Wogonin Suppresses Arrhythmias, Inflammatory Responses, and Apoptosis Induced by Myocardial Ischemia/Reperfusion in Rats

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Abstract: Wogonin is a flavonoid isolated from *Scutellaria baicalensis* Georgi, a traditional Chinese medicine, and it possesses antioxidant and anti-inflammatory effects. The aim of this study is 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-minute left coronary artery occlusion and 2-hour reperfusion. Rats were treated with wogonin (5, 10, and 20 mg/kg, intraperitoneal) 40 minutes before ischemia or treatment with 10 mg/kg of wogonin 15 minutes after occlusion. Pretreatment with 10 mg/kg of wogonin significantly delayed the occurrence of ventricular premature contractions and tachycardia, and it suppressed the incidence of ventricular tachycardia and ventricular fibrillation, and mortality elicited by ischemia when compared with that in the control group, accompanied by reducing the arrhythmia scores. After 2-hour reperfusion, pretreatment and posttreatment with wogonin 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 the myocardium with ischemia/reperfusion. The expression of monocyte chemoattractant protein-1, phosphorylated p38 mitogen-activated protein kinase, p65 and IκBα, and active caspase-3 in ischemic myocardium pronouncedly increased in the control group; these were significantly attenuated by treatment with wogonin. In conclusion, wogonin demonstrated in vivo cardioprotective effects by the attenuation of the severity of ischemia-induced arrhythmias and irreversible ischemia/reperfusion injury, which is associated with its antioxidant capacity and anti-inflammatory effects. The suppression of nuclear factor-κB and p38 mitogen-activated protein kinase activation and the inhibition of monocyte chemoattractant protein-1 expression contribute to the beneficial effects of wogonin.

Key Words: wogonin, myocardial ischemia/reperfusion injury, arrhythmias, apoptosis, monocyte chemoattractant protein-1, nuclear factor-κB, p38 mitogen-activated protein kinase

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INTRODUCTION

Myocardial ischemia caused by acute myocardial infarction results in metabolic and electrophysiological changes that render the heart more vulnerable to ventricular arrhythmias and sudden death. Several major changes that are 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.[1]

After acute myocardial infarction, early and successful myocardial reperfusion with the use of thrombolytic therapy or primary percutaneous coronary intervention is the most effective strategy for reducing the size of a myocardial infarct and improving the clinical outcome. Animal models of sustained ischemia have shown exacerbation of myocardial injury during reperfusion, mediated largely by cytotoxic effects of free-radical generation, complement activation, and inflammation.[2] Reperfused myocardial infarction is associated with an inflammatory response leading to leukocyte recruitment, healing, and scar formation. Chemokines have been demonstrated to possess a potential role in myocardial ischemia/reperfusion via the regulation of neutrophil and mononuclear cell trafficking. Mononuclear cell chemoattractants, such as the CC chemokine monocyte chemoattractant protein-1 (MCP-1), are expressed, leading to monocyte and lymphocyte recruitment in the ischemic area.[3] In addition, the activation of nuclear factor-κB after ischemia/reperfusion has been documented previously,[4] and its inhibition has been shown to be cardioprotective.[5,6] Ischemia/reperfusion also activates cell-signaling cascades leading to apoptosis.[7]

Wogonin is a plant flavonoid and an active ingredient of *Scutellaria baicalensis* Georgi (Huang Qui), which is a traditional Chinese herbal medicine, and is widely used for the

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treatment of various allergic and inflammatory diseases. In in vitro studies, wogonin possesses the free-radical scavenging and antioxidant capacity[8] and reduces inducible enzyme expression inducible nitric oxide synthase and cycloxygenase-2, leading to the inhibition of nitric oxide and prostaglandin E_2 production, respectively, in lipopolysaccharide-activated marcorphages.[9,10] Interestingly, wogonin inhibits MCP-1 gene expression in human endothelial cells.[11] Furthermore, wogonin can inhibit interleukin-1β-induced interleukin-6 and interleukin-8 mRNA expression via the suppression of nuclear factor-κB binding activities in the human retinal pigment epithelial cell line.[12] In in vivo studies, wogonin shows an anti-inflammatory effect on 12-*O*-tetracanoyphorbol-13-acetate-induced skin inflammation[13] and lipopolysaccharide-induced inflammation in mice.[14] Therefore, in this study, wogonin was evaluated in an open-chest model of ischemia/reperfusion in anesthetized rats to observe (1) whether pretreatment with wogonin can suppress the occurrence of ischemia-induced ventricular arrhythmias; (2) whether the antioxidant and anti-inflammatory effect is related to the beneficial effect of wogonin on myocardial ischemia/reperfusion injury.

