Inducible Nitric Oxide Synthase Expression and Plasma Bilirubin Changes in Rats under Intermittent Hypoxia Treatment

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Abstract

It has been reported that intermittent hypoxia treatment prevents oxidative injuries to the brain and protects the heart against ischemia-reperfusion injury. Both anti-oxidative defensive systems and prevention of free intracellular calcium overload might be the result of intermittent hypoxia. Thus, the purpose of this study was to explore the effects of intermittent hypoxia (8 h at 12 % O_2 per day) for 0, 7 or 14 days on inducible nitric oxide synthase (iNOS) expression in the spleen and on splenic calcium response to the mitogen phytohemagglutinin (PHA). The results demonstrated that administration of intermittent hypoxia for 7 days caused severe hemolysis of erythrocytes in the spleen and the hemolytic condition was ameliorated by intermittent hypoxia for 14 days. However, a significant decline in splenic weight and an increase in plasma total bilirubin levels appeared in rats after hypoxia for 14 days. No calcium response to PHA was observed in splenocytes obtained from rats after intermittent hypoxia for 7 days. After intermittent hypoxia for 14 days, the calcium response to PHA was restored to the level of the controls. Intermittent hypoxia for 7 days was able to induce higher iNOS expression in splenic tissues than hypoxia for 14 days. These results suggested that intermittent hypoxia for 14 days appeared to involve acclimatization that protects the rats from oxidative injury through less hemolysis and iNOS expression in splenic tissues and by the presence of more bilirubin in the plasma. The increase in plasma total bilirubin levels might be the cause of induced adaptation to chronic intermittent hypoxia.

Key Words: intermittent hypoxia, hemolysis, bilirubin, Ca^{2+}, iNOS, spleen, splenocytes, rat.

Introduction

Acute hypoxia can alter the regional blood flow that normally ensures adequate O_2 supply to vital organs (14). The organ O_2 supply is maintained by an increase in blood hemoglobin concentration secondary to the polycythemia of intermittent hypoxia. The increase in blood hemoglobin is usually achieved by splenic contraction *via* an increase in sympathetic activity during intermittent hypoxia. Splenic contraction induces a reversible increase in hemoglobin concentration of rats during intermittent hypoxia (15).

Senile or osmotic fragile damaged erythrocytes that undergo hemolysis are sequestered and destroyed in the spleen. Heme oxygenase (HO) is the primary enzyme responsible for heme catabolism and is found in several tissues with significant activity levels in the liver, spleen, and erythropoietic tissue (23). HO is the rate-limiting enzyme in the conversion of heme into carbon monoxide, iron, and biliverdin, which is immediately reduced to bilirubin. The spleen plays a primay role in the degradation of hemoglobin and in

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bilirubin excretion. The capacity of the normal human spleen to handle any accumulation of bilirubin accounts for 50% of the normal daily production from erythroid sources (24). Since bilirubin is a scavenger of reactive oxygen species (ROS), splenectomization of dogs reduces their circulatory adjustments to hypoxaemia and decreases oxygen availability (7). The incident of fatal pneumococcal septicaemia is increased in man after splenectomy (1). Acute splenectomy reduces by 80% the bilirubin production of rats during hypoxia (20). Therefore, plasma total bilirubin levels need to be investigated in rats to explore whether the level is affected by intermittent hypoxia.

It has been reported that intermittent hypoxia treatment prevents oxidative injuries to the brain and protects the heart against ischemia-reperfusion injury (3, 16). Inducible nitric oxide synthase (iNOS) catalyzes the formation of nitric oxide (NO) from L-arginine and O_2 (6, 17). A delicate balance between the injurious and protective action determines the role played by iNOS in tissue pathology. Nitric oxide (NO) has been shown to be a mediator of hypoxic injury in rat renal proximal tubules. Using knockout studies, it has been shown that the iNOS gene is able to protect rats against hypoxic injury (18). GW274150, a potent and highly selective inhibitor of iNOS, reduces experimental renal ischemia/reperfusion injury (4). In contrast, iNOS is also responsible for the long term protection afforded to the kidney by ischemic preconditioning and renders the kidney resistant to subsequent ischemia (21). iNOS is required not only for hypoxic adaptation but also has the ability to invade into veins and into renal tumor thrombi (5, 12).

