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High fat diet exacerbates vascular endothelial dysfunction in rats exposed to continuous hypobaric hypoxia

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ABSTRACT

Independently, a high fat diet and hypoxia are associated with vascular endothelial dysfunction (VED) and often occur concurrently in patients. Nevertheless, the effects of a high fat diet on vascular endothelial function combined with hypoxia, a situation occurring with increasing frequency in many parts of the world, remain largely unknown. We investigated the effects of a high fat diet on vascular endothelial function in rats exposed to continuous hypoxia for 4 weeks. Seventy two male Sprague-Dawley rats were randomly divided into 3 groups: a hypoxia group fed regular chow, a combined hypoxia and high fat diet (HFD) group, and for comparison, rats maintained in normoxia, regular chow conditions were set as baseline (BL) group. The experimental data of BL group were obtained at beginning of hypoxia given in the other groups. Continuous hypoxia was induced in a hypobaric chamber maintained at an altitude of 5000 m. Compared to hypoxic conditions alone, hypoxia plus a HFD prevented adaptive changes in plasma nitric oxide (NOx) levels and caused earlier and more severe changes in aortic endothelial structures. Functionally, hypoxia plus a HFD resulted in impaired endothelium-dependent vasorelaxation responses to acetylcholine and altered the bioavailability of the nitric oxide synthase (NOS) substrate L-Arginine. At the molecular level, hypoxia plus a HFD blunted increases in endothelial NOS (eNOS) mRNA and protein in aortic endothelial tissue. Taken together, our findings demonstrate that in the setting of hypoxia, a high fat diet leads to earlier and more severe VED than hypoxia alone. These data have important implications for populations residing at high-altitude, as dietary patterns shift towards increased fat intake.

1. Introduction

Nitric oxide (NO) is continuously synthesized by vascular endothelial cells via nitric oxide synthase (NOS). Endothelial NOS (eNOS) derived NO is crucial for vascular endothelium to perform functions such as vasodilatation, inhibition of vascular inflammation, leucocyte adhesion, and control of vascular smooth muscle proliferation [1], thereby regulating vascular structure and tone. Vascular endothelial dysfunction (VED) includes dysregulation of vascular remodeling and impairment of endothelium-dependent vasorelaxation. VED may be caused by reducing NO bioavailability and production as well as eNOS activation, leading to an increase in the generation of reactive oxygen species (ROS) [2,3]. VED is associated with the pathogenesis of many cardiovascular disease, such as hypertension [4], atherosclerosis [5], coronary artery disease [6] and diabetes mellitus [7].

Hypercholesterolemia, a traditional risk factor for cardiovascular disease, induces VED. More recently, hypoxia has also been identified as a risk factor for VED, through similar mechanisms of decreasing NO generation and bioavailability, leading to increased ROS generation [8]. Moreover, chronic intermittent hypoxia can induce hypercholesterolemia, suggesting interrelated pathways between these two risk factors for VED [9]. Nevertheless, most of the published literature to date has focused on the influence of single factor [10,11] and the influence of a high fat diet on VED in body exposed to continuous hypobaric hypoxia— an issue of significant clinical importance in many regions of the world — remains incompletely understood. Therefore, the aim of this study was to investigate the effects of high fat diet on vascular endothelial

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function in settings of exposure to long-term, continuous hypobaric hypoxia.

