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EXPERIMENTALLY INDUCED DISEASE

Role of Angiopoietin-like 4 on Bone Vascularization in Chickens Exposed to High-altitude Hypoxia

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Summary

The aim of this study was to investigate the role and expression of a novel angiogenic factor (angiopoietin-like 4, ANGPTL4) in tibial growth plates of broiler chickens exposed to high-altitude hypoxia. One-day-old healthy broiler chickens ($n = 120$) were transported from lowland to a high-altitude hypoxic region (nearly 3,000 m above sea level) and were reared under hypoxic- (natural lower oxygen content) and normoxic conditions (nearly 21% oxygen content) for 14 days. The effect of hypoxia on angiogenesis in the tibial growth plates and hypoxia-inducible factor (HIF)-1α and ANGPTL4 expressions were determined by histological examination, quantitative reverse transcriptase polymerase chain reaction (qRT–PCR), western blot and enzyme-linked immunosorbent assay (ELISA) techniques. The increase in vascular distribution to the hypertrophic chondrocyte zone of tibial growth plates contributed to promoting growth and development of the tibia under hypoxic conditions, which was highly correlated with the upregulation of ANGPTL4 at both the mRNA and protein levels together with activation of HIF-1α under hypoxic conditions. These findings demonstrate that angiogenic factor ANGPTL4 upregulation is involved in tibial growth plate angiogenesis to promote the development of the tibia in broiler chickens under hypoxic conditions. They also suggest that ANGPTL4 may serve as a new molecular therapeutic target for ameliorating tibial dyschondroplasia chicken bone vascularization.

Keywords: ANGPTL4; bone vascularization; chicken; high-altitude hypoxia

Introduction

Tibial dyschondroplasia (TD) is a common disorder of poultry caused by unvascularized and uncalcified cartilage accumulating in the tibial growth plates. Increasing evidence shows that the occurrence of TD is attributed to tibial chondrocyte death due to low or no blood supply that results in apparent locomotion problems. TD has a rising prevalence and up to 30% of broiler flocks may be affected (Rath *et al.*, 2007; Herzog *et al.*, 2011; Pelicia *et al.*, 2012; Huang *et al.*, 2017a, 2018a). The exact prevalence of TD is unknown as the clinical signs of the disorder are mostly subclinical. However, it can still result in leg weakness and reduced mobility, which contributes to reduce production performance and compromised welfare (Groves and Muir, 2017). Furthermore, broilers suffering from TD are likely susceptible to fractures during the feeding process (Genin *et al.*, 2012). However, the aetiopathogenesis of TD is not well understood, but is suggested to be related to the growth plates, with lack of adequate supply of oxygen and nutrients via the blood vessels (Herzog *et al.*, 2011; Iqbal *et al.*, 2016).

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Hypoxia or low oxygen tension, occurs in physiological situations such as during embryonic development, as well as in pathological conditions such as ischaemia, wound healing and cancer (Hickey and Simon, 2006). A number of studies have found that hypoxia may upregulate the expression of hypoxia-inducible factor (HIF)-1α and vascular endothelial growth factor (VEGF) and promote angiogenesis, especially in tumours (Costache *et al.*, 2017; Nan and Jiang, 2017; Schmittnaegel *et al.*, 2017). Xiong *et al.* (2015) reported that high-altitude hypoxic stress could alter mRNA expression and DNA methylation of HIFs in yaks (*Bos grunniens*). Simultaneously, hypoxia is also a potent stimulator of VEGF synthesis and subsequently promotes angiogenesis (Shweiki *et al.*, 1992). However, tibial vascular growth with the exposure to high-altitude hypoxia in chickens remains largely unknown (Huang *et al.*, 2017b).

