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Original Article

Chronic intermittent hypobaric hypoxia protects the heart against ischemia/reperfusion injury through upregulation of antioxidant enzymes in adult guinea pigs

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Aim: To investigate the protection and the anti-oxidative mechanism afforded by chronic intermittent hypobaric hypoxia (CIHH) against ischemia/reperfusion (I/R) injury in guinea pig hearts.
Methods: Adult male guinea pigs were exposed to CIHH by mimicking a 5000 m high altitude (p_B=404 mmHg, p_{O2}=84 mmHg) in a hypobaric chamber for 6 h/day for 28 days. Langendorff-perfused isolated guinea pig hearts were used to measure variables of left ventricular function during baseline perfusion, ischemia and the reperfusion period. The activity and protein expression of antioxidant enzymes in the left myocardium were evaluated using biochemical methods and Western blotting, respectively. Intracellular reactive oxygen species (ROS) were assessed using ROS-sensitive fluorescence.
Results: After 30 min of global no-flow ischemia followed by 60 min of reperfusion, myocardial function had better recovery rates in CIHH guinea pig hearts than in control hearts. The activity and protein expression of superoxide dismutase (SOD) and catalase (CAT) were significantly increased in the myocardium of CIHH guinea pigs. Pretreatment of control hearts with an antioxidant mixture containing SOD and CAT exerted cardioprotective effects similar to CIHH. The irreversible CAT inhibitor aminotriazole (ATZ) abolished the cardioprotection of CIHH. Cardiac contractile dysfunction and oxidative stress induced by exogenous hydrogen peroxide (H_2O_2) were attenuated by CIHH and CAT.
Conclusions: These data suggest that CIHH protects the heart against I/R injury through upregulation of antioxidant enzymes in guinea pig.

Keywords: chronic intermittent hypobaric hypoxia; cardioprotection; antioxidant enzymes; ischemia/reperfusion; guinea pig

Introduction

Increasing evidence has shown that chronic intermittent hypobaric hypoxia (CIHH) effectively protects the heart against ischemia/reperfusion (I/R) or hypoxia/reoxygenation injury[1, 2]. The protective effects promote recovery of cardiac contractile function from I/R, limiting cardiac infarct and arrhythmia induced by I/R[3–5]. Several potential factors have been proposed to be involved in the protective mechanism afforded by CIHH, including regulation of myocardial heat shock protein expression, amelioration of coronary circulation and angiogenesis, activation of protein kinase C and involvement of K_{ATP} channels[6–9]. However, the precise mechanisms

underlying the protective effects of CIHH on ischemic hearts are far from clear.

It is well known that oxidative stress and oxygen-derived free radicals [primarily reactive oxygen species (ROS)] contribute to I/R injury[10, 11]. Many proteins that have key roles in the homeostasis of cardiomyocytes, such as the Na^+/Ca^{2+} exchanger and the sodium pump, are modified during massive ROS release[12, 13]. Intercellular antioxidant enzymes, which can eliminate the oxygen-derived free radicals, include glutathione peroxidase (GPX), catalase (CAT) and superoxide dismutase (SOD). SOD includes a cytosolic dimeric Cu/ZnSOD (SOD-1) and a mitochondrial tetrameric MnSOD (SOD-2)[14] enzyme, which contribute to the first line of antioxidant defenses by catalyzing the conversion of O_2^- into hydrogen peroxide (H_2O_2). Both GPX and CAT belong to the secondary line of antioxidant defenses by catalyzing the con-

version of H_2O_2 to H_2O. Thus, it is generally considered that manipulations that enhance the antioxidant activity in heart tissue may be useful in preventing I/R injury. Previous findings have shown that renal SOD levels in rats were increased during hypoxic preconditioning[15]. Hyperbaric oxygenation pretreatment can induce CAT and reduce infarct size in the ischemic rat myocardium[16]. Previous studies on the cardioprotection of CIHH have shown that antioxidant activity increases, suggesting a role for antioxidation in the cardioprotective mechanism of CIHH[4, 17, 18]. It was reported, however, that antioxidant enzymes in rats submitted to CIHH (7000 m altitude, 8 h/day and 5 days/week for 24−30 days) were not significantly altered[19]. Whether or not antioxidation contributed to the cardioprotection of CIHH as well as the details of the underlying mechanisms are yet to be elucidated.

The cardioprotective nature of CIHH is affected by many factors, such as the level, duration and protocol of hypoxia[2]. It also depends on resistance to hypoxia, which varies between animals. For example, guinea pigs are relatively resistant to hypoxia[20, 21]. To date, using CIHH as a cardioprotective measure has been approved in some animals, such as rats and dogs[5]; however, it has yet to be approved for use in guinea pigs.

The aim of the present study was to evaluate (i) whether CIHH could protect the hearts of adult guinea pigs against I/R injury by using recovery of ventricular function as an end point, and (ii) whether antioxidation is involved in the cardioprotection afforded by CIHH.

Materials and methods
Animal CIHH treatment
Adult male guinea pigs (250±20 g, *n*=134) were provided by the Experimental Animal Center of Hebei Medical University (Shijiazhuang, China). All animals were treated in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health, and divided into non-CIHH and CIHH groups. Guinea pigs submitted to CIHH were exposed to intermittent high-altitude hypoxic conditions of 5000 m (p_B=404 mmHg, p_{O2}=84 mmHg) in a hypobaric chamber for 6 h/day for 28 days, and non-CIHH guinea pigs were kept in the same environment without hypoxic exposure as a control. All animals were used for further experiments on the day following the pretreatment described above. Both groups of guinea pigs were raised at room temperature (20–24 °C) with a natural light-dark cycle (12 h:12 h) and had free access to water and food throughout pretreatment.

