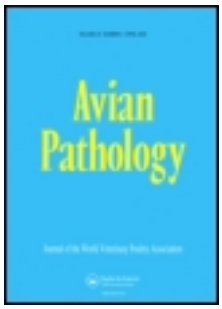

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ORIGINAL ARTICLE

Expression of endothelin-1 and its receptors in the lungs of broiler chickens exposed to high-altitude hypoxia

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To investigate the influence of exposure to high-altitude (HA) hypoxia on the expressions of endothelin-1 (ET-1), endothelin type A (ETA) and endothelin type B (ETB) receptors in broiler chickens, immunohistochemistry studies were performed in the lungs. Six hundred 1-day-old male broiler chickens were randomly divided into two groups: group A, birds maintained under rich oxygen conditions (oxygen content 21%); and group B, birds exposed to HA hypoxia (oxygen content 13%). Our data showed that exposure to altitude elevated ET-1 and ETA gene expressions at 21 and 28 days of age when compared with the rich oxygen group. Meanwhile, a marked decline in ETB expression was observed at 28 days of age in the course of HA, although there were no significant changes ($P > 0.05$) at 7, 14 and 21 days of age. The increased response was accompanied by adverse effects on weekly body weight gain and ascites mortality. These observations suggested that ET-1, ETA and ETB genes are normally expressed in the lungs of birds. Increased levels of ET-1 and ETA and decreased ETB gene expression in the lungs are probably involved in the lung dysfunction of broiler chickens with developmental ascites.

Introduction

Endothelin-1 (ET-1) is a 21-amino-acid peptide with diverse biological actions. ET-1 was first isolated from porcine aortic endothelial cells in 1988 as an endothelium-derived factor that exerted prolonged vasoconstriction and an increase in arterial blood pressure (Yanagisawa *et al*., 1988). ET-1 acts through endothelin type A (ETA) and endothelin type B (ETB) receptors to induce contraction of blood vessels and relaxation (Miller *et al*., 1993). The ETA receptor has a high affinity for ET-1 and is responsible for the vasoconstrictor response of vascular smooth muscle cells, whereas the ETB receptor has equal affinity for all ET isoforms and mediates the vasodilatation via the release of nitric oxide from endothelial cells (Chen *et al*., 2002b). Recent studies have shown that ET-1 is a key player of endothelial dysfunction in pulmonary vascular diseases ranging from pulmonary hypertension (PH), to acute lung injury and to pulmonary fibrosis (Stow *et al*., 2011). Moreover, PH followed by ascites has been recognized as a serious cause of economic loss in commercial broiler production (Singh *et al*., 2011) and accumulating research suggests a role for ET-1 and its receptors in these pathophysiological processes (Cacoub *et al*., 1997; Giaid, 1998; Chen & Oparil, 2000; Gomez *et al*., 2008).

It is well known that the expression of ET-1 is regulated by many physiological stimuli (Rubanyi & Polokoff, 1994) and hypoxia has been revealed to stimulate the expression of endothelin genes in various pathological processes (Gao *et al*., 2012). Chronic hypoxia leads to increased circulating levels of ET-1 (Bialecki *et al*., 1998), and ET-1 mRNA and protein have been shown to increase in the rat lung under hypoxic conditions (Chen *et al*., 1995). Also, immunohistochemical studies have shown that sustained hypoxia increases the expression of ETA in the rat carotid body (Chen *et al*., 2002a). However, limited information is available on the expression of ET-1, ETA and ETB receptors in response to hypobaric hypoxia in the broiler lungs . The aim of the present study was thus to evaluate the response of ET-1 and its receptors to high-altitude (HA) hypoxia in lungs of broiler chickens maintained chronically (4 weeks) under conditions of normoxia or of HA hypoxia. In addition, we determined the feed conversion ratio and the cardiac index in the birds during rearing.

