Respiratory Physiology & Neurobiology 242 (2017) 19–29

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

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

The cessation of breathing in the chicken embryo during cold-hypometabolism

Ryoji Ide, Satoko Tomita Ide, Jacopo P. Mortola*

Department of Physiology, McGill University, Montreal, QC, Canada

ARTICLE INFO

Article history:
Received 14 September 2016
Received in revised form 22 February 2017
Accepted 8 March 2017
Available online 21 March 2017

Keywords:
Breathing pattern generator
Breathing frequency
Control of breathing
Hypothermia
Respiratory arrest
Respiratory rhythm

ABSTRACT

The avian embryo toward end-incubation combines gas exchange through the chorioallantoic membrane (CAM) and pulmonary ventilation ($\dot{V}E$). The main experiments examined breathing activity during cold-hypometabolism. Chicken embryos close to hatching were prepared for simultaneous measurements of oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$; open-flow methodology) and breathing frequency (*f*; barometric technique). As ambient (Ta) and egg temperature (Tegg) dropped, breathing eventually ceased at ~18 °C, when $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were 22–28% of the normothermic values. With the eggshell experimentally covered to reduce CAM gas exchange breathing ceased at slightly lower $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ (17–18% of normothermia). Once breathing had stopped, egg exposure to hypoxia (10% or 5% O_2) or hypercapnia (3% or 8% CO_2) did not resume breathing, which recovered with re-warming. In normothermia, 10% O_2 caused hypometabolism and tachypnea; differently, in 5% O_2 $\dot{V}_{O_2}$ dropped as much as with hypothermia and breathing stopped, to recover upon return in air. Correlation analysis among Ta, Tegg, $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and *f* during cooling and re-warming indicated that *f* followed more closely the changes in $\dot{V}_{O_2}$ and, especially, in $\dot{V}_{CO_2}$ than the changes in Ta or Tegg. Some considerations suggest that in this experimental model the cessation of breathing in hypothermia or severe hypoxia may be due to hypometabolism, while the lack of chemo-responses may have a different mechanistic basis.

1. Introduction

In many vertebrates pulmonary ventilation ($\dot{V}E$) is the sole mode of gas exchange. Therefore, one should expect a close relationship between changes in metabolic rate and $\dot{V}E$. Indeed this was recognised more than a century ago (Haldane and Priestley, 1905) and confirmed thereafter, predominantly by measurements during muscle exercise, which is the most extensively studied condition of a rise in metabolic rate. For exercise intensities of moderate degrees, that is, before lactic acidosis provides an additional stimulus on $\dot{V}E$, arterial CO_2 remains nearly constant (Forster et al., 2012), meaning that changes in $\dot{V}E$ adequately match the increased metabolic activities. The coupling between metabolism and $\dot{V}E$ has since been recognised in many other hypermetabolic conditions, from hormonal and pharmacological stimulations, to hypernutrition, pregnancy and cold-induced thermogenesis. In addition, the closeness of the metabolism-$\dot{V}E$ relationship is observed in various cases of oscillatory or decreased metabolic rate, like hypoxic hypometabolism, daily metabolic rhythms, undernutrition, hypothyroidism and, perhaps, in some mammals during torpor and hibernation (Mortola and Maskrey, 2011). Despite the abundance of data on the correlation, the fundamental issue that remains unresolved is the mechanism that permits the link between the body's metabolic activities and breathing.

