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Role of brain nitric oxide in the thermoregulation of broiler chicks

A.C. Coleone [a], K.A.A. Torres [a], E.C. Carnio [b], L.H. Gargaglioni [a], M. Macari [a], R.L. Furlan [a], K.C. Bícego [a,*]

[a] Department of Animal Morphology and Physiology, College of Agricultural and Veterinarian Sciences, São Paulo State University, Jaboticabal, SP, 14884-900, Brazil
[b] Nursing School of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, 14040-902, Brazil

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ABSTRACT

Nitric oxide (NO) plays a key role in body temperature (Tb) regulation of mammals, acting on the brain to stimulate heat loss. Regarding birds, the putative participation of NO in the maintenance of Tb in thermoneutrality or during heat stress and the site of its action (periphery or brain) is unknown. Thus, we tested if NO participates in the maintenance of chicks' Tb in those conditions. We investigated the effect of intramuscular (im; 25, 50, 100 mg/kg) or intracerebroventricular (icv; 22.5, 45, 90, 180 μg/animal) injections of the non selective NO synthase inhibitor L-NAME on Tb of 5-day-old chicks at thermoneutral zone (TNZ; 31–32 °C) and under heat stress (37 °C for 5–6 h). We also verified plasma and diencephalic nitrite/nitrate levels in non-injected chicks under both conditions. At TNZ, 100 mg/kg (im) or 45, 90, 180 μg (icv) of L-NAME decreased Tb. A significant correlation between Tb and diencephalic, but not plasma, nitrite/nitrate levels was observed. Heat stress-induced hyperthermia was inhibited by all tested doses of L-NAME (im and icv). Tb was correlated neither with plasma nor with diencephalic nitrite/nitrate levels during heat stress. These results indicate the involvement of brain NO in the maintenance of Tb of chicks, an opposite action of that observed in mammals, and may modulate hyperthermia.

1. Introduction

Nitric oxide (NO) is a free radical gas that mediates a wide range of physiological events including regulation of arterial blood pressure, macrophage defense mechanisms, intercellular communication, pro-inflammatory and anti-inflammatory effects as well as thermoregulation (*cf.* Toda and Ayajiki, 2006; *cf.* Bícego et al., 2007; Guerra et al., 2008). It is well established that NO plays a key role in body temperature (Tb) regulation of mammals, acting on the central nervous system (CNS) to stimulate autonomic heat dissipation responses during heat stress, fever and anapyrexia (Eriksson et al., 1997; Steiner et al., 2001; 2002; Schwimmer et al., 2004). Recently, we demonstrated in toads that NO plays an important role in the CNS to modulate the behavioral thermoregulatory mechanism involved in the development of hypoxia-induced anapyrexia, a regulated reduction of Tb (Guerra et al., 2008).

Endogenous NO is produced by the conversion of L-arginine into L-citrulline and NO, a reaction catalyzed by the enzyme NO synthase (NOS) (Moncada et al., 1991), which can be found as three different isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (Bredt et al., 1991; Moncada et al., 1991). NOS can be found throughout the mammalian brain including neurons and glia (Forstermann et al., 1990; Wiesinger, 2001).

In birds, NO causes vasodilatation (Weidong et al., 2002; Wang et al., 2002), similar to that observed in mammals (Palmer et al., 1987), and its decreased release in pulmonary vessels has been implicated in the endotoxin-induced pulmonary hypertension in broilers (Martinez-Lemus et al., 1999; Wideman et al., 2004). Additionally, sensory transmission, such as olfactory and visual, and memory consolidation are important functions modulated by NO in the CNS of birds (for a review see Toda and Ayajiki, 2006). Regarding thermoregulation, Gray et al. (2005) performed intra abdominal injection of 200 mg/kg of L-NAME in ducks and observed no effect on Tb but inhibition of the lipopolysaccharide-induced fever. However, no data exists about the putative role of NO in thermoregulation of young birds and their response to heat stress as well as the site of NO action (periphery or the CNS). In view of these considerations, the aim of the present study was to test the hypothesis that NO is important for the maintenance of Tb of chicks at the thermoneutral zone and when submitted to heat stress. It is specifically at the beginning of life – immediately after hatching – that heat stress constitutes a critical factor for the development of thermoregulatory mechanisms in these animals, influencing the thermogenic and thermolytic responses in the adult phase (Arjona et al., 1988; 1990; Yahav and Hurwitz, 1996). Thus, we evaluated the effect of intramuscular injection and intracerebroventricular microinjection of the nonselective NO synthase inhibitor L-NAME on Tb of chicks in thermoneutrality and when exposed to a high ambient temperature. Furthermore, we

* Corresponding author. Departamento de Morfologia e Fisiologia Animal, Faculdade de Ciências Agrárias e Veterinárias de Jaboticabal, Universidade Estadual Paulista, Via de Acesso Prof. Paulo D. Castellane, s/n, 14884-900, Jaboticabal, São Paulo, Brazil. Tel.: +55 16 3209 2656; fax: +55 16 3202 4275.
E-mail address: keniacb@fcav.unesp.br (K.C. Bícego).

