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Functional argument for the existence of an avian nitric oxide synthase in muscle mitochondria: Effect of cold acclimation

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ABSTRACT

We report the first evidence of a mitochondrial NO synthase (mtNOS) in bird skeletal muscle. In vitro, mtNOS activity stimulated by L-arginine reduced intermyofibrillar mitochondrial oxygen uptake and ATP synthesis rates, stimulated endogenous H_2O_2 generation, but had no effect on oxidative phosphorylation efficiency. Arginine-induced effects were fully reversed by L-NAME, a known NOS inhibitor. When ducklings were cold exposed for 4 weeks, muscle mitochondria displayed an increased state 3 respiration, a reduced H_2O_2 generation but no significant alteration in mtNOS activity. We conclude that mtNOS is expressed in avian skeletal muscle.

1. Introduction

With few exceptions, most of bird species develop great capacities for adaptive thermogenesis when chronically exposed to cold [1]. In birds, skeletal muscle is a major thermogenic tissue involved in both shivering activity and, in some species, in non-shivering thermogenesis (NST) [2]. The adaptive responses of skeletal muscle to long term cold exposure involve changes in oxygen and substrates delivery, mitochondrial content and oxidative phosphorylation activity, and heat production [3–6]. Non-contractile heat generation in birds may rely on the up-regulation of the avian uncoupling protein (avian UCP) gene [4,6–9], the activity of which is increased by cold-acclimation in muscle tissues [4,9]. Most of the attention in this field has thus been focused on this mitochondrial protein that might divert energy from ATP synthesis to thermogenesis when properly activated [4,9]. Although this theory has been recently questioned [5,10], little is known about other physiological modulators of the mitochondrial energy transduction in cold-exposed birds. In particular, the role of nitric oxide (NO) as a modulator of mitochondrial functions has not been investigated in avian models. NO is formed by the enzymatic oxidation of L-arginine [11] via NO synthase [12] which is present in most mammalian tissues [13]. In mammals, NO plays a crucial role in metabolic adjustments by virtue of its influence on mitochondrial electron transfer through the respiratory chain [14], and the presence of a NO synthase isoform in mitochondria (mtNOS) [15,16]. NO also controls mitochondrial biogenesis and metabolic rate [17–19], suggesting that it plays a key role in cell response to metabolic stress such as prolonged cold exposure. In this context, it has been reported that the regulation of mtNOS activity in liver mitochondria of rat exposed to cold was biphasic, being low in the early phase (1–2 weeks) favouring a calorigenic increase in oxygen uptake; while it was markedly increased after 3–4 weeks of cold exposure [19]. The modulation of mtNOS may thus contribute to regulate energy expenditure during cold exposure as it does in thyroid-dependent liver oxygen uptake in rats [17].

The first goal of this study was to provide functional arguments for the existence of a mtNOS activity in mitochondria isolated from duckling skeletal muscle by activation/inhibition of the mitochondrial functions using L-arginine as substrate and N^{ω}-nitro-L-arginine methyl ester (L-NAME) as nitric oxide synthase inhibitor [20]. In particular, we determined whether activation of mtNOS influenced mitochondrial oxidative phosphorylation efficiency and H_2O_2 production. Finally, we investigated whether chronic exposition to cold affects the activity of mtNOS and its effects upon mitochondrial metabolism.

2. Materials and methods

Birds were cared for following the French Code of Practice for the Care and Use of Animals for Scientific Purposes and the experimental protocols were approved by the French Ministry of Agriculture Ethics Committee. Newly hatched male Muscovy ducklings (*Cairina moschata*) were kept at thermoneutrality (30 °C at that age) for one week with food and water ad libitum before being randomly assigned to two experimental groups in which ducklings were reared either at 25 °C (thermoneutral controls, TN, $n = 6$) or at 4 °C (cold-acclimated group, CA, $n = 6$). After 4 weeks of thermal acclimation, a protocol known to stimulate the development of adaptive NST in skeletal muscle [2], ducklings were euthanized and gastrocnemius muscle was removed for mitochondrial extraction.

Intermyofibrilar mitochondria were isolated as described in Rey et al. using a classical procedure involving potter homogenization, protease digestion and differential centrifugations in isolation buffer containing 100 mM sucrose, 50 mM KCl, 5 mM EDTA, 50 mM Tris-base (pH 7.4) [4].

