KR-31378 Protects Cardiac H9c2 Cells from Chemical Hypoxia-Induced Cell Death via Inhibition of JNK/p38 MAPK Activation

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Abstract: Using a metabolic inhibition buffer as an ischemic model, we show here that KR-31378, a cardioselective ATP-sensitive potassium channel opener, protects H9c2 cells from chemical hypoxia (CH)-induced cell death. Our previous study showed that CH downregulated caspase activities, but led to differential activation of mitogen-activated protein kinases (MAPKs) in H9c2 cells. The repression of CH-induced c-jun N-terminal kinase (JNK)/p38 MAPK activation resulted in partial protection against CH-induced cell death, implying JNK/p38 MAPK’s causative role in CH-induced cell death. This study further that research and examines if KR-31378’s protective effect came from modulating MAPK activity and/or caspase activity in H9c2 cells. Although KR-31378 did not restore downregulated caspase-3 activity, it did block the activation of JNK and p38 MAPK in a dose-dependent manner. Extracellular signal-regulated kinase activity was not recovered by KR-31378 treatment. CH-induced reactive oxygen species (ROS) generation was suppressed by KR-31378. Thus our results indicate that the cardioprotective effect of KR-31378 in CH is due, at least in part, to the differential inhibition of MAPKs. [The Japanese Journal of Physiology 54: 575–583, 2004]

Key words: KR-31378, potassium channel opener, JNK, p38 MAPK, H9c2 cell.

Ischemia/reperfusion (I/R) injury has been identified as a major stress for cellular death in several disease states, including myocardial infarction [1]. I/R causes tissue injury as a result of both apoptosis and necrosis [2]. Although the mechanism of cardiomyocyte cell death is currently the subject of intensive investigation, a detailed study of signaling pathways leading to myocardial cell death following I/R has yet to be performed.

A brief episode of ischemia renders the heart more tolerant to a subsequent prolonged ischemic injury, an effect known as preconditioning [3, 4]. ATP-sensitive potassium channel (K<sub>ATP</sub>) has been suggested to serve as an endogenous cardioprotector because preconditioning is thought to be mediated by an activation of K<sub>ATP</sub> channels [5]. Although cromakalim was discovered as a K<sub>ATP</sub> channel activator, it opens K<sub>ATP</sub> channels also under nonischemic conditions [6]. In this regard, BMS-180448 ([3S-trans]-N-[4-chlorophenyl]-N′-cyano-N″-[6-cyano-3,4-dihydro-3-hydroxy-2,2-dimethyl-2H-1-benzopyran-4-yl] guanidine) was the first compound to demonstrate selective K<sub>ATP</sub> channel-opening activity under hypoxic conditions [7]. However, since cromakalim and BMS-180448 have vasodilator activity, the need for a cardioselective K<sub>ATP</sub> channel activator without this activity remained, prompting the development of KR-31378.

A novel benzopyran derivative, KR-31378 ([2S,3S,4R]-N″-cyano-N-[6-amino-3,4-dihydro-3-hydroxy-2-methoxymethyl-2H-benzopyran-4-yl]-N'-
benzylguanidine) (Fig. 1), was synthesized by the Korea Research Institute of Chemical Technology (Daejeon, Korea) as a chemical preconditioning agent [8]. KR-31378 is an antioxidant KATP channel opener that has been shown to protect rat cortex neurons against iron-induced oxidative injury, and it significantly reduces infarct size in a model of rat transient cerebral ischemia [9–11]. KR-31378 is a potent reactive oxygen species (ROS) scavenging agent and demonstrates an antiapoptotic effect in smooth muscle cells [12]. Recently, KR-31378 was reported to act as a potential inhibitor against atherosclerosis in a mouse model [13]. KR-31378 ameliorates atherosclerosis by inhibiting NF-κB–dependent cellular adhesion and the expression of chemotactic molecules such as the cell adhesion molecule and interleukin-8. The above-mentioned KR-31378 studies were performed in a cerebral ischemic model and endothelial cells, making the present study the first report to examine the protective effect of KR-31378 in heart-derived H9c2 cells.

