Homeothermy in neonatal chicks exposed to low environmental temperature with or without intracerebroventricular administration of corticotropin-releasing factor

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Abstract To determine the mechanism of sensitivity to low-temperature exposure (20 °C for 3 h) and corticotropin-releasing factor (CRF) induced increased homeothermy, we investigated gene transcripts of putative thermogenic proteins and mitochondrial fatty acid (FA) oxidation enzymes in neonatal chicks. The hypothalamic–pituitary–adrenal (HPA) axis in low-temperature-exposed neonatal chicks was activated by central administration of CRF. Neonatal chicks showed hyperthermia on exposure to low-temperature, with no enhancement of HPA axis and gene transcripts of avian adenine nucleotide translocator, avian uncoupling protein, avian peroxisome-proliferator-activated receptor-γ co-activator-1α, and mitochondrial FA transport and oxidation enzymes in vital organs. However, central administration of CRF activated the HPA axis under low environmental temperature and induced increased homeothermy that was associated with the enhancement of gene transcripts and activities of mitochondrial FA-oxidation enzymes in the liver and heart.

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1. Introduction

Maintenance of body temperature in a cold environment is crucial for the survival of homeotherms. On exposure to low-temperature, a significant decrease in core temperature occurs when increases in heat production are not sufficient to compensate for increases in heat loss. At the transcriptional level, regulation of metabolic genes enables cells with not only the basic machinery for energy metabolism, but also tissue-specific components that expedite specific physiological functions to survive in ever-changing environments. As compared to adults, however, neonatal chicks cannot survive exposure to low-temperature without adequate shelter and heating. Although shivering is considered to be the primary mechanism for thermogenesis in birds, non-shivering responses may also play roles in heat production. Many studies have used cold-acclimated muscovy ducklings to examine avian non-shivering thermogenesis [1,2]. Because it is well established that bird species have no distinct stores of brown adipose tissue (BAT) or related tissues [3,4], other organs must mediate thermogenesis. Due to its high capacity for O2 consumption and energy expenditure [2,5,6], skeletal muscle is considered to be the major organ for thermogenesis in birds as it is in large mammals. Thus, skeletal muscle appears to be involved in non-shivering responses under conditions of low-temperature exposure.

Uncoupling proteins (UCPs) belong to a family of transporter proteins present in the mitochondrial inner membrane. UCPs generate heat by regulating inducible mitochondrial proton leak, a highly exergonic process, which ‘uncouples’ the free energy stored in the electrochemical proton gradient from ATP synthesis. First identified more than three decades ago, UCP1 is both necessary and sufficient for the thermogenic adaptation to cold in mammals [7]. UCP1 is present mainly in BAT, which is the major site of regulatory thermogenesis in small rodents [8]. Five additional uncoupling protein homologs, UCP2-4, brain mitochondrial carrier protein type 1 (BMCP 1), and kidney mitochondrial carrier protein 1 (KMCP 1), have been identified to date. UCP2 is expressed ubiquitously [9], while UCP3 gene expression is seen in skeletal muscle, adipose tissue, and heart [10]. UCP 4, BMCP 1, and KMCP 1, all of which have recently been identified [11–13], are expressed primarily in the brain and other neural tissues and within kidney cortex.

Thermogenesis via environmental cold-induced uncoupling in the skeletal muscle of pigeon was described by Skulachev’s group in the early 1960s [14,15]. There is evidence that non-shivering mechanisms associated with non-phosphorylating oxidation might play an important role in 4-week-old ducklings during cold exposure [5], along with an increase of avian (av) adenine nucleotide translocator (ANT) [16] or both avANT and avUCP gene transcripts in the skeletal muscle of 3- to 4-week-old chickens exposed to cold for 1–12 days [17,18]. Peroxisome-proliferator-activated receptor-γ co-activator-1α (PGC1α), a transcriptional co-activator, plays a role in mitochondrial biogenesis, muscle fiber specialization, and adaptive thermogenesis. PGC1α mRNA in skeletal muscle is important in coordinating the expression of mitochondrial uncoupling protein in response to cold exposure: exposure of chickens to a cold environment results in the upregulation of

