Abstract

Background: Hyperosmotic solutions have been used successfully in different shock resuscitations with cardioprotection. This study was to examine the effects of hyperosmotic sodium chloride on isolated heart function and heart responses to ischemia/reperfusion in normotensive and hypertensive rats. The roles of hyperosmolality-induced antioxidants including hyperosmolality-relevant heat shock proteins as well as vasodilating endothelial nitric oxide synthase (eNOS) and vasoactive catecholamines were investigated.

Methods: Hearts of normal rats and stroke-prone spontaneously hypertensive rats were isolated and perfused for 30 min with control Krebs-Henseleit buffer (osmolarity 300 mOsm/L) or hyperosmotic buffer of different sodium chloride concentrations (320, 350 and 400 mOsm/L) before subjected to 40-min global ischemia followed by 10-min hyperosmotic reperfusion and 30-min normal buffer reperfusion. Heart function, creatine phosphokinase leakage and myocardial antioxidants were examined. Myocardial antioxidants after hyperosmotic perfusion with different osmolytes were assayed with Western blotting.

Results: Pre-ischemic hyperosmotic sodium chloride perfusion enhanced heart contractility and diastole function and reduced coronary vascular resistance in both normal and hypertensive hearts. Post-ischemic recoveries of heart function were improved in hyperosmotic perfused hearts, associated with lower creatine phosphokinase leakage, higher coronary flow, reduced coronary resistance and lower norepinephrine overflow. At the end of reperfusion, the myocardial activities of total superoxide dismutase and catalase, glutathione content as well as osmosis-relevant heat shock protein 32 and 90 were increased in hyperosmotic hearts. In addition to sodium chloride, in vitro hyperosmotic mannitol, glucose and raffinose also increased protein expressions of antioxidants including superoxide dismutase, catalase, heat shock protein 32 and 90 and vasodilating eNOS.

Conclusion: Hyperosmotic perfusion enhanced heart function and preconditioned normal and hypertensive hearts against ischemia/reperfusion injury. The hyperosmolality-induced up-regulations in myocardial antioxidants including heat shock proteins and eNOS may play an important role in the hyperosmolality-induced cardioprotection.

Keywords: Hyperosmotic sodium chloride; Isolated heart function; Ischemia and reperfusion; Antioxidants; Heat Shock protein; Endothelial nitric oxide synthase; Stroke-prone spontaneously hypertensive rat

Introduction

Hyperosmolality, usually defined as solutions that have a higher osmolarity compared with the normal extracellular fluid (around 300 mOsm/L), can be found in different physiological and pathophysiological conditions, such as dehydration, the high osmolarity of the interstitial fluid in the medulla of the kidney or hyperglycemic hyperosmol syndrome in diabetic patients. The osmolarity can be as high as 1200 to 1400 mOsm/L in such states, 3 times higher than that of normal interstitial fluid around most body cells. Although detrimental to some cell function, acute hyperosmolarity has also been found capable of exerting protection to some extent. Previous studies have shown that small amount of hyperosmotic solutions such as hypertonic saline (7.5% NaCl, 2400 mOsm/L), especially when given in dextran, were effective in shock resuscitations of different causes including sepsis, burn and pancreatitis [1-5]. In our previous study in diabetes, we found that severe hyperglycemia rendered diabetic.
hearts resistant to ischemia and reperfusion injury, with significant up-regulations of hyperosmolarity-related antioxidants, giving rise to our interest in the innate or adaptive responses of cardiovascular system to hyperosmolarity [6-8].

The major benefit of hyperosmotic solutions given in vivo is the rapid expansion of plasma volume, which leads to an improvement in cardiovascular function and tissue microcirculation. However, the detailed responses of normal and hypertensive hearts to different hyperosmolarity irrespective of plasma volume expansion are not clearly elucidated. Studies including ours have shown that hyperosmotic NaCl or glucose in vitro could improve myocardial function in thermal injury as well as coronary flow after ischemia/reperfusion injury [3, 9-12]. We have found that hyperosmotic glucose or mannitol could induce endothelial nitric oxide synthase (eNOS) and heme oxygenase (HO, also called heat shock protein 32, HSP32) in rat heart and aorta with enhanced vasodilation [6,7,12]. We therefore hypothesized that hyperosmolarity may play an important role in the observed protection of hyperglycemia. However, glucose can pass through cell membrane easily to counteract the osmolarity gradient applied and have metabolic effects as well. Therefore, we chose hyperosmotic NaCl, which does not have metabolic effects as glucose does, to test whether it could have similar cardioprotection as hyperglycemia.

