



L-Citrulline acts as potential hypothermic agent to afford thermotolerance in chicks



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ABSTRACT

Recently we demonstrated that L-citrulline (L-Cit) causes hypothermia in chicks. However, the question of how L-Cit mediates hypothermia remained elusive. Thus, the objective of this study was to examine some possible factors in the process of L-Cit-mediated hypothermia and to confirm whether L-Cit can also afford thermotolerance in young chicks. Chicks were subjected to oral administration of L-Cit along with intraperitoneal injection of a nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester HCl (L-NAME), to examine the involvement of NO in the process of hypothermia. Food intake and plasma metabolites were also analyzed after oral administration of L-Cit in chicks. To examine thermotolerance, chicks were orally administered with a single dose of L-Cit (15 mmol/10 ml/kg body weight) or the same dose twice within a short interval of 1 h (dual oral administration) before the exposure to high ambient temperature (35 ± 1 °C) for 180 min. Although the rectal temperature was reduced following administration of L-Cit, L-NAME caused a greater reduction. L-NAME reduced total NO₂ and NO₃ (NO_x) in plasma, which confirmed its inhibitory effect on NO. A single oral administration of L-Cit mediated a persistent state of hypothermia for the 300 min of the study without affecting food intake. It was further found that plasma glucose was significantly lower in L-Cit-treated chicks. Dual oral administration of L-Cit, but not a single oral administration, afforded thermotolerance without a significant change in plasma NO_x in chicks. In conclusion, our results suggest that L-Cit-mediated hypothermia and thermotolerance may not be involved in NO production. L-Cit-mediated thermotolerance further suggests that L-Cit may serve as an important nutritional supplement that could help in coping with summer heat.

1. Introduction

Endothermic species, including birds and mammals, generally maintain a constant body temperature. However, environmental temperature and nutritional condition can influence body temperature. For example, body temperature can be increased in chicks exposed to high ambient temperature (HT; Ito et al., 2015; Yang et al., 2016; Han et al., 2017; Bahry et al., 2017). Recently, we also found that oral administration of a nutrient, L-citrulline (L-Cit), can reduce body temperature in chicks (Chowdhury et al., 2015). L-Cit was first encountered as a constituent of watermelon juice (*Citrullus vulgaris*; Koga and Ohtake, 1914; Wada, 1930). Watermelon is a rich natural source of the dietary nonessential amino acid L-Cit (Rimando and Perkins-Veazie, 2005; Tarazona-Díaz et al., 2011). Endogenous L-Cit is a physiological amino acid in most living systems (Curis et al., 2005). L-Cit is well known to enhance the synthesis of L-arginine (L-Arg), the endothelial substrate for the production of nitric oxide

(NO), and ultimately to increase endogenous NO production (Schwedhelm et al., 2008). NO synthase (NOS) converts L-Arg to NO. NO has been suggested as a regulator of various types of behavior (Szabo, 1996), including thermoregulation (De Luca et al., 1995; Gourine, 1995). However, it is still not known whether L-Cit can induce hypothermia through stimulating NO production.

Body temperature can be regulated by the physiological process of non-shivering thermogenesis and by metabolic processes. It has been suggested that mitochondrial uncoupling protein (UCP) and the adenine nucleotide translocator (ANT) function as a mitochondrial anion carrier in thermogenesis (Skulachev, 1991). Cold acclimation was found to induce an increase in the level of avian ANT (avANT) mRNA and, to a lesser degree, in the level of avian UCP (avUCP) in skeletal muscle (Toyomizu et al., 2002). Peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α), a transcriptional coactivator, plays a role in mitochondrial biogenesis and in adaptive thermogenesis (Ueda et al., 2004). Recently, it

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Table 1
Primers used for real-time PCR.

Gene	Accession no.	Sequences 5'–3' (forward/reverse)	Annealing temperature (°C)	Product size (bp)
<i>avUCP</i>	AF433170.2	5'-GATGTGGTGAAGACGCGTA-3'/ 5'-GTCCGCCATCACTGCTTTGT-3'	60	169
<i>avANT</i>	NM_204231.2	5'-TGCTGCCAGATCCAGAAAC-3'/ 5'-CATCCCTTGCAATCTCCGC-3'	60	192
<i>PGC-1α</i>	NM_001006457.1	5'-GCGTCGTGTGATTTACGTGG-3'/ 5'-TCTCAAGAGCAGCAAAGCA-3'	60	176
<i>RP-II</i>	NM_001006448.1	5'-CGACGGTTTGATTGCACCTG-3'/ 5'-CAATGCCAGTCTCGCTAGTTC-3'	64	161

Primers were designed with Primer-Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) for avian uncoupling protein (*avUCP*), avian adenine nucleotide translocator (*avANT*), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1α*) and RNA polymerase II (*RP-II*).

was demonstrated that oral administration of the medium containing both D-aspartate-producing live bacteria and D-aspartate significantly reduced body temperature and the mRNA abundance of the *avUCP* in the breast muscle, but led to a significant increase in *avANT* mRNA in the same muscle (Do et al., in press). In addition, birds have a thermoregulatory capability enabling them to control body temperature by regulating the metabolic rate (Mozo et al., 2005). Examination of plasma metabolites in chicks allows metabolic activity to be tracked under different physiological conditions (Chowdhury et al., 2012a, 2012b; Erwan et al., 2014; Ito et al., 2015). For example, a low level of blood glucose can be related to a hypothermic state in mammals (Buchanan et al., 1991; Almeida and Branco, 2001). Besides plasma glucose, plasma total cholesterol and triacylglycerol can also represent the physiological condition of chicks (Chowdhury et al., 2012a, 2012b), and aspartate amino transferase (AAT) serves as a marker of liver damage (Kalmansohn and Kalmansohn, 1961). Reduced food intake and hypothermia have been reported previously in endotherms (Hoshino, 1996; Sakurada et al., 2000). Therefore, food intake, metabolic status and mitochondrial biogenic gene expression can provide some clue about the existence of hypothermia.

