### Title
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Title: Direct effect of mild hypothermia on the coronary vasodilation induced by an ATP-sensitive K channel opener, a nitric oxide donor and isoflurane in isolated rat hearts

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Short title: Hypothermia and coronary vasodilators

Key Words: hypothermia, coronary, nitric oxide, ATP-sensitive potassium channel, isoflurane
Abstract

Purpose: Deliberate mild hypothermia (MHT) is applied for cerebroprotection after cardiopulmonary resuscitation and during cardiac surgery. MHT has been shown to alter both contractility and relaxation of blood vessels in the brain. However, the effects of MHT on drug-induced vasodilation are not fully understood. The aim of this study was to clarify the effects of MHT on the coronary vasodilation induced by cromakalim: an ATP-sensitive K channel opener, S-nitroso acetyl-penicillamine (SNAP): a nitric oxide donor, and isoflurane in isolated rat hearts.

Methods: Male SD rat hearts were isolated and perfused with Krebs-Henseleit buffer. Coronary flow was measured with the coronary perfusion pressure kept at 60 mmHg, and coronary vascular resistance (CVR) was calculated. After cardiac arrest was induced by tetrodotoxin, the hearts were allocated to one of three temperature groups: 37, 34, and 31°C (n = 7 for each). All groups received 0.01, 0.1, and 1.0 μM of either cromakalim or SNAP or were exposed to isoflurane at 1MAC and 2MAC. Finally, 50 mM of adenosine was administered to obtain maximal coronary vasodilation.

Results: CVR significantly increased after cardiac arrest, but did not change after application of each temperature. Cromakalim, SNAP and isoflurane significantly decreased CVR in each temperature group. There were no significant differences in CVR among the three temperature groups with any of the test drugs.

Conclusion: These results indicate that cromakalim-, SNAP-, and isoflurane-induced coronary vasodilation are not affected by MHT. (235 words)
Introduction

Therapeutic mild hypothermia (MHT), defined as cooling the core body temperature to 32-34°C, is widely applied for cerebroprotection during cardiac surgery. Investigations in animals have shown that MHT can provide significant protective effects against cerebral ischemia [1]. Furthermore, it has been reported that MHT improves neurological outcomes in cerebral injury caused by cardiac arrest [2, 3] and cerebral trauma [4, 5] in humans. Conversely, however, even a mild degree of hypothermia might cause complications such as myocardial ischemia. Frank et al. [6] reported that there was a significantly higher incidence of postoperative myocardial ischemia and angina attacks in patients with a temperature lower than 35°C as compared to those with normothermia.

Nitroglycerin and nicorandil, widely recognized as drugs for the treatment of myocardial ischemia, are commonly used during MHT for the treatment of myocardial ischemia. Nitroglycerin-induced coronary vasodilation involves nitric oxide (NO) stimulation of the guanylate cyclase (GC) pathway, leading to increased cellular levels of cyclic guanosine 3',5'-monophosphate (cGMP) [7]. Nicorandil is characterized as a hybrid between nitrates and ATP-sensitive K channel (K_{ATP} channel) openers. K_{ATP} channel openers exert coronary vasodilating effects by modifying the membrane potential of vascular smooth muscle [8]. Isoflurane, a volatile anesthetic agent, induces coronary vasodilation. Cason et al. [9] and Crystal et al. [10] demonstrated that glibenclamide, a K_{ATP} channel blocker, prevented isoflurane-induced coronary vasodilation, and suggested that coronary vasodilation induced by isoflurane could be associated with the activation of K_{ATP} channels.

Clear understanding of coronary vascular reactivity under hypothermia is
required for precise cardiovascular management during MHT. However, the influence of hypothermia on the effects of coronary vasodilators is not fully understood. The present study was carried out to clarify the influence of MHT on the coronary vasodilation induced by cromakalim, a $\mathrm{K}_{\text{ATP}}$ channel opener, S-nitrosoacetyl-penicillamine (SNAP), a nitric oxide donor, and isoflurane. We used the isolated perfused rat heart arrested with tetrodotoxin citrate (TTX) to exclude indirect effects such as myocardial contraction and work, as described previously [11, 12].
Materials and Methods