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Abbreviations: 3HADH, 3-hydroxyacyl CoA dehydrogenase; ANT, adenine nucleotide translocator; BAT, brown adipose tissue; CPT-I, carnitine-palmitoyl-transferase-I; CPT-II, carnitine-palmitoyl-transferase-II; CRF, corticotropin-releasing factor; CS, citrate synthase; EB, Evans Blue; FA, fatty acid; HPA, hypothalamic–pituitary–adrenal; ICV, intracerebroventricular; LCAD, long-chain acyl CoA dehydrogenase; NEFA, non-esterified fatty acid; PGC1, peroxisome-proliferator-activated receptor-γ co-activator-1; ROS, reactive oxygen species; SA, sympathoadrenal; UCP, uncoupling protein.
avPGC1α expression, which precedes increments in avUCP and avANT expression in skeletal muscle [18].

Although adult birds have a high tolerance for cold, very young birds cannot maintain homeothermy in low-temperature environments until they acquire a capacity for thermogenesis and mature thermogenic organs. The ability of chicks to regulate body temperature during the post-hatching period increases in an age-dependent manner and birds become completely homeothermic by the age of 10 days [19]. During development, birds increase their capacity to regulate body temperature, as the size and maturity of the skeletal muscles and other organs that generate heat in response to decreasing body temperature increase [20,21]. The development of thermoregulation is also promoted by a decrease in the thermal conductance of the chick resulting from increased size and insulation of the body [22]. Although neonatal chicks are particularly sensitive to low-temperature exposure, the role of putative thermogenic proteins and their up-regulator and the changes in mitochondrial enzymes involved in fatty acid (FA) transport and oxidation that may explain this lack of homeothermy are not well known.

In neonatal chicks, it has been proposed that the hypothalamic–pituitary–adrenal (HPA) axis plays role in thermogenesis [23]. Corticotropic-releasing factor (CRF), a 41-amino acid peptide hormone produced in the hypothalamus, has multiple biological effects and plays a central regulatory role in the biological effects and plays a central regulatory role in the hypothalamic–pituitary–adrenal (HPA) axis. This may be explained in part by increased locomotion by central CRF [26]. Although neonatal chicks are particularly sensitive to low-temperature exposure, the role of putative thermogenic proteins and their up-regulator and the changes in mitochondrial enzymes involved in fatty acid (FA) transport and oxidation that may explain this lack of homeothermy are not well known.

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2.7. 3HADH and CS activity

3HADH and CS activities were measured according to the methods of Bradshaw and Noyes [33] and Srere [34], respectively.

2.8. Statistical analysis

Data were analyzed using the StatView program (SAS Institute, NC, USA). Data for core temperature were statistically analyzed with repeated two-way analysis of variance (ANOVA) with respect to exposure time and treatment, while means were compared using Tukey's test. All other data were first analyzed by one-way ANOVA and the means were compared using Tukey's test. All data are expressed as means ± standard error (S.E.). Differences were considered significant for values of \( P < 0.05 \).

3. Results

3.1. Core temperature

As shown in Fig. 1A, the core temperature of neonatal chicks significantly decreased on exposure to low-temperature with or without ICV-CRF \( [F(2, 45) = 57.4, P < 0.0001] \) in a time-independent manner. The core temperature was decreased with exposure to low-temperature, but recovered to some extent by ICV-CRF. The core temperature of low-temperature-saline chicks was decreased even after 1 h and remained low throughout the experimental period. After 1 h of low-temperature exposure, the core temperature of CRF-administered neonatal chicks was similar to that of thermoneutral chicks and then decreased as the duration of low-temperature exposure increased; although it was higher than low-temperature-exposed chicks without CRF.