MATERIALS AND METHODS

Male Sprague–Dawley rats, weighing 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 were 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 US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Surgical Procedures of Ischemia/Reperfusion

Rats were anesthetized with intraperitoneal (ip) 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.[15–18] The animal preparations and surgical procedures to induce ischemia and reperfusion were performed as described previously.[19] The left coronary artery was occluded for 45 minutes followed by 2 hours of reperfusion to induce an irreversible ischemia/reperfusion injury.

Experimental Groups

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

Ventricular Arrhythmias

Diagnosis and quantification of arrhythmias conformed with the guidelines of the Lambeth Conventions.[20] Ventricular arrhythmias were recorded by the time to onset of the first arrhythmia, incidence of ventricular tachycardia and ventricular fibrillation (all types), incidence of sustained ventricular fibrillation, and arrhythmia score.[21] Sustained ventricular fibrillation was defined as ventricular fibrillation lasting continuously for >120 seconds (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).[22,23] All arrhythmias were scored on a 0–8 arrhythmia scoring scale for 0- to 30-minute postligation period.[21] 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 1 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 <60 seconds (4), for reversible ventricular tachycardia and/or ventricular fibrillation episodes lasting 60–120 seconds (5), for ventricular tachycardia and/or ventricular fibrillation episodes lasting >120 seconds (6), for fatal ventricular fibrillation starting at >15 minutes after occlusion (7), for fatal ventricular fibrillation starting at between 4 minutes and 14 minutes 59 seconds after occlusion, and (8) for fatal ventricular fibrillation within 4 minutes. The mortality in each group was also evaluated.

Area at Risk and Infarct

At the end of the 2-hour reperfusion, the left coronary artery was reoccluded and 0.3 mL Evans blue (3%) was injected intravenously to denote the area at risk. The heart was then excised and frozen for 90 minutes (−20°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 minutes (37°C). The surviving tissue turns a deep red, whereas the infarct portion is white. The slice was fixed in 10% formalin overnight. The areas of risk and infarct were taken with a digital camera. The areas were then measured and analyzed using Image-Pro plus analysis software. The infarct size is presented as a percentage of the area at risk (infarct: area at risk).

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 (LDH) levels 60 minutes after reperfusion.[24] Plasma levels of creatine kinase muscle–brain and LDH were measured using an analyzer of Fuji DRI-CHEM FDC 3000 (Fuji Photo Film, Japan). The tissue necrosis factor-α (TNF-α) level was determined by an enzyme-linked immunoadsorbent assay (rat TNF-α Immunoassay Kit, R&D

Systems, United States) according to the manufacturer's instructions.

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.[25] In brief, myocardium samples (3 × 3 mm) were taken from the ischemic regions 30 minutes after reperfusion. Scintillation plates containing Krebs–2-[4-(2-hydroxethyl)-1-piperazinyl]ethanesulfonic acid buffer with lucigenin (1.25 mM) were placed into a microplate luminometer (Hidex, Microplate Luminometer, Finland). Counts were obtained in duplicate at a 15-second interval. The plates containing all the 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 the samples were dried in a 90°C (16 hours) oven for expressing results on a milligram myocardium dry weight basis. These results were expressed as count per second per milligram of myocardium dry weight.

Western Blot Analysis

To elucidate the effect of wogonin on the protein expression of MCP-1, activation of nuclear factor-κB, and p38 mitogen-activated protein kinase (p38 MAPK) signaling pathway, and apoptosis elicited by ischemia/reperfusion, Western blot analysis was used. After 45-minute ischemia and 2-hour reperfusion, the ischemic region of the 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 antiphospho-IκBα antibody (Cell signaling, United States; 1:1000), mouse antiphospho-p65 antibody (Epitomics, United States; 1:1000), mouse monoclonal antiphospho-p38 MAPK antibody (Cell Signaling; 1:1000), mouse polyclonal anti-MCP-1 antibody (eBioscience, United States; 1:1000), and rabbit monoclonal anticaspase-3 (active) antibody (Epitomics; 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.

Statistical Analyses

The Chi square test with the 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 the first arrhythmia was $\log_{10}$ transformed to generate a Gaussian-distributed variable.[23] Data are expressed as group mean ± SEM. Statistical evaluation was performed with 1-factor analysis of variance followed by the Newman–Keuls post hoc comparison test. A P value of <0.05 was deemed statistically significant.

RESULTS

Hemodynamics

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

TABLE 1. Summary of Hemodynamic Parameters During the Experiments

Group	5 min after Thoracotomy	1 min before Occlusion	Time for ischemia (min)			Time for reperfusion (min)		
			1	5	40	10	60	120
Mean Blood Pressure (mm Hg)								
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 (ip) was administered 40 minutes before left coronary artery occlusion; Post-Wog 10, wogonin 10 mg/kg was administered 15 minutes 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 that of the control group; †$P < 0.05$ compared with that of the Pre-Wog 10 group.