Perfusion of the isolated hearts and measurement of cardiac function
Both CIHH and non-CIHH guinea pigs were anesthetized with sodium pentobarbital [60 mg/kg, intraperitoneally (ip)] as previously described[22]. The heart was quickly excised, mounted on a Langendorff apparatus and perfused with Krebs-Henseleit buffer (K-H buffer) gassed with 95% O_2−5% CO_2 and

kept at 37 °C at a constant pressure of 80 mmHg. Composition of the K-H buffer (pH 7.4) was (in mmol/L): NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 11.0. A water-filled latex balloon connected to a pressure transducer (Gould P23Db) was introduced into the left ventricle via the mitral valve to record isovolumic left ventricular pressure. The balloon volume was adjusted to achieve a stable left ventricular end-diastolic pressure (LVEDP) of 5–10 mmHg during the initial equilibration. Coronary flow rate (CF) was obtained by collecting the coronary effluent for 1 min. Heart rate (HR), left ventricular peak systolic pressure (LVPSP) and peak rate of pressure that developed (±dp/dt_{max}) were monitored using a PowerLab system (AD Instrument Ltd, Castle Hill, Australia) before and after I/R. Pressure-rate product (PRP) and left ventricular developed pressure (LVDP) were calculated as LVDP=LVPSP–LVEDP and PRP=HR×LVDP, respectively.

Experimental protocol and groups
Six hearts from each group (non-CIHH and CIHH) used for the analysis of myocardial hypertrophy were rapidly excised and washed in saline. Incisions were made into the right ventricle and left ventricle through the interventricular septum. All parts were weighed and the corresponding ratios were calculated.

Seventy guinea pigs were divided into five groups: (1) non-CIHH (CON), (2) CON+antioxidant enzyme, (3) CON+aminotriazole (ATZ), (4) CIHH only, and (5) CIHH+ ATZ. To detect the effect of antioxidant on the cardioprotection of CIHH, 35 hearts (*n*=7 in each group) were subjected to I/R injury using a protocol of 20-min baseline perfusion, 30 min no-flow global ischemia, followed by a 60 min reperfusion. Hearts in the CON+antioxidant enzyme (CON+SOD+CAT) group were perfused with an antioxidant mixture containing SOD (100 U/mL)[23] and CAT (120 U/mL)[23] for 10 min before ischemia until the end of I/R. To examine whether the beneficial effects of CIHH on I/R injury are a result of its increased antioxidant activity, 35 hearts (*n*=7 in each group) were subjected to a protocol of 20 min baseline perfusion and 30 min H_2O_2 (300 μmol/L, a potent oxidant)[32] perfusion. Hearts in CON+antioxidant enzyme (CON+CAT) group were perfused with CAT (120 U/mL) for 10 min before H_2O_2 treatment until the end of the protocol. In all CON+ATZ and CIHH+ATZ groups, ATZ (1.0 g/kg)[29, 42] was administered to the guinea pig intraperitoneally 1 h before heart isolation.

To observe the effects of CIHH on antioxidant enzyme activity and protein level in guinea pig myocardial tissue before and after I/R, 24 hearts were divided into four groups (*n*=6 in each group): (1) non-CIHH baseline, (2) non-CIHH I/R, (3) CIHH baseline, and (4) CIHH I/R. All I/R hearts were subjected to a protocol of 20 min baseline perfusion, 30 min no-flow global ischemia, followed by a 60 min reperfusion. All baseline hearts were subjected to 20 min baseline perfusion. The hearts were then frozen in liquid nitrogen and stored at -80 °C.

Measurement of antioxidant enzyme activity and lipid superoxide level

The myocardium was rinsed and then homogenized in buffer composed of (in mmol/L)[24]: Tris-HCl 10, NaCl 137, Na_2EDTA 1, dithiotreitol (DTT) 0.5, and sucrose 250 at pH 7.4 using a homogenizer (T 18 basic Ultra-Turrax®; Mandel Scientific Company Inc, Guelph, Canada). The homogenate was centrifuged at $1000 \times g$ for 15 min at 4 °C. The supernatants were used for biochemical assay and Western blot analysis. The activity of total SOD, SOD-1, SOD-2, CAT and GPX and the content of malondialdehyde (MDA) were used as indices of antioxidant capacity and lipid superoxide level, respectively. Measurements were made using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China) with a spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). Total SOD activity was determined by inhibition of pyrogallol antioxidation. To determine SOD-2 activity, the assay was repeated in the presence of potassium cyanide (1 mmol/L), which inhibits the activity of SOD-1. The activity of SOD-1 was calculated as the difference between the total SOD activity (without potassium cyanide) and the SOD-2 activity (with potassium cyanide). A unit of the enzyme is generally defined as the amount of enzyme that inhibits the reaction by 50%[17]. MDA was determined by a variation of the thiobarbituric acid method. The activity of CAT and GPX was measured by the method described by Zang *et al*[25]. Protein concentration was measured using the bicinchoninic acid method (Pierce, Rockford, USA) with bovine serum albumin as a standard.

