

Cardioprotective effect of resveratrol, a natural antioxidant derived from grapes

Li-Man Hung^a, Jan-Kan Chen^b, Shiang-Suo Huang^a, Ren-Shen Lee^c, Ming-Jai Su^{a,*}

^a*Institute of Pharmacology, College of Medicine, National Taiwan University, No. 1, Sec. 1, Jen-Ai Road, Taipei, Taiwan*

^b*Department of Physiology, College of Medicine, Chang Gung University, Taoyuan, Taiwan*

^c*Center of General Studies, Chang Gung University, Taoyuan, Taiwan*

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Abstract

Background: The major objective of the present study was to examine the cardioprotective effect of resveratrol, an antioxidant presents in red wines, in the rat after ischemia and ischemia–reperfusion (I–R). **Methods:** The left main coronary artery was occluded for 30 or 5 min followed by a 30-min reperfusion in anesthetized rats. Animals were preinfused with and without resveratrol before occlusion and the severity of ischemia- and I–R-induced arrhythmias and mortality were compared. **Results:** Resveratrol pretreatment had no effect on ischemia-induced arrhythmias nor on mortality. In contrast, a dramatic protective effects were observed against I–R-induced arrhythmias and mortality. Resveratrol pretreatment both reduced the incidence and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF). During the same period, resveratrol pretreatment also increased nitric oxide (NO) and decreased lactate dehydrogenase levels in the carotid blood. **Conclusions:** Resveratrol is a potent antiarrhythmic agent with cardioprotective properties in I–R rats. The cardioprotective effects of resveratrol in the I–R rats may be correlated with its antioxidant activity and upregulation of NO production. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The Southern French have a very low mortality rate due to coronary heart disease (CHD) despite having a high-fat diet and smoking habits. This so-called ‘French paradox’ has been attributed in part to wine consumption, particularly red wines [1,2]. Resveratrol (3,5,4′-trihydroxystilbene), a polyphenol present in red wine, has been thought to be responsible for the cardiovascular benefits associated with moderate wine consumption [3]. In purified or synthetic form, resveratrol has been shown to reduce the synthesis of lipids in rat liver [4], to inhibit the synthesis of eicosanoids in rat leukocytes [5], to interfere with arachidonate metabolism [6], to inhibit platelet activation/aggregation [7], to inhibit the activity of some protein kinases [8], to exert a strong inhibitory effect on reactive

oxygen species produced by human polymorphonuclear leukocytes [9], and is an antioxidant more powerful than vitamin E in preventing LDL oxidation [10]. However, because of its unpredictable effect on other organ systems, it is unwise to recommend a glass or two of wine to someone with a known predisposition to CHD.

It is now well established that cardiomyocytes cannot survive under severe ischemic conditions for prolonged period. Regardless of its cause (thrombosis, arterial spasm, atheroma, etc.), ischemia induces an imbalance between oxygen supply and demand for the metabolizable substrates in the cardiac tissue, rapidly leading to functional, metabolic, electrophysiological, and morphological alterations of the myocardium, and may eventually cause cellular necrosis [11].

Reperfusion of the ischemic myocardium is associated with a host of distinctive pathophysiologic derangements, including reperfusion arrhythmias, transient mechanical

*Corresponding author. Tel.: +886-2-2356-2221; fax: +886-2-2391-5602.

E-mail address: mjsu@ha.mc.ntu.edu.tw (M.-J. Su).

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dysfunction or ‘myocardial stunning’, and cell death [12]. The underlying pathophysiological mechanisms have not been fully elucidated. It has been suggested that an overproduction of reactive oxygen intermediates (superoxide anion, hydroxyl radical, hydrogen peroxide, singlet oxygen) [13], and intracellular calcium overload or redistribution [14] might be involved. Whether there is a causal relationship between the overproduction of reactive oxygen derivatives and I–R injury is a question with physiological and medical significance. If such a relationship exists, would then pretreatment of ischemic heart with antioxidant before reperfusion be beneficial in decreasing the risk of CHD mortality? The antioxidant property of resveratrol and its capacity to stimulate endothelial NO production [15] prompted us to investigate whether it is capable of exerting any protective effects in face of I–R injury. Most of the studies thus far have focused on the beneficial effects of resveratrol in the prevention of atherosclerosis and coronary heart disease, and little has been considered for its possible use as a therapeutic drug in treating acute scenarios such as I–R injury. In the present study, we evaluate the possible protective effect of resveratrol on ischemia or I–R injury in anesthetized rat hearts subjected to transient regional ischemia. Our results show that preinfusion of resveratrol effectively suppresses I–R-induced arrhythmias and cardiac cell damage and these effects may be attributable to its antioxidant activity and upregulation of NO production.