Isolated Heart Preparation

All experimental procedures and protocols described in this study were approved by the Institutional Animal Care and Use Committee. In each experiment, male Sprague-Dawley rats (weighing 434 ± 53 [mean ± SD], range 430–500 g, and aged 13–16 weeks) were anesthetized with diethyl ether and sodium pentobarbital (50 mg/kg IP), and then heparinized (2000 U/kg IP). A midline sternotomy was performed, and the heart was rapidly excised, submerged into the oxygenated perfusate (37°C, composition provided below) and immediately perfused via the ascending aorta using a non-recirculating Langendorff coronary perfusion system [13]. Coronary perfusion pressure (CPP) was kept at 60 mmHg by an adjustable-speed rotary pump (Masterflex model 7520-50, Cole-Parmer Instruments Co). This was continuously monitored from the sidearm of the perfusion line near the aortic root, using a pressure transducer (blood pressure monitor link sck-9082 Becton Dickinson) and blood pressure amplifier (AP-641G Nihon-Kohden, Tokyo, Japan), and was shown on the Polygraph system (Nihon-Kohden) throughout the experiment. Coronary flow (CF) was monitored by measuring the flow of the perfusate supplied to the heart using an in-line ultrasound flow meter (MFV3200; Nihon-Kohden). The left ventricular apex was punctured with a thin piece of Tygon tubing to discharge the Thebesian drainage [13], and the heart was submerged into the perfusate at 37°C. This drain prevented ventricular distension and permitted identification of aortic valve insufficiency. The perfusate used was Krebs-Henseleit buffer (KHB) composed of (in mM) NaCl 120, KCl 5.8, NaHCO₃ 25, NaH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 1.0, and D-glucose 10. The perfusion buffer was continuously bubbled with 95% O₂ + 5% CO₂, and the pH was adjusted to 7.40-7.45 at
37˚C with HCL and monitored by Cyber scan pH 510 (Kagaku Kyoeisha Ltd.). A filter (0.45 µm, Millipore) was used for the perfusate before perfusion into the coronary arteries. After instrumentation, recirculating perfusion was instituted for the duration of the experimental protocol [Fig. 1].

Protocol

The experimental protocol is shown in Fig. 1. The rats were allocated to one of three groups (n = 21 for each group) receiving cromakalim (group CRO), SNAP (group SNP) or isoflurane (group ISO). Each group was then subdivided into three groups (n = 7 for each group) corresponding to set Krebs-Henseleit buffer (KHB) temperatures of 31°C (subgroup a), 34°C (subgroup b) or 37°C (subgroup c). All rats were allowed to stabilize for at least 20 min. After 9.0 µg/ml TTX (28 µM) was added to the perfusion circuit to induce cardiac arrest, each group was kept perfused with KHB at the same temperature. Thereafter, groups CRO and SNP received 0.01, 0.1, and 1.0 µM of cromakalim and SNAP, respectively, each dose being administered for 10 min. Group ISO was exposed to 1MAC (1.7% end-tidal) and 2MAC (3.4%) [12] isoflurane, each for 15 min, in an O₂:CO₂ mixture through a calibrated vaporizer (Isotec 3, Ohmeda, Tokyo, Japan). The exposure period to isoflurane was set on the basis of previous data that CF responses to isoflurane reach 90% of the steady-state value within 8 min [14]. Finally, 50 mM of adenosine was administered to all groups to investigate maximal coronary vasodilation.

CF and CPP were measured before and after TTX-induced cardiac arrest, at each temperature, in the presence of test compounds, and finally after adenosine. After completion of measurements, the hearts were removed from the perfusion device,
and heart tissue trimmed off connective tissue was dried to obtain dry heart weight.

**Drug preparation**

TTX (Wako Pure Chemical Co.) was dissolved in water and directly added to the KHB. Cromakalim and adenosine (Sigma Chemical Co.) were dissolved in dimethyl sulfoxide and directly added to the KHB.

**Data analysis**

Coronary vascular resistance (CVR) in dyn·s·g·cm⁻⁵ was calculated as CPP/CF·dry heart weight.

All values are expressed as mean ± SD. Statistical comparisons within groups were made using repeated measures analysis of variance, followed by a paired t-test. Statistical comparisons among groups were analyzed using a two-way analysis of variance, followed by Turkey-Kramer test. A p value less than 0.05 was considered statistically significant.
Results

The mean dry weight of all hearts was 0.282 ± 0.023 g, with no significant differences among groups. CPP values were between 58 to 62 mmHg throughout the experiment. Perfused KHB temperature was maintained between 30.8-31.1, 34.0-34.2 and 36.8-37.1°C in subgroups a, b and c, respectively. Baseline CVR values at the predetermined set temperatures under cardiac arrest, and after adenosine in each group were as shown in Table 1; there were no significant differences in baseline CVR values among subgroups within each drug group. TTX successfully induced cardiac arrest in all hearts, following which the temperature was set at predetermined levels in each group. CVR increased significantly after cardiac arrest compared to baseline in each group (data not shown); there were no significant differences among subgroups within each group. CVR at the set temperatures were not different from those after arrest in any group. Adenosine decreased CVR significantly compared to values after setting the temperature in all groups; there were no significant differences among subgroups within each group (Table 1).