3.2. Plasma NEFA and corticosterone concentrations

As shown in Fig. 1B, exposure to low-temperature with or without ICV-CRF significantly increased the plasma NEFA concentrations \( [F(2, 15) = 39.8, P < 0.0001] \). Plasma NEFA levels increased 2.3-folds of control levels after exposure to low-temperature, while low-temperature-exposed chicks with CRF showed significantly higher NEFA levels compared to chicks without CRF. Exposure to low-temperature did not increase the plasma corticosterone levels (Fig. 1C), while chicks that received centrally administered CRF and were exposed to low-temperature showed significantly higher levels \( [F(2, 15) = 15.1, P < 0.0001] \).

3.3. Gene transcripts for avUCP, avANT, and avPGC1α

Fig. 2A–C shows the expressions of avUCP, avANT, and avPGC1α mRNA transcripts in the skeletal muscle (gastrocnemius), as analyzed by real-time RT-PCR. Levels of these gene transcripts did not change in chicks with either low-temperature exposure or following ICV-CRF injection and exposure to low-temperature.

3.4. Gene transcripts for mitochondrial FA transport and oxidation enzymes

Expression of the genes related to mitochondrial FA transport and oxidation (CPT-I, CPT-II, LCAD, 3HADH, and CS) were analyzed in skeletal muscle (gastrocnemius and pectoralis superficialis), liver, and heart by real-time RT-PCR (Figs. 2D–H, 3A–E and 4A, B). Expression of these genes did not change in skeletal muscle on exposure to low-temperature with or without ICV-CRF (Figs. 2D–H, 3A–E). In the liver and heart, the expression of genes were significantly changed by different treatments \( [F(2, 15) = 3.8–68.9, P < 0.003–0.0001] \). Chicks that received centrally administered CRF and were exposed to low-temperature had increased expression of genes, while no change was observed in low-temperature-exposed chicks without CRF (Fig. 4A and B).
3.5. Enzyme activity

Fig. 5A and B shows 3HADH and CS enzyme activities in skeletal muscle (gastrocnemius), liver, and heart. Activity of these enzymes did not change in skeletal muscle of chicks on exposure to low-temperature with or without ICV-CRF. However, in the liver and heart of low-temperature-exposed chicks with CRF, the activities of these enzymes were 1.2- to 1.4-fold higher than control treatments \( F(2,15) = 8.2–19.8, P < 0.001–0.0001 \), while no change was observed in low-temperature-exposed chicks without CRF.

Fig. 2. Expression of avUCP (A), avANT (B), avPGC1α (C), CPT-I (D), CPT-II (E), LCAD (F), 3HADH (G), and CS (H) transcripts in the gastrocnemius muscle of neonatal chicks exposed to either thermoneutral (TN) or low-temperature (LT) conditions. Chicks were exposed to low-temperature with or without intracerebroventricular injection of corticotropin-releasing factor (CRF). Real-time RT-PCR was performed to determine gene expression. Results were normalized to 18s rRNA transcript levels. Values are means ± S.E. of six chicks.

Fig. 3. Expression of CPT-I (A), CPT-II (B), LCAD (C), 3HADH (D), and CS (E) transcripts in the pectoralis superficialis muscle of neonatal chicks exposed to either thermoneutral (TN) or low-temperature (LT) conditions. Chicks were exposed to low-temperature with or without intracerebroventricular injection of corticotropin-releasing factor (CRF). Real-time RT-PCR was performed to determine gene expression. Results were normalized to 18s rRNA transcript levels. Values are means ± S.E. of six chicks.
Fig. 4. Expression of CPT-I, CPT-II, LCAD, 3HADH, and CS transcripts in the liver (A) and heart (B) of neonatal chicks exposed to either thermoneutral (TN) or low-temperature (LT) conditions. Chicks were exposed to low-temperature with or without intracerebroventricular injection of corticotropin-releasing factor (CRF). Real-time RT-PCR was performed to determine gene expression. Results were normalized to 18s rRNA transcript levels. Values are means ± S.E. of six chicks. *For each treatment, values with different letters are statistically different ($P < 0.003-0.0001$).