2. Materials and methods

2.1. Animals

The procedures in this study were approved by the Institutional Animal Ethical Committee. 6 week-old male Sprague-Dawley (SD) rats (n = 72) weighing about 180–200 g were purchased from China Pharmaceutical University (altitude of 10 m). Rats were randomly divided into three equal groups: a hypoxia (H) group fed regular chow, a combined hypoxia and high fat diet (H + HFD) group, and for comparison, rats maintained in normoxia (altitude of 10 m, Nanjing), regular chow conditions were set as baseline (BL) group. As our study was aimed to investigate the effects of high fat diet on VED in settings of exposure to hypoxia, other than the interaction effects of these two factors, so a combined normoxia and high fat diet group was not set. The experimental data of BL group were obtained at beginning of hypoxia given in the other groups. The H + HFD and H group rats were maintained in hypobaric hypoxic chamber in our laboratory in Xining, China. The hypobaric hypoxic chamber was used to create an altitude of 5000 m where rats were maintained during the entire study. Rats were gavaged with fat emulsion (10 ml/kg/d) or equal volume of saline solution once daily for 4 weeks. High-fat emulsion preparation was done as previously reported [12]. Animals were exposed to a normal light–dark cycle of the day. Blood pressure and heart rate were assessed by a noninvasive blood pressure cuff (rat tail pressure, Softron Beijing Incorporated, Beijing, China) and body weights were recorded. Blood was collected from the inferior vena cava, following anesthetization with urethane. Hemoglobin (HGB) concentration was determined with a fully automatic blood cell analyzer (BC-2300, Mairui Biotec, Shenzhen, China). Plasma superoxide dismutase (SOD) activity was measured by the WST-1 method. The plasma content of malondialdehyde (MDA) was measured by the TBA method. Nitric oxide was estimated in plasma by detecting the stable metabolite nitrates and nitrites (NOx) with the nitrate reductase method. The SOD, MDA, and NOx kits were purchased from Nanjing Jiancheng Bio-Engineering Research Institute, China. Commercially available kits (Beijing Leadman Biochemical Co., LTD, Beijing, China) were used to estimate the plasma total cholesterol (TCH) and triglyceride (TG) and high-density lipoprotein (HDL) and low density lipoprotein (LDL) levels.

2.2. Isolated aorta studies

After 1 week of treatment, aortas were separated from a subgroup of anaesthetized rats, placed in ice-cold buffer, cleaned of adherent connective tissue, and then cut into 5 mm lengths to measure vascular tone. Aortic rings were mounted on a pair of steel hooks connected to an isometric tension transducer in an organ bath (Multi Wire Myograph, Model MP150, BIOPAC System, Inc. USA) filled with 40 ml of Krebs–Henseleit (K–H) buffer (37 °C, pH 7.4) consisting of NaCl (118.29 mM), KCl (4.69 mM), $MgSO_4$ (2.39 mM), KH_2PO_4 (1.19 mM), $NaHCO_3$ (25 mM), $CaCl_2$ (2.52 mM), and glucose (12.11 mM), bubbled with 95%O_2/5%CO_2. The aortic rings were equilibrated for 60 min with a rest tension of 1 g. Phenylephrine (Phe; 10^{-6} M) was added to the baths to test the viability of rings. Baths were then washed three times to make sure tension returned to baseline levels. The aortic rings were contracted with Phe (10^{-6} M). When constriction reached a plateau, acetylcholine (ACh, 10^{-8}–$10^{-4.5}$ M) or sodium nitroprusside (SNP, 10^{-8}–$10^{-4.5}$ M) (Sigma Aldrich Inc, USA) was added to measure endothelium-dependent and endothelium-independent relaxation responses separately, and changes in aortic tension were recorded. The endothelium-dependent relaxation response to ACh was assessed in the presence or absence of L-Arginine (L-Arg; a nitric oxide synthase substrate dosed at 10^{-3} M for 15 min before Phe pre-contraction) or Nω-Nitro-L-Arginine Methyl Ester (L-NAME; a nitric oxide synthase inhibitor; 5×10^{-6} M; incubation for 20 min before Phe pre-contraction). The vasorelaxant effects were expressed as the change in tension with ACh or SNP, relative to maximal Phe contraction.

2.3. Histopathological analysis of the aorta

For the light microscopy analyses, the aorta of the rats were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. The paraffin block was sectioned at a thickness of 5 μm and sections were stained with hematoxylin–eosin (H&E). Aortic morphology was assessed using an Olympus BX53F microscope (Tokyo, Japan) and MShot digital imaging system.