Materials and Methods

Experimental birds. The experiments were conducted in Nyingchi County of Tibet, China (with an average elevation of 3100 m, the oxygen content is declined by 40% compared with sea level). Six hundred 1-day-old male broiler chickens were obtained from a commercial hatchery and reared in a temperature, humidity and air flow controlled building, using standard nutritional and management procedures for commercial operations. The birds were randomly divided into two groups: group A, birds maintained under rich oxygen conditions (oxygen content 21%, $n = 300$); and group B, birds exposed to HA hypoxia (oxygen content 13%, $n = 300$). The concentrations of

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oxygen were monitored by a gas detector through the duration of the study. Feed and water were provided for *ad libitum* consumption, and lighting was continuous. The temperature was initially 34°C during the first 7 days, and gradually decreased to a range between 18 and 21°C after the third week of the growing period. Suggested ethical guidelines for the care of laboratory animals were taken into consideration.

Measurement of growth performance. At weekly intervals, the body weight of 20 birds was measured and the ratio of right ventricular to total ventricular weight (Rv/Tv) was examined on an individual basis in both group A and group B. An Rv/Tv ratio above 0.299 was classed as right ventricular hypertrophy (Julian, 1987). The incidence of ascites was recorded daily according to abdominal fluid accumulation and the Rv/Tv ratio, and the feed conversion was corrected for mortality. Necropsies and cause of death were conducted on all birds that died during the experiments. For histological analysis, six bird lungs of each group were fixed by immersion in 10% formalin in 0.1 M phosphate buffer (pH 7.4) for 24 h. Samples were then snap-frozen in liquid nitrogen and stored at 70°C until they were used.

Quantitative immunohistochemistry. For quantitative immunohistochemical studies, 5 μm paraffin pulmonary sections were cut, mounted onto Superfrost Plus glass slides, and placed in a 60°C degree oven for 60 min. Sections were then deparaffinized and incubated with target retrieval solution (Boster, Wuhan, China) diluted 1:10 with double-distilled water in a pressure cooker in the microwave on high for 10 min. Sections were allowed to come to room temperature over 30 min and then washed with distilled water three times for 3 min each. This was followed by quenching with peroxidase blocking reagent (Boster) for 30 min at room temperature to eliminate endogenous peroxidase activity. Sections were then washed three times with Tris buffer (2 min each) and incubated with normal goat serum (diluted 1:10 with Tris buffer) overnight in a 4°C refrigerator. Sections were then incubated with primary antibody (diluted 1:80 in phosphate-buffered saline for ET-1, and 1:100 for ETA and ETB according to the manufacturer's instructions; Boster) in a humidity chamber placed in 4°C refrigerator overnight. After primary incubation, sections were rinsed three times with Tris buffer as above and incubated with anti-goat IgG biotinylated secondary antibody (Boster) for 30 min at room temperature. This was followed by three rinses with Tris buffer as above and incubation with substrate-chromagen for 5 min. Sections were then washed with tap water, rinsed twice with distilled water, and coverslipped using cytoseal.

Quantitative image analysis. Immunohistochemically-stained slides were scanned using a light microscope at 400 × magnification. The microscopic images were captured with a digital camera. In each section, the five mostly intense microscopic fields were submitted for the quantitative analysis. Image ProPlus 6.0 (Media Cybernetics, Silver Spring, Massachusetts, USA) software was used to segment and measure the intensity of pixels in the multichannel, representing the cytoplasm of ET-1, ETA and ETB. Statistical analysis was performed using SPSS 14.0 (SPSS Inc., Chicago, IL, USA) for Windows software. Data are expressed as means ± standard deviations.

Results

Effects of high-altitude hypoxia on endothelin-1 expression in the lungs. The influences of altitude hypoxia exposure on ET-1 expression in the lungs of the birds are presented in Table 1. Group B had a significant ($P < 0.05$) increase in ET-1 production when compared with group A at 21 and 28 days of age, although the main effects of altitude hypoxia exposure on the examined variables were not observed at 7 and 14 days of age. Moreover, noteworthy is the strong positive correlation between the elevated expression of ET-1 and the ascites incidence.

Table 1. *Mean optical density of ET-1 expression in birds' lungs.*

Group	7 days	14 days	21 days	28 days
A	0.33 ± 0.13^{A}	0.33 ± 0.12^{B}	0.33 ± 0.08^{A}	0.40 ± 0.06^{A}
B	0.37 ± 0.10^{A}	0.41 ± 0.06^{B}	0.47 ± 0.13^{B}	0.58 ± 0.06^{B}

Compared with group A, data with the same superscript uppercase letters are not significantly different ($P > 0.05$); different superscript uppercase letters are significantly different ($P < 0.05$).