METHODS

Chemicals and reagents. KR-31378 (Fig. 1), KR-31981 (1-methyl-5-nitroisatin), KR-31982 (1-benzyl-5-nitroisatin) [15], and BMS-180448 were synthesized and dissolved in dimethyl sulfoxide as 100 mM stock solutions. SP600125, an inhibitor of c-Jun N-terminal kinase (JNK), was obtained from TOCRIS (Ellisville, MO, USA). SB203580 (a highly specific inhibitor of p38 MAP kinase), U0126 (an inhibitor of MEK1), and z-Val-Ala-Asp (Ome)-CH2F (zVAD-fmk, a pan caspase inhibitor) were purchased from Calbiochem (La Jolla, CA, USA). α-Tocopherol was obtained from Sigma (Sigma, St. Louis, MO, USA).

Cell culture. Rat heart-derived H9c2 cells were cultured in Dulbeco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin [Gibco-BRL, Gaithersburg, MD, USA]). The cells were subcultured after trypsinization with a solution of trypsin-EDTA. They were grown to confluency at 37°C in the presence of 95% air and 5% CO2 in culture dishes.

Metabolic inhibition model. The cells were washed once with phosphate-buffered saline (PBS) before an addition of metabolic inhibition buffer (106 mM NaCl, 4.4 mM KCl, 1 mM MgCl2 · 6H2O, 38 mM NaHCO3, 2.5 mM CaCl2, 20 mM 2-deoxy-D-glucose, 1 mM NaCN, pH 6.6) [16] and placed in the metabolic inhibition buffer for indicated times (Fig. 2).

Measurement of cell viability and lactate dehydrogenase (LDH) release. Cell viability was quantified by the use of XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis[4-methoxy-6-

![Figure 1. Chemical structure of KR-31378.](image)

We have previously shown that chemical hypoxia (CH) induces myocardial cell death through the protein kinase C ε (PKCe)-JNK/p38 MAPK signaling pathway [14]. CH-induced cell death occurred in a caspase-independent manner. c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) were activated, and extracellular signal-regulated kinase (ERK) and caspases were inactivated under conditions of chemical hypoxia. Thus the present study examined if KR-31378’s protective effect came from modulating MAPK activity and/or caspase activity. Our data show that KR-31378’s cardioprotective effect is due, at least in part, to its ability to inhibit the JNK/p38 MAPK activation in CH-induced cell death.

![Figure 2. Experimental protocol for chemical hypoxic studies.](image)
Cardioprotection by KR-31378

Effect of KR-31378 on cell viability. In the present study, we examined the cell viability and death of H9c2 cells after 2 h of CH using XTT assay and LDH assay, respectively. In H9c2 cells exposed to CH for 2 h, cell viability was markedly reduced, and cell death was gradually increased. The decrease in cell viability was inhibited by treatment with KR-31378 (1–20 μM) in a concentration-dependent manner (Fig. 3, upper panel). After the application of KR-31378 (20 μM), the cells showed 65.3 ± 3.1% viability when compared to the untreated cells. Similarly, the LDH...
release was inhibited and decreased from 56.4 ± 2.4 (vehicle treated) to 25.9 ± 1.2% (20 µM, KR-31378) (Fig. 3) through a concentration-dependent treatment of KR-31378 (1–20 µM). BMS-180448 behaved in a similar fashion, but it was less potent than KR-31378 in cardioprotection.

**KR-31378 acts independent of the caspase cascade.** In this study, we tested whether the protective signaling pathway of KR-31378 was mediated through the caspase cascade. The specific inhibitors of caspase-3, KR-31981, and KR-31982, were used as controls for a caspase-3 activity assay [15]. In our previous report, we demonstrated that CH decreased the activity of caspase-3, caspase-8, and caspase-9 [14]. As shown in Fig. 4, caspase-3 activity was not affected by KR-31378 in vitro (Fig. 4, upper panel). This lack of change in caspase-3 activity in H9c2 cells indicates that KR-31378 acts independently of the caspase cascade (Fig. 4, lower panel).

