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Different intracellular signalling properties induced by human and porcine growth hormone

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ABSTRACT

Growth hormone (GH) is reportedly species-specific. Primate growth hormone can trigger non-primate growth hormone receptor (GHR), but primates GHR cannot be activated by non-primate GH. However, it is also unclear that why primate GH and non-primate GH have different biological activities. Thus, we analysed primate growth hormone (human growth hormone (hGH)) or non-primate GH (porcine growth hormone (pGH))-induced intracellular signalling in 3T3-F442A cells and rat hepatocytes in a dose- and time-dependent manner to explore the different biological activities between them. The results revealed that both hGH and pGH can activate Janus kinase 2 (JAK2), Signal transducers and activators of transcription 1, 3 and 5 (STATs 1, 3 and 5) and extracellular signal-regulated kinase 1/2 (ERK1/2). There were no significant differences in JAK2 or ERK1/2 tyrosine phosphorylation after hGH and pGH treatment, but there were different between hGH and pGH in STAT/1/3/5 tyrosine phosphorylation, and JAK2, STAT/1/3/5 tyrosine phosphorylation was time-dependent and dose-dependent, whereas ERK1/2 was not. Both hGH and pGH demonstrated similar kinetics for STATs 1, 3 and 5 phosphorylation, but the pGH-mediated tyrosine phosphorylation was weaker than that mediated by hGH. Our observations indicated that the levels of main signalling proteins phosphorylation triggered by hGH or pGH were not exactly the same, which may explain the different biological activities showed by primate GH and non-primate GH.

1. Introduction

Growth hormone (GH) is a polypeptide hormone that is produced in the anterior pituitary. It consists of 191 amino acids with a predicted molecular weight of 22 kDa (Brooks and Waters, 2010; Li et al., 2013). GH plays an important role in the regulation of postnatal growth (Brooks and Waters, 2010), and it regulates substance metabolism (Møller and Jørgensen, 2009). GH exerts its effects by binding to growth hormone receptor (GHR), which is a member of the class 1 cytokine receptor family. GHR is a transmembrane glycoprotein with three parts: an extracellular domain (ECD), a transmembrane domain (TMD) and an intracellular domain (ICD) (Leung et al., 1987). There are two conservative sequences in the intracellular domain of GHR, which are termed Box 1 and Box 2. The former is a proline-rich sequence that is important for JAK2 association (Kaabi, 2012). GHR functions as a homodimer to activate JAK2 and a Src family kinase, which activates several intracellular signalling pathways, especially JAK2, STAT transcription factors (representatives are STATs 1, 3 and 5) and ERK1/2 to regulate gene transcription (Waters and Brooks, 2012).

GH is species specific. Rivero et al. compared the native conformations of the growth hormone family (Rivero et al., 1990), and they observed no conformational differences between any two primates or any two non-primates. Among primate and non-primate GHs, hGH is "evolutionarily optimized" because: (1) it can activate primate and non-primate GHR, whereas non-primate hormones have no physiological effect on human GHR (Behncken et al., 1997; Lauterio et al., 1988); (2) Pearce et al. determined that there were no increases in hGH biological activity, even if the affinity of hGH site 1 increased 400-fold or the affinity of hGH site 2 increased 40-fold (Pearce et al., 1999). The biological activity of hGH is also reportedly higher compared with non-primate GH, e.g., porcine and rat GH in the non-primate GHR model. In the porcine GHR model, pGH biological activity is weaker than human GH; however, the biopotency of porcine GH for its receptor was increased up to 5-fold when site 1 binding residues were substituted with hGH residues (Wan et al., 2004). In the rat GHR model, human GH also demonstrated high biopotency compared with rat GH (Ram et al., 1996). Until now, the mechanism underlying the different

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biological activities of primate GH (such as human GH) and non-primate GH has been unclear.

In the present study, we explored the reason for the different biological activities of primate GH (human GH) and non-primate GH (porcine GH) from the angle of GH-induced intracellular signalling. To assess this, we analysed hGH or pGH-induced intracellular signalling in 3T3-F442A cells and rat hepatocytes, which endogenously express mouse GHR or rat GHR. These cells are a valuable system for studying the mechanism of action of GH and GH-induced signalling pathways (Li et al., 2013; Lan et al., 2014a, b; Wang et al., 2009; Smit et al., 1996; Jin et al., 2008). We observed that activation of intracellular signalling was different between hGH and pGH, which can provide an explanation for the different biological activities induced by primate GH and non-primate GH.

2. Materials and methods

2.1. Antibodies and reagents

Rabbit monoclonal antibodies for phospho-JAK2 (Tyr1007/1008) and total JAK2, phospho-STAT5 (Tyr694) and total STAT5, phospho-STAT3 (Tyr705) and total STAT3, phospho-STAT1 (Tyr701) and total STAT1, total ERK1/2 and phospho-ERK1/2 (Thr202/Tyr204) were obtained from Cell Signalling Technology (Danvers, MA, USA). HRPHOSPHO-conjugated goat anti-rabbit antibodies, hGH and pGH were obtained from Sigma (USA). Cell lysis buffer (RIPA kit), Difco skim milk, enhanced chemiluminescence (ECL) and BCA kits were obtained from Pierce (USA). Dulbecco's modified Eagle's medium (DMEM), newborn calf serum (NBCS) and foetal bovine serum (FBS) were obtained from Gibco (USA). Bovine serum albumin (BSA) and polyvinylidene fluoride (PVDF) membranes were obtained from Millipore (Bedford, MA, USA). Unless otherwise stated, other reagents were obtained from Sigma (USA).