Fig. 5. Changes in the 3HADH (A) and CS (B) enzyme activities in skeletal muscle (gastrocnemius), liver, and heart of neonatal chicks exposed to either thermoneutral (TN) or low-temperature (LT) conditions. Chicks were exposed to low-temperature with or without intracerebroventricular injection of corticotropin-releasing factor (CRF). Values are means ± S.E. of six chicks. *For each treatment, values with different letters are statistically different ($P < 0.0001$).
4. Discussion

Results from the present study indicate that neonatal chicks were unable to maintain thermostability under low environmental temperature (20 °C). This inability to maintain core temperature may be caused by failure in metabolic adjustments upon low-temperature exposure. We hypothesized that the inability to enhance putative thermogenic proteins expression and their up-regulator, and mitochondrial FA oxidation enzyme activity may explain this lack of homeothermy in neonatal chicks. Considering the possible role of avUCP and avANT in avian thermogenesis on cold exposure, we studied the gene transcript levels of these avian putative thermogenic proteins in low-temperature-exposed neonatal chicks with or without central administration of CRF. Central administration of CRF was carried out to increase homeothermy by HPA axis activation and to increase the expression of avUCP, central administration of urocortin (a member of CRF family) in rodents has been reported to increase UCP1 expression in BAT after 2 h [35].

We studied the changes in PGC1α, because exposure of chickens to a cold environment results in the up-regulation of avPGC1α expression, which precede increases in avUCP and avANT expression in skeletal muscle [18]. The expression of PGC1α mRNA in skeletal muscle is an important factor for coordinating the expression of mitochondrial UCP in response to cold exposure. Expression of PGC-1α increased in the skeletal muscle of 7-day-old chicks on exposure to 4 °C for 24 h and was associated with muscle fiber transformation in chicks during the acquisition of cold tolerance [36]. However, the results of the present study revealed that under low environmental temperature (20 °C), the expression of skeletal muscle avPGC1α was unchanged, indicating that exposure of chicks to low-temperature for a short duration (3 h) did not stimulate avPGC1α expression. In addition, central administration of CRF and exposure to low-temperature increased homeothermy that was not associated with the enhancement of gene transcripts of avPGC1-α in skeletal muscle.

The results showed that low-temperature exposure does not increase the gene transcripts of avANT and/or avUCP in skeletal muscle. This lack of increase in putative thermogenic protein gene transcripts may ultimately result in the inability to maintain body temperature upon low-temperature exposure during the post-hatching period. In the present study, no change in the gene transcript levels of avANT and avUCP was observed in chicks even with central administration of CRF, although their core temperature increased under low-temperature exposure as compared to that of low-temperature-exposed chicks without CRF. These results suggest that in neonatal chicks, the HPA axis-induced increased homeothermy is not associated with the enhancement of avUCP and avANT gene transcripts.

Recent extensive data support the idea that the new UCPs, those discovered after 1997, are involved in the control of reactive oxygen species (ROS) generation rather than thermogenesis. UCP1 in thymus [37] and other low-abundance UCPs (UCP2 to UCP5) attenuate mitochondrial ROS production [38–40]. In fact, in birds whose mitochondrial avUCP gene expression and protein levels were downregulated, ROS was produced more from skeletal muscle mitochondria compared to control birds [32,41]. Previous findings and results of the present study in which the gene transcript of avUCP was unchanged upon exposure to low-temperature suggest that avUCP under certain conditions may have a key regulatory role in scavenging mitochondrial ROS production rather than thermogenesis. This inability to increase gene transcripts of avUCP on exposure to low-temperature as observed in the present study may make neonatal chicks prone to oxidative damage, especially during CRF-induced increased homeothermy.

