Hypertonic perfusion reduced myocardial injury during subsequent ischemia and reperfusion in normal and hypertensive rats[1]

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ABSTRACT

AIM: To determine the effects of hypertonic solution on myocardial ischemia and reperfusion injury in normal and stroke-prone hypertensive rat hearts *in vitro*. **METHODS:** Hearts were perfused in an isolated-perfused Langendorff apparatus and perfused with normal or hypertonic solution (360 mOsm/L, by addition of NaCl to the normal perfusate of 300 mOsm/L) before subjected to 30 min ischemia followed by 40 min isotonic reperfusion. Heart function, myocardial creatine kinase leakage, norepinephrine release, and ventricular calcium content were determined. **RESULTS:** Normal rat hearts with hypertonic perfusion showed higher recovery rate of spontaneous beating than control hearts after ischemia. Hypertensive rat hearts perfused with hypertonic solution also had better recovery in diastolic function and less creatine kinase leakage than hypertensive controls. Concomitantly, myocardial release of norepinephrine was also reduced from hypertensive hearts perfused with hypertonic solution. There was no significant difference in myocardial calcium content between normal and hypertonic perfused hypertensive hearts. **CONCLUSION:** Hypertonic perfusion may precondition the hearts and protect them from ischemia and reperfusion injury in both normal and hypertensive rats. The modulation of hypertonic perfusion on myocardial norepinephrine release and its role in cardioprotection needs further investigation.

INTRODUCTION

Many biochemical and neurohormonal alterations contributed to irreversible cell injury during myocardial ischemia and reperfusion. It has been long recognized that the most significant early changes are depletion of high-energy phosphates, accumulation of glycolytic and other metabolic products, and massive calcium accumulation in myocytes ultimately leads to irreversible myocardial damage. Sympathetic overexcitation is also an important factor in determining the outcomes in both complicated and non-complicated myocardial ischemia, and large amounts of norepinephrine released during

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ischemia may affect the myocardium at risk, resulting in increased myocyte necrosis and lethal arrhythmias[1-3]. Hypertensive hearts have increased susceptibility to ischemia and reperfusion and release more catecholamines[4], which could accelerate progression of cell damage by an increase in cellular energy demand and by stimulation of calcium influx into the myocytes[5,6]. Manipulations of these pathophysiological changes including ischemic preconditioning before sustained ischemia could alleviate cell damage and preserve heart function after ischemia[7,8]. Catecholamine reduction before ischemia preserves postischemic heart function, improves coronary flow and cardiac metabolism by preventing the drop of myocardial ATP to a critical low level and subsequent irreversible myocardial changes[9,10]. And ischemic preconditioning also attenuates myocardial norepinephrine release during myocardial ischemia via ATP-sensitive potassium channels[11].

Our previous study showed that stroke-prone spontaneously hypertensive rat (SHRSP) hearts released more norepinephrine during ischemic injury and reduction of myocardial norepinephrine prior to an ischemic insult could preserve postischemic heart function[4,10], indicating an important role of catecholamines in ischemic myocardial injury. In this study we tried hypertonic solution to see whether it could protect hypertensive hearts from ischemic injury and interfere with cardiac catecholamine release. The impetus for this study came in part from the basis that hypertonic solution has beneficial effects in shock resuscitation[12], and from our previous findings that severe hyperglycemia, which put the hearts under a hypertonic state, paradoxically rendered diabetic hearts resistant to ischemia[13].

In the present study, we first observed the response of normal Sprague-Dawley rat hearts to ischemia after hypertonic perfusion *in vitro* and found reduced myocardial injury in this model. Then we perfused SHRSP hearts with hypertonic solution and the results showed that hypertonic perfusion prior to ischemia also alleviated ischemic damage in hypertensive hearts and concomitantly reduced myocardial norepinephrine release after ischemia.

