Original Article

NADPH Oxidase Accounts for Changes in Cerebrovascular Redox Status in Hindlimb Unweighting Rats*

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Abstract

Objective The roles of cerebrovascular oxidative stress in vascular functional remodeling have been described in hindlimb-unweighting (HU) rats. However, the underlying mechanism remains to be established.

Methods We investigated the generation of vascular reactive oxygen species (ROS), Nox2/Nox4 protein and mRNA levels, NADPH oxidase activity, and manganese superoxide dismutase (MnSOD) and glutathione peroxidase-1 (GPx-1) mRNA levels in cerebral and mesenteric smooth muscle cells (VSMCs) of HU rats.

Results ROS production increased in cerebral but not in mesenteric VSMCs of HU rats compared with those in control rats. Nox2 and Nox4 protein and mRNA levels were increased significantly but MnSOD/GPx-1 mRNA levels decreased in HU rat cerebral arteries but not in mesenteric arteries. NADPH oxidases were activated significantly more in cerebral but not in mesenteric arteries of HU rats. NADPH oxidase inhibition with apocynin attenuated cerebrovascular ROS production and partially restored Nox2/Nox4 protein and mRNA levels, NADPH oxidase activity, and MnSOD/GPx-1 mRNA levels in cerebral VSMCs of HU rats.

Conclusion These results suggest that vascular NADPH oxidases regulate cerebrovascular redox status and participate in vascular oxidative stress injury during simulated microgravity.

Key words: Microgravity; Oxidative stress; NADPH oxidase; Manganese superoxide dismutase; Glutathione peroxidase-1

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INTRODUCTION

Exposure to microgravity results in cardiovascular deconditioning and consequent orthostatic intolerance in astronauts when they return to Earth. Evidence from spaceflight/ground-based human studies and simulated microgravity animal studies has linked vascular structural and functional adaptations to post-flight cardiovascular deconditioning[1-2]. Although much progress has been made in the past two decades, the underlying mechanisms remain to

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be elucidated.

Fluid and pressure gradient shifts result in different vascular structural and functional changes in hindlimb-unweighting (HU) rodent models[3-4]. Changes in the local renin-angiotensin system, vascular smooth muscle cells (VSMCs) ion-channel remodeling, inflammation, oxidative stress, and the nitric oxide synthase (NOS)-nitric oxide (NO) system in arteries from different regions have been implicated in this process[5-9]. Our previous studies suggested that inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) oxidases with apocynin reversed the abnormal cerebrovascular responses to vasoconstrictors and vasodilators by regulating the NOS-NO system[10]. However, the characteristics and underlying molecular mechanism of cerebrovascular oxidative stress injury in HU rats remain unclear.

NADPH oxidases are major sources of reactive oxygen species (ROS) other than mitochondria and include the Nox1-5 and Duox1 and Duox2 catalytic subunits, the p22phox, p47phox, Noxo1, p67phox, Noxa1, and p40phox regulatory subunits, and the major binding partner Rac[11]. Of these, Nox2 and Nox4 are expressed in cardiovascular tissues, participate in normal vascular and cardiac function, and also contribute to the development of cardiovascular diseases. Nox2 produces superoxide, which promotes endothelial dysfunction caused by a high-fat diet, predisposes arteries to a reduced vasodilator response, and contributes to altered cerebral blood flow[12]. Nox4 is the primary source of inflammation-and tumor necrosis factor (TNF)-alpha-induced oxidative stress leading to apoptosis in cerebrovascular endothelial cells[13]. However, the individual roles of the Nox subtypes in the vasculature are unclear and controversial. Although inhibiting NADPH oxidases with apocynin improves vasoreactivity[10], whether NADPH oxidases account for the cerebrovascular oxidative injury induced by HU merits further investigation.

Vascular manganese superoxide dismutase (MnSOD) and glutathione peroxidase-1 (GPx-1) scavenge ROS and protect cells from oxidative damage. Decreased expression and activity of mitochondrial MnSOD in pulmonary artery endothelial cells increases oxidative stress[14] and MnSOD deficiency increases mitochondrial oxidative stress and aggravates age-dependent vascular dysfunction[15]. GPx-1 expression and activities play major roles in protecting against angiotensin II-induced endothelium-dependent relaxation in carotid arteries[16]. A GPx-1 deficiency results in increased formation of oxidants, which triggers endothelial NOS dysfunction and endothelial dysfunction in aged GPx-1(−/−) mice[17]. High mitochondrial MnSOD/GPx-1 protein levels and activities are attenuated in cerebral arteries of HU rats[18]; however, whether vascular cell cytoplasmic levels of MnSOD and GPx-1 change during simulated microgravity is unclear. In addition, it is unclear whether NADPH oxidases regulate MnSOD and GPx-1 mRNA expression.