Figure 2 shows the effects of cromakalim on CVR. In groups CRO-a, CRO-b and CRO-c, cromakalim significantly decreased CVR at doses of 0.1 and 1.0 μM, but not at 0.01 μM; there were no significant differences in CVR among the 3 subgroups at each does level. Figure 3 shows the effects of SNAP on CVR. In groups SNP-a, SNP-b and SNP-c, SNAP significantly decreased CVR at 0.1 and 1.0 μM but not at 0.01 μM; there were no significant differences in CVR among the 3 subgroups at each does level. Figure 4 shows the effects of isoflurane on CVR. In groups ISO-a, ISO-b and ISO-c, isoflurane significantly decreased CVR at both 1MAC and 2MAC; there were no significant differences in CVR among the 3 subgroups at each does level.
Discussion

The present results show that cromakalim, SNAP and isoflurane significantly dilate the coronary arteries during MHT to the same extent as during normothermia in isolated perfused rat hearts arrested with TTX under constant CPP, and suggest that the direct coronary dilating effects of NO and K$_{ATP}$ channels are preserved even during MHT.

Smits et al. [15] reported that lemakalim, a K$_{ATP}$ channel opener, caused coronary vasodilation in a dose-dependent manner in anesthetized rats. Larach et al. [12] reported that isoflurane induced a dose-dependent decrease in CVR through activation of K$_{ATP}$ channels in isolated rat hearts. Walia et al. [16] showed that SNAP caused potent coronary vasodilation in pig coronary arteries. The present results during normothermia are consistent with these previous reports, and further demonstrate that the coronary vasodilatory effects of cromakalim, SNAP and isoflurane are preserved even during MHT.

The effects of hypothermia on K$_{ATP}$ channel opener-induced vasodilation are controversial. Atalik et al. [17] investigated the effects of cooling (28°C) on the vasodilation induced by diazoxide on the carbachol-pre-contracted calf coronary artery and cardiac vein. They reported that pIC$_{50}$ values, but not maximal responses to diazoxide, were attenuated by cooling. Saito et al. [18] reported that hypothermia (23°C) attenuated the vasorelaxation induced by K$_{ATP}$ channel openers, NIP-121, cromakalim and pinacidil, in guinea pig aorta without endothelium. In contrast, it was reported that MHT could enhance pial arteriolar vasodilation induced by cromakalim, isoflurane, and sevoflurane in cats [19, 20]. On the other hand, Dojo et al. [21] reported that MHT (33°C) did not alter the vasodilation induced by a K$_{ATP}$ channel...
opener, levromakalim, in rat aorta without endothelium. Thus, it appears that the influence of hypothermia on $K_{ATP}$ channel opener-induced vasodilation varies according to the kind of artery being studied, temperature and experimental models.

Booth et al. [22] reported that hypothermia (27°C) significantly decreased nitroglycerin-induced relaxation of rabbit aorta, and suggested that biotransformation of nitroglycerin and the release of NO, and the tissue response to NO were impaired by hypothermia. Atalik et al. [17] reported that cooling (28°C) did not attenuate sodium nitroprusside-induced relaxation in the calf coronary artery and cardiac vein with intact endothelium. Although the reason for this discrepancy is unclear, it seems possible that the coronary artery, but not the aorta, can function in response to NO even under hypothermia.

The experimental model used in the present study was isolated perfused rat hearts arrested with TTX under constant CPP. The advantages of this model are as follows. 1) A constant CPP can prevent autoregulatory changes in resistance [11]. 2) Use of arrested hearts negates the influence of myocardial oxygen consumption (MVO$_2$) on coronary vasoresistance. In the beating heart, negative inotropic drugs reduce cardiac work and MVO$_2$, resulting in indirect vasoconstriction via the process of coronary flow-metabolism coupling [14, 23]. Larach et al. [11] showed that hearts arrested with TTX had decreased MVO$_2$ and increased CVR, while halothane, isoflurane and/or adenosine administration decreased CVR without causing any significant change in MVO$_2$. The results of the present study are consistent with those of Larach et al in that CVR increased significantly after cardiac arrest at 37°C. 3) Use of arrested hearts with drainage of the ventricular cavities can prevent changes in intramyocardial vascular compressive forces [11]. 4) TTX blocks the influence of the autonomic nerve
terminal located in the coronary artery. Kawada et al. [24] reported that electrical vagal stimulation-induced acetylcholine release at cardiac vagal nerve terminals was suppressed by local administration of TTX. Cohen et al. [25] showed that beta adrenergically-mediated relaxation induced by electrical stimulation was suppressed by TTX during contractions evoked by prostaglandin F2 in the canine coronary artery.

