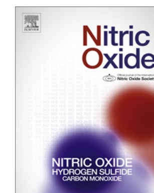
Nitric Oxide 38 (2014) 38–44

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox



Inhibition of inducible nitric oxide synthase expression and nitric oxide production in plateau pika (*Ochotona curzoniae*) at high altitude on Qinghai-Tibet Plateau

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A R T I C L E I N F O

Article history:
Received 11 August 2013
Revised 19 February 2014
Available online 12 March 2014

Keywords:
Inducible nitric oxide synthase
Endothelial nitric oxide synthase
Nitric oxide
Plateau pika
Qinghai-Tibet Plateau

A B S T R A C T

Nitric oxide (NO), a potent vasodilator, plays an important role in preventing hypoxia induced pulmonary hypertension. Endogenous NO is synthesized by nitric oxide synthases (NOSs) from L-arginine. In mammals, three different NOSs have been identified, including neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Plateau pika (*Ochotona curzoniae*) is a typical hypoxia tolerant mammal that lives at 3000–5000 m above sea level on the Qinghai-Tibet Plateau. The aim of this study was to investigate whether NOS expression and NO production are regulated by chronic hypoxia in plateau pika. Quantitative real-time PCR and western blot analyses were conducted to quantify relative abundances of iNOS and eNOS transcripts and proteins in the lung tissues of plateau pikas at different altitudes (4550, 3950 and 3200 m). Plasma NO metabolites, nitrite/nitrate (NO_x^-) levels were also examined by Ion chromatography to determine the correlation between NO production and altitude level. The results revealed that iNOS transcript levels were significantly lower in animals at high altitudes (decreased by 53% and 57% at altitude of 3950 and 4550 m compared with that at 3200 m). Similar trends in iNOS protein abundances were observed (26% and 41% at 3950 and 4550 m comparing with at 3200 m). There were no significant differences in eNOS mRNA and protein levels in the pika lungs among different altitudes. The plasma NO_x^- levels of the plateau pikas at high altitudes significantly decreased (1.65 ± 0.19 μg/mL at 3200 m to 0.44 ± 0.03 μg/mL at 3950 m and 0.24 ± 0.01 μg/mL at 4550 m). This is the first evidence describing the effects of chronic hypoxia on NOS expression and NO levels in the plateau pika in high altitude adaptation. We conclude that iNOS expression and NO production are suppressed at high altitudes, and the lower NO concentration at high altitudes may serve crucial roles for helping the plateau pika to survive at hypoxic environment.

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1. Introduction

Exposure to high altitudes is associated with reduced oxygen tension. At high altitude, the oxygen partial pressure (PO_2) gradient extends from the atmosphere to the tissues and reaches the cellular level to impact the basic units of life [1,2]. Low PO_2 can lead to oxidative damage and disruption of the efficiency of the antioxidant system [3]. It is well known that healthy low altitude mammals exposed to high altitude often develop pulmonary hypertension with increased muscularization of pulmonary arterioles, vascular resistance and vasoconstriction [4–7]. Previous studies have shown that the native species to high altitude, such as yaks [8–10,11], snow pigs [6], and llamas [12], maintain a slight pulmonary arterial pressure compared with low altitude animals exposed to high altitude.

Hypoxia directly affects cell function by regulating the expression of hypoxia responsive genes in mammals. Hypoxia can induces gene expression by controlling the activities of transcription factors hypoxia inducible factor 1α (HIF1α) and HIF2α. HIF1α and HIF2α bind to promoters of several genes that play essential roles in

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http://dx.doi.org/10.1016/j.niox.2014.02.009
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oxygen metabolism and angiogenesis such as erythropoietin (EPO) [13], vascular endothelial growth factor (VEGF) [14,15]. Recent studies have indicated that hypoxia also controls nitric oxide (NO) production through HIF1α [16,17] and HIF2α [18] dependent mechanisms. NO is an endothelium derived vasodilator that possesses the ability to mediate hypoxia response by eliciting blood vessel relaxation [19–21]. Endogenous NO is synthesized by nitric oxide synthases (NOSs) from L-arginine in a reaction that requires oxygen, NADPH, and the cofactors FAD, FMN, and tetrahydrobiopterin [22–24]. At least three isoforms of NOS have been isolated and cloned, including neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) [23,24]. Each NOS gene is located on different chromosome and can be accessed by different transcription factors. It has been demonstrated that HIF1α directly regulates iNOS expression [16,17] while HIF2α controls the transcription of eNOS [18].