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evaluated the NO production by measuring plasma and diencephalic levels of the products of the NO metabolism, nitrite and nitrate, during these two conditions.

2. Material and methods

2.1. Animals

One-day-old male broiler chicks (*Gallus gallus* — Cobb 500®) were purchased from a commercial hatchery (Sertanejo Alimentos, Ipiguá, SP, Brazil). Chicks were reared as a group in a temperature controlled chamber (32 °C) with a 13 h:11 h light/dark cycle, and they were provided with a standard broiler starter diet (Rostagno et al., 2000) and water *ad libitum*. The study was conducted with 5-day old chicks (body mass of 60–90 g) in compliance with the guidelines set by SBCAL (Sociedade Brasileira de Ciência em Animais de Laboratório) and with the approval of the São Paulo State University Animal Care and Use Committee (Protocol # 019971-06).

2.2. Body temperature measurement

The body temperature of chicks was measured with a sensor (Yellow Springs Instrument, Co., OH, USA) that was inserted 3 cm through the cloaca inside the animal's rectum and connected to a telethermometer (model 45TUC, Yellow Springs Instrument Co.).

2.3. Drug and intracerebroventricular (icv) microinjection

L-NAME (N^G-nitro-L-arginine methyl ester; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline containing a 1% Evans blue solution (vehicle) and administered into the lateral ventricle using a Hamilton microsyringe according to the method of Davis et al. (1979) and based on the Tomonaga et al. (2005) study**.** For the microinjections, a dental injection needle (Mizzy, 200 μm OD) was connected to a 5 μL Hamilton syringe by a PE-10 tube. An acrylic device was used to hold the head of chicks. The injection (1 μL) was performed over a period of 30 s followed by a period of 20 s before the injection needle was removed to avoid reflux. This method seems not to be a stressful procedure since post injected chicks was previously demonstrated to present no change in feeding behavior and plasma corticosterone levels (Furuse et al., 1999; Saito et al., 2005). Furthermore, that procedure (except the insertion of the injection needle in the brain) was repeated during 50 s for 3 days before the day of the experiment in order to avoid stress of holding the animals' head.

All injections were performed using a microinjector machine (model 310, Stoelting, Wood Hale, IL, USA). The vehicle group was injected with a saline containing 1% Evans blue solution. A third group, the sham chicks, was similarly prepared, but no solution was injected.

At the end of the experiment, birds were deeply anesthetized with ketamine and brain removed to verify injection location. Data from individuals that did not present Evans blue in the lateral ventricle were not considered for the analyses.

2.4. Determination of nitrite plus nitrate (nitrite/nitrate) levels in plasma and in the central nervous system

Trunk blood and brain of chicks under thermoneutrality and submitted to heat stress were collected after decapitation. The blood sample was centrifuged (1000*g* for 10 min at 4 °C) and plasma stored at −70 °C. On the day of the assay, plasma samples were thawed and deproteinized with ethanol 95% at 4 °C for 30 min and then centrifuged (12,000*g* for 5 min at 4 °C). The supernatant was used to measure nitrite/nitrate, according to the NO/ozone chemiluminescence technique (Archer, 1993), using a Sievers NO Analyzer (Sievers 280 NOA; Sievers, Boulder, CO, USA). Sodium nitrate (Sigma) was used as standard reference.

To determine diencephalic concentrations of nitrite/nitrate, brain was dissected based on the stereotaxic atlas of chicks (Kuenzel and Masson, 1988), which was frozen under liquid nitrogen and stored at −70 °C. To determine nitrite/nitrate levels, the samples were homogenized on ice in 0.1 N acetic acid solution using a digital 600-W microprocessor cell disrupter (Virtis, Gardiner, NY, USA). The resulting homogenates were then centrifuged (12,000*g*, 10 min, 2 °C) and the pellets were processed for protein measurement (Perez et al., 1991). Sixty μL of ethanol (100 %) was then added to 30 μL of the supernatant, and after 30 min at 0 °C, the samples were centrifuged again (12,000*g*, 5 min, 25 °C). Five microliters of the supernatant obtained was injected into the NO analyzer (Sievers 280 NOA; Sievers, Boulder, CO, USA) for the determination of nitrite/nitrate. For protein determination, the pellets were diluted in 4 mL of sodium hydroxide (0.1 N) using a digital 600-watt microprocessor cell disrupter (Virtis). The solution obtained was assayed for protein determination using a Bio-Rad protein assay (Bio-Rad Laboratories; code number 500–0006). The diencephalic nitrite/nitrate levels are expressed as fmol/μg of protein.