In the present study, we examined heart function responses to hyperosmotic NaCl in isolated normotensive and hypertensive hearts, which were subsequently subjected to ischemia/reperfusion injury. Systolic and diastolic heart function, myocardial creatine phosphokinase leakage and myocardial antioxidants after ischemia/reperfusion were examined. We also test the hypothesis that hyperosmolarity might be responsible for the cardioprotection and hyperosmotic solutions with different osmolaryte would induce myocardial antioxidants including osmosis-related heat shock proteins 32 and 90 (HSP32 and HSP90) as well as vasodilating eNOS. The myocardial overflow of vasoactive catecholamines, i.e., epinephrine and norepinephrine were also measured after ischemia.

Materials and Methods

Animals

Forty male normotensive Wistar-Kyoto rats (WKY) and forty male stroke-prone spontaneously hypertensive rats (SHRSP) from Animal Center of Kinki University School of Medicine, Osaka, Japan (donated by Professor Tsuneyuki Suzuki) were used in the present study. They were housed in groups up to two rats in a temperature-controlled room (23±1°C) on a regular 12-hour light and dark cycle and had free access to tap water and chow until sacrifice at the age of 5 months. The mean body weight levels of WKY and SHRSP before heart experiments were 403±8 g and 280±5 g, respectively. Systolic blood pressure levels in conscious state were measured by tail-cuff method, which were 120±2 mmHg in WKY group and 225±5 mmHg in SHRSP group, respectively. All experiments conformed to the Guide for the Care and Use of Laboratory Animals by the Chinese Academy of Science of Shanghai.

Isolated heart preparation

Isolated heart perfusion was performed as published previously [6,7] with modifications. Briefly, under anesthesia with sodium pentobarbital (60 mg/kg, i.p.), 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 (KH buffer) (mmol/L): NaCl 118.0, KCl 4.7, CaCl$_2$ 1.2, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.2, NaHCO$_3$ 25.0, glucose 11.0) kept at 37°C 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 for filling of left ventricle. Perfusion pressure in the aorta and left atrium was set at 70 mmHg and 15 mmHg, respectively.

A catheter (PE-50) was inserted into the left ventricular cavity through the apex and connected to a pressure transducer (PT-140D, Fudan University, Shanghai, China). The intra-ventricular pressure changes including left ventricular systolic pressure, left ventricular end-diastolic pressure (LVEDP) and heart rate were recorded throughout the experiment with computerized data acquisition system (MPA 2000, Alcott Biotech Co., Shanghai, China). Cardiac contractile and diastole function as represented by maximum increase and decrease rates of left ventricular pressure (dP/dt$_{max}$ and dP/dt$_{min}$, respectively) was derived by the data acquisition system automatically from the intra-ventricular pressure changes. Left ventricular developed pressure (LVPD) was calculated as left ventricular systolic pressure minus LVEDP. The rate-pressure product (RPP) was calculated as heart rate times LVPD. The timed 5-min collections of coronary effluent were recorded as coronary...
flow corrected for wet ventricular weight and coronary vascular resistance was calculated as perfusion pressure divided by coronary flow. Samples of coronary effluent and myocardium collected after ischemia/reperfusion were stored at –70°C until assay.

**Hyperosmotic perfusion and induction of ischemia/reperfusion**

After 10-min equilibration and 30-min hyperosmotic perfusion (the osmolarity of KH buffer adjusted to 320, 350 and 400 mOsm/L by addition of different concentrations of NaCl, with normal KH buffer of 300 mOsm/L as control), the hearts were subjected to global ischemia at 37°C by clamping both atrial inflow and aortic outflow for 40 min. The thermostatic glassware, in which the hearts were suspended, was covered to maintain temperature and prevent the hearts from drying out during ischemia. Ischemia was followed by 10-min hyperosmotic reperfusion and 30-min normal buffer reperfusion. The reperfusion was started by opening of both atrial and aortic cannulae and the hearts were allowed to restore beating spontaneously.

**Biochemical assays**

Creatine phosphokinase (CPK) was measured from the timed 5-min collections of coronary effluent using standard spectrophotometric method with CPK assay kit (Jian-Cheng Biomedical Engineering Co., Nanjing, China), following the manufacturer’s instructions. Total integrated CPK activity over the 40-min reperfusion was calculated for each heart and corrected for wet ventricular weight. Myocardial lipid peroxide product maleic dialdehyde (MDA) and antioxidants including activities of total superoxide dismutase (SOD), catalase and glutathione (GSH) were determined after reperfusion using respective assay kits (Jian-Cheng Biomedical Engineering Co.) [10].