The present study was designed to investigate whether microgravity simulated by HU alters NADPH oxidase expression and activity and MnSOD/GPx-1 mRNA levels in HU rat cerebral and mesenteric VSMCs. We also determined whether inhibiting NADPH oxidases with apocynin attenuates vascular oxidative injury by regulating the expression of Nox2/Nox4-containing NADPH oxidases, total activities of NADPH oxidases, and MnSOD and GPx-1 mRNA expression during HU.

METHODS

Animal and Tissue Treatments

Handling and treatment of animals were in accordance with the Guiding Principles for the Care and Use of Animals in Physiological Sciences and were approved by the Chinese guidelines for experimental animals. The care and use of experimental rats were supervised and approved by the Animal Ethical Committee of Chinese PLA General Hospital.

Male Sprague-Dawley rats (6-7 weeks old) were assigned randomly to four groups (n=8): control (CON), HU, apocynin-treated HU (HU+APO), and apocynin-treated control (CON+APO). HU+APO and CON+APO rats received distilled water containing 50 mg/(kg·day) apocynin by gavage. The rats in the other groups received an equal volume of vehicle (distilled water). The HU technique has been described previously in detail[8] and was used to simulate microgravity in rats.

After 28 d of treatment, all rats were anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneally) and sacrificed by exsanguination via the abdominal aorta. The cerebral and mesenteric arteries were removed rapidly and placed in cold Krebs buffer solution in mmol/L: NaCl, 118.3; KCl, 14.7; KH_2PO_4, 1.2; $MgSO_4{\cdot}7H_2O$, 1.2; $CaCl_2{\cdot}2H_2O$, 2.5; $NaHCO_3$, 25; dextrose, 11.1; and

EDTA, 0.026; pH 7.40.

Reactive Oxygen Species Detection

ROS levels were determined using the DCFH-DA fluorescent probe (Molecular Probes, Inc., Eugene, OR, USA)[19]. DCFH-DA is a general indicator of ROS that reacts with H_2O_2, $ONOO^-$, lipid hydroperoxides, and $O_2^{\bullet-}$. VSMCs were dissociated from cerebral and mesenteric arteries as described previously[7,9] and incubated with DCFH-DA (10 μmol/L) at 37 °C for 1 h, as the DCFH-DA was hydrolyzed by endogenous esterases to DCFH. After the incubation, the cells were resuspended in Krebs buffer. Flow cytometric analysis (BD FACS Calibur Flow Cytometer, BD Biosciences, San Jose, CA, USA) was performed to analyze ROS levels. The results are expressed as mean fluorescence intensity (MFI).

Expression of the Nox2 and Nox4 NADPH Oxidase Isoforms

Western blot was used to identify Nox2/Nox4 protein levels in cerebral and mesenteric arteries. The cerebral and mesenteric arteries were homogenized in 10 mmol/L HEPES lysis buffer (320 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L DTT, 10 μg/mL leupeptin, and 2 μg/mL aprotinin, pH 7.40). The lysates were centrifuged at 12,000 ×*g* for 10 min at 4 °C. Protein concentrations were determined with a bicinchoninic acid assay kit (Pierce, Rockford, IL, USA). The proteins (loading mass: 25 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis, and the fractionated proteins were electrophoretically transferred to nitrocellulose membranes (Amersham, Piscataway, NJ, USA). The membranes were incubated with mouse anti-Nox2 monoclonal (1:1,500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-Nox4 polyclonal (1:1,000; Santa Cruz Biotechnology) primary antibodies. The membranes were incubated with goat anti-mouse or goat anti-rabbit secondary antibody (1:10,000; Jackson Immuno Research, West Grove, PA, USA). The enhanced chemiluminescence detection reagents (Amersham, Cleveland, OH, USA) were added, and the membranes were exposed to Hyperfilm (Amersham). An anti-GAPDH monoclonal antibody (1:10,000; Abcam, Cambridge, MA, USA) was used to normalize for loading variations. The protein bands were analyzed using Quantity One ver. 4.62 software.

