to the late phase of endotoxaemia. However, the possibility that a sustained rise in heart rate is a potential side effect of baicalein in sepsis cannot be ruled out.

Sepsis leads to the expression of iNOS, which produces high levels of NO, in the myocardium (Preiser et al., 2001; Khadour et al., 2002). This is responsible for direct effects on vascular tone, depression of mitochondrial respiration and further release of pro-inflammatory cytokines, leading to myocardial depression (Rudiger and Singer, 2007). NO reacts with superoxide anion to generate a cytotoxic product, peroxynitrite (Pacher et al., 2007), which

also contributes to myocardial dysfunction. In our previous study, baicalein suppressed iNOS expression in aorta and plasma levels of NO metabolites in sepsis (Cheng et al., 2007). In the present study, we also showed that baicalein suppressed iNOS induction by LPS in cardiac tissues (Fig. 3A and 3B), which contributed to improvement of the LPS-induced myocardial dysfunction by baicalein.

The innate immune response is activated in sepsis (O'Brien et al., 2007). A systemic response to infection brought about by various inflammatory mediators, such as cytokines and chemokines, leads to the infiltration of specific leukocyte populations including neutrophils and monocytes into host tissues. MCP-1, a prototype CC chemokine, is a potent chemoattractant and a regulatory mediator involved in a variety of inflammatory diseases (Luster, 1998). MCP-1 expression is regulated at the transcriptional level by stimulatory agents such as TNF-α, IFN-γ, platelet-derived growth factor and stress factors (Melgarejo et al., 2009). Recently, anti-MCP-1 treatment has been proposed to be of potential therapeutic value in the treatment of sepsis and endotoxaemia (Ramnath et al., 2008). In the present study, we demonstrated for the first time that baicalein suppresses MCP-1 expression (Fig. 3C and 3D), which could reduce the influx

of macrophages into tissues and alleviate the inflammatory responses during sepsis.

NF-κB is clearly one of the most important regulators of pro-inflammatory gene expression. Triggering through toll-like receptors by bacterial ligands, e.g., LPS, initiates signalling cascades that result in the activation of NF-κB, which drives transcription of a range of important pro-inflammatory cytokines and chemokine genes, such as TNF-α, IL-1β, IL-6, IL-8 and iNOS. In fact, NF-κB can be rapidly activated by many pathogenic stimuli, including TNF-α and IL-1 (Xie et al., 1994; Sriskandan and Altmann, 2008). Post-treatment with baicalein attenuated the LPS-induced NF-κB activation in myocardium evidenced by suppression of phospho-IκBα and phospho-p65 levels. This contributed to the reduction of iNOS and MCP-1 expression, leading to amelioration of cardiac inflammation.

In addition, ROS have been shown to be involved in NF-κB activation (Schreck et al., 1991; Adcock et al., 1994). Anti-oxidants have been reported to possess beneficial effects in sepsis (Berger and Chiolero, 2007). LPS-induced cardiac dysfunction may be in part the result of ROS production induced by inflammatory mediators such as TNF-α (Rudiger and Singer, 2007). Elevated oxidative stress can induce HO-1 protein expression, which can produce

protective effects in various diseases (Takahashi et al., 2004). The porphyrin ring of haem can be broken by HO-1 to yield equimolar amounts of biliverdin IXα, free iron (Fe^{2+}) and carbon monoxide (CO). Iron, an oxidant, is directly sequestered and inactivated by co-induced ferritin (Harrison and Arosio, 1996). Biliverdin IXα is rapidly converted by biliverdin reductase to bilirubin IXα, which has been reported to be an anti-oxidant (Stocker et al., 1987). CO can suppress inflammatory responses and apoptosis (Otterbein et al., 2000). Beneficial effects of the HO-1/CO system in patients with severe sepsis/septic shock have recently been reported (Takaki et al., 2010). It has been suggested that manipulation of the HO-1 pathway may represent a future therapeutic strategy to counteract oxidative stress in endotoxaemia (Tamion et al., 2010). In the present study, baicalein was able to induce cardiac HO-1 expression at the late stages of sepsis, which may attenuate free radical formation and contribute to its anti-inflammatory effect.

Inhibition of apoptosis in animal models of sepsis has resulted in improved cardiac function (Fauvel et al., 2001; Neviere et al., 2001; Buerke et al., 2008). The cysteine proteases comprising the caspase family have been considered one of the major executioners of programmed cell death or apoptosis (Yaginuma et al., 2001). Caspase-3 is involved in a wide variety of functional responses in

ventricular myocytes including a negative inotropic response (Laugwitz et al., 2001). Caspase-3 activation directly targets the three main components of the myofilament machinery, namely, α-actin, α-actinin and troponin T, and induces the breakdown of myofibrillar proteins, leading to a decrease in ATPase activity and force development (Communal et al., 2002). Apoptosis is potentially triggered by cytokines, TNF-α, ROS and NO released by infiltrating polymorphonuclear leukocytes or macrophages (Zhao & Vinten-Johansen, 2002). Baicalein reduces plasma levels of TNF-α (Cheng et al., 2007), attenuates superoxide anion formation and suppresses iNOS and MCP-1 expression in cardiac tissue, which would all lead to a decrease in LPS-induced apoptosis of cardiomyocytes.

Chapter 5 Conclusion and Perspectives

Wogonin and baicalein, two major components of Scutellaria baicalensis Georgi, show cardioprotective actions *in vivo*. They exhibited anti-inflammatory effects evidenced by reducing free radical production and cytokine release, suppressing inflammation-related proteins, MCP-1 and iNOS expression, and by inducing HO-1 expression in myocardium with acute inflammatory responses. Suppression of the activation of p38 MAPK and NF-κB signaling pathways in cardiomyocytes is involved in these beneficial effects. The anti-apoptosis effect by wogonin and baicalein may contribute to improvement of cardiac function. Wogonin also showed antiarrhythmic actions in myocardial ischemia. The underlying mechanisms are still uncertain. Reducing heart rate and intracellular Ca^{2+} overload (see Appendix Fig. 9) are involved in this effect. Further investigation in the mechanisms of regulation of Ca^{2+} homeostasis, e.g. Na^{+}-Ca^{2+} exchange and L-type Ca^{2+} currents, Ca^{2+} concentration in sarcoplasmic reticulum (SR), sarco-endoplasmic Ca^{2+}-ATPase (SERCA) and phospholamban expression, by wogonin or baicalein will be taken. This is also helpful to know whether baicalein can regulate intracellular Ca^{2+} to improve contractile function of heart. Furthermore, to examine the electrophysiological characteristics of wogonin and baicalein in ventricular cardiomyocytes or tissues will contribute to understand the underlying mechanisms of their anti-arrhythmic effect.

In *in vitro* studies, baicalein reduced the levels of free radicals and LDH release caused by hypoxia-reoxygenation in cardiomyocytes of chicks and rats (Shao et al., 2002; Woo et al., 2005). The *in vivo* effect is uncertain. Based on the results of wogonin in myocardial ischemia, it is considerable to evaluate the antiarrhythmic, anti-infarct and anti-apoptotic effects of baicalein in animal model. The comparison of cardioprotective effects of wogonin and baicalein in a same acute inflammatory model can afford useful information about the potency and efficacy of these two drugs.

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Chapter 7 Tables &Figures

Table 1 Summary of hemodynamic parameters during the experiments

	5 min after	1 min before	Time for ischemia (min)			Time for reperfusion (min)		
Group	Thoracotomy	Occlusion	1	5	40	10	60	120
Mean blood pressure (mmHg)								
Control	94.2±4.1	84.2±4.1	67.9±3.6	81.3±4.5	83.6±4.1	86.9±4.9	84.4±3.5	82.0±3.0
Pre-Wog 5	90.7±3.4	83.3±4.5	68.1±3.7	85.8±4.4	80.2±3.5	83.3±2.4	78.4±1.6	78.8±1.9
Pre-Wog 10	90.1±2.5	86.9±2.8	71.2±3.0	82.0±3.6	91.2±5.3	88.9±2.7	87.6±2.1	86.7±1.9
Pre-Wog 20	92.7±5.7	90.3±4.1	76.1±3.1	86.8±8.4	95.2±9.5	90.3±7.0	88.4±6.4	83.8±6.6
Post-Wog 10	91.2±1.3	92.8±4.6	74.6±3.2	87.0±4.8	94.8±3.2	86.2±5.2	84.8±5.1	88.2±1.5
Heart rate (beats/min)								
Control	433.0±4.1	381.1±11.1	403.6±12.6	420.7±8.5	399.9±11.4	413.5±8.9	384.7±10.2	374.6±10.9
Pre-Wog 5	436.7±7.7	390.8±11.4	405.5±10.6	410.6±9.5	399.6±12.5	400.5±9.9	388.7±7.8	384.6±12.9
Pre-Wog 10	410.0±8.3	345.7±9.7*	358.2±10.3*	371.2±10.8*	371.4±9.8	378.4±9.3	359.1±11.3	339.4±14.2
Pre-Wog 20	434.5±10.6	368.8±14.4	390.0±11.8	402.6±8.2	396.6±10.5	398.5±12.0	389.8±9.8	380.6±8.9
Post-Wog 10	420.0±7.7	389.0±11.2#	397.6±7.5#	404.0±4.6#	399.8±10.1	388.8±9.8	381.0±9.1	372.2±7.4

Pre-Wog 5, 10, and 20: wogonin 5, 10, and 20 mg/kg (i.p.) was administered 40 min before left coronary artery occlusion; Post-Wog 10: wogonin 10 mg/kg was administered 15 min after occlusion; n=12 in the control, n=5 in Pre-Wog 5; n=20 in Pre-Wog 10, n=5 in Pre-Wog 20, and n=5 in the Post-Wog 10 group; Values are expressed as mean ± SEM, * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the Pre-Wog 10 group.

Table 2 The effect of wogonin on the time to onset of first ischemia-induced ventricular arrhythmias

	VPC		VT		VF	
Treatment	N	$\log_{10}$ (sec)	N	$\log_{10}$ (sec)	N	$\log_{10}$ (sec)
Control	30	2.54±0.01	27	2.59±0.01	20	2.67±0.02
Pre-Wog 5	15	2.57±0.04	15	2.63±0.04	10	2.71±0.03
Pre-Wog 10	30	2.63±0.02*	19	2.66±0.02*	10	2.66±0.04
Pre-Wog 20	10	2.54±0.02	9	2.61±0.02	7	2.63±0.02

N, number of rats; Pre-Wog 5, 10 and 20: pretreatment with wogonin, i.p., 5, 10 or 20 mg/kg 40 min prior to ischemia; VPC: ventricular premature contraction; VT: ventricular tachycardia; VF: ventricular fibrillation; Values are expressed as mean ± SEM; * $P < 0.05$ compared with the control group.

Table 3 The effect of wogonin on the incidence of ischemia-induced arrhythmias

Treatment	N	VPC	VT	Total VF	Sustained VF	Mortality
		Incidence (%)	Incidence (%)	Incidence (%)	Incidence (%)	(%)
Control	30	100.0	90.0	66.7	40.0	40.0 (12/30)
Pre-Wog 5	15	100.0	100.0	66.7	33.3	33.3 (5/15)
Pre-Wog 10	30	100.0	63.3*	33.3*	13.3*	13.3* (4/30)
Pre-Wog 20	10	100.0	90.0	70.0	50.0#	50.0# (5/10)

N, number of rats; Pre-Wog 5, 10 and 20: pretreatment with wogonin, i.p., 5, 10 or 20 mg/kg 40 min prior to ischemia; VPC: ventricular premature contraction; VT: ventricular tachycardia; VF: ventricular fibrillation; * $P < 0.05$ compared with the control group, # $P < 0.05$ compared with the Pre-Wog 10 group.

Table 4 Effects of wogonin on arrhythmias during 2 h-reperfusion period

		VPC		VT		VF	
Treatment	N	log_{10} VPC counts	incidence (%)	log_{10} duration	incidence (%)	log_{10} duration	incidence (%)
Control	12	1.96 ± 0.15	100 (12/12)	0.91 ± 0.22	25.0 (3/12)	0.51	8.3 (1/12)
Pre-Wog 10	20	1.58 ± 0.19	100 (20/20)	0.70 ± 0.08	15.0 (3/20)	--	--
Post-Wog 10	5	2.55 ± 0.12[#]	100 (5/5)	0.97	20.0 (1/5)	--	--

N, number of rats; Pre-Wog 10: pretreatment with wogonin, i.p., 10 mg/kg 40 min prior to ischemia; Post-Wog 10: treatment with wogonin 15 min after left coronary artery occlusion; VPC: ventricular premature contraction; VT: ventricular tachycardia; VF: ventricular fibrillation; Values are expressed as mean ± SEM; [#] $P < 0.05$ vs. Pre-Wog 10 group.