**Heart perfusion with hyperosmotic solutions of different osmolytes**

Sodium chloride, mannitol, glucose or raffinose were added to KH buffer separately to obtain perfusate osmolarity of 350 mOsm/L and normal hearts were perfused with these hyperosmotic perfusates for 2 h continuously. Then left ventricular apex was sampled and myocardial...
antioxidants including copper zinc SOD (CuZnSOD), manganese SOD (MnSOD), catalase, eNOS and inducible NOS (iNOS) as well as HSP32 and HSP90 were determined with Western blotting.

**Determination of catecholamines in coronary effluent**

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. Epinephrine and norepinephrine present in the effluent were concentrated by adsorption on acid-activated alumina adjusted to pH 8.6 with 1 mol/L Tris-2% EDTA buffer. Then the catecholamines were eluted into acetic acid for assay. Total cumulated catecholamines over the entire reperfusion period was calculated for each heart and corrected for wet ventricular weight. Dihydroxybenzylamine was added to each sample as an internal standard before alumina extraction and used for recovery rate calculation. Norepinephrine and epinephrine were measured with high performance liquid chromatography coupled with electrochemical detection (Agilent 1100 Series, HP1049, HP Co., USA) [9].

**Western blotting**

Middle parts of left ventricles after ischemia were freeze-clamped and homogenized in lysis buffer (pH 7.4) for protein assay as previously described (6). Protein samples were loaded onto SDS-PAGE, and transferred to polyvinylidene difluoride membrane after electrophoresis. Blots were incubated overnight at 4°C with antibodies against CuZnSOD (1:3000; Calbiochem, Darmstadt, Germany), MnSOD (1:3000; BD Biosciences Pharmingen, USA), catalase (1:2000; Calbiochem), eNOS (1:2000; Sigma-Aldrich, Saint Louis, Missouri,
USA), iNOS (1:2000; Santa Cruz Biotechnology, USA), HSP32 and HSP90 (1:3000; Stressgen, Victoria, BC, Canada). Immunoreactive bands were visualized using enhanced chemiluminescence detection and relative levels of proteins were semiquantified with densitometry, normalizing to tubulin (1:5000; Sigma-Aldrich).

Statistical analyses and calculations

Data are expressed as mean±SEM. Two-way ANOVA followed by Student-Newman-Keuls post hoc analysis. Repeated measurement ANOVA was used for values of time-dependent trends of functional parameters during reperfusion. Significance was defined as \( P<0.05 \).

Results

Isolated heart responses to hyperosmotic perfusion

As shown in Figure 1, hyperosmotic NaCl perfusion increased coronary flow and reduced coronary vascular resistance in both WKY and SHRSP, which was more prominent in WKY. Hyperosmotic...
perfusion also enhanced myocardial contractility indicated by elevation in \( \frac{dP}{dt_{\text{max}}} \) (Figure 2a). The myocardial diastole dysfunction in SHRSP was ameliorated by 30-min hyperosmotic perfusion indicated by better \( \frac{dP}{dt_{\text{min}}} \) and lower LVEDP (Figure 2b and 2c). Hyperosmotic perfusion of 320, 350 and 400 mOsm/L reduced heart rate in SHRSP but was only effective in WKY at 400 mOsm/L (Figure 2d). Hyperosmotic perfusion was also effective in reducing workload in SHRSP at higher osmolarity (Figure 2e).

Coronary flow and heart function after ischemia/reperfusion injury

WKY hearts perfused with hyperosmotic NaCl showed higher coronary flow and reduced coronary vascular resistance after ischemia/reperfusion in a dose-dependent way, with the most significant effect being in 350 mOsm/L group (Figure 3a and 3c). Coronary norepinephrine overflow was reduced in hyperosmotic perfused WKY hearts, which shared a similar pattern with the reduction in coronary resistance (Figure 3e), indicating the optimal osmolality level for coronary relaxation at 350 mOsm/L. There were no significant changes in epinephrine release. SHRSP had a similar trend in coronary flow and catecholamines (Figure 3b and 3f). Coronary resistance was reduced significantly in SHRSP at the osmolarity of 400 mOsm/L (Figure 3d).

The post-ischemia/reperfusion recovery of heart contractility, diastolic function and work product were better in both hyperosmotic perfused WKY and SHRSP hearts (Figure 4).

Myocardial injury

As shown in Figure 5, both WKY and SHRSP hearts perfused with hyperosmotic NaCl lowered myocardial CPK leakage and MDA levels dose-dependently, indicating less myocardial necrosis and lipid peroxide damage.