In conclusion, cromakalim, SNAP and isoflurane significantly dilate the coronary artery during MHT to the same extent as during normothermia in isolated perfused rat hearts arrested with TTX under constant CPP, suggesting that the direct coronary dilating effects of NO and K\textsubscript{ATP} channels are preserved even during MHT.
References


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12. Larach DR, Schuler HG. Direct vasodilation by sevoflurane, isoflurane, and halothane alters coronary flow reserve in the isolated rat heart. Anesthesiology. 1991; 75:268-78.


Table 1 Coronary vascular resistance in cromakalim, SNAP and isoflurane groups

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>After cardiac arrest, and at predetermined temperature levels</th>
<th>After adenosine</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(dyn·s·g·cm⁻⁵)</td>
<td>(dyn·s·g·cm⁻⁵)</td>
<td>(dyn·s·g·cm⁻⁵)</td>
</tr>
<tr>
<td>CRO-a</td>
<td>109 ± 4</td>
<td>131 ± 10*</td>
<td>91 ± 7†</td>
</tr>
<tr>
<td>CRO-b</td>
<td>108 ± 5</td>
<td>129 ± 11*</td>
<td>96 ± 9†</td>
</tr>
<tr>
<td>CRO-c</td>
<td>107 ± 7</td>
<td>129 ± 15*</td>
<td>94 ± 12†</td>
</tr>
<tr>
<td>SNP-a</td>
<td>114 ± 8</td>
<td>137 ± 5*</td>
<td>81 ± 4†</td>
</tr>
<tr>
<td>SNP-b</td>
<td>117 ± 6</td>
<td>138 ± 8*</td>
<td>83 ± 10†</td>
</tr>
<tr>
<td>SNP-c</td>
<td>118 ± 8</td>
<td>140 ± 4*</td>
<td>81 ± 10†</td>
</tr>
<tr>
<td>ISO-a</td>
<td>118 ± 4</td>
<td>140 ± 7*</td>
<td>88 ± 9†</td>
</tr>
<tr>
<td>ISO-b</td>
<td>110 ± 9</td>
<td>138 ± 7*</td>
<td>85 ± 7†</td>
</tr>
<tr>
<td>ISO-c</td>
<td>108 ± 7</td>
<td>147 ± 8*</td>
<td>82 ± 7†</td>
</tr>
</tbody>
</table>

All values are presented as mean ± SD (n = 7 for each group).

Group CRO, SNP and ISO received cromakalim, S-nitroso acetyl-penicillamine and isoflurane, respectively. Subgroups were maintained at KHB temperatures of 31°C (subgroup a), 34°C (subgroup b) and 37°C (subgroup c).

*Significant (P < 0.05) differences between baseline levels and those after cardiac arrest and at the predetermined temperature level.

†Significant (P < 0.05) differences between values after arrest and at the predetermined set temperature level and after adenosine.
Figure 1 Experimental protocol.

TTX = tetrodotoxin citrate; SNAP = S-nitroso acetyl-penicillamine.
Figure 2 The effect of mild hypothermia on cromakalim-induced coronary vasodilation

Data are expressed as mean ± SD (n = 7 for each group).

*Significantly (P < 0.05) different after cardiac arrest and at the predetermined temperature level.

†Significantly (P < 0.05) different from the level with 0.01 μM cromakalim.

#Significantly (P < 0.05) different from the level with 0.1 μM cromakalim.
Figure 3 The effect of mild hypothermia on SNAP-induced coronary vasodilation

Data are expressed as mean ± SD (n = 7 for each group). SNAP = S-nitroso acetyl-penicillamine.

*Significantly (P < 0.05) different after arrest and at the predetermined temperature level.

†Significantly (P < 0.05) different from the level with 0.01 μM SNAP.

#Significantly (P < 0.05) different from the level with 0.1 μM SNAP.
Figure 4 The effect of mild hypothermia on isoflurane-induced coronary vasodilation

Data are expressed as mean ± SD (n = 7 for each group).

*Significantly (P < 0.05) different after arrest and at the predetermined temperature level.

†Significantly (P < 0.05) different from 1MAC isoflurane.