The functional activity of mtNOS was assessed taking advantage of the mtNOS-dependent inhibition of mitochondrial state 3 oxygen uptake by addition of L-arginine [20]. By adding increasing doses of L-arginine, we thus describe the kinetic inhibitory action of the mtNOS substrate upon state 3 respiration rates and calculated the half maximum inhibitory effects (K_i) in both groups of ducklings.

Oxygen uptake was determined polarographically at 38 °C, in an air-saturated respiration buffer (120 mM KCl, 1 mM EGTA, 3 mM Hepes, 2 mM MgCl$_2$, 5 mM KH$_2$PO$_4$, 0.3% BSA, pH 7.3). State 3 phosphorylative respiration was obtained with 5 mM succinate, 5 μM rotenone and 0.5 mM ADP. A second set of experiments was conducted with 0.3 mM TMPD and 4 mM ascorbate as substrate providing electrons to cytochrome-*c* oxidase. When indicated, activity of mtNOS was stimulated by increasing doses of L-arginine [20] and then inhibited by a specific NOS inhibitor (L-NAME) to verify the reversibility and specificity of the activation. We also measured the effect of L-NAME alone on state 3 respiration rate and determined a mtNOS functional activity index [21].

The ATP/O ratio was determined from the rate of ATP synthesis and oxygen consumption of isolated muscle mitochondria (0.3 mg/ml) respiring on succinate (5 mM plus 5 μM rotenone) in an hexo-kinase (1.5 U/ml) plus glucose (20 mM) ADP-regenerating system (ADP ranging from 5 to 100 μM) as previously described for bird mitochondria [5].

Mitochondrial H$_2$O$_2$ generation was measured as described previously [4] following the linear increase in fluorescence (λ_{ex} 312 nm and λ_{em} 420 nm) due to the oxidation of homovanilic acid (0.1 mM) by H$_2$O$_2$ in the presence of horseradish peroxidase (6 U/ml). Mitochondria (0.05 mg/ml) where incubated with 5 mM succinate in respiration buffer (38 °C). Effect of mtNOS activity on mitochondrial H$_2$O$_2$ production was assessed by addition of 3 mM L-arginine.

Statistical significance was determined by analysis of variance (ANOVA) for independent (effect of cold acclimation) or repeated (effect of L-arginine or L-NAME) values followed by PLSD tests or paired Student's t tests. Data are presented as mean ± S.E.M. with significance considered at $P < 0.05$.

3. Results

3.1. Activity of mtNOS in skeletal muscle mitochondria

As shown in Fig. 1A, state 3 respiration rate of intermyofibrillar mitochondria was significantly higher in CA than in TN ducklings
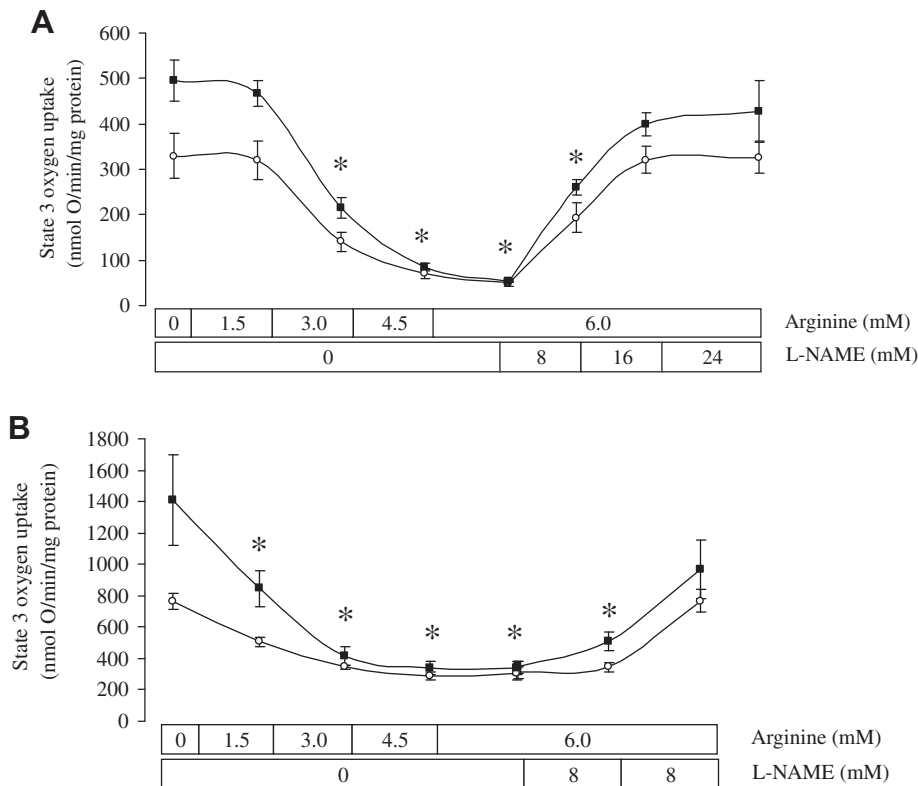