Arrhythmias During the Ischemic Period

Ventricular arrhythmias commenced within 4–30 minutes of occlusion, manifesting as ventricular premature contractions, ventricular tachycardia, and ventricular fibrillation. All the rats developed arrhythmias during the 30-minute postligation 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 that in 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 that of the control group ($P > 0.05$) (Table 3 and Fig. 1). The mortality of the Pre-Wog 20 group was significantly higher than that in the Pre-Wog 10 group. In the Post-Wog 10 group, because wogonin was given 15 minutes after occlusion, the incidence of ventricular fibrillation (66.7%) and mortality (33.3%; 5 rats died, 10 rats survived) were similar to those of the control group. In addition, the arrhythmias also occurred in the reperfusion period. Pretreatment or posttreatment with 10 mg/kg of wogonin did not significantly reduce the counts of ventricular premature contractions when compared with those of 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).

Plasma Levels of Creatine Kinase muscle–brain and Lactate Dehydrogenase

After 1-hour reperfusion, the creatine kinase muscle–brain level was significantly elevated in the control group (5858 ± 436 U/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 the control group (Pre-Wog 5: 3610 ± 213, n = 5; Pre-Wog 10: 3500 ± 479, n = 10; Post-Wog 10: 3320 ± 377 U/L, n = 5; $P < 0.05$). Pretreatment with a high dose of wogonin (20 mg/kg) did not attenuate the creatine kinase muscle–brain level when compared with that of the control group (Pre-Wog 20: 4620 ± 535 U/L, n = 5). There was no significant difference in the creatine kinase muscle–brain level among wogonin-treated groups (Fig. 2A). The LDH data of the control group were 2562 ± 215 U/L (n = 10). Pretreatment with 5 and 10 mg/kg of wogonin and posttreatment with 10 mg/kg of wogonin significantly attenuated the levels of LDH 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 U/L, n = 5; $P < 0.05$). Pretreatment with a high dose of wogonin (20 mg/kg) did not significantly reduce the plasma level of LDH when compared with that of the control group (Pre-Wog 20: 2268 ± 236 U/L, n = 5). There was no significant difference in the LDH level among wogonin-treated groups (Fig. 2B).

TABLE 2. The Effect of Wogonin on the Time to Onset of First Ischemia-induced Ventricular Arrhythmias

		VPC		VT		VF
Treatment	N	Log_{10} (s)	N	Log_{10} (s)	N	Log_{10} (s)
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

*$P < 0.05$ compared with that of the control group.

N, number of rats; Pre-Wog 5, 10, and 20, pretreatment with wogonin, ip, 5, 10, or 20 mg/kg 40 minutes before ischemia; VF, ventricular fibrillation; VPC, ventricular premature contraction; VT, ventricular tachycardia.

TABLE 3. The Effect of Wogonin on the Incidence of Ischemia-induced Arrhythmias

		Incidence (%)				Mortality
Treatment	N	VPC	VT	Total VF	Sustained VF	(%)
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)

*$P < 0.05$ compared with that of the control group.

†$P < 0.05$ compared with that of the Pre-Wog 10 group.

N, number of rats; Pre-Wog 5, 10 and 20, pretreatment with wogonin, ip, 5, 10, or 20 mg/kg 40 minutes before ischemia; VF, ventricular fibrillation; VPC, ventricular premature contraction; VT, ventricular tachycardia.

Size of Infarction After 2 Hours of Reperfusion

No significant differences in the area at risk, expressed as percentage of the total left ventricle, were noted among the groups (Fig. 2D). A significant reduction in infarct size, expressed as the percentage of the area at risk, was noted in groups of pretreatment and posttreatment with 10 mg/kg of wogonin (Pre-Wog 10 and Post-Wog 10 groups), when

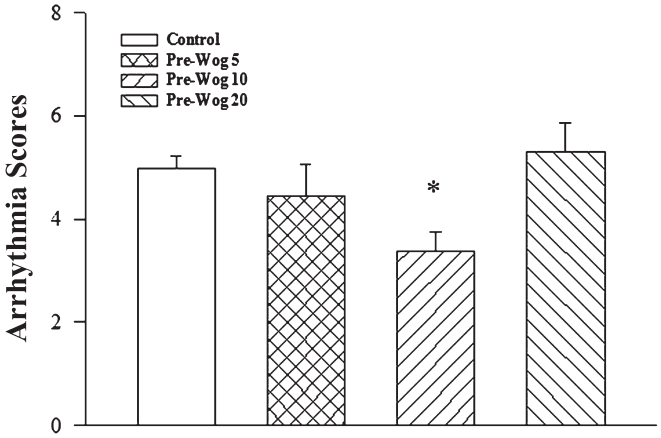

FIGURE 1. Effects of pretreatment with wogonin on arrhythmia scores during 30-minute left coronary artery occlusion in anesthetized rats. Values are expressed as mean ± SEM. *$P <$ 0.05 versus the control, n = 10–30.