2.2. Cell line and cell culture

The 3T3-F442A mouse pre-adipocyte cell line (kindly provided by WeiXing, Huacheng biological Co. Ltd, Changchun) were maintained in Dulbecco's modified Eagle's medium with 4.5 g/L glucose (Cellgro, Inc.) supplemented with 10% newborn calf serum, streptomycin (0.05 mg/mL), and penicillin (0.1 mg/mL) (Biofluids, Rockville, MD) at 37 °C in a 100% humidified atmosphere of 5% CO_2 and passaged every 3 or 4 days.

2.3. Primary rat hepatocyte culture

Rat hepatocytes were isolated using the two-step collagenase perfusion technique with slight modifications (Li et al., 2013; Salavert and Iynedjian, 1982; Ribaux et al., 2002). The isolating method was approved by the Animal Ethical Committee of Jilin Agricultural University. Briefly, the rats (Sprague–Dawley, 150–200 g body weight, provided by the Experimental Animal Center, Jilin University) were anaesthetised using pentobarbital (100 mg/kg). The abdominal cavity and chest was opened, the heart and liver were fully exposed, the abdominal fat and small intestine were poked, and the inferior vena cava and hepatic portal vein were exposed. The rat livers were perfused in two steps, first using the calcium- and magnesium-free Hank's buffer (pH 7.4) at 37 °C for 10 min and subsequently a recirculating collagenase buffer containing 0.05% (w/v) collagenase at 37 °C for 10 min. Then, the liver was transferred to a petri dish, minced and filtered through a 70-micron sterile cell strainer nylon mesh. The resulting cell suspensions were centrifuged twice at 1000 RPM for 5 min, and the precipitate was resuspended in culture medium (DMEM with 10% FBS, glutamine, penicillin and streptomycin). Hepatocyte viability was approximately 90% as assessed by trypan blue dye exclusion. After 3 h, the floating hepatocytes were attached to collagen-coated plastic culture dishes, and the cells were cultured with maintenance medium (DMEM with 4% FBS, penicillin and streptomycin) for the next experiment.

2.4. Cell treatment and protein extract

The cell density was 1×10^6, and the cells were cultured as previously described. Before the experiment, the 3T3-F442A cells were starved with culture medium containing 0.5% (wt/vol) BSA in the absence of calf serum, or the isolated hepatocytes were cultured with maintenance medium (DMEM with 4% FBS, penicillin and streptomycin) for 16–20 h. Pretreatments and stimulations were performed at 37 °C in binding buffer consisting of 25 mM Tris–HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.1% (wt/vol) BSA, and 1 mM dextrose. For the dose-dependent experiments, the stimulation doses of hGH and pGH were 0, 0.4, 2, 4, 10, 20 and 40 nM, which continued for 20 min. For the time course experiments, the cells were stimulated by hGH (4 nM) or pGH (4 nM) and terminated at 0, 3, 10, 20, 40, 60 and 90 min. Stimulations were terminated by washing the cells three times with ice-cold PBS, and the cells were subsequently solubilised for 30 min at 4 °C with lysis buffer and harvested by scraping. The cells were centrifuged at 4500 RPM for 15 min at 4 °C, and the supernatant was collected for Western blot analyses.

2.5. Analysis of Intercellular Signalling by Western Blot in 3T3-F442A or hepatocytes

Protein samples were fractionated using 12% polyacrylamide gels. The proteins were then transferred onto PVDF membranes, and the membranes were blocked for 1 h at 37 °C with TBST blocking buffer (0.1% Tween 20, 5% bovine serum albumin). The membranes were then washed three times for 10 min in TBST buffer (10 mM Tris–HCl (pH 7.5), 0.1 M NaCl, 0.1% Tween 20) and incubated overnight at 4 °C with phospho- or total-JAK2, STAT5, STAT3, STAT1, or ERK1/2 antibodies according to the manufacturer's protocols. After washing three times for 10 min in TBST buffer, the membranes were incubated with secondary goat anti-rabbit IgG-conjugated horseradish peroxidase antibodies at room temperature for 1 h and then washed in TBST buffer as previously described. Proteins were detected using the ECL detection system. To detect signals from other primary antibodies, the blots were stripped by incubation in 2% sodium dodecyl sulphate, 100 mM 2-mercaptoethanol, and 62.5 mM Tris–HCl (pH 6.7) for 50 min at 37 °C and then rinsed three times for 10 min in TBST prior to reprobing. The results presented in the individual figures are based on grey-scale scans of portions of X-ray films for each membrane. The scans were obtained using a Cannon IX-4015 scanner.

2.6. Densitometric analysis

Densitometric analysis of the immunoreactive protein bands was performed using Quantity One® software (developed by Bio-Rad Technical Service Department, USA; LSG.TechServ.US@Bio-Rad.com).

2.7. Statistical analysis

The data are represented as the mean values ± standard error (S.E.). Statistical analysis was performed by independent-samples *T* test using Statistical Analysis System (SAS) software (SAS version