Cholesterol-enriched diet inhibits cardioprotection by ATP-sensitive K⁺ channel activators cromakalim and diazoxide

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1Cardiovascular Research Group, Department of Biochemistry, University of Szeged, Szeged, Hungary; 2Pharmahungary Group, Szeged, Hungary; 3Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary; and 4Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, Hungary

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Cholesterol-enriched diet inhibits cardioprotection by ATP-sensitive K⁺ channel activators cromakalim and diazoxide. Am J Physiol Heart Circ Physiol 306: H405–H413, 2014. First published November 27, 2013; doi:10.1152/ajpheart.00257.2013.—It has been previously shown that hyperlipidemia interferes with cardioprotective mechanisms. Here, we investigated the interaction of hyperlipidemia with cardioprotection induced by pharmacological activators of ATP-sensitive K⁺ (KATP) channels. Hearts isolated from rats fed a 2% cholesterol-enriched diet or normal diet for 8 wk were subjected to 30 min of global ischemia and 120 min of reperfusion in the presence or absence of KATP modulators. In normal diet-fed rats, either the nonselective KATP activator cromakalim at 10⁻⁵ M or the selective mitochondrial (mito)KATP opener diazoxide at 3 × 10⁻⁵ M significantly decreased infarct size compared with vehicle-treated control rats. Their cardioprotective effect was abolished by coinadministration of the nonselective KATP blocker glibenclamide or the selective mitoKATP blocker 5-hydroxydecanoate, respectively. However, in cholesterol-fed rats, the cardioprotective effect of cromakalim or diazoxide was not observed. Therefore, we further investigated how cholesterol-enriched diet influences cardiac KATP channels. Cardiac expression of a KATP subunit gene (Kir6.1) was significantly downregulated in cholesterol-fed rats; however, protein levels of Kir6.1 and Kir6.2 were not changed. The cholesterol significantly decreased cardiac ATP, increased lactate content, and enhanced myocardial oxidative stress, as shown by increased cardiac superoxide and dityrosine formation. This is the first demonstration that cardioprotection by KATP channel activators is impaired in cholesterol-enriched diet-induced hyperlipidemia. The background mechanism may include hyperlipidemia-induced attenuation of mitoKATP function by altered energy metabolism and increased oxidative stress in the heart.

hyperlipidemia; infarct size; ATP-sensitive potassium channel; oxidative stress

ISCHEMIC HEART DISEASE developing as a consequence of several risk factors, including hyperlipidemia, remains one of the major causes of morbidity and mortality in civilized societies. Therefore, therapeutic strategies to protect the ischemic myocardium have been extensively studied (15, 47). Activation of ATP-sensitive K⁺ (KATP) channels is a well-known cardioprotective cellular mechanism (for reviews, see Refs. 3, 20, 34, 46, and 64). KATP channels, first described by Noma (43), are expressed at high density in the sarcolemma of cardiomyocytes. KATP channels operate as molecular biosensors for coupling cellular energy metabolism and excitability (64). The KATP channel opens at low intracellular ATP levels, which may occur during ischemia, thereby facilitating increased K⁺ influx and shortening of action potential duration, which leads to a reduction of Ca²⁺ overload during ischemia-reperfusion-induced injury (42, 44, 59). In 1991, another form of KATP channels was identified in the mitochondrial inner membrane (33). The KATP complex is a unique combination of pore forming inward rectifier K⁺ (Kir) channels (Kir6.1 or Kir6.2), an ATP-binding cassette protein, and the sulfonylurea receptor (SUR1, SUR2A, or SUR2B), a regulatory subunit. Both sarcolemmal and mitochondrial KATP (mitoKATP) channels are activated by pinacidil and cromakalim and inhibited by glibenclamide (21, 48). Selective inhibitors and activators of cardiac mitoKATP channels include 5-hydroxydecanoate (5-HD) and diazoxide (21, 39). Both KATP channels have been demonstrated to play a key role in endogenous cardioprotective mechanisms, such as preconditioning and postconditioning (15, 35, 58, 60).

