Detection of Leptin Activity in Living Cells Expressing Chicken Leptin Receptor and STAT3

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The chicken leptin receptor (chLEPR) activates Janus kinase (JAK) - signal transducers and activator of transcription (STAT) signaling pathway after leptin stimulation. We have previously developed a bioassay using leptin inducible reporter gene in cultured cells. However, we failed to detect leptin-like activity in chicken blood. In the present study, we expressed green fluorescent protein (GFP) fused chSTAT3 (GFP-chSTAT3) in cells expressing chLEPR and analyzed leptin dependent activation of the chSTAT3. Leptin phosphorylated GFP-chSTAT3 and by which fluorescent signal translocated into nuclei in COS-7 cells transiently expressing GFP-chSTAT3 with chLEPR. Furthermore, we established CHO-K1 cells stably expressing chLEPR and chSTAT3 (CHO-chLEPR/chSTAT3), and in which detected time- and dose-dependent activation of chSTAT3 by leptin. Therefore, the CHO-chLEPR/STAT3 cells would be an excellent tool to detect and monitor leptin-like activity in avian tissues.

Key words: bioassay, chicken, leptin, signal transduction, STAT3

J. Poult. Sci., 49: 46-50, 2012

Introduction

The chicken (ch) leptin receptor (chLEPR) was cloned by our laboratory and others (Horev *et al.*, 2000; Ohkubo *et al.*, 2000) as the first LEPR cloned from non-mammalian vertebrates. In addition, chLEPR gene has been mapped on the chromosome 8 of chicken genome (Dunn *et al.*, 2000). Beside existence of chLEPR gene, identification of chicken leptin gene is still in conflict (Friedman-Einat *et al.*, 1999; Amills *et al.*, 2003; Richards and Proszkowiec-Weglarz, 2007). Nevertheless the evidence for the expression of functional LEPR in avian target tissues comes from the observation that exogenous leptin treatment modulates physiological process including food intake, immune response and ovarian function in birds (Ohkubo and Adachi, 2008, Review). In addition, infusion of radiolabeled leptin to chicken led to detection of radioactivity in multiple tissues (McMurtry *et al.*, 2004). Western blot analysis has identified the presence of LEPR protein in the chicken brain and chicken hepatoma cell, LMH (Ohkubo *et al.*, 2007).

Following leptin binding with LEPR, activated Janus kinase 2 (JAK2) rapidly phosphorylates signal transducers

and activator of transcription 3 (STAT3). Phosphorylated STAT3 then translocates to the nucleus to modulate transcription of the target genes in mammals (Banks *et al.*, 2000). Recently it has been shown by us and others that chLEPR expressed in mammalian cells activates JAK - STAT signaling pathway (Adachi *et al.*, 2008; Hen *et al.*, 2008), indicating thereby that endogenous LEPR in bird is capable of processing leptin-like signals in a way similar to higher vertebrates. However, it has not been possible to detect leptin-like activity in the avian blood by the *in vitro* bioassay (Adachi *et al.*, 2008; Hen *et al.*, 2008; Yosefi *et al.*, 2010). This may be due to plasma leptin levels being below the detection threshold of the quantitative bioassay using luciferase gene. Therefore, the possibility that there is no natural ligand of the LEPR in birds cannot be completely ruled out.

An alternative way to detect endogenous leptin-like activity in birds is to monitor intracellular localization of STAT3. It is known that fluorescent protein fused STAT3 migrates into nucleus after cytokine stimulation (Kretschmar *et al.*, 2004; Pranada *et al.*, 2004; Watanabe *et al.*, 2004). In addition, leptin activates chLEPR which induces STAT dependent activation of luciferase gene (Adachi *et al.*, 2008; Hen *et al.*, 2008). Therefore, detection of translocation of GFP fused STAT3 into nuclei would be an alternate bioassay in detecting endogenous leptin-like activity in birds. To test this possibility, we expressed chicken STAT3 as GFP tagged protein and then detecting its translocation into nuclei.

Received: August 10, 2011, Accepted: October 26, 2011

Released Online Advance Publication: November 25, 2011

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Materials and Methods

Antibodies

Anti-STAT3 monoclonal antibody and anti-mouse STAT3 (pY705) monoclonal antibody were obtained from BD Biosciences (Tokyo, Japan). These antibodies were suitable for the detection chicken STAT3 (Adachi *et al.*, 2008). Anti-GFP monoclonal antibody was purchased from Nacalai Tesque (Kyoto, Japan). Anti-chLEPR monoclonal antibody was described previously (Ohkubo *et al.*, 2007).