Table 2. *Mean optical density of ETA expression in birds' lungs.*

Group	7 days	14 days	21 days	28 days
A	0.26 ± 0.06^{A}	0.32 ± 0.09^{B}	0.35 ± 0.07^{A}	0.38 ± 0.14^{A}
B	0.30 ± 0.28^{A}	0.41 ± 0.09^{B}	0.55 ± 0.13^{B}	0.59 ± 0.07^{B}

Compared with group A, data with the same superscript uppercase letters are not significantly different ($P > 0.05$); different superscript uppercase letters are significantly different ($P < 0.05$).

Effects of high-altitude hypoxia on endothelin type A expression in the lungs. The influences of altitude hypoxia exposure on ETA expression in the lungs of the birds are presented in Table 2. Similar to ET-1 expression data, group B presented a noticeable ($P < 0.05$) increase in ETA production when compared with group A at 21 and 28 days of age, although there were no statistically significant changes observed at 7 and 14 days of age. These results reveal that altitude hypoxia has significant effects on the expressions of ETA, in a time-dependent way.

Effects of high-altitude hypoxia on endothelin type B expression in the lungs. The influences of altitude hypoxia exposure on ETB expression in the lungs of the birds are presented in Table 3. ETB production was strikingly lower in group B than the corresponding ones in group A at 28 days of age ($P < 0.05$). However, no statistical differences were found when comparing the corresponding values at 7, 14 and 21 days of age.

Effect of high-altitude hypoxia on birds' weekly body weight gain and ascites mortality. The influences of altitude hypoxia exposure on growth performance and ascites mortality of the birds are presented in Table 4. Exposure of the birds to HA hypoxia (group B) presented a marked ($P < 0.05$) reduction in weekly body weight gain, along with a significant ($P < 0.05$) increase in ascites mortality when compared with those maintained under rich oxygen conditions (group A) at

Table 3. *Mean optical density of ETB expression in birds' lungs.*

Group	7 days	14 days	21 days	28 days
A	0.29 ± 0.06^{A}	0.37 ± 0.09^{B}	0.38 ± 0.17^{C}	0.49 ± 0.08^{A}
B	0.39 ± 0.12^{A}	0.43 ± 0.16^{B}	0.48 ± 0.13^{C}	0.29 ± 0.06^{B}

Compared with group A, data with the same superscript uppercase letters are not significantly different ($P > 0.05$); different superscript uppercase letters are significantly different ($P < 0.05$).

Table 4. *Effects of HA hypoxia on body weight gain and ascites mortality.*

	Body weight gain (kg)		Mortality (%)	
Age (days)	Group A	Group B	Group A	Group B
14	0.26 ± 0.04^{A}	0.12 ± 0.03^{B}	0^{A}	6.7 ± 0.06^{B}
21	0.37 ± 0.05^{A}	0.21 ± 0.14^{B}	$6.7\% \pm 0.06^{A}$	33.3 ± 0.06^{B}
28	0.49 ± 0.19^{A}	0.34 ± 0.07^{B}	$50.0\% \pm 0.05^{A}$	90.0 ± 0.11^{B}

Compared with group A, data with the same superscript uppercase letters are not significantly different ($P > 0.05$); different superscript uppercase letters are significantly different ($P < 0.05$).

14, 21 and 28 days of age, respectively. The findings demonstrate that altitude hypoxia can significantly reduce a bird's weekly body weight gain and increase the ascites incidence, and artificial oxygen can be effective against these adverse effects.