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; Post-Wog 10, treatment with wogonin 15 minutes after left coronary artery occlusion; Pre-Wog 10, pretreatment with 10 mg/kg of wogonin, ip, 40 minutes before ischemia; VF, ventricular fibrillation; VPC, ventricular premature contraction; VT, ventricular tachycardia.
*$P < 0.05$ versus Pre-Wog 10 group.

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 ($P > 0.05$).

Superoxide Anion Production in Ischemic Myocardium After Reperfusion

Myocardial superoxide anion production was measured in the ischemic regions of the control and wogonin-treated groups after 45-minute ischemia/30-minute reperfusion (Fig. 3A). Pretreatment and posttreatment 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 per milligram tissue weight, n = 5), when compared with that of the control group (Control: 15.3 ± 3.5 counts per second per milligram tissue weight, n = 6; $P < 0.05$). There is no significant difference in the superoxide anion level among wogonin-treated groups.

Plasma Tissue Necrosis Factor-α Levels After Ischemia/Reperfusion

The plasma levels of TNF-α were measured 1 hour after reperfusion. Pretreatment with 5 and 10 mg/kg of wogonin significantly decreased the ischemia-/reperfusion-induced elevation of plasma TNF-α level as compared with that of 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 TNF-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. 3B).

Protein Expression After Ischemia/Reperfusion

Western blots on homogenates of the ischemic myocardium after 2-hour 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 posttreatment with 10 mg/kg of wogonin significantly reduced ischemia-/reperfusion-induced elevation of MCP-1, phospho-IκBα, phospho-p65, phospho-p38

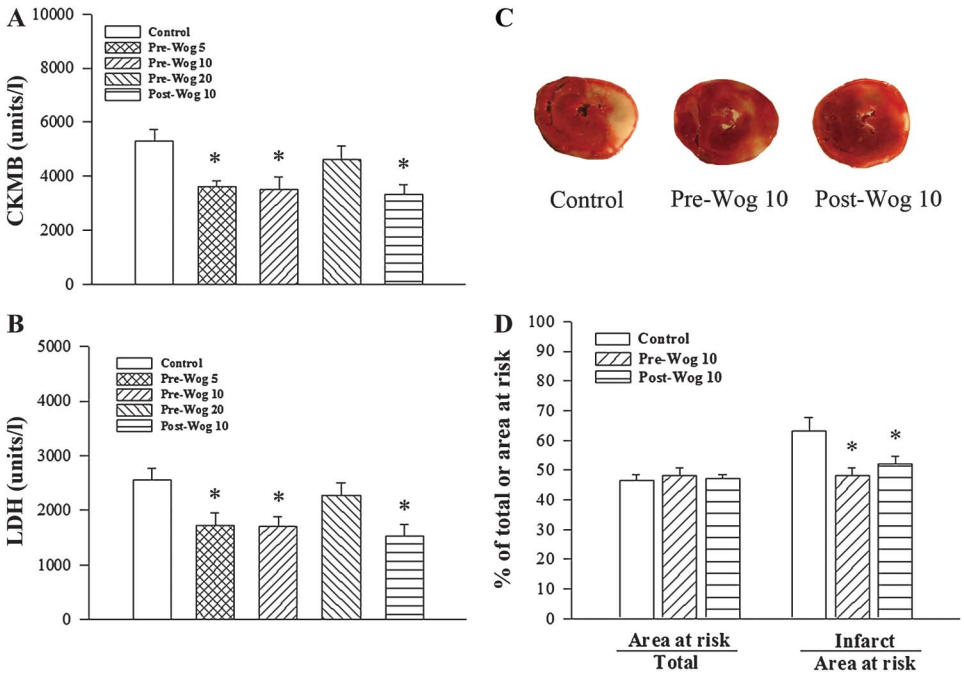

FIGURE 2. Effects of wogonin on the plasma levels of CKMB (A) and LDH (B) with 45-minute ischemia per hour of reperfusion and infarct size (C, D) of the rats with 45-minute ischemia per 2 hours of reperfusion. Representative cross sections after triphenyltetrazolium chloride staining of each group were shown. Pre-Wog 5, 10, and 20: pretreatment with 5, 10, and 20 mg/kg of wogonin 40 minutes before ischemia; Post-Wog 10: treatment with 10 mg/kg of wogonin 15 minutes after ischemia. Data are given as mean ± SEM. *$P < 0.05$ versus the control, n = 5–10.

