Effects of melatonin on the nitric oxide system and protein nitration in the hypobaric hypoxic rat hippocampus

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Abstract

Background: It is well documented that the nitric oxide (NO) might be directly involved in brain response to hypobaric hypoxia, and could contribute to memory deficiencies. Recent studies have shown that melatonin could attenuate hypoxia or ischemia-induced nerve injuries by decreasing the production of free radicals. The present study, using immunohistochemical and immunoblot methods, aimed to explore whether melatonin treatment may affect the expression of nitric oxide system and protein nitration, and provide neuroprotection in the rat hippocampus injured by hypobaric hypoxia. Prior to hypoxic treatment, adult rats were pretreated with melatonin (100 mg/kg, i.p.) before they were exposed to the altitude chamber with 48 Torr of the partial oxygen concentration (pO2) for 7 h to mimic the ambience of being at 9000 m in height. They were then sacrificed after 0 h, 1, and 3 days of reoxygenation.

Results: The results obtained from the immunohistochemical and immunoblotting analyses showed that the expressions of neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), nitrotyrosine (Ntyr) and Caspase 3 in the hypoxic hippocampus were increased from 0 h to 3 days of reoxygenation. Interestingly, the hypoxia-induced increase of nNOS, eNOS, iNOS, Ntyr and Caspase 3 protein expression was significantly depressed in the hypoxic rats treated with melatonin.

Conclusions: Activation of the nitric oxide system and protein nitration constitutes a hippocampal response to hypobaric hypoxia and administration of melatonin could provide new therapeutic avenues to prevent and/or treat the symptoms produced by hypobaric hypoxia.

Keywords: Hippocampus, Hypobaric hypoxia, Nitric oxide, Protein nitration, Melatonin

Background

Sudden exposure to high altitude (HA) (i.e., rapid ascent without acclimatization, as in mountain climbing) results in the development of hypobaric hypoxia (HBH). Hypobaric hypoxia leads to appearance of neuropsychological disorders and mental dysfunctions such as insomnia, dizziness, and memory deficiencies which are consequences of the decreased partial pressure of oxygen (pO2) available to the central nervous system (CNS) [1, 2]. It has been established that CNS is highly sensitive to hypoxia and that some areas, such as the hippocampus, are especially vulnerable to hypoxic damage [3]. Several studies have determined that hypobaric hypoxia in the CA1 region of the hippocampus provokes metabolic, electrophysiological, and morphological modifications related to neuronal death [4–7]. The obvious cell damage in the hippocampus and learning/memory deficits were evidenced after exposure to HBH [8]. The exact mechanisms of neuronal damage in hypobaric hypoxia remain to be elucidated. Growing evidence showed that nitric oxide (NO) system involved in certain neuronal modifications and could...
contribute to memory deficiencies related to ischemic hypoxia, normobaric and hypobaric hypoxia [9–11]. NO is a short-lived bioactive molecule that participates in the physiology and pathophysiology of various systems in mammals. NO is produced by nitric oxide synthases (NOS) that constitute a family of enzymes that catalyze the oxidation of l-arginine and nicotinamide adenine dinucleotide phosphate by oxygen to yield l-citrulline and NO [12, 13]. Three distinct NOS isoforms have been identified: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). It has been suggested that the formation of NO is directly linked to the glutamate, which can cause post-synaptic calcium influx and trigger a cascade of events leading to cell damage following N-methyl-D-aspartate (NMDA) receptors activation during hypoxia insults [14–16]. Part of the released NO after hypoxic injury can rapidly react with superoxide produced in excess during reoxygenation, forming peroxynitrite, a potent oxidizing agent with neurotoxic actions [17, 18]. Peroxynitrite can act on tyrosine residues in proteins to form the stable end product 3-nitro-l-tyrosine (nitrotyrosine; Ntyr). This compound can thus be used as a marker for the potentially cytotoxic effect of NO production in the presence of superoxide [18, 19].

