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European Journal of Pharmacology 506 (2004) 27–35

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KR-31378, a novel benzopyran analog, attenuates hypoxia-induced cell death via mitochondrial K_{ATP} channel and protein kinase C-ε in heart-derived H9c2 cells

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Received 8 April 2004; received in revised form 13 October 2004; accepted 14 October 2004
Available online 11 November 2004

Abstract

A novel compound 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*-benzopyran-4-yl)-*N*-benzylguanidine] has been demonstrated as an anti-ischemic agent in rat heart and brain. Here, we report the effects of this compound on hypoxia-induced cell death and possible signaling pathways in heart-derived H9c2 cells. Treatment with KR-31378 (3–30 μM) 1 h before and during hypoxia significantly reduced hypoxia-induced cell death in a concentration-dependent manner. In addition, increase in hypoxia-induced transferase UTP nick end labeling (TUNEL)-positive cells was reduced by KR-31378, suggesting its antiapoptotic potential in H9c2 cells. The protective effect conferred by KR-31378 (10 μM) was abolished by cotreatment with 5-hydroxydecanoate (5HD), a specific blocker of the mitochondrial K_{ATP} (mtK_{ATP}) channel, but not by HMR-1883 (1-[[5-[2-(5-chloro-*o*-anisamido)ethyl]-methoxyphenyl]sulfonyl]-3-methylthiourea), a specific blocker of the sarcolemmal K_{ATP} channel. We observed that the treatment with KR-31378 could increase the expression of protein kinase C (PKC)-ε protein, but not other PKC isotypes (-α, -β, -δ, -ζ), in the particulate fraction. This increased level of PKC-ε was sustained during the hypoxic period up to 8 h. In addition, our results showed that treatment with KR-31378 induced the expression of PKC-ε mRNA as early as 15 min after the treatment. A specific inhibitor for PKC-ε isoform, εV1-2, completely blocked the protective effect of KR-31378 against hypoxia-induced cell death. In conclusion, our results suggest that KR-31378 can protect cultured H9c2 cells from hypoxia-induced death via the mtK_{ATP} channel and PKC-ε.

Keywords: KR-31378; Potassium channel; Protein kinase C-ε; Cardiac cell; Hypoxia

1. Introduction

Ischemic preconditioning is a well-known phenomenon in which brief episodes of ischemia/reperfusion render the myocardium resistant against a subsequent, more sustained ischemic insult (Murry et al., 1986). Although the precise signaling pathways involved in ischemic preconditioning have not been fully elucidated, several molecules, including protein kinase C (PKC) and an ATP-sensitive potassium (K_{ATP}) channel, are regarded as essential elements for triggering and maintenance of protection (Light et al., 2001). Although protection by K_{ATP} channels was initially thought to occur via the K_{ATP} channels in the sarcolemma ($sarcK_{ATP}$ channels) of myocytes, several studies have shown that K_{ATP} channels in the mitochondrial inner membrane mitochondrial K_{ATP} (mtK_{ATP}) channels are more important in this regard (Garlid et al., 1997; Gross and

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doi:10.1016/j.ejphar.2004.10.037

Fryer, 1999; Liu et al., 1998; Liu et al., 1999). Recently, an interlink between the mtK_{ATP} channels and PKC has been reported in the signaling process of cardioprotection against ischemia (Wang et al., 2001).

We previously found that benzopyranyl indole-2-carbocylic ester analog (KR-31466) showed good cardioprotective efficacy in vitro possibly through the opening of mtK_{ATP} (Jung et al., 2003). Unfortunately, however, KR-31466 showed little effect on infarct size in rat myocardial infarct model in vivo, and these results seem to be attributed to the susceptibility of its chemical structure, such as an ester group, to metabolic cleavage in vivo. We have, therefore, focused on developing compounds with better phramacokinetic profiles as well as good efficacies. In such attempts, a novel anti-ischemic cyanoguanidine analogue, 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] was found to be metabolically stable, leading to a beneficial pharmcokinetic profile (Kim et al., 2000). We previously demonstrated the anti-ischemic effects of KR-31378 in several in vivo studies including rat and dog myocardial infarct models (Yoo et al., 2001; Lee et al., 2002). In vitro effects of KR-31378, however, have not been elucidated in cultured cardiomyocytes. Therefore, the present study was designed to investigate the effect of KR-31378 on hypoxia-induced cell death in heart-derived H9c2 cells.

2. Materials and methods

2.1. Cell culture and hypoxia system

Heart-derived H9c2 cells were purchased from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM glucose supplemented with 10% fetal bovine serum before experimentation. For hypoxic challenges, H9c2 cells were transferred into an anaerobic chamber (Forma Scientific, Marietta, OH, USA) maintained at 37 °C with humidified atmosphere of 5% CO_2, 10% H_2 and 85% N_2 as described before (Kim et al., 2003). In the anaerobic chamber, the culture medium was replaced with serum-free, glucose-free DMEM that had been saturated with N_2 gas for 1 h. Normoxic incubation of the cells in the serum-free DMEM was conducted in a water-jacked incubator gassed with 95% O_2 and 5% CO_2 at 37 °C.