Since our original observations in hyperlipidemic rabbits and rats (17, 54), it has been well established that hypercholesterolemia, independently of the development of coroanary atherosclerosis, interferes with the cardioprotective mechanisms of ischemic before and after conditioning in animal models and in humans as well (14, 15, 56). The exact mechanism by which hyperlipidemia may disrupt cardioprotective signaling cascades is still a question of debate; however, several mechanisms have been suspected. Hyperlipidemia decreases expression of the cardioprotective 70-kDa heat shock protein (10), modifies the cardiac nitric oxide-cGMP-PKG pathway (13, 24) and the mevalonate pathway (13), interferes with endogenous inhibition of matrix metalloproteinases (25), and influences tetrahydrobiopterin synthesis (55). Moreover, hyperlipidemia results in an alteration of the gene expression pattern of the heart (36, 49). Therefore, to find valid drug targets for cardioprotection, cardioprotective mechanisms should be studied in the presence of cardiovascular risk factors such as, e.g., hyperlipidemia (15, 47).

The effect of hyperlipidemia on cardioprotection by KATP activation has not been studied yet; however, it is plausible to speculate that hyperlipidemia may affect KATP channel function by several mechanisms. First, due to hyperlipidemia, expressions of metabolic genes are downregulated (49); thus, ATP (the main regulator of KATP channels) production is decreased. KATP channels are also believed as metabolic sensors; however, the expression of cardiac KATP channels in hyperlipidemia has not been studied yet. We have previously shown that hyperlipidemia increases oxidative stress in hearts (23, 45), which may modify KATP channel function (29).
The aim of the present study was to investigate if hypercholesterolemia interferes with the cardioprotection induced by pharmacological activation of sarcolemmal $K_{ATP}$ and mito$K_{ATP}$ channels by cromakalim and diazoxide, respectively.

**METHODS**

This investigation conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996) and was approved by the local animal ethics committee.

**Experimental Protocol**

Male Wistar rats (120–160 g), housed in a room maintained at 12:12-h light-dark cycles and a constant temperature of 22 ± 2°C, were fed laboratory chow enriched with 2% cholesterol or standard chow for 8 wk as previously described (10, 18). At the end of the diet period, hearts were isolated and perfused in a constant-pressure (100 cmH2O) Langendorff system. Hearts were subjected to a 15-min equilibration period followed by 30 min of global ischemia and 120 min of reperfusion.

The nonselective $K_{ATP}$ opener cromakalim (final concentration: 10−5 M, SmithKline Beecham, Essex, UK), the nonselective $K_{ATP}$ blocker glibenclamide (10−7 M, Sigma, St. Louis, MO), their combination, the selective mito$K_{ATP}$ opener diazoxide (3 × 10−5 M, Sigma), the selective mito$K_{ATP}$ blocker 5-HD (10−4 M, Sigma), and their combination were dissolved in DMSO (final concentration in the perfusion fluid: 50 μL/L, Sigma). The perfusion fluid contained the drugs throughout the experiments. The concentrations of the most cardioprotective dose of cromakalim (10−5 M) and the nonvasoactive concentration of glibenclamide (10−7 M) were chosen to block the protective effect of cromakalim, which was consistent with our previous findings (9, 16, 17). The doses of mito$K_{ATP}$ modulators were selected according to the literature (35, 46, 58). The concentration of DMSO used in this study did not influence cardiac function (9, 16, 17).

To measure infarct size (8), 5 mL of 1% triphenyltetrazolium chloride (TTC; Sigma) dissolved in phosphate buffer (pH 7.4) was slowly injected for 5 min into the aorta to stain infarcted myocardial tissue at the end of reperfusion. TTC-stained hearts were frozen (−20°C), cut into ~2-mm-thick slices, transferred to 10% formalin solution for 12 min, rinsed, and then scanned between glass plates. TTC-stained red and unstained pale areas of images were quantified by digital planimetry (Infarctsize, Pharmahungary Group, Szeged, Hungary). Evaluation of all images was carried out in a blinded manner by one experienced person throughout the study.

To study the effect of cholesterol-enriched diet on cardiac Kir6.x expression, metabolic changes, and oxidative stress, in separate experiments, hearts from normal and cholesterol-fed animals were perfused for 5 min to eliminate blood. Fresh ventricular tissue was sampled and used for different measurements.

