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***In vivo* upregulation of nitric oxide synthases in healthy rats**

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Abstract

Periodic acceleration (pGz), sinusoidal motion of the whole body in a head–foot direction in the spinal axis, is a novel noninvasive means for cardiopulmonary support and induction of pulsatile shear stress. pGz increases plasma nitrite levels, *in vivo* and *in vitro*. Additionally, pGz confers cardioprotection in models of ischemia reperfusion injury. We hypothesize that pGz may also confer a cardiac phenotypic change by upregulation of the expression of the various NO synthase (NOS) isoforms *in vivo*. pGz was applied for 1 h to awake restrained male rats at 2 frequencies (360 and 600 cpm) and acceleration (Gz) of $\pm 3.4 \text{ m/s}^2$. pGz did not affect arterial blood gases or electrolytes. pGz significantly increased total nitrosylated protein levels, indicating increased NO production. pGz also increased mRNA and protein levels of eNOS and nNOS, and phosphorylated eNOS in heart. pGz increased Akt phosphorylation (p-AKT), but not total Akt, or phosphorylated ERK1/2. Inducible (i) NOS levels were undetectable with or without pGz. Immunoblotting revealed the localization of nNOS, exclusively in cardiomyocyte, and pGz increased its expression. We have demonstrated that pGz changes myocardial NOS phenotypes. Such upregulation of eNOS and nNOS was still evident 24 h after pGz. Further studies are needed to understand the biochemical and biomechanical signal transduction pathway for the observed NOS phenotype changed induced by pGz.

Keywords

Nitric oxide; Nitric oxide synthase (NOS); Periodic acceleration; Cardioprotection

Introduction

Nitric oxide (NO) is a pluripotent signaling molecule synthesized by a family of nitric oxide synthase isoforms (NOS) found in most tissues [1]. NO possesses numerous biologic properties in many cell types ranging from bactericidal effects of macrophages, signal transduction during inflammation, cytoprotection, vasodilation, and regulation of apoptosis, to long term potentiation in neural networks. Three distinct NOS isoforms are recognized that enzymatically produce NO from L-arginine. These isoforms are identified as endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). Endothelial NOS and nNOS which were first identified in endothelial cells and neurons, respectively, are constitutively present in many cell types and are activated by calcium [2,3]. The nNOS has been reported to be localized to cardiomyocytes in both sarcolemma [4] and sarcoplasmic

reticulum [5–7]. The expression of iNOS is induced in a wide range of cell types during inflammation and the activity of iNOS is calcium independent. NO derived from all NOS isoforms activates soluble guanyl cyclase [1].

In the cardiovascular system, eNO (NO derived from eNOS) is present at a nanomolar concentration and regulates resting vascular tone and blood pressure. In contrast, iNO (NO derived from iNOS) can reach micromolar levels after stimulation [8]. The nitric oxide synthase inhibitor L-NAME increases resting blood pressure [9,10] while nitric oxide donors produce vasodilation and are used to control hypertension and reduce after load in patients with cardiovascular disease [11,12]. NO modulates interactions between the circulation and vessel wall. Platelet aggregation and leukocyte- endothelial cell adhesion are both decreased by NO derived from eNOS after ischemia–reperfusion and inflammation [13–15]. NO also reduces mitogenesis and proliferation of vascular smooth muscle cells and inhibits vascular protein and collagen formation. These effects serve to limit vessel wall inflammation, maintain vessel patency and increase oxygen delivery to tissues [1].

Periodic acceleration (pGz), motion of the whole body in a head–foot direction in the spinal axis [z-direction], has been demonstrated to be a novel noninvasive method of cardiopulmonary support [16]. pGz applied to anesthetized paralyzed animals maintains ventilation, increases regional blood flow to vital organs, and decreases pulmonary and systemic vascular resistance [17]. pGz induces pulsatile shear stress on the vascular endothelium *in vivo* and *in vitro*, and, acutely increases plasma nitrite (an indirect measure of nitric oxide) and prostaglandins [18,19]. Additionally, pGz applied during and after global ischemia induced by cardiac arrest is cardioprotective [16,20–22]. Inhibition of NO and prostaglandin synthesis individually and combined abrogated protection, implicating NO as the mediator of pGz-induced cardioprotection [23,24]. We hypothesized that pGz may also confer a cardiac phenotypic change by upregulation of the expression of the various NO synthase (NOS) isoforms *in vivo*.