Summer heat stress is a growing global challenge which has severe negative impacts on living organisms, including chickens (Cooper and Washburn, 1998) and growing chicks (Chowdhury et al., 2014). We found that L-Cit concentration declined in the plasma in heat-exposed chicks (Chowdhury et al., 2014). However, it is still not known whether L-Cit-dependent hypothermia (Chowdhury et al., 2015) can provide thermotolerance in any species. Birds can generate a stress response to help them adjust to changes in their immediate environment (Cockrem, 2007). Stress responses involve secretion of corticosterone in birds. Plasma corticosterone was increased in heat-exposed chicks (Ito et al., 2015). An analysis of rectal temperature under HT, plasma corticosterone and NOx would clarify the effect of L-Cit on thermotolerance in heat-exposed chicks.

In the present study, three experiments were conducted to investigate some possible factors involved in the hypothermic effect of L-Cit. In particular, we evaluated the effect of a NOS inhibitor, *N*^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), with or without L-Cit, on body temperature in chicks, and we also analyzed the effect of L-Cit on plasma metabolites, food intake, and mitochondrial biogenic gene expression in skeletal muscle in chicks. Finally, we analyzed the effect of oral administration of L-Cit on body temperature under HT and on plasma corticosterone and plasma NOx concentrations in chicks.

2. Materials and methods

2.1. Animals

One-day-old male layer chicks (Julia) (*Gallus gallus domesticus*) were purchased from a local hatchery (Murata hatchery, Fukuoka, Japan) and housed in a wire-mesh cage (50 × 35 × 33 cm) in a group (20–25 birds) at a constant temperature of 30 ± 1 °C and with continuous light. Chicks were housed without any adult present. Food (AX and Adjust

diets; Toyohashi Feed and Mills Co. Ltd., Aichi, Japan) and water were provided *ad libitum*. AX diet [Commercial starter diet (metabolizable energy: 12.77 MJ/kg and protein: 24%; food ingredients: grain 61% (mainly maize), defatted meal 25% (soybean meal and maize gluten meal), fish meal 9%, rice bran 1% and others 4%)] was used in Experiments 1 and 2 and replaced by Adjust diet (metabolizable energy: > 12.55 MJ/kg, protein: > 23%) in Experiment 3 because the commercial supply of AX diet ceased. There was virtually no other difference in composition between the two diets that was of any significance. For acclimatization, chicks were reared individually and assigned to treatment and control groups 48 h prior to the day of the experiment on the basis of their body weight in order to produce uniform groups. The chicks had free access to food and water during the whole experimental period. This study was performed in accordance with the guidelines for animal experiments carried out in the Faculty of Agriculture and on the Graduate Course of Kyushu University, and adhered to Law no. 105 and Notification no. 6 of the government.

2.2. Preparation of drugs

L-Cit and L-NAME were purchased from Wako Pure Chemical Industries (Osaka, Japan). Since L-Cit is hard to dissolve in water, L-Cit was suspended in 0.25% methyl cellulose solution, and these suspensions were stirred well in a vortexer before oral administration. L-NAME was dissolved in 0.85% saline and administered to chicks through intraperitoneal (i.p.) injection. These solutions were kept at room temperature (30 ± 1 °C) during the experiments.

2.3. Experimental design

Experiment 1 was conducted to examine the involvement of NO in L-Cit-dependent changes in body temperature. In this experiment, we used 7-day-old chicks on the basis of findings in our previous report to study the effect of L-Cit on body temperature (Chowdhury et al., 2015). Following an acclimatization period, 7-day-old chicks (average body weight 65 g) were divided into five groups based on their initial body weight in order to produce uniform groups. The ambient temperature was 30 ± 1 °C during the experimental period. One group of chicks received oral administration of L-Cit (15 mmol/10 ml/kg body weight) based on the findings of our recent report (Chowdhury et al., 2015), using an elastic plastic needle on a small syringe. The control group was administered in the same way with 0.25% methyl cellulose solution. The other three groups received oral administration of L-Cit (15 mmol/10 ml/kg body weight) plus i.p. injection with L-NAME (50, 100 or 150 mg/0.1 ml/kg body weight or 11, 26 or 37 μmol/chick) based on a previous report involving the use of L-NAME on rats in the amount of 50 mg/kg body weight (Ding et al., 2006). Rectal temperature was measured immediately before dosing with L-Cit or L-NAME, which was considered as the data at 0 min, followed by the measurements at 30, 60, 90 and 120 min after the treatments. Rectal temperature was measured by using a digital thermometer with an accuracy of ± 0.1 °C

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