2.2. Chemicals and treatment

KR-31378 and HMR1883 (1-[[5-[2-(5-chloro-*o*-anisamido)ethyl]-methoxyphenyl]-sulfonyl]-3-methylthiourea), which were synthesized at the Bio-organic Division of Korea Research Institute of Chemical Technology (Daejon, Korea), and myristoylated PKC-ε V1-2 (εV1-2, Biomol, Plymouth Meeting, PA, USA) were dissolved in dimethyl sulfoxide (DMSO) as 100 mM stock solutions. The final concentration of DMSO was 0.1% and this concentration of DMSO was found to have no effect on H9c2 cell viability. 5-hydroxydecanoate (5HD), which was purchased from Sigma (St. Louis, MO, USA), was dissolved in distilled water and diluted with media to give a final concentration of 100 μM. H9c2 cells were treated with KR-31378 (3, 10 or 30 μM) according to experimental protocols shown in Fig. 1. 5HD, HMR-1883 and εV1-2 were used as specific inhibitors for mtK_{ATP} channel (Sato and Marban, 2000), sarcolemmal (sarc) K_{ATP} channel (Wirth et al., 1999) and PKC-ε isoform, respectively. The concentrations of inhibitors' treatment were chosen on the basis of preliminary studies (data not shown).

2.3. Propidium iodide staining

To examine the extent of cell death, we stained the cells with propidium iodide (10 μg/ml), which is normally taken up by dead cells, but not by living cells, after 8 h of hypoxic period (Moon et al., 2000). The percent cell death was calculated from the number of propidium iodide-stained cells divided by total cell count.

2.4. In situ terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assay

To examine the extent of apoptotic cell death, we performed TUNEL-staining after 8 h of hypoxia as described

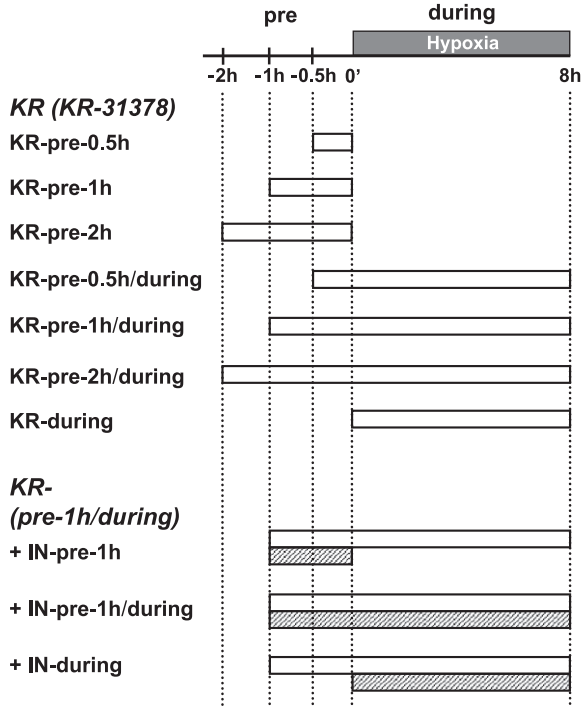

Fig. 1. Experimental protocols for study. ☐ KR (KR-31378); ▨ IN (inhibitors: 5HD, εV1-2); pre represents pretreatment before hypoxic insult; during represents treatment during hypoxic insult; pre/during represents pretreatment before hypoxic insult and treatment during hypoxic insult. See text for details.

previously (Jung et al., 2003). In situ labeling of fragmented DNA was performed by TUNEL assay with the commercially available ApopTag Plus kit (Oncor, Gaithersburg, MD, USA). Monolayers of H9c2 cells were grown on 24-well plates and fixed with 4% paraformaldehyde. Then nucleosome-sized DNA fragments were tailed with digoxigenin nucleotide and reacted with fluorescein-conjugated antidigoxigenin antibodies. The nucleus was counterstained with hematoxyline. The percent cell death was calculated from the number of TUNEL-positive cells divided by total cell count.

2.5. Western blot analysis

To determine whether KR-31378 can modulate the expression of PKC isozymes before and during hypoxia, Western blot analysis was performed. The cells in 6-well plates were lysed using 150 μl/well of lysis buffer (20 mM Tris–HCl, 2 mM EDTA, 5 mM EGTA, 5 mM dithiothreitol, 6 mM β-mercaptoethanol, 1 mM PMSF (pheylmethylsulfonyl fluoride), 20 mM leupeptin, 50 μg/ml aprotinin, pH 6.8). H9c2 cells have grown up to 99% confluence at a density of 2×10^5 cells/well in 6-well plates. The particulate (membrane) and soluble (cytosolic) fractions of the lysates were separated by centrifugation at 100,000×*g* for 1 h at 4 °C, and the supernatant was collected for the soluble fraction. Pellets resuspended in the same volume of lysis buffer containing 1% Triton X-100 were centrifuged at 10,000×*g* for 10 min at 4°C, and the supernatant was collected for the particulate fraction (Jiang et al., 1996; Rogers et al., 2001; Mohammadi et al., 2001; Kim et al., 2003). A quantity of 40 μg protein of either the soluble fraction or particulate fraction was separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MD). The membrane was blocked with 5% nonfat dry milk for 90 min, and incubated overnight with PKC isotype antibodies for PKC-α, -β, -δ, -ε and -ζ (Santa Cruz, Delaware Avenue, CA, USA). The membrane was then incubated with a secondary immunoglobulin antibody conjugated with alkaline phosphatase for 4 h, and the PKC band was visualized using the NBT/BCIP method (Sigma-Aldrich, St. Louis, MO, USA).