2.4. Western Blot analysis of endothelial nitric oxide synthase

The frozen aortas were homogenized by polytron homogenizer on ice in RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China). After aortic tissues were fully lysed, the samples were centrifuged at 12000 × *g* for 5 min at 4 °C. Supernatants were collected and protein concentration was measured by the BCA Protein Assay Kit (Pierce Chemical Company, USA). The supernatants were then denatured at 90 °C for 10 min. Proteins were examined by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred protein to polyvinylidene fluoride (PVDF) membranes by Bio-Rad Trans-Blot SD (Bio-Rad Laboratories, Inc, USA). The membranes were washed in TBST and blocked with 5% nonfat milk in TBST for 2 h at room temperature. Membranes were incubated overnight at 4 °C with the primary antibodies (polyclonal rabbit β-actin, 1:1000, or polyclonal rabbit eNOS, 1:250. abcam Ltd., Hong Kong, China). The membranes were washed and incubated with horseradish peroxidase goat anti rabbit secondary antibody (1:6000; Santa Cruz Biotechnology Inc., California, USA) for 2 h at room temperature. The membranes were washed in TBST and then incubated in chemiluminescent substrate (ECL Western Blotting Kit, Pierce Chemical Company, USA) for 30 s. Protein bands were exposed onto X-ray film. ImageJ software was used to measure the integral optical density value of protein bands from the scanned film and calculate the relative ratio of eNOS and β-actin bands' integral optical density value.

2.5. Quantitative real-time PCR analysis of endothelial nitric oxide synthase

Total RNA of iced aortas was prepared using RNA simple Total RNA Kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. 1 ug of total RNA was reverse transcribed to cDNA using random primers (Takara Biotechnology, Dalian, China). Real-time PCR was performed using an ABI PRISM 7500 (Applied Bio systems, Foster City, CA, USA). The reaction contained 10 μl SYBRR Select Master Mix (Life technologies, Carlsbad, USA), 2 μl cDNA, 0.4 μl of each primer and 7.2 μl of RNase-free water. The cycling condition consisted of a holding stage (50 °C for 2 min, then 95 °C for 2 min) and cycling stage (95 °C for 15 s, then 60 °C for 1 min; repeated 40 times). Forward and reverse primers were as follows: eNOS [13]: 5′- CTACCGGGACGAGGTACTGG -3′and 5′- GGAAAAGGCGGTGAGGACTT -3′,iNOS: 5′- TCTTTGCTTCTGTGCTAA TG-3′and 5′- CAGTAGTTGTTCCTCTTCCA-3′, β-actin [14]: 5′- TCCCGGCAGCACCAGTAAC-3 -3′and 5′- CCCAGATGC ATAATCGCT GC

-3′, Real-time PCR results were analyzed using the method of $2^{-\triangle\triangle Ct}$.

2.6. Statistical analysis

Values were expressed as mean ± SD. SPSS version 17.0 statistical analysis package (SPSS Inc., Chicago, IL, USA) was used to analyze variance by one-way ANOVA followed by post-hoc test for multiple comparisons with the significant level pre-determined at $p < 0.05$.

3. Results

3.1. The effects of treatment on heart rate, blood pressure, hemoglobin and body weight

Compared to baseline, hypoxia led to an initial decrease in heart rates. In rats subject to hypoxia alone, heart rates normalized by week 3 of treatment. However, in rats subject to hypoxia + HFD, heart rates continued to decrease through week 4 of treatment, suggesting an inability to compensate to hypoxia (Fig. 1A). The systolic and diastolic blood pressures did not differ between groups at baseline or during the course of the study (data not shown). Hemoglobin (HGB) levels increased in both the H and H + HFD groups compared to baseline, consistent with continuous hypoxia at a simulated altitude of 5000 m (Fig. 1B). Body weight increased gradually in rats subjected to hypoxia, while there was no significant weight gain in rats subjected to hypoxia plus a high fat diet (H + HFD group; Fig. 1C).

3.2. Effects of treatment on plasma NOx, SOD, and MDA levels

Compared to baseline, plasma NOx levels increased significantly and then gradually declined in rats exposed to continuous hypoxia, consistent with an adaptive response to hypoxia. In contrast, plasma NOx levels were unchanged in rats exposed to hypoxia and a HFD, suggesting an inability to compensate (Fig. 1D). Plasma levels of SOD were significantly higher in rats exposed to hypoxia. However, in animals subject to hypoxia and a HFD, SOD levels normalized at 4 weeks (Fig. 1E). Similarly, hypoxia alone or hypoxia plus a HFD resulted in significantly higher plasma MDA content by 2 weeks. However, in rats exposed to hypoxia + HFD, by 4 weeks into treatment, MDA levels were significantly lower than MDA levels in hypoxia alone conditions (Fig. 1F).

3.3. The effects of treatment on blood lipids

In rats exposed to continuous hypoxia and a HFD, there were significant increases in both TCH and LDL levels. These changes were evident as early as 2 weeks into treatment. In contrast, in rats exposed to hypoxia alone, TCH and LDL levels remained similar to baseline. HDL levels significantly decreased in rats exposed to either hypoxia alone or hypoxia + HFD, with changes most marked at 4 weeks (Fig. 1G).