**Measurement of Cardiac ATP and Lactate Content**

To study the effect of cholesterol-enriched diet on cardiac ATP and lactate content, fresh tissue samples were vacuum dried. ATP and lactate were extracted from 3–5 mg of vacuum-dried heart tissue by 0.5 M perchlorate followed by 2.3 M KHCO3 neutralization and elimination of perchlorate, and the supernatants were used for further investigations. ATP content was calculated from the formation of the coenzyme NADPH in an assay system based on hexokinase- and glucose-6-phosphate dehydrogenase-catalyzed reactions. Lactate content was measured by ultraviolet photometry after the conversion of lactate and NAD to pyruvate and NADH in the presence of lactate dehydrogenase (LDH). Lactate content was calculated from the formation of NADH. Both ATP and lactate were normalized to tissue dry weight and expressed in micromoles per gram dry weight.

**Cardiac Gene Expression**

To study the effect of cholesterol-enriched diet on the cardiac expression of $K_{ATP}$ Kir subunits and the enzymes responsible for ATP and lactate synthesis (ATP synthase and LDH), quantitative real-time PCRs were performed. Two micrograms of total RNA from each sample were reverse transcribed in the presence of SuperScript II RNase H-Reverse transcriptase (Invitrogen, Carlsbad, CA) and random primer sequences as previously described (65).

The reverse transcription reactions were carried out in a final volume of 20 μL in the presence of 3.5 mM random hexamer primer, 1× first-strand buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, and 15 mM MgCl2], 500 nM dNTP mix (500 nM of each), 10 mM DTT, 40 units prime RNase inhibitor (Eppendorf), and 200 units SuperScript II RNase H-Reverse transcriptase (Invitrogen) at 42°C for 2 h. After dilution with 30 μL of water, 1 μL of the diluted reaction mix was used as template for quantitative PCR. Quantitative PCR was performed on a RotorGene 3000 instrument (Corbett Research, Sydney, NSW, Australia) with gene-specific primers (Table 1) using the SYBR green protocol. Reactions were done with ABSolute quantitative PCR SYBR Green mix (ABGene, Epsom, UK) according to the manufacturer’s instructions at a final primer concentration of 250 nM under the following conditions: 15 min at 95°C and 45 cycles at 95°C for 15 s, 60°C for 25 s, and 72°C for 25 s. The fluorescence intensity of SYBR green dye was detected after each amplification step. Melting temperature analysis was done after each reaction to check the quality of the reaction. A nontemplate control sample was used for each PCR run to check the primer-dimer formation. The quality of the primers was verified by mass spectroscopy analysis provided by Bioneer. Relative expression ratios were calculated after normalization to the mRNA level of three rat housekeeping control genes: cyclophillin, hypoxanthine-guanine phosphoribosyltransferase, and tubulin. The final rela-

**Table 1. Primers used in quantitative PCR analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{ATP}$ channels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kir6.1</td>
<td>5'-GATTTCGACGCGCATGTCT-3'</td>
<td>5'-CCGACGCGAACGTGAG-3'</td>
</tr>
<tr>
<td>Kir6.2</td>
<td>5'-ATGTTTGCCTGCGACAGG-3'</td>
<td>5'-CCGAACTCTCAGGAAAG-3'</td>
</tr>
<tr>
<td>u$K_{ATP}$-1</td>
<td>5'-GCCAGGCCCCACAGAAG-3'</td>
<td>5'-CGGAGAAATTACAGCAT-3'</td>
</tr>
<tr>
<td>ATP synthase, H+ transporting, mitochondrial F0 complex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subunit c (subunit 9), isoform 1</td>
<td>5'-CCAGGAAGGACTGCTCAT-3'</td>
<td>5'-GCCGTGATTAGACCCCTGTGA-3'</td>
</tr>
<tr>
<td>Subunit F6 (Atp5)</td>
<td>5'-GGCTGGAGCCTGAGGAAAG-3'</td>
<td>5'-GAAGGACAGAGAGAGGGCGCAA-3'</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>5'-TCACTGCAGCGTTGATTACC-3'</td>
<td>5'-TGTTGAAGGTTGCTGGAGC-3'</td>
</tr>
</tbody>
</table>

$K_{ATP}$ channels, ATP-sensitive K$^+$ channels.
tive gene expression ratios were calculated as ΔΔCt values (where Ct is threshold cycle).