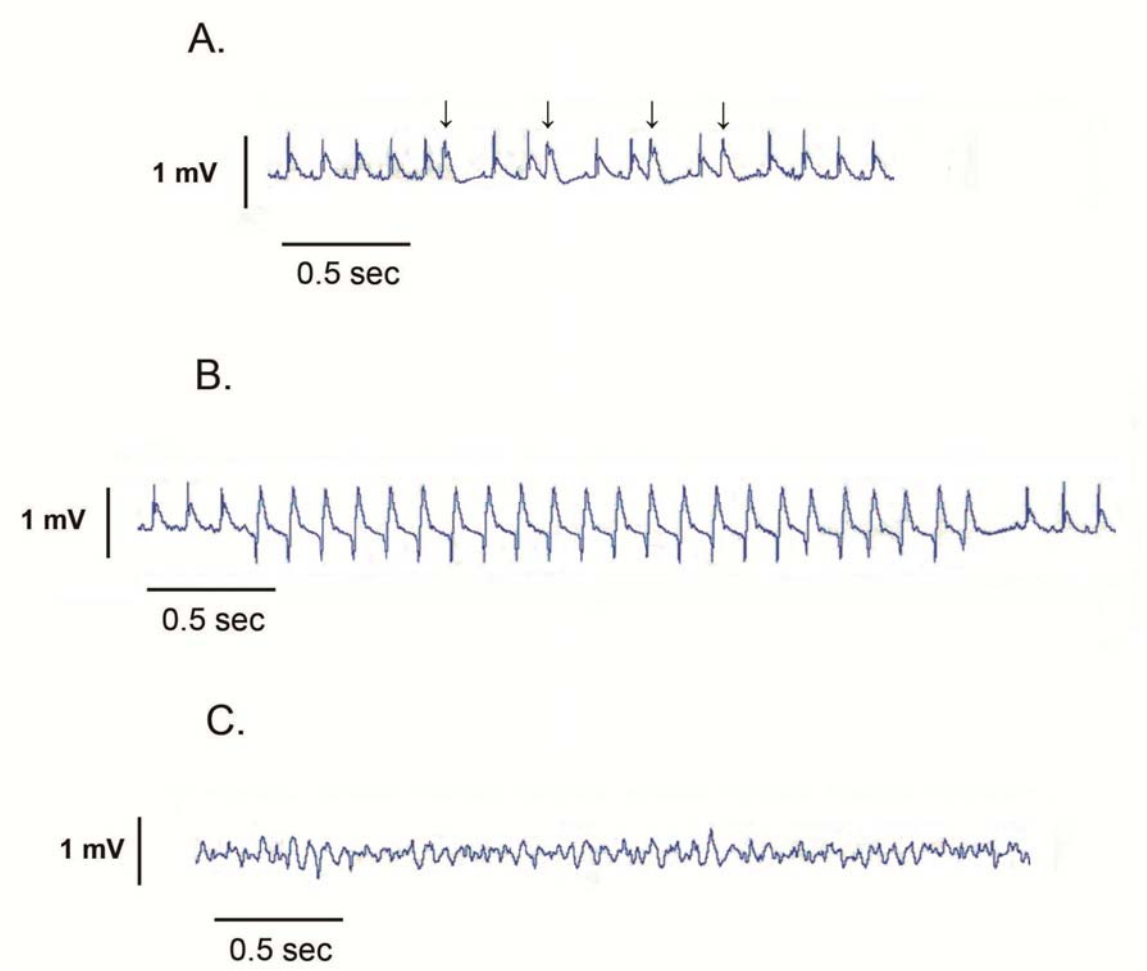


Figure 1 Characteristic electrocardiogram of an anesthetized rat with myocardial ischemia. (A) ventricular premature contractions (VPC); (B) a burst of ventricular tachycardia (VT); (C) ventricular fibrillation (VF).

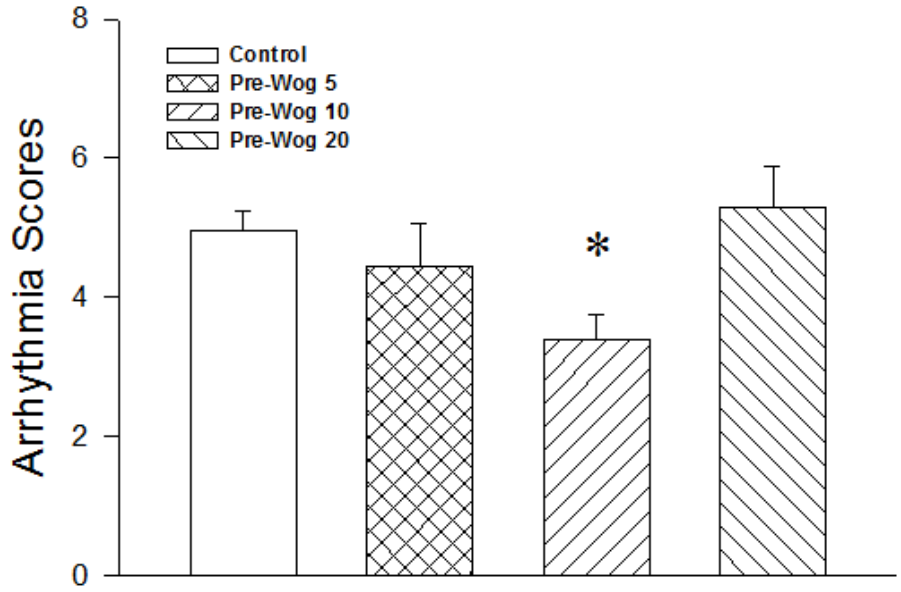


Figure 2 Effects of pretreatment with wogonin on arrhythmia scores during 30-min left coronary artery occlusion in anesthetized rats. Values are expressed as mean ± SEM. *$P < 0.05$ vs. the control, n = 10-30.

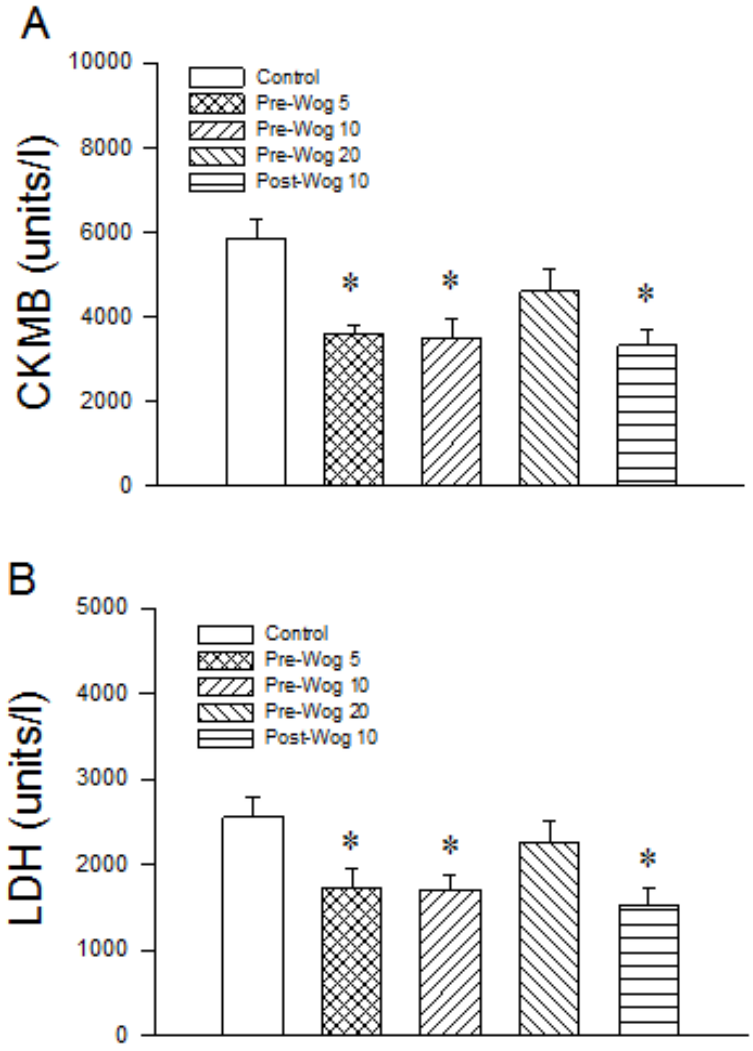


Figure 3 Effects of wogonin on plasma levels of creatine kinase-muscle-brain (CKMB) (A), and lactate dehydrogenase (LDH) (B) in rats with 45-min ischemia/1 h of reperfusion. Pre-Wog 5, 10 and 20: pretreatment with 5, 10 and 20 mg/kg wogonin 40 min prior to ischemia; Post-Wog 10: treatment with wogonin 10 mg/kg 15 min after ischemia. Data are given as mean ± SEM. * $P < 0.05$ vs. the control, n = 5-10.

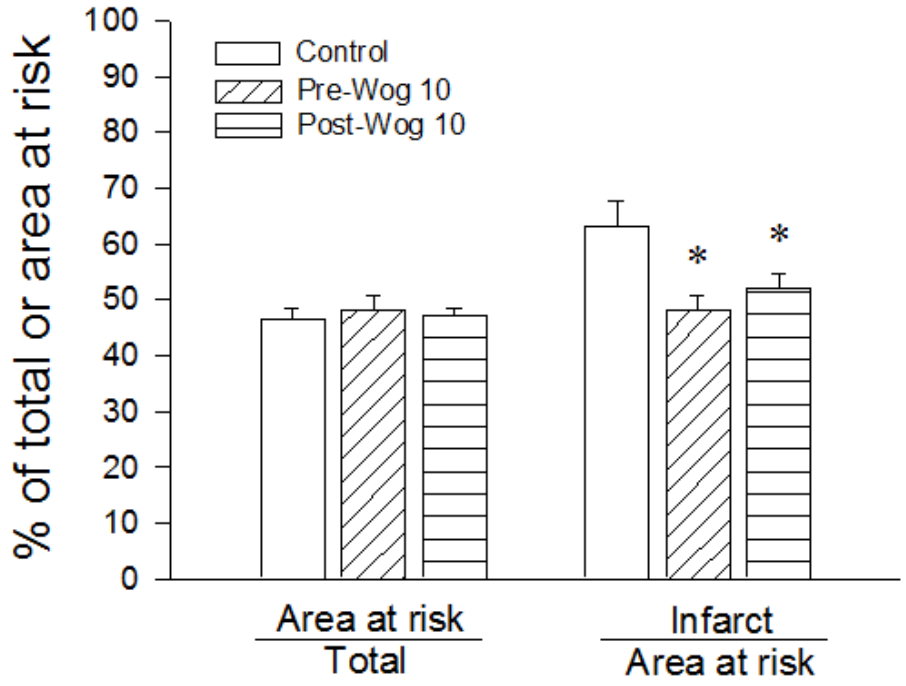
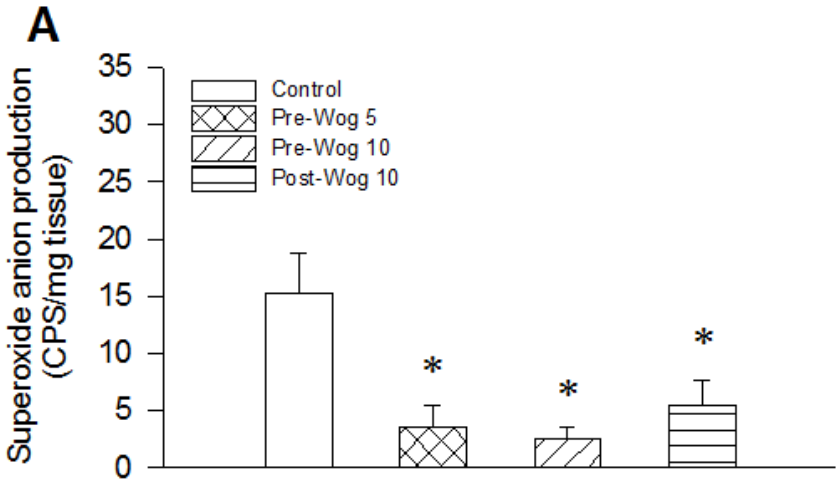


Figure 4 Effects of wogonin on infarct size in rats with 45-min ischemia/2 h of reperfusion. Pre-Wog 5, 10 and 20: pretreatment with 5, 10 and 20 mg/kg wogonin 40 min prior to ischemia; Post-Wog 10: treatment with wogonin 10 mg/kg 15 min after ischemia. Data are given as mean ± SEM. * $P < 0.05$ vs. the control, n = 5-10.