Myocardial antioxidants

Hyperosmotic perfusion enhanced myocardial activities of SOD, catalase with higher GSH contents in both WKY and SHRSP hearts after ischemia/reperfusion injury (Figure 6), suggesting that beneficial effect of hyperosmolarity on myocardial anti-oxidative defense.

Hyperosmolarity-related heat shock proteins

Two hyperosmolarity-related anti-oxidative heat shock proteins, HSP32 and HSP90, were elevated in hyperosmolarity-perfused WKY and SHRSP hearts at the end of ischemia/reperfusion (Figure 7), indicating direct and significant responses of myocardium to hyperosmolarity encountered in vitro.

Heart antioxidants after stimulation with hyperosmotic solutions of different osmolytes

To determine whether antioxidants up-regulations are induced exclusively by hyperosmotic NaCl, hyperosmotic solutions with NaCl, mannitol, glucose or raffinose as osmolytes were tested. As demonstrated in Figure 8, all of these hyperosmotic solutions could induce protein expressions of myocardial antioxidants including MnSOD, catalase, eNOS, HSP32, HSP90, with the later three having properties of promoting vasodilation.

Discussion

The major findings of the present study include: 1) hyperosmotic NaCl perfusion reduced coronary vascular resistance and increased...
coronary flow with enhanced myocardial contractility at the same time in normal and hypertensive rat hearts in vitro; 2) up to 400 mOsm/L, hyperosmotic perfusion ameliorated myocardial ischemia/reperfusion injury and heart dysfunction in a dose-dependent manner, which was associated with elevation in myocardial antioxidants; 3) hyperosmolarity up-regulated myocardial antioxidants in normal and hypertensive hearts with or without ischemia/reperfusion.

Hyperosmotic solutions, especially NaCl plus dextran, have been used successfully in different shock resuscitations since the early report by Velasco and his colleagues who described the use of small volumes of hypertonic NaCl solution (NaCl 7.5%, 2400 mOsm) for treating hemorrhagic shock [13]. The underlying mechanisms have been proposed due to its effectiveness in expanding blood volume, improving cardiac function and myocardial microcirculation as well as anti-inflammation effect [1-5,14]. In the present study, we found that hyperosmotic NaCl enhanced myocardial contractility and coronary flow within 30-min perfusion in vitro, i.e., without the influences of blood volume and autonomic nerve system-induced inotropic response, indicating that hyperosmotic NaCl has a rapid and independent action on heart function and coronary circulation, whether in normotensive or hypertensive hearts. Our data are also in consistent with the previous reports that hypertonic saline enhanced isolated heart function and hyperosmotic mannitol or sucrose could increase myocardial contraction [3,15,16]. The elevated intracellular calcium concentration due to cell dehydration or Na⁺-Ca²⁺ exchange

Figure 6: Myocardial antioxidants at the end of ischemia/reperfusion with and without hyperosmotic perfusion. Hyperosmotic NaCl increased myocardial antioxidants dose-dependently in normotensive Wistar-Kyoto rats (WKY) and stroke-prone spontaneously hypertensive rats (SHRSP). SOD, superoxide dismutase; GSH, glutathione. n=7-10. *P<0.05, **P<0.01 versus respective controls.
may be related to the positive inotropic action by hyperosmotic stimulation, which might involve better filling of calcium stores and availability for release from these stores [15-17].

Hyperosmotic solutions have also been found to be protective in myocardial injury after ischemia or hypoxia [9,11,18-21]. The present results showed that hyperosmotic NaCl perfusion ameliorated ischemia/reperfusion injury in both normotensive and hypertensive rat hearts, demonstrated by better recovery of heart function and coronary

Figure 7: Hyperosmolarity-related heat shock proteins (HSP) at the end of ischemia/reperfusion after hyperosmotic NaCl perfusion. Hyperosmolarity-related anti-oxidative HSP32 and HSP90 were increased in hyperosmotic NaCl-perfused myocardium of both normotensive Wistar-Kyoto rats (WKY) and stroke-prone spontaneously hypertensive rats (SHRSP). The osmolarity was 350 mOsm/L versus control of 300 mOsm/L. n=7-10. **P<0.01 versus respective controls.