2.6. mRNA expression of PKC

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to assess mRNA expression of PKC-ε. Total RNA was extracted from the cells with RNAsol (InTron, Sungnam, Korea) according to the manufacturer's suggested protocol. The total RNA (1 μg) was synthesized into cDNA using random hexamers, avian myeloblastosis virus reverse transcriptase (Promega, Serva, Heidelberg, Germany) and PCR buffer (20 mM Tris–HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 μg/ml bovine serum albumin). The cDNA (2 μl) was amplified by PCR in a total volume of 20 μl using 0.5 U Taq DNA polymerase (Bioneer, Seoul, Korea), 100 μM dATP, dCTP and dGTP, 50 μM dTTP (Boehringer-Mannheim, Mannheim, Germany), and 0.5 μM of each primer in PCR buffer. One-minute cycles were performed at 95, 60 and 72 °C in a microprocessor-driven thermal cycler (Perkin Elmer-Cetus, Emeryville, CA, USA). Primers for PKC-ε were 5′-ACT GCT CCC ACT GCA GAG AT 3′ (sense) and 5′-TAG TTC CTG GTC ACA AGG GG-3′ (antisense), while the primers for GAPDH were 5′-CCA TGG AGA AGG CTG GG-3′ (sense) and 5′-CAA AGT TGT CAT GGA TGA CC-3′ (antisense).

2.7. Statistical analysis

All data were expressed as mean±S.D. The numerical data were compared using a one-way ANOVA followed by a posttest such as Bonferoni test. A *P*-value of <0.05 was considered significant.

3. Results

3.1. Protective effect of KR-31378 against hypoxia-induced cell death

To evaluate whether KR-31378 protects H9c2 cells from hypoxia, we performed propidium iodide-staining as an indicator for cell death, after 8 h of hypoxic period. To examine the effect of KR-31378 against hypoxic injury, we treated H9c2 cells with KR-31378 by several protocols (7 treatments), as shown in Fig. 1. In H9c2 cells exposed to hypoxia for 8 h, propidium iodide uptake was increased up to about 40% compared to that in the normoxic condition (7.5±2.0%). When KR-31378 (3, 10 and 30 μM) was treated by pretreatment (Fig. 2A) or during-treatment (Fig. 2B), there was no change in hypoxia-induced cell death. On the other hand, hypoxia-induced cell death (42.3±4.32%) was significantly reduced by combination of pretreatment and during-treatment (KR-pre-1 h/during, KR-pre-2 h/during) of 10 μM KR-31378 (13.3±1.21% and 14.7±0.84%, respectively, Fig. 2C). When cells were treated with several concentrations (3, 10 and 30 μM) of KR-31378 by 1 h pretreatment and during-treatment (KR-pre-1 h/during), the uptakes of propidium iodide during hypoxia (37.8±3.4%) were significantly decreased (31.6±2.81%, 12.2±2.53% and 8.9±0.42%, respectively, Fig. 2D). These results indicate that some modulation by KR-31378 should precede hypoxic insult to trigger a protective effect during hypoxia. Thus, we thereafter focused on the protective effect of KR-31378 treated by KR-pre-1 h/during.

3.2. The role of the mtK_{ATP} channel in KR-31378-induced cardioprotection

To investigate whether the protective effect of KR-31378 was mediated through the mtK_{ATP} channel or $sarcK_{ATP}$, we examined the effects of specific inhibitors for the mtK_{ATP}

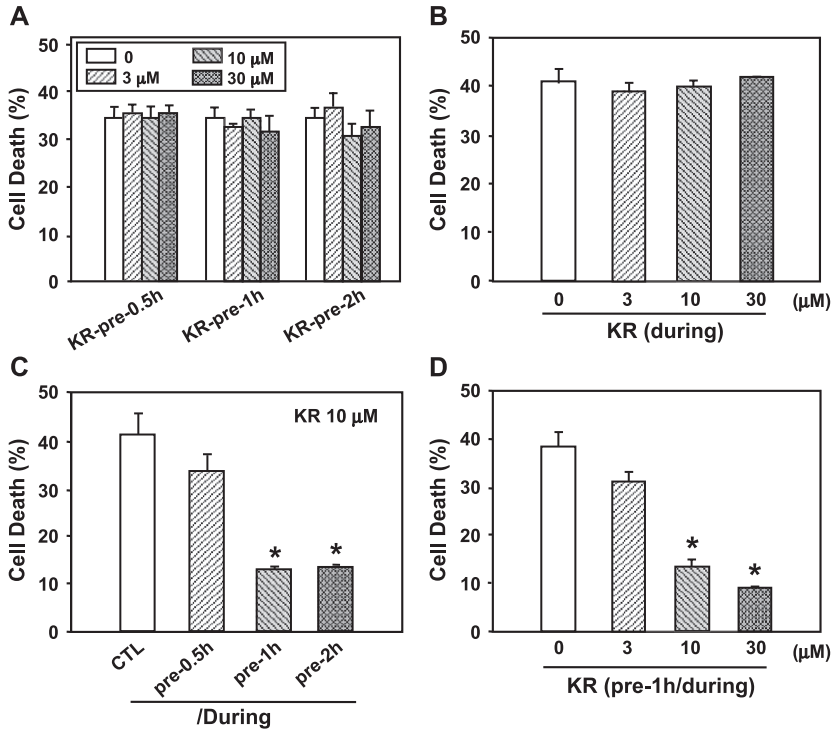

Fig. 2. Effect of KR-31378 on hypoxia-induced cell death in H9c2 cells. (A) H9c2 cells were treated with KR-31378 (3, 10 and 30 μM) 0.5, 1 or 2 h before hypoxia (KR-pre-0.5 h, KR-pre-1 h and KR-pre-2 h, respectively). (B) H9c2 cells were treated with KR-31378 (3, 10 and 30 μM) during hypoxia (KR-during). (C) H9c2 cells were treated with 10 μM KR-31378 by combination of pretreatment and during treatment (KR-pre-0.5 h/during, KR-pre-1 h/during, KR-pre-2 h/during). (D) H9c2 cells were treated with KR-31378 (3, 10 and 30 μM) by 1 h of pretreatment and during treatment (KR-pre-1 h/during). Cell death (%) was calculated by dividing the number of propidium iodide-stained cells by total number of cells 8 h after hypoxic insult. All data represent the mean±S.D. (n=4–5). *P<0.01 vs. hypoxic control (CTL, 0).