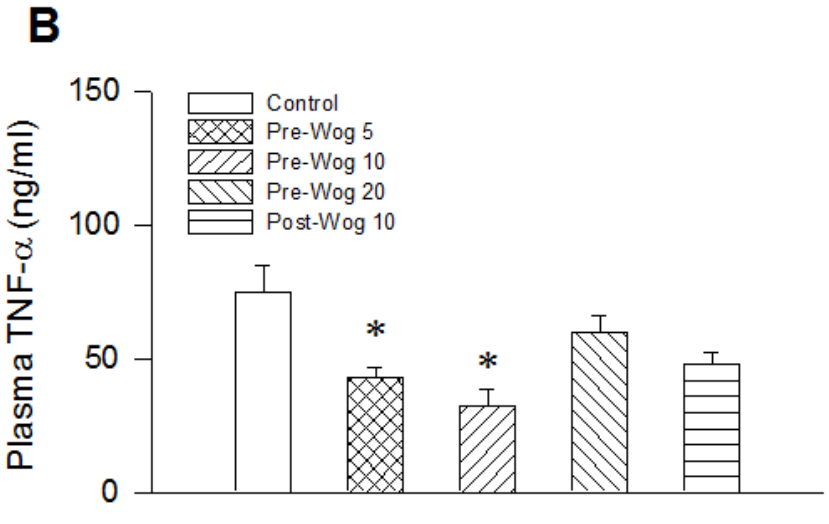


Figure 5 Effects of wogonin on levels of superoxide anion production (A) in ischemic myocardium of rats with 45-min myocardial ischemia followed by 30 min of reperfusion, and plasma tissue necrosis factor (TNF)-α (B) measured at 1 h after reperfusion. Pre-Wog 5, 10 and 20: pretreatment with wogonin 5, 10 and 20 mg/kg 40 min prior to ischemia; Post-Wog 10: treatment with wogonin 10 mg/kg 15 min after ischemia. CPS: counts per second; Data are given as mean ± SEM. * $P < 0.05$ versus the control, n = 5-6.

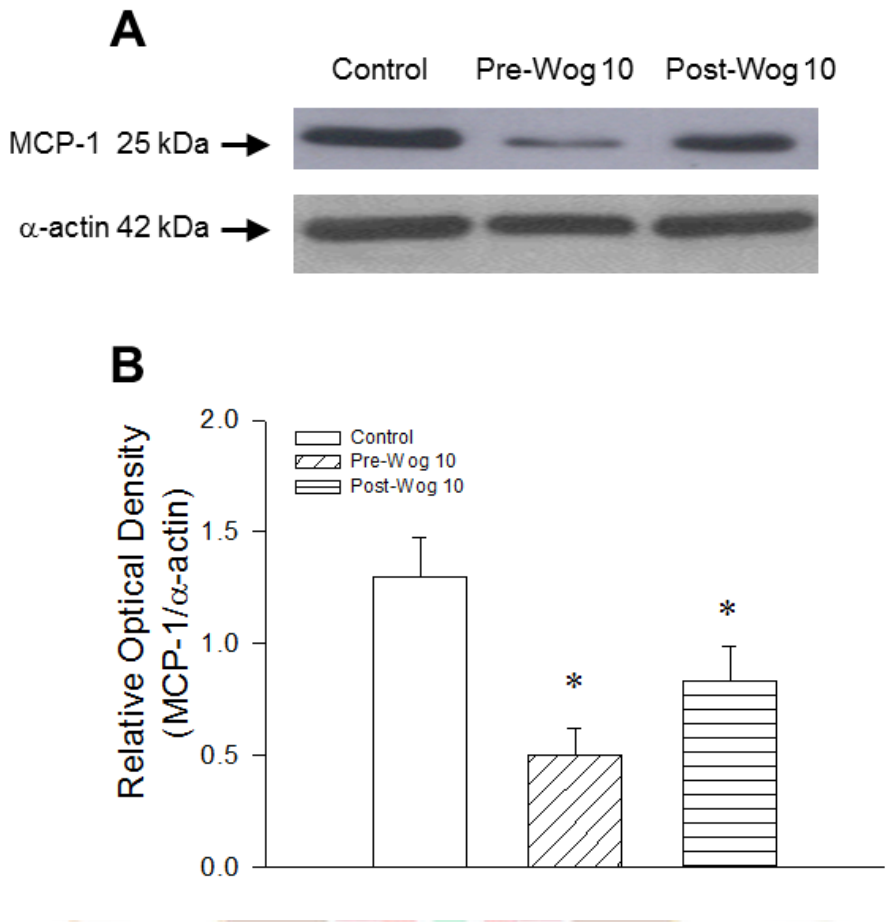


Figure 6 Effects of wogonin on monocyte chemoattractant protein-1 (MCP-1) protein expression in ischemic myocardium of rats with 45-min ischemia followed by 120 min of reperfusion. A: representative Western blots, B: mean MCP-1 Western blot densitometry relative to respective α-actin densitometry for each group. Pre-Wog 10 and Post-Wog 10 mean pre- and post-treatment with wogonin 10 mg/kg, respectively. Data are given as mean ± SEM. * $P < 0.05$ versus the control. n = 10 in the control and Pre-Wog 10 groups, n = 5 in the Post-Wog 10 group.

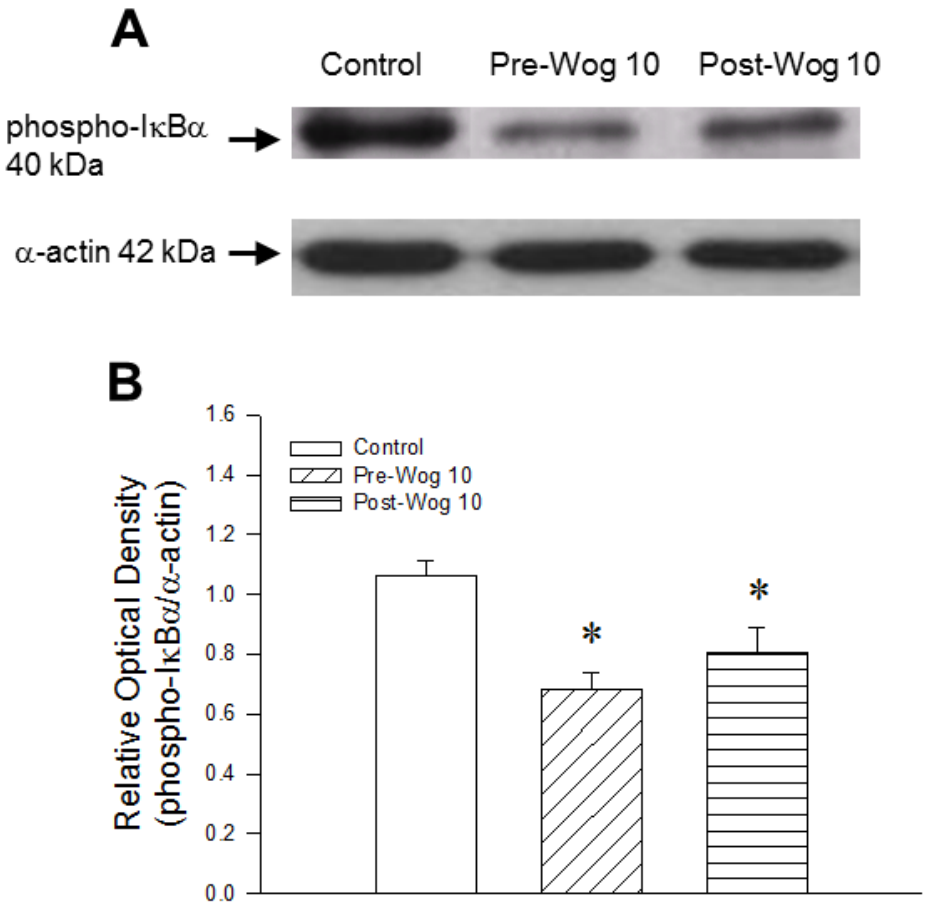


Figure 7 Effects of wogonin on phospho-IκBα protein expression in ischemic myocardium of rats with 45-min ischemia followed by 120 min of reperfusion. A: representative Western blots, B: mean phospho-IκBα Western blot densitometry relative to respective α-actin densitometry for each group. Pre-Wog 10 and Post-Wog 10 mean pre- and post-treatment with wogonin 10 mg/kg, respectively. Data are given as mean ± SEM. * $P < 0.05$ versus the control. n=10 in the control and Pre-Wog 10 groups, n=5 in the Post-Wog 10 group.

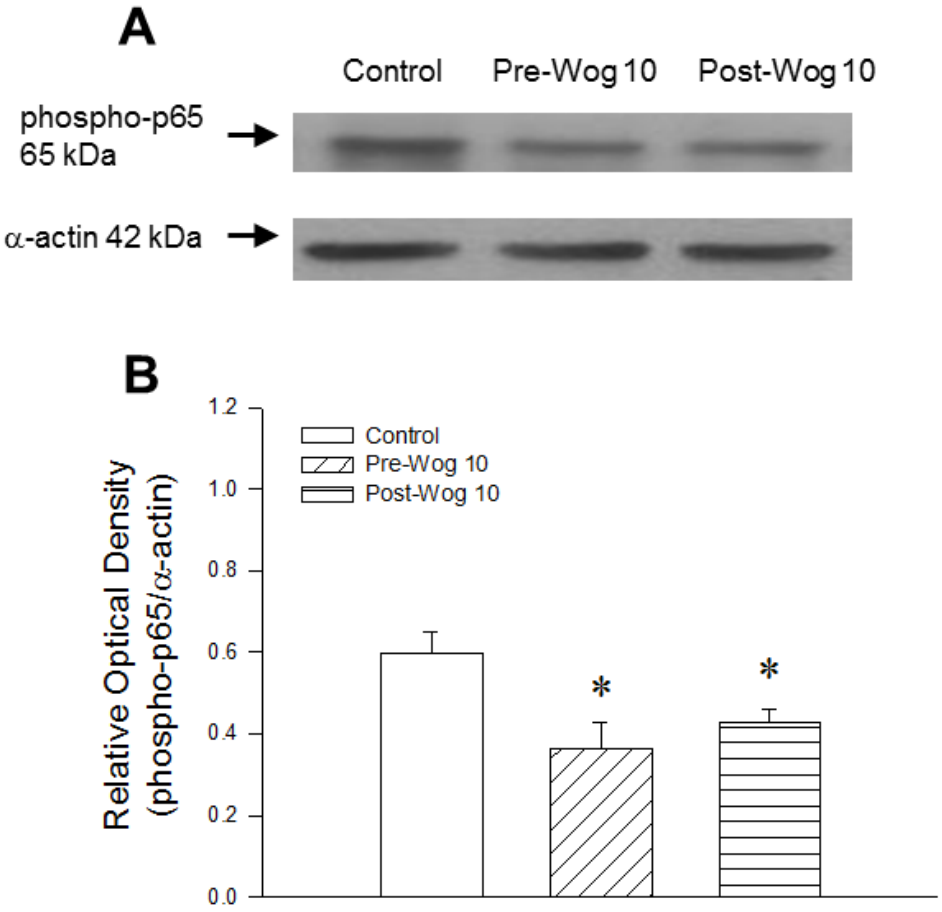


Figure 8 Effects of wogonin on phospho-p65 protein expression in ischemic myocardium of rats with 45-min ischemia followed by 120 min of reperfusion. A: representative Western blots, B: mean phospho-p65 Western blot densitometry relative to respective α-actin densitometry for each group. Pre-Wog 10 and Post-Wog 10 mean pre- and post-treatment with wogonin 10 mg/kg, respectively. Data are given as mean ± SEM. * $P < 0.05$ versus the control. n=10 in the control and Pre-Wog 10 groups, n=5 in the Post-Wog 10 group.