The activities of NOSs are controlled by different extrinsic and intrinsic cues. eNOS and nNOS isoforms, designated as constitutive NOSs, are activated by calcium and calmodulin (CaM) [22]. These two NOSs are constantly present in resting cells. However, iNOS is independent of calcium and CaM, instead, its expression level increases dramatically in response to inflammatory or pro-inflammatory mediators, including IL-1, IL-6, TNF-α, NF-κB and LPS [22,23]. iNOS also responds to prolonged hypoxia in a HIF1α dependent manner. Under hypoxic condition, HIF1α binds to the hypoxia responsive element (HRE) in the iNOS promoter and regulates its transcription [25,26].

Plateau pika (*Ochotona curzoniae*) is a small non-hibernating rodent mammal that is native to Qinghai-Tibet Plateau. This species has been adapted to high altitude for more than 37 million years as indicated by fossil samples found the north edge of the Qinghai-Tibet Plateau [27]. Cold climate and hypoxia are two most important ecological factors determine the distribution and survival of high altitude animals including plateau pika. The plateau pika has developed its own mechanisms to resist hypoxic environmental stress during their evolution. They can survive extreme hypoxia environment by increasing the efficiency of oxygen utilization, decreasing hypoxic pulmonary vascular response and reducing hemoglobin (Hb) and Hemotocrit (Hct) concentrations [28–30]. Anatomically, the lungs of these animals contain thin-walled pulmonary arteries, which are crucial for the blunted hypoxic pulmonary vasoconstriction response [29].

The majority of studies in the past were conducted to examine mechanisms underlying hypoxia tolerance in plateau pika at the physiological level. Recently, researchers have begun to decipher this puzzle with molecular biology tools. The hemoglobin α (HbA) and β (HbB) chains from the plateau pika have been cloned and it suggests that the genetic diversity in pika HbA may be one of the key factors responsible for the hypoxia resistance [31]. The HIF1α gene has been cloned from the plateau pika [32], and HIF1α protein increases in lungs of pikas at high altitude compared with that of low altitude counterparts [33]. Despite these findings, the effect of chronic hypoxia on NOSs expression and NO production in natural plateau pika has not been determined. The objective of the present study was to detect the expression of iNOS and eNOS in the lung tissues and plasma NO concentrations of plateau pikas that lived at different altitudes to better understand the roles of NO signal in the adaptation of plateau pikas to chronic hypoxia.

2. Materials and methods

2.1. Plateau pika

Healthy plateau pikas were captured near the Haibei Alpine Meadow Research Station, Chinese Academy of Sciences (HB, altitude of 3200 m), Dawu town, Guoluo (DW, altitude of 3950 m) and Huashixia town, Guoluo (HSX, altitude of 4550 m) of the Qinghai province, China. Adult plateau pikas, weighing 150–180 g, were used in this study. Animals were lightly anesthetized with chloral hydrate (3 mg/kg) by intraperitoneal injection and sacrificed at the spot. Dissected lung tissues were snap frozen in liquid nitrogen and stored at −80 °C until use. The blood samples were collected from abdominal venous, then the heparinized blood samples were immediately spun at 4000 rpm for 5 min and plasma removed and frozen for NO measurements. All animal studies were in accordance with China's Practices for the Care and Use of Laboratory Animals and were approved by the Chinese Zoological Society.

2.2. RNA isolation and the first strand cDNA synthesis

Total RNA was extracted from the frozen lung tissues using RNAVzol (Vigorous Biotechnology, Beijing, China) according to the manufacturer's instruction. RNA samples were treated with RNase-free DNase (TaKaRa Biotechnology Co., Ltd., Dalian, China) to remove genome DNA. Samples with $A_{260}/A_{280} \geqslant 1.8$ were used. Reverse transcription for the first strand cDNA was performed in a volume of 20 μL containing 4 μg of total RNA, 200 units of M-MLV reverse transcriptase, 1 mM of dNTP Mix, 5 μM of oligo$(dT)_{18}$ primer, 1× reaction buffer and 20 units of RNase inhibitor (Fermentas, Opelstr. Germany). The reaction was carried out at 65 °C for 5 min, 42 °C for 60 min and 70 °C for 10 min. The cDNA samples were diluted (1:2) and stored in −20 °C.