Measurement of antioxidant enzyme protein level

The protein of supernatants was separated using SDS-PAGE on 12% acrylamide gels. They were then transferred onto polyvinylidene fluoride (PVDF) membranes and blocked with 5% nonfat dry milk in Tris Buffered Saline Tween-20 (TBST) buffer (50 Tris-HCl, 150 mmol/L NaCl, 0.1% Tween, pH 7.4) for 1 h at room temperature. Membranes were then incubated with the polyclonal IgG for SOD-1, SOD-2, and CAT (dilution 1:200) overnight at 4 °C, washed with TBST three times for 10 min each, and then incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) for 1 h at 37 °C. After washing, blots were detected using an enhanced chemiluminescence plus system (ZhongShan Bioengineering Institute, Beijing, China). Quantification of Western blot signals was performed using densitometric measurements. The data were normalized to the ratios of β-actin (dilution 1:800) detected on the same blot to control for possible variations in protein loading.

Measurement of ROS in isolated ventricular myocytes

Myocytes were enzymatically isolated from the left ventricles of non-CIHH or CIHH guinea pigs as previously described[26]. Briefly, guinea pigs were anesthetized with sodium pentobarbitone solution (60 mg/kg, ip). The heart was quickly excised and perfused in a retrograde fashion with oxygenated Ca^{2+}-free Tyrode's solution (pH 7.4, 37 °C) (in mmol/L): NaCl 135.0, KCl 5.4, $MgCl_2$ 1.0, NaH_2PO_4 0.33, HEPES 5.0 and glucose 10.0,

and gassed with pure O_2 for 5 min. The perfusion solution was then switched to Ca^{2+}-free Tyrode's solution containing 0.4 mg/mL collagenase II (Worthington Biochemical Co, Lakewood, NJ, USA) for 15 min. The left ventricle was removed and agitated mechanically in high-K^+ Kraft-Bruhe (KB) solution to obtain single ventricular myocytes, which were used within 6 h after isolation. The composition of the high-K^+ KB solution was (in mmol/L): KOH 80, KCl 40, $MgSO_4$ 3, KH_2PO_4 25, glutamic acid 50, taurine 20, HEPES 10, EGTA 1 and glucose 10, at pH 7.2.

Intracellular oxidative stress was monitored by using the probe 29, 79-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, USA). Cells were loaded with 10 μmol/L DCFH-DA for 30 min at room temperature in the dark. After loading, cells were washed twice and maintained in Tyrode's solution (Ca^{2+}-free Tyrode's solution+$CaCl_2$ 1.8 mmol/L) until the assay was performed. For confocal imaging, a Leica (Wetzlar, Germany) DM-IRBE inverted microscope fitted with a TCS-SP2 scan head was used. Excitation of DCFH-DA was achieved with a 488 nm argon ion laser line, and emissions were collected at 500–565 nm. The fluorescent intensity (FI) was measured before (FI_0) and after (FI) adding H_2O_2 into the Tyrode's solution. The change of ROS was calculated using the ratio of $(FI-FI_0)/FI_0$.

Myocytes were divided into five groups (*n*=18 in each group, from 3−4 different hearts): (1) non-CIHH (CON1), (2) CON1+CAT, (3) CON1+ATZ, (4) CIHH1, and (5) CIHH1+ATZ group. In the present study, we used 10 min baseline perfusion and a 10 min H_2O_2 (1 mmol/L) treatment protocol. The myocytes in the CON1+CAT group were treated with CAT (100 U/mL) for 5 min before and 10 min during H_2O_2 treatment. Aminotriazole was administered intraperitoneally to the guinea pigs at 1 h before isolation of the hearts from the CON1+ATZ and CIHH1+ATZ groups.

Reagents

The SOD, CAT, ATZ, and H_2O_2 were purchased from Sigma Chemical Company (St Louis, MO). Antibodies against SOD-1, SOD-2, CAT, and β-actin (Santa Cruz Biotechnological Co Santa Cruz, CA) were used.

Statistical analysis

All data are expressed as mean±SEM. Statistical analysis was carried out using a one-way ANOVA or Student's *t* tests when appropriate. Differences were considered significant at $P<0.05$.

Results

Effects of CIHH on body weight and heart weight

The body weight of guinea pigs in the CIHH groups had no significant change compared with the non-CIHH group (Table 1). No differences were found in the ratio of heart weight to body weight, the ratio of left ventricular plus interventricular septum weight to body weight, the ratio of right ventricular weight to left ventricular plus interventricular septum weight or the ratio of right ventricular weight to body weight between

Table 1. Effects of CIHH on body weight and heart weight of guinea pigs. *n*=6. Values are means±SEM.

Group	BW (g)	HW/BW (g/kg)	(LVW+ISW)/BW (g/kg)	RVW/(LVW+ISW) (g/g)	RVW/BW (g/kg)
non-CIHH	365.4±5.3	3.60±0.16	2.57±0.06	0.25±0.01	0.64±0.05
CIHH	355.6±5.8	3.65±0.14	2.51±0.09	0.21±0.01	0.71±0.04

BW, body weight; HW/BW, ratio of heart weight to body weight; (LVW+ISW)/BW, ratio of left ventricular plus inter-ventricular septum weight to body weight; RVW/(LVW+ISW), ratio of right ventricular weight to left ventricular plus inter-ventricular septum weight; RVW/BW, ratio of right ventricular weight to body weight. non-CIHH, control group; CIHH, chronic intermittent hypobaric hypoxia group.

the CIHH groups and the non-CIHH group. These findings indicate that CIHH in this experimental paradigm may not result in heart hypertrophy in guinea pigs.