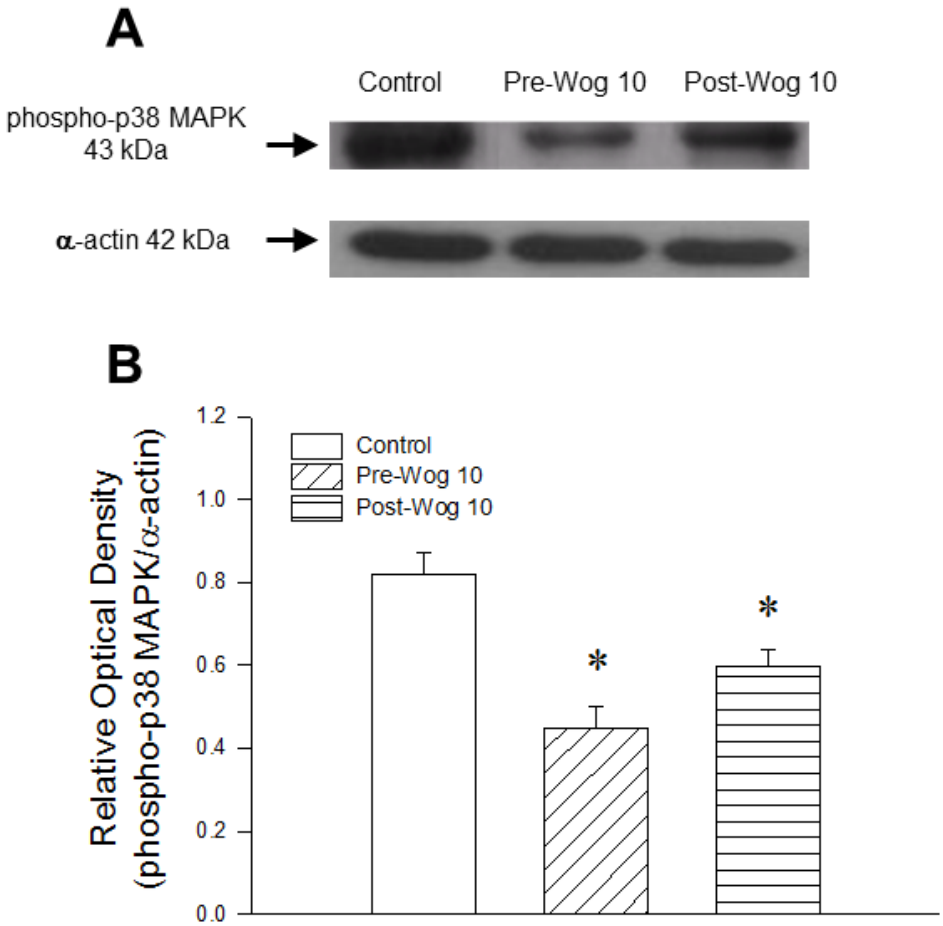


Figure 9 Effects of wogonin on phospho-p38 mitogen-activated protein kinase (p38 MAPK) protein expression in ischemic myocardium of rats with 45 min-ischemia followed by 120 min of reperfusion. A: representative Western blots, B: mean phospho-p38 MAPK Western blot densitometry relative to respective α-actin densitometry for each group. Pre-Wog 10 and Post-Wog 10 mean pre- and post-treatment with wogonin 10 mg/kg, respectively. Data are given as mean ± SEM. * $P < 0.05$ versus the control. n=10 in the control and Pre-Wog 10 groups, n = 5 in the Post-Wog 10 group.

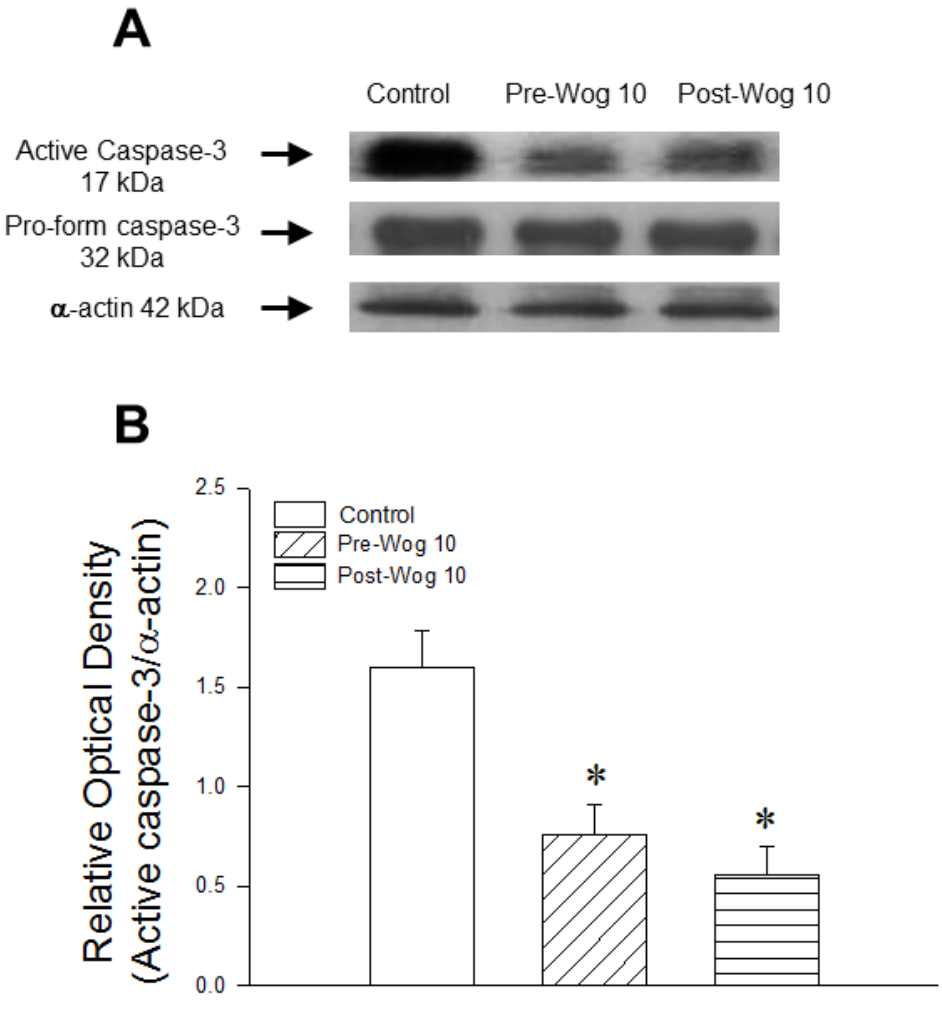


Figure 10 Effects of wogonin on active caspase-3 protein expression in ischemic myocardium of rats with 45-min ischemia followed by 120 min of reperfusion. A: representative Western blots are shown, B: mean active caspase-3 Western blot densitometry relative to respective α-actin densitometry for each group. Pre-Wog 10 and Post-Wog 10 mean pre- and post-treatment with wogonin 10 mg/kg, respectively. Data are given as mean ± SEM. * $P < 0.05$ versus the control. n=10 in the control and Pre-Wog 10 groups, n=5 in the Post-Wog 10 group.

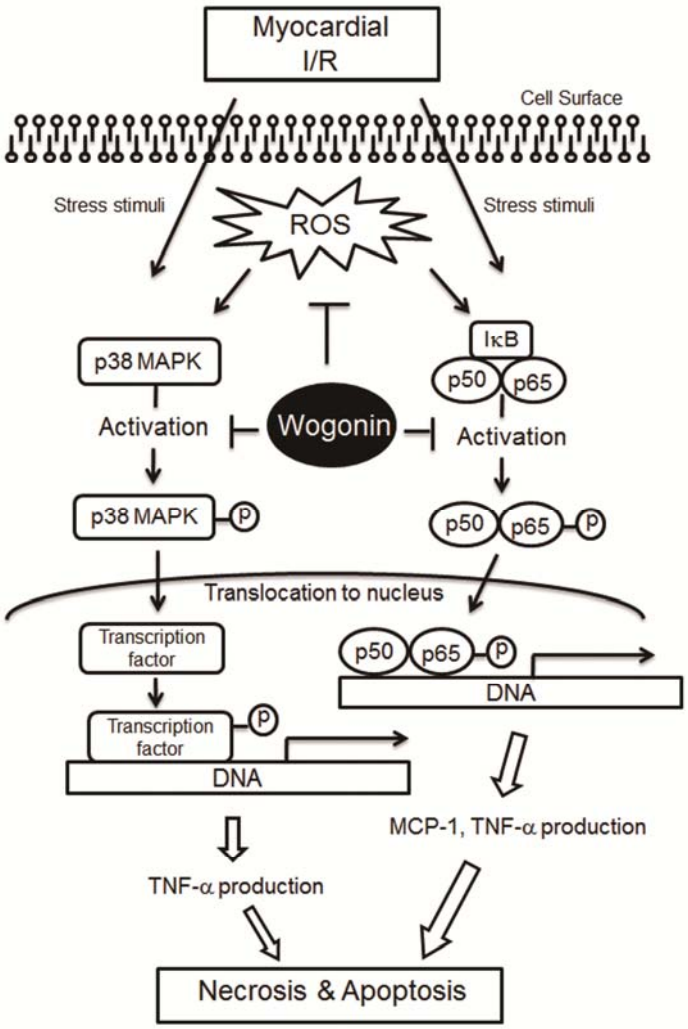


Figure 11 Schematic diagram of the possible mechanisms responsible for the effectiveness of wogonin in myocardial ischemia/reperfusion injury. It is hypothesized that wogonin suppresses: (i) oxidative stress, and (ii) activation of p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor-κB signaling pathways, leading to reduction in monocyte chemoattractant protein-1 (MCP-1), tissue necrosis factor-α (TNF-α), apoptosis, and necrosis in cardiomyocytes.

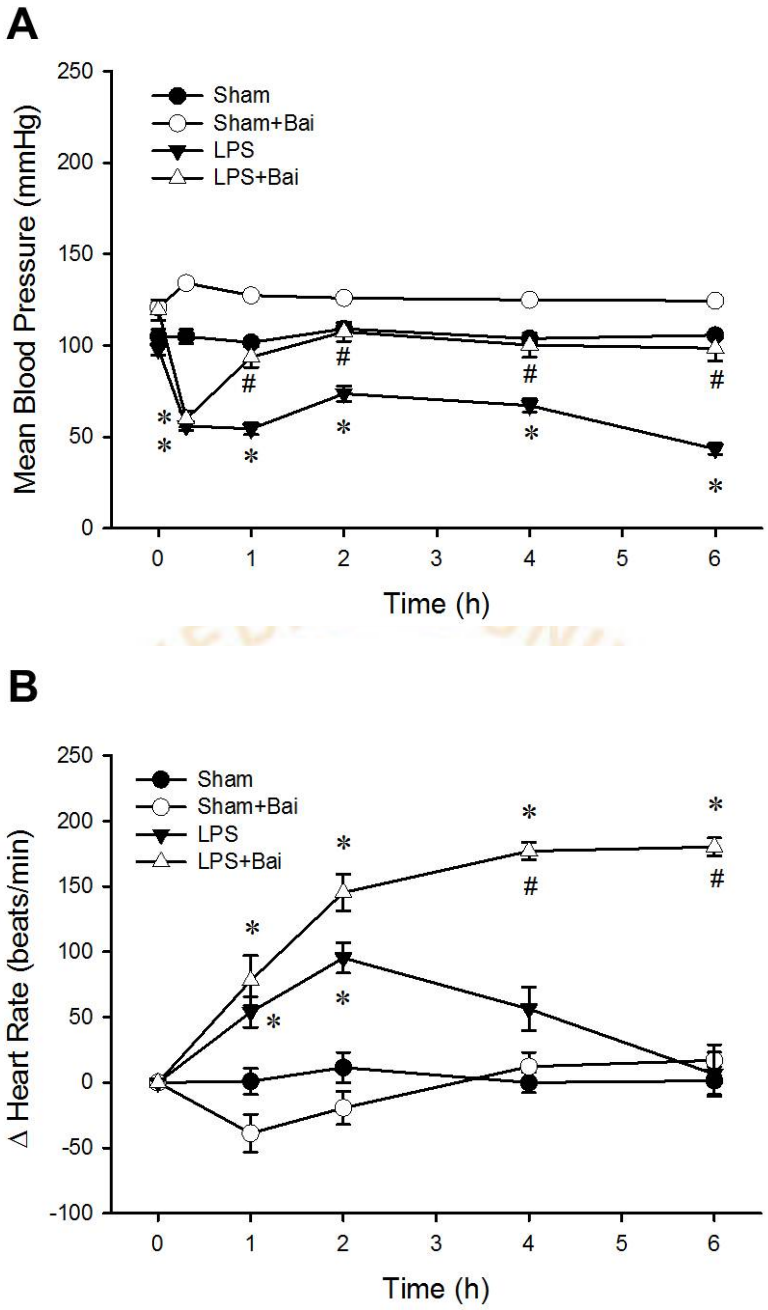


Figure 12 Effects of post-treatment with baicalein on mean arterial blood pressure (A) and changes in heart rate (B) in conscious rats with sepsis induced by LPS injection. Baicalein (Bai) 10 mg/kg was given 30 min after LPS 10 mg/kg injection. Values are expressed as mean ± SEM. **P* < 0.05 vs. the sham group; $^{\#}P$ < 0.05 vs. the LPS group, n = 6.