Cardiac Oxidative Stress

To study the effect of cholesterol-enriched diet on cardiac oxidative stress, we measured superoxide production in freshly minced ventricles of normal and cholesterol-fed animals assessed by lucigenin-enhanced chemiluminescence (11). Approximately 100 mg of the apex of the heart were placed in 1 mL air-equilibrated Krebs-Henseleit solution containing 10 mM HEPES- NaOH (pH 7.4) and 5 μM a-lucigenin. Chemiluminescence was measured at room temperature in a liquid scintillation counter using a single active photomultiplier positioned in out-of-coincidence mode in the presence or absence of the superoxide scavenger nitroblue tetrazolium (200 μM). Nitroblue tetrazolium-inhibitable chemiluminescence was considered an index of myocardial superoxide generation.

In a separate set of experiments, we also measured the formation of another cardiac oxidative stress marker (dityrosine). The perfusion buffer was supplemented with l-tyrosine, and dityrosine was detected in the coronary effluent as previously described (11). Dityrosine formation was normalized to coronary flow and wet weight of the hearts and expressed as picomoles per minute per gram of protein.

Mitochondria Isolation for Western Blot Analysis

Isolation of mitochondria was performed as previously described (26). Briefly, after washout of blood, fresh cardiac tissue was minced with scissors in ice-cold isolation buffer (250 mM sucrose, 10 mM HEPES, and 1 mM EGTA; pH 7.4) and homogenized with an ultrasound homogenizer for 3 × 5 s. The homogenate was centrifuged at 480 g for 10 min, and the supernatant was filtered through a nylon filter (250-μm pore size) and then centrifuged again at 10,780 g for 10 min. The pellet containing mitochondria was resuspended in isolation buffer and then centrifuged again at 7,650 g for 20 min. The pellet was resuspended in 50 μL isolation buffer, layered onto 30% Percoll solution, and then ultra centrifuged at 35,000 g for 30 min. The lower band, reflecting intact mitochondria, was collected and washed in isolation buffer by centrifugation at 8,000 g for 5 min.

Western Blot Analysis of Kir6.1 and Kir6.2

Protein levels of KATP subunits Kir6.1 and Kir6.2 in the whole heart and in heart mitochondrial samples were determined by Western blot analysis. Heart tissue and mitochondria samples were homogenized by an ultrasonic homogenizer for 2 × 20 or 2 × 8 s, respectively. After centrifugation, protein concentrations of the supernatants were determined by the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Samples (20 μg) were loaded for SDS-PAGE (10%) followed by transfer of proteins onto a nitrocellulose membrane (400 mA, 1 h). Membranes were then blocked overnight at 4°C in Tris-buffered saline-0.1% Tween 20 with 5% BSA. Membranes were incubated either with rabbit polyclonal anti-rat Kir6.1 or Kir6.2 antibodies (both 1:500, Santa Cruz Biotechnology, Dallas, TX) for 1.5 h at room temperature and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Dako, Glostrup, Denmark) for 40 min. Membranes were then developed with an enhanced chemiluminescence kit (GE HealthCare, Little Chalfont, UK), exposed to X-ray (Kodak, Rochester, NY) film, and scanned. After being stripped, each membrane was incubated with either an internal control of heart tissue (rabbit monoclonal anti-GAPDH, 1:10,000, Cell Signaling Technology, Danvers, MA) or an internal control of mitochondria (monoclonal anti-succinate dehydrogenase, 1:1,000, Molecular Probes). Band density was calculated by integrating the area (in pixels × intensity) and expressed as the percent ratio of Kir6.2 and GAPDH or Kir6.2 and succinate dehydrogenase fluorescence, respectively.

Thallium Assay to Measure Functional KATP Protein

In a separate set of experiments, we measured functional KATP protein levels by a thallium assay according to Wojtovich et al. (62) in hearts from normal and cholesterol-fed rats. This assay measures KATP function in vitro conditions; therefore, it provides information on the functional KATP protein level but does not give insight into KATP function in situ, in the cardiac tissue environment.

Ventricular tissue was homogenized in heart mitochondria isolation media (HMIN; 300 mM sucrose, 20 mM Tris, and 2 mM EGTA; pH 7.35 at 4°C) and centrifuged at 600 g for 5 min at 4°C. The resulting supernatant was filtered (300-μm plastic mesh), and the suspension was centrifuged for 10 min at 7,000 g at 4°C. The mitochondria-enriched pellet was resuspended in 800 μL HMIM [supplemented with the fluorescent indicator benzothiazole coumarin (BTC)-AM and pluronic F-127 solutions; see below] and placed on ice until the assay.