Fig. 1. Effect of NOS activity on the oxygen uptake of muscle mitochondria from thermoneutral controls (TN, open circles) or cold acclimated (CA, filled squares) ducklings. Experiments were carried out in metabolic state 3 with energized intermyofibrillar mitochondria respiring on succinate (A) or TMPD-ascorbate (B) as respiratory substrates. Sequential doses of L-arginine were then added in the incubation medium, followed by several additions of L-NAME. Values are means ± S.E.M. for $n = 5$ independent mitochondrial preparations.

(+50%, $P < 0.05$). Additions of increasing doses of L-arginine, aiming at activating mtNOS, gradually reduced state 3 respiration rate in both TN and CA groups, reaching 84% and 89% of inhibition in the presence of 6 mM L-arginine, in TN and CA ducklings, respectively (Fig. 1A). Note that a significant inhibitory effect of arginine on state 3 respiration was already observed from 3 mM. In both groups, the inhibitory effect of L-arginine was fully reversed by increasing doses of L-NAME (Fig. 1A). Interestingly, without prior addition of L-arginine, L-NAME (8 mM) alone led to a slight increase in mitochondrial state 3 respiration rate (+9% and +13% in TN and CA groups, respectively, $P < 0.05$), suggesting a persistent basal inhibition by mtNOS in isolated duckling mitochondria.

Similar patterns of variations were observed in both groups using TMPD-ascorbate as substrate, i.e., L-arginine-induced a dose-dependent inhibition of state 3 respiration rate (Fig. 1B). At 6 mM of L-arginine, mitochondrial respiration was reduced by 60% and 75% in TN and CA ducklings respectively. This inhibitory effect was slightly higher in CA than in TN ducklings ($P < 0.05$) and was entirely reversed by addition of L-NAME (Fig. 1B).

However, the half maximum inhibitory effects of L-arginine upon state 3 respiration (K_i) were not significantly different between TN and CA mitochondria respiring on either succinate ($K_i = 2.6 \pm 0.1$ vs 2.8 ± 0.2 mM in TN and CA, respectively; $P = 0.4$) or TMPD-ascorbate ($K_i = 1.4 \pm 0.2$ vs 1.6 ± 0.2 mM in TN and CA, respectively; $P = 0.4$). In addition, the mtNOS functional activity index [21] calculated with succinate as substrate was also not statistically different ($P = 0.15$) between TN (93 ± 5%) and CA (103 ± 4%) ducklings.

3.2. ATP/O ratios of skeletal muscle mitochondria

In both TN (Fig. 2A) and CA ducklings (Fig. 2B), 3 mM L-arginine significantly reduced the maximal rates of oxygen consumption and ATP synthesis (the highest points to the right of the linear relations) when compared with basal condition. The addition of L-NAME restored the rates of oxidative phosphorylation (oxygen consumption and ATP production) up to basal condition, in both TN and CA birds.

Fig. 2 also shows that the linearity of the relation between the rates of ATP synthesis and oxygen consumption was not affected by the presence of L-arginine or L-arginine plus L-NAME compared with basal condition. The superimposition of the three relationships in each experimental group clearly indicates that L-arginine-induced mtNOS activity did not alter the efficiency of mitochondrial oxidative phosphorylation, i.e., the amount of ATP molecules synthesized from ADP per amount of oxygen consumed.