Materials and methods

Platform design

The motion platform which imparts whole body periodic acceleration has been previously described. Briefly this platform consist of a linear displacement motor powered by an amplifier (APS Dynamics, Inc., Carlesbad, CA, model 400, 12 V) that is regulated by a sine wave controller (NIMS, Miami, FL, model 140-072). The regulator permits control of the frequency of the table and subsequent acceleration of the stroke. The table platform is directly driven by the underlying motor and articulates across the frame on stainless steel tracks and nylon wheels. The unit controller operates at a frequency between 30 and 600 cycles/min (cpm) at a *G* force of ± 1 –9.8 m/s². The table and its characteristics have been previously described [16,17,20,21].

Animal preparation and treatment

All animal studies were approved by the Institutional Animal Care and Use Committee of Mount Sinai Medical Center and were in compliance with the Animal Welfare Act 26. Sprague–Dawley rats (300 g) were used in this study.

To study blood pressure, blood gases and electrolytes, 6 animals were anesthetized with ketamine (10 mg/kg, i.m.) and xylazine (2 mg/kg), and intravascular catheters were placed into the carotid artery to measure systemic blood pressure by connecting the fluid filled catheter to a pressure transducer (Transpac®, Abbott Critical Care Systems, North Chicago, IL). Two hundred and fifty microliters of arterial blood was drawn for blood gas analysis,

before and after the treatment of pGz of 360 cpm, Gz of $\pm 3.4 \text{ m/s}^2$, using a blood gas analyzer (Rapid Lab TM348, Bayer Diagnostics, Tarrytown, NY).

To study the effect of pGz on conscious rats, the rats were placed in a restrainer (Kent Scientific, CT). The restrainer was placed longitudinally on the platform to allow the head-foot movement. Twenty-two conscious rats were divided into three groups: (1) control group (control) ($n = 6$), rats were placed in the restrainers for 1 h; (2) pGz 360 cpm group ($n = 8$), rats were treated with 1 h pGz at a frequency of 360 cpm and Gz of $\pm 3.4 \text{ m/s}^2$; and (3) pGz 600 cpm group ($n = 8$), rats were treated with 1 h pGz at a frequency at 600 cpm and Gz of $\pm 3.4 \text{ m/s}^2$. Four hours after treatment, all 6 rats of the control group and, 4 rats of both pGz of 360 cpm and pGz of 600 cpm groups were sacrificed. The remaining rats in both pGz groups were sacrificed 24 h after treatment. The heart was harvested for protein and mRNA analysis.

Detection of S-nitrosylated proteins

The level of S-nitrosylated proteins was measured using an S-Nitrosylated protein detection Assay Kit (Cayman Chem, Ann Arbor, MI), according to manufacturer's instruction with some modifications. Briefly, the heart tissue was washed three times with wash buffer and homogenized in Buffer A (provided in the kit) containing blocking reagent, and the homogenate was cleared by centrifugation after a 30-min incubation at 4°C . The total protein isolated by incubation with acetone and centrifugation was incubated with Buffer B (provided in the kit) containing reducing and labeling reagents for 1 h at room temperature. The labeled protein was isolated by incubation with acetone and centrifugation, resuspended in wash buffer, and measured for S-nitrosylated protein levels.

Western blot

Our methods for analyzing proteins by Western blots have been described previously [25]. Briefly, tissues were homogenized in lysis buffer (50 mM potassium phosphate buffer containing 1% Triton X-100, protease inhibitor cocktail, phosphatase inhibitor cocktail, 4 mM EDTA, pH 7.2) and cleared by centrifugation. Sixty micrograms of protein were separated on 4–12% SDS-PAGE and transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked (Tris-NaCl buffer containing 3% (W/V) dry milk and 0.1% Tween 20, pH 7.2), probed with primary and HRP-conjugated secondary antibodies, and visualized by Enhanced Chemiluminescence (ECL) (GE, Piscataway, NJ). Antibodies of eNOS, nNOS, iNOS and GAPDH were from Santa Cruz Biotech., CA. Signals were quantified using Image J 1.36b (NIH, Bethesda, MD).