2.3. Quantitative real-time PCR

PCR amplification was performed by using SYBR® Premix Ex Taq™ (Perfect Real Time) (TaKaRa Biotechnology Co., Ltd., Dalian, China) in a final reaction volume of 20 μL. Each reaction contained 1.5 μL of diluted cDNA sample. The amplification was operated for 40 cycles at the following conditions: 95 °C for 5 s (denaturation) and 59.5 °C for 30 s (annealing). Each sample was tested in triplicate. Gene quantification was carried out using the BIO-RAD iCycler iQ® Muticolor Real-Time PCR detection system (BIO-RAD). Primer pairs for the real-time PCR were designed based on the plateau pika sequences of iNOS cDNA (Accession No. KC707553) and eNOS cDNA sequence (Accession No. KC707554) deposited in GenBank (Table 1). To compare among groups, mRNA levels of target genes were measured as relative expression using $2^{-\triangle\triangle CT}$ values and normalized to β-Actin generated from the same sample [33]. The mRNA levels of iNOS and eNOS in pika lungs were normalized to the results obtained from tissues collected at altitude of 3200 m, respectively. Normalized iNOS and eNOS expressions were calculated by

$$T_{i/e} = 2^{-\Delta\Delta C_T}$$

where $\Delta\Delta C_T = (C_{T1} - C_{T1\beta}) - (C_{T0} - C_{T0\beta})$. $C_{T0\beta}$: C_T for β-Actin in animals at 3200 m; $C_{T1\beta}$: C_T for β-Actin in animals at a different altitude; C_{T0}: C_T for target gene iNOS or eNOS in animals at altitude

Table 1
Primers used for real-time PCR.

Primers name	Primer sequence (5′–3′)	Product size (≈bp)
iNOS-qRT-PCR-F	CAACCTGCACGCCAAGAAC	
iNOS-qRT-PCR-R	TCCACAACTCGCTCCAAGATG	204
eNOS-qRT-PCR-F	GTGGCCAATGCCGTGAAGAT	
eNOS-qRT-PCR-R	GCACAGGACCCGGGGATCAA	162
β-actin-qRT-PCR-F	CACGGGTATCGTGATGGACT	
β-actin-qRT-PCR-R	CAAGAAGGAGGGCTGGAAGA	340

Abbreviations: qRT-PCR, quantitative reverse transcription-polymerase chain reaction; F, forward; R, reverse.

of 3200 m; C_{T1}: C_T for target gene iNOS or eNOS in animals at a different altitude.

2.4. Western blot

Frozen tissues were homogenized and lysed for about 1 h at 4 °C in the lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 and 25 mM sodium pyrophosphate) in the presence of protease inhibitors. The tissue lysates were centrifuged at 4 °C for 20 min (16,000*g*) and the supernatants containing proteins were collected as described previously [33]. The protein concentration was determined using the Bradford Protein Assay Kit (Beyotime Biotechnology, Beijing, China). 90 μg of total protein was separated by electrophoresis in a 10% SDS–PAGE and transferred to the PVDF membrane in glycine–methanol buffer. The membrane was blocked in 5% non-fat dry milk in Tris-tween 20-buffer saline (TBST) at room temperature for 1 h. The membrane was then incubated in the primary monoclonal anti-human iNOS antibody (1.5 μg/mL, R&D Biotechnology), primary anti-human eNOS biotinylated affinity purified PAb antibody (0.15 μg/mL, R&D Biotechnology), or anti-β-Actin [HRP] monoclonal antibody (Mouse) (0.1 μg/mL, Genscript) for 1 h at room temperature. After incubation, the membrane was probed with a secondary HRP-conjugated goat anti-mouse IgG polyclonal antibody (1:5000, GeneTex Biotechnology) or steptavidin-HRP (1:10000, GeneTex Biotechnology) for 1 h at room temperature. The membrane was washed 3 times in TBST and the immunoreactive signal was detected by the substrate luminescent reaction using an ECL Kit according to the manufacturer's instruction (BeyoECL Plus Kit, Beyotime, Beijing, China). Protein bands were quantified using densitometry Image J software. Each sample isolated from individual animal was tested in triplicate. The β-Actin was used as an internal standard. Normalization was carried out by dividing the average gray value of target protein, iNOS or eNOS by the levels of β-Actin in each sample. The protein expression levels of iNOS and eNOS in animals at 3950 and 4550 m were then normalized to these of 3200 m, respectively.