However, we still had the following unanswered questions: (1) in the absence of increased gene transcripts of putative thermogenic proteins in skeletal muscle of neonatal chicks on exposure to low-temperature, do other mechanisms in muscle or other organs try to contribute in enhanced homeothermy? and (2) although the low-temperature-exposed neonatal chicks that received central administration of CRF showed no change in the gene transcript levels of avUCP and/or avANT, and their up-regulator, how were they able to induce enhanced homeothermy? So, we investigated the gene transcript levels of CPT-I and CPT-II (involved in the mitochondrial capacity of FA transport), LCAD and 3HADH (which act on the fatty acyl-CoAs to generate FADH2 and NADH), and CS (causes condensation of acetyl-CoA and oxaloacetate to form citrate, which is the first reaction of the tricarboxylic acid cycle), not only in skeletal muscles but also in the liver and heart.

The results revealed that neonatal chicks upon exposure to low-temperature were unable to increase gene transcripts of enzymes involved in mitochondrial FA transport and oxidation in different tissues in spite of increased plasma NEFA levels. On the other hand, low-temperature-exposed neonatal chicks with central administration of CRF showed increased homeothermy, increased plasma NEFA levels, and enhancement of gene transcripts for mitochondrial FA transport and oxidation enzymes in the liver and heart. The rise in plasma fatty acids in saline- and CRF-administered low-temperature-exposed chicks might be due to increased absorption and/or mobilization of stored fat relative to their rate of FA utilization. Although neonatal chicks have a limited amount of adipose tissues, they have a good reservoir of fat in the yolk sac. Making up 20% of the body weight of newly hatched chicks, yolk provides the immediate post-hatching energy needs for maintenance and growth [42]. In the post-hatching period, fat is more completely absorbed in the intestine than glucose or methionine and an in situ study showed that duodenal mucosal uptake and serosal transport of fatty acids from yolk were more efficient [43]. Therefore, the increased levels of plasma fatty acids in saline- or CRF-administered low-temperature-exposed chicks might be the result of fat mobilization from adipose tissue and/or increased absorption from the yolk sac providing the energy substrate for oxidation and enhanced homeothermy.

To confirm the results of FA oxidation enzyme gene transcripts in different tissues, we also studied the activities of the two key enzymes, 3HADH and CS, involved in mitochondrial substrate oxidation. The patterns of these enzyme activities in different tissues were similar to those of gene transcripts of mitochondrial enzymes involved in FA transport and oxidation. These results indicate that upon low-temperature exposure, neonatal chicks were unable to induce enhanced homeothermy, possibly due to their inability to increase the expression of genes involved in mitochondrial FA transport and oxidation, as well as the FA oxidation enzyme activities, in spite of higher plasma NEFA levels.
Central administration of CRF, on the other hand, increased the expression of genes involved in mitochondrial FA transport and oxidation, and subsequently the FA oxidation enzyme activities in a tissue-specific manner, along with higher levels of plasma NEFA. Thus, the low-temperature-exposed neonatal chicks with centrally administered CRF could more efficiently utilize FA for enhanced homeothermy and increase their body temperature as compared to low-temperature-exposed chicks without CRF. However, these chicks were unable to maintain their core temperature after 2–3 h of low-temperature exposure. This indicates that CRF induced increased homeothermy, but this was not sufficient to keep core temperature within the normal range after 3 h; although it was higher than the low-temperature-exposed chicks without CRF. To induce increased homeothermy, the ability of CRF to change the core temperature of chicks by preventing the heat loss has not yet been validated and needs further investigation.

To sustain core body temperature, an increase in metabolism is required immediately upon exposure to low ambient temperatures. When the preoptic region of the hypothalamus detects a reduction in body temperature, the sympathetic nervous system is stimulated [44]. Stimulation of the sympathetic nervous system results in a release of epinephrine and norepinephrine from the adrenal medullae, which increases the cellular metabolic rate, resulting in an elevation of heat production [44]. The heart rate increases, as do cardiac output, mean arterial pressure, and ventilation [45]. If the body’s primary response to low-temperature is not adequate, there will be a continued drop in core temperature. In neonatal chicks, the lack of gene transcript enhancement of mitochondrial FA transport and oxidation, and FA oxidation enzyme activities in the liver, heart, and skeletal muscle in spite of high plasma NEFA levels indicates an insufficient metabolic response to low environmental temperature and results in hypothermia. On the other hand, ICV-CRF increases the gene transcript and enzyme activities in the liver and heart that probably utilizes the high plasma NEFA levels. This may result in enhancement of cellular metabolism in liver and heart, and contributes to increase homeothermy of neonatal chicks. The liver and heart are more responsive to CRF-induced modulation in mitochondrial FA oxidation enzyme gene transcripts and activities compared to skeletal muscle. The skeletal muscle of neonatal chicks does not seem to play an efficient role in CRF-induced increased homeothermy compared to the liver and heart.

