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KB-R7943 Reduces Necrosis and Apoptosis in Hyperlipidemic Animals through the Activation of K+ATP Channels

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Abstract

Reverse-mode activation of the Na^+-Ca^{2+} exchanger (NCX) at the time of reperfusion following ischemia contributes to Ca^{2+} overload and cardiomyocyte injury, KB-R7943, as a selective reverse-mode NCX inhibitor, reduces lethal reperfusion injury under normal conditions, but its effectiveness under certain pathological states is in dispute. In the present study, we sought to determine the effect of KB-R7943 in hyperlipidemic animals and assess if the K^+_{ATP} are involved in the protective mechanisms. Anesthetized rats were randomized into five groups, as follows, and were subjected to 25min global ischemia (GI) followed by 120 min reperfusion (R). Normally fed animals: a Control group (NC) with no additional intervention. Cholesterol fed (6weeks) animals: a Chol group (HC) with no additional interventions, a KB-R7943 group (HKB) treated with KB-R7943, a KB-R7943 and glybenclamide group (HGKB) treated with KB-R7943 and glybenclamide (K^+_{ATP}, blocker, 0.3μM), a glybenclamide group (HGLY) treated with glybenclamide

The infarct size was measured by triphenyltetrazolium. The infarct size was 35±5.0% in NC, 46±8.7% in HC and 47±8.5% in HGLY (NC vs. HC, P<0.05; HC vs. HGLY, P>0.05). KB-R7943 reduced the infarct size (28.6±3.3% in HKB vs. HC, P<0.05), In addition, KB-R7943 attenuated apoptotic cell (HKB vs. HC, p<0.05), glybenclamide abolished the effect reached by KB-R7943. Thus, diet-induced hypercholesterolemia enhances myocardial injury; KB-R7943 reduces infarct size and apoptosis in hyperlipidemic animals through the activation of K^+_{ATP} channels.

Keywords: Myocardial infarction; Hypercholesterolemia; Apoptosis; KB-R7943; K^+_{ATP}

Introduction

Ischemic heart disease (IHD), a major cause of mortality in industrialized societies, is characterized by insufficient blood supply to regions of the myocardium which leads to tissue necrosis (infarction). Patients with IHD are likely to have a number of co-morbid conditions at the time of presentation, many of which can influence the sensitivity of the myocardium to certain cardioprotective strategies [1]. In this study, we investigated the effects of hypercholesterolemia alone on the ischemic myocardium in an isolated rat model of acute ischemia-reperfusion (IR).

Only a few studies have used hypercholesterolemic animals and the findings are still controversial. Some investigators reported that diet-induced hypercholesterolemia enhances myocardial injury by increasing oxidative stress [3], upregulation of inflammation [4], inhibition of nitric oxide synthesis [5], vascular obstruction [6], and increased cardiomyocyte apoptosis [7], while others have found no additional harm after reperfusion injury [2,7], or have shown that hypercholesterolemia improves myocardial function and may even be cardioprotective [8].

Reverse-mode activation of the Na^+-Ca^{2+} exchanger (NCX) at the time of reperfusion following ischemia contributes to Ca^{2+} overload and cardiomyocyte injury [9]. KB-R7943 (2-[2-[4-(4-nitrobenzyloxy) phenyl] ethy1] -isothiourea methanesulfonate) is a relatively new compound that was developed as a selective reverse-mode NCX inhibitor. A number of studies have investigated whether KB-R7943 reduces ischemia and reperfusion injury in the intact heart. KB-R7943 has been shown to decrease enzyme release [10,11] and reduce infarct size following myocardial ischemia [12,13]. KB-R7943 reduces lethal reperfusion injury under normal conditions, but its effectiveness under certain pathological states is in dispute. In the present study, we sought

to determine the effect of KB-R7943 in hypercholesterolemic animals and assess if the K^+_{ATP} channel is involved in the protective mechanisms.

Methods

Animals

Male Wistar rats were randomly assigned to 2 different dietary groups: 12 of them weighing 300-350 at the time performing I/R experiment, were fed standard rat food (NC) for 6 weeks and 48 others weighing 380-450 at the time performing I/R experiment ,received a hypercholesterolemic diet (1.5% cholesterol) for 6 weeks (HC, HKB, HGKB and HGLY). All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH). The protocol was approved by the committee of experimental animals of China Medical University.