3.3. Mitochondrial H_2O_2 production

As shown in Table 1, intermyofibrillar mitochondria from CA ducklings produced less H_2O_2 than mitochondria from TN birds (−42%, $P < 0.05$). Stimulation of mtNOS by addition of 3 mM L-arginine enhanced mitochondrial H_2O_2 production in both TN and CA ducklings. The relative increase in H_2O_2 production was higher in CA (+311%) than in TN (+177%) birds. Therefore, in the presence of L-arginine, the difference in H_2O_2 production between groups was abolished ($P = 0.43$). Nevertheless, in absolute values, the increases in H_2O_2 production were equivalent in both groups ($\Delta H_2O_2 = +2.7 \pm 0.3$ vs $+2.8 \pm 0.4$ nmol $H_2O_2 \cdot min^{-1} \cdot mg$ protein^{-1} in TN and CA birds respectively; $P = 0.71$).

4. Discussion

In the present study, we describe for the first time the effect of arginine and its inhibition on bird mitochondrial bioenergetics, an
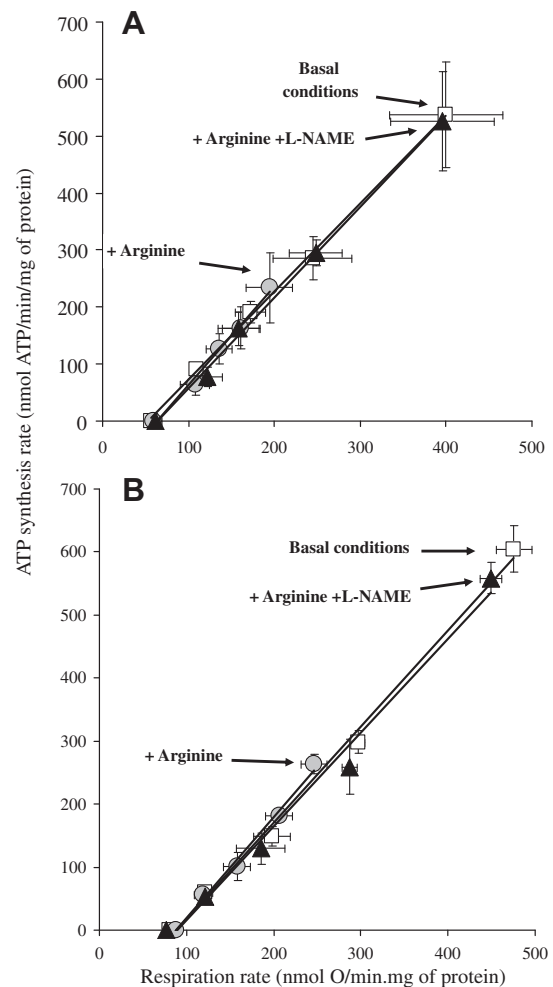

Fig. 2. Effect of L-arginine and L-NAME on the oxidative phosphorylation efficiency of muscle intermyofibrillar mitochondria from (A) thermoneutral controls (TN) or (B) cold-acclimated (CA) ducklings. Basal conditions (open squares) are obtained with succinate (plus rotenone) as respiratory substrate. Measurements were repeated in the presence of 3 mM arginine (grey circles) or 3 mM arginine plus 8 mM L-NAME (black triangles). Values are means ± S.E.M. for $n = 4$–5 independent mitochondrial preparations.

effect that can be ascribed to a functional mitochondrial NOS. We found that L-arginine-induced mtNOS activity reversibly inhibited oxidative phosphorylation and increased basal radical oxygen species production in intermyofibrillar mitochondria isolated from duckling skeletal muscle. These effects were not differentially affected by cold acclimation.

As previously reported for mammalian mitochondria [20], incubation of duckling muscle mitochondria with the substrate of mtNOS reversibly inhibited state 3 oxygen uptake. The inhibition was observed whatever the respiratory substrate (succinate or TMPD-ascorbate, Fig. 1). Since TMPD-ascorbate is a respiratory substrate that provides electrons directly to complex IV, this result suggests that NO competes with oxygen for cytochrome-*c* oxidase leading to a reduction in mitochondrial respiration in bird muscle mitochondria. These data in birds extend previous observations in mammals [22]. In relation with the lower activity of cytochrome-*c* oxidase, L-arginine increased mitochondrial endogenous H_2O_2 generation (Table 1). In line with previous studies conducted in mammalian models [20,23], this data suggests that L-arginine-induced NO production modifies the redox state of cytochromes within the electron transport chain, leading to an upregulation of superoxide production in bird muscle mitochondria. These reversible

Table 1
Activation of mitochondrial H$_2$O$_2$ production by L-arginine (3 mM) addition in thermoneutral (TN) or cold acclimated (CA) ducklings. Mitochondria were treated as described in Section 2. Values are means ± S.E.M. for n = 5–6 independent mitochondrial preparations.