channel and sarcK_{ATP}, 5HD and HMR-1883, respectively, on KR-31378-induced protection. Fig. 3 shows the effects of 5HD on KR-31378-induced protection against hypoxia-induced cell death shown by propidium iodide-uptake. Fig. 3A (KR-pre-1 h/during+5HD-pre-1 h/during group) shows that the protective effect by 10 μM KR-31378 against hypoxia-induced cell death (12±1.4%) was completely abolished by cotreatment with 100 μM 5HD (31±0.5%), while it remained unaltered by cotreatment with 10 μM HMR-1883 (13±1.1%). These results indicate that KR-31378 protects cardiac cells against cell death during hypoxia via the mtK_{ATP} channel rather than the sarcK_{ATP} channel. Interestingly, an inhibitory effect of 5HD was also revealed when 5HD was treated with KR-31378 by pretreatment only (Fig. 3B, KR-pre-1 h/during+5HD-pre-1 h group). On the other hand, hypoxia-induced cell death remained unaltered when 5HD was treated by during-treatment (Fig. 3C, KR-pre-1 h/during+5HD-during group).

3.3. Protective effect of KR-31378 against hypoxia-induced apoptosis

To investigate the effect of KR-31378 on hypoxia-induced apoptotic cell death, we performed TUNEL-staining. As shown in Fig. 4, the amount of TUNEL-positive cells were 20.9±2.4% in vehicle-treated cells exposed to hypoxia for 8 h. This amount was decreased by treatment with 10 μM KR-31378 (4.71±1.24%), suggesting an antiapoptotic potential of KR-31378 in H9c2 cells. This antiapoptotic effect of KR-31378 was completely blocked by 100 μM 5HD (21.1±1.72%).

3.4. Effect of KR-31378 on PKC isozyme expression

We examined whether the treatment of H9c2 cells with 10 μM of KR-31378 could stimulate the expression of PKC isozymes. As shown in Fig. 5A and B, treatment with KR-31378 alone for 1 h produced an approximately twofold increase in the expression of PKC-ε in the particulate fraction (189±11% of the vehicle-treated control), while PKC-ε in the soluble fraction remained unchanged. KR-31378, however, had no effect on other types of PKC (-α, -β, -δ, -ζ) in either the particulate or the soluble fractions (Fig. 5A). We further examined the time course effect of the KR-31378 treatment (0.5, 1 and 2 h) and found that KR-31378 alone tended to increase PKC-ε protein as early as 30 min after treatment (135±2.5%), reaching a peak after 1 h (276±3.1%, Fig. 6A and B). To clarify whether this stimulatory effect of KR-31378 on PKC-ε protein is associated with its effect on mRNA, we determined the mRNA level of PKC-ε by RT-PCR. The mRNA levels of PKC-ε remarkably increased at 15 min after the KR-31378

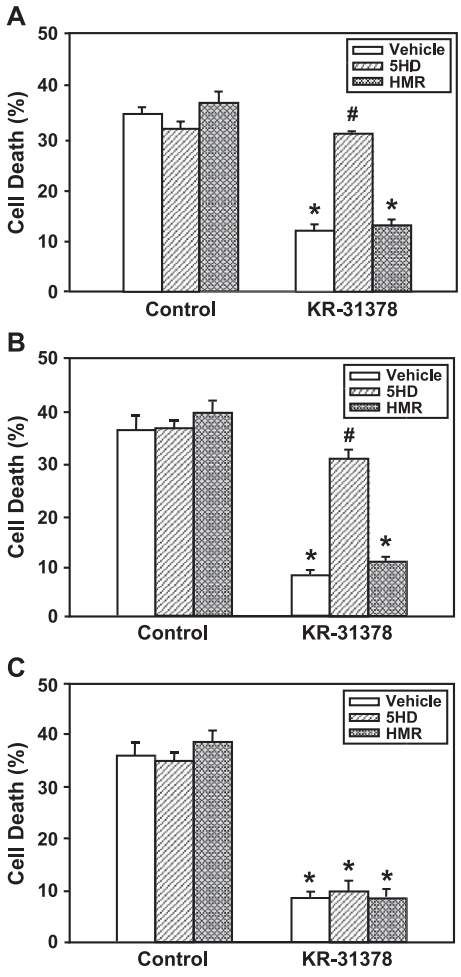

Fig. 3. Effect of 5-hydroxydecanoate (5HD) and HMR-1883 on KR-31378-induced protection against hypoxic cell death. (A) 'KR-pre-1 h/during+IN-pre-1 h/during', H9c2 cells were cotreated with KR-31378 and K_{ATP} channel inhibitors (IN; 5HD or HMR-1883) 1 h before and during hypoxia. (B) 'KR-pre-1 h/during+IN-pre-1 h', H9c2 cells were cotreated with KR-31378 and K_{ATP} channel inhibitors (IN; 5HD or HMR-1883) 1 h before hypoxia, thereafter, treated with KR-31378 alone during hypoxia. (C) 'KR-pre-1 h/during+IN-during', H9c2 cells were treated with KR-31378 alone 1 h before hypoxia, thereafter, cotreated with KR-31378 and K_{ATP} channel inhibitors (IN; 5HD or HMR-1883) during hypoxia. Concentrations for KR-31378, 5HD and HMR-1883 were 10, 100 and 10 μM, respectively. Cell death (%) was calculated by dividing the number of propidium iodide-stained cells by total number of cells 8 h after hypoxic insult. All data represent the mean±S.D. (n=4–5). *P<0.01 vs. vehicle-treated hypoxic control (Control). $^{\#}P$<0.01 vs. KR-31378-treated hypoxia.

treatment and this increased level was sustained until 45 min after the treatment, followed by a decrease in its level (Fig. 6C).