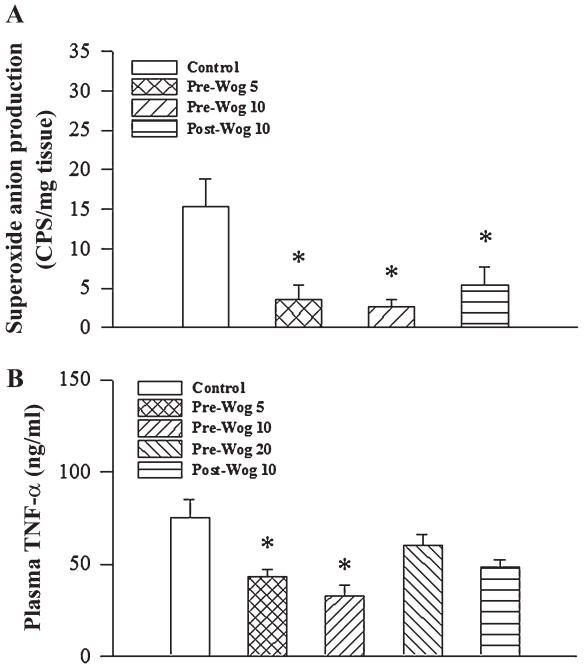

FIGURE 3. Effects of wogonin on levels of superoxide anion production (A) in the ischemic myocardium of the rats with 45-minute myocardial ischemia followed by 30 minutes of reperfusion and plasma tissue necrosis factor (TNF)-α (B) measured at 1 hour after reperfusion. Pre-Wog 5, 10, and 20: pretreatment with 5, 10, and 20 mg/kg of wogonin 40 minutes before ischemia; Post-Wog 10: treatment with 10 mg/kg of wogonin 15 minutes after ischemia. CPS: counts per second; Data are given as mean ± SEM. *$P < 0.05$ versus the control, n = 5–6.

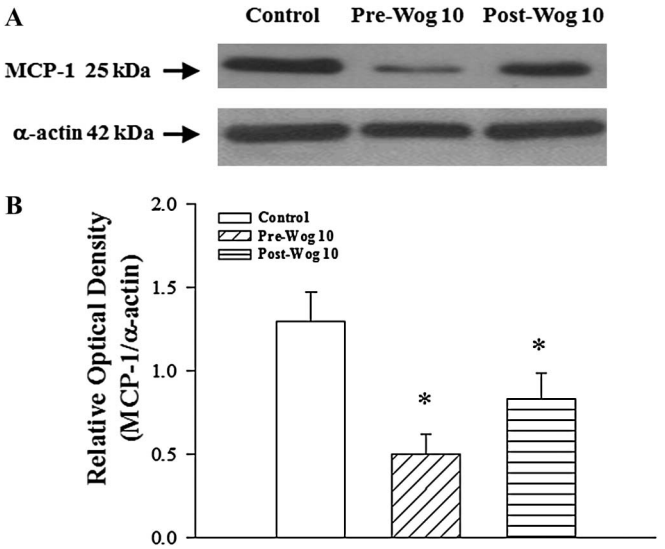

FIGURE 4. Effects of wogonin on MCP-1 protein expression in the ischemic myocardium of rats with 45-minute myocardial ischemia followed by 120 minutes of reperfusion. Representative Western blots are shown. Pre-Wog 10 and Post-Wog 10 mean pretreatment and posttreatment with 10 mg/kg of wogonin, respectively. 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.

MAPK, and active caspase-3 protein expression (Figs. 4–7) ($P < 0.05$). There is no significant difference in the expression levels of all observed proteins between the Pre-Wog 10 and Post-Wog 10 groups ($P > 0.05$).

DISCUSSION

In this study, we showed the in vivo evidence for the first time that wogonin markedly suppresses ischemia-induced lethal ventricular arrhythmias, contributing to reduced mortality. Besides pretreatment, even given after the occurrence of ischemia (posttreatment), 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 TNF-α level, and protein expression of chemokine MCP-1, which may be mediated by the suppression of activation of nuclear factor-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 an intracellular Ca^{2+} overload, spontaneous increases in membrane potential that manifest as delayed afterdepolarizations.[1] This triggered activity from Ca^{2+} overload may successfully propagate throughout the myocardium and form lethal arrhythmias. In this study, wogonin showed an antiarrhythmic effect during ischemic insult and reduced mortality. The antiarrhythmic mechanism of wogonin is still unknown. In a 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 (data not shown). Therefore, wogonin may reduce Ca^{2+} overload in ischemic myocardium by restoring the changes in Ca^{2+} handling. Further experiments to explore the possible antiarrhythmic 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.[26] Wogonin has been reported to possess free-radical scavenging effects and suppress nicotinamide adenine dinucleotide phosphate-dependent lipid peroxidation.[8] Meanwhile, in this study, we showed the inhibitory effect of wogonin on superoxide anion production in the myocardium after 45-minute ischemia/30-minute 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 the time of diastole and myocardial blood supply, which result in the development of myocardial