Mitochondria were then loaded with 20 μM BTC-AM and 0.05 (wt/vol) pluronic F-127 dissolved in HMIM for 10 min at room temperature. After the incubation, the mitochondrial suspension was washed with 35 mL HMIM followed by a centrifugation at 7,000 g for 5 min at 4°C for two times, respectively. Finally, the pellet was resuspended in 800 μL HMIM.

BTC-AM-loaded mitochondria (~0.3 mg/mL) were added to a 96-well plate containing chloride-free thallium assay buffer (195 mM mannitol, 10 mM HEPES, 2 mM MgSO4, 2 mM Na2HPO4, 2 mM succinate, and 1 μg/mL oligomycin; pH 7.2 at 37°C) on the plate reader. After the measurement of baseline fluorescence, thallium sulphate solution (2 mM Tl2SO4 in thallium assay buffer) was injected into the wells, and a subsequent kinetic fluorescence intensity measurement was performed at 488-nm excitation and 525-nm emission.

Statistical Analysis

Results are expressed as means ± SE. Changes in gene expressions are expressed as log2 (upregulation if log2 > 0, downregulation if log2 < 0). ANOVA followed by a post hoc test or t-test was used to evaluate differences between groups as appropriate. Differences were considered significant at P values of <0.05. In the case of gene expression changes, P values of <0.05 and log2 > 0.85 or log2 ≤ −0.85 were accepted as statistical significant differences.

RESULTS

Cardioprotection by KATP Channel Activators in Normal and Hypercholesterolemic Rats

To assess the cardioprotective effect of pharmacological activation of KATP channels, we measured infarct size after test ischemia-reperfusion in normal and cholesterol-fed rats in the presence of nonspecific KATP modulators. In normal hearts, cromakalim, a nonspecific KATP activator at 10−5 M concentration, significantly decreased infarct size compared with control hearts. Glibenclamide, a nonspecific KATP blocker at its nonvasoactive 10−7 M concentration, did not influence infarct size; however, when it was applied in combination with cromakalim, it abolished its cardioprotective effect, showing that the cardioprotective effect of cromakalim was most likely mediated by KATP activation (Fig. 1). In hearts of cholesterol-fed animals, nonspecific KATP opening by cromakalim failed to decrease infarct size significantly (Fig. 1).

To investigate the cardioprotective effect of pharmacological activation of mitoKATP channels, we measured infarct size after test ischemia-reperfusion in normal and cholesterol-fed rats in the presence of the selective mitoKATP opener diazoxide, the selective mitoKATP blocker 5-HD, and their combination. In normal hearts, diazoxide at 3 × 10−5 M concentration
significantly decreased infarct size compared with control hearts. 5-HD at 10^{-4} M concentration did not influence infarct size; however, when it was applied in combination with diazoxide, it abolished the cardioprotective effect of diazoxide, showing that the cardioprotective effect of diazoxide was most likely mediated by mitoK_{ATP} activation (Fig. 2). In hearts of cholesterol-fed animals, opening of mitoK_{ATP} channels by diazoxide failed to decrease infarct size significantly (Fig. 2).

As to background hemodynamics, the cholesterol-enriched diet did not change coronary flow; however, both cromakalim and diazoxide significantly increased preischemic coronary flow in both normal and cholesterol-fed animals. Administration or coadministration of glibenclamide or 5-HD did not change coronary flow (Table 2). Heart rate was not significantly changed by the different treatments (data not shown).

Effect of Cholesterol Diet on Cardiac Gene Expression, Energy Metabolism, and Oxidative Stress

To study how cholesterol diet may influence cardiac K_{ATP} channels, we measured cardiac gene expression as well as markers of cardiac energy metabolism and oxidative stress, known regulators of K_{ATP} channels.

Cardiac gene expression. To investigate the gene expression of K_{ATP} subunits Kir6.1 and Kir6.2 as well as ATP synthase and LDH (the enzymes responsible for ATP and lactate syn-
and log2

DMSO)-treated control animals. (Fig. 3). To further investigate the functional KATP protein of cardiac mitochondria of normal and cholesterol-fed rats and Kir6.2 protein contents were not different in cardiac tissue in normal and cholesterol-fed groups. We found that Kir6.1 cardiac mitochondria were measured by Western blot analysis further investigate K ATP channel expression at the protein level in vitro did not change significantly (Table 3).