Effects of CIHH on recovery of coronary flow and left ventricular function after I/R

All cardiac function parameters in each group were altered after I/R ($P<0.05$ or 0.01), with the exception of HR (Table 2). Among them, CF, LVPSP, $\pm\mathrm{d}p/\mathrm{d}t_{\mathrm{max}}$ and PRP were greatly reduced, whereas LVEDP was significantly increased. However, I/R-induced harmful changes of these parameters, with the exception of LVPSP, were modestly improved by CIHH adaptation ($P<0.05$ or 0.01). A similar improvement was observed in non-CIHH hearts treated with SOD+CAT ($P<0.05$ or 0.01). Moreover, treatment with ATZ (1.0 g/kg) completely abolished the improvement of CIHH on cardiac function impaired by I/R ($P<0.01$), suggesting that the beneficial effects of CIHH on cardiac performance in hearts that underwent I/R may be related to its antioxidant capacity. Pretreatment with CIHH did not change cardiac function parameters before I/R, except for CF, which was increased by CIHH and not abolished by ATZ.

Effects of CIHH on antioxidant enzymes and MDA in the left ventricle

The biochemical experiment demonstrated that the baseline activity of total SOD, SOD-2, and CAT in CIHH hearts was higher than those in non-CIHH hearts (Figure 1, $P<0.01$); however, SOD-1, and GPX activity and MDA content did not significantly change. After I/R, total SOD, SOD-1, SOD-2, and CAT activity decreased and MDA content significantly increased in non-CIHH and CIHH hearts ($P<0.01$), whereas the activity of total SOD, SOD-2, and CAT in CIHH hearts was still higher than those in non-CIHH hearts ($P<0.01$). Moreover, the content of MDA was lower in CIHH hearts than in non-CIHH hearts ($P<0.01$). Western blot analysis demonstrated that the baseline expression of SOD-2 (25 kDa band) and CAT

Table 2. Effects of CIHH on hemodynamic parameters in guinea pigs suffered from ischemia and reperfusion. *n*=7. Values are means±SEM. [b]$P<0.05$, [c]$P<0.01$ *vs* corresponding baseline perfusion; [e]$P<0.05$, [f]$P<0.01$ *vs* corresponding of CON; [h]$P<0.05$, [i]$P<0.01$ *vs* corresponding of CIHH.

	CON	CON+SOD+CAT	CON+ATZ	CIHH	CIHH+ATZ
Baseline					
CF (mL/min)	24.3±1.5	22.6±1.1	23.3±1.4	29.9±1.3[e]	28.8±1.4[e]
LVPSP (mmHg)	97.8±2.4	92.5±3.9	102.6±5.9	97.6±7.9	94.8±6.3
LVEDP (mmHg)	8.2±1.0	8.2±0.7	8.2±2.0	6.6±1.3	7.4±1.0
HR (beat/min)	244±16	256±14	250±19	256±19	262±17
+d*p*/d*t* (mmHg/s)	1497±148	1533±143	1559±83	1694±75	1441±140
-d*p*/d*t* (mmHg/s)	−1262±96	−1482±91	−1140±71	−1426±107	−1249±100
PRP (10^3 mmHg/min)	21.2±1.6	21.4±1.8	23.7±2.2	23.3±3.5	23.1±2.4
Reperfusion					
CF (mL/min)	7.9±0.6[c]	12.8±0.8[cf]	7.3±0.7[c]	15.6±1.4[cf]	9.9±0.7[ci]
LVPSP (mmHg)	79.8±5.0[b]	71.1±2.3[b]	82±5.4[b]	75.2±6.3[b]	77.7±3.0[b]
LVEDP (mmHg)	52.7±8.3[c]	26.9±4.9[cf]	57.2±9.0[c]	23.6±3.0[cf]	45±3.0[ci]
HR (beat/min)	226±15	242±14	222±10	254±21	252±13
+d*p*/d*t* (mmHg/s)	463±82[c]	799±65[ce]	401±90[c]	923±45[ce]	485±66[ci]
-d*p*/d*t* (mmHg/s)	−353±84[c]	-658±48[ce]	−312±84[c]	−683±63[ce]	−389±53[ci]
PRP (10^3 mmHg/min)	7.2±0.8[c]	11.8±0.7[cf]	6.1±0.9[c]	12.9±1.0[cf]	8.9±0.6[ch]