3.5. Effect of KR-31378 on PKC-ε expression during hypoxia

We investigated the effect of KR-31378 on PKC-ε for short (2 h) and long (8 h) periods following the onset of hypoxia. In agreement with our previous report (Kim et al., 2003), the expression of PKC-ε in particulate fraction was significantly reduced 2 and 8 h after the onset of hypoxia (Fig. 7). In H9c2 cells treated with KR-31378, the protein expression of PKC-ε in particulate remained almost unaltered at 2 h of hypoxia, and only slightly decreased at 8 h of hypoxia (Fig. 7). PKCε in soluble fraction appears to decrease in normoxia with KR-31378 treatment, as well as in hypoxia with and without KR-31378, but not to a significant degree.

3.6. The role of PKC-ε in KR-31378-induced cardioprotection

To elucidate the role of PKC-ε in KR-31378-induced protection, we examined the effect of the pharmacological blockade of PKC-ε on hypoxia-induced cell death by using εV1-2, a specific cell-permeable inhibitor for PKC-ε (Gray et

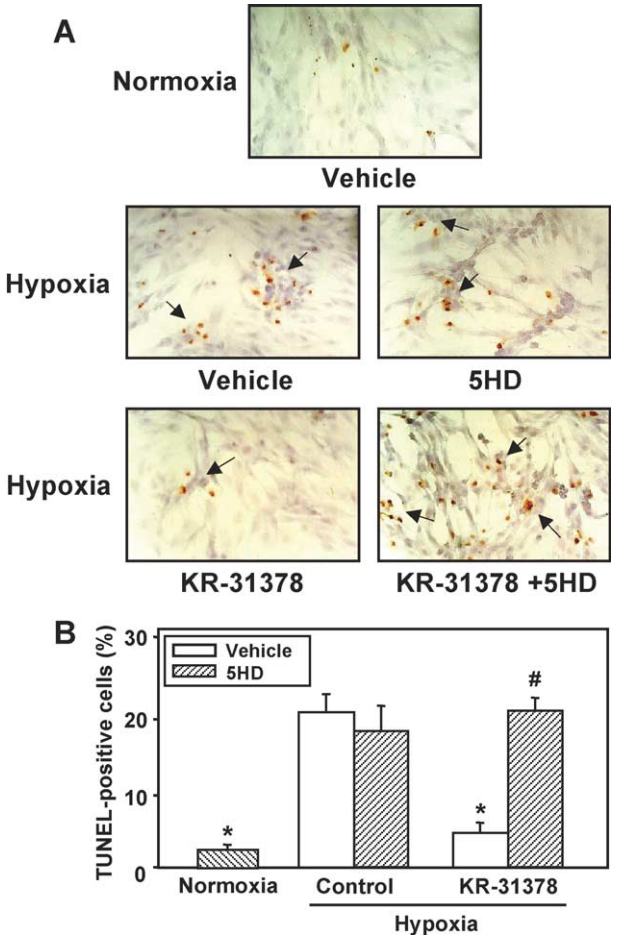

Fig. 4. Effect of 5-hydroxydecanoate (5HD) on KR-31378-induced protection against hypoxia-induced apoptosis. Apoptotic cell death was examined by TUNEL-staining. H9c2 cells were treated with KR-31378 (10 μM) 1 h before and during hypoxia in the absence or presence of 100 μM 5HD (KR-pre-1 h/during+5HD-pre-1 h/during). (A) TUNEL-stained photographs. Arrows indicate TUNEL-stained H9c2 cells. Data shown are representative of four experiments. (B) Percent TUNEL-positive cells of total cell count, mean±S.D. (n=5). *P<0.01 vs. vehicle-treated hypoxic control (Control). $^{\#}P$<0.01 vs. KR-31378-treated hypoxia (Vehicle-KR-31378).

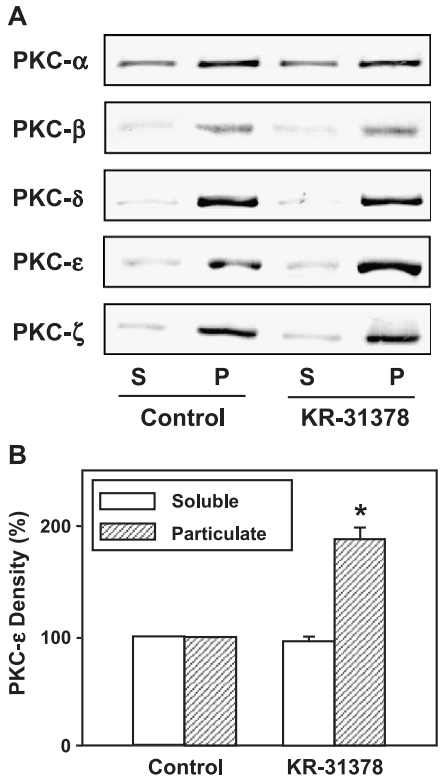

Fig. 5. Western blot analysis and quantitative analysis after the treatment with H9c2 cells with KR-31378. (A) Western blots for PKC isotypes (α, β, δ, ε and ζ) detected in the soluble (S) and the particulate (P) fraction after 1 h of KR-31378 (10 μM) treatment. Shown are representative Western blots of three separate experiments. (B) Quantitative analysis for PKC-ε in the soluble and particulate fraction, mean±S.D. (n=5). *P<0.01 vs. vehicle-treated control (Control) in particulate fraction.