2.5. Experimental protocols

Chicks were maintained in groups of approximately 20 animals per chamber (56.5 cm × 69.5 cm × 18 cm; width × length × height) during the experiments. For all protocols, the measurements of rectal temperature were repeated for 3 days before the day of the experiment in order to avoid handling stress. The number of animals used in each experimental group is presented between parentheses in the figures.

Protocol 1. Effect of intramuscular (im) injection of L-NAME or saline on body temperature (Tb) of chicks maintained in the thermoneutral zone (TNZ) or submitted to heat stress.

Experiments were conducted with animals maintained at 31–32 °C (TNZ) or 37 ºC (heat stress). L-NAME (25, 50 and 100 mg kg^{-1}) or saline was injected intramuscularly (hind limb). The control group received no injection. The doses of L-NAME were chosen on the basis of previous studies in rats (Steiner et al., 1998) and ducks (Gray et al., 2005). Tb was measured before and 1, 2, 3, 4, 5 and 6 h after injections. In the heat stressed group, L-NAME or saline were injected 30 min before the exposure to high temperature.

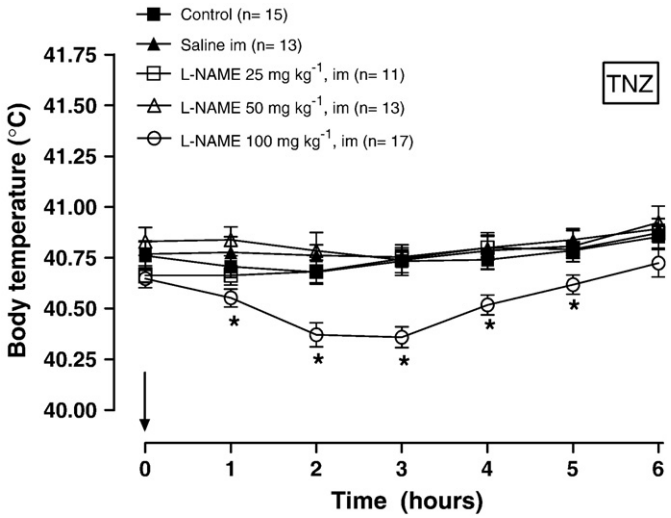

Fig. 1. Effect of intramuscular (im) injection of L-NAME (25, 50 and 100 mg kg^{-1}) or saline on body temperature of 5-day-old chicks maintained in the thermoneutral zone (TNZ; 31–32 °C; *Protocol 1*). Control: animals that received no injection. *n*: number of animals. Arrow indicates time of injection. All values are presented as mean ± S.E.M. *Significant ($p<0.05$) difference from saline group within each time.

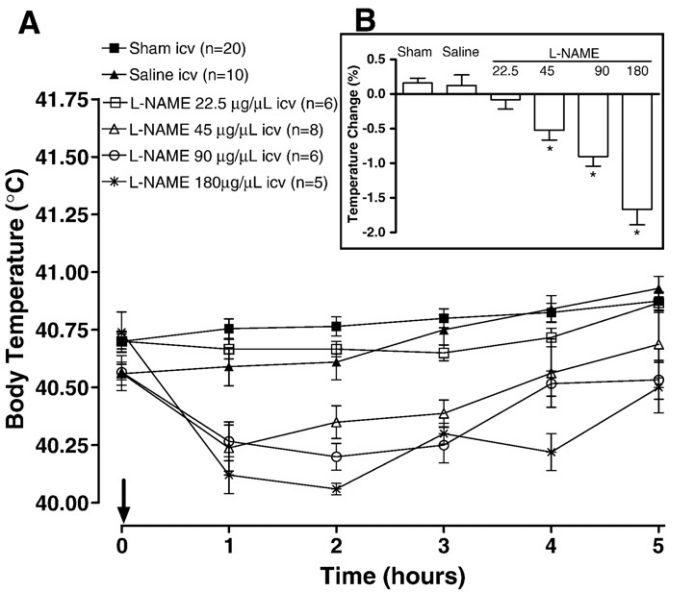

Fig. 2. A) Effect of intracerebroventricular (icv) microinjection of L-NAME (22.5, 45, 90 and 180 µg/µL/animal) or vehicle (saline containing a 1% Evans blue solution; 1µL) on body temperature of 5-day-old chicks maintained in the thermoneutral zone (TNZ; 31–32 °C; *Protocol 2*). B) Percentage change of body temperature from baseline (time 0) for each treatment at time 2 h. Sham: needle was introduced in the brain but no solution was injected. *n*: number of animals. Arrow indicates time of injection. All values are presented as mean ± S.E.M. *Significant ($p<0.0001$) difference from saline group (one-way ANOVA).

Protocol 2. Effect of intracerebroventricular (icv) microinjection of L-NAME or vehicle on Tb of chicks maintained in the TNZ or submitted to heat stress.