CF, coronary flow; LVPSP, left ventricular peak systolic pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; $\pm\mathrm{d}p/\mathrm{d}t_{\mathrm{max}}$, peak rate of pressure developed; PRP, pressure-rate product. CON, control hearts; SOD, superoxide dismutase; CAT, catalase; ATZ, aminotriazole; CON+SOD+CAT, antioxidant mixture containing SOD and CAT was infused into the perfusion stream in control hearts; CIHH, chronic intermittent hypobaric hypoxia hearts; CON+ATZ, control guinea pigs pretreated with ATZ (1.0 g/Kg). CIHH+ATZ, CIHH guinea pigs pretreated with ATZ.
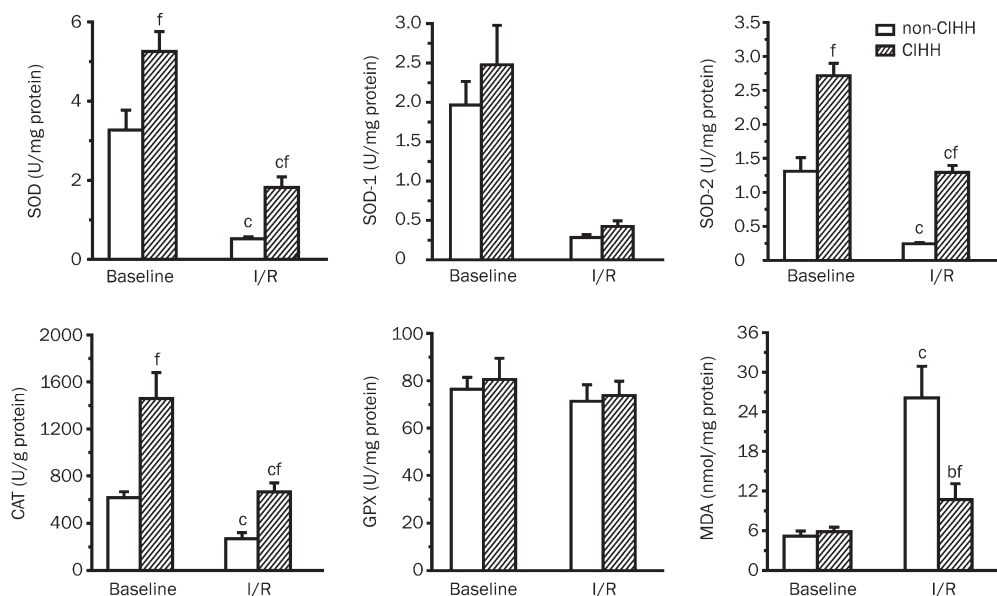
Figure 1. Effects of CIHH on anti-oxidant enzyme activity and MDA concentration in guinea pig hearts. SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; MDA, malondialdehyde; non-CIHH, control group; CIHH, chronic intermittent hypobaric hypoxia group; baseline, pre-ischemia; I/R, 60 min after reperfusion. $n=6$. Values represent the means±SEM. $^cP<0.01$ *vs* corresponding baseline values; $^fP<0.01$ *vs* the corresponding non-CIHH group.

(64 kDa band) protein was higher in CIHH hearts than in non-CIHH hearts ($P<0.05$) (Figure 2). However, the expression of SOD-1 protein did not change. The protein expression of SOD-1, SOD-2, and CAT did not significantly change after I/R in the non-CIHH and CIHH hearts, whereas the expression of SOD-2 and CAT in CIHH hearts was still higher than those in non-CIHH hearts ($P<0.01$). These results indicate that CIHH increases the activity of SOD-2 and CAT by upregulating their respective protein expression.

Effects of CIHH on H$_2$O$_2$-induced heart contractile dysfunction

Data shown in Table 3 indicated that H$_2$O$_2$ perfusion for 30 min, like I/R, induced a similar alteration in all cardiac funtion parameters except for HR in all groups. Significant

decreases in CF, LVPSP, ±dp/dt_{max} and PRP and an increase in LVEDP were observed ($P<0.05$ or 0.01). Compared with the CON group, CF, ±dp/dt_{max} and PRP were higher and LVEDP was lower in CIHH treated rats ($P<0.05$ or 0.01), suggesting that CIHH possesses significant protective effects on H$_2$O$_2$-induced contractile dysfunction. A similar improvement was also observed in H$_2$O$_2$-perfused non-CIHH hearts pretreated with CAT (100 U/mL) ($P<0.05$ or 0.01). Administration of ATZ also completely abolished the protective effect of CIHH on cardiac function ($P<0.05$ or 0.01), but not before treatment with H$_2$O$_2$. Furthermore, ATZ had no effect on hearts in the non-CIHH group before or after H$_2$O$_2$ treatment. Pretreatment with CIHH did not change any cardiac function parameters before H$_2$O$_2$ perfusion, except for CF, which was increased by
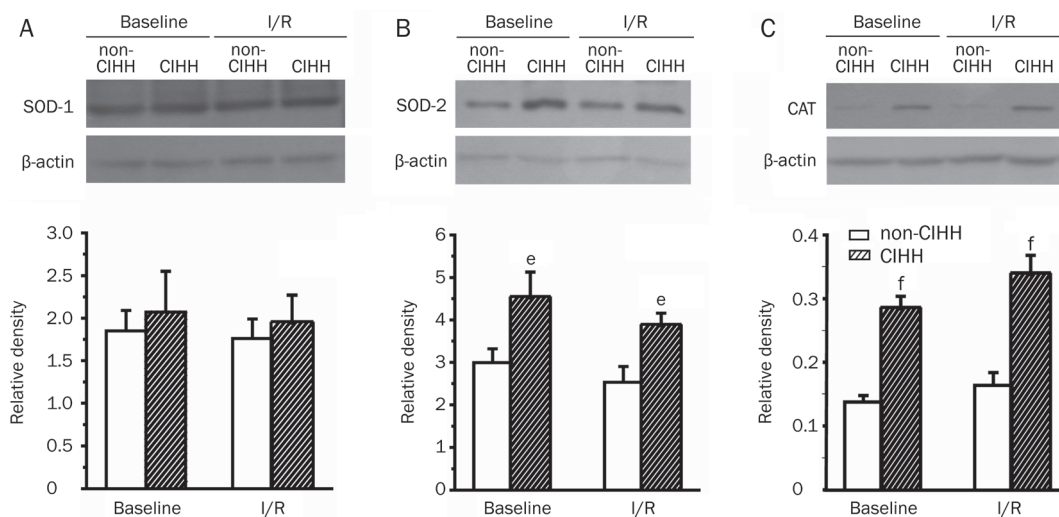