A quantitative real-time quantitative polymerase chain reaction (qPCR) analysis was performed using a Bio-Rad IQ5 system (Hercules, CA, USA). The forward and reverse primer sequences are shown in Table 1. Total RNA was prepared with a total RNA Kit (R6934; Omega Bio-tek Inc., Norcross, GA, USA), and cDNA was synthesized with a cDNA Synthesis Kit (K1622; Fermentas International Inc., Burlington, ONT, Canada) according to the manufacturer's instructions. Each PCR was performed in triplicate in a 20 μL final volume of solution: 10 μL of SYBR Green dye, 1 μL diluted cDNA products, 0.2 μmol/mL of each paired primer, and 8.6 μL deionized water under the following conditions: initial denaturation for 10 min at 95 °C, followed by 40 cycles denaturation for 15 s at 95 °C, and extension for 30 s at 60 °C. The last cycle for dissociation of the SYBR Green probe was 15 s at 95 °C, 30 s at 60 °C, and 15 s at 95 °C. Threshold cycle (Ct) values of Nox2 and Nox4 mRNA were measured and normalized to that of *β*-actin and expressed as a relative ratio.

Measurement of NADPH Oxidase Activities

NADPH oxidase activities were measured using a NADPH oxidase activity assay kit (Genmed Scientifics Inc., Wilmington, DE, USA), according to the manufacturer's instructions. In brief, cerebral and mesenteric VSMCs were washed and incubated with NADPH. NADPH oxidase activity was measured by monitoring the rate of consumption of NADPH that was inhibited after adding diphenyliodonium. NADPH oxidase activity was determined by spectrophotometry (Thermo Fisher Scientific Inc., Madison, WI, USA) at 340 nm, and the results are expressed as percent enzyme activity compared to that of the control.

Table 1. Nox2, Nox4, β-actin, Manganese Superoxide Dismutase (MnSOD) and Glutathione Peroxidase-1 (GPx-1) Primer Sequences Used in the Present Study

Genes	Primer Sequences (5′-3′)
Nox2	Forward: GTGGAGTGGTGTGTGAATGCC Reverse: ATGCCAGCCAACCGAGTCACA
Nox4	Forward: TAGCTGCCCACTTGGTGAACG Reverse: TGTAACCATG AGGAACAATACCACC
β-actin	Forward: TGACAGGATACAGAAGGAGA Reverse: TAGAGCCACCAATCCACACA
MnSOD	Forward: ATTAACGCGCAGATCATGCA Reverse: CCTCGGTGACGTTCAGATTGT
GPx-1	Forward: TATAGAAGCCCTGCTGTCCA Reverse: CAAGCCCAGATACCAGGAA

MnSOD and GPx-1 mRNA Levels

Cerebral and mesenteric VSMCs were dissociated, and total RNA was extracted with the phenol/chloroform method using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA, USA). RNA (5 μg) was reverse-transcribed to cDNA in a 50 μL reaction mixture with Superscript II RNase H-reverse transcriptase and random primers (Life Technologies, Rockville, MD, USA). The forward and reverse primer sequences are shown in Table 1. Threshold cycle (Ct) values of MnSOD and GPx-1 mRNA were measured and normalized to that of β-actin and expressed as a relative ratio.

Statistical Analysis

The results are expressed as mean±standard error. GraphPad Prism 5.0 software was used for statistical analyses and figure presentation. The statistical evaluation was performed using one-way analysis of variance. $P<0.05$ was considered significant.

RESULTS

General Data

HU resulted in significantly lower soleus muscle mass compared to that of control rats ($P<0.001$). Soleus muscle-to-body mass ratios decreased ($P<0.001$) in HU and HU + APO rats, which confirmed the efficacy of simulated microgravity and reliability of the animal model used in the present study. Data are summarized in Table 2.

Effects of Apocynin on Vascular ROS Generation

Vascular ROS levels are shown in Figure 1. Compared with their age-matched controls, 4-week-treated HU rat cerebral VSMCs showed significantly higher ROS levels ($P<0.001$), and chronic treatment with apocynin significantly attenuated ROS production ($P<0.001$) (Figure 1A). However, HU did not affect ROS generation in mesenteric VSMCs (Figure 1B).