Plasmids and Vector Construction

An expression vector for chicken leptin receptor (pCI-chLEPR) has been described previously (Ohkubo *et al.*, 2007). Each pGEM-T Easy, pcDNA3.1Hygro (+) and pEGFP-N1 was obtained from Promega (Tokyo, Japan), Invitrogen (Tokyo, Japan) and Clontech (Palo Alto, CA, USA), respectively.

The entire coding sequence of chicken STAT3 (chSTAT3) was amplified by PCR using a pair of primers, 5'-GCTCG-AGGATGGCGCAGTGGAAACCA-3' (sense: nucleotide 18–35 of NM_001030931 with *Xho* I site) and 5'-TGGATCCA-TTGGTGAGGAAGCACACT-3' (antisense: nucleotide 2312–2331 of NM_001030931 with *Eco* RI site) with White Leghorn hypothalamic cDNA library (Ohkubo *et al.*, 2000). PCR was carried out for 25 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 2 min 30 s. Thereafter, the amplified fragment was cloned into pGEM-T Easy (Promega) and sequenced. This was followed by introduction of chSTAT3 cDNA into pEGFP-N1 (pEGFP-chSTAT3). Subsequently, chLEPR cDNA removed from pCI-chLEPR was cloned into pcDNA3.1 Hygro (+) (pcDNA-chLEPR).

Cell Culture

CHO-K1 cell and CHO-K1 cell stably expressing chLEPR (CHO-chLEPR) (Adachi *et al.*, 2008) were maintained in nutrient mixture Ham's F-12 medium (Sigma-Aldrich Japan, Tokyo, Japan) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in an atmosphere of 95% air and 5% CO₂. COS-7 cell was maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin under 5% CO₂ and 95% air at 37°C.

Expression of GFP-fused chSTAT3 in COS-7 Cells

COS-7 cell grown in 35 mm² dish (3.0×10^5 cells/well) was transfected with 2 µg of pEGFP-chSTAT3 or pEGFP-N1 as control using TransFectin Lipid Reagent (Bio-Rad Laboratories, Tokyo, Japan) according to the manufacturer's instructions. Twenty-four hours after the transfection, cells were harvested and lysed with 100 µl of Mild Lysis Solution (CytoSignal, Irvine, CA, USA). Protein concentration of the cell lysates was measured by using Pierce BCA Protein Assay Kit (TaKaRa Bio, Shiga, Japan) and these were stored at –20°C until used for Western blot analysis for STAT3 and GFP.

Leptin Dependent Activation of GFP-fused chSTAT3 in COS-7 Cells

COS-7 cells seeded in 24 well plate (0.8×10^5 cells/well) were transfected with 0.25 µg each of pCI-chLEPR and

pEGFP-chSTAT3 by TransFectin Lipid Reagent (Bio-Rad Laboratories) under manufacturer's protocol. These were then incubated for 4 h and again for another 16 h in serum free medium. Subsequently the cells were treated with 125 ng/ml recombinant murine leptin (rmleptin; Sigma Aldrich Japan, Tokyo, Japan) for 10, 30, 60 and 120 min. At the end of each treatment, cells were harvested and stored at –20°C until use for Western blot analysis for STAT3.

Western Blot Analysis

Total cell lysates were separated by SDS-PAGE under reducing conditions and then transferred to Hybond-P membrane (GE Healthcare Japan, Tokyo, Japan) using Trans-Blot SD semi-dry transfer cells (Bio-Rad Laboratories) at 15 V for 30 min in 25 mM Tris, 192 mM glycine, and 20% methanol. After transfer, the blot was treated with PBS containing 0.1% Tween 20 (PBST) including 5% non-fat dry milk for 1 h at room temperature. The membrane was incubated with appropriate primary antibody for 1 h at room temperature followed by washing with PBST for 10 min, twice. Then the blot was with secondary antibody conjugated with horseradish peroxidase for 1 h and washed with PBST for 10 min, twice. After washing, the membrane was then immersed in SuperSignal West Dura Extended Duration Substrate (TaKaRa Bio) and the signals detected by LAS-3000 mini (Fuji Film Co. Ltd., Tokyo, Japan).

Determination of Leptin Induced Nucleus Accumulation of chSTAT3 in COS-7 Cells Transiently Expressing chLEPR

Seeding of 0.8×10^5 of COS-7 cells were made in 24 well plate dish with a glass coverslip and were transfected with 0.25 µg of pcDNA-chLEPR and pEGFP-chSTAT3 using TransFectin Lipid Reagent (Bio-Rad Laboratories). Four hours after transfection, the medium was replaced by serum-free medium and was left for 16 h. Then the cells were stimulated with 125 ng/ml of rmleptin (Sigma-Aldrich Japan) for 0, 10, 30, 60 and 120 min. After the stimulation, the cells were fixed in 4% formaldehyde and stained with 2-(4-amidinophenyl)-1H-indole-6-carboxamide (DAPI). Then, the coverslip was mounted on a glass slide, and the fluorescent signal was detected by a fluorescence microscope (Nikon Labophot, Nikon, Tokyo, Japan).