Pharmacological agents can reduce NO production or prevent its biological effects by a variety of mechanisms, including the inhibition of l-arginine uptake into the cell, the reduction of cellular availability of necessary cofactors by preventing their formation or promoting their breakdown, or inhibition of the cellular mechanisms leading to induction of different NOS isoforms [12]. Of the many substances identified, recent studies suggest that the melatonin and its metabolites are highly effective physiological antioxidants and free radical scavengers [20, 21]. Many biochemical and histopathological findings have revealed that melatonin exerts neuroprotective effects in suppressing NO production and enhancing superoxide dismutase (SOD) activity following numerous experimental and clinical oxidative injury [22–24]. Lowering circulating levels of melatonin also exaggerates the oxidative damage to tissues that are subjected to increased oxidative stress [25]. Thus, melatonin serving as a powerful agent in the treatment of various neurotoxicities is anticipated. To our knowledge, the potential effects of melatonin on nitric oxide system and protein nitrination following hypobaric hypoxic insults in the hippocampus have not yet been explored. Therefore, this study was using immunohistochemical and immunoblot methods, aimed to explore the time course alteration of NOS and nitrotyrosine expression in the hippocampus of HBH rats. We also sought to elucidate whether melatonin treatment would have any beneficial effects to prevent and/or treat the symptoms associated with hypobaric disease.

Methods

Experimental animals

Healthy adult male Wistar rats weighing 150–250 g obtained from the Laboratory Animal Center of the National Taiwan University were used in this study. These animals were housed in conditions with controlled temperature (22 °C) and exposed to an automatically regulated light/dark cycle of 12:12 h (light on at 07:00–19:00 h), with ad libitum access to food and water throughout the study period. For the care and handling of all experimental animals, the guidelines as stated in the Guide for the Care and Use of Laboratory Animals (1985) as stated in the United States NIH guidelines (NIH publication No. 86-23), were followed. All the experiments were approved by our Laboratory Animal Center, China Medical University, Taiwan (No. 96-181-B). All efforts were made to minimize animal suffering and the smallest numbers of animal were used for the experiments presented. The experimental animals were carried out to evaluate the post-treatment effect of melatonin in hypoxic exposure. The animals (n = 180) were divided into four groups (I–IV) with 45 rats each. Groups I and II served as controls, the rats were subjected to normoxic breathing by receiving intraperitoneal administrations of vehicle (normal saline) and melatonin (100 mg/kg body weight in saline), respectively. Rats of groups III and IV were pretreated with intraperitoneal injections of normal saline and melatonin, respectively, 30 min before the hypobarc hypoxic insult. Melatonin (Sigma, St Louis, MO, USA) was dissolved freshly in pure absolute ethanol and later liquidized with isotonic sodium chloride (0.9 % NaCl) amounting to final concentration of 1:10 in a freshly prepared solution form, under sterile conditions. Pretreatment of both melatonin and normal saline was carried out at 10 am. Hypoxia was achieved by keeping the rats in an altitude chamber at 9000 m with the partial pressure of oxygen set at the level of 0.303 atm (pO2 = 48 Torr) for 7 h. Following hypobaric hypoxic exposure, each of the experimental groups was further divided into three subgroups (n = 15 each) sacrificed at 0 h, 1, and 3 days, respectively.

Perfusion and tissue preparation

At each of the respective time points, both the hypoxic treated and control rats were deeply anesthetized with an intramuscular injection of mixtures of zoletil (30 mg/kg) and xylazine (10 mg/kg) and perfused transcardially with 100 ml of normal saline followed by 300 ml of 4 % paraformaldehyde in 0.1 M phosphate buffer (PB), pH
7.4. After perfusion, the hippocampus were removed and postfixed in the same fixative for 2 h. Tissue samples were then rinsed in 0.1 M PB and placed overnight in sucrose buffer (10–30 %) for cryoprotection at 4 °C. Serial 30 μm thick sections of the hippocampus were cut transversely with a cryostat (Bright 5040, Bright Instrument Company, Huntingdon, UK) on the following day and were alternatively placed into six wells of a cell culture plate, such that each well ultimately contained a group of sections, with each spaced a distance of 180 μm apart from the others. Sections collected in the first, second, and third wells were processed for nNOS, eNOS, and Ntyr immunohistochemistry.