RT-PCR

RNA was extracted using TRIReagent (Sigma, St. Louis, MO). One step RT-PCR was performed using a commercially available kit (Qiagen, Valencia, CA). The cDNA obtained was amplified for 30 cycles. Specific primers were: (1) eNOS: sense: 5'-TGACCCTCACC GATACAACA-3' and antisense: 5'-CTGGCCTTCTGCTCAT TTTC-3'; (2) nNOS: sense: 5'-CTGCAAAG CCCTAAGTCCAG-3' and antisense: 5'-AGCAGTGTTCTCTCC TCCA-3'; (3) glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control, sense: 5'-ATGGAAGCTG GTCATCAAC-3' and antisense: 5'-TTCACACCCA ACACAAGCAT-3'. PCR products were separated on 1% agarose gel, visualized under UV, and quantified using Image J 1.36b (NIH, Bethesda, MD).

Detection of nNOS using immunohistochemistry

The cellular localization of nNOS was detected using immunohistochemistry by Zyagen (San Diego, CA). Rat hearts were harvested and embedded in OCT immediately after

control or pGz (360 cpm). Frozen hearts were sectioned by cryostat at a thickness of 7 μm , mounted on slides treated for adhesion and fixed in 10% neutral buffer formalin for 30 min. After three wash with PBS, the sections were blocked in 10% normal serum diluted in PBS for 1 h at room temperature to reduce nonspecific binding of antibody. The sections were then incubated with primary antibody (1:25) for 1 h, diluted biotinylated secondary antibody for 30 min, and streptavidin-FITC conjugate for 30 min at room temperature in the humidity chamber. The sections were washed three times with PBS after each incubation. The sections were then examined under fluorescent microscope.

Statistical analysis

The results were expressed as means $\pm$ SD. The *p* value was determined by ANOVA when three groups were compared or Student's *t*-test when two groups were compared. Unless otherwise stated, statistical comparisons were made between the control and experimental groups. Statistical significance was established at *p* values less than 0.05.

Results

One hour of pGz at frequency of 360 cpm and *G* force of $\pm 3.4 \text{ m/s}^2$, did not significantly change arterial pH, PaCO_2 , PaO_2 , oxygen saturation and electrolytes including sodium, potassium, calcium and chloride (Table 1).

Since the formation of S-nitrosylated protein is a NO-dependent process, the S-nitrosylated protein level was used as an indirect indicator of NO production [26,27]. As shown in Fig. 1, pGz increased the total S-nitrosylated protein by 30% ($p < 0.05$), indicating increased NO production.

eNOS can be activated by phosphorylation of Ser-1177. eNOS phosphorylation (Ser-1177) was increased to 261% of control ($n = 4$) by 1 h of pGz (360 cpm) ($n = 4$) (Fig. 2, $p < 0.05$).

Compared to control ($n = 6$), a single 1-h exposure to pGz (360 cpm) ($n = 4$) increased the protein level of eNOS in the heart by $393 \pm 85\%$ ($p < 0.05$) and $461 \pm 78\%$ ($p < 0.01$) at 4 and 24 h, respectively (Fig. 3A). The increases of nNOS were $167 \pm 9\%$ ($p < 0.01$) and $189 \pm 36\%$ ($p < 0.05$) at 4 and 24 h, respectively (Fig. 3B). Consistent with these results, mRNA levels of eNOS and nNOS were also significantly increased by pGz (Fig. 4).

Periodic acceleration at a frequency of 600 cpm ($n = 4$) caused a more sustained increase of eNOS at 4 and 24 h, $392 \pm 46\%$ ($p < 0.01$) and $534 \pm 57\%$ ($p < 0.01$) (Fig. 3A), but significantly less induction of nNOS levels ($135 \pm 19\%$ at 4 h and no change at 24 h) (Fig. 3B) compared to pGz at a frequency of 360 cpm. Inducible nitric oxide synthase (iNOS) was not detected in control or pGz at either frequency (data not shown).