Langendorff perfusion and experimental design

Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (100mg kg^{-1}). Heparin (1,500IU kg^{-1}) was administered intravenously to prevent intracoronary clot formation. The heart was rapidly excised and immediately immersed in ice-cold heparinized-modified Krebs-Henseleit buffer containing (in $mmol^{-1}$)

127 NaCl, 17.7 NaHCO$_3$, 5.1 KCl, 1.5 CaCl$_2$, 1.26 MgCl$_2$, 11 D-glucose (pH7.4). The heart was mounted on a Langendorff-perfusion apparatus and retrogradely perfused through the aorta with recirculating buffer saturated with 95%O2-5% CO$_2$ at 37°C. The heart was maintained in a thermostatic chamber at 37°C. Perfusion was maintained at a constant pressure of 75 mmHg. A fluid-filled latex balloon was inserted in the left ventricle (LV) via the left atrium for pressure measurement. The balloon was connected to a pressure transducer and inflated to an initial LV end-diastolic pressure between 8 and 10 mmHg.

The hearts were divided into five groups: 1) Group 1(NC, n=12) - global ischemia for 25 min, by interrupting the coronary perfusion, and reperfusion for 120 min; 2) Group 2(HC, n=12) –The same protocol as in Group 1 was performed, but the animals were fed with a hypercholesterolemic enriched diet (1.5% cholesterol) for 6 weeks. 3) Group3 (HKB, n=12) -The same protocol as in Group 2 was performed, but 1μM KB-R7943 was added to the perfusate for 10 min from the start of reperfusion. 4) Group4 (HGKB, n=12) - The same protocol as in Group3 was performed and glybenclamide (K$^+_{ATP}$ blocker, 0.3μM) was administered for 10 min from the start of reperfusion. 5) Group5 (HGLY, n=12) –The same protocol as in Group 2 was performed, but 0.3μM glybenclamide was added to the perfusate for 10 min from the start of reperfusion.

Biochemical analyses

Blood samples were obtained both at the beginning of the protocol and on the day of the sacrifice to determine serum cholesterol. Cholesterol (C) was determined using commercial enzymatic kits (BHKD, Beijing, China), C-HDL (high-density lipoprotein) and C-LDL (low-density lipoprotein) were determined by selective precipitation methods.

Measurement of myocardial function

Homodynamic assessment included heart rate (HR), +dp/dt, -dp/dt, and LVDP. These parameters were continuously monitored throughout the experimental protocol. The HR, +dp/dt, -dp/dt and LVDP were sampled and digitally processed via a homodynamic system (BIOPAC MP150, USA)

Measurement of infarct size

Infarct size was determined as previously described in five consecutive randomly selected rats from each groups [14]. After 2h of reperfusion, the hearts were harvested and the LVs were sectioned from apex to base into 2-3 mm sections. Following 20 min of incubation at 37°C in 1% triphenyltetrazolium chloride (TTC; Sigma, St Louis, MO, USA), unstained tissue was carefully separated from stained tissue by an independent observer. In fact, while unstained tissue represents the amount of death cells, the stained tissue represents the viable cells. The unstained mass was expressed as a percentage of total left ventricular mass. In fact, the total left ventricle mass also corresponds to the risk area because a global ischemia was performed.

TUNEL Staining

Apoptotic cells were identified using the ApopTag detection kit according to manufacturer's specifications (Calbiochem, La Jolla, CA). Three sections from each myocardial sample were randomly selected, and 10 microscopic fields per section were evaluated by two independent blind observers. In each field, the nuclei were counted and the percentage of TUNEL positive nuclei was calculated.

Statistical analysis

All values are expressed as means ± SD. Statistical analyses were performed by using Sigma Stat software version 3.5 (Systat software). Differences between groups were evaluated using one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls post hoc test. For the Langendorff data, ANOVA for repeated measurements was used. A P value < 0.05 was considered to be statistically significant.

Results

Biochemical analyses

Cholesterol plasma levels according to the assigned diet are shown in Table 1. There was a statistically significant increase in total cholesterol and LDL-cholesterol levels in animals fed with cholesterol enriched diet compared with the control group (p <0.05).

Changes of hemodynamics

Table 2 shows the values of HR, +dp/dt, -dp/dt and LVDP at baseline and during the different times of reperfusion. A hypercholesterolemic enriched diet did not affect myocardial function following I/R injury. Using KB-R7943 significantly protected cardiac function of LVDP and

	Total Cholesterol (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
Before cholesterol-enriched diet	68.6±10.5	47.6±7.2	13.7±0.5
After cholesterol-enriched diet	106.1±3.6 *	55.8±13.0	25.2±1.3 *

*p< 0.05 vs. before cholesterol-enriched diet

Table 1: Biochemical Analysis (n=6).

