The effects of age and resveratrol on the hypoxic preconditioning protection against hypoxia–reperfusion injury: studies in rat hearts and human cardiomyocytes

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Abstract

OBJECTIVES: The loss of effectiveness of ischaemic preconditioning in protecting old hearts from ischaemia/reperfusion damage is thought to be due to low sirtuin 1 levels in old hearts. We sought to determine whether resveratrol (RES), an activator of sirtuin 1, would restore this protection to that seen with ischaemic preconditioning in young hearts.

METHODS: A Langendorff heart perfusion model was established in 80 old and 80 adult rats to test the effects of hypoxic preconditioning (HPC) and/or RES on preventing hypoxia–reperfusion (H/R) injury. The effects were further tested by comparing the effects of HPC and RES on cell survival rate and lactate dehydrogenase (LDH) in cardiomyocytes from 15 old and 15 young humans.

RESULTS: The HPC + RES group performed better in both adult and old groups than the corresponding H/R, HPC and RES groups, causing ≏50% in the adult and 40% in the old group restoration of left ventricular developed pressure and ≏90% in the adult and 80% in the old group restoration of \( \frac{dp}{dt_{\max}} \). HPC and RES each reduced apoptosis in both groups. The HPC + RES treatment showed an additive benefit in reducing apoptosis in the adult group but not in the old group. In H/R-treated young and old human cardiomyocytes, cell survival and LDH level were significantly improved in the RES + HPC group compared with the HPC group.

CONCLUSIONS: This study showed that RES lessened the ageing effect and enhanced the cardioprotective effect of HPC in older individuals.

Keywords: Resveratrol • HIF-1 • SIRT1 • Myocardial ischaemic preconditioning • Hypoxia–reperfusion injury

INTRODUCTION

It is well established that myocardial ischaemic preconditioning may provide cardioprotection in ischaemia reperfusion (I/R) injury (for a review, see [1]). However, young and old hearts differ in myocardial structure and endogenous cardioprotective mechanisms, and the myocardium of elderly individuals is more sensitive than that of the young to I/R injury [2]. Animal studies have also shown older hearts to be less responsive to preconditioning [3].

One important gene in conveying the cardioprotective effect of ischaemic preconditioning is sirtuin 1 (SIRT1), an NAD+-dependent histone/protein deacetylase that plays an important role in gene silencing, DNA repair, metabolism and prolongation of lifespan, and is related to the ageing of cells [4].

Resveratrol (RES), a polyphenolic substance found in wine and grapes [5], can activate SIRT1 to exert antioxidative, anti-apoptotic, anti-arteriosclerotic and anti-ageing effects. A natural substance that is available as a dietary supplement, RES has been shown to have a cardioprotective effect in humans [6]. Perioperative adverse events often occur in elderly individuals with coronary heart disease, but myocardial ischaemic preconditioning is less effective in protecting old hearts than young hearts [3]. Therefore, it is important to explore treatments that can enhance the protective effect of myocardial ischaemic preconditioning in elderly patients. The aim of this study is to examine whether RES shows any protective effects against myocardial I/R injury in old hearts, substituting hypoxic for ischaemic preconditioning, because the experiments were performed in vitro and no blood was used. We therefore compared the effects of RES and hypoxic preconditioning (HPC) on cardiac function and apoptosis in isolated old and adult rat hearts and on cell survival and cell damage in cultured old and young human cardiomyocytes.
METHODS

Isolated rat hearts

Animals. The hearts of male adult rats (4–9 months old; n = 80) and old rats (12–18 months old; n = 80) were obtained and used in an in vitro Langendorff myocardial perfusion model [7]. Animals were purchased from the Experimental Animal Center of Xinjiang Medical University, and animal processing was done according to the Guide of the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

Establishment of isolated heart perfusion model. Rats were fasted for 12 h before surgery. For surgery, the rat was placed in a supine position, intraperitoneally anesthetized with pentobarbital at 60 mg/kg and heparin (200 U), and intratracheal intubation was performed for mechanical ventilation. After thoracotomy, the heart was harvested and placed at 4°C in Krebs–Henseleit (K-H) solution (11.8 mM NaCl, 4.7 mM KCl, 12 mM MgSO4, 2.5 mM CaCl2, 24.7 mM NaHCO3, 11 mM glucose). The aorta was suspended in a Langendorff perfusion system (ADInstruments Shanghai Trading Co., Ltd.), and perfused in a retrograde manner [7] with K-H solution (37°C, pH = 7.37–7.45) saturated with 95% O2 and 5% CO2 at a perfusion pressure of 75 cmH2O. During the perfusion, the heart was immersed in a constant temperature system (37°C). When the heart rate was stable for 30 min, a latex bladder was inserted into the left ventricle and connected to a multichannel signal acquisition system via a pressure transducer. The heart rate and left ventricular developed pressure (LVDP) were recorded.