Determination of Leptin Induced Intra-nucleus Translocation of chSTAT3 in CHO-K1 Cells Stably Expressing GFP-tagged chSTAT3 with chLEPR

Stable cell line expressing chLEPR and GFP-fused chSTAT3 was established by transfecting CHO-K1 cells with each 0.25 µg of pcDNA-chLEPR and pEGFP-chSTAT3 using TransFectin Lipid Reagent (Bio-Rad Laboratories). Twenty-four hours after transfection, the cells were exposed to a medium containing 500 µg/ml G418 (Nacalai Tesque, Kyoto, Japan) and 200 µg/ml of HygroGold (InvivoGen, San Diego, CA, USA). Cells expressing both chLEPR and GFP-tagged chSTAT3 were selected by Western blot using anti-chLEPR monoclonal antibody and anti-STAT3 monoclonal antibody, and established cells stably expressing chLEPR and chSTAT3 (CHO-chLEPR/STAT3). At the same time, CHO-K1 cells stably expressing GFP-fused chSTAT3 were constructed as control of CHO-chLEPR/STAT3.

The CHO-chLEPR/STAT3 were treated with rmleptin, and nuclear accumulation of chSTAT3 was monitored for 45 min by BZ-1000 All-in-One Fluorescence Microscope (KEYENCE Japan, Tokyo, Japan).

Results

Expression of GFP Tagged chSTAT3 in COS-7 Cell

GFP-tagged chSTAT3 was detected as approximately 110 kDa protein by Western blot analysis with anti-STAT3 and anti-GFP antibodies (Fig. 1). The molecular weight of the protein was similar to the estimated molecular mass of chSTAT3 with GFP.

Leptin Dependent Activation of GFP-fused chSTAT3 in COS-7 Cells

In Western blot analysis, it was found that leptin phosphorylated the GFP-chSTAT3 in COS-7 cells expressing chLEPR. The phosphorylated chSTAT3 was detected in 10 min and this is observed by 2 hours after stimulation (Fig. 2).

In fluorescence microscopic analysis, green fluorescence dispersed in the cytoplasm in COS-7 cells co-transfected with GFP-chSTAT3 and pCI-chLEPR as well as in control cells before leptin stimulation (Fig. 3). Leptin induced translocation of GFP-chSTAT3 into nuclei was detected by 10 min and intranuclear chSTAT3 was found by 2 h after leptin treatment. However, green fluorescence in the COS-7 cells exposed 2 h with rmleptin was found in the cytoplasm as well

as in the nuclei (Fig. 3), which may indicate increased in-activated or dephosphorylated form of chSTAT3 in the cells.

Activation of chSTAT3 by leptin in CHO-K1 cells stably expressing GFP-fused chSTAT3.

Green fluorescence signals accumulated into nucleus after rmleptin stimulation in CHO-chLEPR/STAT3 cells, whereas no such dynamic change was found in CHO-chSTAT3 cells (Fig. 4). Frequency of nucleus condensation of the GFP signals was more with stimulation by 10 ng/ml of rmleptin than by 5 ng/ml (Fig. 4).

Discussion

Since identification of chicken leptin gene is still in conflict (Friedman-Einat *et al.*, 1999; Amills *et al.*, 2003; Richards and Proszkowiec-Weglarz, 2007), we had earlier established a bioassay to detect leptin activity using chicken LEPR (Adachi *et al.*, 2008). However, we could not detect leptin-like activity in chicken serum. Other researchers had also failed to detect serum leptin activity in chicken and other birds using similar bioassay (Hen *et al.*, 2008; Yosefi *et al.*, 2010).