In the thermoneutral condition, animals received icv microinjection of 22.5, 45, 90 and 180 µg (volume of 1 µL) of L-NAME or vehicle. These doses were chosen on the basis of a previous study in rats (Steiner et al., 1998). In the heat stressed group, vehicle or the minor dose of L-NAME (22.5 µg/animal), which did not affect Tb in the TNZ, were injected 30 min before the exposure to high temperature. Tb was measured before and 1, 2, 3, 4, and 5 h after injections in both conditions. In the sham group the needle was introduced in the brain, but no solution was injected. Animals were habituated to the acrylic device during the three days before the experiment. Their head was held in the device for 50 s each day.

Protocol 3. Tb and plasma and diencephalic nitrite/nitrate levels of chicks maintained in the TNZ or submitted to heat stress.

Body temperature measurements and blood and diencephalon samples collection for nitrite/nitrate determination were performed on non injected animals in the TNZ (time 0; 31–32 °C) and 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 and 6 h after the beginning of high ambient temperature exposure (Ta = 37 °C).

2.6. Statistical analysis

The results are presented as means ± S.E.M. The effect of L-NAME on Tb of animals was analyzed through the analysis of variance (two-way ANOVA) for repeated measures. The effect of heat stress (times 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6 h) on the plasma and diencephalic nitrite/nitrate levels was analyzed through the one-way ANOVA. The Duncan's test was used for multiple comparisons. The relationship between Tb and nitrite/nitrate levels in the plasma and in the diencephalon of animals at the TNZ and submitted to heat stress was analyzed by a Correlation test. A pool of all times was taking into account for the Correlation tests during heat stress, since there was no significant correlation between Tb and nitrite/nitrate levels in each time analyzed one by one. Statistical analyses were conducted using a software program (SAS®; Littell et al., 2002). Differences were considered significant for values of $p<0.05$.

3. Results

In all experimental protocols the mean Tb of the 5-day-old chicks in resting condition was 40.7 ± 0.01 °C.

3.1. Role of nitric oxide in the Tb maintenance of chicks in the TNZ

Fig. 1 depicts the effect of im injection of L-NAME on Tb of chicks in the TNZ. Saline or L-NAME at the doses of 25 and 50 mg kg^{-1} did not affect Tb of chicks while the higher dose of L-NAME, 100 mg kg^{-1}, caused a significant reduction in Tb with a nadir at 2 and 3 h after injection (effect of treatment: $p<0.001$, $F_{4,64}=61{,}18$; effect of time: $p<0.001$, $F_{4,64}=15{,}42$; interaction treatment-time: $p<0.001$, $F_{4,64}=3{,}0$).

The effect of icv microinjection of L-NAME on Tb of chicks is shown in Fig. 2A. Body temperature of sham and vehicle groups did not change at the TNZ. Similarly, L-NAME at the lower dose (22.5 µg/animal) did not alter Tb of chicks when compared to control animals (sham and vehicle). On the other hand, icv microinjection of 45, 90 and 180 µg of L-NAME decreased Tb of chicks with a nadir between 1 and 2 h after injection (effect of treatment: $p<0.0001$, $F_{5,49}=18.19$; effect of time: $p<0.0001$, $F_{5,49}=24.33$; interaction treatment-time: $p<0.0001$, $F_{5,49}=4.35$). As can be seen in Fig. 2B, L-NAME caused a dose dependent reduction in Tb at the time 2 h ($p<0.0001$, $F_{(5,49)}=25.12$; one-way ANOVA).

Mean values and standard errors of Tb and nitrite/nitrate concentration in plasma and diencephalon of chicks that received no injection in thermoneutrality are shown in Table 1 (time 0). As can be seen in Fig. 3, diencephalic nitrite/nitrate levels correlate significantly with Tb values ($p<0.01$; Pearson $r=0.9187$; Fig. 3 top) while no correlation between Tb and plasma concentration of these metabolites was observed (Fig. 3 bottom).

3.2. Role of nitric oxide in the Tb regulation of chicks exposed to heat stress

Heat stress induced a significant increase in Tb of control and saline (im) treated animals with a peak 30 min after the beginning of heat

Table 1
Body temperature (Tb) and plasma and diencephalic nitrite/nitrate concentrations of chicks in the thermoneutral zone (time 0; Ta = 31–32 °C) and submitted to heat stress (times 0.25–6 h; Ta = 37 °C).