If at any time during the first 30 min of stable perfusion, the heart rate was <180 bpm, left ventricular systolic pressure was <75 mmHg or coronary flow was <8 ml/min, perfusion was considered to be failed. Hearts with perfusion failure were excluded from this study.

Grouping. After stable perfusion for 30 min was completed, hearts were randomly assigned into four groups (20 adult and 20 old hearts in each group): (i) hypoxia-reperfusion (H/R) group: a 30-min hypoxia treatment followed by 60-min reperfusion; (ii) HPC group: a 10-min hypoxia, 10-min reperfusion treatment cycle repeated three times, followed by the —H/R procedure described above; (iii) RES group: a 10-μM RES treatment for 15 min followed by the H/R procedure and (iv) RES and hypoxic-preconditioning (RES + HPC) group: a 10-μM RES treatment for 15 min, then three H/R preconditioning cycles, followed by the H/R procedure.

Ventricular function assessment and sample collection. To assess the effect of RES and HPC on cardiac function, heart rate, LVDP and dp/dtmax were recorded with the signal acquisition system at the end of stable perfusion (T1), after preconditioning (T2), and after 5-min (T3), 30-min (T4) and 60-min (T5) reperfusion. At the end of the 60-min reperfusion, the tissues from left ventricle were rapidly harvested, fixed in 4% formalin and embedded in paraffin.

Detection of apoptotic myocytes. To assess the effect of RES and HPC on apoptotic cell death, TUNEL staining (Roche Diagnostics, Indianapolis, IN, USA) was performed on processed heart sections to detect apoptotic cells judged by the appearance of yellow to yellow-brown granules in the nuclei. The detection was done at the magnification of ×400, and the apoptosis index (Al) was calculated as follows:

\[ AI = \frac{\text{apoptotic cells}}{\text{total cells}} \times 100\% \]

The average Al values were obtained from five randomly selected fields.

Human cardiomyocytes

Culture of primary human myocytes. Young human cardiomyocytes were obtained from 15 patients (aged 5–20 years) with congenital heart disease who underwent surgery under extracorporeal circulation. Old human cardiomyocytes were obtained from 15 patients (aged 60–80 years) with rheumatic heart disease who underwent valve replacement surgery under extracorporeal circulation. This study was approved by the Ethics Committee of Affiliated First Hospital of Xinjiang Medical University, and informed consent was obtained from each patient.

Atrial tissue (0.5 cm3) from each patient was collected during surgery before insertion of the superior vena cava cannula, and immediately placed in cold (4°C), blood-containing crystallloid cardioplegic solution (20 mM KCl, 2.6 mM CaCl2, 16 mM MgCl2, 154 mM NaCl, 0.5 mM procaine, pH: 7.2, osmolality 340 mM). Two-step digestion was employed to collect myocytes: tissues were first digested with collagenase V (259 U/ml) and protease (0.4 mg/ml) for 50 min in the presence of bovine serum albumin, and then with collagenase V (200 U/ml) with bovine serum albumin for 30 min. The cell suspension was then diluted in calcium-free solution at a 1 : 5 (v/v); then the calcium concentration was gradually increased to the final of 0.6 mM. A differential adhesion method was employed to remove fibroblasts, and myocytes were seeded into plates followed by incubation (5% CO2, 95% air, 37°C) in a CO2 incubator (Thermo311 incubator, Thermo Fisher Corp., USA).

Hypoxic injury and preconditioning of primary human myocytes and grouping. To induce hypoxia-reoxygenation injury, the buffer (0.9 mM NaH2PO4, 6 mM NaHCO3, 1.8 mM CaCl2, 1.2 mM MgSO4, 20 mM, HEPES, 98.5 mM NaCl, 10 mM KCl, pH 6.8) was saturated with a gas mixture with low-oxygen concentration (94% N2, 5% CO2 and 1% O2) for 30 min, and then it was used to replace the normal M199/EBSS medium (HyClone) in the culture flasks. Cells were incubated in the low-oxygen buffer for 8 h, after which the low-oxygen buffer was replaced with normal medium and the cells were incubated in a normal environment for 1 h.