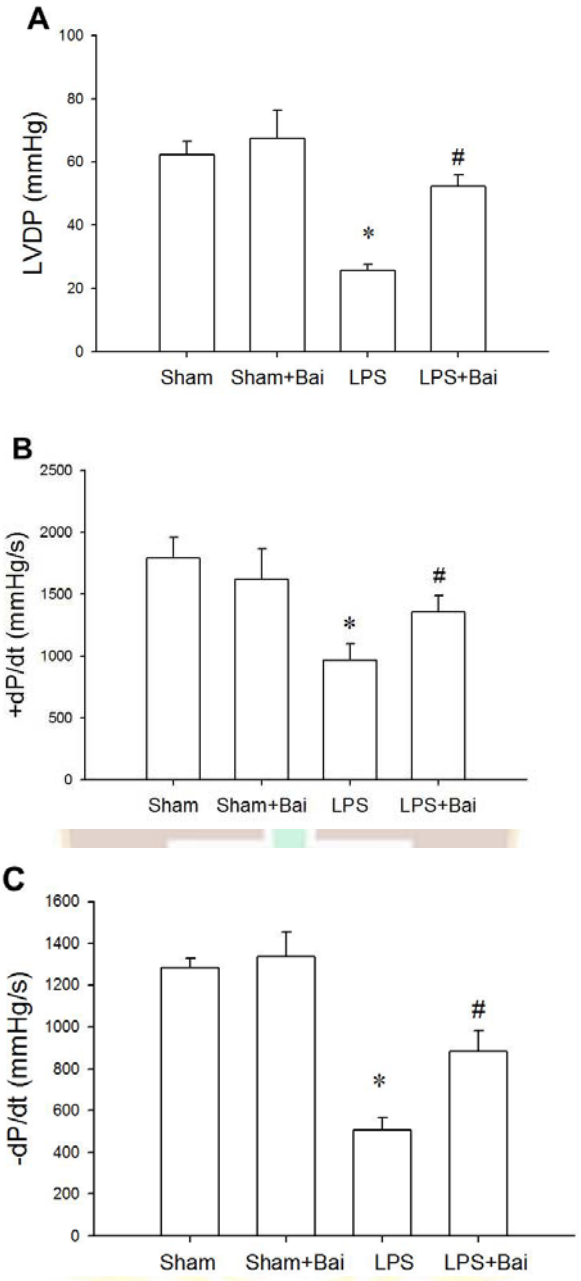


Figure 13 Effects of post-treatment with baicalein on cardiac contractile dysfunction caused by LPS. A: left ventricular developed pressure (LVDP); B and C: +dP/dt and –dP/dt in hearts 6 h after being subjected to LPS administration. Baicalein (Bai) 10 mg/kg was given 30 min after LPS 10 mg/kg injection. Data are given as mean ± SEM. * $P < 0.05$ vs. the sham group, $^{\#}P < 0.05$ vs. the LPS group, n = 6.

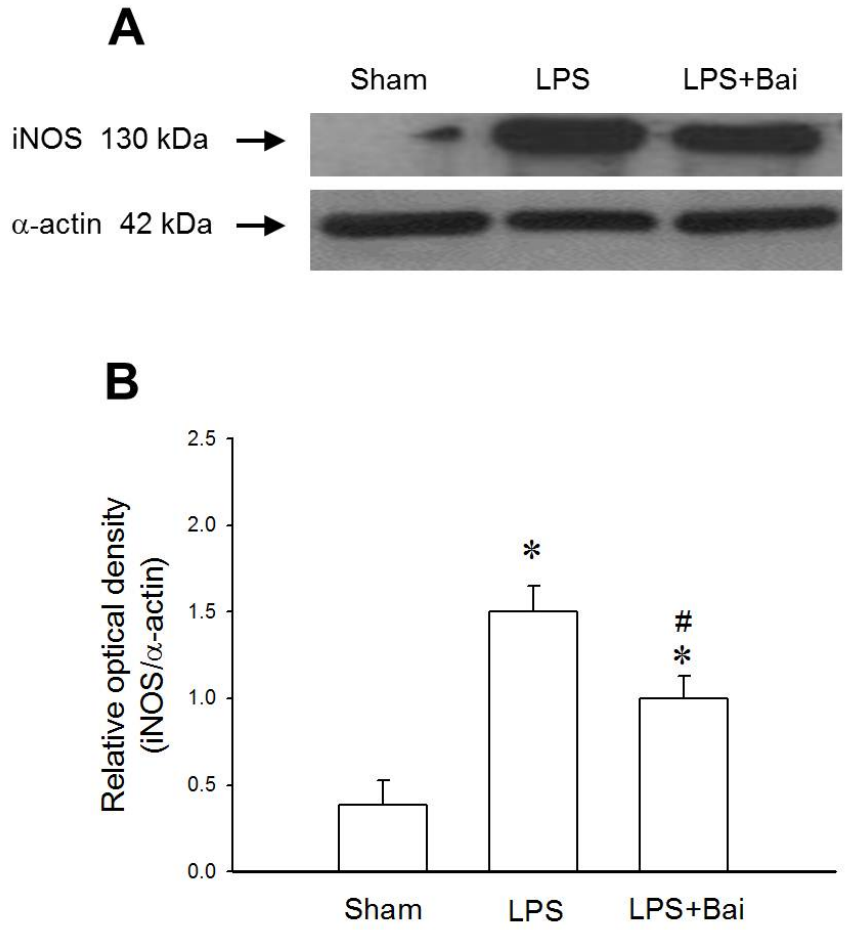


Figure 14 Effects of post-treatment with baicalein on iNOS protein expression in left ventricular myocardium of rats 6 h after being subjected to LPS administration. A: representative Western blots, B: B: mean iNOS Western blot densitometry relative to respective α-actin densitometry for each group. Baicalein (Bai) 10 mg/kg was given 30 min after LPS 10 mg/kg injection. Data are given as mean ± SEM. * $P < 0.05$ vs. the sham group, $^{\#}P < 0.05$ vs. the LPS group, n = 6.

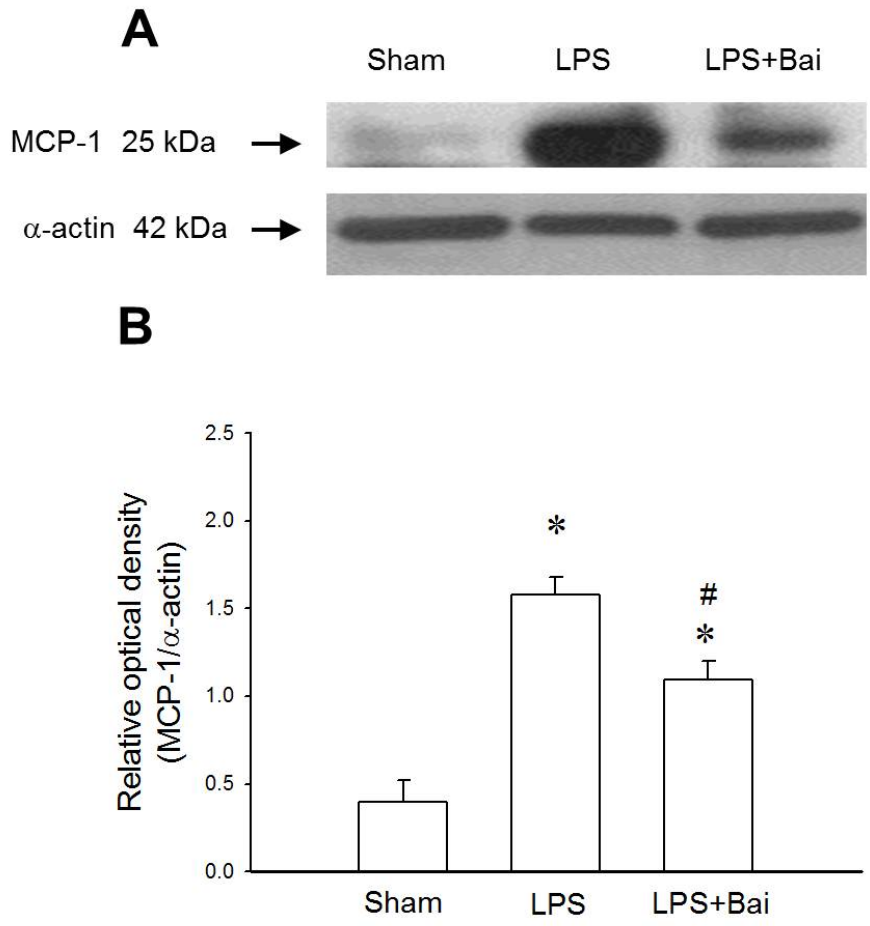


Figure 15 Effects of post-treatment with baicalein on MCP-1 protein expression in left ventricular myocardium of rats 6 h after being subjected to LPS administration. A: representative Western blots, B: mean MCP-1 Western blot densitometry relative to respective α-actin densitometry for each group. Baicalein (Bai) 10 mg/kg was given 30 min after LPS 10 mg/kg injection. Data are given as mean ± SEM. * $P < 0.05$ vs. the sham group, $^{\#}P < 0.05$ vs. the LPS group, n = 6.

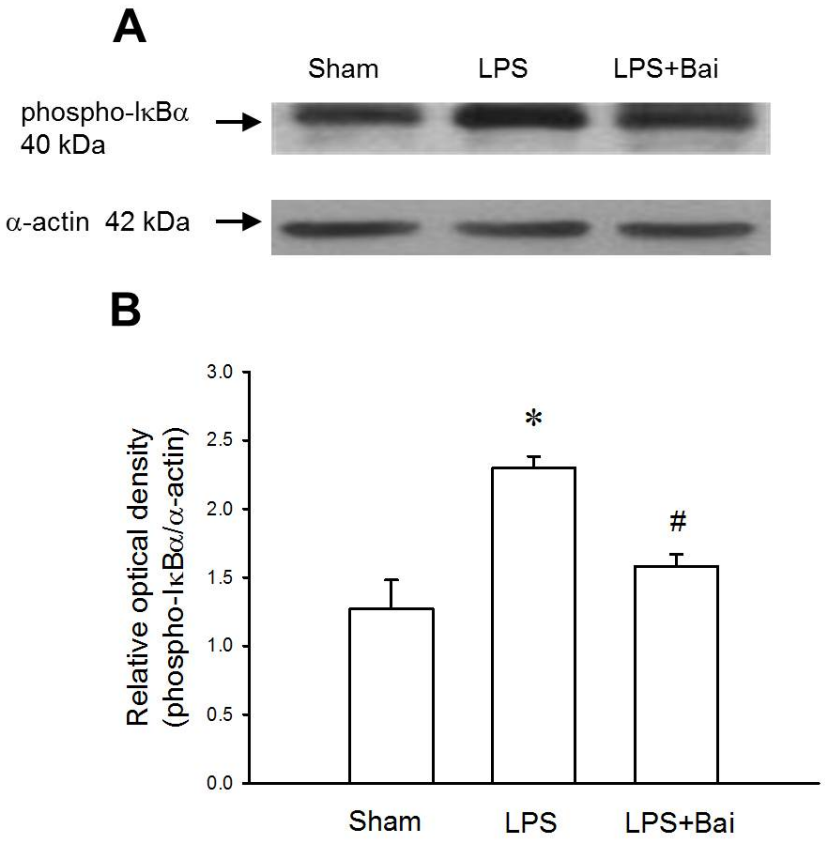


Figure 16 Effects of post-treatment with baicalein on phospho-IκBα protein expression in left ventricular myocardium of rats 6 h after being subjected to LPS administration. A: representative Western blots, B: mean phospho-IκBα Western blot densitometry relative to respective α-actin densitometry for each group. Baicalein (Bai) 10 mg/kg was given 30 min after LPS 10 mg/kg injection. Data are given as mean ± SEM. * $P < 0.05$ vs. the sham group, $^{\#}P < 0.05$ vs. the LPS group, n = 6.