2.5. NO measurement

Nitrite and nitrate are stable chemical degradation products of NO and have been used as indices of NO production [34]. Plasma nitrite/nitrate (NO_x^-) were measured by Ion chromatography. Briefly, the plasma samples were thawed and filtered through Ag/Na columns to remove chloride ion. Samples were then diluted in deionized water (1:3 v/v) and measured using ICS-1500 (U.S.A.) with the following conditions at 35 °C: flow rate of 1.2 mL/min, 3.5 mM Na_2CO_3/1.0 mM $NaHCO_3$ of mobile phase and nitrate for 6.13 min as well as nitrite for 4.15 min of the peak time.

2.6. Statistical analysis

The results were expressed as means ± SEM. Statistical analysis was performed using analysis of variance (ANOVA). The probability of $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered highly significant.

3. Results

3.1. Quantifications of iNOS and eNOS mRNA levels in the lung tissues of plateau pikas at different altitudes

We tested relative expression levels of iNOS and eNOS mRNA in the lung tissues of plateau pikas captured at different altitudes by real-time PCR. The iNOS mRNA levels in the 3950 and 4550 m animals were highly significantly decreased by 53% and 57% ($P < 0.01$), compared with that in the 3200 m ones (Fig. 1A and Table 2). The relative abundance of iNOS transcript did not differ between 3950 and 4550 m animals. The eNOS mRNA levels in 3950 and 4550 m animals were slightly lower than that of animals lived at 3200 m, but the differences were not statistically significant (Fig. 1B and Table 2). These results show that chronic hypoxia inhibits iNOS mRNA expression in the plateau pika and has no effect on eNOS mRNA expression.

3.2. Quantifications of iNOS and eNOS protein levels in the lung tissues of plateau pikas at different altitudes

Expression levels of iNOS and eNOS proteins in the lung tissues of these animals were determined by Western blot. The iNOS protein levels in the 3950 and 4550 m animals were highly significantly lower than that in 3200 m counterparts, which were decreased by 26% and 41% ($P < 0.01$), respectively. Moreover, the iNOS protein level at 4550 m was significantly less than that at 3950 m ($P < 0.05$) (Fig. 2A and Table 2). However, there were no significant differences in the eNOS protein expression in plateau pika lungs among different altitudes (Fig. 2B and Table 2). These results suggest that iNOS not eNOS protein level is suppressed in plateau pika that lives at high altitudes.

3.3. NO measurement

We measured plasma nitrite and nitrate concentration to analyze NO production. Because the half-life of NO is very short, with