To produce HPC, the buffer described previously was saturated with a gas mixture with low-oxygen concentration for 30 min, and then used to replace the normal medium. Cells were incubated in the low-oxygen buffer for 1 h, and then switched to a normal medium and incubated in a normal environment for 1 h. This low/normal oxygen cycle was repeated three times.

For grouping, myocytes were randomly assigned into the following six groups: (i) control group: cells were cultured under normal condition and had no treatment; (ii) dimethyl sulfoxide (DMSO) group: cells were incubated in the medium with DMSO; (iii) hypoxia–reoxygenation (H/R) group: cells were cultured in the medium with low oxygen 8 h followed by 1 h in normal oxygen as described above; (iv) HPC group: cells underwent hypoxic preconditioning as described above followed by H/R; (v) RES and HPC (RES + HPC) group: cells underwent HPC in 40 μM of the SIRT activator RES (Sigma-Aldrich, Germany) in DMSO followed by H/R; and (vi) sirtinol and HPC (Sirtinol + HPC) group: cells underwent
HPC in 50 µM of the SIRT inhibitor sirtinol (Sigma-Aldrich, Germany) in DMSO followed by H/R. DMSO was used to dissolve RES; therefore, in order to exclude the possible/interference of DMSO on the experimental results for RES, a DMSO group was included.

Detection of cardiomyocyte viability by the MTT assay. Cells were seeded into a 96-well plate at a density of 1 x 10⁵/well, and the treatments described in the previous section were administered. After the treatments, MTT (MP Biomedicals) at 5 mg/ml was added to each well (20 µl), incubated for 4 h, then the reaction was stopped and the supernatant was removed. The crystals were dissolved by adding 150 µl DMSO to each well and incubated for 10 min with vortexing. The optical density (OD) of each well was measured at 490 nm with a microplate reader. In blank controls, DMSO alone was added to each well. Data were recorded as cell viability = OD_{treatment}/OD_{control} × 100%.

Detection of myocyte injury using the lactate dehydrogenase assay. Cell injury was evaluated by detecting the release of lactate dehydrogenase (LDH). In brief, 0.1 ml of culture medium was harvested and LDH was detected according to the manufacturer’s instruction (Nanjing Jiancheng Biotech Co., Ltd).

Statistical analysis
Sample sizes were planned according to previous reports [3, 8]. Continuous data were presented as means and standard deviations (SDs). Due to the repeated measurements of the changes in the heart Langendorff model, the linear mixed model was performed to investigate the effect of groups (denoted as Group Effect), test times (denoted as Time Effect) and their interaction (denoted as Group × Time Effect). The variances of the experimental animals were transformed so that the raw data could be normally distributed with constant variance. No other transformations were performed on the raw data. Data were transformed in this manner before analysis, and the results given in the tables and figures are all the transformed outcome. When the main effects or interactions were found significant, Bonferroni correlations were used for controlling type I error during multiple comparisons. One-way ANOVA with Bonferroni post hoc tests was performed to compare differences between groups in pressure restoration, cell survival rate and LDH. Statistical analyses were performed with the SAS software version 9.2 (SAS Institute, Inc., Cary, NC, USA) and analysis of the data in the figures was performed with the SPSS software version 17 (SPSS, Inc., Chicago, IL, USA). A two-tailed P-value <0.05 indicated statistical significance.

RESULTS
Cardiac function and myocyte death in rat heart
Changes in heart rate in the Langendorff model. Heart rate was significantly decreased 5 min into the reperfusion (T3) and then increased as reperfusion continued in all groups (Table 1). For adult hearts, heart rates increased by the end of reperfusion to levels indistinguishable from basal rates in the HPC, RES and HPC + RES groups (comparing T5 and T1, P = 0.986, 0.484 and 0.127, respectively). These results showed that either HPC or RES treatment was sufficient to bring heart rates back to basal levels in adult hearts, and that no further improvement occurred when the two treatments were combined. Basal heart rates were slower in old than in adult hearts in all groups. HPC treatment alone had no effect in old hearts, but RES treatment alone or RES combined with HPC significantly increased the heart rate. However, only RES treatment used alone increased heart rate to basal levels by the end of reperfusion (T5 vs T1 (P = 0.672)).