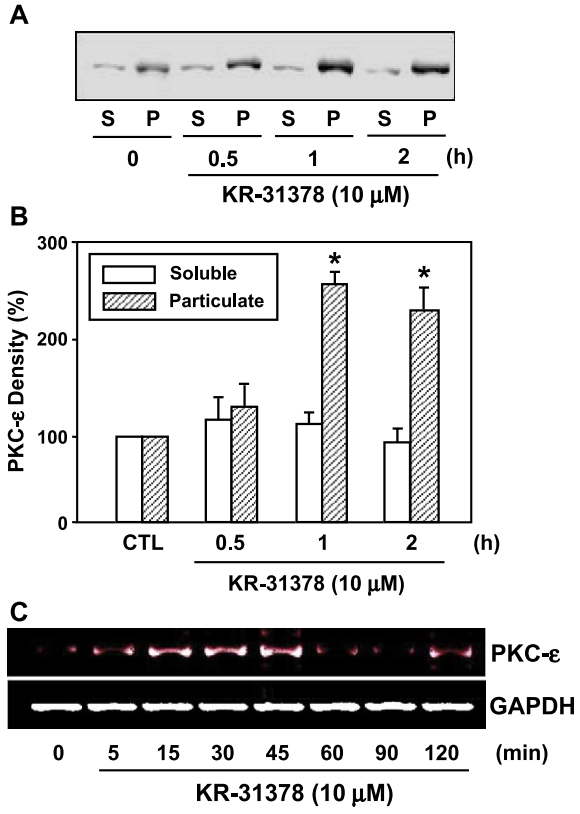

Fig. 6. Western blot analysis and RT-PCR analysis after the treatment with H9c2 cells with KR-31378. (A) Western blots for PKC-ε detected in the soluble (S) and the particulate (P) fraction after various periods of treatment (0, 0.5, 1 and 2 h) with KR-31378 (10 μM) in H9c2 cells. Shown are representative Western blots of three separate experiments. (B) Quantitative blot analysis for PKC-ε in a soluble and particulate fraction. (C) RT-PCR products after various periods of treatment (0, 5, 15, 30, 45, 60, 90 and 120 min) with KR-31378 (10 μM). Total RNAs extracted from the H9c2 cells were reverse transcribed with hexanucleotide primers; cDNA was amplified with specific primers for PKC-ε. Shown are representative of three separate experiments. *P<0.01 vs. vehicle-treated control (CTL, 0 time) in particulate fraction.

al., 1997). εV1-2 peptide, derived from the first unique region (V1) of PKC-ε (amino acids 14–21), is known to inhibit PKC-ε translocation and activity (Gray et al., 1997; Johnson et al., 1996). In addition, we have previously demonstrated that εV1-2 decreases the amount of PKC-ε expression in cardiomyocytes (Kim et al., 2003). As shown in Fig. 8A (KR-pre-1 h/during+εV1-2-pre-1 h/during group), we found that the protective effect of KR-31378 (9.8±0.3%) against hypoxic cell death was completely abolished by cotreatment with 10 ìM εV1-2 (29.1±2.9%). These results indicate that KR-31378 protects cardiac cells against cell death during hypoxia via activation of PKC-ε. We further found that the inhibitory effect of εV1-2 was also revealed when 10 ìM εV1-2 was treated by pretreatment only (Fig. 8B, KR-pre-1 h/during+εV1-2-pre-1 h group). On the other hand, hypoxia-induced cell death remained unaltered when εV1-2 was treated by during-treatment (Fig. 8C, KR-pre-1 h/during+εV1-2-during group).

4. Discussion

In the present study, we have demonstrated that a novel cyanoguanidine analogue KR-31378 can protect heart-derived H9c2 cells from hypoxic injury via the mtK$_{ATP}$ channel and PKC-ε.

We previously demonstrated the anti-ischemic effect of KR-31378 in various in vivo models, including rat (Yoo et al., 2001) and dog (Lee et al., 2002) myocardial infarct models and a rat cerebral ischemia model (Hong et al.,

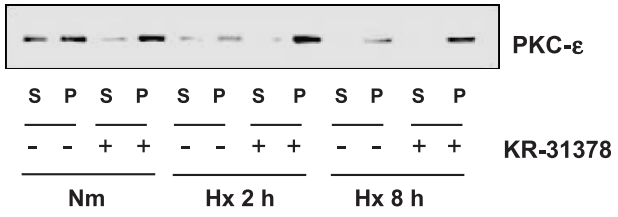

Fig. 7. Effect of KR-31378 on PKC-ε expression during hypoxia. H9c2 cells were treated with KR-31378 (10 μM) for 1 h and then exposed to normoxia (Nm) or hypoxia (Hx) for 2 or 8 h. Shown are Western blots for PKC-ε detected in the soluble (S) and the particulate (P) fraction, and representatives of three experiments.