Hypoxia induces leukocytes to change their ROS and nitric oxide (NO) balance, which are restored during acclimatization (9). A few reports have discussed the effect of intermittent hypoxia on iNOS expression in rat spleens, whether iNOS plays a role in adaptation and if changes in expression of iNOS are restored during acclimatization. Chronic stress has been associated with impaired immune function, a reduction of the intracellular calcium ($[Ca^{2+}]_i$) rise and an impairment of protein kinase C-dependent NF-κB activation in mitogen stimulation of T lymphocytes (25). It is known that hypoxia can modulate $[Ca^{2+}]_i$ in non-excitable cells and, most importantly, it can evoke Ca^{2+} release from intracellular stores (22). Thus, splenocytes were isolated to study their responses in terms of $[Ca^{2+}]_i$ changes to the mitogen phytohemagglutinin (PHA). $[Ca^{2+}]_i$ was determined using the fluorescent dye Fura-2. The iNOS expression levels were studied by immunohistochemistry. Bilirubin is a potent free radical scavenger and its total plasma levels were determined in this study.

Materials and Methods

Animal Treatment

All animal experiments in this study were approved by the Animal Research Committee, National Yang-Ming University, and strictly adhered to the guideline for animal experimentation, National Yang-Ming University. Male Sprague-Dawley rats weighing 250-300 g were employed in this study. Animals were random divided into three groups (each consisting of 6-7 rats) and exposed to intermittent hypoxic challenges (8 h at 12% O_2 per day) for 0, 7 or 14 days. This experiment was repeated at least 5 times. The rats were weighed and sacrificed to collect the plasma for measurement of total bilirubin level. The spleens were weighed and transferred into sterilized physiological saline for splenocytes isolation or fixed in 4% formaldehyde solution for immunostaining of inducible nitric oxide synthase (iNOS). These tissue sections were counterstained with conventional Prussian blue or hematoxylin and eosin.

Chemicals

Fura-2/AM was purchased from Molecular Probes (Eugene, OR, USA). Phytohemagglutinin (PHA), RPMI 1640 medium (RPMI), Hank's balanced salt solution (HBSS) and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY, USA). Poly-L-lysine, 3, 3,-diamino-benzidine and Mayer hematoxylin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-iNOS antibody was purchased from Lab Vision Co. (Fermont, CA, USA). PHA was dissolved in distilled water. The culture media were supplemented with 10% heat-inactivated FCS (v/v).

Measurement of $[Ca^{2+}]_i$

The isolated rat splenocytes (2×10^7 cells/ml) were loaded with Fura-2/AM (5 mM) in medium RPMI 1640 with 10% fetal calf serum (FCS, v/v) for 30 min at 25°C, washed free of extracellular Fura-2/AM with RPMI 1640 three times and resuspended (4×10^8 cells/ml) in RPMI 1640 with 10% FCS. To determine the $[Ca^{2+}]_i$, portions of the cell suspension (2×10^6 cells) were washed twice, resuspended in 2.5 ml of loading buffer (152 mM NaCl, 1.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, 5 mM KCl, 10 mM glucose, 10 mM HEPES, pH 7.4) and placed in a plastic cuvette at 37°C in a dual-wavelength spectrofluorometer (Spex Industries Inc., model CM1T11I, Edison, NJ, USA). Fluoresecence emission was measured at excitation wavelengths of 340 nm and 380 nm, and an emission wavelength of 505 nm. The $[Ca^{2+}]_i$ was determined by

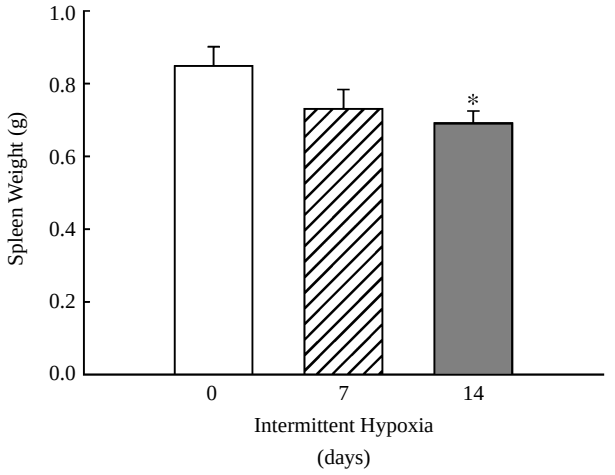

Fig 1. Effects of intermittent hypoxia on the weight of rat spleens. After exposure to intermittent hypoxic challenges (8 h at 12% O_2 per day) for 0, 7 or 14 days (each group consisting of 6-7 rats), the rats were sacrificed and effects on spleen weight were observed. This is one representative of 5 experiments. *, $P < 0.05$ compared to 0 day controls. Each value represents mean ± SEM (n = 6-7).