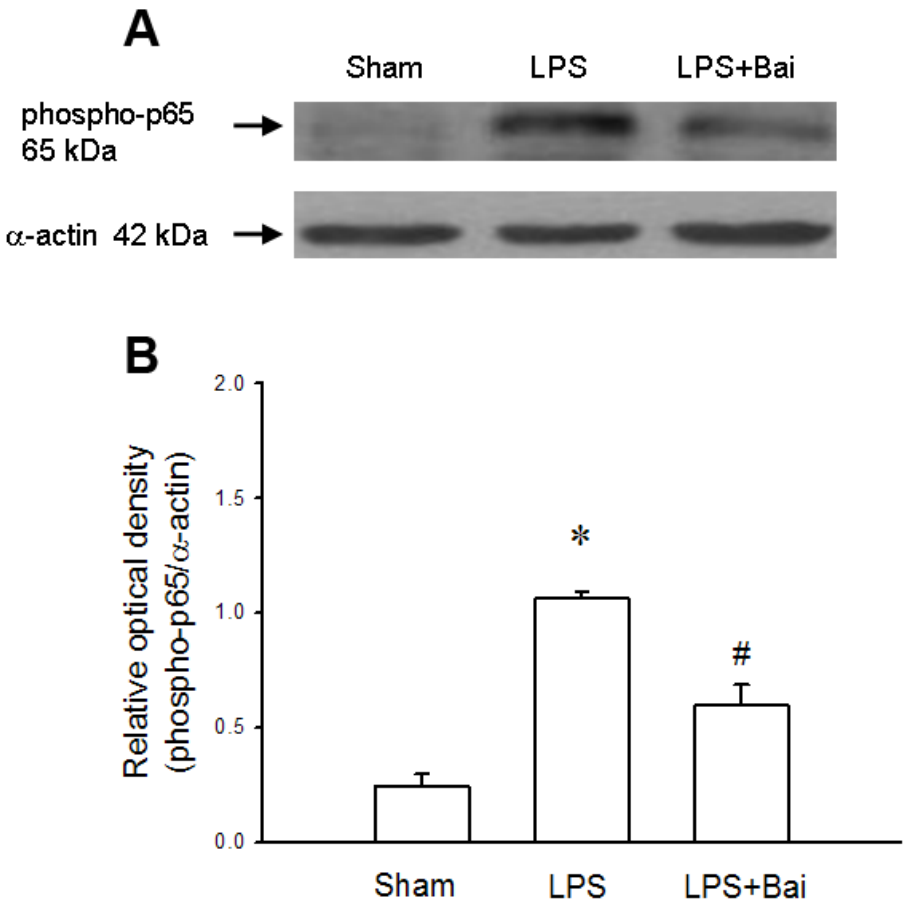


Figure 17 Effects of post-treatment with baicalein on phospho-p65 protein expression in left ventricular myocardium of rats 6 h after being subjected to LPS administration. A: representative Western blots, B: mean phospho-p65 Western blot densitometry relative to respective α-actin densitometry for each group. Baicalein (Bai) 10 mg/kg was given 30 min after LPS 10 mg/kg injection. Data are given as mean ± SEM. * $P < 0.05$ vs. the sham group, $^{\#}P < 0.05$ vs. the LPS group, n = 6.

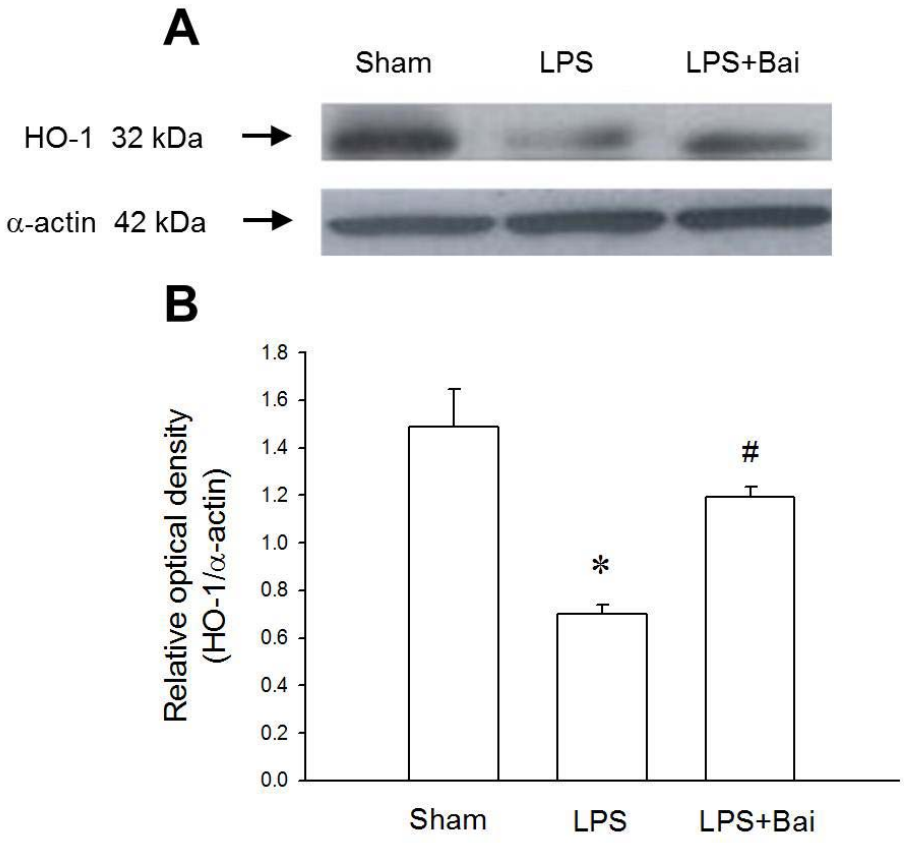


Figure 18 Effects of post-treatment with baicalein on heme oxygenase-1 (HO-1) protein expression in left ventricular myocardium of rats 6 h after being subjected to LPS administration. A: representative Western blots, B: mean HO-1 Western blot densitometry relative to respective α-actin densitometry for each group. Baicalein (Bai) 10 mg/kg was given 30 min after LPS 10 mg/kg injection. Data are given as mean ± SEM. * $P < 0.05$ vs. the sham group, $^{\#}P < 0.05$ vs. the LPS group, n = 6.

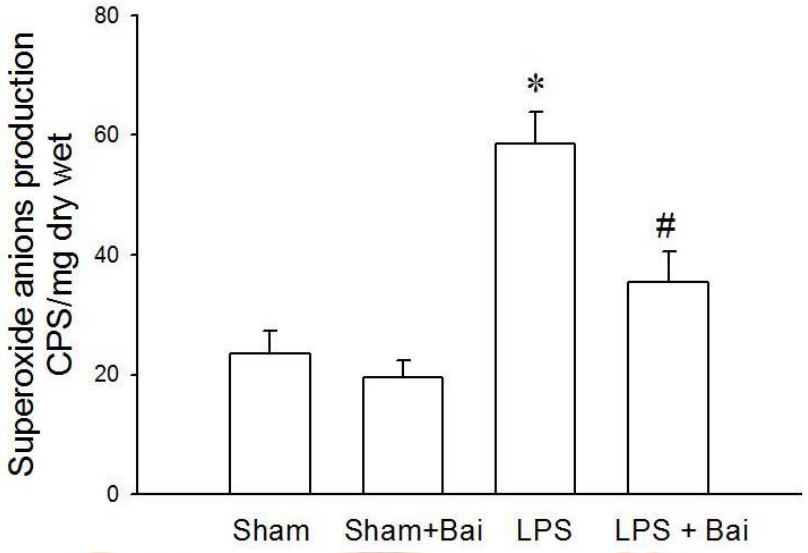


Figure 19 Effects of post-treatment with baicalein on superoxide anions production in left ventricular myocardium of rats 6 h after being subjected to LPS administration. Baicalein (Bai) 10 mg/kg was given 30 min after LPS 10 mg/kg injection. Data are given as mean ± SEM. CPS: counts per second, * $P < 0.05$ vs. the sham group, $^{\#}P < 0.05$ vs. the LPS group, n = 6.

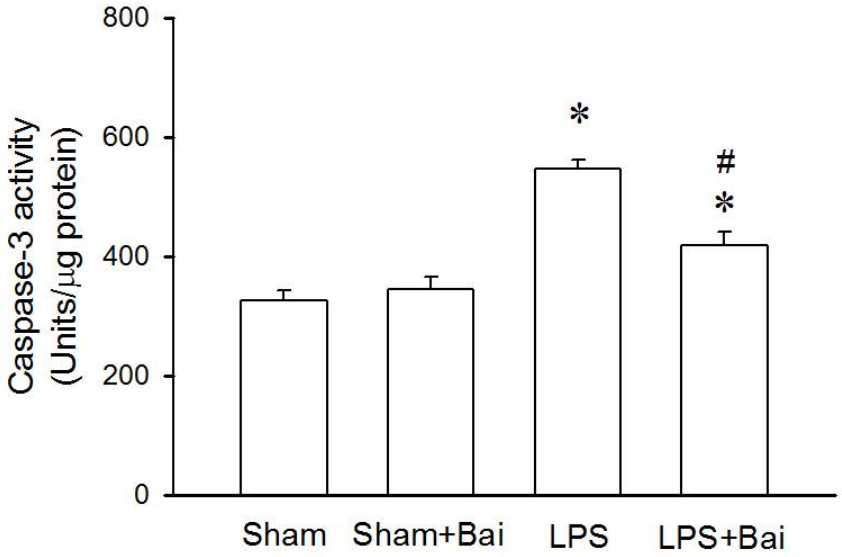


Figure 20 The effect of post-treatment with baicalein on caspase-3 activity in left ventricular myocardium of rats 6 h after being subjected to LPS administration. Baicalein (Bai) 10 mg/kg was given 30 min after LPS 10 mg/kg injection. Data are given as mean ± SEM. * $P < 0.05$ vs. the sham group, $^{\#}P < 0.05$ vs. the LPS group, n = 6.

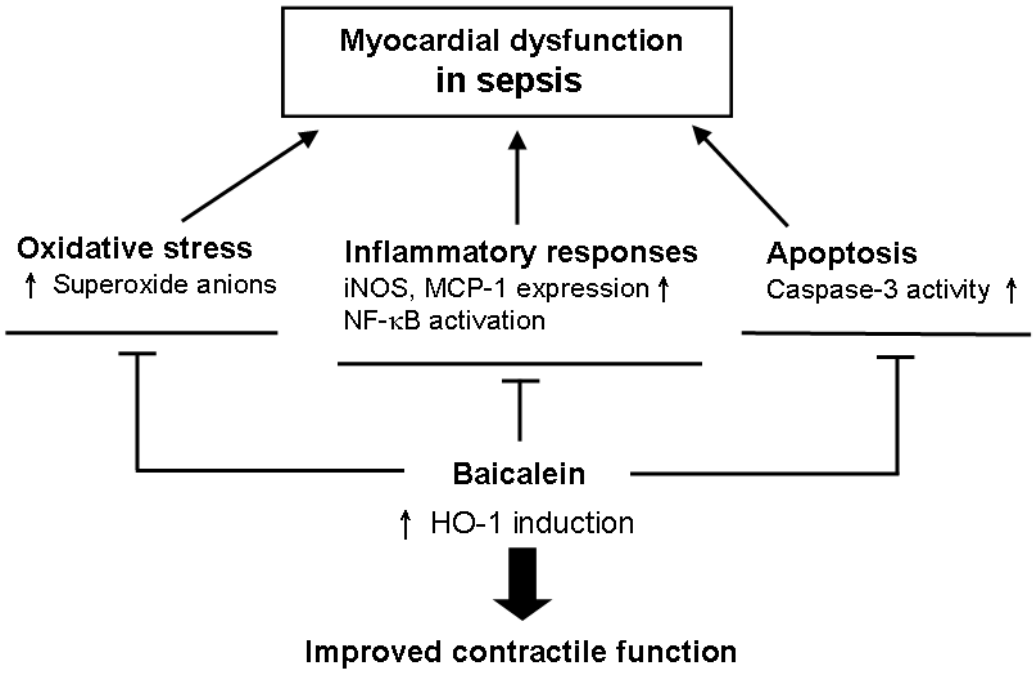


Figure 21 Schematic diagram of the possible mechanisms responsible for the protective effect of baicalein on myocardial dysfunction induced by sepsis. It is hypothesized that baicalein induces HO-1 production and suppresses: (i) oxidative stress, and (ii) nuclear factor-κB signaling pathways, leading to attenuation of inflammatory respnses and apoptosis.