Figure 2. Effects of CIHH on the protein expression of SOD-1, SOD-2, and CAT in the left ventricle. SOD, superoxide dismutase; CAT, catalase. Top: representative lanes of a Western blot for SOD-1 (A), SOD-2 (B), or CAT (C). Bottom: densitometric analysis of SOD-1, SOD-2, and CAT bands normalized to β-actin. non-CIHH, control group; CIHH, chronic intermittent hypobaric hypoxia group; baseline, pre-ischemia; I/R, 60 min after reperfusion. $n=5$. Values represent the means±SEM. $^eP<0.05$, $^fP<0.01$ *vs* the corresponding non-CIHH group.

Table 3. Effects of CIHH on H_2O_2-induced changes in cardiac performance. $n=7$. Values are means±SEM. [b]$P<0.05$, [c]$P<0.01$ *vs* corresponding baseline perfusion; [e]$P<0.05$, [f]$P<0.01$ *vs* corresponding of CON; [h]$P<0.05$, [i]$P<0.01$ *vs* corresponding of CIHH.

	CON	CON+CAT	CON+ATZ	CIHH	CIHH+ATZ
Baseline					
CF (mL/min)	22.3±1.4	21.9±1.1	24.1±2.2	27.9±1.6[e]	28.0±1.7[e]
LVPSP (mmHg)	92.5±7.2	95.7±3.5	102.0±5.8	91.6±9.8	100.8±8.0
LVEDP (mmHg)	5.1±1.4	8.4±1.0	7.0±1.3	6.3±1.3	7.8±1.0
HR (beat/min)	257±20	261±18	246±19	255±15	260±18
+dp/dt (mmHg/s)	1249±62	1393±124	1646±155	1445±118	1589±168
-dp/dt (mmHg/s)	−1005±127	−1175±100	−1175±100	−1111±136	−1097±121
PRP (10^3 mmHg/min)	22.5±1.9	22.8±2.4	23.4±3.1	21.8±1.7	24.2±2.0
H_2O_2					
CF (mL/min)	5.7±0.5[c]	13.1±0.6[cf]	5.3±0.7[c]	14.7±1.1[cf]	6.8±0.8[ci]
LVPSP (mmHg)	58.0±4.6[c]	70.6±4.0[b]	69.4±3.3[c]	65.6±6.2[b]	72.4±4.0[b]
LVEDP (mmHg)	49.0±5.6[c]	28.6±4.8[be]	56.6±7.5[c]	25.6±5.8[be]	56.4±4.2[ch]
HR (beat/min)	251±17	257±21	244±17	251±18	253±20
+dp/dt (mmHg/s)	142±49[c]	638±88[bf]	294±73[c]	769±177[be]	323±76[ch]
-dp/dt (mmHg/s)	−115±40[c]	−493±58[bc]	−207±45[c]	−522±152[be]	−224±40[ch]
PRP (10^3 mmHg/min)	2.3±0.5[c]	10.8±0.9[cf]	3.1±0.6[c]	10.1±1.0[cf]	4.1±0.6[ci]

CF, coronary flow; LVPSP, left ventricular peak systolic pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; ±dp/dt_{max}, peak rate of pressure developed. PRP, pressure-rate product. CON, control hearts; CAT, catalase; ATZ, aminotriazole; CON+CAT, CAT treatment was started 10 min before H_2O_2 perfusion and continued throughout the H_2O_2 perfusion period in control hearts; CIHH, chronic intermittent hypobaric hypoxia; CON+ATZ, control guinea pigs pretreated with ATZ (1.0 g/kg) one hour before isolation of heart. CIHH+ATZ, CIHH guinea pigs pretreated with ATZ (1.0 g/kg) one hour before isolation of heart.

CIHH and was not abolished by ATZ.

Effects of CIHH on oxidative stress induced by H_2O_2 in single cardiac myocytes

The fluorescent intensity was considerably higher and the staining was strikingly stronger in CON1 cardiac myocytes treated with 1 mmol/L H_2O_2 for 10 min. These results suggested an elevated level of ROS, which was significantly attenuated by CAT (100 U/mL) ($P<0.01$) (Figure 3). The fluorescent intensity in CIHH1 cardiac myocytes was significantly lower than that of CON1 cardiac myocytes, and pretreatment with ATZ significantly increased the fluorescent intensity in CIHH1 cardiac myocytes ($P<0.01$).

Discussion

We have shown that, although cardiac hypertrophy may not occur in the right and left ventricles, the adaptation of guinea pigs to CIHH significantly increased the cardiac tolerance to I/R and H_2O_2 injury. We observed an improved recovery of contractile function, increased CF and a reduced level of ROS in cardiomyocytes, indicating that CIHH has a cardioprotective effect on isolated hearts subjected to I/R and H_2O_2 injury and on isolated cardiomyocytes subjected to H_2O_2 injury. These results are consistent with studies showing the cardioprotection of CIHH in dogs and rats[5, 9], demonstrating that CIHH also has a protective effect on the hearts of guinea pigs, which are resistant to hypoxia and I/R injury. These results suggest that CIHH cardiac protection universally exists in ani-

mals.