TIME	baseline	R-10	R-20	R-30
HR				
NC	256±23*	150±18	174±19	170±14
HC	157±20	125±26	155±23	148±15
HKB	175±12	158±22	178±27	176±22
HGKB	160±18	126±17	146±12	148±16
HGLY	155±16	126±21	160±15	155±12
LVDP(mmHg)				
NC	86±6.4	34±5.4	38±5.1	34±3.2
HC	84±7.3	32±7.7	38±6.5	33±4.3
HKB	83±6.6	45±7.9*	47±6.0*	40±3.4*
HGKB	88±7.5	31±8.4	34±5.7	30±4.9
HGLY	84±6.9	34±6.7	39±4.2	34±3.4
+dp/dt				
NC	2322±233	1589±103	1622±208	1362±198
HC	2318±187	1466±259	1589±217	1284±114
HKB	2472±274	1946±202*	2066±287*	1754±203*
HGKB	2231±262	1489±301	1584±211	1335±158
HGLY	2330±198	1476±225	1601±201	1378±167
-dp/dt				
NC	1476±222	1378±178	1422±117	1238±156
HC	1378±107	1109±167	1271±189	979±134
HKB	1408±190	1689±312*	1608±202*	1556±234*
HGKB	1400±156	1176±232	1298±345	1078±198
HGLY	1401±165	1188±198	1274±237	1056±159

Values are means ±SD. +dp/dt, Positive first order derivative of ventricular pressure; -dp/dt, Negative first order derivative of ventricular pressure LVDP, left ventricular developed pressure. *P<0.05 vs.G2. n=7

Table 2: The measure and behavior of HR, +dp/dt, -dp/dt, and LVDP before and during reperfusion.

+/- dP/dt in HC group. However, this protection was reversed by using glybenclamide (K+ATP blocker), whereas glybenclamide did not have effect in I/R+hypercholesterolemia group, suggesting that inhibition of NCX would result in increased K+ATP.

Measurement of infarct size

As illustrated in Figure 1. The infarct size was 35±5.0% in NC, 46±8.7% in HC (NC vs. HC, P<0.05). Using KB-R7943 significantly reduced infracted size up to 28.6±3.3% in HC group (P<0.05 vs. HC). However, glybenclamide (K+ATP blocker) abolished the protective effect, increasing the infarct size to 42.8±5.1%, whereas glybenclamide did not have effect in I/R+hypercholesterolemia group (47±8.5%), suggesting that inhibition of NCX would result in increased K+ATP

TUNEL staining for apoptosis

Quantitative analysis of DNA fragmentation was performed by using the TUNEL method at the single-cell level. TUNEL-positive cardiomyocyte nuclei are shown in Figure 2. Percentage of TUNEL-positive cardiomyocytes was 7.1±1.4% in NC, 14.8±3.0% in HC (NC vs. HC, p<0.05). Using KB-R7943 significantly reduced the percentage of apoptotic cells up to 28.6±3.3% in HC group (P<0.05 vs. HC). However, glybenclamide (K+ATP blocker) abolished the protective effect, increasing the percentage of apoptotic cells to 13.1±1.7%, whereas glybenclamide did not have effect in I/R+hypercholesterolemia group (13.6±3.0%), suggesting that inhibition of NCX would result in increased K+ATP.
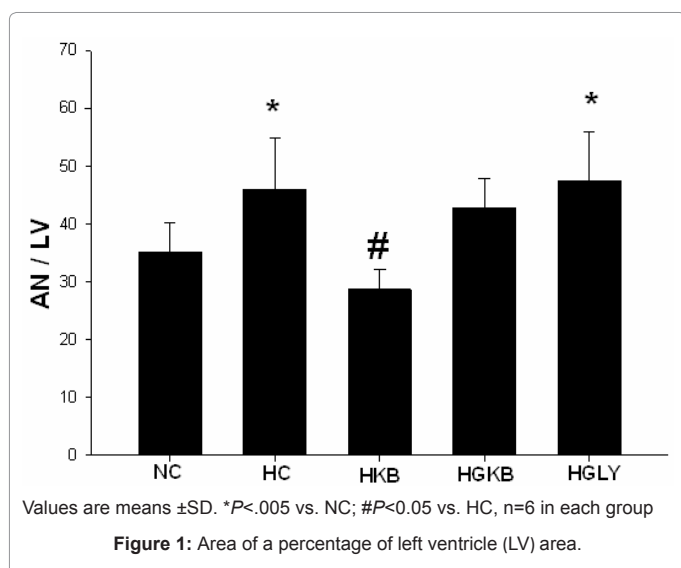
Discussion

