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Protective Effect of KR-31378 on Oxidative Stress in Cardiac Myocytes

Mi-Young Kim, Sunkyung Lee[1], Kyu Yang Yi[1], Sung Eun Yoo[1,2], Dong-Ha Lee[3], Hong Lim[3], Ho Soon Kim[3], Soo Hwan Lee, Eun Joo Baik, Chang-Hyun Moon, and Yi-Sook Jung

Department of Physiology, School of Medicine, Ajou University, Suwon 442-749, [1]Medical Science Division, [2]Center for Biological Modulators of the 21st century Frontier Program, Korea Research Institute of Chemical Technology, Daejon 305-600, and [3]AgroPharma Research Institute, Dongbu Hannong Chemical Co. Ltd., Daejon 305-708, Korea

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In this study, we investigated whether a novel anti-ischemic K_{ATP} opener KR-31378 [(2*S*,3*S*,4*R*)-*N*''-cyano-*N*-(6-amino-3,4-dihydro-3-hydroxy-2-methly-2-dimethoxymethly-2*H*-benzopyran-4-yl)-*N*'-benzylguanidine] has protective effect against oxidative stress-induced death in heart-derived H9c2 cells. Cell death was induced by BSO, butionine sulfoximine, which inhibits GSH synthesis and subsequently increases reactive oxygen species (ROS) level. Cell death was quantitatively determined by measuring lactate dehydrogenase (LDH) activity and stained by Hoechst 33258. BSO-induced ROS production and mitochondrial membrane potential (MMP) were measured using 2',7'-dichlorofluorescein diacetate oxidation and rhodamine 123, respectively. Both the LDH release and the ROS elevation induced by treatment of H9c2 cells with 10 mM BSO, were significantly decreased by KR-31378. These protective effect and antioxidant effect of KR-31378 appeared to be independent on K_{ATP} channel opening. Cells exposed to BSO showed an early reduction in MMP, and this reduction in MMP was significantly reversed by treatment with KR-31378. Caspase-3 activity in BSO treated H9c2 cells was remarkably increased, and this increased caspase-3 activity was significantly reversed by KR-31378. In conclusion, our results suggest that KR-31378 can produce cardioprotective effect against oxidative stress-induced cell death through antioxidant mechanism.

Key words: KR-31378, Oxidative stress, Cardioprotection, Antioxidant, ROS

INTRODUCTION

Oxidative stress refers to the cytopathologic consequences of an imbalance between the production of free radicals and the antioxidant system (Kumar and Jugdutt, 2003). A number of studies in the heart suggest that oxidative stress, associated with the production of reactive oxygen species (ROS), triggers cardiac myocyte death in a variety of diseases including myocardial infarction and ischemia/reperfusion injury (MacLellan and Schneider, 1997; Haunstetter and Izumo, 1998; Singal *et al.*, 1998). An increase in ROS is quite often accompanied by depletion of glutathione (GSH), a key cellular antioxidant (Carmody and Cotter, 2001). In ischemic heart, GSH deficiency has been demonstrated to result in enhanced vulnerability of cardiac myocytes to oxidative damage (Ceconi *et al.*, 1988). As shown in previous reports including ours (Jung *et al.*, 2004), experimental depletion of cellular GSH can be produced by using L-buthionine-*S*,*R*-sulfoximine (BSO), which inhibits γ-glutamylcysteine synthetase, an essential enzyme in glutathione biosynthetic cascade (Ettorre *et al.*, 2003).