It is known that myocardial I/R induces an injurious cascade of ROS[27]. In our study, CIHH was able to markedly promote the recovery of guinea pig cardiac function from a 30 min ischemic episode followed by 60 min reperfusion, suggesting the effect of antioxidant enzymes in the cardioprotection of CIHH in I/R injury. Hydrogen peroxide is an important product of oxidative stress that could reach 1 to 2 mmol/L in the microenvironment during I/R injury[28]. Oxidative stress can therefore be induced by direct perfusion of H_2O_2. In the present study, we perfused the isolated hearts and single cardiomyocytes of guinea pigs with H_2O_2 to mimic I/R injury induced by ROS. We found that CIHH promoted the recovery of heart function and reduced the elevated level of ROS in cardiomyocytes, which further indicated that antioxidant enzymes had a key role in the cardioprotection of CIHH against I/R injury. When the hearts were pretreated with the CAT inhibitor ATZ, we observed that the cardioprotection offered by CIHH was abolished in either myocardial tissue or single cardiomyocytes subjected to I/R or H_2O_2 injury. These findings are consistent with previous studies showing the administration of CAT inhibitors attenuated ischemic tolerance induced by hyperbaric oxygen[29]. Superoxide dismutase only converts O_2^- generated in enzymatic reactions to H_2O_2, which is still harmful to tissue. Hydrogen peroxide is then converted to H_2O by CAT or GPX; therefore, inhibition of CAT may also partly eliminate the action of SOD. When we pretreated the hearts and single cardiomyocytes subjected to I/R or H_2O_2 injury from non-
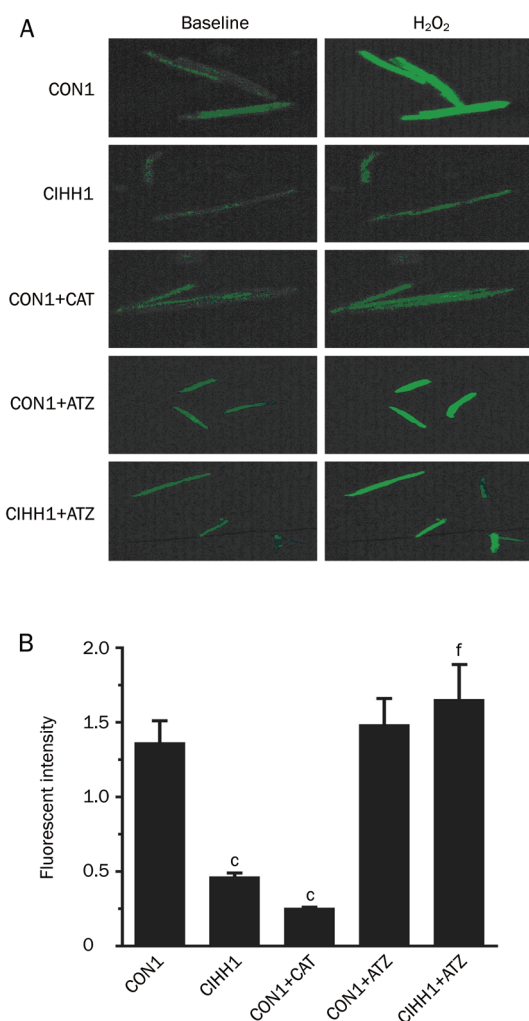
Figure 3. Effects of CIHH on H_2O_2-induced oxidative stress in isolated cardiac myocytes. CAT, catalase; ATZ, aminotriazole; CON1, non-CIHH group; CON1+CAT, CON1+CAT group; (3) CON1+ATZ, CON1+ATZ group; CIHH1, CIHH group; CIHH1+ATZ, CIHH1+ATZ group. Myocytes were subjected to H_2O_2-induced injury using a protocol of 10 min baseline perfusion, followed by 10 min of H_2O_2 treatment. The myocytes in the CON1+CAT group were treated with CAT (100 U/mL) for 5 min before and 10 min during H_2O_2 treatment. Aminotriazole was administered intraperitoneally to the guinea pig 1 h before isolation of the heart in the CON1+ATZ and CIHH1+ATZ groups. (A) Fluorescent intensity changes in cardiac myocytes exposed to H_2O_2 (10 min). (B) The bar graph shows the fluorescent intensity that represents the ratio of $(Fl-Fl_0)/Fl_0$. n=18, from 3−4 different hearts. Values represent the means±SEM. [c]P<0.01 *vs* the CON1 group; [f]P<0.01 *vs* the CIHH1 group.