MATERIALS AND METHODS

Animals Male Sprague-Dawley rats (SD, body weight 250-300 g) from Shanghai Experimental Animal Center, Chinese Academy of Sciences, and SHRSP and their age-matched normal controls Wistar-Kyoto rats (WKY) (4 months of age) from Animal Center of Kinki University School of Medicine were used in the present study. They were housed in groups up to three rats in a temperature-controlled room (23±1 ºC) on a regular 12-h light and dark cycle and had free access to tap water and chow. Systolic blood pressure was measured by tail-cuff method under conscious status[4]. All experiments conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No 85-23, revised 1996).

Isolated heart preparation Isolated heart perfusion was performed as published[4,10] with modifications. Briefly, under anesthesia with sodium pentobarbital (60 mg/kg, ip), the hearts were excised and connected rapidly to the aortic cannula of a Langendorff apparatus. The retrograde perfusion was instantly started with Krebs-Henseleit buffer (in mmol/L: NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.0) kept at 37 ºC and bubbled constantly with 95 % O_2 and 5 % CO_2 (pH 7.4) throughout the perfusion period. The left atrium was connected to a cannula and perfused for filling of left ventricle. Perfusion pressure in the aorta and left atrium was set at 90 cmH_2O and 10 cmH_2O, respectively.

A catheter (PE-50) was inserted into the left ventricular cavity through the apex and connected to a pressure transducer (PT 140DM, Fudan University, Shanghai, China) to measure changes in intraventricular pressure. Left ventricular pressure (LVP), left ventricular end-diastolic pressure (LVEDP) and heart rate were recorded with the computerized data acquisition system (MPA 2000, Alcott Biotech Co, Shanghai, China) throughout the experiment. Cardiac performance as represented by maximum rates of increase and decrease in left ventricular pressure (dp/dt_{max} and dp/dt_{min}) derived by the data acquisition system from intraventricular pressure changes. Coronary outflow was recorded during perfusion and samples were collected and stored at -70 ºC until norepinephrine assay.

Induction of ischemia and reperfusion After 15 min equilibration and 15 min hypertonic perfusion (perfusate adjusted to 360 mOsm/L by addition of NaCl), the hearts were subjected to normothermic global ischemia by clamping both atrial inflow and aortic outflow for 40 min in SD rats or 30 min in SHRSP and WKY rats. The thermostatic glassware, in which the hearts were suspended, was covered to prevent the hearts from drying out during ischemia. The ischemia was then followed by 40 min reperfusion. Reperfusion

was started by opening of both atrial and aortic cannulae and the hearts were allowed to restore beating spontaneously.

Determination of norepinephrine in coronary effluent[10] The timed 5-min collections of coronary effluent were stabilized by the addition of perchloric acid and Na_2-EDTA to final concentrations of 0.01 mol/L and 0.025 %, respectively. Norepinephrine present in the effluent was concentrated by adsorption on to acid-activated alumina adjusted to pH 8.6 with 1 mol/L Tris-2 % edetic acid buffer. Then norepinephrine was eluted into 0.2 mol/L acetic acid for assay. Total cumulated norepinephrine over the entire reperfusion period was calculated for each heart and corrected for wet heart weight. Dihydroxybenzylamine was added to each sample as an internal standard before alumina extraction and used for recovery rate calculation. Norepinephrine was measured with high performance liquid chromatography coupled with electrochemical detection (Agilent 1100 Series, HP1049, HP Co, USA). The detection limit for norepinephrine was 50 pg.

Creatine kinase assay Creatine kinase (CK) was measured from the collected samples of coronary effluent using the standard spectrophotometric method with CK assay kit (Jian-Cheng Biomedical Engineering Co, Nanjing, China), following the manufacturer's instructions. Total integrated CK activity over 40 min reperfusion was calculated for each heart and corrected for wet heart weight[10].