	Time (hours)									
	0	0.25	0.5	1	1.5	2	3	4	5	6
Tb (°C)	40.4 ± 0.08	41.3 ± 0.09*	41.4 ± 0.06*	41.4 ± 0.06*	41.3 ± 0.08*	41.3 ± 0.06*	41 ± 0.05*	41.2 ± 0.06*	41.2 ± 0.05*	41.2 ± 0.06*
Plasma nitrite/nitrate (µM)	18.2 ± 1.68	16.9 ± 1.9	18.2 ± 1.72	15.1 ± 2.16	13.5 ± 1.13	13.4 ± 0.78	14.5 ± 1.15	14.2 ± 1.62	12 ± 1.08	13.7 ± 1.97
Diencephalon nitrite/nitrate (fmol/µg protein)	3.2 ± 0.24	3.3 ± 0.26	2.7 ± 0.15	3.7 ± 0.45	3.4 ± 0.35	3.5 ± 0.42	3.8 ± 0.34	3.6 ± 0.57	3.3 ± 0.36	3 ± 0.39

All values are presented as mean ± S.E.M.
* Denotes difference from time 0 ($p<0.0001$).

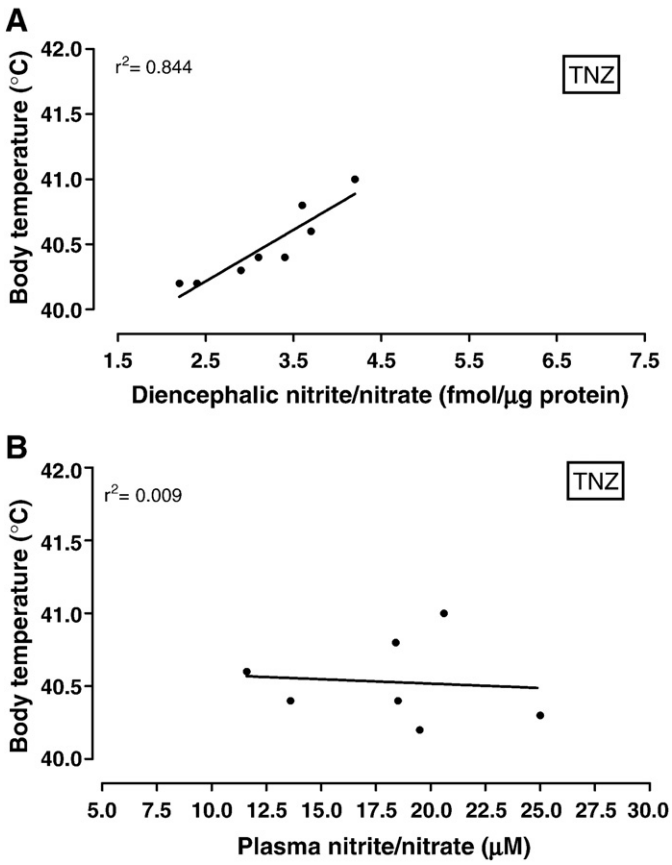

Fig. 3. Relationship between body temperature and nitrite/nitrate levels in plasma (A) and in the diencephalon (B) of individual 5-day-old chicks in the thermoneutral zone (TNZ; 31–32 °C; *Protocol 3*).

exposure (effect of time: $p<0.001$, $F_{4,74}=74.58$; Fig. 4). L-NAME (im) caused a dose dependent reduction of this hyperthermia (effect of treatment: $p<0.001$, $F_{4,74}=75.82$; interaction treatment-time: $p<0.001$, $F_{4,74}=4.14$). More specifically, L-NAME reduced the initial

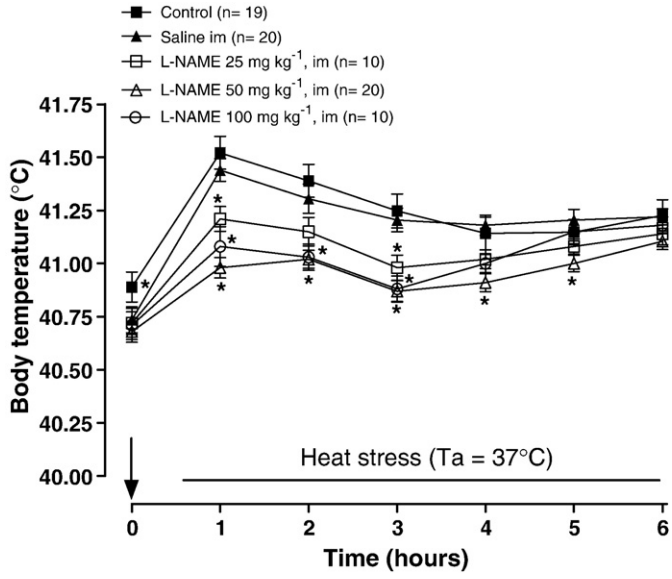

Fig. 4. Effect of intramuscular (im) injection of L-NAME (25, 50 and 100 mg kg^{-1}) or saline on body temperature of 5-day-old chicks exposed to heat stress (37 °C; *Protocol 1*). Control: animals that received no injection. *n*: number of animals. Arrow indicates time of injection. All values are presented as mean ± S.E.M. *Significant ($p<0.05$) difference from saline group within each time.