Previously, we have demonstrated that a novel anti-ischemic K_{ATP} channel opener, KR-31378 [(2*S*,3*S*,4*R*)-*N*''-cyano-*N*-(6-amino-3,4-dihydro-3-hydroxy-2-methly-2-dimethoxy-methly-2*H*-benzo-pyran-4-yl)-*N*'-benzylguanidine], has cardioprotective effects against ischemia/reperfusion injury in rat myocardial infarct model (Yoo *et al.*, 2001). Our previous study has also reported that KR-31378 protects heart-derived H9c2 cells from hypoxia-induced death *via* the mitochondrial K_{ATP} channel opening (Moon *et al.*, 2004). Furthermore, our colleagues have demonstrated

Correspondence to: Yi-Sook Jung, Department of Physiology, School of Medicine, Ajou University, Suwon 442-749, Korea
Tel: 82-31-219-5044, Fax: 82-31-219-5049
E-mail: yisjung@ajou.ac.kr

that KR-31378 protects neuronal cells and smooth muscle cells from ROS-induced death (Yoo *et al.*, 2001; Kim *et al.*, 2001) through antioxidant mechanism. However, the antioxidant potential of KR-31378 in oxidative stress-induced injury in cardiac myocytes has not been examined. Therefore, the present study was designed to investigate whether KR-31378 can protect heart-derived H9c2 cells against cell death induced by oxidative stress using BSO.

MATERIALS AND METHODS

Cell cultures and drug treatment

Rat heart-derived H9c2 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM glucose supplemented with 10% fetal bovine serum. For hypoxic challenges, H9c2 cells were transferred into an anaerobic chamber (Forma Scientific, Marietta, OH, U.S.A.) maintained at 37°C with a humidified atmosphere of 5% CO_2, 10% H_2 and 85% N_2 as previously described (Moon *et al.*, 2000).

Chemicals

KR-31378 [(2*S*,3*S*,4*R*)-*N*-(6-amino-3,4-dihydro-2-dimethoxymethyl-3-hydroxy-2-methly-2*H*-benzopyranyl)-*N'*-benzyl-*N'*-cyanoguanidine] and HMR-1883 (1-[[5-[2-(5-chloro-*O*-anisamido)ethyl]-methoxyphenyl]-sulfonyl]-3-methylthiourea) were synthesized at Bio-organic Division of Korea Research Institute of Chemical Technology (Daejon, Korea). KR-31378 and HMR-1883 were dissolved in dimethyl sulfoxide (DMSO) to form stock solutions. The final DMSO concentration of 0.1% was found to have no effect on H9c2 cell viability. 5-hydroxydecanoate (5-HD) was purchased from Sigma Co. (St. Louis, MO, U.S.A.), was dissolved in distilled water and diluted with media to give a final concentration of 100 μM. Cells were treated with KR-31378 (3, 10 or 30 μM) simultaneously with BSO (Sigma Co., St. Louis, MO, U.S.A.).

Lactate dehydrogenase (LDH) assay

Cell death was analyzed by measuring LDH release as previously described (Moon *et al.*, 2000). The percent LDH was calculated from the maximum LDH release (100%) induced by lysing cells with 0.1% Triton X-100.

Hoechst staining

Hoechst 33258 has been widely used to obtain nuclear condensation, which is one of distinct morphological changes observed in apoptotic cells. After BSO treatment, cells were fixed with 4% PFA, and then incubated with 1 μg/mL Hoechst 33258 for 10 min at room temperature in dark. The cells were observed with a fluorescent microscope (Olympus, Japan).

Measurements of intracellular ROS

Intracellular ROS level was measured by fluorescence using 2'7'-dichlorofluorescein diacetate (DCFDA). This nonfluorescent dye freely permeates into cells, where it deesterifies to the ionized free acid (DCFH), which reacts with ROS to form fluorescent 2'7'-dichlorofluorescein (DCF). In brief, cells were washed with HCSS, loaded with 20 μM of DCF-DA and 20% Pluronic F-127 for 30 min at 37°C and then washed again with HCSS. For DCF measurement, excitation was monitored at 490 nm and fluorescence at 526 nm using a fluorescence plate reader.

Mitochondrial membrane potential (MMP) analysis

MMP was assayed by measuring the accumulation of rhodamine 123 (Molecular probes, Eugene, OR, U.S.A.), a membrane-permeable cationic fluorescent dye (Emaus *et al.*, 1986). H9c2 cells were loaded with 1 μM rhodamine 123 in HCSS. The cells were incubated for 10 min at 37°C and washed three times with HCSS. The cells were observed with a confocal microscope (Olympus, Japan) and the fluorescence intensity of rhodamine 123 was quantified using image-analysis computer software (Fluoview FV300; Olympus, Japan).