Measurement of myocardial calcium content The myocardial calcium content was determined by atomic absorption spectrophotometry modified according to the method of Oshiro *et al*[15]. Upon completion of 40 min reperfusion, the hearts were perfused through aorta with ice-cold washout solution containing sucrose 0.35 mol/L and histidine 5 mmol/L at an injection rate of 10-15 mL/min for 5 min. The solution was filtered through a 0.5 μm membrane before perfused into coronary artery. The ventricles were then blotted, weighted and stored at -70 ºC until assay. Before the calcium determination the ventricular samples were put into platinum pot and heated to 200 ºC for 1 h and then into muffle furnace of 700 ºC for 1 h and allowed to cool to room temperature afterwards. Then 50 % nitric acid 10 mL was added and the samples was nitrified to clear and transparent solution under low electrical oven. The solution was then cooled and transferred to a volumetric flask, diluted to 50 mL and mixed for the determination of calcium with Atomic Absorption Spectrophotometer (Z-5000 Zeeman, Hitachi Inc, Japan)

Statistical analysis Data are expressed as mean±SD. Differences among groups were evaluated with analysis of variance (ANOVA). Unpaired Student's *t*-test was used when data between two groups were compared and Fisher's Exact test for the rates of spontaneous recovery of heart beating after ischemia. Statistical significance was defined as $P<0.05$.

RESULTS

Myocardial injury in SD rats and effect of hypertonic perfusion Hypertonic perfusion significantly reduced CK leakage after 30 min ischemia (CK in hypertonic perfusion group: 427±56 IU/g wet heart weight, $n=6$, versus 883±125 IU/g in control group, $n=7$, $P<0.01$). Hypertonic solution also improved restoration of spontaneous beating of the hearts after severe ischemia. The spontaneous beating rate after 40 min ischemia in control was 14 % and 100 % in hypertonic perfused group ($P=0.0041$).

Heart function in hypertensive rats with and without hypertonic perfusion Systolic blood pressure was (198±26) mmHg in SHRSP rats and (137±30) mmHg in WKY rats before isolated heart experiment. There was a transient increase in dp/dt_{max} after hypertonic perfusion in SHRSP before ischemia. During postischemic reperfusion, SHRSP had marked impairment of diastolic function represented by significantly elevated LVEDP and depressed recovery of dp/dt_{min} as compared with normal WKY rats (Fig 1A and 1C). However, perfusion with hypertonic solution before ischemia alleviated this impairment in SHRSP (Fig 1). There was no significant difference of systolic function (dp/dt_{max}) between groups (Fig 1B).

CK leakage during reperfusion after 30 min ischemia The hypertensive hearts had more CK leakage after ischemia as compared with normal WKY controls (Fig 2), demonstrating increased cell damage in hypertensive rats during ischemia and reperfusion. With hypertonic perfusion pretreatment, hypertensive hearts showed increased resistance to ischemia and reperfusion injury with less CK leakage ($P<0.05$).

Cardiac norepinephrine release after ischemia During 40 min reperfusion after ischemia, the hypertensive hearts released more norepinephrine than WKY hearts, while hypertonic perfusion reduced cardiac norepinephrine release from hypertensive hearts (Fig 3, $P<0.05$).