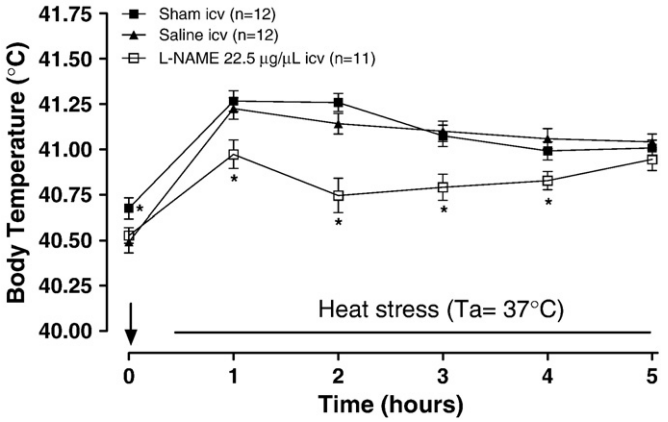

Fig. 5. Effect of icv microinjection of L-NAME (22.5 µg/µL/animal) or vehicle (saline containing a 1% Evans blue solution; 1µL) on body temperature of 5-day-old chicks exposed to heat stress (37 °C; *Protocol 2*). Sham: needle was introduced in the brain but no solution was injected. *n*: number of animals. Arrow indicates time of injection. All values are presented as mean ± S.E.M. *Significant ($p<0.05$) difference from saline group within each time.

response to heat stress but had little effect on the magnitude of the hyperthermia during the last 2 h (Fig. 4).

Exposure to high ambient temperature also caused a significant increase in Tb of sham and vehicle (icv) groups with a plateau between 30 and 90 min of heat stress (effect of time: $p<0.0001$, $F_{2,32}=56.74$; Fig. 5). As can be seen in Fig. 5, icv microinjection of

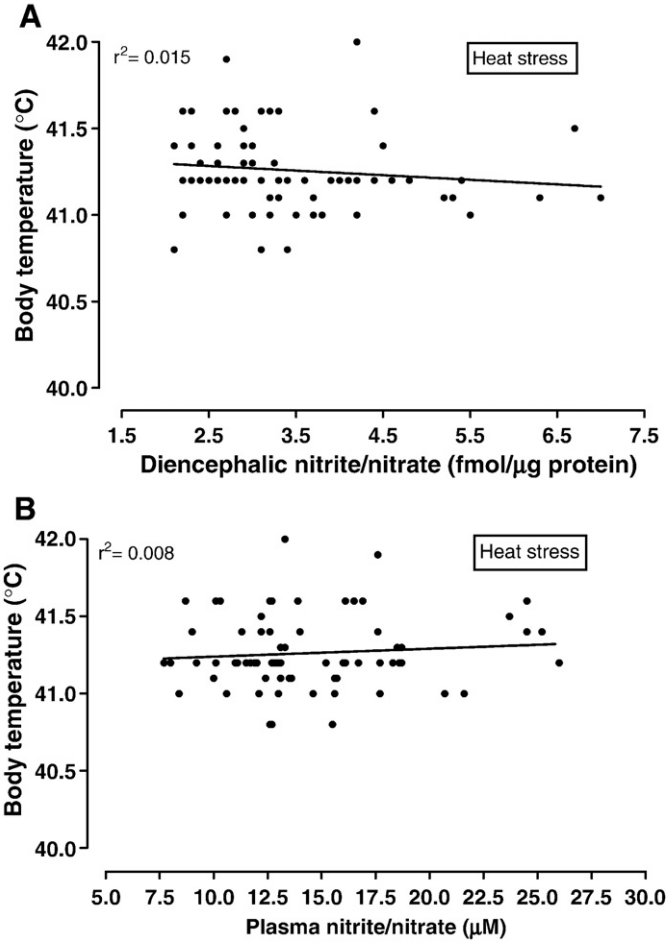

Fig. 6. Relationship between body temperature and nitrite plus nitrate levels in plasma (A) and in the diencephalon (B) of individual 5-day-old chicks exposed to heat stress (37 °C; *Protocol 3*).

22.5 µg of L-NAME inhibited significantly the heat stress-induced hyperthermia (effect of treatment: $p<0.001$, $F_{2,32}=10.04$; interaction treatment-time: $p<0.0001$, $F_{2,32}=4.45$).

Table 1 shows the effect of heat stress on Tb and plasma and diencephalic nitrite/nitrate levels of chicks that received no treatment. Tb of animals submitted to heat stress was higher than those at the TNZ ($p<0.0001$; $F_{9,68}=15.96$) but no significant effect of high temperature was observed in the plasma and diencephalic nitrite plus nitrate concentrations. Furthermore, there was no correlation between Tb and the levels of these metabolites in both diencephalon (Fig. 6 top) and plasma (Fig. 6 bottom) during heat stress.

