Respiratory Physiology & Neurobiology 184 (2012) 86–96

Contents lists available at SciVerse ScienceDirect

Respiratory Physiology & Neurobiology

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

Acute regulation of hematocrit and blood acid–base balance during severe hypoxic challenges in late chicken embryos (*Gallus gallus*)

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ARTICLE INFO

Article history:
Accepted 2 August 2012

Keywords:
Acid–base balance
Chicken embryo
Lactate
Hct, [RBC] and [Hb]
Mean corpuscular indices
Severe hypoxia

ABSTRACT

Acid–base and hematocrit (Hct) responses of vertebrate embryos to severe hypoxia are as yet unknown, but may reveal the maturation process of physiological regulatory mechanisms. The present study elucidated how acute, severe hypoxia (10% O_2, with and without 5% CO_2) affects Hct and blood acid–base balance in late prenatal (days 11–19) chicken embryos. The time-course of the resulting Hct changes and blood acid–base disturbances was examined in detail in day 15 (d15) embryos to further understand the magnitude and time-components of these physiological changes. We hypothesized that Hct of developing embryos increases during severe hypoxia (10% O_2) and hypercapnic hypoxia (5%CO_2, 10%O_2), due to increased mean corpuscular volume (MCV) and red blood cell concentration ([RBC]). We additionally hypothesized that 10% O_2 would induce anaerobic glycolysis and the attendant increase in lactate concentration ([La^-]) would create a severe metabolic acidosis. Hct increased in all embryos (d11–d19) during severe hypoxia (2 h) but, with the exception of d19 embryos, the increase was due to increased MCV and was therefore unlikely related to O_2 transport. The time-course of the d15 embryonic Hct response to hypoxic or hypercapnic hypoxic exposure was very rapid with MCV increasing within 30 min. Severe metabolic acidosis occurred in all developing embryos (d11–d19) during 2 h hypoxic exposure. Additionally, respiratory acidosis was induced in d15 embryos during hypercapnic hypoxia, with acid–base status recovering within 120 min in air. Throughout hypoxic exposure and recovery, changes in [HCO_3^-] were matched by those in [La^-], indicating that anaerobic glycolysis is a key factor determining the metabolic alterations and overall acid–base status. Further, the blood gas and Hct values recovered in air and unchanged embryo mass suggest that the hypoxia-induced disturbances were only transient and may not affect long-term survival.

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1. Introduction

Hematocrit (Hct) is a function of mean corpuscular volume (MCV), red blood cell concentration ([RBC]) and plasma volume (PV). Increasing [RBC], and thus Hct, during hypoxia to maintain adequate tissue O_2 delivery would appear to be a logical response. Yet, previous studies have demonstrated that the Hct increase during 1 day of moderate hypoxia in developing embryos is largely created through changes in MCV and is therefore unlikely related to O_2 transport (e.g., Ackerman, 1970; Burggren et al., 2012). In addition, contribution of PV to changes in Hct, i.e., $\Delta Hct = \Delta[RBC] + \Delta MCV + \Delta PV$, was little (Burggren et al., 2012). Hypoxia-induced disturbances of blood acid–base balance can alter Hct through changes in MCV, which is under the influence of a multitude of factors. These include potassium-chloride co-transport, taurine transport and sodium-dependent beta-amino acid transport systems modulated via changes in osmolality (Osm), P_{O_2}, P_{CO_2} and pH (for reviews see Cossins and Gibson, 1997; Nikinmaa, 1992 and Hoffmann et al., 2009).