Cardiac and mitochondrial Kir6.1 and Kir6.2 protein. To further investigate KATP channel expression at the protein level, Kir6.1 and Kir6.2 protein content in the myocardium and cardiac mitochondria were measured by Western blot analysis in normal and cholesterol-fed groups. We found that Kir6.1 and Kir6.2 protein contents were not different in cardiac tissue of cardiac mitochondria of normal and cholesterol-fed rats (Fig. 3). To further investigate the functional KATP protein level in mitochondria, we performed a thallium assay in isolated mitochondria from hearts of normal and cholesterol-fed rats. We found that the functional KATP protein level in vitro was not different in isolated mitochondria from hearts of normal and hyperlipidemic rats (change in fluorescence: 11.8 ± 0.9% and 11.2 ± 1.2% of control after injection of thallium, respectively), which confirmed the results of the Western blot analysis.

Cardiac energy metabolism. To study changes in myocardial energy metabolism due to the cholesterol-enriched diet, we measured cardiac ATP and lactate levels in hearts of normal and cholesterol-fed groups. In hyperlipidemic rats, myocardial ATP content was significantly lower and lactate content was significantly higher compared with normal control rats (Fig. 4).

Cardiac oxidative stress. To study myocardial oxidative stress, we measured cardiac superoxide and dityrosine formation. We found that both cardiac superoxide and dityrosine production were significantly increased in cholesterol-enriched diet-fed rats compared with normal rats (Fig. 5).

DISCUSSION

In the present study, we found that the infarct-size reducing effect of either the nonselective KATP activator cromakalim or the selective mitoKATP activator diazoxide was lost in hearts of hyperlipidemic rats, showing that hyperlipidemia may influence KATP channel function in the heart. Although cholesterol feeding led to a downregulation of KATP subunit Kir6.1 gene expression, Kir6.1 and Kir6.2 proteins in the myocardium and isolated cardiac mitochondria were not changed. However, experimental hypercholesterolemia led to a decrease in cardiac ATP and an increase in lactate levels and oxidative stress, which may modify KATP channel function in situ. This is the first demonstration that hypercholesterolemia interferes with the cardioprotection elicited by both the nonselective KATP activator cromakalim and the mitoKATP activator diazoxide. Although the mechanism(s) of the loss of cardioprotection by cromakalim and diazoxide due to hyperlipidemia was not fully explored in the present study, we provided important data suggesting that this might be due to an altered energy metabolism and increased oxidative stress in the myocardium, which may lead to an impairment of in situ KATP channel function.

Activation of KATP channels is a well-characterized cardioprotective mechanism (for reviews, see Refs. 20, 34, 46, 59, and 64). Based on our earlier study (17), in the present study, we demonstrated that the most effective cardioprotective concentration of the nonselective KATP opener cromakalim signifi-

Table 2. Effects of the nonselective KATP blocker glibenclamide (10⁻⁷ M), the nonselective KATP opener cromakalim (10⁻⁵ M), and their combination as well as the selective mitoKATP blocker 5-hydroxydecanoate (10⁻⁴ M), the selective mitoKATP opener diazoxide (3 × 10⁻⁵ M), and their combination on coronary flow measured before ischemia and at the first 5 min of reperfusion in hearts of rats fed a normal diet or a cholesterol-enriched diet