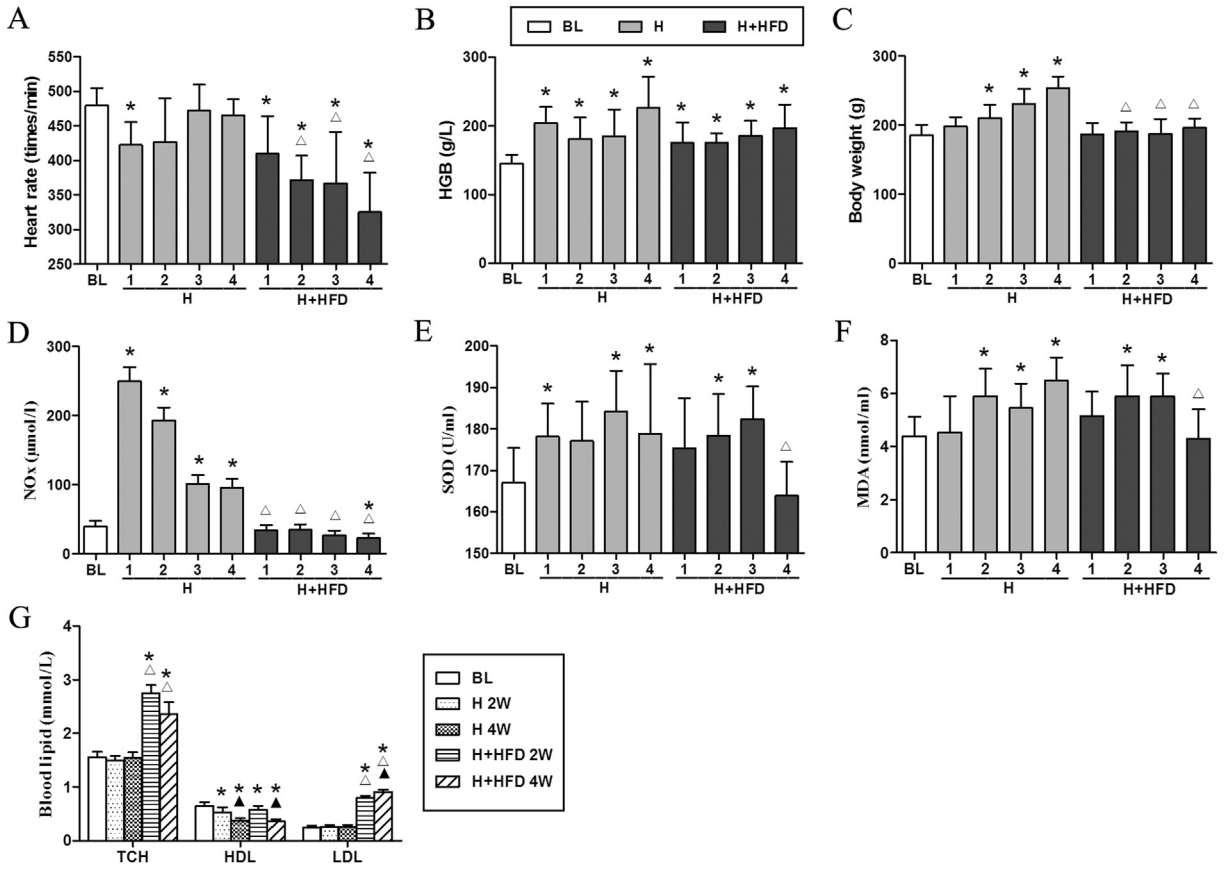

Fig. 1. Changes in heart rate (A), hemoglobin (B), body weight (C), plasma levels of metabolic nitrates and nitrites (NOx) (D), superoxide dismutase (SOD) (E), malondialdehyde (MDA) (F) and blood lipid (G) in rats subject to either continuous hypoxia alone (H) or continuous hypoxia plus a high fat diet (H + HFD). Data are shown at 1, 2, 3, and 4 weeks (blood lipid at 2 and 4 weeks) of treatment. For comparison, data from baseline (BL) group is also shown. Values represent mean ± SD (n = 8.*$P < 0.05$ vs. BL group, △$P < 0.05$ vs. H group of same week, ▲$P < 0.05$ vs. 2w of same group).