**Characterization of CH-induced H9c2 cell death.** We performed a FACS analysis to characterize the protective mechanism of KR-31378. Rat heart derived H9c2 cells showed an increase of propidium iodide (PI) staining after CH, indicating the rapid onset of necrosis, and the increased cell population stained annexin V negative, excluding the possibility of apoptosis (Fig. 5). This method allows differentiation between apoptotic (annexin V positive, PI negative) and necrotic (annexin V negative, PI positive) cells. The amount of necrotic cells was decreased from 89.6 ± 1 (vehicle treated) to 17.4 ± 0.8% by the treatment with 20 µM of KR-31378 (Fig. 5). However, the number of necrotic cells was not significantly affected by treatment with zVAD-fmk, a pan caspase inhibitor. The failure of cardioprotection by zVAD-fmk treatment implies that caspases are not essential for the CH-induced cell death of H9c2 cells.

**Inhibition of CH-induced JNK/p38 MAPK activation by KR-31378 in H9c2 cells.** Several reports have shown that the activation of MAPKs is responsible for apoptosis [17, 18]. In our previous
report [14], we revealed causal relationship between CH-induced JNK/p38 MAPK activation and myocardial cell death. To investigate whether KR-31378 inhibits CH-induced JNK/p38 MAPK activation and cell death, H9c2 cells were incubated with KR-31378 30 min before and during the CH treatment. Figure 6 shows that CH treatment significantly induced the activation of JNK and p38 MAPK and that activation levels dropped dramatically upon treatment with KR-31378. In contrast, KR-31378 did not prevent the dephosphorylation of ERK1/2 in response to CH. The quantitative analysis of MAPK activities was performed by densitometry (Fig. 6B).

Fig. 5. Characterization of CH-induced H9c2 cell death. CH-induced myocardial cell death was analyzed by flow cytometry. The cells were double-stained by annexin V and PI 2 h after CH. The data shown are representative of three independent experiments (left panel), and the fluorescence-positive cells were quantitated and graphed (right panel). Ctrl, untreated cells; CH, chemical hypoxia; Veh, vehicle treated; zVAD, zVAD-fmk. *P < 0.05 vs. Ctrl.

KR-31378 (10 µM) and SP600125 (10 µM) downregulated JNK activity to 0.77- (Fig. 6B) and 1.53-fold (Fig. 7A), respectively, when compared to untreated cells upon CH. IC50 of KR-31378 in the suppression of CH-induced JNK activity in cardiomyocytes is 3.5 µM, which is comparable to the SP600125’s IC50 of 5–10 µM for the PMA-induced JNK activity suppression in Jurkat T cells [19]. KR-31378 was also more effective than SB203580 in p38 MAPK inhibition. Upon CH, KR-31378 (10 µM), and SB203580 (10 µM) downregulated p38 MAPK activity to 0.75 (Fig. 6B) and 1.08-fold (Fig. 7B), respectively, compared to the activity of untreated cells. Even though

Fig. 6. Regulation of MAPK activations by KR-31378 during CH. A: To investigate the effect of KR-31378 on MAPK phosphorylation, the cells were treated with KR-31378 at various concentrations (1, 5, 10, and 20 µM), as indicated. The levels of MAPKs were assayed by in vitro kinase assays as described under "METHODS." The activation of ERK1/2 was determined by a Western blot analysis using an antiphospho-ERK1/2 antibody. The activations of JNK and p38 MAPK were shown by radio-labeled substrates. The data shown are representative of three independent experiments. B: The relative levels of MAPK phosphorylation (shown by phosphorylated-c-Jun, -ATF-2, and -ERK1/2) were quantified with a bioimaging analyzer system (BAS-1800, Fujifilm Photofilm, Tokyo, Japan) and were graphed. The results were normalized to 1 for the untreated cells.
KR-31378 was more effective in MAPK suppression than SP600125 and SB203580, protective efficacies against cardiomyocyte death by KR-31378 and MAPK inhibitors were relatively similar because 10 µM of SP600125, SB203580, and KR-31378 increased the cell viability to 1.43-, 1.49-, and 1.55-fold of the untreated control, respectively.