CIHH guinea pigs with the antioxidant enzymes SOD and/or CAT, similar cardioprotection to CIHH adaptation was also observed. These data further confirm that, although the mechanism of the protective role is not precisely understood, a key part is played by antioxidant enzymes. It is important to note that we observed the increases in activity and protein expression of major antioxidant enzymes SOD-2 and CAT in the CIHH guinea pig myocardial tissue. Although the activity of these antioxidant enzymes decreased after myocardial I/R,

a significantly higher activity of SOD-2 and CAT and a lower expression of MDA were observed in the CIHH group than those in the non-CIHH group. Taken together, our results strongly support the suggestion that antioxidant enzymes are involved in the cardioprotection of CIHH against I/R insult. These results are consistent with previous findings showing that administration or overexpression of antioxidant enzymes can improve the recovery of the heart contractile function from I/R injury[30–32], indicating that CAT has an important role in cardiac protection against I/R insult even though the its activity in the myocardium is low[33]. Some studies[16, 17, 34], however, suggest a close correlation between the increase in antioxidant enzyme activity and the cardioprotection induced by various ischemic and non-ischemic preconditioning methods. These methods have the characteristic of repetitive cycles of hypoxia and reoxygenation or ischemia and reperfusion, like CIHH. A previous report also found that intermittent hypoxia mimicking obstructive sleep apnea changed the susceptibility of the heart to oxidative stress, partly by the alteration of thioredoxin, but not SOD or CAT[35]. By contrast, some studies were unable to demonstrate the protective effects of antioxidant therapy or the use of some endogenous antioxidant enzymes against I/R injury[36, 37]. Koler *et al* reported that antioxidant enzymes in CIHH rats were not significantly changed[19]. Although some studies contradict our findings, our results strongly suggest that antioxidant enzymes may be critical for the cardioprotective effect against oxidative stress under some pathological conditions. Moreover, our results suggest that the induction and activation of SOD-2 and CAT could be the underlying mechanism of the cardioprotective effects associated with CIHH.

ROS contributed to I/R injury and appeared to play the part of activator in processes in which CIHH upregulated the antioxidant enzymes, which may be a double-edged sword. Furthermore, ROS has been shown to be a second messenger for the induction of antioxidant enzymes in both prokaryotes and eukaryotes[33]. During adaptation to CIHH, repetitive cycles of hypoxia and reoxygenation (sub-lethal stresses) may lead to the production of ROS in the hypoxic myocardium, the latter triggering a cascade of events that lead to increased antioxidant enzyme activity. It has been reported that in prokaryotes, ROS modifies the protein structure of the transcription factor oxyR to an activated form, which induces the expression of the CAT gene[38, 39]. These findings indicate that the ROS produced in myocardial tissue during sub-lethal stress may serve as a signal transduction pathway[33] that forms a positive feedback loop consisting of ROS, transcriptional factors and cytokines and even a sequence of signaling events linking ROS, PKC-δ, and mitoK$_{ATP}$ channels[40]. Because mitochondria are particularly susceptible to oxidative damage from ROS, and because SOD-2 is located in mitochondria[40], mitochondrial SOD-2 is more likely to be induced than cytosolic SOD-1. This not only indicates that the induction of SOD-2 in CIHH may have a critical role in the acquisition of CIHH against I/R injury, such as in the delayed preconditioning[34], but also explains why CIHH only increased the activity and protein expression

of SOD-2 and not SOD-1. In addition to SOD-1, no significant change in GPX activity was observed in CIHH hearts in our experiment. Similar to our results, others have shown an increase in the activity of CAT and SOD in spinal cord tissue without affecting GPX activity[29]. These findings suggest that the relationship of GPX with ischemic tolerance is yet to be elucidated.

The present study showed that the baseline and recovery of CF after ischemia were higher in CIHH hearts compared with non-CIHH hearts, which is consistent with findings that capillary density and CF are higher in CIHH hearts [7]. These data suggest that the increase of CF is one possible mechanism for the protection conferred by CIHH. In addition, other studies have found that the burst of vascular ROS production following I/R can lead to dysfunction of coronary microvessels to reduce CF[24, 41]. These results are consistent with our findings showing that ATZ only abolished the recovery of cardiac function involving CF after I/R or H_2O_2 perfusion, and did not affect the baseline values of cardiac function in CIHH hearts or of any parameters of cardiac function in non-CIHH hearts. This indicates that ATZ exerted its antagonistic role only in the presence of higher levels of CAT and oxidative stress, which may be due to the lower doses of ATZ that we used and the lower level of CAT in myocardium[33]. Previous studies have shown that a similar dose of ATZ did not deteriorate spinal cord ischemia or myocardial infarction in control rats[29, 42].

In previous studies, antioxidant capacity was only detected in ventricular tissue[4, 17]. Here, the DCFH-DA method was used to detect the levels of intracellular ROS. Because the single cardiomyocyte does not contain neutrophils, endothelial cells or other potential sources of oxidants, our study directly demonstrates that the cardiomyocytes from CIHH hearts have an enhanced antioxidant capacity to scavenge ROS.

Injury that is induced by I/R is an inevitable complication that depresses cardiac function and expands myocardial infarction. Although some studies have demonstrated adverse effects or controversial results of intermittent hypoxia[35], the present findings imply that CIHH could be useful in the clinical setting for the prevention of I/R injury in ischemic diseases. Moreover, the antioxidant supplementation in I/R injury may also have beneficial consequences.

In conclusion, the present study shows that CIHH upregulates the activity and protein expression of the antioxidant enzymes CAT and SOD-2, thereby leading to an increase in antioxidant capacity. This may play an important part in the cardioprotection of CIHH against I/R injury in the guinea pig.

Acknowledgements

Author contribution

Hui-cai GUO and Yong-li WANG designed the study; Hui-cai GUO and Zhe ZHANG performed the research; Li-nan ZHANG, Chen XIONG, Chen FENG, Qian LIU, Xu LIU, and Xiao-lu SHI contributed new analytical reagents and tools; Hui-cai GUO, Yong-li WANG, Li-nan ZHANG, Qian LIU, and Chen XIONG analyzed data; Yong-li WANG and Hui-cai GUO wrote the paper.

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