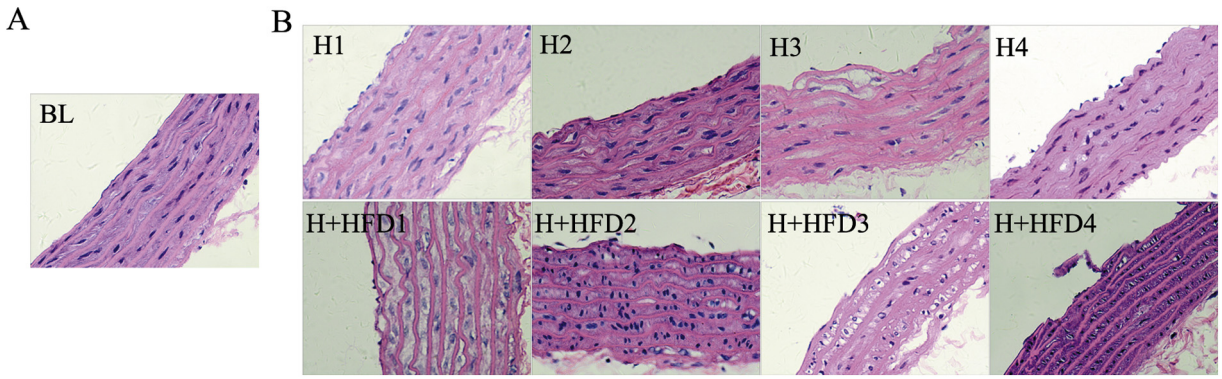

Fig. 2. Light microscopic images of the H&E stained aortic sections of rats subject to either continuous hypoxia alone (H) or continuous hypoxia plus a high fat diet (H + HFD) at weeks 1, 2, 3, and 4 weeks of treatment. For comparison, an image from baseline (BL) group is also shown. (magnification, ×400).

3.4. Histological changes of aortas

In the baseline group, the aorta intima layer was smooth and intact with the continuous arrangement of endothelial cells (EC) and the smooth muscle cells (SMC) were arranged in the normal structure and form in tunica media. In rats exposed to hypoxia only, aortas were relatively normal at week 1 and at week 2 began to show mild EC swelling and hyperplasia, SMC proliferation, and cytoplasmic loosening. These changes became more severe by 4 weeks of treatment with intima layer desquamation from the vascular wall, rounded EC nuclei, and disarranged SMCs with oval nuclei. In comparison, the aortas of the hypoxia + HFD group exhibited more marked changes than hypoxia alone group with rounded EC nuclei and mild SMC disarrangement. These changes were also apparent earlier during the course of continuous hypoxia exposure. These alterations became progressively more severe phenotypically by week 4 with intima layer desquamation, SMC proliferation, round nuclei, vacuolar degeneration (Fig. 2).

3.5. Relaxation and contraction in aorta

Aortic ring contraction to Phe was similar between all groups at 1 week. In the rats exposed to hypoxia alone, aortic ring contraction