**Immunohistochemistry**

Following fixation and incubations as described above, sections collected in the wells were rinsed in 0.05 M Tris-buffer saline (TBS, pH 7.4), and then were treated in TBS containing 10 % methanol and 3 % hydrogen peroxide for 1 h to abolish the endogenous peroxidase activity. For blocking nonspecific binding, sections were first rinsed three times in TBS and then were reacted in an incubation medium containing 10 % normal goat serum or horse serum and 0.1 % Triton X-100 (all from Sigma) for 1 h. After several washes in TBS, the sections were then incubated separately in the primary monoclonal antibodies: nNOS (1:100; Santa cruz), Ntyr (1:3000; Santa cruz), and polyclonal antibodies: eNOS (1:1000; Santa cruz) overnight at 4 °C, respectively. After that they were treated separately with biotinylated horse anti-mouse and goat anti-rabbit antibodies (1:200; Vector) for 1 h at room temperature. After incubation in secondary antibody, the sections were processed by the standard Strepatavidin/HRP (DAKO) procedure with diaminobenzidine as a substrate of peroxidase.

**Western blot analysis**

At a designated time point following experimental protocols rats from groups I–IV were deeply anesthetized and then the hippocampus were rapidly removed and kept in liquid nitrogen. After that, they were rinse with PBS and then were homogenized with 100 ml lysis buffer using a grinder on ice. For the tissue processing and western blot analysis, we followed the methods as described previously [26]. Briefly, 100 mg of solubilized proteins were separated by electrophoresis in a 10 % polyacrylamide gel, transferred to nitrocellulose membranes, and they were stained with Ponceau Red to confirm equal protein loading. The membranes were blocked with 5 % nonfat dry milk for 1 h, then immunoreacted with mouse monoclonal antibodies: nNOS (1:10,000; Santa cruz), Ntyr (1:500; Santa cruz), iNOS (1:1000; Santa cruz) and rabbit polyclonal antibodies: eNOS (1:1000; Santa cruz), Caspase 3 (1:1000; Millipore) overnight at 4 °C, respectively. The nitrocellulose sheet was further processed for chemiluminescence detection (Santa Cruz) using horse-radish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit and anti-sheep secondary antibodies (Santa Cruz) for 1 h at room temperature. Equal protein loading was confirmed by stripping the membranes, then immunoreacted with Beta-actin (1:1000; Sigma). Optical densities were quantified with a computer-assisted program (Gel-Pro Analyzer software).

**Nitrite assay**

NO production was measured by the accumulation of nitrates (NO3⁻) in supernatant from different brain regions. The total amount of NO in hippocampus was assessed by the Griess reagent: 0.1 % N-(1-naphthyl) ethylene diamine dihydrochloride (Acros Organics), 1 % sulfanilamide (Cica) and 2.5 % H3PO4 (Cica) that detects nitrite, a stable reaction product of NO. Homogenates as described above for Western blotting were prepared and centrifuged at 15,000×g for 15 min and the supernatant was collected. The reagent was added to an equal volume of tissue supernatant (50 μL) and incubated for 10 min at room temperature. The optical density of each group was measured at 550 nm. Sodium nitrite dissolved in the lysis buffer was used as the standard.