<table>
<thead>
<tr>
<th></th>
<th>Normal diet</th>
<th>Glibenclamide</th>
<th>Cromakalim</th>
<th>Glibenclamide + Cromakalim</th>
<th>5-Hydroxydecanoate</th>
<th>Diazoxide</th>
<th>5-Hydroxydecanoate + Diazoxide</th>
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</thead>
<tbody>
<tr>
<td>Before ischemia</td>
<td>16.1 ± 0.9</td>
<td>15.4 ± 1.4</td>
<td>24.3 ± 1.5*</td>
<td>22.8 ± 1.0*</td>
<td>15.7 ± 1.5</td>
<td>24.3 ± 1.5*</td>
<td>21.5 ± 2.4*</td>
</tr>
<tr>
<td>After ischemia</td>
<td>13.2 ± 1.0</td>
<td>15.3 ± 1.5</td>
<td>16.0 ± 1.6</td>
<td>18.9 ± 1.6*</td>
<td>16.4 ± 1.2</td>
<td>17.0 ± 0.7*</td>
<td>14.5 ± 0.9</td>
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<tr>
<td>Cholesterol-enriched diet</td>
<td>17.7 ± 0.7</td>
<td>15.5 ± 1.9</td>
<td>28.3 ± 1.8*</td>
<td>23.0 ± 1.1*</td>
<td>15.4 ± 2.3</td>
<td>25.2 ± 0.8*</td>
<td>22.0 ± 1.7*</td>
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<tr>
<td>After ischemia</td>
<td>13.8 ± 1.0</td>
<td>13.7 ± 1.2</td>
<td>13.8 ± 1.4</td>
<td>14.2 ± 2.1</td>
<td>12.9 ± 1.1</td>
<td>17.5 ± 1.0</td>
<td>17.9 ± 0.4</td>
</tr>
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</table>

Results are means ± SE of coronary flow values (in ml/min); n = 6–8. mitoKATP channels, mitochondrial KATP channels. *P < 0.01 vs. vehicle (50 μl/l DMSO)-treated control animals.

Table 3. Gene expression changes due to high cholesterol diet-induced hyperlipidemia as assessed by quantitative PCR in rat hearts

<table>
<thead>
<tr>
<th>Gene</th>
<th>GeneBank Accession Number</th>
<th>Log2</th>
<th>SD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KATP channels</td>
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<td></td>
</tr>
<tr>
<td>Kir6.1</td>
<td>AB043637</td>
<td>−0.95*</td>
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<td>Kir6.2</td>
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<td>−0.15</td>
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<td>0.62</td>
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<tr>
<td>uKATP-1</td>
<td>D42145</td>
<td>−0.89*</td>
<td>0.25</td>
<td>0.03</td>
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<td>ATP synthase, H⁺ transporting, mitochondrial F₀ complex</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Subunit c (subunit 9), isoform 1</td>
<td>NM_017311</td>
<td>0.19</td>
<td>0.25</td>
<td>0.18</td>
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<tr>
<td>Subunit F6 (Atp5j)</td>
<td>NM_053602</td>
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<td>0.25</td>
<td>0.21</td>
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<td>Lactate dehydrogenase</td>
<td>X01964</td>
<td>0.07</td>
<td>0.25</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Changes in gene expression are expressed as log2 (upregulation if log2 > 0, downregulation if log2 < 0) compared with the normal diet group. *P < 0.05 and log2 ≥ 0.85 or log2 ≤ −0.85 (1.8-fold) were accepted as significant differences.
significantly reduced infarct size, which was abolished by a nonvasoactive dose of glibenclamide that alone did not affect infarct size. Here, we obtained similar results with the selective mitoK\textsubscript{ATP} modulators diazoxide and 5-HD. However, treatment with either cromakalim or diazoxide failed to reduce infarct size in hyperlipidemic heart. Here, we demonstrate, for the first time, that a cholesterol-enriched diet inhibits cardioprotection induced by K\textsubscript{ATP} activators and show that a cholesterol diet may impair cardiac K\textsubscript{ATP} channels. This is in accordance with the observation that hypercholesterolemia attenuates cardioprotection induced by preconditioning, postconditioning, heat stress, or cGMP-PKG signaling (for an extensive review, see Ref. 15), although the exact mechanism of this phenomenon is unknown. Others (22) have shown in rabbits that protection against no-reflow phenomenon by the nitric oxide donor and KATP opener nicorandil is impaired by hypercholesterolemia; however, the role of K\textsubscript{ATP} channels in this phenomenon was not probed in that study.

The mechanism by which hyperlipidemia may inhibit the cardioprotective effect of K\textsubscript{ATP} modulators is not known. Therefore, in the present study, we investigated the possible mechanisms by which hyperlipidemia may influence K\textsubscript{ATP} channels in the heart: 1) cardiac K\textsubscript{ATP} expression at the gene and protein levels, 2) markers of cardiac energy metabolism (ATP and lactate content), and 3) ROS production as well-known metabolic regulators of K\textsubscript{ATP} channels.