Chapter 8 Appendix

Table 1 Definition of diseases

Systemic inflammatory response syndrome	Two or more of the following: ● Body temperature >38·5°C or <35·0°C ● Heart rate >90 beats per minute ● Respiratory rate >20 breaths per minute or arterial CO_2 tension <32 mm Hg or need for mechanical ventilation ● White blood cell count >12 000/mm³ or <4000/mm³ or immature forms >10%
Sepsis	Systemic inflammatory response syndrome and documented infection (culture or gram stain of blood, sputum, urine, or normally sterile body fluid positive for pathogenic microorganism; or focus of infection identified by visual inspection—eg, ruptured bowel with free air or bowel contents found in abdomen at surgery, wound with purulent discharge)
Severe sepsis	Sepsis and at least one sign of organ hypoperfusion or organ dysfunction: ● Areas of mottled skin ● Capillary refilling time ⩾3 s ● Urinary output <0·5 mL/kg for at least 1 h or renal replacement therapy ● Lactates >2 mmol/L ● Abrupt change in mental status or abnormal electroencephalogram ● Platelet counts <100 000/mL or disseminated intravascular coagulation ● Acute lung injury—acute respiratory distress syndrome ● Cardiac dysfunction (echocardiography)
Septic shock	Severe sepsis and one of: ● Systemic mean blood pressure <60 mm Hg (<80 mm Hg if previous hypertension) after 20–30 mL/kg starch or 40–60 mL/kg serum saline, or pulmonary capillary wedge pressure between 12 and 20 mm Hg ● Need for dopamine >5 µg/kg per min or norepinephrine or epinephrine <0·25 µg/kg per min to maintain mean blood pressure above 60 mm Hg (80 mm Hg if previous hypertension)
Refractory septic shock	Need for dopamine >15 µg/kg per min or norepinephrine or epinephrine >0·25 µg/kg per min to maintain mean blood pressure above 60 mm Hg (80 mm Hg if previous hypertension)

(Annane et al., Lancet 2005;365:63-78)

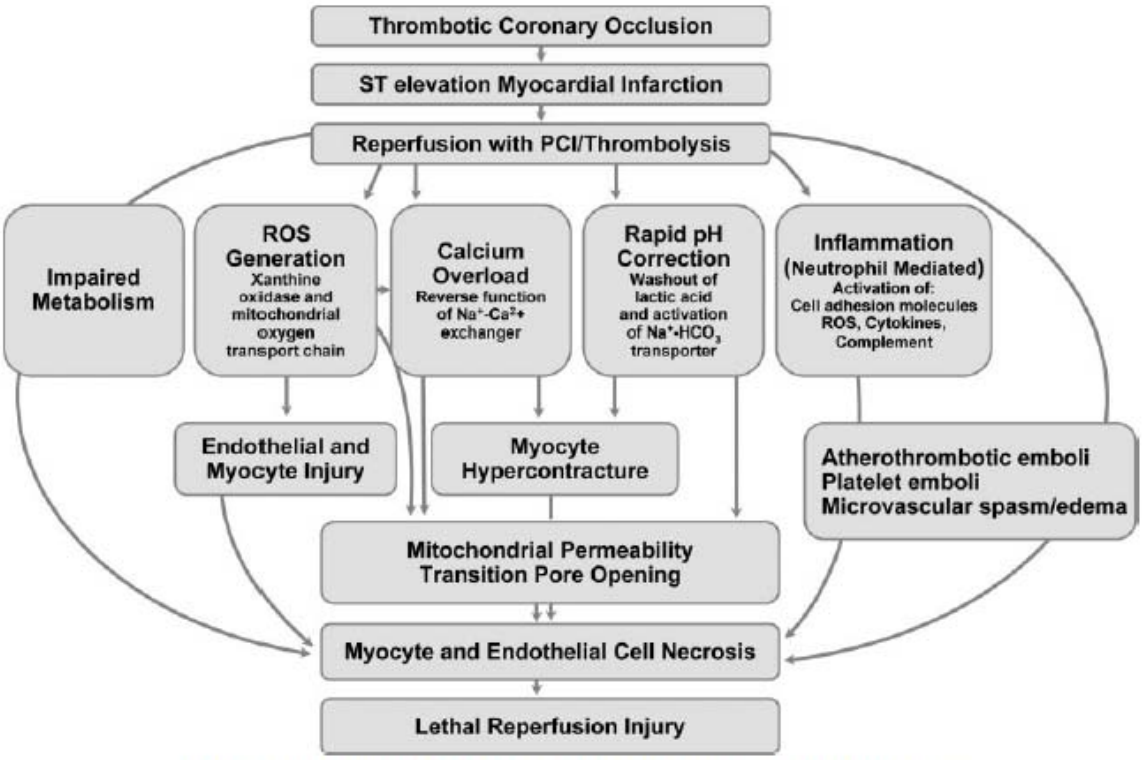


Figure 1. Pathophysiology of lethal RI. ROS indicates reactive oxygen species.

Figure 1 Pathophysiology of lethal reperfusion injury.

(Prasad et al., Circulation 2009;120;2105-2112)

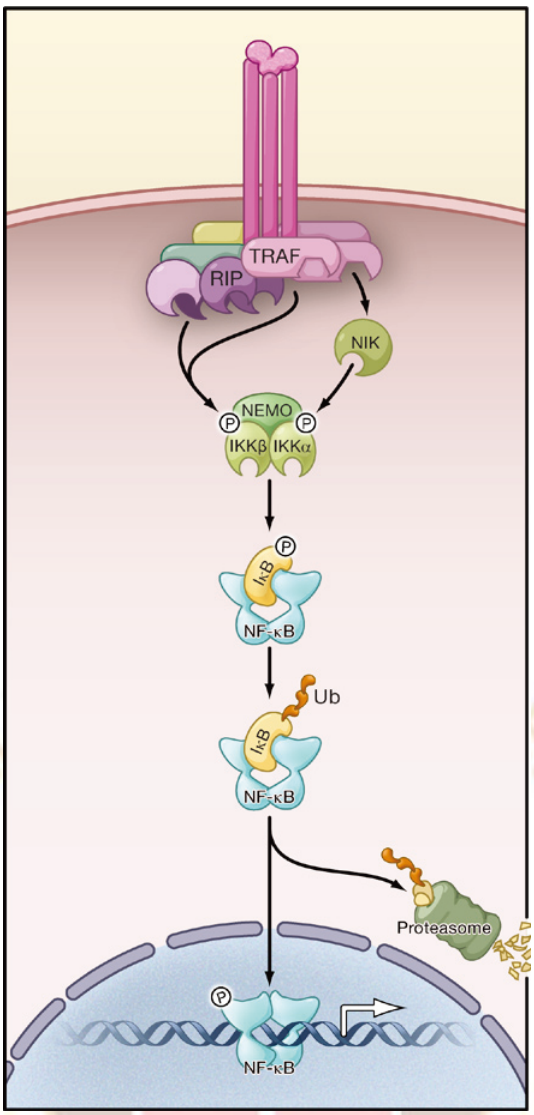


Figure 2 NF-κB Signaling Pathways Following receptor ligation and recruitment of receptor proximal adaptor proteins, signaling to IKK proceeds through TRAF/RIP complexes, generally in conjunction with TAK1, leading to canonical NF-κB signaling, or through TRAFs and NIK leading to the noncanonical NF-κB pathway. IKK activation results in IκB phosphorylation and degradation in the canonical pathway or p100 processing to p52 in the noncanonical pathway. Phosphorylated NF-κB dimers bind to κB DNA elements and induce transcription of target genes.

(Hayden & Ghosh, Cell 2008;132:344-362)

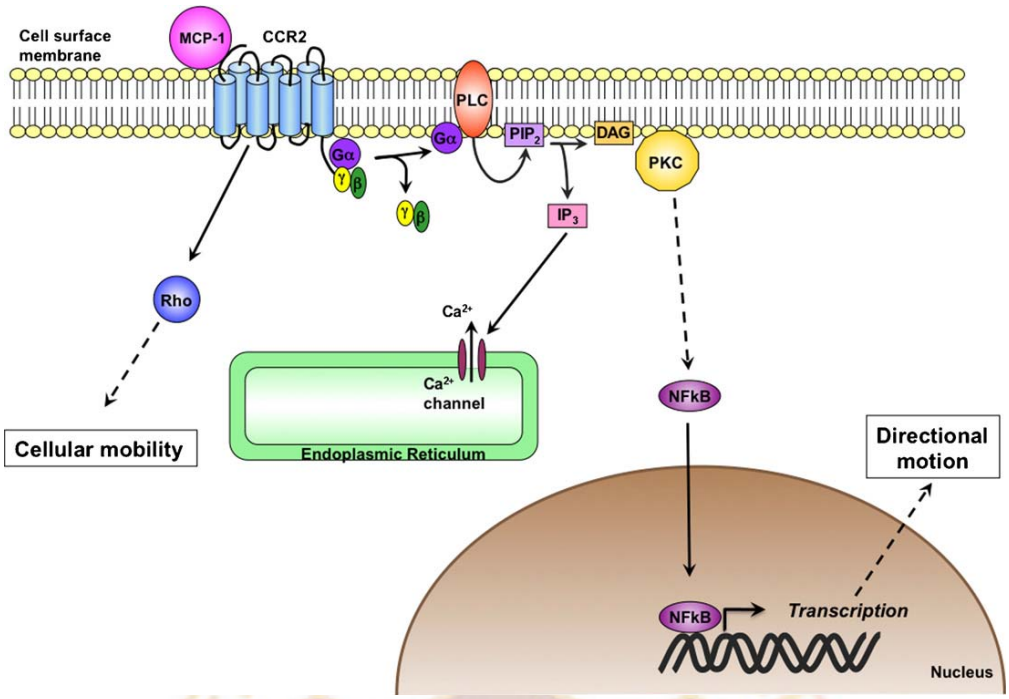


Figure 3 Overviewof the signaling pathway activated upon MCP-1 ligation to CCR2. Activation of the G protein induces the PLC-IP3 pathway that produces intracellular calcium release. In addition to inducing IP3, PLC causes the activation of PKC that activates PKC-dependent NF-κB. NF-κB upregulates several genes that produce directional cell motion. Rho is also activated, which results in induction of cell mobility. Abbreviations: DAG, diacylglycerol; IP3, inositol trisphosphate; NF-κB, nuclear factor-kappa B; PIP2, phosphatidylinositol-bisphosphate; PKC, protein kinase C; PLC, phospholipase C.

(Melgarejo et al., Int J Biochem Cell Biol 2009;41:998-1001)

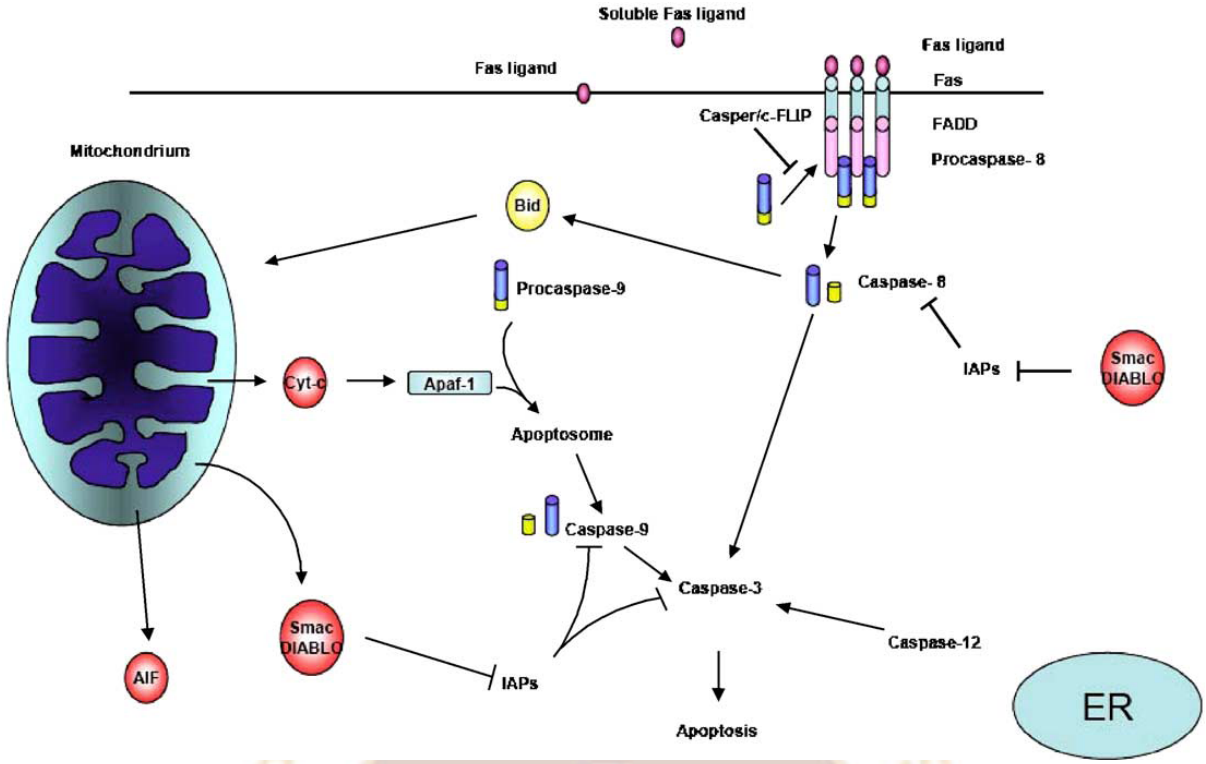


Figure 4 Pathways of apoptosis.