2. Methods

2.1. Materials

trans-Resveratrol (*trans*-3,4',5-trihydroxystilbene), a natural antioxidant present in red wine, was synthesized as described in [16], and purified by silica-gel column chromatography (ethylacetate–*n*-hexane, 1:2, v/v) and recrystallization (ethylacetate–*n*-hexane, 1:1, v/v). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and sodium nitrite were purchased from Sigma (St. Louis, Mo. USA). Vanadium (III) was obtained from Merck (Darmstadt, Germany).

2.2. 1,1-Diphenyl-2-picrylhydrazyl test

An ethanolic solution of the stable nitrogen centered free radical DPPH (100 μ M) was incubated with the test compounds and the absorbance was monitored spectrophotometrically at 517 nm. The concentration ($IC_{0.200}$) of the test compounds that induced a decrease of 0.200 in absorbance during a 30-min observation period was taken as the free radical-scavenging potency [17].

2.3. Animals

All studies were performed in accordance with guide-

lines described in the NIH *Guide for the Care and Use of Laboratory Animals* (DHHS publication No. [NIH] 85-23, revised 1996). Male Sprague–Dawley rats (200–300 g b.wt.) underwent myocardial ischemia by a temporary occlusion of the left main coronary artery as previously described [18]. Briefly, rats were anesthetized with urethane (1.25 g/kg i.p.) and placed on an operating table. Polyethylene catheters (PE 50) were inserted from internal carotid artery into the common carotid artery for the measurement of blood pressure and heart rate. After tracheotomy, the animals were ventilated with room air by a respirator for small rodents (model 131, NEMI, USA) with a stroke volume of ≈ 12 ml/kg body weight and at a rate of 60 strokes/min to maintain normal pO_2 and pH parameters. The chest was opened and the ribs were gently spread. The heart was quickly expressed out of the thoracic cavity, inverted and a 7/0 silk suture was placed under the left main coronary artery. The heart was repositioned in the chest and the animal was allowed to recover for 15 min. A small plastic snare formed from a Portex P-270 cannula was threaded through the suture and placed in contact with the heart. Tightening the suture could then occlude the artery and reperfusion was achieved by releasing the tension applying to the suture (Operated groups). Sham operated animals underwent all the above described surgical procedures, apart from the fact that the 7/0 silk, passing around the left coronary artery, was not tied (Sham groups). Animals were infused with a bolus of resveratrol (2.3×10^{-7} , 2.3×10^{-6} , 2.3×10^{-5} g/kg) or vehicle (dimethyl sulfoxide–0.9% NaCl, 1:10³; v/v) from a jugular vein 15 min before coronary occlusion. Coronary artery was occluded for 30 min or 5 min followed by 30 min reperfusion and animals were randomized in the following groups: (1) sham+vehicle; (2) sham+resveratrol (2.3×10^{-5} g/kg); (3) operated+vehicle; (4) operated+resveratrol (2.3×10^{-7} g/kg); (5) operated+resveratrol (2.3×10^{-6} g/kg); (6) operated+resveratrol (2.3×10^{-5} g/kg).

Before and during the ischemia or reperfusion period, heart rate (HR), blood pressure (BP) and ECG changes were recorded on a personal computer with a WAVE FORM data analysis software (MacLab data acquisition system, AD Instruments, Castle Hill, NSW, Australia). Ventricular ectopic activity was evaluated according to the diagnostic criteria advocated on the Lambeth Convention [19]. The number of ventricular premature beats (VPB) and the incidence and duration of ventricular tachyarrhythmias, including VT and VF, in surviving animals were determined.

2.4. Plasma LDH and NO analysis

Cellular damage was evaluated by measuring the plasma LDH. The blood samples were drawn from the carotid artery at the end of reperfusion, collected in heparinized tubes. The blood was kept at 4°C until it was centrifuged at

2000 g for 15 min. The plasma was recovered and aliquots were used for determination of LDH activity with a commercial kit from Sigma.