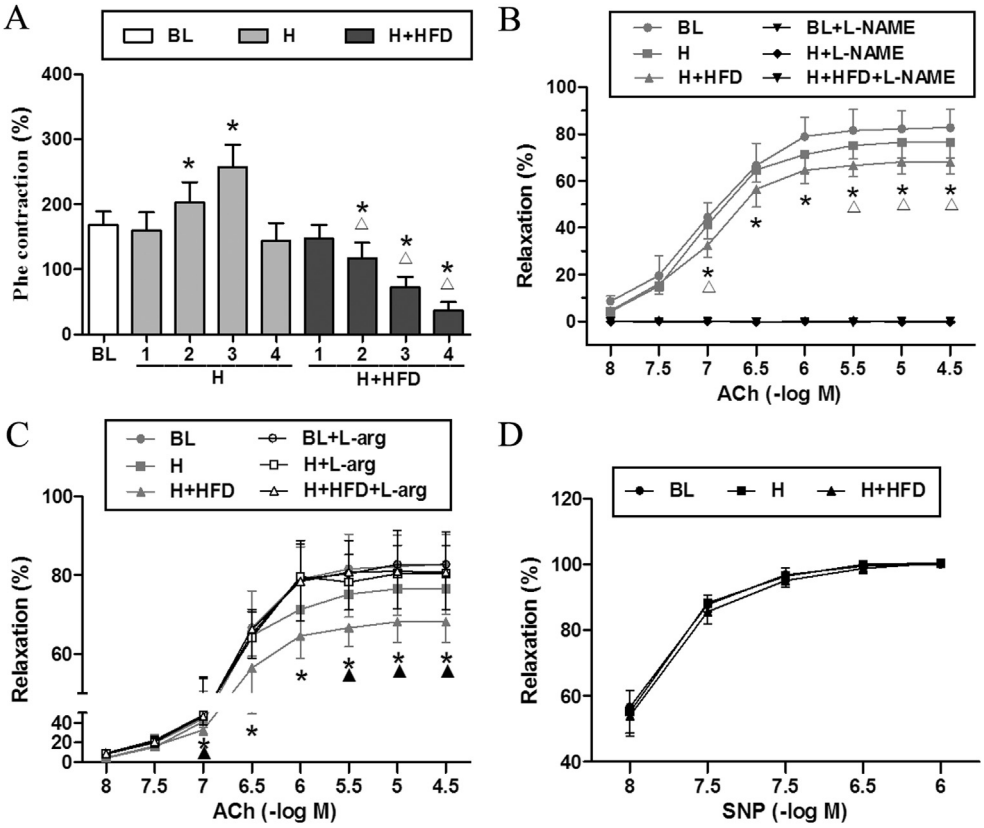

Fig. 3. The effects of L-NAME and L-arg on endothelium-dependent relaxations and Phe on contraction in aortic rings from rats of baseline (BL) group, hypoxia (H) group and hypoxia plus a high fat diet (H + HFD) group. (A) Assessment of aortic ring contraction response to Phe (10^{-6} M) at 1, 2, 3, and 4 weeks of treatment (n = 6 animals/group). (B) Endothelium-dependent relaxation response of aortic rings to ACh (10^{-8}–$10^{-4.5}$M) with or without the NOS inhibitor L-NAME (5×10^{-6} M). (C) Endothelium-dependent relaxation response to ACh of aortic rings with L-arginie (L-arg, 10^{-3} M). (D) Endothelium-independent relaxation response to SNP of aortic rings (10^{-8}–$10^{-4.5}$M). Aortic rings of (B, C and D) were from baseline group rats or rats treated with either continuous hypoxia alone or hypoxia plus an HFD for 1 week (n = 6 animals/group). Values represent mean ± SD (*$P < 0.05$ vs. BL group, △$P < 0.05$ vs. H group of same week or same concentration of ACh, ▲$P < 0.05$ vs. H + HFD + L-arg).

increased during weeks 2–3 and then were similar to baseline at week 4, suggesting adaptive changes had occurred in response to hypoxia. In contrast, in rats exposed to hypoxia + HFD, there was a steady decline in aortic ring contraction through week 4 (Fig. 3A). Similarly, aortic rings from the hypoxia + HFD group demonstrated impaired endothelium-dependent vasorelaxation following ACh stimulation. ACh-mediated vasorelaxation was fully inhibited from all groups when pre-incubated with the NOS inhibitor L-NAME (Fig. 3B), consistent with our hypothesis that ACh-stimulated vasorelaxation was NO dependent. Impairments in endothelium-dependent vasorelaxation were rescued when we pre-incubated the aortic rings with the NOS substrate L-arginie (Fig. 3C). Endothelium-independent relaxation in response to SNP, an NO donor, was similar in all groups (Fig. 3D).

3.6. eNOS mRNA and protein, iNOS mRNA

Adaptive increases in eNOS were significantly blunted during the first two weeks of treatment in rats exposed to continuous hypoxia and a HFD compared to rats exposed to hypoxia alone (Fig. 4A, B, and D). Transcript expression levels of iNOS were similar among hypoxia alone and hypoxia + HFD groups during the first 3 weeks of treatment, but by 4 weeks, the expression of iNOS in aortic tissues was significantly higher in hypoxic rats given a HFD than hypoxic rats only (Fig. 4C).