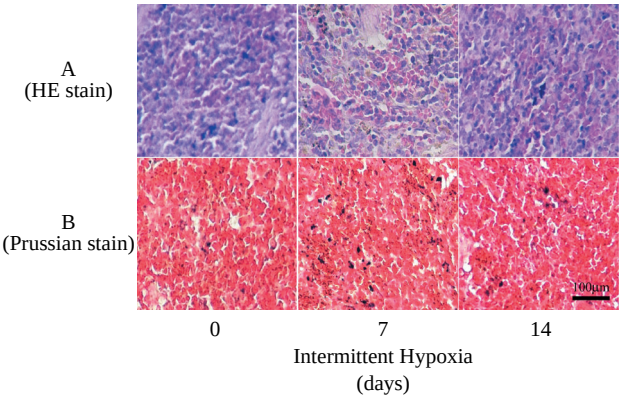

Fig. 2. Effects of intermittent hypoxia on rat splenic tissues. After exposure to intermittent hypoxic challenges (8 h at 12% O_2 per day) for 0, 7 or 14 days, rats were sacrificed to prepare spleens for histochemistry studies. (A) Photomicrographs of HE staining of the spleen after intermittent hypoxic challenges for 0, 7, 14 days. (B) The photomicrographs of Prussian blue staining of the spleen after intermittent hypoxic challenges for 0, 7, 14 days. The bar in the photograph is 200 μm.

monitoring the Fura-2 fluorescence-ratio signal. The intracellular Ca^{2+} concentration was calculated using Spex DM3000 software (10).

Immunohistochemistry

Paraffin sections of the spleen were deparaffinized, and endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxide for 15 min at room temperature. The sections were washed in 0.01 M phosphate-buffered saline, pH 7.2, containing 10 % normal goat serum and incubated overnight at 4°C with rabbit anti-iNOS antibody (1: 100 dilution). Horseradish-conjugated goat anti-rabbit Ig antibody was used at 1:200 after washing. Immune complexes were detected with a solution of 3, 3,-diamino-benzidine (0.2 mg/ml) and hydrogen peroxide in 0.05 M Tris-HCl buffer. Sections were counterstained with Mayer hematoxylin and mounted. In the negative control sections, rabbit antiserum against ovalbumin (1 : 500) was used as a control non-relevant antibody. Histo-pathological examination and photographic records were made using a Zeiss Axioskop MC 80 Microscope (Göttingen, Germany) equipped with a camera.

Statistical Analysis

The data were analyzed by Student's *t* test with a significance level set at $P < 0.05$. All values are quoted as the mean ± SEM.

Results

Effects of Intermittent Hypoxia on Rat Spleens

The weight of the spleens decreased significantly in rats after intermittent hypoxia for 14 days (Fig. 1). When compared to normoxia (0 day), severe congestion and hemolysis were observed in the red pulp of the spleen tissue after intermittent hypoxia for 7 days (Fig. 2A). However, the incidence of congestion and hemolysis became less severe in the splenic tissues after hypoxia for 14 days (Fig. 2A). Senile erythrocytes are sequestered and destroyed in the spleen and these osmotically fragile hemolytic erythrocytes containing hemosiderin-laden could be detected in the normoxia rat splenic tissues within the red pulp after Prussian blue staining (Fig. 2B). After 7 days of intermittent hypoxia, the number of hemosiderin-laden erythrocytes had increased dramatically (Fig. 2B). However, this effect was reduced in splenic tissues from rats after intermittent hypoxia for 14 days (Fig. 2B).

Effects of Intermittent Hypoxia on iNOS Expression in Spleen Tissue

Expression of iNOS was not observed in normoxic rat splenic tissues (Fig. 3). However, the expression of iNOS was induced in rat splenic tissues after intermittent hypoxia. There was more iNOS expression in splenic tissues from rats that had undergone intermittent hypoxia for 7 days than for 14 days (Fig. 3).