The deproteinized plasma samples were frozen and kept until analysis. For measurement of NO we employed the NO/ozone chemiluminescence technique (280 NOA™, Sievers Instruments, Boulder, CO, USA). This method has previously been described in detail [20]. Briefly, the detection of plasma NO level is based on its reaction with ozone, which leads to the emission of red light ($\text{NO} + \text{O}_3 \rightarrow \text{NO}_2^* + \text{O}_2$; $\text{NO}_2^* \rightarrow \text{NO}_2 + h\nu$). The photons from this reaction are detected and transformed to an electrical signal by a photomultiplier tube (PMT). Due to the use of filters in front of the PMT, NO/O₃ chemiluminescence recorded with the Sievers NOA 280 is highly specific for NO. The current from the PMT is A/D converted and fed into a PC running the ASYST software (Sievers NO Analysis Liquid Program, USA). The amount of light produced by NO/O₃ chemiluminescence is proportional to the amount of NO sampled. Hence, the calculated area under the curve of the PMT current for each determination is proportional to the amount of NO. This was verified before each experiment by standard curves (1, 5, 10, 20, 40 and 100 $\mu\text{mol/l}$) which were produced using freshly prepared solutions of sodium nitrite in distilled water, which was reduced to NO in an equimolar manner by the reducing agent. We chose to measure the level of nitrite or nitrate in blood samples, by using a reaction vessel containing a reducing system (Vanadium (III) dissolved in 1 M HCl), to which the sample was injected and NO was generated from nitrite or nitrate in an equimolar manner. A continuous stream of Helium (99.999%) purged the resultant NO from the reaction vessel to the chemiluminescence chamber.

2.5. Statistics

Data were expressed as mean \pm standard error (S.E.). Mann–Whitney rank-sum test was used to analyze the differences in the duration of VT and VF between vehicle and drug treated groups. The BP and HR changes between vehicle and drug infused rats in arrhythmia study were analyzed by ANOVA (analysis of variance) followed by Bonferroni's test. The difference in the percentage incidence of VT, VF and mortality rate was analyzed with a χ^2 test. DPPH radical scavenging activity was evaluated by paired Student's *t*-test. The IC_{0.200} value was obtained by regression analysis. Plasma NO and LDH levels were statistically evaluated by unpaired Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Interaction of resveratrol with stable free radical DPPH

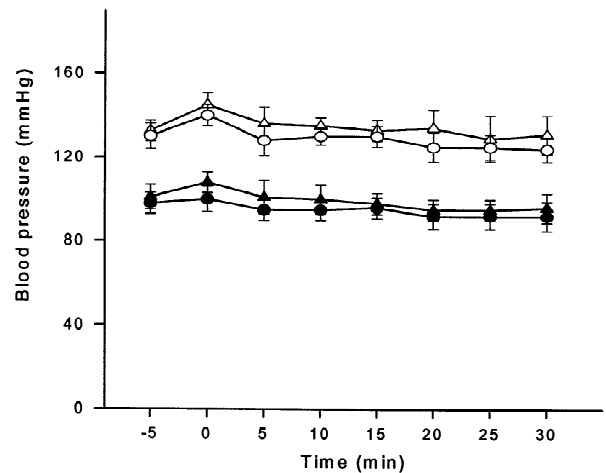
In the DPPH assay, the free radical scavenging activity

of resveratrol was expressed as IC_{0.200}. The decrease in optical absorbance at 517 nm after addition of resveratrol was monitored following the trapping of the unpaired electron of DPPH. Resveratrol scavenged DPPH in a concentration-dependent manner with an IC_{0.200} of 7.0 μM .

3.2. Infusion of resveratrol had no effect on hemodynamic parameters

Jugular vein bolus injection of resveratrol did not modify the diastolic, systolic blood pressure and heart rate in rats (Fig. 1). No significant difference was seen among

A.



B.