4. Discussion

The objective of this study was to investigate the effects of a high fat diet on vascular endothelial function in rats exposed to continuous hypobaric hypoxia for up to 4 weeks. We discovered that aortic endothelial structures were more markedly disrupted and in an earlier fashion in hypoxia + HFD rats, compared to rats subject to hypoxia alone (Fig. 2). Consistent with these histological changes, hypoxia in combination with a HFD impaired endothelium-dependent vasorelaxation response to ACh (Fig. 3). Finally, these changes in VED were associated with dysregulation of the NO pathway. Specifically, hypoxia plus a HFD prevented adaptive increases in plasma NOx levels, blunted the increase of eNOS mRNA and protein expression, and altered the bioavailability of NOS substrate L-Arg in hypoxia exposed rats at early stage (1 week).

Taken together, our findings demonstrate that in the setting of hypoxia, a high fat diet leads to earlier and more severe VED than hypoxia alone. ACh-induced, endothelium-dependent vasorelaxation is mediated by endothelium-derived relaxing factors including NO, prostacyclin and endothelium-derived hyperpolarizing factor [15,16]. The relative contribution of these factors to vasorelaxation varies by species, organs and vessels. In our study, ACh-induced relaxation was completely suppressed by the NOS inhibitor L-NAME. Our findings are consistent with those of Balarini et al. [17] and is consistent with the supposition that ACh-mediated vasorelaxation of the aorta in rats is dominated by NO.

NO exerts protective effect on adaptation to hypobaric hypoxia. By increasing blood flow and oxygen delivery, high levels of plasma NOx are adaptive to hypoxia in Tibetan highlanders [18] and hypoxia-exposed lowlanders [19]. Similar adaptive responses are seen in rats under experimental conditions of hypoxia [20]. An inability to compensatorily increase NO levels could increase the risk of maladaptive responses to hypoxia, including the life threatening condition of high altitude pulmonary edema.