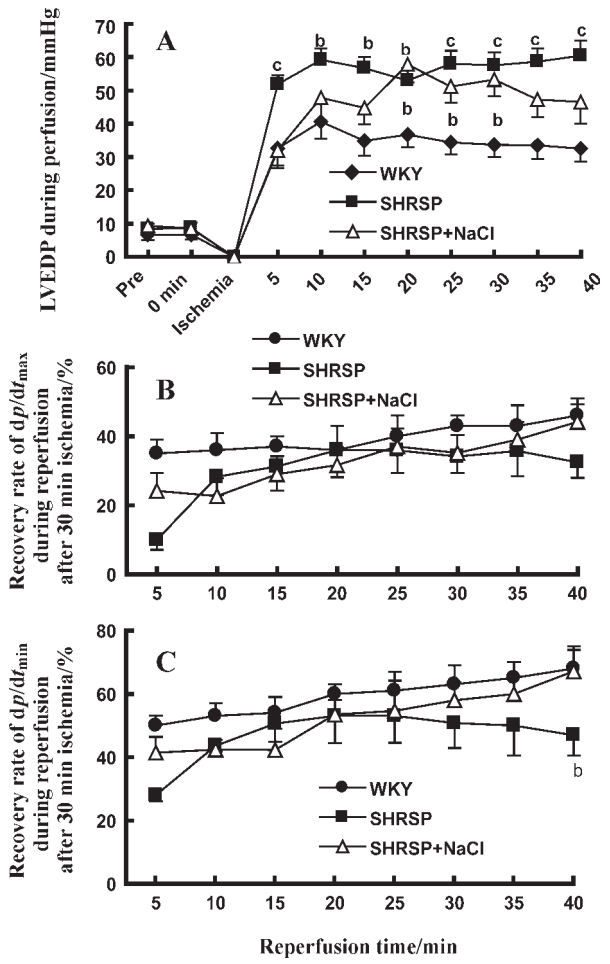

Fig 1. Heart function after ischemia injury in stroke-prone spontaneously hypertensive rats (SHRSP) with and without hypertonic perfusion. Fig 1A shows the trend in left ventricular end-diastolic pressure (LVEDP) during normal perfusion and after reperfusion. As compared with normal Wistar-Kyoto rats (WKY), SHRSP had marked impairment of diastolic function after ischemia represented by significantly elevated LVEDP and depressed recovery of dp/dt_{min}. After perfusion of hypertonic solution (SHRSP+NaCl), there was less increase in LVEDP and better recovery of dp/dt_{min} as compared with SHRSP without hypertonic perfusion (Fig 1A and 1C). There was no significant difference of systolic function between groups (Fig 1B). *n*=5-7. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* WKY.

Cardiac calcium content The ventricles were collected for calcium measurement after completion of 30 min ischemia and 40 min reperfusion. There was no difference in total ventricular calcium content between normal and hypertonic perfused hearts (24.5±3.4 μg/g ventricular weight in SHRSP control and 25.1±2.6 μg/g in SHRSP with hypertonic perfusion, $P>0.05$).

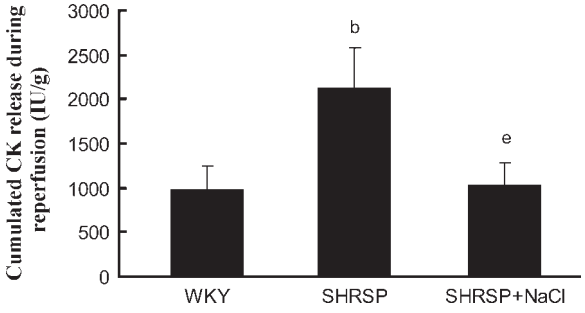

Fig 2. Creatine kinase (CK) leakage during reperfusion after 30 min ischemia. The stroke-prone spontaneously hypertensive rat (SHRSP) hearts had more CK leakage after ischemia as compared with normal Wistar-Kyoto rat (WKY) controls, demonstrating increased injury in hypertensive rats during ischemia and reperfusion. With hypertonic perfusion before ischemia, hypertensive hearts showed increased resistance to ischemia and reperfusion injury (less CK leakage, SHRSP+NaCl). *n*=5-7. Mean±SD. $^bP<0.05$ *vs* WKY control. $^cP<0.05$ *vs* SHRSP control.