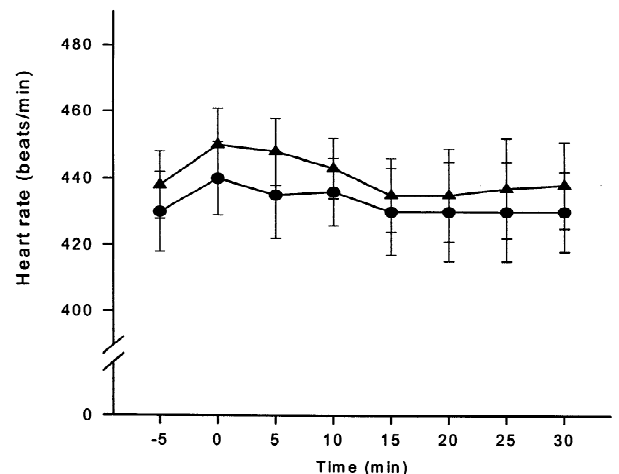


Fig. 1. (A) Systolic (open symbols) and diastolic (closed symbols) blood pressure in vehicle (0.1% DMSO) (○, ●) and 2.3×10^{-5} g/kg (△, ▲) resveratrol-infused rats. (B) Heart rate (HR) in vehicle (●), 2.3×10^{-5} g/kg (▲) resveratrol-infused rats. The differences between vehicle and resveratrol infused animals were not statistically significant (ANOVA).

Table 1
Effect of resveratrol on ischemia (30 min) induced arrhythmias in anesthetized rats^a

	Ventricular tachycardia		Ventricular Fibrillation		Mortality (%)
	Incidence (%)	Duration (s)	Incidence (%)	Duration (s)	
Sham					
Vehicle (4)	–	–	–	–	–
Resveratrol 2.3×10^{-5} g/kg (4)	–	–	–	–	–
Operated					
Vehicle (7)	100	38.2±8.5	63	15.6±10.5	38
Resveratrol 2.3×10^{-5} g/kg (6)	100	36.9±10.5	50	20.9±11.1	33

^a Vehicle is 0.1% DMSO in normal saline; (n), number of experiments; values for duration of VT and VF are shown as the mean±S.E..

control and drug groups (2.3×10^{-5} g/kg resveratrol, $n = 4$).

3.3. Ischemia-induced rhythm disturbances

Occlusion of the coronary artery induced severe ventricular arrhythmias in animals of the control (operated+vehicle) group. It started to occur at 6–7 min, peaked at 10 min, and normally subsided by approximately 15 min after occlusion. Pretreatment with resveratrol had no effect on ischemia-induced arrhythmias nor on mortality (Table 1).

3.4. Reperfusion-induced rhythm disturbances

The severity of reperfusion-induced arrhythmias is critically dependent on the duration of the preceding period of ischemia. Thus, we selected a 5-min period of ischemia followed by a 30-min period of reperfusion; this protocol could produce the highest incidence of rhythm disturbance. The same I–R protocol has been reported by Manning et al. [21] and Kusama et al. [22]; both groups showed that the arrhythmia induced is related with superoxide anion generation. Table 2 shows that in the control group (operated+vehicle), about 80% of the animals exhibited VF in the reperfusion period. Some of the VF converted spontaneously to VT or normal sinus rhythm. As a consequence, only 50% of them died. In

animals preinfused with resveratrol at 2.3×10^{-6} and 2.3×10^{-5} g/kg, a drastic reduction in mortality and in the incidence and duration of VT and VF was observed.

3.5. Plasma LDH

The biochemical indicator of cellular damage (LDH release) was examined in animals with a 5-min coronary artery occlusion followed by a 30-min reperfusion period. Blood samples were collected at the end of the 30-min reperfusion period. LDH activities was low in sham-operated animals with and without resveratrol preinfusion (102.5 ± 34.6 and 103.9 ± 15.9 U/l, respectively). In the operated animals without resveratrol preinfusion, the LDH activity was increased to 298.4 ± 21.4 U/l. Resveratrol dose dependently reduced the LDH activity; at a resveratrol dose of 2.3×10^{-5} g/kg, the LDH activity was reduced to 126.3 ± 17.6 , a value that was quite close to that of the sham-operated animals (Table 3).

