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Role of prolactin-like protein (PRL-L) in cold-induced increase of muscle mass in chicks

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ABSTRACT

This study examined the hypothesis that a novel prolactin-like protein gene (PRL-L) is involved in cold-induced growth of skeletal muscle in chicks. Six-day-old chicks (*Gallus gallus domesticus*) were exposed to cold at 4 °C or kept warm at 30 °C for 24 h. Cold exposure induced significant increases in PRL-L expression that coincided with increases in the weight of the sartorius muscle, which comprises both fast- and slow-twitch fibers. Meanwhile, no induction of PRL-L mRNA was observed in the heart, liver, kidney, brain, or fat. Myoblast cells that expressed PRL-L mRNA grew faster than untransduced cells in media containing 2% serum. These results suggested that PRL-L might be involved in in controlling cold-induced muscle growth of chicks.

1. Introduction

Newborn chicks cannot maintain their body temperature in a cold environment because of their immature thermogenic ability (Nichelmann and Tzschentke, 2002; Olson, 1994), but they acquire the capacity for thermogenesis as they post natally develop skeletal muscle. Most chicks (*Gallus gallus domesticus*) older than 6 days tolerate 24 h of cold exposure (4 °C) and maintain their body temperature over this time, accompanied by both increasing mass in their leg muscles (sartorius and quadriceps muscle) and a transformation of muscle fibers from fast- to slow-twitch (Hirabayashi et al., 2005; Ijiri et al., 2009b). From the quadriceps of the cold-exposed chicks, 16 independent cold-induced genes were obtained by subtraction and differential display analysis (Hirabayashi et al., 2005). One of them was in agreement with a novel prolactin like protein (PRL-L) gene (Wang et al., 2010). Wang et al. (2010) reported that PRL-L was widely expressed at sites outside the pituitary gland of adult chickens. The predicted PRL-like protein precursor is 225 amino acids in length; however, the role of the PRL-L has not yet been reported.

The objective of this study was to examine the role of PRL-L in the skeletal muscle of neonatal chicks. We previously reported that the leg muscle mass of neonatal chicks was increased in response to 24 h of cold exposure, whereas the pectoral muscle mass was not changed (Ijiri et al., 2009b). The leg muscles of chicks (i.e., sartorius muscle and gastrocnemius muscle) are composed of both fast- and slow-twitch fibers (Maier, 1998; Nishida et al., 1997), while the pectoralis muscle is composed of only fast-twitch fibers (Nikovits et al., 2001). In this study, the sartorius muscles and gastrocnemius muscles were defined as mixed muscle, and the pectoral muscle was defined as white muscle. Therefore, in the current study, we examined whether PRL-L is involved in cold-induced growth of mixed muscles using this animal model. We herein show that cold exposure induced a marked increase in the expression of PRL-L mRNA in mixed muscle of chicks. Although this gene was increased in white muscle in response to cold exposure, the degree of increase was 50 times higher in mixed muscle compared to that in white muscle. We also observed that myoblast cells transduced with the PRL-L gene showed high proliferation rates under low serum conditions. Finally, our additional study on the localization of PRL-L protein provides evidence that this protein exists in the extracellular matrix in the mixed muscle of chicks.

Abbreviations: PRL-L, prolactin-like protein; NT, no treatment; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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2. Materials and methods

2.1. Animals and treatment

Chicks hatched from genetically identical Rhode Island Red (*Gallus gallus domesticus*) eggs were bred at the Agricultural and Forestry Research Center, University of Tsukuba, in accordance with institutional guidelines. All chicks were kept at a thermoneutral temperature (30 °C and 60% relative humidity) until they were exposed to cold temperature (4 °C and 60% relative humidity). All chicks had free access to food and water under constant 24 h lighting. To examine the effects of cold exposure on PRL-L mRNA expression in chicks, two separate experiments were performed. (i) Twelve 6-day-old chicks were randomly divided into two groups (cold exposure and no treatment [NT]). The cold exposure group was exposed to cold temperature for 24 h, and the NT group was kept at a thermoneutral temperature for 24 h as a control. After measurement of body temperature, chicks were killed by cervical dislocation under ether anesthesia after each treatment. Sartorius muscle, pectoral muscle, heart, liver, kidney, brain, and fat were collected and immediately frozen in liquid nitrogen and stored at -80 °C until use. (ii) Thirty-six 6-day-old chicks were randomly divided into five groups. Four of the five groups were exposed to cold temperature for 0.5, 1, 3, and 6 h, respectively, and the remaining group served as controls (0 h). All chicks were killed by cervical dislocation under ether anesthesia after each treatment. Sartorius muscles collected from both legs were weighed, immediately frozen in liquid nitrogen, and stored at −80 °C until use for mRNA expression and protein expression. The gastrocnemius muscle was collected from the NT groups and embedded for immunofluorescence in OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and rapidly frozen in isopentane chilled in liquid nitrogen. The experimental protocols and procedures were reviewed and approved by the Animal Care and Use Committee of the University of Tsukuba, Japan.