To explore the signal transduction pathway through which pGz acts, two pathways were investigated: the Akt pathway and the MAPK (ERK1/2) pathway. As shown in Fig. 5, the phosphorylation of Akt at Thr 308 was increased to 217% of control ($n = 4$), while the total Akt was not changed by pGz (360 cpm) ($n = 4$) treatment for 1 h. pGz (360 cpm) treatment for 1 h did not significantly change the level of total and phosphorylated ERK1/2 (data not shown).

To determine the localization of nNOS in the heart, immunohistochemical staining was performed. As shown in Fig. 6, nNOS is exclusively present in the cardiomyocyte. Compared to control group, pGz upregulated nNOS levels in cardiomyocytes as indicated by increased fluorescence intensity (Fig. 6).

Discussion

This study shows the effects of a single 1-h exposure to pGz, on the expression of various NOS isoforms. We confirmed that pGz increased NO production, as indicated by increased nitrosylated proteins, and upregulated eNOS and nNOS, both protein and mRNA, in heart. The effects persisted for at least 24 h after cessation of the stimulus. pGz did not affect iNOS protein levels. Additionally, pGz also increased phosphorylation of eNOS and Akt (active forms), without change in either total Akt or MAP kinase pathway.

eNOS upregulation is known to occur in both isolated vessels, and endothelial cell cultures after exposure to pulsatile shear stress [28,29]. Periodic acceleration imposes low amplitude additional pulsation on the intrinsic pulsations produced by the beating heart, and induces pulsatile shear stress both *in vivo* and *in vitro* [20,21]. Physical exercise also increases pulsatile shear stress and increases eNOS expression. This pathway may be an important component of cardioprotection conferred by exercise [30–33]. Hambrecht et al. [34] recently demonstrated that eNOS mRNA levels increase by 97%, 36 h after exercise, similar to our results of 75% increase 24 h after pGz treatment. Therefore, pGz may afford equivalent cardioprotection possibly through a similar pathway to exercise training.

Two signal transduction pathways, Akt pathway and MAPK (ERK1/2) pathway were examined in this study. pGz increased Akt phosphorylation, without effecting total Akt, or total and phosphorylated ERK1/2. Shear stress has been shown to increase eNOS phosphorylation through Akt pathway [35–37]. Therefore, we postulate that pGz-induced eNOS phosphorylation is at least in part mediated through Akt pathway by increasing pulsatile shear stress. However, the exact mechanism how pGz upregulates the expression of eNOS and nNOS needs to be further elucidated.

nNOS is emerging as an important regulator of cardiac contractility. Hare and co-workers [6] found nNOS immunoprecipitated with the ryanodine receptor of sarcoplasmic reticulum in mouse heart homogenates, suggesting a role in the regulation of calcium handling. nNOS has been demonstrated to regulate excitation–contraction coupling in the heart [6,38]. Additionally, nNOS derived NO changes the cardiac autonomic balance with enhanced vagal activity and reduced β -adrenergic responsiveness [39]. nNOS deficient animals appear to have compromised vagal control of heart rate, impaired diastole [40–42], and increased mortality after experimental myocardial infarction [43]. Adams et al. [44] reported that pigs with selective inhibition of nNOS before global ischemia/reperfusion do not have return of spontaneous circulation (ROSC) whereas 75% of control group have ROSC. In a myocardial infarction model, Dawson [45] and Saraiva [43] showed that nNOS knock-out mice developed more pronounced left ventricular dilatation and dysfunction compared to wild type. Taken together these data suggest that nNOS is of vital importance to cardiovascular function in normal and diseased hearts. We investigated the localization of nNOS in the heart using immunohistochemical staining. nNOS is present exclusively in the cardiomyocytes and pGz increased its expression. The latter might account for the improved cardiac function during post-resuscitation when pGz is employed as a CPR method or when used in delayed post-conditioning tactic [20–22].