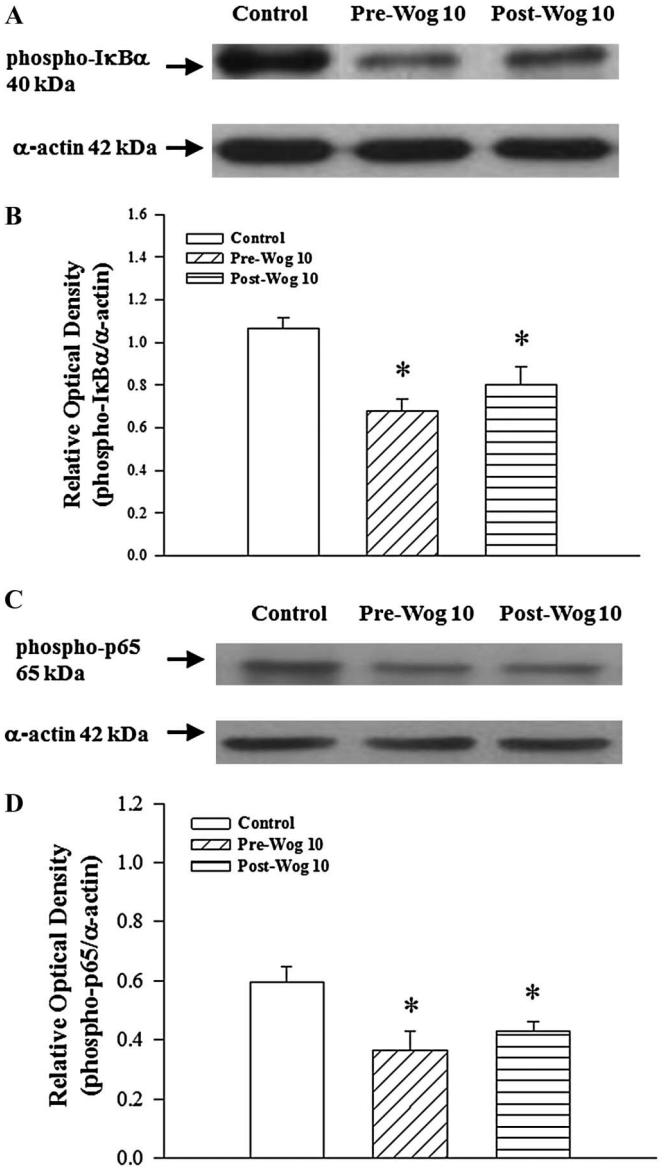

FIGURE 5. Effects of wogonin on phospho-IκBα protein expression in the ischemic myocardium of rats with 45-minute myocardial ischemia followed by 120 minutes of reperfusion. Representative Western blots are shown. Pre-Wog 10 and Post-Wog 10 mean pretreatment and posttreatment with 10 mg/kg of wogonin, 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.

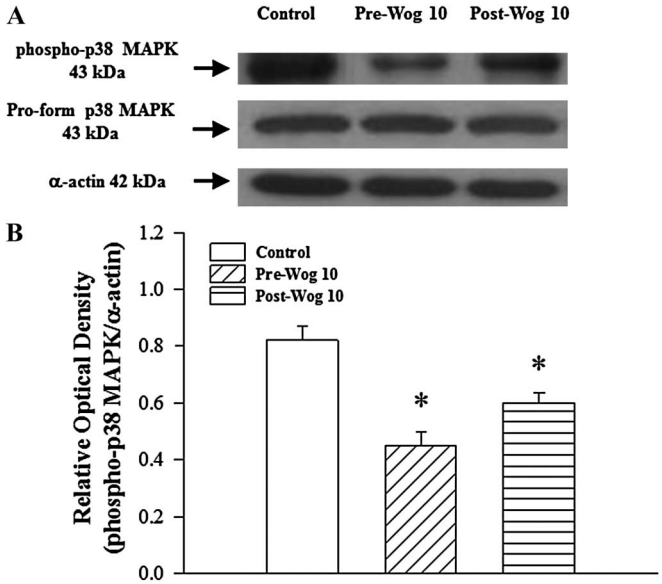

FIGURE 6. Effects of wogonin on phospho-p38 MAPK protein expression in the ischemic myocardium of rats with 45-minute myocardial ischemia followed by 120 minutes of reperfusion. Representative Western blots are shown. Pre-Wog 10 and Post-Wog 10 mean pre and posttreatment with 10 mg/kg of wogonin, respectively. 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.