STAT proteins are mediators of cytokine-regulated genes and exist in cytoplasm as monomers. STATs are phosphorylated by JAK after cytokine stimulation, and activated STATs dimerize and translocate into nucleus. The use of GFP as a tag for shuttling protein is valuable to study the trafficking of signaling molecule in individual living cells (Cubitt *et al.*, 1995; Htun *et al.*, 1996; Misteli *et al.*, 1997). In the present study, we developed a new bioassay using GFP tagged chSTAT3 to detect leptin activity in living cells. GFP-fused chSTAT3 was transiently expressed in COS-7 cells (Fig. 1) and was phosphorylated after leptin treatment (Fig. 2). In addition, intracellular localization of GFP-chSTAT3 changed after leptin treatment. After leptin stimulation, fluorescence signals were observed around cell membrane as well as in nuclei in 10 min after leptin stimulation and intranuclear GFP-chSTAT3 was observed by 2 h after leptin treatment (Fig. 3). However, most of the signals condensed in the nuclei spread to the cytoplasm 2 h after leptin treatment (Fig. 3). It is suggested that activated STAT6, which belongs to the same STAT family with STAT3, is deactivated by dephosphorylation and which initiates recruitment of the molecule (Andrews *et al.*, 2002). Data obtained from the fluorescence microscopic analysis may therefore reflect physiological process of STAT3 after leptin stimulation. In addition, we observed that GFP-chSTAT3 activated STAT3-responsive reporter gene on stimulation by leptin (data not shown). Therefore, GFP-chSTAT3 acts as transcription factor in activating target genes. We then therefore established cells stably expressing chLEPR and GFP-chSTAT3 (CHO-chLEPR/STAT3) to develop a new bioassay monitoring leptin activity using fluorescence microscope in living cells. Leptin specifically induced nuclear transition of GFP-chSTAT3 in CHO-chLEPR/STAT3 in a dose dependent manner (Fig 4). Reporter gene assays developed by us and others require a long time to determine specific leptin activity (Adachi *et al.*, 2008; Hen *et al.*, 2008), and it is noteworthy

Fig. 1. **Western blot analysis of GFP fused chSTAT3 expressed in COS-7 cells.** Whole cell lysates prepared from COS-7 transfected with pEGFP-N1 (control) or pEGFP-chSTAT3 (chSTAT3) were subjected to Western blot using anti-GFP monoclonal antibody (GFP mAb) or anti-STAT3 monoclonal antibody (STAT3 mAb).

Fig. 2. **Phosphorylation of GFP-fused chSTAT3 by leptin stimulation.** COS-7 transfected with pEGFP-chSTAT3 was treated with 125 ng/ml rmleptin. Whole cell lysates prepared at 0, 10, 30, 60 and 120 min after leptin treatment was analyzed by Western blot using anti-STAT3 monoclonal antibody and anti-mouseSTAT3 (pY705) monoclonal antibody.

Fig. 3. Cellular localization of GFP-fused chSTAT3 after leptin stimulation in COS-7 cells. COS-7 transfected with pEGFP-chSTAT3 was stimulated with 125 ng/ml of rmleptin for 0, 10, 30, 60 and 120 min. Arrowhead indicates chSTAT3 migrated into nucleus by leptin stimulation. Nucleus condensation of the STAT3 was confirmed by DAPI stain. The representative photo confirming condensation of STAT3 in the nucleus after 30 min rmleptin stimulation is shown as merge.

Fig. 4. Time dependent change of intracellular GFP-fused chSTAT3 by rmleptin in CHO-chLEPR/STAT3 cells. CHO-chLEPR/chSTAT3 (chLEPR/STAT3) and CHO-chSTAT3 (chSTAT3) as control were stimulated with 5 ng/ml (A) or 10 ng/ml (B) rmleptin and in which intracellular behavior of GFP-fused chSTAT3 was monitored by fluorescence microscope. Arrowhead indicates cells chSTAT3 was activated by leptin treatment.

that in the present study, leptin dependent activation of STAT3 was found in less than one hour.

Physiological leptin concentrations in blood of mammals range from 3 to 80 ng/ml (Tomimatsu *et al.*, 1997; Friedman-Einat *et al.*, 2003; Nkrumah *et al.*, 2007). We confirmed that rat plasma specifically activated chLEPR in CHO-chLEPR/STAT3 cells (data not shown). This result indicates that CHO-chLEPR/STAT3 cells seem to be sensitive to the physiological dose of leptin in mammals. As xenopus leptin activates chLEPR (Hen *et al.*, 2008), it is likely that CHO-chLEPR/STAT3 specifically binds with leptin-like molecule in lower vertebrates. Therefore, CHO-chLEPR/STAT3 would be useful as a qualitative bioassay to determine leptin-like activity in blood and tissues in vertebrates including birds. Further experiment is required to confirm whether leptin-like activity is detected from avian derived sample using this bioassay system to rule out existence of leptin-like molecules in bird.

In conclusion, we established a cell stably expressing chicken LEPR and GFP-tagged chSTAT3 and this makes it possible to determine leptin-like activity in living cells. Therefore, the CHO-chLEPR/STAT3 cells are excellent tools to detect and monitor leptin-like activity in avian tissues.

Acknowledgment

This study was supported in part by a Grant-in-Aid for Scientific Research (C) from the Promotion of Sciences to T.O. (No. 18580290) and Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists to H.A.

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