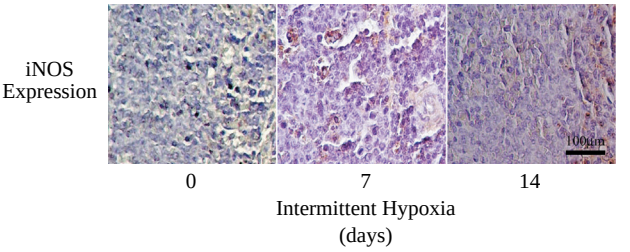

Fig. 3. Effects of intermittent hypoxia on iNOS expression in rat splenic tissue. After exposure to intermittent hypoxic challenges (8 h at 12% O_2 per day) for 0, 7 or 14 days, rats were sacrificed to prepare spleens for immunohistochemistry study. The photomicrographs are iNOS staining of the spleens after intermittent hypoxic challenges for (A) 0, (B) 7, (C) 14 days. The bar in the photograph is 200 μm.

Effects of Intermittent Hypoxia on Splenic $[Ca^{2+}]_i$ Responses to PHA

An increase of PHA-induced $[Ca^{2+}]_i$ could not be observed in splenocytes isolated from rats that had undergone intermittent hypoxia for 7 days. However, an increase of PHA-induced $[Ca^{2+}]_i$ response appeared in the splenocytes isolated from normoxic rats or from those had undergone intermittent hypoxia for 14 days (Fig. 4).

Effects of Intermittent Hypoxia on Plasma Total Bilirubin Levels in Rats

The plasma total bilirubin levels of rats were not altered after intermittent hypoxia for 7 days. However, plasma bilirubin levels were significantly increased in rats undergone hypoxia for 14 days (Fig. 5).

Discussion

The weight of the rat spleens was decreased after the exposure to intermittent hypoxia (Fig. 1). There is an increase in sympathetic activity that is stimulated by intermittent hypoxia. This results in splenic contraction, which reduces the splenic weight by lowering the number of erythroid colonies in spleen (15). The less congestion and hemolysis in spleen caused a significant decrease in weight after hypoxia for 14 days (Fig. 1 and 2A). The erythrocytes are under intermittent hypoxia and many of them are able to adapt to such a stressful environment. However, senile erythrocytes are vulnerable and damaged. Thus, congestion and hemolysis can be observed in splenic tissues in the intermittent hypoxia-exposed animals. The damaged erythrocytes were sequestered in the red pulp of the spleen, where the entire cell is phagocytized by macrophages (Fig. 2A). However,

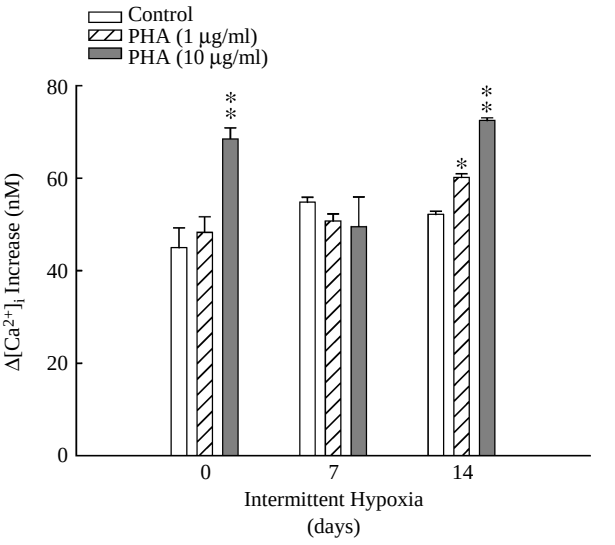

Fig. 4. Effects of intermittent hypoxia on the dose-response of PHA-induced $[Ca^{2+}]_i$ increase in rat splenocytes. After exposure to intermittent hypoxic challenges (8 h at 12% O_2 per day) for 0, 7 or 14 days, the rats were sacrificed to isolate the splenocytes. Fura-2-loaded cells were stimulated with 0, 1, or 10 μg/ml PHA. *, $P < 0.05$; **, $P < 0.01$ compared to the vehicle control. Each value represents mean ± SEM (n = 5-6).