ischemia and arrhythmias in ischemic areas.[27] Treatment with 10 mg/kg of wogonin gradually reduced heart rate of the rats (Table 1), which last to even onset of ischemia and, at least, 5 minutes 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 5 mg/kg of wogonin did not significantly reduce the arrhythmia score and mortality of the rats subjected to ischemia, the antireperfusion-injury effect was pronounced (Figs. 2A, B), which may be associated with its antioxidant and anti-inflammatory effects (Fig. 3). Unlike 10 mg/kg of wogonin, pretreatment 5 mg/kg of wogonin did not reduce the heart rate during the baseline and early ischemic period (Table 1). This also implies that 5 mg/kg of wogonin may not alleviate ischemia-induced Ca^{2+} overloading and triggered activity; therefore, it did not afford protective effects during the ischemic period. Preteatment with a 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 that in the Pre-Wog 10 group. However, 20 mg/kg of wogonin did not significantly worsen ischemic insult when compared with in the control group. This may be a result that the toxic effect of high dose wogonin counteracted its beneficial effects. Moreover, the antireperfusion injury effects of posttreatment with 10 mg/kg of wogonin did not significantly differ from that of pretreatment indicating that wogonin mainly exerted its protective actions during the reperfusion period. However, the ventricular premature counts during the 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.[28] As aforementioned, wogonin is a polyhydroxyflavonoid, and it

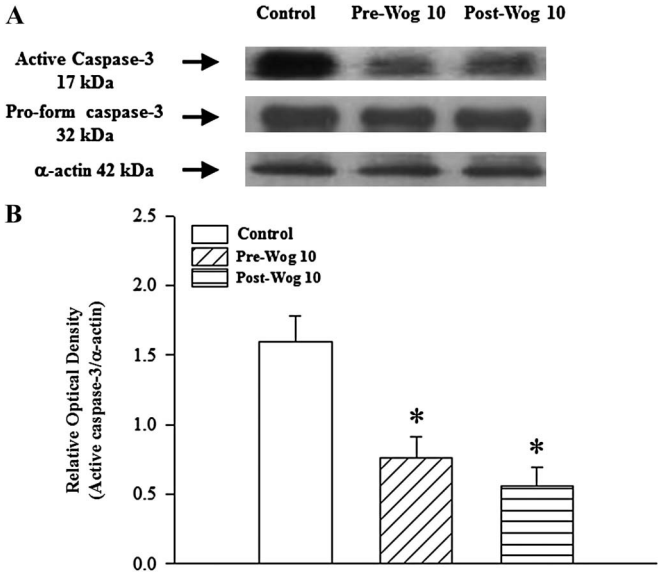

FIGURE 7. Effects of wogonin on the active caspase-3 protein expression in the ischemic myocardium of rats with 45-minute myocardial ischemia followed by 120 minutes of reperfusion. Representative Western blots are shown. Pre-Wog 10 and Post-Wog 10 mean pretreatment and posttreatment with 10 mg/kg of wogonin, 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.

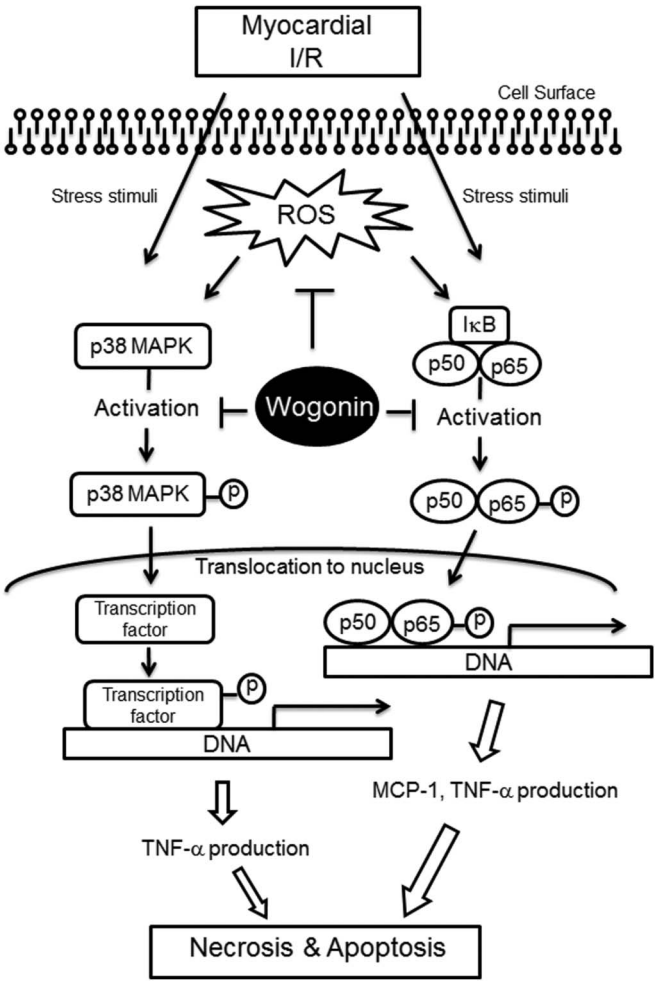

FIGURE 8. Schematic diagram of the possible mechanisms responsible for the effectiveness of wogonin in myocardial ischemia/reperfusion injury. It is hypothesized that wogonin suppresses (1) oxidative stress and (2) activation of p38 MAPK and nuclear factor-κB signaling pathways, leading to the reduction in MCP-1, TNF-α, apoptosis, and necrosis in cardiomyocytes.