An interesting deviation from the metabolism-$\dot{V}E$ relationship occurs when the lung is not the sole mechanism for gas exchange, because extra-pulmonary structures contribute to gas exchange. In conscious and resting adult sheep with extracorporeal circulation through a membrane lung, $\dot{V}E$ decreased and eventually stopped as the extracorporeal exchanger removed CO_2 at the same rate of the animal's metabolic production, with normal arterial blood gases (Phillipson et al., 1981). Newborn goats and lambs similarly instrumented produced analogous results (Kolobow et al., 1977, 1978; Kuipers et al., 1992, 1997; Praud et al., 1997; Kozuma et al., 1999). One interpretation could be that the relevant factor in the control of $\dot{V}E$ was that component of gaseous metabolism that exceeded the fraction exchanged by the extracorporeal circuitry. The same conclusion emerged from measurements in the Julia Creek dunnart, a marsupial that for the first weeks after birth exchanges the respiratory gases through both skin and lungs (Mortola et al.,

* Corresponding author at: Dept. of Physiology, McGill Univ., 3655 Promenade Sir William Osler, Room 1121, Montreal, Quebec, H3G 1Y6 Canada.
E-mail address: jacopo.mortola@mcgill.ca (J.P. Mortola).

http://dx.doi.org/10.1016/j.resp.2017.03.004
1569-9048/

1999). In this newborn a drop in temperature decreased oxygen consumption ($\dot{V}_{O_2}$) and $\dot{V}E$; the latter eventually stopped when $\dot{V}_{O_2}$ was sufficiently low for the skin to fulfil the animal's gas exchange requirements (Frappell and Mortola, 2000).

The avian embryo toward end incubation is another natural case of a dual gas exchanger. For most of incubation the chorioallantoic membrane (CAM) guarantees the gas exchange of the embryo inside the egg. The CAM is a predominantly vascular structure that, in combination with the porosity of the eggshell, permits diffusion and convection of the respiratory gases (Tullett and Deeming, 1982). Toward end incubation the embryo pips into the egg air cell and $\dot{V}E$ begins, initiating pulmonary gas exchange. Differently from mammals, which have simultaneous gas exchange through placenta and lungs for only a few minutes at birth, in birds the dual gas exchange of CAM and lungs lasts the many hours of the hatching process (Mortola, 2009). Because avian embryos are nearly ectotherms, decreasing the ambient temperature lowers the embryo's $\dot{V}_{O_2}$. The expectation was that in this animal model, similarly to the experiments mentioned above, breathing activity would decrease as $\dot{V}_{O_2}$ dropped and would eventually cease when non-pulmonary means entirely fulfilled the gas exchange requirements. Hence, the relationship between the total embryo's $\dot{V}_{O_2}$ and $\dot{V}E$ should have an intercept on the $\dot{V}_{O_2}$ axis that corresponds to the $\dot{V}_{O_2}$ through the CAM. This test, and a parallel one with the eggshell partially covered to reduce the diffusion capacity of the CAM, represented the initial object of the current study; the experimental results confirmed the expectation. Cessation of breathing could reflect absence of adequate stimuli to the chemoreceptors or a central inability to generate a respiratory output despite the presence of chemical stimuli. Therefore, once breathing had stopped, we asked whether respiratory stimuli (hypoxia and hypercapnia) could get breathing to re-start. The answer to this question was negative. Finally, because the cessation of breathing of the main study group occurred by lowering body temperature, we tried to sort out whether in this animal model the hypothermia or the hypometabolism was the more likely cause of the breathing arrest. The results could be interpreted either way; hence, this last question did not obtain a definitive answer.

2. Methods

The core of the experiment was on 40 chicken embryos close to hatching (internal pipping phase, IP, defined as the presence of the embryo's beak inside the air cell after piercing the inner membrane). It consisted in measurements of the gaseous component of metabolism (oxygen consumption, $\dot{V}_{O_2}$, and carbon dioxide production, $\dot{V}_{CO_2}$) and breathing frequency (*f*) as ambient (Ta) and egg (Tegg) temperatures were progressively lowered until breathing stopped. Then, in separate groups of embryos, we examined the *f* response to changes in chemical stimuli in normothermia and at breathing cessation in hypothermia. Finally, the comparison of the iso-time loops between Ta, Tegg, $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and *f*, constructed during cooling and re-warming were examined to sort out the role of temperature versus gaseous metabolism on the embryo's *f*.