A limitation to the present study relates to the analysis of heart tissue homogenates for the expression of NOS isoforms. Since heart homogenates also contain endothelial cells from vascular and endothelial lining of the heart and cardiomyocytes it is possible that eNOS expression could be primarily localized in endothelial cells. In contrast we found that nNOS was localized exclusively in the cardiomyocytes and pGz increased its expression.

In conclusion, this study provides a molecular mechanism for the effects of a single exposure of pGz on NO production and its respective NOS isoforms. A single pGz exposure

produces a robust change in cardiac NOS isoform phenotype. Whether or not additional exposures to pGz can result in greater output and thus a more robust phenotype, remains to be determined. The clinical applicability of such a noninvasive method extends from preconditioning prior to a planned ischemia reperfusion event (cardiac surgery, revascularization and others) to post-conditioning strategies.

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Fig. 1. Effect of pGz on total nitrosylated proteins in heart. * $p < 0.05$ pGz ($n = 4$) vs control ($n = 4$).

Fig. 2. Effect of pGz on phosphorylated eNOS (Ser-1177) in heart. Upper panel, representative Western blot image; lower panel, relative phosphorylated eNOS levels using control as 100%. * $p < 0.05$ pGz ($n = 4$) vs control ($n = 4$).

Fig. 3. Effect of pGz on protein levels of eNOS and nNOS in the heart. (A) Effect of pGz on eNOS. Upper panel, representative Western blot image; lower panel, relative eNOS levels using control as 100%. (B) Effect of pGz on nNOS. Upper panel, representative Western blot image; lower panel, relative nNOS levels using control as 100%. ** $p < 0.01$, * $p < 0.05$ pGz ($n = 4$) vs control ($n = 6$).

Fig. 4. Effect of pGz of 360 cpm and 600 cpm on mRNA of eNOS and nNOS in heart. (A) eNOS; (B) nNOS. ** $p < 0.01$, * $p < 0.05$ pGz ($n = 4$) vs control ($n = 6$).

Fig. 5. Effect of pGz on total and phosphorylated Akt in heart. Upper panel, representative Western blot images; lower panel, relative total Akt and phosphorylated Akt levels using control as 100%. * $p < 0.05$ pGz ($n = 4$) vs control ($n = 4$).

Fig. 6. Localization of nNOS in heart. Immunohistochemical staining of nNOS in the heart with magnification as indicated. Upper: control; lower: pGz. (A and C) Magnification 20 $\times$ and (B and D) magnification 40 $\times$. nNOS was localized to cardiomyocytes. pGz induced greater immunofluorescence of nNOS.

Table 1

Effect of pGz (360 cpm) on blood gas and electrolyte. The values are expressed as means $\pm$ SD.

	Control		pGz	
	Baseline	1 h treatment	Baseline	1 h treatment
pH	7.46 $\pm$ 0.05	7.46 $\pm$ 0.03	7.49 $\pm$ 0.02	7.48 $\pm$ 0.08
PaCO ₂ (torr)	31 $\pm$ 1	32 $\pm$ 1	31 $\pm$ 6	31 $\pm$ 1
PaO ₂ (torr)	92 $\pm$ 2	90 $\pm$ 4	92 $\pm$ 12	99 $\pm$ 16
O ₂ saturation (%)	97 $\pm$ 0.50	96 $\pm$ 1.26	98 $\pm$ 0.89	98 $\pm$ 0.76
Sodium (mg/dl)	139 $\pm$ 0.21	138 $\pm$ 1	140 $\pm$ 1	137 $\pm$ 2
Potassium (mg/dl)	3.7 $\pm$ 0.56	3.4 $\pm$ 0.01	3.5 $\pm$ 0.75	4.3 $\pm$ 0.81
Calcium (meq/l)	1.10 $\pm$ 0.16	1.17 $\pm$ 0.07	1.16 $\pm$ 0.20	1.17 $\pm$ 0.16
Chloride (mg/dl)	106 $\pm$ 6	109 $\pm$ 8	106 $\pm$ 7	103 $\pm$ 1