Effects of Apocynin on Nox2 and Nox4 Protein and mRNA Expression

Nox2 and Nox4 protein and mRNA levels in HU rat cerebral and mesenteric VSMCs are shown in Figures 2 and 3. Nox2 protein and mRNA levels increased ($P<0.001$ and $P<0.001$) in HU rat cerebral arteries compared with those in the control, whereas Nox2 expression did not vary in mesenteric arteries between control and HU rats (Figures 2A and 3A). Nox4 protein and mRNA levels also increased significantly in HU rat cerebral arteries ($P<0.001$ and $P<0.001$), whereas their expression levels did not vary in mesenteric arteries (Figures 2B and 3B). Treatment with apocynin partially restored Nox2/Nox4 protein and mRNA expression levels in cerebral arteries ($P<0.001$ and $P<0.05$ for Nox2, respectively; $P<0.001$ and $P<0.001$ for Nox4, respectively), whereas it had no effect on Nox2/Nox4 expression in mesenteric arteries.

Effects of Apocynin on NADPH Oxidase Activity

We measured NADPH oxidase activity to investigate whether apocynin attenuated cerebral oxidative stress by inhibiting NADPH oxidase (Figure 4). The 4-week HU treatment activated NADPH oxidase in cerebral VSMCs. In contrast, HU had no stimulatory effect on NADPH oxidase activity in mesenteric VSMCs. Apocynin decreased NADPH oxidase activity in HU rat cerebral arteries, whereas apocynin did not affect NADPH oxidase activity in mesenteric arteries.

Effects of Apocynin on MnSOD and GPx-1 mRNA Levels

To investigate whether vascular antioxidative enzymes were altered during HU, we detected MnSOD and GPx-1 mRNA expression (Figure 5A and 5B) in cerebral and mesenteric VSMCs. MnSOD and GPx-1 mRNA levels decreased significantly ($P<0.001$ and $P<0.001$) in 4-week-treated HU rat cerebral arteries,

Table 2. Body Mass, Soleus Mass, and Soleus: Body Mass Ratio (mg/g) of Rats from Control, Apocynin-treated Control, Hindlimb Unweighted, and Apocynin-treated Hindlimb Unweighted (n=8/group)

Items	Initial Mass (g)	Final Mass (g)	Soleus Mass (mg)	Soleus: Body Mass (mg/g)
CON	194.53±4.14	343.29±4.27	133.71±0.83	0.39±0.02
HU	196.34±4.36	341.96±4.24	64.97±0.47	0.18±0.01***
CON+APO	197.25±4.23	341.17±3.12	138.75±1.10	0.41±0.01
HU+APO	198.34±4.21	346.51±4.26	74.12±0.64	0.22±0.02***

Note. CON, control; HU, hindlimb unweighted; CON+APO, apocynin-treated control; HU+APO, apocynin-treated HU. Values are means±standard errors; *** $P<0.001$ *vs.* control. n=8/group.