2.1. Egg preparation

Measurements were conducted on chicken (*Gallus gallus*) embryos of the White Leghorn variety. At midday (embryonic day zero, E0) the freshly laid eggs were placed in a still air incubator (Hova-Bator model 1602, Savannah, GA, USA) at the temperature of 37.5 °C and 60% relative humidity, both monitored by a data logger (Hobo®, Onset Computer Corp., Bourne, MA), with a 90° egg rotation eight times a day. On the day of the experiment, E20 (out of ~20.5 days of total incubation), the air cell was identified by transillumination and its contour marked. A small opening (~5 mm ID) was drilled through the eggshell at the blunted end of the egg to access the air cell and to verify visually that the embryo was at the IP stage. A fine tungsten-constantan thermocouple measured egg temperature (Tegg) as a proxy for the embryo's body temperature. To this end, the thermocouple, via the eggshell hole, was threaded through the inner membrane along the embryo's body and secured with its tip at approximately half egg height. If the process caused bleeding, the embryo was disregarded. A plastic cap covered the region of the eggshell hole, secured with dental cement. The cap had four leads (Fig. 1); two were for passage of gases, one for recording the breathing-related pressure (P) oscillations and the remaining one for calibration of the P signal with a micro-syringe.

The egg was placed in a 120-ml plastic container, totally sealed, and submerged into a circulating water bath that provided the desired temperature (37.5 °C). A transmitter powered by an energizer-receiver unit (4000E, Minimitter, Sunriver, OR) measured the temperature of the chamber (ambient temperature, Ta) by telemetry. The chamber had inflow-outflow leads for the passage of gas flow. At the end of the measurements, irrespective of the experimental protocol adopted, the egg was opened to verify its wellbeing.

2.2. Measurements of $\dot{V}_{O_2}$

Oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were measured by an open flow methodology adapted to the chicken embryo (Mortola and Labbè, 2005; Szdzuy et al., 2008). When the circuit through the air cell and its cap was blocked by two stopcocks (Fig. 1), a negative pressure pump located downstream the circuitry maintained a flow of 100 ml/min through the respirometer, under the control of a mass flow meter (Sable Systems International Fox, Henderson, NV). The turning of the stopcocks directed the gas flow either through the respirometer or through the air cell. Calibrated gas analyzers (Sable Systems International Fox, Henderson, NV) arranged in series recorded continuously the outflow O_2 and CO_2 concentrations, after the gas had passed through a drying column (anhydrous calcium sulfate, Drierite®, Hammond, Xenia, OH); the inflow concentrations were monitored intermittently. The inflow lid of the respirometer was connected to an impermeable 15 l or 30 l bag filled with the gas mixture of interest. A computer monitor continuously displayed the gas flow, O_2 and CO_2 concentrations and Ta. The gas fractional concentrations were mathematically corrected for the error introduced by a respiratory exchange ratio different from unity (Depocas and Hart, 1957; Mortola and Besterman, 2007); then, $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ corresponded to the product of flow rate and inflow-outflow O_2 (or CO_2) concentration difference. These values were calculated at standard temperature, pressure and dry conditions in μl/min. Depending on whether the gas flow was directed through the respirometer or through the air cell, $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were taken to represent the values of, respectively, total or pulmonary gas exchange. The time needed by the analyser to sense a rapidly injected bolus of CO_2 into the respirometer was ~20 s. The time for complete washout of the respirometer (with the egg inside) and connecting tubings was ~4.5 min. Both times were adequate for the measurements performed, which occurred in steps of about 45 min (see "*2.4. Protocols*").

2.3. Breathing

A high-sensitivity P transducer recorded the pressure (P) signal originated by the embryo's breathing, displayed on a computer monitor and acquired on line (100 Hz) after appropriate analog-digital conversion. The number of oscillations per unit time