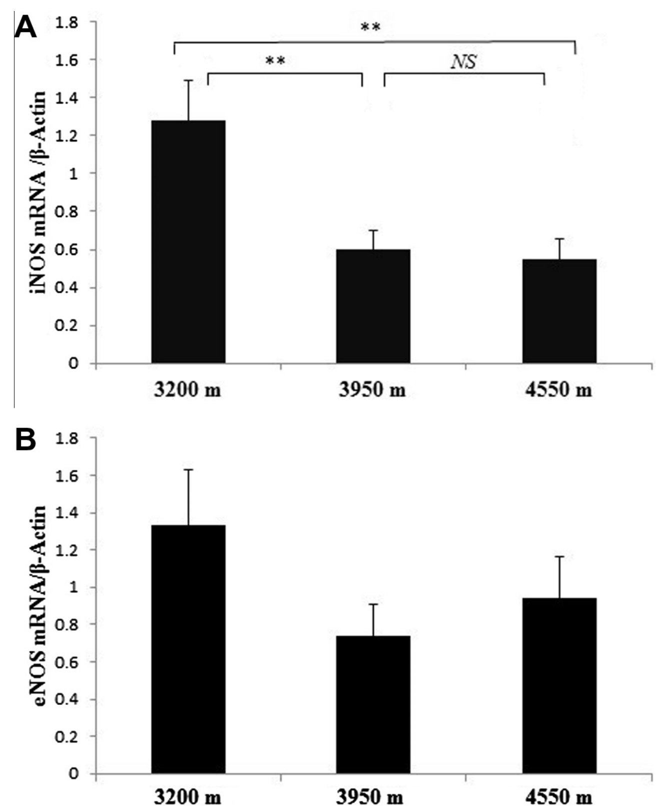

Fig. 1. Quantifications of iNOS and eNOS mRNA in the lung tissues of plateau pika at different altitudes (HB, 3200 m; DW, 3950 m; HSX, 4550 m). Panel A: iNOS mRNA level of plateau pika lungs in different altitudes. Panel B: eNOS mRNA level of plateau pika lungs in different altitudes. Expression levels were normalized to β-Actin mRNA levels. Graphs represent relative iNOS and eNOS mRNA levels. Value represents as means ± SEM ($n = 9$). Asterisks indicate significant differences (**$P < 0.01$), and *NS* means no significant differences.

Table 2
The measurements of samples collected from 3200, 3950 and 4550 m above sea level (the data show at means ± SEM).

Contents		3200 m	3950 m	4550 m
iNOS	RT-PCR	1.28 ± 0.22[a]	0.60 ± 0.10[b]	0.55 ± 0.10[b]
	WB	0.81 ± 0.02[a]	0.60 ± 0.04[b]	0.48 ± 0.03[c]
eNOS	RT-PCR	1.33 ± 0.30	0.74 ± 0.17	0.94 ± 0.22
	WB	0.68 ± 0.02	0.69 ± 0.02	0.67 ± 0.02
Plasma nitrite/nitrate (μg/mL)		1.65 ± 0.19[a]	0.44 ± 0.03[b]	0.24 ± 0.01[b]

Note: The abbreviations are WB for Western blot of the iNOS protein and eNOS protein contents, RT-PCR for iNOS mRNA and eNOS mRNA amplified by real-time reverse transcriptase-polymerase chain reaction. Means with different superscript letters are significantly different at the 5% significant level within different altitudes.

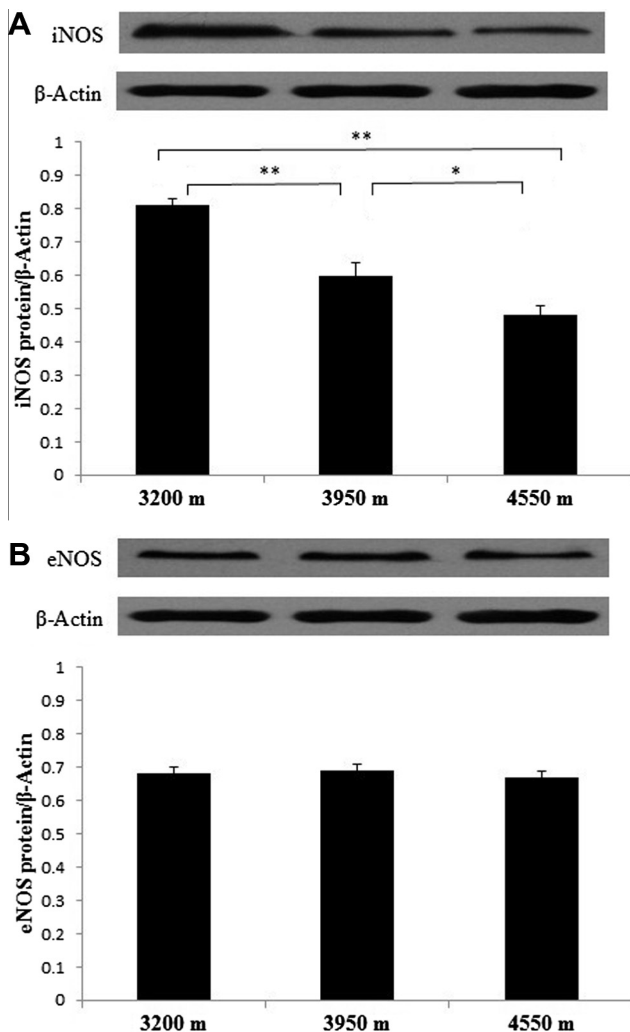

Fig. 2. Quantifications of iNOS and eNOS protein in the lung tissues of plateau pika at different altitudes (HB, 3200 m; DW, 3950 m; HSX, 4550 m). Panel A: iNOS protein level of plateau pika lungs in different altitudes. Panel B: eNOS protein level of plateau pika lungs in different altitudes. iNOS and eNOS protein content are expressed as relative levels and quantified using Image J software. Expression levels were normalized to β-Actin protein levels. Graphs represent relative iNOS and eNOS protein levels. Value represents as means ± SEM ($n = 9$). Asterisks indicate significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$).