3.6. Release of NO

NO release was measured by the presence of nitrite (NO_2^-) and nitrate (NO_3^-) in the plasma. In sham-operated animals, plasma NO was 6.9 ± 0.6 and 13.2 ± 2.2 $\mu\text{mol/l}$ without and with resveratrol preinfusion, respectively. In the operated animals, resveratrol exerted a dose-dependent

Table 2
Effect of resveratrol on reperfusion (30 min) induced arrhythmias in anesthetized rats^a

	Ventricular tachycardia		Ventricular fibrillation		Mortality (%)
	Incidence (%)	Duration (s)	Incidence (%)	Duration (s)	
Sham					
Vehicle [4]	–	–	–	–	–
Resveratrol 2.3×10^{-5} g/kg (4)	–	–	–	–	–
Operated					
Vehicle (10)	100	35.9±8.9	80	71.6±26.4	50
Resveratrol 2.3×10^{-7} g/kg (8)	50*	22.6±17.8	38	17.9±9.1	13
Resveratrol 2.3×10^{-6} g/kg (8)	50*	4.7±2.2**	13*	0.5±0.5*	0*
Resveratrol 2.3×10^{-5} g/kg (10)	20**	0.4±0.4**	0**	0.0±0.0**	0*

^a Vehicle is 0.1% DMSO in normal saline; (n), number of experiments; values for duration of VT and VF are shown as the mean±S.E. Statistical difference at the level of * $P < 0.05$ and ** $P < 0.01$, as compared with vehicle.

Table 3
Effects of resveratrol on NO ($\mu\text{mol/l}$) and LDH (U/l) release during the end period of reperfusion^a

Treatment	NO	LDH
Sham		
Vehicle	6.9 \pm 0.6	103.9 \pm 15.9
Resveratrol 2.3×10^{-5} g/kg	13.2 \pm 2.2*	102.5 \pm 34.6
Operated		
Vehicle	7.9 \pm 1.2	298.4 \pm 21.4
Resveratrol 2.3×10^{-7} g/kg	13.0 \pm 3.8	284.9 \pm 41.4
Resveratrol 2.3×10^{-6} g/kg	17.5 \pm 1.4**	178.4 \pm 20.2**
Resveratrol 2.3×10^{-5} g/kg	27.1 \pm 2.6**	126.3 \pm 17.6**

^a Vehicle is 0.1% DMSO in normal saline; data are presented as mean \pm S.E. ($n=6$). Statistical difference at the level of * $P<0.05$ and ** $P<0.01$, as compared with vehicle.

increase of plasma NO; at a resveratrol dose of 2.3×10^{-5} g/kg, there was a 4-fold increase compared to that of the uninfused animals (Table 3).

4. Discussion

Several epidemiological studies have suggested that the mortality rate from coronary heart disease can be decreased by moderate consumption of alcohol, particularly red wines [1,2]. Resveratrol, a polyphenolic antioxidant found in the skin of grapes and relatively abundant in red wine, has been thought to be the major component responsible for such epidemiological observations [3]. In the present study, we explored the possible use of resveratrol as a therapeutic drug in treating acute scenarios such as I–R injury.

An important consequence of both myocardial ischemia and reperfusion is the occurrence of various disturbances of cardiac rhythm, including the potential lethal condition of VF [23]. What remains to be established is which of many complex molecular changes that occur during I–R is critical to the initiation of electrophysiological instability. In recent years, many cardiac biochemical changes have been suggested as potential culprits, these include unfavorable redistribution and accumulation of ions through Na^+ / H^+ exchange [24], Na^+ , Ca^{2+} and ATP-sensitive K^+ channels [25], the release of catecholamines, cAMP [26], changes in the availability of glycolytically produced ATP [27], the accumulation of fatty acids and membrane lysophosphatides [28], expression of platelet activating factor [29] and production of free radicals [13], and the activation of the α, β -receptor [30].

In the present study, we showed that in anesthetized rats the administration of resveratrol prior to coronary artery occlusion and I–R had no protective effect on ischemia-induced arrhythmias, nor on mortality. In contrast, a dramatic protective effect was observed in I–R-induced arrhythmias and mortality. Though small amounts of free radical formation could occur under normal or ischemic