The avian embryo allows acid–base responses to hypoxia and hypercapnic hypoxia to be studied in a relatively simple vertebrate system that lacks mechanical ventilation (gas exchange is via diffusion only) and possesses a relatively underdeveloped renal system (e.g., metanephric development continues after hatching – Romanoff, 1960; Carretero et al., 1995). Accordingly, the embryo cannot employ respiratory compensation and potentially renal compensation to combat acid–base imbalances. Uncompensated metabolic acidosis results when day 15 (d15) and d17 embryos are exposed to moderate hypoxia (15% O_2) for 1 day (Burggren et al., 2012). In contrast, uncompensated respiratory acidosis results from additionally exposing embryos to hypercapnia (i.e., hypercapnic hypoxia: 5%CO_2, 15%O_2). In both cases, an increase in Hct occurred due to an increase in both MCV and [RBC] in d15 embryos (Burggren et al., 2012). These data indicate that low O_2 concentration is a strong driver for Hct regulation in the presence or absence of CO_2. However, Hct changes during alterations of ambient O_2 or CO_2

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http://dx.doi.org/10.1016/j.resp.2012.08.002

should be interpreted within a blood acid–base balance framework because of the complex interactions between the acid–base and hematological respiratory systems. Further, because both 'normal' blood acid–base balance and Hct and their responses during perturbations (such as hypoxia) change as maturation progresses (e.g., Tazawa, 1980; Tazawa et al., 1992, 2011; Burggren et al., 2012), full understanding of the effects of acute hypoxia (or hypercapnic hypoxia) requires a developmental context.

The acid–base and Hct responses of embryos when challenged by severe hypoxia are as yet unknown. Early chicken embryos (<d6) tolerate severe hypoxia (10%) surviving >240 min. However, after ~day 6, profound bradycardia develops during severe hypoxic exposure, often ending in death. Additionally, survival time in such conditions declines with age (e.g., ~160 min on d9) (Akiyama et al., 1999 and Andrewartha et al., 2011a for review). It is likely that severe acid–base disturbances during this time-course (>3 h on d9) prove to be ultimately fatal and that the relatively greater metabolism of older embryos will result in critically low pH and/or bicarbonate levels. Thus, the present study elucidates how acute, severe hypoxia (10% O_2, with and without 5% CO_2) affects Hct and blood acid–base balance in late prenatal (d11–d19) embryos. The time-course of the resulting Hct changes and blood acid–base disturbances are examined in detail in d15 embryos, with the goal of further revealing the scope and nature of embryonic acid–base and hematocrit regulatory mechanisms. We hypothesize that the Hct of developing embryos will increase during severe hypoxia (10% O_2) and hypercapnic hypoxia (10%O_2, 5%CO_2) exposure due to an increase in both MCV and [RBC], as occurs at milder hypoxia levels in d15 embryos (Burggren et al., 2012). However, because 10% O_2 is low enough to induce anaerobic glycolysis, it is additionally hypothesized that the metabolic acidosis will be more predominant in severe hypoxia (and hypercapnic hypoxia) due to increased lactate. In some bird species, eggs are laid in a burrow nest where developing embryos are exposed to a hypercapnic environment. Thus, the effect of hypercapnia (5%CO_2) in hypoxia (10%CO_2) is not only of use in revealing emerging regulatory mechanisms, but is additionally an interesting subject to be examined from comparative point of view.

2. Materials and methods

2.1. Incubation of eggs

Fertile eggs of the domestic fowl (*Gallus gallus domesticus*) were obtained weekly from Texas A&M University (College Station, TX, USA). Eggs were weighed (±0.01 g) and then incubated at 37.5 ± 0.1 °C and relative humidity of ~55% in a forced draught incubator (1502, G.Q.F. Manuf. Co., USA). The eggs were placed vertically on an automatic turning tray which rotated the eggs every 3 h.

2.2. Blood collection and analysis

Blood was collected from the allantoic vein. A 6–8 mm diameter region of the eggshell was removed and the underlying allantoic vein gently lifted by forceps through the hole in the eggshell. Allantoic venous values represent "arterialized" values, since an allantoic vein is analogous to a vein in the pulmonary circulation (Piiper et al., 1980). Consequently, values measured are presented with the subscript "a" as for systemic arterial values. Approximately 0.15–0.35 mL of blood was collected from d11, d13 and d19 embryos and ~0.3–0.6 mL from d15 and d17 embryos, using a 1 mL plastic syringe with a fixed needle to minimize a dead-space and flushed in advance with heparinized saline (100 mg in 100 mL saline).