Measurement of caspase-3 activity

The cells were lysed with lysis buffer (10 mM Tris/HCl, 0.32 M Sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 1 μg/mL aprotinin, 10 μg/mL leupeptin, 5 mM EDTA, and 10 mM dithiothreitol (DTT), pH 8.0) for 30 min and the lysates were centrifuged (10,000×g at 4°C for 5 min). Samples (200 μg) of the extracted protein were incubated with the reaction buffer (100 mM HEPES, 10% sucrose, 0.1% 3-[3-cholamidopropylammonio]-1-propanesulfonate (CHAPS), pH 7.5, 10 mM DTT and 10 μg/mL leupeptin) to 100 μL volume containing 200 mM Ac-DEVD-*p*-Na (Biomol, Plymouth meeting, PA, U.S.A.). Enzyme-catalyzed release of *p*-NA was measured at 405 nm using microplate reader (Molecular Devices, Palo Aldo, CA, U.S.A.).

Statistical analysis

All data were expressed as mean±S.D. Numerical data were compared using Students' *t*-test for paired observations between two groups. A P value of <0.05 was considered significant.

RESULTS

Effect of KR-31378 on BSO-induced cell death

To investigate the effect of KR-31378 on BSO-induced cell death, H9c2 cells were exposed to 10 mM BSO for 24 h in the presence or absence of KR-31378 (3, 10 or 30 μM). In H9c2 cells exposed to BSO alone for 24 h, LDH

release was remarkably increased by 73.2 ± 2.2% compared to that in control. This increase in LDH release was inhibited by treatment with KR-31378 (Fig. 1).

Effect of KR-31378 on BSO-induced elevation of ROS level

We investigated the effect of KR-31378 on BSO-induced elevation of intracellular ROS level. The level of intracellular ROS was assessed using a peroxide-sensitive fluorescent probe, DCFDA. In H9c2 cells exposed to BSO for 16 h, DCFDA intensity was maximally increased up to 170.4 ± 6.7% compared to that in the control. This increased intensity of DCFDA was significantly decreased by co-treatment with several concentrations (3, 10, 30 μM) of KR-31378 (138.1 ± 7.4%, 116.7 ± 2.2%, 114.3 ± 1.1, respectively, Fig. 2).

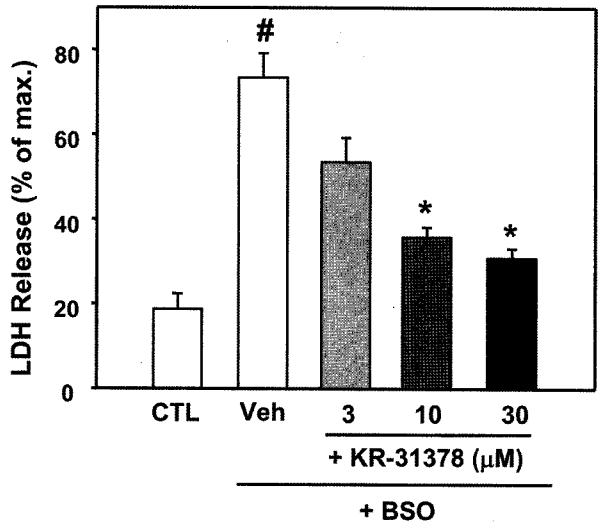

Fig. 1. Effect of KR-31378 on BSO-induced cell death. H9c2 cells were treated with KR-31378 (3, 10, and 30 μM) simultaneously with 10 mM BSO. LDH release was measured 24 h after treatment with BSO. Percent LDH was calculated from the maximum LDH release (100%) induced by 0.1% Triton X-100. CTL, control; Veh, vehicle. The data are mean±S.D. (n=5). #P<0.05 vs. CTL. *P<0.05 vs. Veh+BSO group.