conditions, far greater production took place during the early period of reperfusion [13]. The absence of protective activities against ischemia-induced arrhythmia and mortality suggests that free radical generation is not an important factor responsible for the induction of cardiac arrhythmia during the ischemic period. Mechanisms such as acidosis, extracellular K^+ accumulation [31], inhomogeneous change of membrane excitability and conductivity in ischemic region [32] and the consequent generation of reentrant and nonreentrant arrhythmia should be considered. During the early postischemic reperfusion period, a burst of free radical generation cannot be adequately counteracted by the cardiac antioxidant mechanism which may then lead to significant myocardial injury [12,23]. In this regard, several reports have shown that some antioxidants, such as xanthine oxidase inhibitors [33], superoxide dismutase [34], and vitamin E [35] possess antiarrhythmic activity during reperfusion. Recently, it has been shown that resveratrol-treated hearts were resistant to I–R injury as evidenced by improved postischemic ventricular function and reduced infarct size on isolated perfused working rat heart and the effect was attributed to its peroxyl radical scavenging activity [36]. Though we do not have direct evidence to prove that enough free radicals were generated during 5 min ischemia followed by the 30-min reperfusion period, a report that used the same experimental protocols proves that the reperfusion damage of cardiac tissue can be prevented by allopurinol, a xanthine oxidase inhibitor [21]. This result indicates that the generation of superoxide anion may occur in this condition. Moreover, a prominent production of free radicals after 15 s of reperfusion was found in the isolated rabbit heart subjected to a short period (i.e. 10 min) ischemia [37]. In the present study, resveratrol was found to have in vitro DPPH scavenging activity and in vivo cardioprotective activity. However, the effective concentrations of resveratrol in DPPH scavenging activity ($\text{IC}_{0.2000}=7.0 \mu\text{M}$) in this study and other antioxidant activities in other reports [38] were far greater than the effective concentration for cardioprotective activity in the I–R rats by assuming that the infused resveratrol was remaining in the circulation. If the animal blood volume is 100 ml/kg by weight, and the highest quantity of infused resveratrol in the present study is 2.3×10^{-5} g/kg, the calculated concentration is 1 μM . One possible reason for the discrepancy in effective concentration for in vitro antioxidant activity and in vivo cardioprotective effect is the selective accumulation of resveratrol in cardiac tissue or membrane which then effectively prevents the membrane damage caused by free radicals. This possibility was proved by a significant cardiac accumulation in rat after chronic oral administration with red wine [39,40]. Another reason for the more effective cardioprotection is the enhancement of NO production by resveratrol (Table 3). In accordance with this speculation, the release of LDH during the same reperfusion period is decreased.

Currently, it is not clear where the increased NO originates (cardiomyocytes and/or endothelial cells of vascular bed and cardiac chamber), nor is it clear whether the increased NO production is due to the inhibition of NO interaction with superoxide anion to form peroxynitrite or enhancement of NOS activity. Numerous studies have shown both beneficial and harmful effects of NO in the cardiovascular system. NO is a free radical itself and can also form peroxynitrite, a potent oxidant that can potentially cause membrane lipid peroxidation leading to myocardial dysfunction [41,42]. In contrast, NO relaxes vascular smooth muscle and could be cardioprotective against I–R injury through coronary vasodilatation and reduction of myocardial oxygen consumption via upregulation of cGMP [43]. Pretreatment with NO donors has been reported to be beneficial in the ischemic myocardium. Both antiarrhythmic and anti-infarction [44] effects of the NO donors have been well documented. More recently NO has been appreciated as the possible key trigger and mediator for ischemic preconditioning [45]. Thus, it may be assumed that part of the anti-I–R-induced arrhythmia effect of resveratrol is probably attributable to its upregulation of NO production. Though NO production in sham-operated animals was increased by infusion with 2.3×10^{-5} g/kg resveratrol, the blood pressure was unchanged. This result suggests that other compensatory vasoconstrictor mechanisms may counterbalance the vasorelaxant effect of NO. Another possibility is the increased NO production in the systemic cardiovascular system does not reach effective concentration for induction of vascular relaxation. In reperfusion animals, the presence of resveratrol would cause a more prominent increase of NO production (Table 3). Whether the increase of NO contributes to the antiarrhythmic activity of resveratrol remains to be clarified. However, the antioxidant activity of resveratrol may prevent the formation more toxic peroxynitrite from the interaction of NO with superoxide anion which then will contribute to the increase of NO level.

5. Conclusions

In conclusion, our data indicate that preinfusion of resveratrol is effective to prevent reperfusion-induced arrhythmias and mortality. This protective effects on arrhythmias and cardiac cells damage by resveratrol may be associated with its antioxidant activity, free radicals scavenging activity and enhanced NO release during the reperfusion period.

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