Mitochondrial H$_2$O$_2$ production (nmol H$_2$O$_2$·min^{-1}·mg protein^{-1})			
Condition	Thermoneutral controls (TN)	Cold acclimated (CA)	% Variation
State 4	1.56 ± 0.26	0.90 ± 0.15	−42%, $P < 0.05$
L-Arginine	4.31 ± 0.52	3.70 ± 0.52	−14%, NS
% Variation	+177%, $P < 0.05$	+311%, $P < 0.01$	

effects of L-arginine on mitochondrial metabolism supports the presence of a functional mitochondrial NO synthase in duckling skeletal muscle.

L-Arginine-dependent negative modulation of oxidative phosphorylation led to decrease mitochondrial ATP production in duckling intermyofibrillar mitochondria. This confirms and extends previous observations in mammals [24,25]. However, in mammals, NO was reported to increase mitochondrial oxidative phosphorylation efficiency in vitro [25], a characteristic that was used to support a physiological role for NO in the improvement of cellular metabolic efficiency in vivo [26]. In birds, by contrast, the superimposition of the relations between the rates of ATP synthesis and oxygen consumption in Fig. 2 clearly indicates that although the mitochondrial fluxes were lowered by L-arginine-induced mtNOS activity, the oxidative phosphorylation efficiency, i.e., the amount of ATP synthesised per oxygen consumed, remained unchanged. Therefore, contrary to rat liver mitochondria [25], NO does not seem to modify the mitochondrial oxidative phosphorylation efficiency in bird skeletal muscle. This finding somehow contradicts the idea that the mitochondrial oxidative phosphorylation efficiency would be inversely proportional to the electron flux through the cytochrome-c oxidase [25]. Whether this discrepancy results from tissue specificity (liver vs skeletal muscle) or phylogenetic difference (mammals vs birds) remains an open question that deserves further investigations.

Considering the role of NO as a regulator of mitochondrial functions, we next explored whether mtNOS activity was modified by acclimating ducklings to cold. As previously reported [4,5], the present cold acclimation schedule enhanced the oxidative phosphorylation activity and decreased the basal production of radical oxygen species of skeletal muscle mitochondria. We found that addition of L-arginine had similar reversible effects upon mitochondrial metabolism in both groups of ducklings, namely a reduction in both oxidative and phosphorylative fluxes, no variation in mitochondrial oxidative phosphorylation efficiency and an upregulation of H$_2$O$_2$ generation. In addition, the inhibition constants (K_i) of L-arginine, as defined by the concentration of L-arginine that elicited 50% of its effects upon active mitochondrial respiration, were not significantly different between groups, suggesting that the affinity of mtNOS for its substrate was not altered by cold acclimation. Similarly, the mtNOS functional activity index was not affected by prolonged cold exposure. These data do not support major intrinsic change in NOS activity after cold-acclimation. Birds therefore differ from mammals in which a transitional modulation of mtNOS could contribute to tissue and animal energy expenditure during the process of cold acclimation [19]. However, present data in vitro do not preclude a regulatory role of bird mtNOS in cold-induced modifications in mitochondrial metabolism in vivo. Indeed, data on mammalian models showed that NO released by mtNOS activity is modulated by mitochondrial membrane potential [27], being stimulated at high potential. Similar effects of membrane potential were also reported for mitochondrial H$_2$O$_2$ production

[28]. Therefore, mechanisms that decrease membrane potential would minimize NO production by mitochondria [27]. In this situation, it is worth noting that cold acclimation enhanced the activity of avian UCP in bird skeletal muscle mitochondria [4,9]. This mitochondrial protein decreases membrane potential once adequately activated. Therefore, we can not rule out the possibility that mtNOS activity may be down regulated when avian UCP, and possibly the ADP/ATP carrier, are activated in the cold [4,6,9], leading to reduced competitive inhibition of NO over the cytochrome-c oxidase. Such UCP-dependent down regulation of mitochondrial NO production would then contribute to increase the oxidative phosphorylation flux, thus reducing the local tension of oxygen and limiting H$_2$O$_2$ production per oxygen consumed in bird muscle mitochondria during long term cold exposure [4]. This hypothesis that warrants specific experiments to be done will provide new insights into the field of bird thermal acclimation and avian bioenergetics.

Acknowledgments

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