In newborn mammals, upon exposure to cold, increases in either sympathetic stimulation or circulating norepinephrine and epinephrine in the blood can cause an immediate increase in the rate of cellular metabolism. It results at least partially from the ability of norepinephrine and epinephrine to uncouple mitochondrial oxidative phosphorylation. This ability in mammals is almost directly proportional to the amount of BAT that contains a large number of specific mitochondria with high expression of UCP. Contrary to this, neonatal chicks have a different thermogenic mechanism upon exposure to low-temperature. As evident in the present study, the inability of avUCP gene transcript upregulation in muscle and the lack of increased gene expression of mitochondrial substrate oxidation enzymes on low-temperature exposure raise a question about the response of the sympathoadrenal (SA) axis to stimulate gene transcription in neonatal chicks. In fact, compared to newborn mammals, the neonatal chicks seem to differ regarding the SA axis-induced transcriptional changes in avANT, avUCP, and mitochondrial FA oxidation enzymes upon exposure to low-temperature. CRF plays a role in the HPA response activation in neonatal chicks under thermoneural conditions [46]. Low-temperature exposure in the present study was unable to reveal the stimulation of the HPA axis in neonatal chicks, as evident by unchanged plasma corticosterone levels (Fig. 1C). Neonatal chicks during early post-hatching development may exhibit a reduced response to low-temperature exposure resulting in lower CRF release and/or a reduced capacity to secrete adrenocorticotropin and corticosterone. The increased SA axis on the other hand may also be responsible for this unchanged corticosterone level upon low-temperature exposure; in neonatal chicks, norepinephrine interacts with and decreases CRF-induced increases in plasma corticosterone and CRF-induced behavior [47]. Significantly high corticosterone levels in CRF-administered low-temperature-exposed chicks, on the other hand, showed that ICV-CRF stimulates the HPA axis even low environmental temperature conditions. These results showed that CRF-induced changes in plasma corticosterone are not suppressed by an increased SA axis under low-temperature exposure when CRF is administered exogenously. Thus, activation of the HPA axis increases the gene transcripts of mitochondrial FA transport and oxidation enzymes in liver and heart, resulting in increased homeothermy.

In summary, the present study showed that gene transcripts of avANT, avUCP, and avPGC1α in skeletal muscle are not upregulated in neonatal chicks upon exposure to low-temperature. In addition, there was no change in the gene transcripts of enzymes involved in mitochondrial FA transport and oxidation, and subsequently in mitochondrial FA oxidation enzyme activities in different tissues, although these chicks had increased plasma NEFA levels. Conversely, low-temperature-exposed neonatal chicks with central administration of CRF showed elevated plasma NEFAs and corticosterone levels and increase homeothermy. The liver and heart of these chicks are characterized by enhanced levels of gene transcripts for mitochondrial FA transport and oxidation enzymes, and increased enzyme activities of 3HADH and CS that might have contributed to increase homeothermy. In conclusion, neonatal chicks show hypothermia on exposure to low-temperature, with no enhancement in gene transcripts of avANT, avUCP, avPGC1α and mitochondrial fatty acid transport and oxidation enzymes in vital organs. However, central administration of CRF activates the HPA axis under low environmental temperature and induces increased homeothermy that is associated with enhancement of gene transcripts and activities of mitochondrial FA oxidation enzymes in the liver and heart.

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