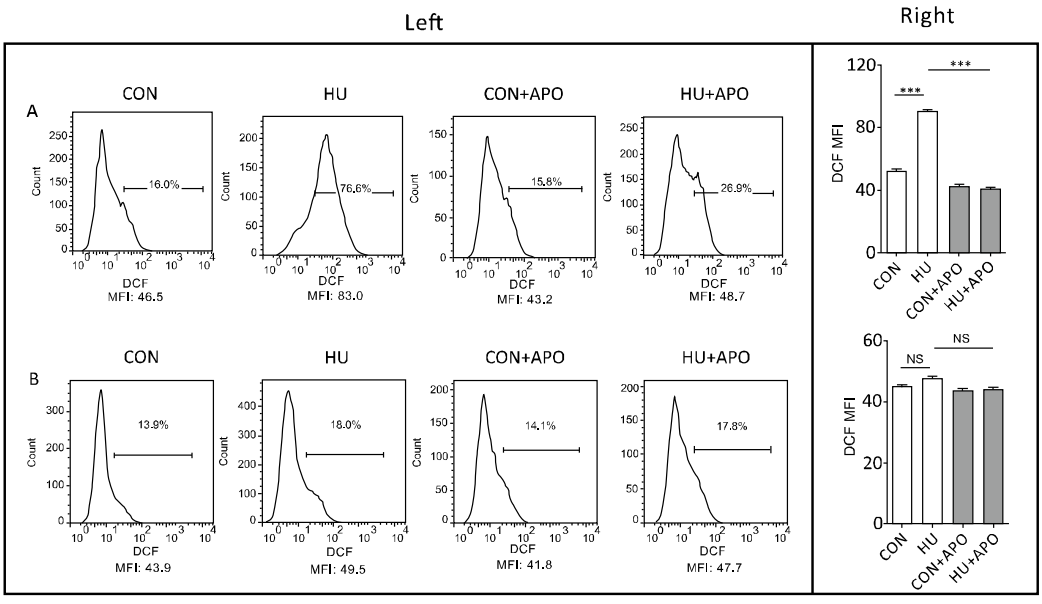

Figure 1. ROS levels in rat cerebral (A) and mesenteric (B) vascular smooth muscle cells. ROS levels were analyzed by flow cytometry after DCFH-DA staining expressed as MFI. Left: Representative quadrantal diagrams from the flow cytometric analysis Right: Mean DCF ROS fluorescence intensity levels. CON, control; HU, hindlimb unweighted; CON+APO, apocynin-treated control; HU+APO, apocynin-treated HU. MFI, mean fluorescence intensity; ROS, reactive oxygen species. NS, no significance. Values are mean±standard error. *** P<0.001 for HU *vs*. control or HU+APO. n=8/group.

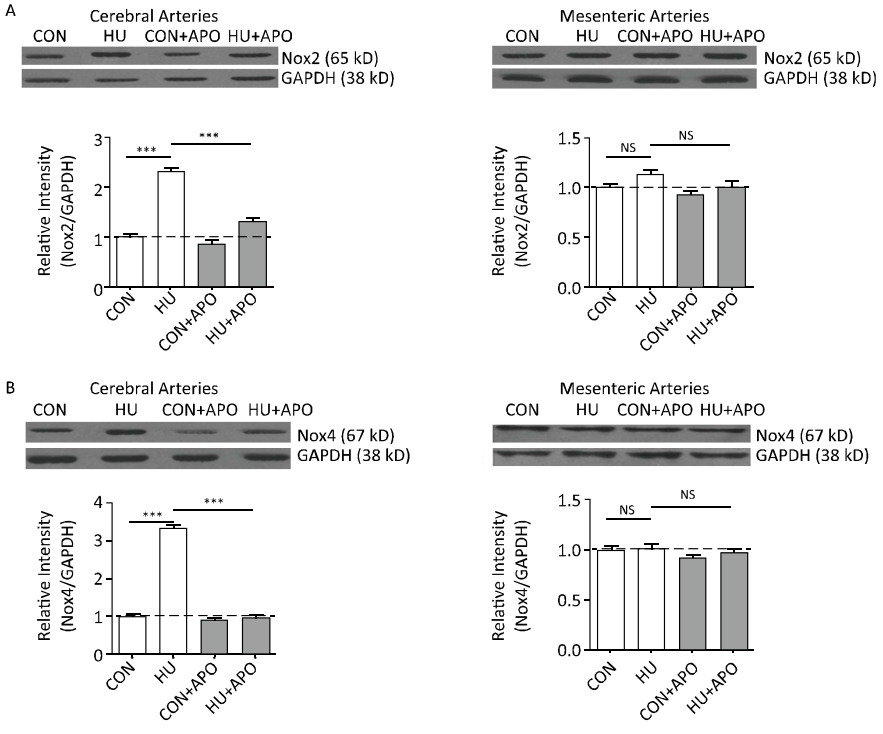

Figure 2. Nox2 (A) and Nox4 (B) protein expression in rat cerebral and mesenteric arteries. Nox2 and Nox4 protein levels were analyzed by Western blot. CON, control; HU, hindlimb unweighted; CON+APO, apocynin-treated control; HU+APO, apocynin-treated HU. NS, no significance. Values are mean±standard error. *** P<0.001 for HU *vs*. CON or HU+APO. n=5/group.