**Quantitative study and image analysis**

The nNOS, eNOS, and Ntyr staining was assessed in sections collected from the wells, and was quantified with a computer-based image analysis system (MGDS) along with the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). A digital camera mounted on the Zeiss microscope imaged sections at 100× magnifications in bright field and displayed them on a higher resolution monitor. At this magnification the optical density (OD), which was used as an index to indicate labeling intensity, of reaction product in the cytoplasm of positive neurons, was measured by using a mouse to draw a line encircling the labeled soma on the digitized image. The OD of the background of each section was measured by averaging five random polygons (area of polygon = 150 μm²) with equal area of the neuropil of the hippocampus. The mean OD is the pixels that comprise the soma reading by densitometer. The actual amounts of staining intensity in a tissue section reflex the enzyme activity which is under the influence of multi-factors. Thus, all the parameters used in the present study were followed by Smolen’s method to ensure to gain a consistent result for gray level adjustment, histogram stretch and minimal optical density [27]. To avoid introducing bias two observers were blinded to examine the immunohistochemical sections for the image analysis for
hippocampus. The OD of positive neurons at various time points in hypoxic animals, with or without melatonin pretreatment was subjected to a two-way ANOVA test. The data collected between the normoxic versus hypoxic groups at each time point were individually further analyzed using Student’s t test. Statistical difference was considered significant if \( P < 0.05 \).

**Control experiments**

Some negative controls have been made to ensure the accuracy of nNOS, eNOS and Ntyr immunohistochemical results obtained from the present study. Thus, omission of nNOS, eNOS and Ntyr immunohistochemistry the primary and secondary antibodies in incubated reaction medium was carried out.

**Results**

**Neuronal NOS immunoreactivity**

In normoxic rats that received normal saline or melatonin administration and sacrificed at various time points, the pattern of nNOS positive neurons detected in the hippocampal CA1 region was consistent; those nNOS positive neurons were weakly stained and predominantly distributed in the pyramidal cell layer of CA1 (Fig. 1a). At the same region of rats subjected to 7 h of HBH, an significantly increased of packing density and immunoreactive intensity of nNOS positive neurons was noticed (Fig. 1a). The majority of the nNOS positive neurons were heavily stained and the staining intensity was drastically enhanced to reach the peak level of 145 % at 1 day of reoxygenation following HBH (Fig. 1b). In rats with longer survival after hypoxic insult, nNOS immunoreactivity was decreased progressively. In the group of animals treated with the melatonin, the pattern and intensity of nNOS immunoreactivity were markedly reduced when compared with that of non-treated rats subjected to HBH. The increased immunoreactivity of nNOS positive neurons induced with HBH significantly was downregulated at 0 h and 1 day of reoxygenation and the prominent downregulation was sustained until 3 days of reoxygenation when the animals were treated with melatonin (Fig. 1b).

Western blot analysis of the hippocampus also revealed a marked increase of nNOS that reached the peak level of 178 % after 1 day of reoxygenation following HBH; these levels were declined to 121 % for those animals having longer survival times (at 3 days of reoxygenation following HBH) (Fig. 1c). In rats receiving melatonin pretreatment, the total nNOS protein levels were drastically decreased in rats surveyed at various time points when compared with those of the rats subjected to HBH but without melatonin pretreatment (\( P < 0.05 \); Fig. 1c).

Results of nNOS immunoblots confirmed those of nNOS immunohistochemistry examination.

**Endothelial NOS immunoreactivity**

In the hippocampus from normoxic rats, eNOS immunoreactivity was observed occasionally in the pyramidal cells of CA1 and blood vessels that were lightly stained (Fig. 2a). After 7 h of HBH, an markedly augmented of cell density and immunoreactive intensity of eNOS positive neurons was noticed in the structures abovementioned. The staining intensity was drastically enhanced to reach the peak level of 145 % after 1 day of reoxygenation following HBH. After 3 days of reoxygenation following HBH, the distribution and intensity of eNOS immunoreactivity was decreased progressively (Fig. 2b). In rats receiving melatonin pretreatment prior to hypoxic insult, eNOS expression was markedly reduced when compared with that of rats with saline pretreatment. Melatonin pretreatment significantly downregulated HBH-induced increase in the immunoreactive intensity of eNOS positive neurons at 1 day of reoxygenation and the downregulation was sustained until 3 days of reoxygenation (Fig. 2b).