To examine the effects of cold exposure on PRL-L mRNA expression in pituitary gland of chicks, 12 male broiler chicks (Chunky strain ROS308, provided by Kajiki Kumiai Hina Center, Kagoshima, Japan) were divided into two groups and exposed to cold temperature or thermoneutral temperature for 24 h. All chicks were killed by cervical dislocation under ether anesthesia after treatment. Pituitary gland was collected, immediately frozen in liquid nitrogen, and stored at −80 °C until use for mRNA expression. The experimental protocols and procedures were reviewed and approved by the Animal Care and Use Committee of the Kagoshima University, Japan.

2.2. RNA extraction and northern blotting

Total RNA was purified using TRIzol reagent (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. Northern blot analysis was performed as described previously (Ijiri et al., 2009a). Image analysis was performed using a Macintosh computer and the public domain NIH Image program. Grey scale thresholding was used to separate positive staining from background, and no visible band was observed in the negative control lane (background).

2.3. Quantitative real-time PCR

Real-time PCR was performed as described previously (Kamizono et al., 2010). In brief, cDNA was synthesized at 40 ng RNA per 10 μL of reaction solution with PrimeScript RT reagent Kit (Takara, Shiga, Japan), which was set at reverse transcription 37 °C for 15 min, inactivation of reverse transcriptase 85 °C for 5 s, and refrigeration 4 °C for 5 min using PC-320 (ASTEC, Fukuoka, Japan). The primers used in this study are listed in Table 1. Gene expression was measured by real-time PCR using the 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) with SYBR Premix Ex Taq (Takara, Shiga, Japan). The thermal cycle was as follows: 1 cycle at 95 °C for 10 s, and 60 cycles at 95 °C for 5 s, 60 °C for 30 s, and 80 °C for 31 s. Expression of GAPDH mRNA was used as an internal standard and was not significantly different between the cold and control groups. Gene expression results are shown as a percentage of the control value.

2.4. Western blot analysis

Skeletal muscles from the chicks were homogenized in 2 ml lysis buffer comprising 20 mM Tris–HCl, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, pH 8.0, and 0.5% Igepal nonionic detergent. The lysate was centrifuged at 14,000*g* for 30 min at 4 °C, and the supernatant was collected. Total protein concentration was estimated by a protein–dye binding assay (Bradford, 1976) using a commercial kit (500–0116; Bio-Rad, Hercules, CA) with mouse IgG as the standard. Aliquots of skeletal muscle were stored at −80 °C until analyzed by western blotting. Western blot analysis was performed as described previously (Ijiri et al., 2009a).

2.5. Retrovirus preparation

The GP2–293 packaging cells (purchased from Clontech, Mountain View, CA) were used to generate retroviruses according to the manufacturer's instructions. A total of 6×10^5 cells were placed in a six-well plate and cultured for 24 h. Six micrograms of retroviral vector pVSVG, pMX GFP, or pMX CTIF-GFP was transfected into the cells by Lipofectamine 2000 (Invitrogen, Tokyo, Japan). After 8 h of incubation under 5% CO_2 at 37 °C, the medium was exchanged with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Tokyo, Japan) and cultured for another 48 h. The supernatant of the culture medium was taken and filtrated through a filter with a 0.45-μm pore size (Toyo Roshi Kaisha Ltd., Tokyo, Japan).

2.6. Cell culture

C2C12 cells were bought from Riken Bioresource Center. One day prior to transfection, C2C12 cells were subcultured at 2×10 cells/well under 5% CO_2 at 37 °C in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The medium was exchanged with DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 4 μg/ml of polybrene, and viral supernatant and cultured for another 24 h. The medium was exchanged every 3 days.

2.7. Cell proliferation assay

Cell number was assessed using the reagent WST-1 (Roche) according to the manufacturer's instructions. Cells were plated at a density of 4×10^2 cells into 96-well plates with DMEM supplemented with 10% FBS (proliferation media) or 2% horse serum (HS) (differentiation media). After 24, 48, and 72 h of incubation, WST-1 reagent was added and incubated for 4 h, then measured at a wavelength of 450 nm using a microplate reader (Model 680; Bio-Rad, Hercules, CA).

2.8. Immunofluorescence

To examine the localization the localization of PRL-L protein in skeletal muscle, the gastrocnemius muscles of the NT group chicks were used in this experiment. Serial cross-sections (7 μm thick) were cut with a microtome (CM30503; Leica Microsystems, Wetzlar, Germany) at −28 °C and collected onto slides. The