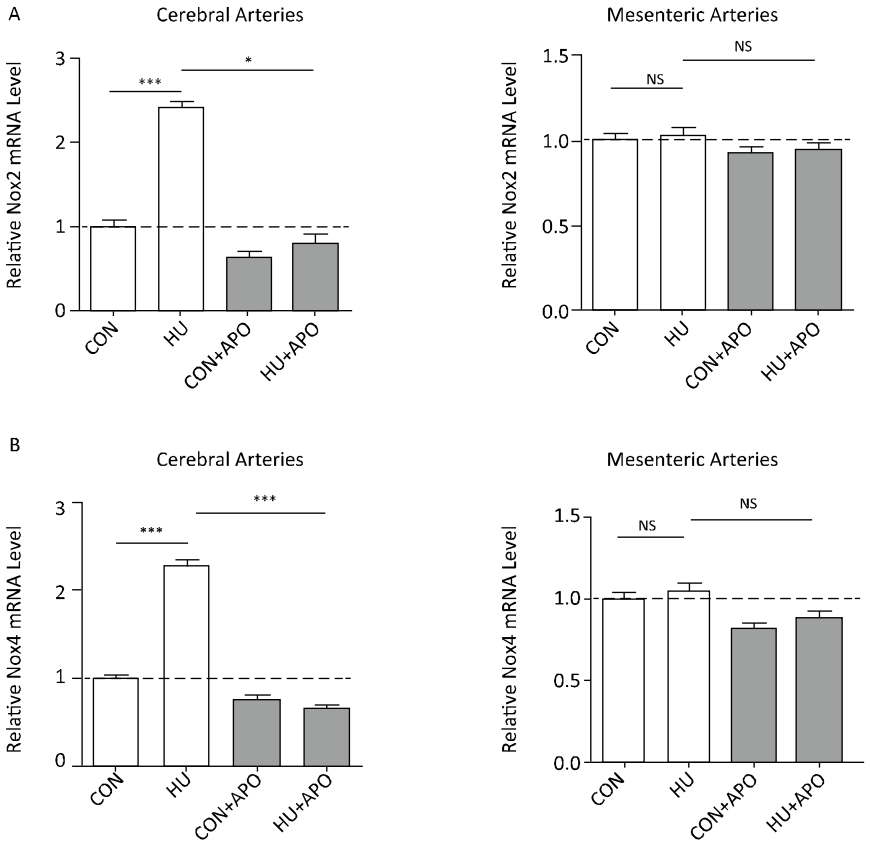

Figure 3. Nox2 (A) and Nox4 (B) mRNA expression in rat cerebral and mesenteric arteries. mRNA levels were analyzed by quantitative polymerase chain reaction. CON, control; HU, hindlimb unweighted; CON+APO, apocynin-treated control; HU+APO, apocynin-treated HU. Ct values of Nox2 and Nox 4 mRNA were measured and normalized to that of β-actin and expressed as a relative ratio. NS, no significance. Values are mean±standard error. $^{*}P$<0.05 for HU *vs*. HU+APO and $^{***}P$<0.001 for HU *vs.* control for Nox2, $^{***}P$<0.001 for HU *vs*. control or HU+APO for Nox4. *n*=6-12/group.

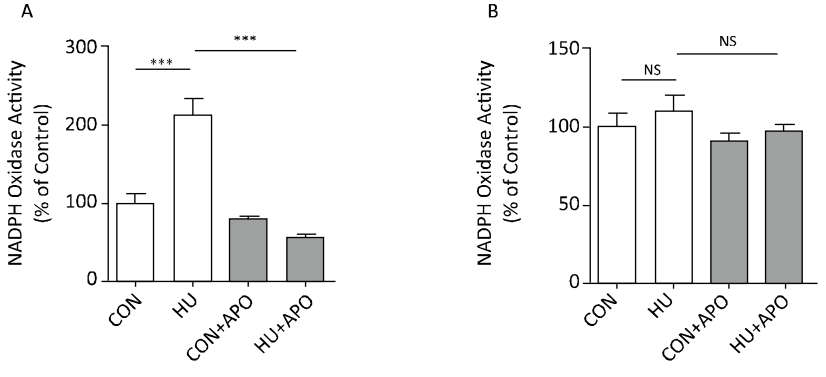

Figure 4. NADPH oxidase activity in rat cerebral (A) and mesenteric (B) vascular smooth muscle cells. CON, control; HU, hindlimb unweighted; CON+APO, apocynin-treated control; HU+APO, apocynin-treated HU. NS, no significance. Values are mean±standard error. $^{***}P$<0.001 for HU *vs*. control or HU+APO. *n*=5/group.

compared with those of control rats and were partially restored by apocynin (P<0.001 and P<0.001). However, MnSOD and GPx-1 mRNA levels in mesenteric VSMCs remained unaltered by HU or apocynin.