To date, myocardial IR injury studies conducted in hypercholesterolemic animals have yielded varied and controversial findings. The reasons for these discrepancies are unknown. There are a few possible explanations: the duration of index ischemia, gender, age, and species. In the present study, we have demonstrated that diet-induced hypercholesterolemia enhances myocardial injury, and the extent of myocyte apoptosis detected by TUNEL assay is also significantly enhanced in HC compared with NC. Therefore, diet-induced hypercholesterolemia enhances myocardial injury by improving myocyte apoptosis. KB-R7943 reduces infarct size and apoptosis in hyperlipidemic animals and glybenclamide abolishes the effect reached by KB-R7943 and it does not increase infarct size on

its own. Thus, we conclude that the K^+_{ATP} channel is involved in the protective mechanisms in hypercholesterolemia enriched animals.
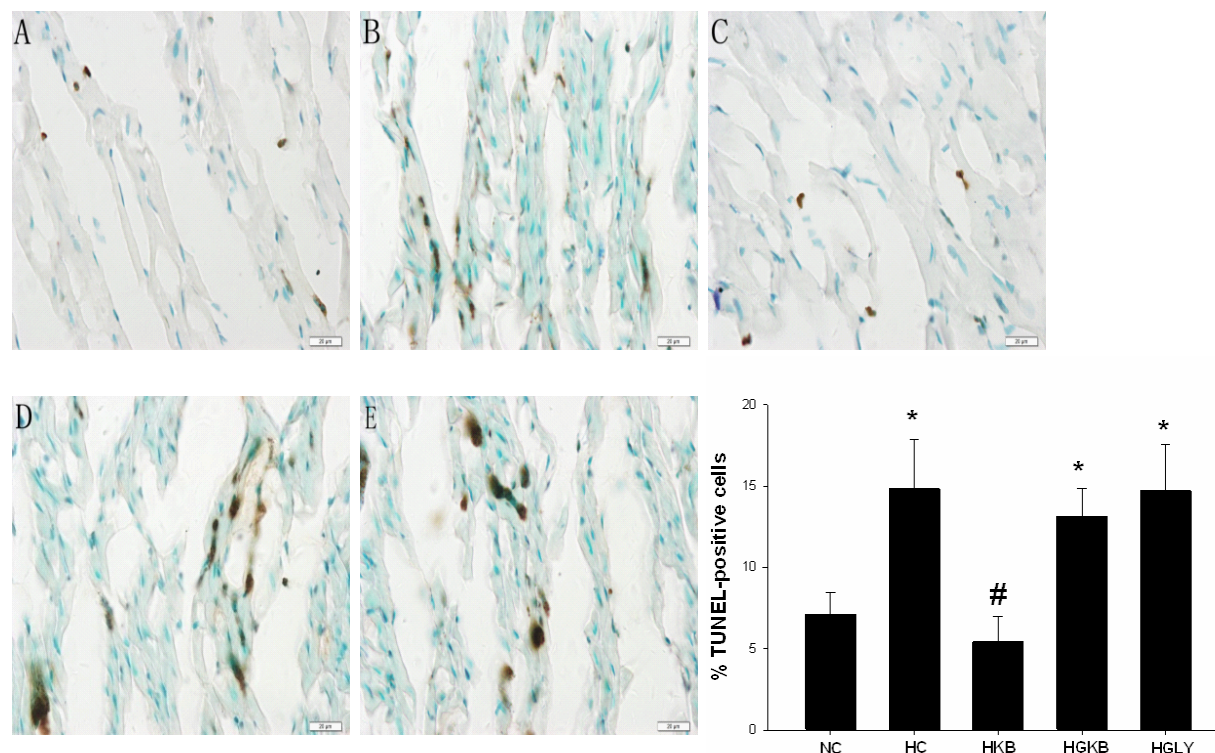
A novel finding in this study was that although diet-induced hypercholesterolemia increased infarct size and decreased HR at baseline, we found that there were no significant difference in +dp/dt, -dp/dt and LVDP at baseline and during the first 30mins of reperfusion among all groups. The +dp/dt, which is a good index of ventricular performance not influenced by afterload, demonstrated that hypercholesterolemia had not a negative inotropic effect. These results show no differences with respect to myocardial function and myocardial necrosis size between normocholesterolemic rats and hypercholesterolemic rats. Our results are in accordance with other studies in a pig model of ischemia/reperfusion [15].