KR-31378 inhibits chemical hypoxia-induced ROS formation. To determine whether KR-31378 suppresses the increase of intracellular ROS levels by CH, we assessed the effect of KR-31378 on ROS generation during CH. ROS was detected with DCFH-DA, which becomes highly fluorescent dichlorofluorescein (DCF) when oxidized by ROS. The exposure of H9c2 cells to 1 h of CH increased DCF fluorescence (Fig. 8A). KR-31378–treated cells (10 µM) showed 58.1 ± 6.2% of DCF intensity when compared to the vehicle-treated cells, indicating that KR-31378 blocked CH-induced ROS generation. BMS-180448, a benzopyran analog (10 µM), also prevented ROS formation during CH (70.5 ± 2.1% of DCF intensity compared to vehicle-treated cells). KR-31378 was better in scavenging ROS than BMS-180448.

KR-31378 demonstrated protective effects in HUVECs by decreasing intracellular ROS levels [12]. In this study, treatment with 10 µM KR-31378 also inhibited the CH-induced intracellular ROS rise in H9c2 cells (Fig. 8B). α-tocopherol was used as a positive control for ROS scavenging. The result implies that KR-31378–mediated protection from CH-induced cell death might be related to the inhibition of ROS formation.
The aim of this study was to examine the mechanism of KR-31378, a benzopyran analog, in cell survival against CH in H9c2 cells derived from rat hearts. The cardioprotective effect of KR-31378 is associated with the inhibition of JNK/p38 MAPK activation. However, KR-31378 restored neither the inactivated ERK1/2 activities nor the downregulated caspase activities in CH. Collectively, our data demonstrate that the cardioprotection of KR-31378 occurs via differential modulation of MAPKs in a caspase-independent manner.

Even though JNK and p38 MAPK activity returns to below the control level with 10 µM KR-31378 treatment, protection from ischemic cell death was not complete (56.1 ± 1.0% of control). A failure of complete protection of ischemic H9c2 cells by the inhibition of JNK and p38 MAPK activations suggests the presence of JNK/p38 MAPK independent ischemic death pathways. ERK activity restoration might act as a complement to provide complete protection, since ERK activity is required for survival signaling in response to growth factors in L929 cells and PC12 cells and for protection against I/R-induced cardiomyocytic cell death [20–22]. It is conceivable that a combination of JNK/p38 MAPK and ERK activities might determine the extent of cardiomyocyte survival.

CH certainly displays characteristics of cell death.
different from those of apoptosis. Typical apoptotic phenomena such as caspase activation and phosphatidyl serine translocation in the cell membrane were not observed in CH-induced cell death (Fig. 5). The transient ATP depletion of cells converts an apoptotic death signal to a necrotic one [23]. In our ischemic model, therefore, the failure of caspase activation might be caused by the depletion of ATP, a component of apoptosis. Thus at least for CH-induced cell death in H9c2 cells, the caspase-independent MAPK signaling module appears to play an important role.

Several benzopyran K\textsubscript{ATP} channel activators such as diazoxide, cromakalim, and BMS-180448 selectively open the MitoK\textsubscript{ATP} channel and demonstrate a cardioprotective effect against I/R, suggesting the upstream and downstream of the MitoK\textsubscript{ATP} channel as a target molecule of cardioprotection [24–26]. And ROS plays an important role both in ischemia and reperfusion, PKC\textepsilon\rightarrow JNK/p38 MAPK signaling module [14]. Moreover, KR-31378 acts as a protective agent against CH in cardiac muscle. Proc Natl Acad Sci USA 85: 8360–8364, 1988.

In the previous report, we newly identified the PKC\textepsilon \rightarrow JNK/p38 MAPK signaling module [14]. The phosphorylations during CH are blocked by KR-31378, it would be interesting to see if KR-31378 could also modulate PKC\textepsilon activation. Moreover, KR-31378 prevents ROS generation induced by CH. Thus the disclosure of the relationships among the ROS generation, PKC\textepsilon, and JNK/p38 MAPK activations would help us to understand the molecular mechanism underlying CH-induced cardiac cell death.

In conclusion, we have demonstrated that KR-31378 acts as a protective agent against CH in cardiomyocytes. This protection is associated with the differential regulation of MAPKs. This study has implications not only for a better understanding of signal pathways in CH-induced cardiomyocyte death, but also for the clinical application of KR-31378 as a cardioprotective agent.

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (02-PJ1-PG10-20905-0007).

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