DISCUSSION

Two major findings were observed in the present study: 1) the 4-week HU-treatment enhanced vascular ROS production, stimulated NADPH oxidase activities, increased Nox2/Nox4 protein and mRNA expression, and decreased MnSOD and GPx-1 mRNA in cerebral VSMCs; 2) inhibiting NADPH oxidase with apocynin attenuated cerebrovascular ROS production and partially restored Nox2/Nox4/MnSOD/GPx-1 levels and NADPH oxidase activity in cerebral arteries.

Exposure to microgravity results in postflight cardiovascular deconditioning and orthostatic intolerance in astronauts, which decreases working capacity and threatens spaceflight safety. Structural and functional remodeling of the vascular system have been implicated in this process. We and other authors have reported that vascular responses to vasoconstrictors are enhanced and endothelium-dependent relaxation is attenuated in HU-treated rat cerebral arteries[1-2,5-6]. We reported that cerebrovascular oxidative stress injury is characterized by increased superoxide production in HU rat cerebral arteries[6] and inhibiting NADPH oxidase with apocynin restores cerebrovascular responses to agents by modulating the NOS-NO system[10]. However, the molecular mechanism(s) is unclear. In the present study, we found that NADPH oxidases were stimulated and apocynin significantly attenuated ROS generation in HU rat cerebral VSMCs. Therefore, we conclude that the NADPH oxidases stimulated by HU contribute to cerebrovascular oxidative stress injury.

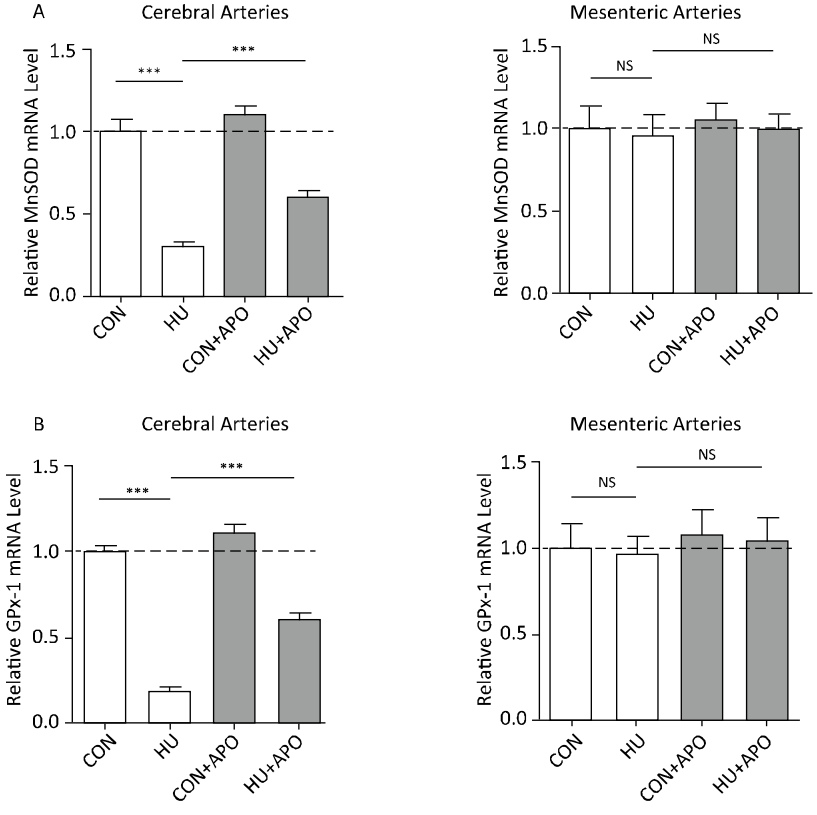

Figure 5. Manganese superoxide dismutase (MnSOD) (A) and glutathione peroxidase-1 (GPx-1) (B) mRNA expression levels in rat cerebral and mesenteric arteries. mRNA levels were analyzed by quantitative polymerase chain reaction. CON, control; HU, hindlimb unweighted; CON+APO, apocynin-treated control; HU+APO, apocynin-treated HU. Ct values of MnSOD and GPx-1 Mrna were measured and normalized to that of β-actin and expressed as a relative ratio. NS, no significance. Values are mean±standard error. *** P<0.001 for HU *vs*. control or HU+APO for MnSOD and GPx-1. n=6-12/group.