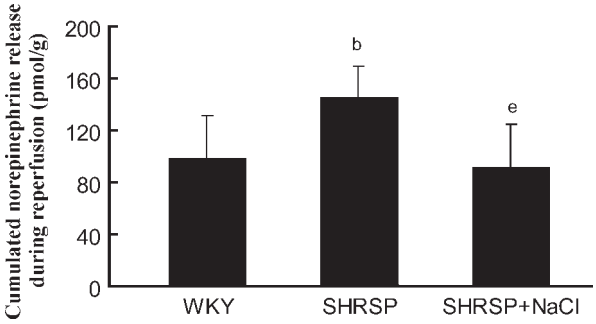

Fig 3. Cardiac norepinephrine release after ischemia. During 40 min reperfusion stroke-prone spontaneously hypertensive rat (SHRSP) hearts released more norepinephrine than Wistar-Kyoto rat (WKY) hearts, while hypertonic perfusion reduced norepinephrine release from hypertensive hearts (SHRSP+NaCl). *n*=5-7. Mean±SD. $^bP<0.05$ *vs* WKY control. $^cP<0.05$ *vs* SHRSP control.

DISCUSSION

This study demonstrates that pretreatment of isolated hearts with hypertonic perfusate reduced myocyte injury (represented by less CK leakage) and improved heart function after ischemia and reperfusion insult in both normal and hypertensive rats. Furthermore, the alleviated myocardial injury was accompanied by less cardiac norepinephrine release.

Commonly used for hemorrhagic shock and sepsis resuscitation, hypertonic saline infusion has been proved to be of potential benefits in various aspects of

the pathophysiology of the above settings, including tissue hypoperfusion, endothelial dysfunction, cardiac depression, and the presence of a broad array of proinflammatory cytokines and various oxidant species[12,16,17]. Since myocardial ischemia also shares some of these pathophysiological aspects, we suggested that hypertonic solution might protect hearts from ischemic injury as well. Just as the results showed here, perfusate adjusted to 360 mOsm/L by addition of NaCl was effective in reducing ischemic myocardial injury in normal hearts.

During myocardial ischemia, acceleration of cell damage and malignant arrhythmias may be induced by sympathetic over stimulation of the heart. This stimulation is due to excessive concentration of catecholamines within the poorly perfused or non-perfused myocardium. Enhanced myocardial sensitivity to adrenergic stimuli increases myocardial oxygen consumption and vasoconstriction through activation of beta- and alpha-adrenergic receptors, and local accumulation of catecholamines can reach a high concentration that is capable of producing direct toxic effect to myocytes, resulting in a vicious cycle in ischemia[18,19]. Hypertensive hearts are more susceptible to ischemia insult with excessive release of catecholamines, mainly norepinephrine, and reduction of myocardial catecholamines before ischemia insult has been shown to provide protective effects, indicating causative effects of catecholamines on heart injury[9,10].

In agreement with our previous reports[4,10], this study also showed increased ischemic heart injury with increased myocardial norepinephrine release in hypertensive hearts. And it is interesting that hypertonic perfusion in this hypertensive hearts alleviated ischemic injury and concomitantly reduced myocardial norepinephrine release. The reduction of cardiac norepinephrine during ischemia and reperfusion may contribute to the cardioprotection of hypertonic perfusion in hypertensive hearts. Since the calcium overload is suggested to be a common path leading to irreversible myocardial injury during reperfusion[3,20] and hypertonic perfusion could inhibit myocardial calcium accumulation during hypoxia[21], we also measured the myocardial calcium after hypertonic perfusion. The result showed that there was no significant difference in myocardial calcium content at the end of reperfusion between normal and hypertonic perfusion. Since calcium overload is most prominent during reperfusion, the hypertonic perfusion applied to the heart before ischemia may be not powerful enough to prevent reperfusion overload of calcium during reperfusion.

In conclusion, our results showed that hypertonic solution could afford protection on ischemic heart function, especially diastolic heart function, and alleviate cell damage during ischemia and reperfusion. Its effect of reducing myocardial norepinephrine release from hypertensive hearts subjected to severe ischemia may participate in the limitation of ischemic cell injury. The exact mechanisms underlying cardioprotection of hypertonic perfusion need further investigation.

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