Western blot analysis of eNOS confirmed the results obtained from eNOS immunohistochemistry. Total eNOS protein levels in whole hippocampus were significantly increased to reach the peak level of 148 % after 1 day of reoxygenation following HBH and declined to 115 % for those animals at 3 days of reoxygenation following HBH (Fig. 2c). Melatonin pretreatment induced similar effect on the expression of eNOS protein that was drastically decreased in rats surveyed at various time points when compared with those of saline-pretreated rats subjected to HBH (\( P < 0.05 \); Fig. 2c).

**Inducible NOS immunoreactivity**

Total iNOS protein levels in whole hippocampus were significantly increased to reach the peak level of approximately 200 % after 1 day of reoxygenation following HBH and declined to 151 % for those animals at 3 days of reoxygenation following HBH (Fig. 3). Similar trend was evidenced in melatonin pretreatment on the expression of iNOS protein that was drastically decreased in rats surveyed at various time points when compared with those of saline-pretreated rats subjected to HBH (\( P < 0.05 \); Fig. 3).

**Nitrotyrosine immunoreactivity**

The rats hippocampus contained Ntyr immunoreactive neurons that were few in number and distributed in the pyramidal layer of CA1. In the normoxic condition, Ntyr immunopositive pyramidal neurons contained
less amount of the protein (Fig. 4a). In rats subjected to 7 h of HBH, pyramidal neurons drastically augmented in number and immunoreactivity. The latter reached the peak level of 162 % after 1 day of reoxygenation following HBH. In rats with longer survival after hypoxic insult, Ntyr immunoreactivity was declined gradually (Fig. 4b). After 3 days of reoxygenation following HBH, the distribution and intensity of the Ntyr immunoreactivity declined as found in normoxic condition. The pretreatment of melatonin again showed similar effect on the downregulation of HBH-induced Ntyr expression to those of nNOS and eNOS (Figs. 1b, 2b, 4b).

The findings of Ntyr immunohistochemistry were then confirmed by Western blot analysis that also showed a significant increase of Ntyr after early reoxygenation and a decline in those animals having longer survival times (Fig. 4c). The blot analysis of Ntyr also revealed a similar trend of the changes of Ntyr expression in rats receiving melatonin pretreatment and was parallel to those of nNOS, eNOS and iNOS (Figs. 1c, 2c, 3, 4c).
Nitrite assay

In normoxic rats that received normal saline or melatonin administration, the whole hippocampus possessed few amount of NO (Fig. 5). In rats subjected to 7 h of HBH, a significant increase of NO production was noticed, reached the peak level of 43 μM after 1 day of reoxygenation following HBH and decreased gradually in rats with longer survival after hypoxic insult (Fig. 5). Downregulation of HBH-induced NO production was significantly observed in animals pre-treated with melatonin and persistent until 3 days of reoxygenation (P < 0.05; Fig. 5).

Caspase 3 immunoreactivity

As Caspase 3 is known to play a central role in the execution-phase of cell apoptosis pathway, the levels of Caspase 3 were measured. Our results showed that HBH increased the levels of Caspase 3 in rat hippocampus following HBH (Fig. 6). The HBH-induced elevation of
Caspase 3 levels were also reduced by pretreatment of melatonin (P < 0.05; Fig. 6).

Discussion

The current study provides the information concerning the melatonin may attenuate HBH induced expression of the nitric oxide system and protein nitration in the hippocampus. We selected the hippocampus as a target for our study considering that the hippocampal susceptibility to hypoxic damage is well established [7, 28]. However, the relationship between exposure to HBH and modifications of the nitric oxide system following melatonin treatment in the hippocampus has rarely been studied, although some reports suggest that NO may be involved in physiological and pathological responses after climbing and air travel as well as in migration to higher altitudes [11]. We report here that HBH boosts NO production and upregulates nNOS, eNOS and iNOS. There is also a modulation of Ntyr and Caspase 3 immunoreactivity that parallels to nNOS, eNOS and iNOS expression after HBH. In addition, our previous and other studies have shown that some antioxidants such as melatonin, green tea and hypoxic strategies may play roles to enhance endogenous antioxidative defense systems to prevent biological organisms from oxidative injuries [29–37]. Interestingly, melatonin selected as an antioxidant for this study showed that melatonin markedly dampened HBH-induced increases in the expressions of nNOS, eNOS and iNOS, NO production and Ntyr formation. Melatonin also effectively protects neurons against HBH-induced neuronal damage in the hippocampus. This protection is supported by a decrease of Caspase 3 levels. The present immunohistochemical results in control and experimental rats subjected to HBH were corroborated by Western blot analysis and NO production measurements.