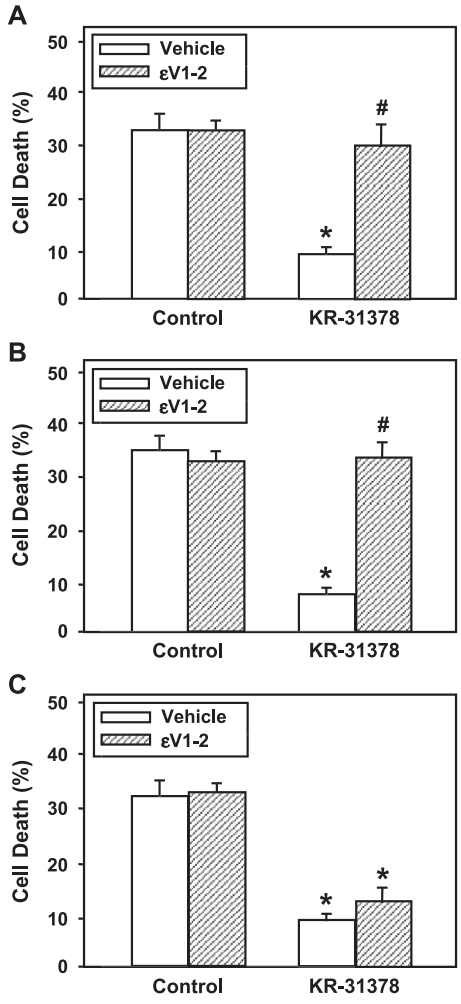

Fig. 8. Effect of PKC-ε inhibitor on KR-31378-induced protection against hypoxic cell death. (A) 'KR-pre-1 h/during+IN-pre-1 h/during', H9c2 cells were cotreated with KR-31378 (10 μM) and PKC-ε inhibitor (IN: εV1-2, 10 μM) 1 h before and during hypoxia. (B) 'KR-pre-1 h/during+IN-pre-1 h', H9c2 cells were cotreated with KR-31378 (10 μM) and PKC-ε inhibitor (IN: εV1-2, 10 μM) 1 h before, thereafter, treated with KR-31378 alone during hypoxia. (C) 'KR-pre-1 h/during+IN-during', H9c2 cells were treated with KR-31378 (10 μM) alone 1 h before hypoxia, thereafter, cotreated with KR-31378 (10 μM) and PKC-ε inhibitor (IN: εV1-2, 10 μM) during hypoxia. Cell death (%) was calculated by dividing the number of propidium iodide-stained cells by total number of cells 8 h after hypoxic insult. All data represent the mean±S.D. (n=4–5). *P<0.01 vs. vehicle-treated hypoxic control (Control). #P<0.01 vs. KR-31378-treated hypoxia (Vehicle-KR-31378).

2002). From results in the present in vitro study demonstrating that KR-31378 (KR-pre-1 h/during) protects H9c2 cells against hypoxia-induced cell death in a concentration-dependent manner (Fig. 2D), it is suggested that cardiomyocytes are cellular targets of KR-31378 responsible for the initiation of protection in the heart. The current results further indicate that the cardioprotection by KR-31378 is elicited, at least partly, via its antiapoptotic activity in cardiomyocytes (Fig. 4). When cells were treated with KR-31378 for several periods of pretreatment (pre-0.5, -1 and -2 h) and subsequently exposed to hypoxia in the presence of KR-31378 (/during), a protective effect by KR-31378 was observed in KR-pre-1 h/during and KR-pre-2 h/during groups (Fig. 2C). On the other hand, we observed that there was no significant protective effect in KR-pre and KR-during groups (Fig. 2A and B). These results are not consistent with our previous in vivo results showing that the cardioprotective efficacy was still demonstrable even when the administration of KR-31378 commenced after occlusion (ischemia) in the rat myocardial infarct model (Lee et al., 2002). This discrepancy may be explained by different environment between in vivo and in vitro systems, and injuries between hypoxia and ischemia/reperfusion. From these results, it is suggested that some modulation by KR-31378 in cardiomyocytes should precede hypoxic insult and be maintained during hypoxia to trigger a protective effect during hypoxia.

Regarding the possible signaling pathways involved in the coupling mechanism by which KR-31378 produces a cardioprotective effect, we focused on the mtK$_{ATP}$ channel. Our previous in vivo study has demonstrated that the reduction in the infarct zone afforded by KR-31378 is blocked by 5HD in the rat infarct model, suggesting the involvement of the mtK$_{ATP}$ channel in cardioprotection (Yoo et al., 2001; Lee et al., 2002). Consistently, a number of studies, including our previous report (Jung et al., 2003; Liu et al., 1999; McCully and Levitsky, 2003), have demonstrated that the mtK$_{ATP}$ channel is an important component in the mechanism of cardioprotection. In addition, a selective mtK$_{ATP}$ channel activator diazoxide is known to exert a cardioprotective effect against ischemia-reperfusion injury in the rat and human heart (Garlid et al., 1997; Ghosh et al., 2000), and the cardioprotection induced by diazoxide is abolished by 5HD (Takashi et al., 1999; Liu et al., 1999). The present in vitro study has provided evidence supporting this concept for the essential role of the mtK$_{ATP}$ in a cultured cell system by showing that the protective effect conferred by KR-31378 (10 μM) was completely abolished by cotreatment with 5HD, but not by HMR-1883 (Fig. 3A and B).

Recently, it has been suggested that the protective effect of the mtK$_{ATP}$ channel opener is associated with PKC (Wang and Ashraf, 1999; Takashi et al., 1999). Evidence for the link between PKC activation and mtK$_{ATP}$ channel opening has been provided by results demonstrating that the PKC activator, phorbol 12-myristate 13 acetate (PMA) potentiates and accelerates the effect of diazoxide and that this effect of PMA is blocked by 5HD (Light et al., 2001). The cardioprotective effect of diazoxide is also abolished by PKC downregulation (Wang et al., 2001). Indeed, PKC has long been known to play an essential role in cardioprotection in a variety of animal models (Speechly-Dick et al., 1994). Consistently, our previous study demonstrated the protective role of PKC in heart-derived H9c2 cells (Moon et al., 2000) and an isolated rat heart model (Moon et al., 1999). PKCs are classified into three subfamilies, which include the conventional (α, βI, βII, γ), novel (δ, ε, η, θ, ι) and atypical (ζ, μ, λ) isoforms based on $Ca^{2+}$and phospholipid sensitivity (Simkhovich et al., 1998).