Ca^{2+} entry via NCX operating in reverse-mode is believed to contribute to intracellular Ca^{2+} overload under pathological conditions such as myocardial ischemia and reperfusion [16]. During ischemia, intracellular Na^+ concentrations rise, in part because intracellular acidosis activates Na^+/H^+ exchange, which promotes Na^+ influx in exchange for H^+ efflux [17]. In addition, Na^+ influx through Na^+ channels and reduced Na^+ removal by the $Na^+–K^+$ ATPase can promote Na^+ loading in ischemia [17]. These elevated levels of intracellular Na^+ alter the driving force on NCX, so that it functions in reverse-mode to remove Na^+ from the cell in exchange for Ca^{2+} [16]. Therefore, activation of reverse-mode NCX is thought to promote intracellular Ca^{2+} overload in ischemia and reperfusion. Increases of cytosolic Ca^{2+} concentration occur, both at early and latest ages of the apoptotic path way [18]. KB-R7943, as an inhibitor of NCX, attenuates apoptosis by inhibiting the cytosolic and mitochondrial Ca^{2+} overload. In the present study, we also found that KB-R7943 reduced infarct size by attenuating induced apoptotic cell death during reperfusion in hypercholesterolemic rats.

We are certain that surface receptors, mitochondrial K^+_{ATP}, free radicals, and protein kinase C all play pivotal roles in the signaling pathway. Under normoxic conditions, the K^+_{ATP} channel exists mainly in a closed, inactive form. However, during myocardial ischemia, as the intracellular ATP concentration falls and ischemic metabolites (ADP, lactate, H^+) accumulate, the probability of the channel being open increases. This results in an enhanced outward repolarizing flow of K^+ and cell membrane hyperpolarization. Consequently, the myocardial action potential duration (APD) is shortened, the voltage-dependent calcium current and myocardial contractility are decreased thereby leading to ATP preservation during ischemia. Thus, it is thought that K^+_{ATP} channels exert a protective property in myocardial ischemic diseases [19]. Previous study [20] showed that ischemic postconditioning (IPost) triggers intracellular signaling kinases (ERK/Akt), mitochondrial K^+_{ATP} channels, and release of nitric oxide (NO) in a rabbit model of ischemia/reperfusion. Although IPost is clinically applicable and has been successful in attenuating infarct size [21], its use as a clinical cardioprotective strategy to decrease ischemia-reperfusion injury is limited to patients with ongoing acute myocardial infarction subjected to coronary angioplasty. In addition, IPost is not effective in hyper-cholesterolemic rabbits subjected to ischemia-reperfusion [22]. Therefore, pharmacological agents administered at the time of reperfusion aim to attenuate reperfusion injury may provide a more amenable approach to cardioprotection. KB-R7943 has been shown to reduce infarct size following myocardial ischemia [12,13], and our previous study in normocholesterolemic rats also showed that K^+_{ATP} channel opener combination with Na^+/Ca^{2+} exchange blockers may further reduce myocardial infarct size [14]. Such effects have probably been achieved by further activating mitochondrial K^+_{ATP} channels. So we

Figure 1: Area of a percentage of left ventricle (LV) area.

Values are means ±SD. *P<.005 vs. NC; #P<0.05 vs. HC, n=6 in each group

Figure 2: Effect of KB-R7943 and glybenclamide on apoptosis after myocardial infarction. A: NC; B: HC; C: HKB; D: HGKB. E: HGLY. Apoptotic cardiomyocyte nuclei appear brown stained, whereas terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-negative nuclei appear green with methyl green.*$p<$ 0.05 vs. NC. #$p<$0.05 vs. HC. n=5 hearts in each group. Bar=20µm.

conclude that K$^+_{ATP}$ may be involved in the protective mechanisms of KB-R7943 in normocholesterolemic rats. Additionally, it is important to consider that all the mechanisms mentioned in this section were described for healthy animals. However its effectiveness under certain pathological states is in dispute. In the present study, glybenclamide abolished the effect reached by KB-R7943 in hyperlipidemic rats and it does not increase infarct size on its own. Thus, we concluded that the K$^+_{ATP}$ channel was involved in the protective mechanisms in hypercholesterolemia animals.

As a limitation of this research, since the Langendorff model was used, and it does not contain protein and is deprived of neutroghil, humoral hormone or autonomic nervous system effects, it cannot be applied in an actual clinic. Further research target in vivo experiments is required.

Conclusion

Diet-induced hypercholesterolemia enhances myocardial injury; KB-R7943 reduces infarct size and apoptosis in hyperlipidemic animals through the activation of K$^+_{ATP}$ channels.

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