It is known that in the hippocampus, NO is involved in several physiologic events as neural plasticity, acting as a retrograde messenger in the case of long-term potentiation and participating in the creation of memory [38–40]. In addition, NO is also involved in physiopathology, playing a role in neuronal damage. Previous evidences that supported a neurotoxic role after hypoxic-ischemic injury in the brain, cerebral cortex, cerebellum, hippocampus and nodose ganglion due to the over-expression of NOS [11, 31, 33–36, 41–43]. hypoxic-ischemic injury causes cell damage in the hippocampus has been associated with memory loss, functional and behavioral deficits [44, 45]. Constitutive NOS expression increased after hypoxic-ischemic damage [11, 33, 34, 42] was also detected in our present study. This increase probably constitutes part of the cascade occurring after hypoxic injury, including glutamate release, calcium influx, activation of NOS, NO synthesis, and reaction with resulted oxygen radicals [46–48]. The over-production of NO or peroxynitrite (ONOO\(^-\)) could readily trigger a series of biochemical reactions to modulate enzyme activities and subsequently lead to lipid peroxidation or DNA damage [48, 49]. Thus the fact that NO is protective or destructive depends on its amount in the organisms; small amounts might have beneficial effects to protect against neuronal damage, and large amounts, which may produce by enhanced activation of NOS, might have detrimental effects to cause cell damage or cell death. The present results showed that HBH induced a significantly increased in nNOS, eNOS and iNOS immunoreactivity and in protein expression in the hippocampus following 0 h and 1 days of reoxygenation. This elevation decreased progressively after 3 days of reoxygenation. The increase in NO production, Ntyr formation and Caspase 3 level followed the same pattern. These results are in agreement with those of Encinas et al. (2003) who found an increase in NOS expression and NO production in the hippocampus immediately after acute hypoxia applied in a hypobaric chamber at 8325 m (260 mmHg) and our previous findings that chronic treadmill running protects hippocampal neurons from hypobaric hypoxia-induced neuronal injury in rats [50]. Although nNOS was reported to be mainly responsible for brain cNOS activity and play the neurotoxic role after hypoxic-ischemic injury in the brain, cerebral cortex,
hippocampus and nodose ganglion [11, 31, 33, 34, 41, 42], however, our major finding showed that eNOS immunoreactivity detected in hippocampal pyramidal cells of CA1 was also augmented and peaked after 1 day of reoxygenation following 7 h of HBH. The eNOS expression in hippocampal pyramidal cells of CA1 is coherent with other studies [51, 52]. The eNOS-derived NO has been suggested as a retrograde messenger of long-term potentiation and implicated in synaptic plasticity [51]. It may also be involved in mitochondrial dysfunction and subsequent pathological changes of prion diseases [52]. Based on the present findings, it is therefore likely that upregulation of eNOS contributes to the incremented cNOS activity and provides a neurotoxic role for NO. Furthermore, the Western blot analysis showed the HBH induced iNOS protein expression in the hippocampus at 0 h, 1 and 3 days post hypoxia. Treatment with melatonin resulted in down-regulation of the iNOS expression and attenuation of the surge of NO in hippocampus due to HBH. The NO burst as a result of iNOS upregulation during acute hypobaric hypoxia interrupts the memory consolidation had been reported [53]. The present study
provided further evidences on the sources of NO surge that are multiply derived from nNOS, eNOS and iNOS.