Changes in left ventricular developed pressure in the Langendorff heart model. Ventricular contractile force, as measured by LVDP also decreased at the beginning of reperfusion (T3) and then increased in all groups, although not to pre-H/R values (Table 2; P < 0.05, data not shown). In adult rat hearts, LVDP was significantly increased by the end of reperfusion (T5) in both the HPC and RES groups. In old rat hearts, only the combination of RES and HPC resulted in significant LVDP improvement. This difference can be clearly seen in Fig. 1, which shows the restoration of pressure as the ratio between T5 and T1.

Table 1: Changes in heart rate (bpm) in a rat heart Langendorff model

<table>
<thead>
<tr>
<th>H/R</th>
<th>HPC</th>
<th>RES</th>
<th>HPC + RES</th>
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<tbody>
<tr>
<td></td>
<td>Adult</td>
<td>Old</td>
<td>Adult</td>
</tr>
</tbody>
</table>
| H/R: hypoxia-reperfusion; HPC: hypoxic preconditioning; RES: resveratrol.
*Significantly different from adult rats at H/R group.
†Significantly different from old rats at H/R group.
‡Significantly different from adult rats at HPC group.
§Significantly different from old rats at HPC group.
¶Significantly different from adult rats at RES group.
#Significantly different from old rats at RES group.
$Significantly different from adult rats at HPC + RES group.
P-value for Time Effect: <0.001.
P-value for Group Effect: <0.001.
and HPC + RES all resulted in more than 50% restoration of LVDP in adult rat hearts. In old rat hearts, HPC or RES alone had no significant effect, but the combined treatment led to nearly 50% restoration of LVDP.

**Changes in dp/dt\text{max} in the Langendorff heart model.** The second indicator of ventricular function, dp/dt\text{max}, decreased at the beginning of reperfusion and then increased, although not to basal values (Table 3; \(P < 0.05\), data not shown). At the end of reperfusion (T5) in adult rat hearts, HPC resulted in the best recovery, although RES and HPC + RES also showed significant improvement over H/R. In old hearts at T5, the HPC and HPC + RES groups had a small but significantly higher dp/dt\text{max} than the corresponding H/R group. The RES + HPC group had a slightly higher effect than the HPC group, but the RES treatment alone had no significant effect on dp/dt\text{max} at T5.

The effect of treatment on dp/dt\text{max} was seen as early as 5 min after the initiation of reperfusion, at which time (T3) the HPC, RES and RES + HPC groups of adult rat hearts all had significantly higher dp/dt\text{max} values than the adult H/R group. HPC or RES given alone had similar effects, and the HPC + RES group had a higher effect. The same trend was true for the old groups. These results suggest that RES enhances the effect of HPC and ameliorates the decrease in dp/dt\text{max} caused by H/R.

**Apoptosis in adult versus old rat hearts.** In hearts from adult rats, HPC and RES treatment significantly decreased the percentage of apoptotic cells seen after hypoxia/reoxygenation (Fig. 2). Using the two treatments together caused an additive effect in adult hearts. In hearts from old rats, HPC and RES treatment also each resulted in a significant decrease in apoptosis, while combining the two treatments did not result in a significant decrease in apoptosis, while combining the two treatments was not additive.

**Cell injury and survival in old and young human cardiomyocytes**

**Effects of hypoxic preconditioning and resveratrol on cell survival rate.** Hypoxia/reoxygenation treatment decreased cell survival in both young and old human cardiomyocytes (Figs 3A and 4A). HPC increased cell survival in both groups. Inhibiting SIRT1 with sirtinol completely blocked the protective effect of HPC, and the addition of the SIRT-1 activator, RES, to HPC further improved survival, to values almost 60% of control values in young cardiomyocytes and almost 50% of control values in old cardiomyocytes.