4. Discussion

The present results indicate that NO is an important molecule acting on the CNS, for the maintenance of the elevated Tb of chicks under thermoneutrality and may participate in the heat stress-induced hyperthermia in chicks. The following facts support this notion, although we cannot exclude a peripheral effect of NO, even if limited: i) there is a positive correlation between Tb and the nitrite/nitrate levels in the diencephalic region of the animals' brain in the TNZ (Fig. 3 top) while no correlation between Tb and plasma nitrite/nitrate is observed (Fig. 3 bottom); ii) L-NAME injected icv causes reduction in Tb (Fig. 2) at doses 80–10 times inferior the lower dose injected im (which does not affect Tb in the TNZ but inhibit hyperthermia; Figs. 1 and 4). Moreover, L-NAME at the dose of 100 mg kg^{-1} (i.m.) decreases plasma nitrite/nitrate levels by 51%, but the resultant decrease in Tb does not correlate with this inhibition of NO production (data not shown).

4.1. Role of nitric oxide in the Tb maintenance of chicks in the TNZ

This scenario observed in chicks contrasts with that demonstrated for mammals, in which NO is considered to act on the CNS to induce heat loss responses and decrease Tb (Eriksson et al., 1997; Mathai et al., 1997; 2004; Steiner et al., 2001; 2002; Begg et al., 2007; Lacerda et al., 2005). Intracerebroventricular infusion of the NO donor SIN-1 in rabbits increases ear skin temperature, the frequency of panting and respiratory evaporative water loss (Eriksson et al., 1997). The opposite effect, i.e., a decrease in the rate of respiratory heat dissipation is observed after L-NAME treatment (Mathai et al., 1997). In rats, during physical exercise brain NO was also demonstrated to be an important molecule involved in the heat loss responses since icv microinjection of L-NAME causes a higher increase in Tb through rising body heat storage and tail thermal threshold for vasodilation (Lacerda et al., 2005). Moreover, NO is known to be an antipyretic molecule during fever caused by endotoxin (Steiner et al., 2001) by acting specifically on the preoptic region, the main thermoregulatory site in the CNS of mammals (Boulant, 1998, 2006) and perhaps of the majority of vertebrates (Bícego and Branco, 2002; see Bícego et al., 2007). Brain NO is also suggested to be a mediator of the typical suppression of the endotoxin-induced fever in near-term rats (Begg et al., 2007, 2008). Besides fever, the opposite response, anapyrexia – a regulated decrease of Tb – induced by hypoxia is dependent on the action of NO in the preoptic region (Steiner et al., 2002). NO is considered to be the most important mediator of hypoxic anapyrexia (see Branco et al., 2006). Interestingly, we recently reported that even in toads, an ectothermic vertebrate, NO is important for the development of hypoxia-induced behavioral anapyrexia by acting on the CNS (Guerra et al., 2008). Perhaps different mechanisms for thermoregulation have evolved in birds, such as an opposite thermoregulatory effect of NO. Supporting this notion, a study performed in ducks seems to corroborate our results since it was demonstrated that intra abdominal injection of 200 mg kg^{-1} of L-NAME reduces the endotoxin-induced fever, without any change in euthermic Tb, indicating a pyrogenic, but not antipyretic, effect of NO (Gray et al., 2005). Nonetheless, in the Gray's study it is still not clear if L-NAME effect is centrally or peripherally mediated.

It is intriguing to note that there is evidence that other systems also present different actions when comparing mammals and birds. In this context, the hormone ghrelin, which is known to cause increases in food intake (Wren et al., 2000; Tschop et al., 2000; Kamegai et al., 2000; 2001; Wren et al., 2001) and in respiratory quotient (Tschop et al., 2000) in rodents, was demonstrated to induce the opposite effects in birds, i.e., inhibition of food intake by acting on the CNS of neonate chicks and adult chicken and quails (Furuse et al., 2001; Saito et al., 2005; Shousha et al., 2005; Kaiya et al., 2009) and decrease in respiratory quotient in neonate chicks (Geelissen et al., 2006). Regarding the present study, as it was performed using young chicks further studies comparing the role of NO in thermoregulation of chicken of different ages are needed to isolate the influence of age in our results. However, it is important to mention that precocial birds, as it is the case of chickens, indeed present thermoregulatory capability just after hatching, since they are covered with down and can respond effectively to cold and to heat (Dawson and Whittow, 2000). Moreover, the study conducted by Gray et al. (2005; see the anterior paragraph) suggesting a contrasting role of NO during fever in ducks compared to mammals, was performed with animals of 2.4–3.5 kg instead of little ones.