All embryos are used only once due to the limited blood capacity of the embryos (i.e., there was no serial sampling in this study). Sampled eggs were subsequently euthanized via exposure to a cold, anoxic environment and the embryos were removed from their eggshell. The yolk and extra-embryonic membranes were then removed, and embryo body mass measured (±0.01 g) with an electronic balance.

Collected blood was gently transferred into a 2 mL plastic vial and ~0.12 mL of blood withdrawn from the bottom of the vial to minimize contact of blood with air. This sample was immediately measured for pH, P_{CO_2} and [HCO_3^-] (calculated by the analyzer from pH and P_{CO_2}) with a blood gas analyzer (ABL5, Radiometer Medical A/S, Copenhagen, Denmark) at 37 °C. The blood was then inverted several times in the vial to ensure thorough mixing, and [RBC] and hemoglobin concentration ([Hb], g%) determined by a blood cell counter (Coulter analyzer, $A^c{\cdot}10$ T, Beckman, USA) and osmolality (Osm, mmol kg^{-1}) by a vapor pressure osmometer (Vapro 5520, Wescor, USA). Duplicate preparations of 60 μL of blood were transferred into sealed hematocrit tubes and centrifuged for 4 min at 10,000 rpm and the mean Hct determined (±0.1%, Readacrit Centrifuge, Becton Dickinson, USA). When the collected blood volume was insufficient to measure all variables (~0.26 mL), either blood gas variables and Osm were measured or the variables determined by the Coulter analyzer ([RBC] and [Hb]). Although Hct could have been determined using the Coulter analyzer, the values reported in this study were determined via centrifugation because the Coulter analyzer underestimates Hct, particularly during hypoxic exposure. Thorough comparison of these two methodologies is undertaken in the Appendix.

Mean corpuscular indices (MCV, μ^3), mean corpuscular hemoglobin (MCH, pg) and mean corpuscular hemoglobin concentration ([MCHb], g%)) were calculated by equations from Tazawa et al. (2011), where Hct is the value obtained via centrifugation (%), [Hb] is the value determined from the Coulter analyzer (g%) and [RBC] (10^6 μL^{-1}) is calculated from an individual value determined by the Coulter analyzer, using a previously obtained regression equation relating values simultaneously determined using the Coulter analyzer and hemocytometry (Tazawa et al., 2011).

A previously constructed Davenport (pH-[HCO_3^-]) diagram (Burggren et al., 2012) was used to depict acid–base balance. Hb, the important non-carbonate buffer in blood, increases during the last half of incubation (Tazawa et al., 2011). Therefore, the blood buffer value is also predicted to increase during embryonic development (as demonstrated in Erasmus et al., 1970/71; Tazawa and Piiper, 1984; Tazawa, 1986). Previous studies have not always demonstrated this increase (e.g., Tazawa et al., 1983; Andrewartha et al., 2011b). A value of −16.0 mmol L^{-1} pH^{-1} was used in this report (as determined in Burggren et al., 2012).

2.3. Acute hypoxic (10%O_2) challenges in developing embryos (d11–d19)

Eggs were candled to locate the allantoic vein on one of d10, d12, d14, d16 and d18, and transferred to a desk-top incubator warmed at 37.5 °C and ventilated with air at a relative humidity of ~60% (Hova-Bator 1590, G.Q.F. Manuf. Co., USA). The eggs were placed on a cardboard holder in the incubator for one further day. On the following target day (one of d11, d13, d15, d17 or d19), the eggs were randomly divided into control or experimental groups. The control eggs remained in the incubator until blood collection. The experimental eggs were transferred to a 3.78 L (26.8 cm × 29.7 cm) plastic Ziploc® bag (referred to as the gas-exposure bag) placed in the incubator. The gas-exposure bag was fitted with diagonally placed inlet and outlet conduits and was ventilated in advance at a rate of ~600 mL min^{-1} with hypoxic gas mixture provided by a Wösthoff gas mixing pump (oHG, Bochum, Germany) (after

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