the presence of nitrite, nitrate or other stable metabolites indicates the level of NO in organisms. Consistent with reduced iNOS mRNA and protein at high altitudes, plasma NO_x^- concentration was highly significantly lower in high altitude pikas. The plasma NO_x^- level was 1.65 ± 0.19 μg/mL at 3200 m, 0.44 ± 0.03 μg/mL at 3950 m and 0.24 ± 0.01 μg/mL at 4550 m, in other words, the NO_x^- levels were significantly decreased by 73% at 3950 m and 83% at 4550 m, compared with that at 3200 m ($P < 0.01$) (Fig. 3 and Table 2). The decreased NO may be caused by hypoxia dependent inhibition of iNOS expression in the plateau pika.

4. Discussion

This study was conducted to investigate the roles of NOS and NO in the adaptation of plateau pika to chronic hypoxia on the Qinghai-Tibet plateau. The relative abundances of iNOS and eNOS transcripts were examined in the lung tissues of plateau pika that lived at different altitudes. Protein levels of these two enzymes were also compared. Finally, the plasma nitrite/nitrate levels of these animals were measured to determine the association between NO production and altitude levels. The transcript and protein levels of iNOS were significantly reduced in animals lived at high altitudes. The plasma NO_x^- levels of plateau pika in high altitudes were lower than that of animals in low altitude. Endogenous NO is mainly synthesized by NOSs, thus we conclude that the decreased NO is possibly caused by suppression of iNOS expression at the transcript and protein levels. The findings contradict our hypothesis that chronic hypoxia will induce iNOS and NO in plateau pika.

It has been known for years that iNOS is a hypoxia-inducible gene that contains a HRE in its promoter region [16]. Compelling evidences have indicated that hypoxia upregulates iNOS-derived NO production [35,36]. Warner and Arstall [37] showed that hypoxia increased iNOS expression in cardiac myocytes and this response requires the HRE and NF-κB binding sites of iNOS promoter. Chronic hypoxia induces iNOS expression at the transcriptional and translational level and enhances NO production in fetal guinea pig heart [38]. The link between hypoxia and iNOS expression is mediated by HIF1α in rat cardiac myocytes [39]. In sharp contrast to these findings, we found iNOS expression and plasma nitrite/nitrate production were reduced in the lung tissues of plateau pika that lived at high altitudes. A possible explanation is that hypoxia alone is not responsible for the decreased iNOS expression and NO production. Regulation of iNOS transcription and NO synthesis by hypoxia is a tissue specific phenomenon that requires different mechanisms in different target cells. Melillo et al. [16] showed that hypoxia alone did not induce iNOS expression in isolated murine macrophages, however, when these cells were treated with IFN-γ and then exposed to hypoxic conditions, they showed higher levels of iNOS expression. Hong et al. [40] found

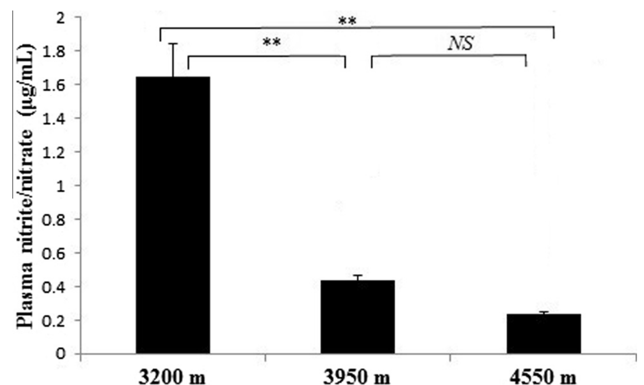

Fig. 3. Plasma nitrite/nitrate (μg/mL) measurements in plateau pika of different habitat altitudes, HB (3200 m) ($n = 24$), DW (3950 m) ($n = 19$), and HSX (4550 m) ($n = 16$). Value represents as means ± SEM. Asterisks indicates significant differences ($^{**}P < 0.01$), and *NS* means no significant differences.