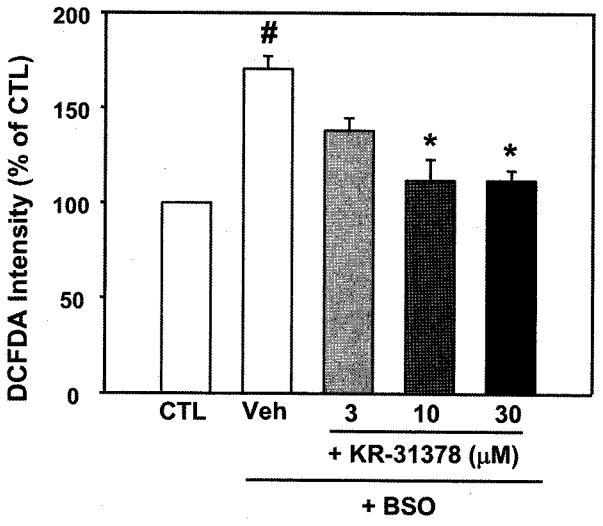

Fig. 2. Effect of KR-31378 on the BSO-induced ROS generation. H9c2 cells were treated with KR-31378 (3, 10, and 30 μM) simultaneously with 10 mM BSO. Intracellular ROS was measured 16 h after BSO treatment. CTL, control; Veh, vehicle. Data shown as mean±S.D. (n=5) represent the percentage of CTL. #P<0.05 vs. CTL. *P<0.05 vs. Veh+BSO group.

Effect of K_{ATP} channel blockers on KR-31378-induced cardioprotection

To investigate whether the protective effect of KR-31378

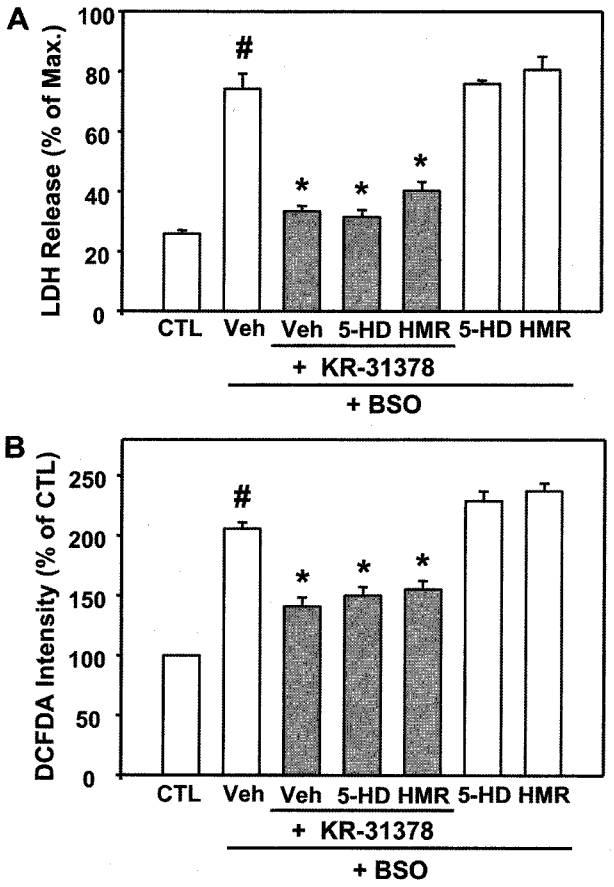

Fig. 3. Effect of K_{ATP} channel blockers on KR-31378-induced cardioprotection. A: The cells were treated with 10 μM KR-31378 simultaneously with 10 mM BSO in the absence or presence of 100 μM 5-HD or 10 μM HMR-1883. LDH release was measured 24 h after BSO treatment. Percent LDH was calculated from the maximum LDH release (100%) induced by 0.1% Triton X-100. CTL, control; Veh, vehicle. The data are mean±S.D. (n=5). #P<0.05 vs. CTL. *P<0.05 vs. Veh+BSO group. B: The cells were treated with 10 μM KR-31378 simultaneously with 10 mM BSO in the absence or presence of 100 μM 5-HD or 10 μM HMR-1883. Intracellular ROS was measured 16 h after BSO treatment. CTL, control; Veh, vehicle. Data shown as mean±S.D. (n=5) represent the percentage of CTL. #P<0.05 vs. CTL. *P<0.05 vs. Veh+BSO group.