**Effects of hypoxic preconditioning and resveratrol on cell injury.** Hypoxia/reoxygenation treatment significantly increased the release of LDH, the biomarker for cell injury, in both young and old cardiomyocytes (Figs 3B and 4B). HPC treatment partly and HPC + RES treatment fully prevented this increased release of LDH. Inhibiting SIRT1 with sirtinol completely blocked the effect of HPC on LDH.
Table 3: Changes of $dp/dt_{\text{max}}$ in a rat heart Langendorff model

<table>
<thead>
<tr>
<th></th>
<th>H/R</th>
<th>HPC</th>
<th>RES</th>
<th>HPC + RES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adult (n = 20)</td>
<td>Old (n = 20)</td>
<td>Adult (n = 20)</td>
<td>Old (n = 20)</td>
</tr>
<tr>
<td>T1</td>
<td>3000 ± 270</td>
<td>2977 ± 130</td>
<td>2988 ± 186</td>
<td>2993 ± 90</td>
</tr>
<tr>
<td>T2</td>
<td>2920 ± 211</td>
<td>2810 ± 141</td>
<td>2922 ± 175</td>
<td>2869 ± 104</td>
</tr>
<tr>
<td>T3</td>
<td>627 ± 108</td>
<td>540 ± 43</td>
<td>903 ± 130</td>
<td>673 ± 54</td>
</tr>
<tr>
<td>T4</td>
<td>1008 ± 121</td>
<td>984 ± 94</td>
<td>2123 ± 173</td>
<td>1350 ± 95</td>
</tr>
<tr>
<td>T5</td>
<td>2019 ± 101</td>
<td>2200 ± 140</td>
<td>2844 ± 164</td>
<td>2303 ± 129</td>
</tr>
</tbody>
</table>

H/R: hypoxia–reperfusion; HPC: hypoxic preconditioning; RES: resveratrol.
*Significantly different from adult rats at H/R group.
†Significantly different from old rats at H/R group.
‡Significantly different from adult rats at HPC group.
§Significantly different from old rats at HPC group.
Significantly different from adult rats at RES group.
Significantly different from old rats at RES group.
Significantly different from adult rats at HPC + RES group.
P-value for Time Effect: <0.001.
P-value for Group Effect: <0.001.

**DISCUSSION**

Previous investigation has shown that aged hearts are less responsive than younger hearts to HPC-induced protection from H/R injury [2]. In our study, HPC was less effective in older than in younger rat hearts in restoring ventricular function, and had no effect on heart rate or LVDP and only a small effect on $d$/d$t_{max}$. The question that needs explanation is why the effects of HPC and RES on cardiac function are different from their effects on cell survival. RES and HPC increased cell survival about 10% in both young and old cardiomyocytes, although this increase only reached statistical significance in old cardiomyocytes. In both groups, inhibiting SIRT1 blocked the prosurvival action of HPC, and adding the SIRT1 activator RES to HPC increased cell survival.

RES is an activator of SIRT1, which is a deacetylase that functions to counteract stress and regulate apoptosis, energy metabolism and cell ageing. Shimura et al. [13] have reported that SIRT1 overexpression increases the tolerance of myocardium to hypoxia. Alcendor et al. [14] used nicotinamide to inhibit the SIRT1 expression in primary rat myocytes and their results showed that reduced SIRT1 expression and/or activity could induce massive apoptosis of myocytes in the presence of hypoxia. We found inhibition of SIRT1 to block the effect of HPC on cell survival, results consistent with those of others.

The question that needs explanation is why the effects of HPC and RES on cardiac function are different from their effects on cell survival. RES and HPC increase cell survival about 50–60% in young and old cardiomyocytes whether rat or human. This combination also increases LVDP to 40–60% of normal in rat hearts, an effect similar in magnitude to its effects on cell survival. RES by itself causes complete recovery but HPC causes no recovery of...
heart rate in old hearts. Further, HPC and RES have no effect on
\( \frac{dp}{dt_{\text{max}}} \) when used alone in old hearts and only a marginally sig-
ificant effect when used together. However, heart rate and ven-
tricular contractile force are determined by electrophysiological
events as well as cell survival. Further study of the molecular
mechanisms involved the HPC and RES-induced cardioprotection
needs to be done in order to find the means through which cell
survival and cardiac function are affected differently.

In conclusion, HPC may attenuate myocardial H/R injury and
exert a cardioprotective effect that is reduced with ageing. Our
study showed that RES lessened the ageing effect and enhanced
the cardioprotective effect of hypoxia preconditioning in older
individuals. RES is likely to work through up-regulating SIRT1 ex-
pression and therefore increasing the expression of its target genes.

Conflict of interest: none declared.

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