Figure 8: Changes of myocardial antioxidants after hyperosmotic perfusion with different osmolytes. After 2-hour perfusion with hyperosmotic solutions of different osmolytes at the osmolarity of 350 mOsm/L, different myocardial antioxidant proteins were enhanced. The up-regulated antioxidants include manganese superoxide dismutase (MnSOD), catalase, endothelial nitric oxide synthase (eNOS), heat shock protein 32 and heat shock protein 90 (HSP32 and HSP90). The latter three have properties of promoting vasodilation. n=6. *P<0.05, **P<0.01 versus respective controls.
flow and reduced myocardial CPK leakage in a dose-dependent manner. There were concomitant increases in several myocardial antioxidants including catalase, SOD and GSH in the hyposmotic perfusion groups. The association of hyperosmolarity-induced myocardial protection with elevated antioxidants and hyperosmolarity-relevant HSP32 and HSP90 suggests that hyperosmotic NaCl exerts cardioprotection partly by enhancing myocardial anti-oxidative capacity [6,10,22].

The reduced coronary vascular resistance by hyposmotic NaCl may also play a role in the better recovery of heart function and less myocardial damage. The present results that hyperosmotic NaCl reduced myocardial catecholamine release during reperfusion is in accordance with our previous findings in hypertensive hearts [9]. Norepinephrine is a strong factor that can induce vasoconstriction and myocardial ischemia. The coincidence of significant reduction in norepinephrine with reduced coronary resistance during reperfusion indicates that myocardial norepinephrine may also play an important role in controlling coronary blood flow and myocardial damage during ischemia/reperfusion. In addition to its vasoactive effect, norepinephrine could also accelerate cell damage by increasing in cellular energy demand and stimulating calcium influx into cardiomyocytes [23], resulting massive calcium accumulation and further myocardium necrosis. We have found that hypertensive hearts released more norepinephrine during ischemia/reperfusion and reduction of myocardial norepinephrine store prior to ischemia/reperfusion preserved post-ischemic heart function [24]. The optimal osmolarity for reducing coronary resistance with lower norepinephrine in normal hearts was up to 350 mOsm/L, since higher osmolarity did not appreciably affect coronary flow before and after ischemia/reperfusion injury. On the other hand, the coronary circulation of hypertensive hearts showing less sensitivity to hyperosmolarity stimulation, responding to hyperosmotic NaCl only at the osmolarity of 400 mOsm/L. The results demonstrate that different physiological and pathophysiological conditions contribute to the responses of cardiovascular system to hyperosmolarity.

Previous study had shown that hypertonic saline with a colloid solution improved myocardial circulation in sepsis [25]. Hypertonic perfusion was also reported to reduce myocardial injury by reducing edema and diminishing calcium accumulation via decreasing Na/Ca exchange-mediated pathway during hypoxia [19,26]. Therefore, the enhanced myocardial anti-oxidative capacity in hyperosmolarity-perfused hearts and enhanced coronary vasodilation may act in concert to reduce myocardial injury.

It is also interesting to find that among the proteins induced by hyperosmolarity, there are three vasodilation-related antioxidants, i.e., eNOS, HSP32 (also named as heme oxygenase, HO) and HSP90. The generation of NO by eNOS exerts powerful endothelium-dependent vasodilation, and HO also exerts vasodilation to some extent, through carbon monoxide (CO), one of the by-products during catalyzing heme. HO could also improve vascular function by enhancing NO bioavailability [27-29]. In addition to eNOS and HO, HSP90 plays a central role in eNOS-generated NO and vasodilation. Both NO and superoxide anion can be generated by eNOS. HSP90 associates with eNOS, shifts NO and superoxide anion generation by eNOS from NO toward superoxide anion, therefore governing vasodilation and reducing radical generation to prevent ischemia/reperfusion injury [30-32]. The present results are in consistence with our previous findings that cardiovascular eNOS, HO and HSP90 were up-regulated in type 1 diabetes and non-obese type 2 diabetic rats with severe hyperglycemia as well as by hyperosmolarity with different osmolites [6,7,12,33-35]. These hyperosmolarity-induced enzymes and heat shock proteins may act in concert to improve endothelium function, resulting in the well-preserved endothelium-dependent vasodilation and elevated coronary flow during reperfusion after ischemia.

In summary, hyperosmotic NaCl enhanced heart function and coronary blood flow in isolated normotensive and hypertensive hearts with and without ischemia/reperfusion injury. Hyperosmolarity up-regulated various antioxidants including vasodilating eNOS/HO/ HSP90 and reduced coronary norepinephrine, which may be parts of the mechanisms underlying the hyperosmotic NaCl-induced cardioprotection. Since hyperosmolarity can be frequently encountered in both physiological and pathophysiological conditions, and a small volume of hyperosmotic solutions have been used successfully in shock resuscitation, hyperosmotic NaCl, with its prompt positive inotropic and vasodilating effects as well as antioxidant property, might also offer a safe and simple choice for the prevention of myocardial ischemia injury.

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References