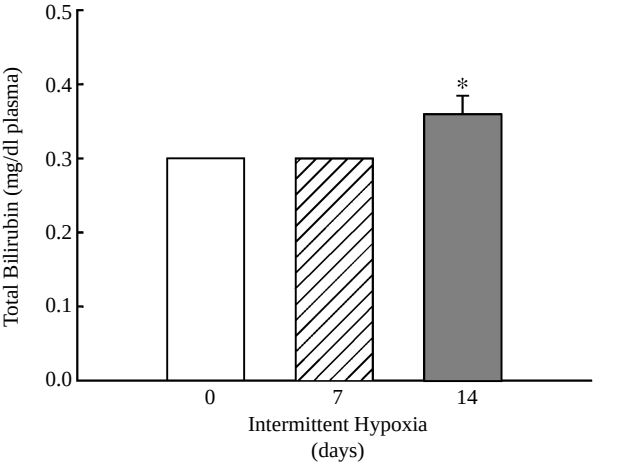

Fig. 5. Effects of intermittent hypoxia on plasma total bilirubin levels in rats. After exposure to intermittent hypoxic challenges (8 h at 12% O_2 per day) for 0, 7 or 14 days, the rats were sacrificed and plasma samples were collected for determination of bilirubin levels. *, $P < 0.05$ compared to 0 day controls. Each value represents mean ± SEM (n = 5-6).

the effect was found to be more severe in animals after intermittent hypoxia for 7 days as shown by an increase in the number of hemosiderin-laden erythrocytes (Fig. 2B).

iNOS was not expressed in the control rat splenic

tissues. The expression of iNOS mRNA is upregulated in rat splenic macrophages after hypoxia (2). In the present study, the expression of iNOS was induced in rat splenic tissues after intermittent hypoxia. Both iNOS expression and the number of hemosiderin-laden erythrocytes present in splenic tissue were higher in rats after 7-day intermittent hypoxia than in rats following 14-day intermittent hypoxia (Figs. 2 and 3). iNOS is an endogenous neuroprotectant after traumatic brain injury in rats and mice (26). The expression of iNOS induced by ischemic pre-conditioning renders the kidney resistant to subsequent ischemia (21). Therefore, more studies are needed to investigate whether the expression of iNOS contributes to cytoprotection against intermittent hypoxic injury in rats.

The results of intermittent hypoxia for 7 days are associated with impairment of the $[Ca^{2+}]_i$ responses to the mitogen PHA in splenocytes (Fig. 4). These results correlate with the finding in splenic tissues that more iNOS expression and an increase in the number of hemosiderin-laden erythrocytes following hypoxia. It is suggested that the intermittent hypoxia for 7 days produced severe effects on rat spleen tissues. However, hypoxia for 14 days led to adaptation by the spleen, which is manifested as less congestion and hemolysis together with an increase in the $[Ca^{2+}]_i$ response to the mitogen PHA; this should be termed splenic acclimatization to intermittent hypoxia. The results for plasma total bilirubin levels showed an increase in rats after intermittent hypoxia for 14 days. It is well known that hemolytic disease plays an important role in creating bilirubin toxicity. The current practice for treating human neonates for jaundice centers on the recommendation that bilirubin levels should be kept below 20 mg/dl (11). In the case of rats with intermittent hypoxia for 14 days, the plasma total bilirubin levels were more below the human jaundice levels. HO-1 can effectively defend hypoxic cardiomyocytes against reoxygenation injury (8). Reoxygenation of hypoxic cardiomyocytes produces marked injury; however, incubation with hemin or bilirubin during hypoxia considerably reduces damage at reoxygenation. Biliverdin, the precursor of bilirubin, also provides enhanced protection against transplant-associated cold ischemia/reperfusion injury of heart and kidney grafts (19).

The question generated by this study is whether bilirubin plays a role in progressive adaptation in rats that undergo chronic intermittent hypoxia. Hemolysis is often concomitant with oxidative stress, which upregulates heme oxygenase expression. It has been well known that erythrocytes are vulnerable and easily damaged because they are anucleate and lack mitochondria. We found severe hemolysis in erythrocytes in rats after intermittent hypoxia for 7 days, but the levels of hemolysis were reduced in rats that had undergone intermittent hypoxia for 14 days. However, the plasma total bilirubin levels were still significantly higher in rats that had undergone intermittent hypoxia for 14 days. The acclimatization to long-term intermittent hypoxia or adaptation to chronic hypoxia stimulates an increase in plasma erythropoietin (13). Our results indicated that intermittent hypoxia for 14 days appeared to start an acclimatization in terms of a reduction in iNOS expression, a reduction in hemolysis in rat splenic tissues and a restoration of the $[Ca^{2+}]_i$ response to the mitogen PHA in splenocytes. According to the present results, the adaptation to chronic intermittent hypoxia might be caused by the increase in the plasma total bilirubin levels in rats.

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