Differences in thermoregulation between birds and mammals and even among the bird species have also already been reported. A number of studies performing lesions and using electrophysiological techniques report the key role of hypothalamus and the preoptic region of several species of birds in the control of Tb. However, the data obtained by thermal stimulation of hypothalamus-preoptic region are somewhat controversial. Hypothalamic warming of sparrows, emus and geese induces heat loss responses while hypothalamic cooling increases heat gain, a pattern similar to that observed in mammals. On the other hand, some species such as pigeons, penguins, quails and even chickens exhibit weak to inappropriate thermoregulatory responses to thermal stimulation of the preoptic region/anterior hypothalamus (for a review see Bícego et al., 2007). What is more, in ducks, appropriate cold and heat defense responses are observed after thermal manipulations of the lower midbrain-upper pontine brainstem level (Martin et al., 1981). Regarding our chicks, although the positive correlation between Tb and diencephalic nitrite/nitrate concentration indicate a central action of NO, preoptic area might not be the putative site where NO acts to increase Tb. In this regard, preliminary results of our laboratory show that there is only a few NADPH-diaphorase labeled neurons in this region of chicks under both conditions, TNZ or submitted to 1 h of heat stress (Bicego et al., in preparation).

4.2. Role of nitric oxide in the Tb regulation of chicks exposed to heat stress

In the present study we observed that L-NAME inhibited the hyperthermia induced by heat stress in chicks, an effect that seems to be mediated in the CNS. Intramuscular injection of 25 mg kg^{-1} and icv microinjection of 22.5 µg/animal of L-NAME had no effect on Tb in the TNZ (Figs. 1 and 2) but inhibited the increase of Tb during heat stress (Figs. 4 and 5). Considering that chicks presented a body weight of 62.1 ± 1.5 g, the dose of 25 mg kg^{-1} is about seventy times higher than that injected icv, indicating that the effect of the NOS blocker during heat stress is likely to be centrally mediated. In fact, at least in pigs, cats and dogs, the activity of brain NOS can be inhibited by intravenous injections of L-NAME at doses higher than 20 mg kg^{-1} (Traystman et al., 1995).

In the present study, we neither observed any significant change of nitrite/nitrate concentrations in both diencephalon and plasma induced by heat exposure (Table 1) nor detected any significant correlation between those variables and Tb (Fig. 6). The reason for these different

results (L-NAME effect on Tb – Figs. 4 and 5 – and no nitrite/nitrate concentration changes – Table 1 and Fig. 6) during heat stress is beyond our understanding. One could argue that L-NAME may affect other systems during heat stress such as a muscarinic receptor blockage (Buxton et al., 1993), besides NOS inhibition, to decrease Tb of chicks. At least in mammals, acetylcholine action in the anterior hypothalamus, preoptic region (Takahashi et al., 2001b) and supraoptic nucleus (Takahashi et al., 2001a) induces a decrease in Tb of rats. Moreover, ventromedial hypothalamus muscarinic cholinoceptors were demonstrated to be involved in the induction of tail heat loss during exercise in rats, preventing excess heat storage in this situation (Wanner et al., 2007). On the other hand, a recent study in pigeons demonstrated that microinjection of muscarinic agonist in the preoptic region increases brain temperature, whereas the antagonist causes the opposite effect (Komarova et al., 2008). Thus, further experiments are certainly needed to clarify this issue, and also verifying the effect of L-NAME on Tb during other thermal states, such as fever and anapyrexia, or on other thermoregulatory effectors, including panting, the major heat loss thermoeffector of birds (Furlan and Macari, 2002).

Intriguingly, even in mammals there are some contradictory results about the role of NO in heat stress. Rats under heat exposure present an increased nNOS expression in the hypothalamus (*cf.* Schwimmer et al., 2004) and an enhanced plasma nitrite/nitrate concentration (Sachidhanandam et al., 2002). However, icv microinjection of 7-NI, a selective nNOS inhibitor, reduces the temperature threshold for heat loss responses such as salivation (euhydrated rats) and salivation and tail vasodilation (hypohydrated rats) in heat stressed anesthetized animals (Schwimmer et al., 2004).

In regard to nitrite/nitrate values, Fig. 6 (stressed animals) shows a larger range of both diencephalic and plasma concentrations compared to Fig. 3 (animals in TNZ). Individual differences among birds may be the reason for this disparity. However, it is important to notice that the majority of the chicks exposed to heat stress (83.3 and 54.2% of chicks considering diencephalic and plasma concentration, respectively) presented nitrite/nitrate concentrations within the value range (2.2–4.2 fmol/μg protein for diencephalon and 11.6–20.6 μM for plasma) of that of thermoneutral ones.

To summarize, based on the present results, it seems that NO plays an important role in the CNS of chicks for the maintenance of Tb under thermoneutrality while its involvement in the thermoregulatory responses to heat stress remains to be determined. It is the first time that NO is demonstrated to act on the CNS of a bird to induce an opposite effect in Tb compared to mammals.

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