that hypoxia inhibited the NO production without up-regulating the transcription of iNOS in rat smooth muscle cells treated with IFN-γ, LPS, or both. Interestingly, exposing cultured rat hepatocytes to hypoxia reduces iNOS expression and the HRE in iNOS promoter is not involved in this process [41]. Pulmonary microvascular endothelial cells (EC) pretreated with IL-1β/TNF-α response to hypoxia exposure, however, in these cells, hypoxia inhibits iNOS expression and its enzyme activity [42]. In summary, the effects of hypoxia on iNOS and NO are species, cell types, and stimulators-dependent. Immigrant Hans are able to undergo similar HIF1α-mediated molecular acclimatization to hypoxia as native Tibetans, because hypoxia induces the mRNA and protein expressions of HIF1α and iNOS in both immigrant Hans and Tibetans [43]. This data suggest that the HIF1α-regulated gene expression may be not a mechanism for assisting the better adaptation of native Tibetan to high altitudes.

It is interesting that the arterial partial oxygen tension (PaO_2) in the plateau pika at high altitude is increased rather than reduced under lower PO_2. Exposing the plateau pikas to hypobaric pressure chambers, Li and Du found that the PaO_2 in the plateau pika blood at 2300 m was 71.9 mmHg but remarkably increased to 93.7 mmHg at 5000 m [44]. This result exhibited a different trend from other mammals, such as rats [45,46], native Tibetans as well as immigrants [47], and Tibetan sheep [48], in which the PaO_2 were reduced under low atmosphere oxygen tension. Because the PaO_2 increased in the plateau pika blood along the climbing altitude, it is reasonable to speculate that iNOS expression is inhibited by environment hypoxia but induced by tissues hypoxia. Consideration for the potential effects of genetic background on the iNOS expression, we estimated the genetic differentiation of the three plateau pika populations from HB, DW and HSX by ARLEQUIN. Analysis of molecular variance (AMOVA) based on Cyt *b* indicated that there were genetic differentiations between plateau pikas from HB and DW, as well as between HB and HSX populations (both the F_{st} P values were 0.0000), but no differentiation was detected between DW and HSX populations (F_{st} P value was 0.10346). However, Global test of eNOS variation among the three populations using ARLEQUIN indicated that these populations were genetics similar (exact P value was 0.12382 ± 0.0066, 0.13410 ± 0.0039, 1.00000 between DW and HB, HSX and HB, as well as DW and HSX populations, respectively). The male-biased dispersal in the plateau pikas [49,50] and a maternal inheritance of mitochondrial DNA [51] may serve to intensify the level of genetic differentiation, which accounts for above paradoxical results. Moreover, the iNOS protein level of HSX is significantly lower than that of DW, which suggests that the down-regulation of iNOS at higher altitude results from low oxygen tension rather than genetic background. HIF1α protein is induced in plateau pika lungs inhabiting high altitudes [33], we suppose that the inhibition of iNOS expression by hypoxia in the lungs of plateau pika at high altitudes is not dependent on HIF1α.

In the study, eNOS expression did not differ in plateau pika at high altitudes and low altitude. Hypoxia up-regulates eNOS in mice with hypoxia-induced pulmonary hypertension [52]. Expression of eNOS increases in the lungs of rats subjected to chronic hypoxia [35]. However, Fike et al. [53] reported that eNOS protein expression and NO concentration decreased in the lungs of newborn pig exposure to chronic hypoxia. The eNOS expression from both Hans and Tibetans is reduced when exposure to high altitude [43]. The eNOS protein level remained unchanged in the pulmonary vascular endothelial and airway epithelial of the plateau pika when exposed to acute hypoxia, but compared with Wistar rats, the basic eNOS level in the pika's airway was significantly higher [54]. Unresponsiveness of eNOS express to chronic hypoxia may be important for protecting the plateau pika from being damaged by low oxygen stress.