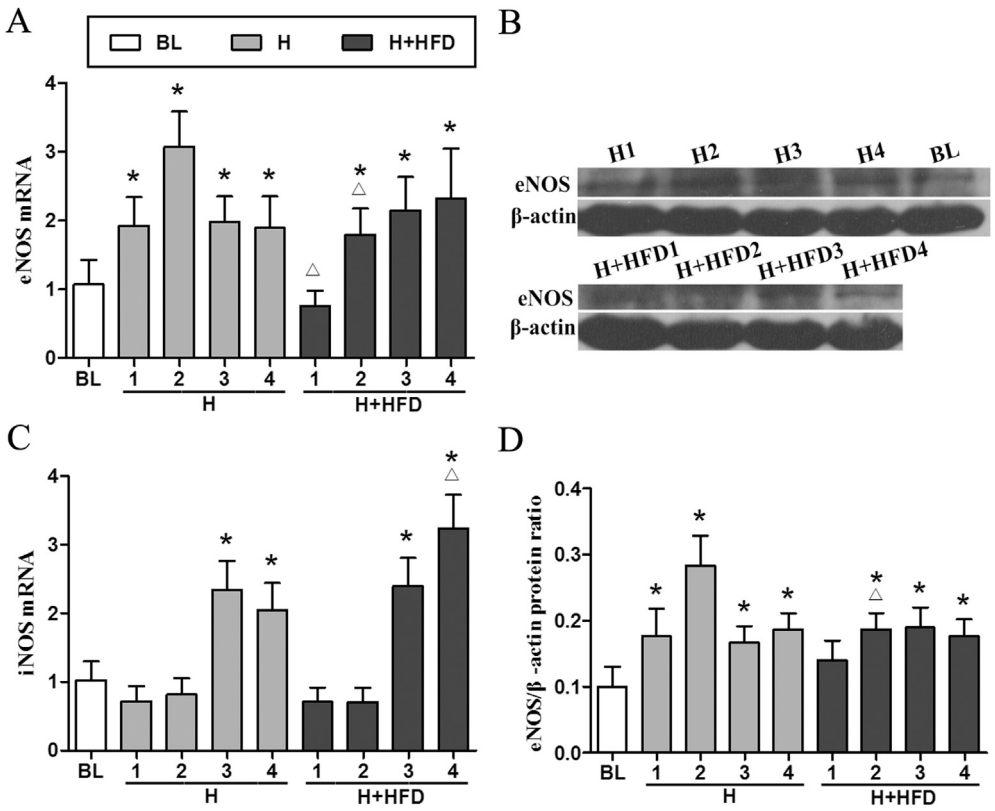

Fig. 4. Expression levels of aortic eNOS mRNA (A) and protein (B, D) and iNOS mRNA (C) in rats subject to either continuous hypoxia alone (H) or continuous hypoxia plus a high fat diet (H + HFD). Data are shown at weeks 1, 2, 3, and 4 weeks of treatment. For comparison, data from baseline (BL) group is also shown. Values represent mean ± SD (n = 5 animals/group. $^{*}P < 0.05$ vs. BL group, $\triangle P < 0.05$ vs. H group of the same week).

We also found that pre-incubating aortic rings with L-Arg, a NOS substrate, improved ACh induced vasorelaxation in H + HFD rats and abolished the differences between hypoxia + HFD, hypoxia only, and baseline. This strongly suggests that the dysfunction of NO-mediated relaxation of aortic rings in hypoxia + HFD rats is due to impairment in L-Arg bioavailability. Also, impairment of L-Arg bioavailability may be responsible for blunted NO responses [21].

Our studies also identify changes in aortic expression of eNOS and iNOS as potential contributors to the impaired NOx production and associated dysfunctional responses in aortic relaxation (Fig. 3). eNOS expressed in endothelial cells is the major source of endothelial NO [22]. Inducible NOS (iNOS) can be induced to express and generate NO in many cell types by stimulated agents [1]. In the present study, compensatory rises in aortic eNOS expression (both mRNA and protein levels) were blunted during the first two weeks of exposure to hypoxia + a HFD compared to hypoxia alone (Fig. 4A, B and D). However, a similar iNOS mRNA expression was showed in three group rats at the same time points (Fig. 4C). These early, impaired responses in eNOS may have contributed to the reduced levels of NOx (Fig. 1D) and dysfunctional aortic relaxation (Fig. 3B) in hypoxic rats given a HFD. Taken together, these data suggest that in the setting of hypoxia, a high fat diet may prevent adaptive physiologic changes necessary for vasodilation.

Previous studies have demonstrated that hypoxia and hypercholesterolemia induce the generation of ROS, thereby decreasing the generation and bioavailability of NO [8,22]. We found that hypoxia increased levels of the oxide metabolite MDA but did not decrease the activity of antioxidant enzyme SOD. As the combination of a high fat diet and hypoxia did not lead to significantly higher MDA levels, oxidative stress does not appear to be the primary reason for the impaired NO responses observed in rats subject to hypoxia plus a HFD.

Elevated plasma TCH and LDL levels may also have contributed to the impaired NO levels observed in hypoxia + HFD rats. Previous studies have shown that oxidized low density lipoproteins impair the dissociation of eNOS from caveolae and the subsequent activation of eNOS [23]. Moreover, hypercholesteremia may induce eNOS uncoupling, resulting in impaired NO generation and VED [24]. The precise mechanism underlying dyslipidemia's effect on eNOS and NO in hypoxic conditions remains unclear and is deserving of further investigation.

The overall effects of hypoxia on hemodynamic responses include an increased cardiac output and decreased systemic vascular resistance [25]. Based on our findings, we hypothesize that a high fat diet might interfere with these adaptive physiologic responses in several ways. The reductions in heart rate and aortic compliance in rats exposed to hypoxia and a HFD may have prevented or blunted the modulation of an increased cardiac output during hypoxia. In addition, as plasma NOx levels were lower in rats exposed to hypoxia and a HFD, compared to hypoxia alone, and NOx is an important mediator of vascular resistance in hypoxic conditions, decreased NOx levels may have prevented adaptive vascular responses. Therefore, high fat diet may disturb the adaptation to hypoxia by absence of elevated NO, which is associated with high levels of blood flow and oxygen delivery [26].

In brief, a high fat diet results in more marked vascular endothelial dysfunction in settings of continuous hypoxia, compared to exposure to hypoxia alone. The VED was associated with alterations in the NO pathway. Our findings support the supposition that high fat diet may be a risk factor for VED in settings of hypoxia. Given changes in the dietary patterns of populations residing at high-altitude, our findings have implications in clinical settings. Future studies should explore the effects and mechanisms of combined high fat diet and hypoxia on vascular endothelial function in humans.

Acknowledgments

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