Accumulating evidence has established important roles for NADPH oxidases in cardiovascular physiology and pathophysiology. Of the NADPH enzymes, Nox1, 2, 4, and 5 are mainly expressed in cardiovascular tissues[20-21]. Nox4 may function as an inducible Nox isoform because of the close correlation between Nox4 mRNA and ROS production[22]. Overexpression of endothelial Nox4 results in vasodilation, which is attributable to increased H_2O_2 production and decreased NO inactivation[23]. Consistent with this finding, Nox4-deficient mice show increased aortic inflammation, media hypertrophy, and endothelial dysfunction, and loss of Nox4 reduces endothelial NOS expression and NO production[24]. Despite these results, Nox4 was considered the primary source of inflammation-and TNF-alpha-induced oxidative stress leading to apoptosis in cerebrovascular endothelial cells in another study[13]. These effects contrast markedly with the effects of Nox2. A mutation in Nox2 results in a significant increase in forearm-mediated vasodilation with increased NO bioavailability[25-26], and the Nox2 deficiency protects against hypercholesterolemia-induced impaired neovascularization, which is linked to decreased ROS production[27]. It also promotes endothelial dysfunction produced by a high-fat diet and predisposes blood vessels to a reduced vasodilator response, and thus may contribute to alterations in cerebral blood flow[12]. In the present study, we found that 4-week HU not only upregulated Nox2/Nox4 mRNA and protein expression but also stimulated NADPH oxidase in cerebral arteries. However, the individual effects of vascular Nox2/Nox4 during microgravity remain to be elucidated. If contradictory effects exist between Nox2 and Nox4, the interpretation of the increased expression of Nox4 and cerebrovascular oxidative stress injury is difficult. Thus, there is an urgent need to unravel the Nox4 paradox.

SOD and GPx eliminate endogenous oxidants[28-29], which are involved in maintaining mitochondrial structural and functional integrity[30], thereby protecting cells from oxidative injury. In the present study, MnSOD and GPx-1 mRNA levels decreased in cerebral VSMCs but were partially restored by the NADPH oxidase inhibitor apocynin. These data suggest that NADPH oxidases not only regulate the cytoplasmic oxidative system, but modulate antioxidative enzymes. Our results demonstrate that NADPH oxidases accounts for the oxidative stress injury in HU rat cerebral arteries.

The vascular responses to vasoconstrictors and vasodilators are both attenuated in mesenteric small arteries[1]. We did not detect oxidative stress in HU rat mesenteric VSMCs in the present study. These results suggest that NADPH oxidases were not involved in mesenteric vascular functional adaptations during microgravity simulated by HU. The expression of sarcoplasmic reticulum Ca^{2+} ATPase 2 and ryanodine 3 receptor mRNA was unaltered, but ryanodine 2 receptor mRNA and protein expression decrease in mesenteric arteries from HU rats[31]. However, human spaceflight reduces mesenteric arterial ryanodine receptor-3 mRNA levels and diminishes contractile responses to norepinephrine in mesenteric veins after flight, suggesting a ryanodine receptor-mediated intracellular Ca^{2+} release mechanism[32]. These controversies also need further data to reach a satisfying interpretation.

Several limitations of this study should be mentioned. First, apocynin has been considered a nonspecific inhibitor of NADPH oxidases, even though it remains a very popularly used. Second, Nox2/Nox4 knockout rodent animals would be a better choice than pharmaceutically inhibiting the NADPH oxidases to clarify these issues due to the lack of very specific NADPH oxidase inhibitors. Third, the individual roles of Nox2/Nox4 during vascular remodeling after HU are unclear and specific Nox2/Nox4 inhibitors should be evaluated in future work. Fourth, whether cerebrovascular redox status is time-dependent during HU remains elusive. These limitations should be clarified in future studies.

In conclusion, we found that microgravity simulated by HU increased the expression of Nox2/Nox4-containing NADPH oxidase, enhanced NADPH oxidase activity, and decreased MnSOD/GPX-1 mRNA levels in cerebral VSMCs. The NADPH oxidases accounted for the cerebrovascular oxidative stress injury and dysfunctional vascular responses to vasoactive agents.

DISCLOSURE

The authors declare no conflicts of interest.

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