was mediated through K_{ATP} channel, we examined the effects of specific inhibitors for the mitochondrial K_{ATP} channel, and sarcolemmal K_{ATP} channel, 5-HD, and HMR-1883, respectively, on KR-31378-induced protection. The protective effect of 10 μM KR-31378 against BSO-induced cell death and its inhibitory effect on BSO-induced elevation of ROS level remained unaltered in the presence of 5-HD or HMR-1883 (Fig. 3A and 3B). These results indicate that cardioprotection by KR-31378 is not contributed to K_{ATP} channel opening.

Effect of KR-31378 on BSO-induced MMP reduction

To examine whether preservation of MMP is associated with cardioprotective effects of KR-31378, we assessed the change of rhodamine 123 fluorescence (Emaus *et al.*, 1986) during oxidative stress-induced injury. The MMP reduction induced by BSO was completely blocked by 10 μM KR-31378 (Fig. 4A and 4B)

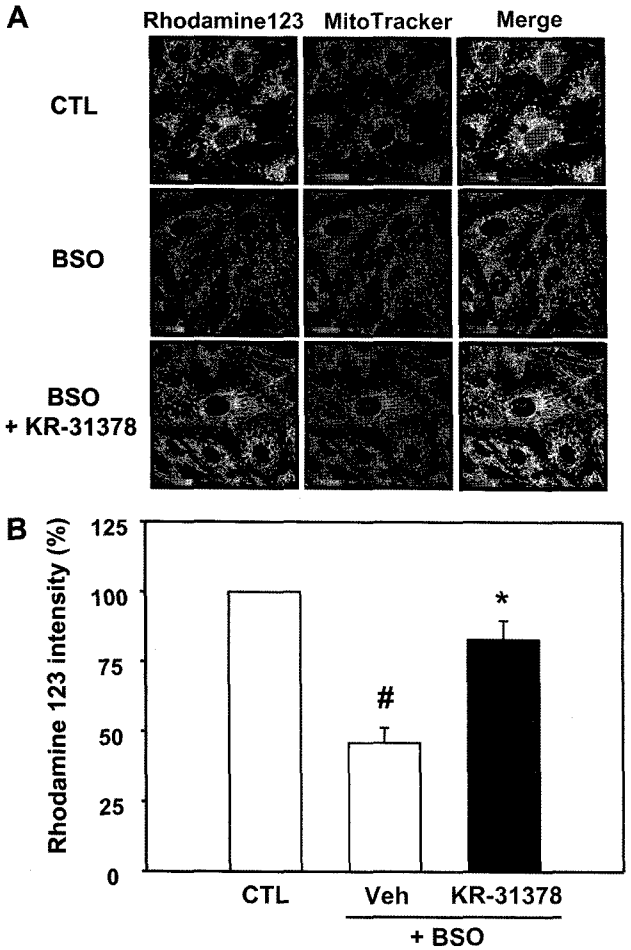

Fig. 4. Effect of KR-31378 on BSO-induced decrease in MMP. A: Fluorescence photomicrographs (stained with rhodamine 123) of cells after 16 h of BSO treatment. H9c2 cells were treated with 10 μM KR-31378 simultaneously with 10 mM BSO. CTL, control. Data shown are representative of 4 experiments. B: Quantitative analysis of rhodamine 123 intensity after 16 h of BSO treatment. H9c2 cells were treated with 10 μM KR-31378 simultaneously with 10 mM BSO. CTL, control; Veh, vehicle. The data are mean±S.D. (n=5). #P<0.05 vs. CTL. *P<0.05 vs. Veh+BSO group.