has been demonstrated to possess antioxidant, free-radical scavenging, and anti-inflammatory activities.[8] In this study, wogonin also suppressed superoxide anion production in the 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 nuclear factor-κB transcription factor and MAPK superfamily that result in altered gene expression profile and generally in an activated and proinflammatory cellular phenotype.[29] Therefore, wogonin-reduced oxidative stress in ischemia/reperfusion is likely to further suppress the activation of nuclear factor-κB transcription factor and p38 MAPK signaling pathway.

Reperfusion injury has to be considered as an inflammatory disease.[30] 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.[31] ROS, cytokines, and shear stress resulting from ischemia/reperfusion injury, stimulate nuclear factor-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.[32] 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 after ischemia/reperfusion.[24] In this study, wogonin can reduce the expression of phospho-IκBα and -p65 showing the antinuclear factor-κB property, which is contributing to the reduction of myocardial stress elicited by ischemia/reperfusion.

The proinflammatory nuclear factor kappa B transcription factor is a key mediator for MCP-1 expression.[33] In the canine model, the induction of MCP-1 mRNA occurred in a previously ischemic area within the first hour of reperfusion, peaked at 3 hours, and persisted throughout the first 2 days of reperfusion.[34] Neutralizing antibody to MCP-1 significantly reduces infarct size, decreasing adhesion molecule expression and macrophage infiltration in rats.[35] Enhanced MCP-1 expression in rat kidney during ischemia/reperfusion injury is mediated by oxidative stress and nuclear factor-κB.[36] In accordance with the results, wogonin inhibits MCP-1 gene expression in human endothelial cells.[11] In this study, we also found that wogonin inhibited MCP-1 protein expression in the ischemic region after ischemia/reperfusion. The inhibitory

effect on MCP-1 may contribute to the beneficial effect of wogonin on ischemia/reperfusion injury, which is likely mediated by the suppression of nuclear factor-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.[37] Activation of p38 MAPK followed by the transcription of genes encoding inflammatory molecules indicates an important role of this stress cascade in 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).[38] The production of cytokines further elicited nuclear factor-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.[39,40] In this study, we showed the cardioprotective effect of wogonin accompanied by suppression of the activation of p38 MAPK, which may be one mechanism of the 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 the severity of ischemia. An abundance of evidence indicates that ischemia-/reperfusion-induced cardiac cell death occurs from both necrosis and apoptosis.[41] Cysteine proteases comprising the caspase family have been considered one of the major executioners of programmed cell death or apoptosis.[42] Apoptosis is potentially triggered by cytokines, tissue necrosis factor-α, ROS, and nitric oxide released by infiltrated polymorphonuclear leukocytes or marcophages.[43] In this study, we measured the levels of active caspase-3 protein expression to reflect the situation of ischemia/reperfusion-induced apoptosis and found that wogonin can attenuate the induction of apoptosis. The antioxidant and anti-inflammatory effects of wogonin likely contribute to this protection.

Although in a previous in vitro report wogonin could not show protection efficacy in a cultured chick cardiomyocyte exposed to ischemia/reperfusion,[44] it ameliorated ischemia/reperfusion injury in vivo in this study. However, an in vivo study is more clinically relevant than an in vitro study of wogonin. Furthermore, the neuroprotective effect of wogonin has been demonstrated in vivo in experimental brain injury models.[45,46] These in vivo results provide a pharmacological basis for the use of wogonin or *S. baicalensis* in the treatment or prevention of stroke and acute myocardial infarction.

In conclusion, wogonin demonstrates the antiarrhythmic effect and improves the survival rate during myocardial ischemia in rats. Administering wogonin before or after onset of ischemia prevents myocardial damage induced by ischemia/reperfusion. It is hypothesized that wogonin suppresses (1) oxidative stress and (2) activation of p38 MAPK and nuclear factor-κB signaling pathways, leading to the reduction in MCP-1, tissue necrosis factor-α, apoptosis, and necrosis in cardiomyocytes (Fig. 8). These in vivo results provide a strong pharmacological basis for the use of wogonin or *S. baicalensis* in the treatment of acute myocardial infarction.

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