Exhibiting a similar trend to the iNOS expression, plasma NO_x^- levels in the pikas at high altitudes were decreased. The hypoxia-dependent reduction in iNOS expression may account for the decreased plasma NO_x^- level in the plateau pikas. However, in addition to blockade of iNOS expression, the NO formation from L-arginine can be impaired by low oxygen, because oxygen is an important substrate of NOS- L-arginine reaction [55–57]. When neonatal pigs were exposed to 10% oxygen tension (hypoxia) for 10–12 days, both plasma NO_x^- and perfusate NO_x^- of lungs were reduced compared with that in the normoxic piglets [53]. In other studies both plasma NO_x^- and perfusate NO_x^- are higher in the chronic hypoxia rats than in normoxic rats [58,59]. It is well known that vast majority of nitrate and some of the nitrite derives from eating plants that are high in nitrates and nitrites. The diet composition and diet-derived nitrate/nitrite level, which has been proved to affect the level of plasma NO_x^- [60,61], may vary between plateau pikas inhabiting different altitudes. Therefore, further studies will help to evaluate the degree of plasma NO_x^- between plateau pikas sampled at three different altitudes affected by diets.

Whether the NO plays an important role in adaptive defense to hypoxia, including preventing hypoxia-associated pulmonary vasoconstriction (HPV) and reducing hypoxia-induced pulmonary pressure is still debatable [62,63]. Suppression of NO production by blocking NOS activity inhibits hypoxic vasoconstriction [64,65]. Other studies, however, have shown that the suppression of NO formation potentiates the lung to hypoxic vasoconstriction [66–68]. iNOS gene transfer reduces hypoxia-induced pulmonary hypertension and vascular remodeling in rats [69]. Hydrogen sulfide (H_2S) synthesized from L-cysteine in various mammalian tissues by several different enzymes, such as cystathionine gamma-lyase (CSE) and cystathionine beta-synthase (CBS) [70], also relaxes vascular smooth muscle cells by opening K_{ATP} [71]. H_2S plays an essential role in the physiological response to low oxygen availability [72,73]. Mice lack of CSE show pronounced hypertension caused by reduced production of H_2S and diminished endothelium-dependent vasorelaxation [74]. The role of NO in preventing hypoxia pulmonary vasoconstriction may be replaced by H_2S. NO/H_2S signaling alone or interaction between NO and H_2S may play an important role in mediating the physiological adaptations to hypoxia [75]. Moreover, iNOS overexpression and NO overproduction by hypoxia can induce cell apoptosis, and inhibition of iNOS protects cells from hypoxia-induced cell injury [76,77]. Thus the down-regulated NO production may be the self-preservation of the plateau pika. The lifetime of NO is significant longer under low oxygen tension [78] and the relaxing effect of NO significantly increases at 28 mmHg oxygen tension compared with it at 480 mmHg [79]. Moreover, higher concentration of NO can inhibit mitochondrial respiration and the inhibition is significantly stronger at physiologically low intracellular oxygen tensions [80]. Taken together, low dose of NO in the plateau pika can perform same functions at high altitude. The relative balance of NO and oxygen tension is very important for maintaining pulmonary arterial functions in the plateau pika.

In summary, there are possible explanations for the inhibition of plateau pika iNOS expression and NO formation at high altitudes as below: (1) most studies that show hypoxia induces iNOS expression are conducted in concomitant cultured cell in the presence of pro-inflammation cytokines rather than exposure to hypoxia alone; (2) the suppression of NO production in the plateau pika is partly dependent on the inhibition of iNOS expression at the mRNA and protein levels; (3) the low PO_2-mediated iNOS-derived NO blockade may be a self-preservation mechanisms for the plateau pika; (4) the vasodilator role of NO in adaptation to hypoxic environment in the plateau pika is possibly replaced by other gasotransmitters, such as H_2S. We suggest that low concentration of NO in the plateau pika at high altitude might be enough for

maintaining adaptation to low oxygen stress and alternative pathways may be responsible for the better adaptation of native plateau pikas. However, the mechanism of the inhibition of iNOS and NO by chronic hypoxia in the plateau pika remains unclear. Further studies using gene knockout or knockdown approaches will be needed to define the role of HIF1α in iNOS expression and NO production under hypoxic conditions in the plateau pika. Functional interactions between H_2S and NO in the lung tissues of plateau pika at different altitudes should be examined to better understand how native species adapt to the harsh environment on Qinghai-Tibet Plateau.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (30800118), the Promoting Innovation Ability Program on Science & Technology in Qinghai province of China (2010-Z-741, 2013-Z-750), the Knowledge Innovation Project of the Chinese Academy of Science (KSCX2-EW-J-26) and the Ministry of Education Spring Bright Plan, China (Z2010077). We are also grateful to Dr. Qi-En Yang for his critical reading of the manuscript and helpful comments.

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