Effect of KR-31378 on BSO-induced apoptotic cell death

Fig. 5A shows the effect of KR-31378 on BSO-induced cell death, which was evaluated by Hoechst 33258 staining, as a well-known indicator of apoptotic cell death. Treatment of H9c2 cells with 10 mM BSO significantly increased the frequency of apoptotic cells with typical fragmented nuclei and condensed chromatin on histochemical nuclear staining with Hoechst 33258, as compared with control cells. The number of apoptotic cells induced by BSO was significantly decreased by 10 μM KR-31378 (Fig. 5A). Caspase-3 activity was analyzed from the cytosolic fraction of H9c2 cells 18 h after BSO treatment (270.8 ± 4.5%). The treatment of cells with 10 μM KR-31378 (165.1 ±8 .6%) markedly decreased the increased activity of caspase-3 induced by BSO (Fig. 5B).

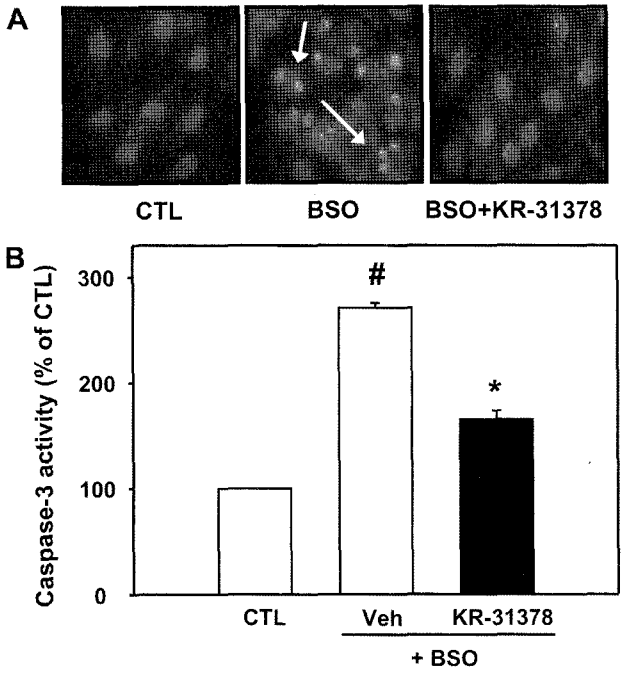

Fig. 5. Effect of KR-31378 on BSO-induced apoptotic cell death and caspase-3 activation. A: Apoptotic cell death was examined by Hoechst 33258 staining. H9c2 cells were treated with 10 μM KR-31378 simultaneously with 10 mM BSO. In Hoechst 33258-stained photographs, arrows indicate BSO-induced apoptotic cells. Data shown are representative of 4 experiments. B: Effect of KR-31378 on BSO-induced caspase-3 activation. H9c2 cells were treated with 10 μM KR-31378 simultaneously with 10 mM BSO. Caspase-3 activity was analyzed from the cytosolic fraction of H9c2 cells 18 h after BSO treatment. CTL, control; Veh, vehicle. The data are mean±S.D. (n=5). #P<0.05 vs. CTL. *P<0.05 vs. Veh+BSO group.

DISCUSSION

In the present study, we demonstrated that the benzopyran analogue KR-31378 protects heart-derived H9c2 cells from oxidative stress-induced cell death by attenuating ROS elevation and maintaining MMP during oxidative stress, independently of K_{ATP} channel.

In patients with chronic heart failure, increased oxidative stress is associated with reduced left ventricular function and correlates with the severity of the disease (Armstrong *et al.*, 2002). The increase in ROS is implicated in the pathogenesis of various cardiovascular diseases (Molyneux *et al.*, 2002). Moreover, cell culture and animal studies suggest that ROS may be important mediators of cardiac hypertrophy and the development of contractile dysfunction (Dhalla *et al.*, 2000). Ischemia results in impaired antioxidant defense, and the subsequent reperfusion results in an increased concentration of ROS (Kim *et al.*, 2001). If the quantity of free radicals exceeds the capacity of the endogenous antioxidant defense mechanisms, oxidative stress occurs (Korantzopoulos *et al.*, 2003). The results from this study showed that the treatment with KR-31378 protected H9c2 cells against BSO-induced death. In addition, KR-31378 significantly attenuated BSO-induced elevation of ROS level, suggesting that the preventive effect of KR-31378 against oxidative stress injury may be attributed to its antioxidant property. These results are consistent with previous studies reporting that KR-31378 protects cultured neurons from $FeSO_4$-induced cell death by attenuating lipid peroxidation (Kim *et al.*, 2004), and that KR-31378 inhibits apoptotic death of A7r5 smooth muscle cells by reducing H_2O_2-induced elevation of ROS level (Kim *et al.*, 2001).