Another consequence of NO production is the generation of nitrotyrosine. NO reacts with superoxide radicals producing peroxynitrite, a new and powerful oxidant with the capacity of nitrating tyrosine residues, thus forming nitrotyrosine, a direct marker of NO synthesis and peroxynitrite formation [48]. In the present model of HBH, coincident with the peaks of NO production and the expressions of nNOS and eNOS is an increase in nitrotyrosine formation. Overproduction of oxygen free radicals including superoxide has been shown in neurons after hypoxia and ischemia [54, 55], which in addition to the overproduction of NO, results in a situation to produce peroxynitrite of cytotoxic concentration. Peroxynitrite has been shown to nitrate tyrosine residues in proteins [18], causing consequent protein-structure changes and alterations of enzymatic activities [19, 56]. Furthermore, mitochondrial proteins have been shown to be a selective target for peroxynitrite, an event directly related to neuronal damage [18]. Our previous studies have shown that inappropriate or excessive NOS expression and NO production are coincident with the death of affected neurons following hypoxia and peripheral nerve injury [33–36]. In this connection, the amount of NOS reactivity and NO levels may be set as an index for the severity of neuronal damage. By reducing the parameters of NOS activity, NO production and Caspase 3 levels could decrease neurological signs and neuronal damage. This detrimental role can be inhibited by the application of melatonin in the nervous tissue. The antioxidative properties of melatonin and its metabolites have been extensively studied and the use of this molecule as a novel neural protector has been widely reported [25, 31, 57–61]. In the present study, coherent with our previous studies [30–32], we further demonstrated the effective high dosage of melatonin (100 mg/kg) to achieve protection against HBH-induced increase in NO production, expressions of nNOS, eNOS and iNOS, and Ntyr formation in the hippocampus. The neuroprotective functions of melatonin are directly attributed to its antioxidant properties and free radical scavenging ability [59, 62]. Intracellular melatonin can bind to calmodulin, which conceivably would suppress the calmodulin-dependent nitric oxide synthase activity [63]. Besides reducing NO formation by restricting the activation of NOS and thereby limiting the subsequent cytotoxicity caused by this free radical, melatonin was recently shown to directly scavenge the highly toxic NO and ONOO⁻ anion as well [64]. Thus, with the reduction in NO synthesis, melatonin can also protect neurons by its ability to scavenge NO as well as ONOO⁻ and associated oxidants.
Conclusion
In summary, this study has demonstrated that hypoxia-induced increases of NO production, nNOS, eNOS and iNOS expressions as well Ntyr formation and Caspase 3 levels in the hippocampus is effectively prevented by melatonin treatment. This novel finding has not only helped to achieve a better understanding of the functional roles of NO involved in the processes of neuronal damage, but also offered the possibilities of potential therapeutic use of melatonin for the preventing and/or treating the symptoms associated with hypobaric exposure.

Abbreviations
HA: high altitude; HBH: hypobaric hypoxia; PO2: partial oxygen concentration; CNS: central nervous system; NO: nitric oxide; NOS: nitric oxide synthases; rNOS: neuronal nitric oxide synthase; eNOS: endothelial nitric oxide synthase; iNOS: inducible nitric oxide synthase; NM: NMMA; A?+methyl-o-arsiparte; Ntyr: 3-nitro-L-tyrosine (nitrotyrosine); SOD: superoxide dismutase; OD: optical density; ONOO·: peroxynitrite.

Authors’ contributions
IHW, CCH, MHT, KTC and CHW conceived this experiment. Animal studies were performed by IHW, JCL, YCW, PIF and KTC. Immunohistochemistry was performed by IHW, JCL, MHT, PIF and MJL. Data acquisition, analysis and manuscript preparation were performed by IHW, CHH, MHT, YCW, KTC and CHW. The manuscript was finally edited by IHW, CCH, MHT and CHW. All authors read and approved the final manuscript.

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Compliance with ethical guidelines
Competing interests
The authors declare that they have no competing interests.

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