Angiogenesis, the formation of new blood vessels from a preexisting primary capillary plexus, is promoted to maintain oxygen homeostasis, deliver nutrients, remove waste products and provide cells and biological mediators (Lienau *et al.*, 2009; Krzykawska-Serda *et al.*, 2017). Furthermore, angiogenesis plays a pivotal role during intramembranous bone formation and endochondral ossification (Harper and Klagsbrun, 1999). Hausman *et al.* (2001) reported that an adequate blood supply to a fracture is a prerequisite for the reconstitution of bone tissue, while insufficient blood supply is likely to result in delayed bone healing.

Angiopoietin-like 4 (ANGPTL4), a multifunctional protein has been shown to be involved in metabolic, physiological and pathological processes including lipid metabolism, glucose metabolism, bone resorption, cartilage degradation and angiogenesis (Oike *et al.*, 2005; Katoh and Katoh, 2006; Hato *et al.*, 2008; Tan *et al.*, 2012; Zhu *et al.*, 2012; Knowles, 2017; La *et al.*, 2017). Angiogenesis is commonly mediated by angiogenic factors (e.g. VEGF, ANGPTL4) and regulated by hypoxia (Pufe *et al.*, 2005; Knowles, 2017). Hu *et al.* (2016) pointed out that ANGPTL4, unlike VEGF, is a second HIF-1-regulated gene product involved in angiogenesis in ocular tumours. ANGPTL4 can induce a strong proangiogenic response independently of VEGF and ANGPTL4 is present in higher concentrations in ischaemic legs than in healthy legs, which suggests that ANGPTL4 has a key role in hypoxia-induced angiogenesis (Le Jan *et al.*, 2003). Although ANGPTL4 protein has been shown to be essential for angiogenesis, its specific role in tibial angiogenesis and tibial growth plate development remains unclear. Hence, the aim of this study was to explore the potential role of ANGPTL4 in angiogenesis in tibial growth plates in chickens.

Materials and Methods

Experimental Animals

One-day-old healthy Arbor Acres chickens (AACs; $n = 120$) were transported from Chengdu to Tibet by plane and the broilers were reared in two-layer metal cages (size 80 × 60 × 50 cm) for 14 days in the Nyingchi region of Tibet, China (nearly 3,000 m above sea level). The temperature was maintained at 33—35°C during the first week and steadily reduced to 29°C at the end of second week. Daily lighting was fixed at a 23 h/1 h (light/dark) cycle throughout the entire experimental period. Additionally, feed and water were provided *ad libitum*.

Animal experiments were approved by the Ethical Committee for Animal Research of Huazhong Agricultural University and conducted based on the state guidelines of the Standing Committee of Hubei People's Congress, China. Birds were sacrificed by the injection of pentobarbital (25 mg/kg, into the pectoral muscle) to minimize suffering.

Hypoxia Experiments

As shown in Fig. 1, all the chicks were weighed and divided randomly into two groups: the hypoxia group and normoxia group (natural low oxygen content [17%] and a nearly 21% oxygen content; $n = 60$/group, 15 chicks per cage) as previously described (Huang *et al.*, 2017b). The oxygen content in the normoxia group was maintained using an oxygenerator (Yuwell, Suzhou, China) and oxygen levels were monitored using a gas detector (CY-7B, Oxygen Analysis Instrument Factory, Jiande, China) until the end of the experiment.

Morphology and Histology of the Tibial Growth Plates

After death, tibial samples ($n = 8$ chicks) were obtained from 3-, 7-, 10- and 14-day-old chicks and fixed in 4% paraformaldehyde prior to decalcification in 10% EDTA at room temperature, routine processing and embedding in paraffin wax. Serial sections (4—5 μm) were taken from the proximal tibia oriented longitudinally and from the lateral or the medial side. Sections were stained with haematoxylin and eosin (HE). In addition, the areas of blood vessels and numbers of blood vessels in the hypertrophic zone adjacent to the tibial growth plate (randomly selected six tibial samples/group, three different microscopical fields/sample) were counted for two isolated groups using Image-Pro® Plus 6.0 (Media Cybernetics,