Although the predominant isozymes in rat heart have been reported to be α, β, δ, ε and ζ subtypes (Disatnik et al., 1994), the roles of PKC isozymes in cardiomyocytes have not been fully characterized. Our results in this study imply that PKC-ε plays an essential role in KR-31378-induced cardioprotection. This conclusion is based on the following data: (1) treatment with KR-31378 increases the expression of PKC-ε protein in the particulate fraction (Fig. 5) and this increased protein level is maintained during hypoxia (Fig. 7); and (2) a specific inhibitor for PKC-ε isoform, εV1-2, completely abolishes the protective effect by KR-31378 against hypoxia-induced cell death (Fig. 8A). In addition, the treatment of H9c2 cells with KR-31378 alone induced the expression of PKC-ε mRNA as early as 15 min after the treatment (Fig. 6C). On the other hand, our results failed to show the translocation of PKC isoforms, a hallmark of PKC activation, in KR-31378-treated cells. As the reason for the absence of alteration of soluble PKC-ε, we suppose that the soluble PKC-ε may be immediately translocated to the particulate fraction, as soon as translated from mRNA in the cytosol. Based on previous studies, including ours (Kim et al., 2003), indicating that the activation of PKC can be elicited by the induction of PKC expression at the protein and mRNA level, it is suggested that the activation of PKC-ε may be involved in KR-31378-induced protection. However, it remains to be investigated whether KR-31378 directly increases PKC-ε activity. Consistent with our results, diazoxide by itself was demonstrated to selectively activate PKC-ε in the particulate fraction before simulated ischemia without any effect on the cytosolic fraction in chick ventricular myocytes (Liu et al., 2002). Others also demonstrated that PKC-ε is translocated to intercalated disks in rat heart, suggesting a correlation between PKC activation and protection by the mtK_{ATP} channel (Wang and Ashraf, 1999; Wang et al., 1999). There are, however, contrasting results to ours about the effect of the mtK_{ATP} channel modulators on PKC-δ. Contrast to our results that PKC-δ remains unaltered by the mtK_{ATP} channel modulator KR-31378 (Fig. 5), Wang and Ashraf (1999) and Wang et al. (1999) have demonstrated that PKC-δ is translocated to mitochondria by diazoxide in rat heart and that cardioprotection by diazoxide is abolished in PKC-δ downregulated hearts, suggesting an essential role of PKC-δ in cardioprotection (Wang et al., 2001). This discrepancy appears to be related with the cell type-specificity and chemical type-specificity, i.e., diazoxide vs. KR-31378.

To investigate the role of KR-31378-induced induction and maintenance of PKC-ε during hypoxia, we examined the influence of a specific blockade of PKC-ε on hypoxia-induced cell death. When cells were treated with εV1-2 together with KR-31378, the protective effect shown in the KR-31378-treated cells was completely blocked (Fig. 8A). These results imply that PKC-ε upregulated by KR-31378 plays an essential role in conferring the resistance on H9c2 cells against hypoxic injury. Although we did not evaluate the ability of KR-31378 to regulate the expression of PKC-ε at the mRNA level during hypoxia, the possibility of reduced degradation of the PKC-ε protein as well as increased expression by KR-31378 during hypoxia cannot be excluded. The present results concerning the role of PKC-ε are consistent with our previous finding that εV1-2 abolished the protective effect of high glucose against hypoxic injury in H9c2 cells (Kim et al., 2003). The beneficial role of PKC-ε against ischemic injury has been reported in various studies, including preconditioning-induced cardioprotection (Gray et al., 1997) and adenosine-induced neuroprotection (Di-Capua et al., 2003). There have been, however, reports that conflict with the present results, documenting the detrimental roles of PKC-ε against hypoxic cell death in cardiomyocyte (Shizukuda and Buttrick, 2001). Indeed, we have demonstrated the proapoptotic role of PKC-ε during chemical hypoxia in cardiomyocytes (Jung et al., 2004). It is, therefore, suggested that the pathophysiological role of PKC-ε may be complicated, and that this enzyme may exert either beneficial or deleterious effects depending on cells and/or injuries.

In this study, we found a good correlation between the duration of KR-31378 pretreatment required for producing a cardioprotective effect and that for affecting the expression of PKC-ε protein. Specifically, we demonstrated that at least 1 h of pretreatment period was required for KR-31378 to produce the maximum protective effect (in KR-pre-1 h/during group) and the maximum expression of PKC-ε protein (in KR-1 h group; Figs. 2C, and 6A and B). On the other hand, KR-31378 did not show any protective effect in the KR-pre group or in the KR-during group. Interestingly, we also found that the inhibitory effect of 5HD or εV1-2 on cardioprotection by KR-31378 was revealed in the KR-pre-1 h/during+5HD(or εV1-2)-pre-1 h group (Figs. 3B and 8B). Taken together, our results suggest that the induction of PKC-ε and activation of the mtK_{ATP} channel by KR-31378 before onset of hypoxic insult are necessary, but not sufficient for triggering a cardioprotective effect during the process of hypoxia-induced cell death in H9c2 cells.

There have been controversial reports about the upstream and downstream relationship between PKC and mtK_{ATP} channel activation during an ischemic insult. Recently, it has been reported that cardioprotection by activation of the mtK_{ATP} is elicited via PKC signaling pathways (Wang et al., 2001; Liu et al., 2002). On the other hand, another study suggests that PKC is an upstream molecule for the activation of the mtK_{ATP} channels (Sato et al., 1998). Thus, further study is needed to clarify the signaling pathway by which KR-31378 induces PKC expression and mtK_{ATP} channel activation leading to cardioprotection.

Acknowledgement

This research was supported by a Grant (CBM1-A100-001-1-0-1) from the Center for Biological Modulators of the 21st Century Frontier R&D Program, the Ministry of

Science and Technology, and from Dongbu Hannong Chemical Company.

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