(van Empel et al., Cardiovasc Res 2005;67:21-29)

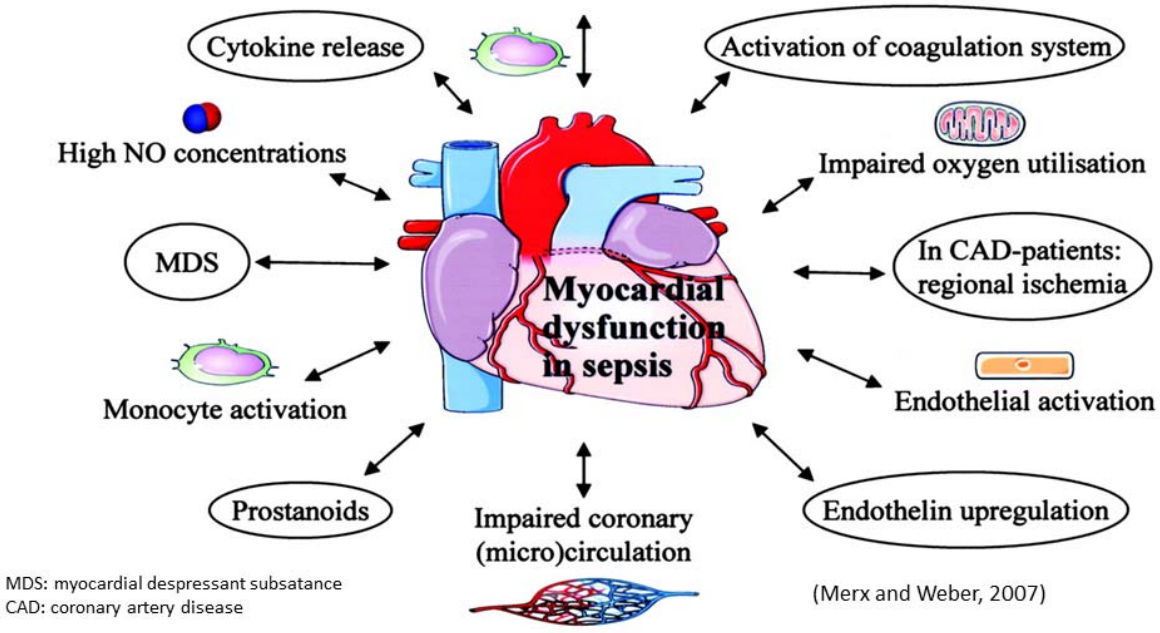


Figure 5 Synopsis of potential underlying mechanisms in septic myocardial dysfunction. MDS indicates myocardial depressant substance.

(Merx & Weber, Circulation 2007;116: 793-802.)

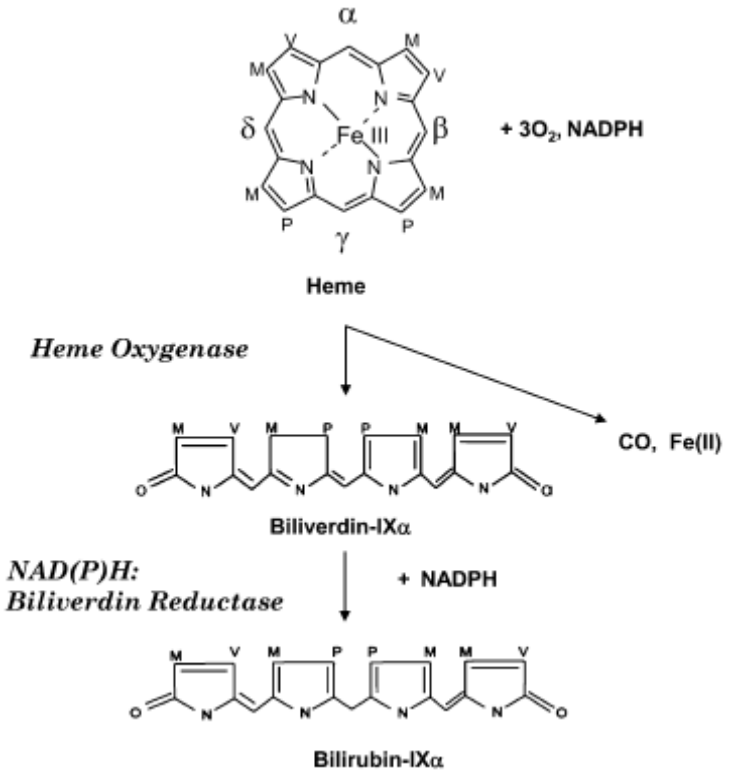


Figure 6 Heme oxygenase (HO) reaction. The HO reaction catalyzes the oxidative degradation of the heme molecule, to generate biliverdin, CO, and ferrous iron. The HO reaction proceeds through three serial monooxygenation cycles in which three molecules of O2 are consumed per heme molecule oxidized. NADPH cytochrome p450 reductase provides electrons for the reduction of the heme iron. The biliverdin released from the HO reaction, which is specific for the a isomer, is enzymatically reduced by NAD(P)H: biliverdin reductase, to form bilirubin.

(Ryter & Choi, Am J Respir Cell Mol Biol 2009;41:251-260)

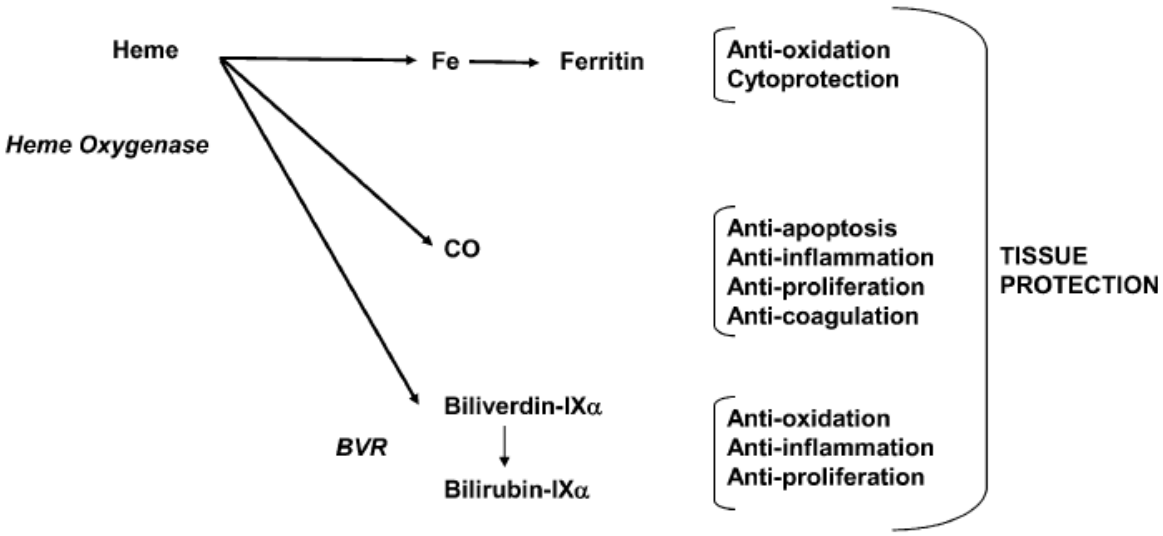


Figure 7 Multimodal effects of HO end-products on tissue protection. The three end-products of HO activity can contribute to cytoprotective mechanisms. CO has been implicated in anti-inflammatory, anti-apoptotic, and anti-proliferative pathways. Biliverdin-IXa and bilirubin-IXa, potent antioxidants, can exert anti-inflammatory and anti-proliferative effects. Iron released from HO activity stimulates a cytoprotective pathway involving the synthesis of ferritin.
(Ryter & Choi, Am J Respir Cell Mol Biol 2009;41:251-260.)

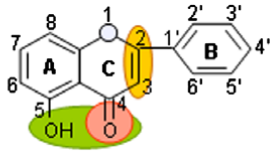
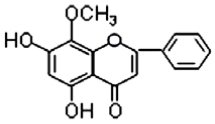
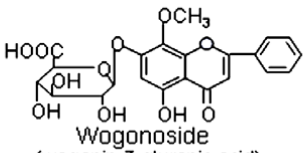
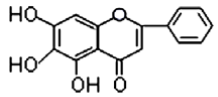
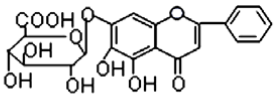
Plant	Name and Chemical Structure	MW	Ratio In raw material	Ref.
 *(Scutellaria baicalensis* Georgi) 	 Wogonin (5,7-dihydroxy-8-methoxyflavone)	284.27	1.3 %	40
	 Wogonoside (wogonin-7-gluronic acid)	460.33	3.55 %	
	 Beicalein (5,6,7-trihydroxyflavone)	270.3	5.41 %	
	 Beicalin (7-glucuronic acid, 5,6-dihydroxy-flavone)	446.36	10.11 %	

Figure 8 Structures of Wogonin, Baicalein and Baicalin derived from S. baicalensis Georgi. 2,3-unsaturation (shaded yellow) in conjugation with a 4-oxo group (shaded red) in the C-ring and 5-hydroxy group in A-ring have been documented to be the structural features important in defining the classical antioxidant potential of flavonoids and in the ability of flavonoids to chelate redox-active metal ions such as copper and iron (shaded green).

(Li-Weber, Cancer Treat Rev 2009;35:57-68)

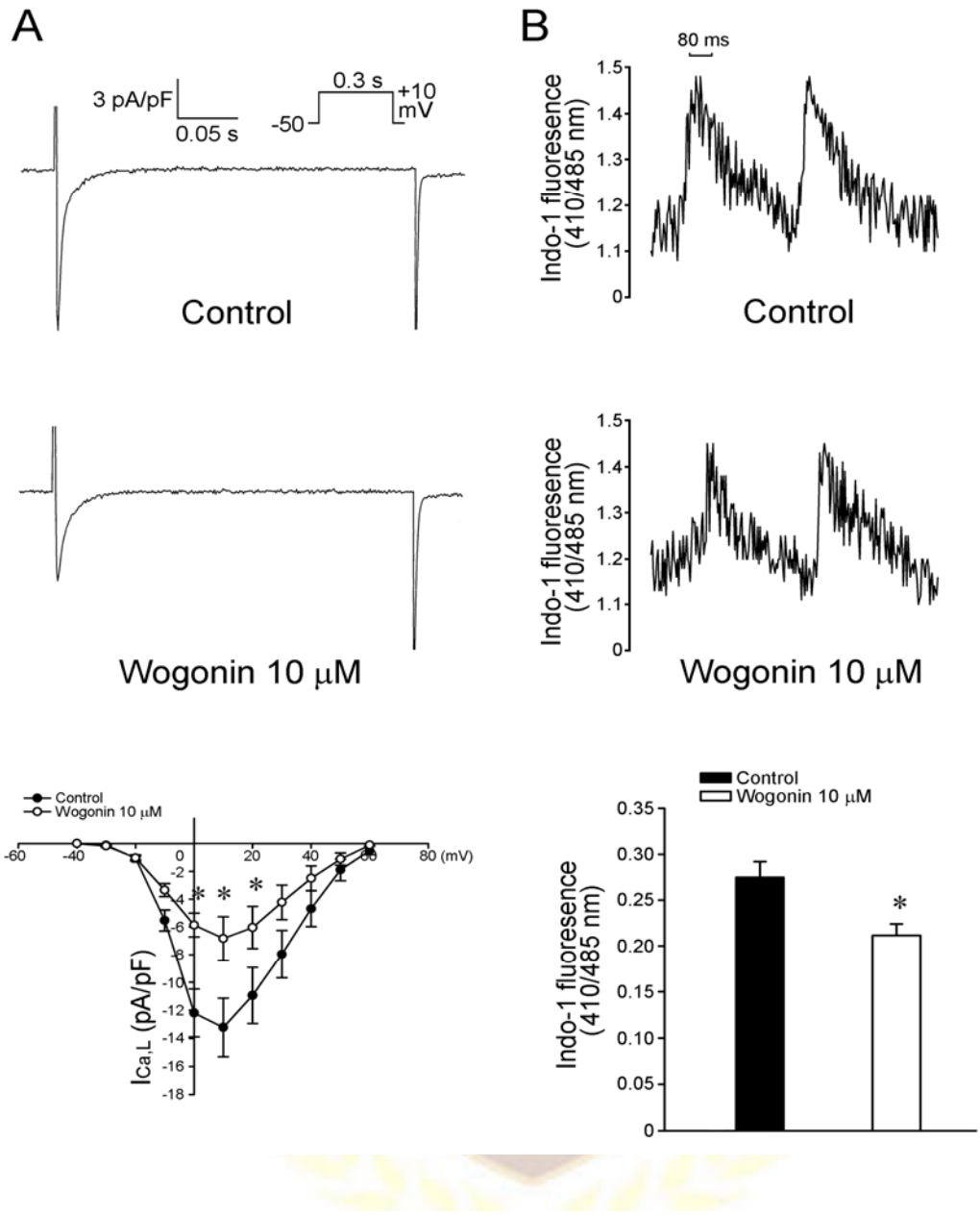


Figure 9 Effects of wogonin on L-type Ca^{2+} currents ($I_{Ca,L}$) (A), and Ca^{2+} transient (B), in the ventricular myocytes of rabbits. Figure A showed the tracings and *I-V* releationship of $I_{Ca,L}$ without (control) or with wogonin (10 μM) administration. Figure B showed the tracings Ca^{2+} transient and the peak systolic $[Ca^{2+}]i$ by electrical stimulation.