In the present study, we have shown that, in hyperlipidemic rats, mRNA levels of Kir6.1 were significantly downregulated. Other studies showed that in Kir6.2 knockout mice, cardioprotection is lost (27, 28); however, upregulation of Kir6.2 (41) or SUR2A (12) confer protection. Others have shown that a reduction in the number of K\textsubscript{ATP} channels results in insufficient K\textsubscript{ATP} channel function in mice (63), rats (6), and humans (44). In the present study, interestingly, despite the downregulated Kir6.1 gene, protein levels of Kir6.1 or Kir6.2 were not changed in the myocardium or cardiac mitochondria due to hypercholesterolemia. We confirmed this negative finding by measurements of functional K\textsubscript{ATP} channel protein in isolated mitochondria in the present study. Nevertheless, these measurements did not provide insights into K\textsubscript{ATP} channel function in situ in myocardial tissue, since K\textsubscript{ATP} channel function can be altered by energy metabolism and ROS formation in the local intracellular microenvironment.

Therefore, we also measured possible metabolic regulators of K\textsubscript{ATP} channels, ATP and lactate levels, and ROS formation. In the present study, we showed that the cholesterol-enriched diet decreased cardiac ATP levels. In the case of low intracellular ATP levels, one can speculate that the open probability of K\textsubscript{ATP} channels in hyperlipidemic animals is high; therefore, further K\textsubscript{ATP} channel activation is less possible. However, in the present study, no aggravation of infarct size in the presence of glibenclamide or 5-HD was observed. In accordance with the fact that in the rat no atherosclerosis develops due to a high-cholesterol diet, we found no change in coronary flow in the hearts in the present study. Therefore, atherosclerosis-induced diminished coronary circulation and a concomitant reduction of oxygen supply can be excluded as reason for low ATP. However, we have previously shown, a cholesterol-enriched diet in rats lead to a redistribution of both sarcolem-
Although we have shown in the present study that a cholesterol-enriched diet interferes with K\textsubscript{ATP} channel activation-induced cardioprotection, possibly via changes in ATP and lactate content and increased oxidative stress, other possible mechanisms cannot be excluded. It is known that normal mitochondrial Cx43 content is important for diazoxide-related cardioprotection (31, 52). Therefore, it can be speculated if altered Cx43 content of the hyperlipidemic myocardium may play a role in the loss of diazoxide-induced cardioprotection in hyperlipidemia. Although diazoxide is known to be a specific opener of mitoK\textsubscript{ATP} channels, it has several K\textsubscript{ATP} channel-independent effects, including the suppression of mitochondrial respiration and free radical formation, inhibition of succinate dehydrogenase, and a reduction of infarct size via a nitric oxide synthase-dependent mechanism (15, 61). Hyperlipidemia might interfere with the aforementioned effects of diazoxide as well, which may be a limitation of our present study. Changes in the composition of membrane components may also interact with the K\textsubscript{ATP} channel and modulate pore function (4, 53). Therefore, we cannot exclude the possibility that the cholesterol-enriched diet influenced K\textsubscript{ATP} channel function at the membrane composition level.

In conclusion, this is the first demonstration that cardioprotection elicited by the nonspecific K\textsubscript{ATP} activator cromakalim or the mitoK\textsubscript{ATP} activator diazoxide is impaired by cholesterol-enriched diet-induced hyperlipidemia. Although the exact mechanism by which hyperlipidemia may influence cardiac K\textsubscript{ATP} channel signaling could not be fully explored in the scope of the present study, we provided here valuable data showing that altered energy metabolism characterized by decreased ATP and increased lactate levels as well as increased oxidative stress but not changes in the expression levels of functional K\textsubscript{ATP} protein expression in the heart due to cholesterol diet may be involved.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.C. conception and design of research; C.C., K.K., P.B., A.G., J.P., and Á.Z. performed experiments; C.C., P.B., and Á.Z. analyzed data; C.C. and Á.Z. interpreted results of experiments; C.C. and T.C. prepared figures; C.C. and T.C. drafted manuscript; C.C., L.G.P., and T.C. analyzed data; C.C. and Á.Z. interpreted results of experiments; C.C. and T.C. wrote manuscript; C.C. and T.C. reviewed and edited manuscript; Á.Z. managed the laboratory; C.C. and Á.Z. assisted with drafting the manuscript; C.C. and Á.Z. provided administrative, technical, or material support; C.C. and Á.Z. supervised the project; C.C. and Á.Z. accessed the data.

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