Discussion

Hypoxia is one of the most potent inducers of ET-1 expression in endothelial cells and may be the primary cause of the increased production of ET-1 during myocardial ischaemia (Watanabe *et al*., 1990). Accumulating evidence has demonstrated that ET-1 mRNA and endothelin peptide levels in rat lung tissue and cultured endothelial cells are elevated after hypoxic exposure (Kourembanas *et al*., 1991; Elton *et al*., 1992; Li *et al*., 1994), further suggesting that increased pulmonary ET-1 expression may contribute to hypoxia-induced hypoxic pulmonary vasoconstriction, pulmonary vascular remodelling, and associated PH. The results of the present work demonstrate that HA stress augments hypoxic induction of pulmonary ET-1 peptide levels and that this response to altitude is attenuated by rich oxygen replacement. This observation confirmed the results of Gomez *et al*. (2008) demonstrating that the ET-1 lung levels were significantly higher in broilers of PH than the corresponding ones in non-PH (NPH) at 42 days. Furthermore, our findings revealed that rich oxygen moderates the development of PH by interfering with increased pulmonary ET-1 expression during HA hypoxic exposure.

There are various reports of ETA expression in the tissues of animals with PH or cardiac failure. In the present study, we found that ETA levels were increased in the lungs of birds with HA hypoxia challenges at 21 and 28 days of age, respectively. The observations of the current study are in agreement with those of Pieske *et al*. (1999) and Balyakina *et al*. (2002) who found up-regulation of ETA in their experiments. However, these contrast with a previous paper by Gosselin *et al*. (1997), who showed in lungs of chronically hypoxic pigs that ETA receptors in the pulmonary arteries were reduced. The reason for this discrepancy is not entirely clear, but one possibility is that it reflects the complexity of endothelin and its receptors in their cardiovascular effects in normal conditions or in diseases, which in turn differ among species (Hassanpour *et al*., 2010). The present data suggest that at HA the augmented release of the potent pulmonary vasoconstrictor peptide ET-1 and its receptor ETA may represent one of the mechanisms underlying the pulmonary vascular remodelling observed in broilers with PH (ascites). For ETB, however, we found a noticeable down-regulation of the gene expression at 28 days of age in the course of HA, although there were no significant changes at 7, 14 and 21 days of age. Our findings are compatible with Hamal *et al*. (2010), who found a decrease of ETB expression in the lung of broilers with idiopathic pulmonary arterial hypertension. Moreover, it has been confirmed that ETB receptors are exclusively involved in the clearance of the circulating ET-1 from the blood (Dupuis *et al*., 1996), which reduces the bioavailability of ET-1, thereby minimizing its pulmonary vasoconstrictor and mitogenic effects. One can therefore speculate that increased levels of ET-1 and ETA and decreased ETB gene expression in the lungs are involved in the lung dysfunction of broiler chickens with developmental ascites.

Our data on the weight parameters showed a considerable decrease of the body weight gain on days 14, 21 and 28 ($P < 0.05$, $P < 0.05$ and $P < 0.05$, respectively) of HA hypoxia and a significant increase of the Rv/Tv ratio on days 14, 21 and 28 ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively). These observations confirmed the findings of Hunter & Clegg (1973) relating to the decrease in the rate of gain in body weight during hypoxia. The fact that there is a reduction in food intake may solely not account for the decrease in body weight and the exact mechanisms of this effect remain unclear. Findings made by Rose *et al*. (1988) demonstrate that hypoxia can be a sufficient reason for the weight loss and decreased food consumption reported by mountain expeditions at HA. Guilland & Klepping (1985) have confirmed negative nitrogen balance at extreme HA. In addition to anorexia, an enhanced basal metabolic rate and energy expenditure during moderate exercise conditions can contribute to body weight loss at extreme altitude (Butterfield *et al*., 1992).

The cardiac index is a valid parameter to diagnose PH at necropsy (Hernandez, 1987), which allowed us to classify broilers as PH or NPH at different ages and at HA . In the present work, we found that exposure of birds to HA hypoxia (group B) presented a striking ($P < 0.05$) increase in ascites mortality when compared with those maintained under rich oxygen conditions (group A) at 14, 21 and 28 days of age. These observations corroborate that HA hypoxia is a major cause of PH in birds (Bartsch *et al*., 2005; Remillard & Yuan, 2005). Furthermore, it is worth noting that increased levels of ET-1 and ETA and decreased ETB expression are closely linked with ascites incidence.

In summary, our findings demonstrated that ET-1, ETA and ETB genes are normally expressed in the lungs of birds subjected to HA hypoxia. Increased levels of ET-1 and ETA and decreased ETB gene expression in the lungs are probably involved in the lung dysfunction of broiler chickens with developmental ascites.

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