A number of our previous studies have shown that KR-31378 possesses K_{APT} channel opening activity (Lee *et al.*, 2001; Yoo *et al.*, 2001) and antioxidant activity, both of which were considered to play important roles in neuroprotection (Hong *et al.*, 2002). Indeed, ROS are also implicated as important mediators of myocardial ischemia/reperfusion injury. We, therefore, first evaluated the effect of KR-31378 on oxidative stress-induced cardiac myocytes death, and found that antioxidant potential of KR-31378 might be involved in cardioprotection mechanisms. Recently, we have reported that KR-31378 possesses mitochondrial K_{APT} channel opening activity rather than sarcolemmal K_{APT} channel opening activity (Moon *et al.*, 2004). Interestingly, the antioxidant effect of KR-31378 shown in the present study appears to be independent on mitochondrial K_{APT} channel opening, as shown by the results that both the protective effect and the antioxidant effect of KR-31378 were remained unaltered in the presence of K_{APT} channel blockers. These results are consistent with the previous studies from our colleagues reporting that neuroprotective effect of KR-31378 is not related to K_{APT} channel opening activity. Our results further suggest that cardioprotective effect of KR-31378 may be contributed to both the K_{APT} channel opening activity and the antioxidant activity, which is similar to the case of the neuroprotection by KR-31378.

Cellular redox potential is largely determined by GSH, which accounts for more than 90% of cellular non-protein thiols (Domenicotti *et al.*, 2000). The majority of GSH is found in the cytosol (Meister, 1994), however a small, but significant, percentage of total cellular GSH (10–15%) is located in the mitochondria, which is the main organelles producing ROS and one of the main cytotoxic targets of ROS. Mitochondrial GSH is of paramount importance in protecting the organelle from ROS produced during coupled mitochondrial electron transport and oxidative phosphorylation (Meister, 1994). Indeed, depletion of GSH by using BSO, which inhibits a key enzyme for the pathway of GSH synthesis, has been demonstrated to allow for the generation of significant quantities of ROS from mitochondria (Langer *et al.*, 1996). Mitochondria are recognized as central regulators of life and death under various stresses in a variety of cells (MacLellan and Schneider, 1997). Cardiac mitochondria are highly vulnerable to injury induced by ischemia, which precipitates myocardial dysfunction in a number of disease conditions. In heart, mitochondrial dysfunction induced by oxidative stress or other stimuli has been demonstrated to produce the mitochondrial permeability transition, the disruption of mitochondrial membrane potential (MMP) and subsequently the cytochrome c release, leading to caspase-3 activation and lethal cell injury (Bialik *et al.*, 1999; Zorov *et al.*, 2000; Dzeja *et al.*, 2001). Thus the decrease in MMP is an early biochemical event in many types of apoptosis, occurring upstream of caspase activation (Ettorre *et al.*, 2003). In this study, we wanted to determine whether KR-31378-induced cardioprotection against BSO injury involves the recovery of MMP decrease and caspase-3 activation. Our results showed that MMP is decreased during BSO-induced oxidative stress, and that caspase-3 activity is increased during that process. KR-31378 significantly inhibited BSO-induced MMP reduction and caspase-3 activation. Although the present study did not involve further experiments to elucidate anti-apoptotic effect of KR-31378, it is suggested that the protective effect of KR-31378 against oxidative stress-induced H9c2 cell death may involve anti-apoptotic potential through maintaining the mitochondrial integrity and subsequently blocking mitochondrial death pathway. Further study remains to be elucidated to clarify the mechanism by which KR-31378 produces anti-apoptotic effect against oxidative stress-induced cardiac myocytes death.

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