

Porcine growth hormone induces the nuclear localization of porcine growth hormone receptor *in vivo*

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Submitted Aug 10, 2017; Revised Sept 16, 2017;

Accepted Sept 21, 2017

Objective: Recent studies have challenged the traditional paradigm that growth hormone receptor (GHR) displays physiological functions only in the cell membrane. It has been demonstrated that GHR localizes to the cell nucleus and still exhibits important physiological roles. The phenomenon of nuclear localization of growth hormone (GH)-induced GHR has previously been described *in vitro*. However, until recently, whether GH could induce nuclear localization of GHR *in vivo* was unclear.

Methods: In the present study, we used pig as an animal model, and porcine growth hormone (pGH) or saline was injected into the inferior vena cava. We subsequently observed the localization of porcine growth hormone receptor (pGHR) using multiple techniques, including, immunoprecipitation and Western-blotting, indirect immunofluorescence assay and electronmicroscopy.

Results: The results showed that pGH could induce nuclear localization of pGHR. Taken together, the results of the present study provided the first demonstration that pGHR was translocated to cell nuclei under pGH stimulation *in vivo*.

Conclusion: Nuclear localization of pGHR induced by the *in vivo* pGH treatment suggests new functions and/or novel roles of nuclear pGHR, which deserve further study.

Keywords: Porcine Growth Hormone; Porcine Growth Hormone Receptor; Nuclear Translocation; *In vivo*

INTRODUCTION

Growth hormone (GH) processes extensive physiological functions [1]. According to statistics, GH has more than 300 functions and roles [2]. GH initiates intracellular signalling by interacting with growth hormone receptor (GHR) localized at the cell membrane. GH binding to GHR induces phosphorylation of the Janus kinase (JAK2), which subsequently triggers a series of intracellular signalling proteins, including signal transducer and activator of transcription (STAT) as well as extracellular regulated protein kinases, and these signalling proteins contribute to all of the actions of GH [1].

The previous paradigm of the action mechanism of GH/GHR is that after GH binds to the membrane-GHR, JAK2, and GHR are activated, and subsequently, down-stream signalling molecules are recruited and activated. These signal molecules transport from the cytoplasm to the nucleus, where they regulate target gene transcription. However, twenty years ago, the phenomenon of GH and/or GHR nuclear localization was reported *in vitro* [3]. However, until recently, researchers had determined the functions of this protein in nuclei. Water et al showed that GHR nuclear localization is associated with the proliferative status of cells and tissues [4]. Subsequently, several studies have reported the new functions of nuclear-GHR [5-6]. Indeed, nuclear translocation of cell membrane GHR can be divided

into three basic processes: GHR internalization, cytoplasmic transport and nuclear localization. Strous et al have indicated that the ubiquitin system is required for GHR internalization. Furthermore, it has been reported that importin α and β (IMP α/β) are responsible for GHR nuclear translocation [4].

Porcine GH possesses extensive biological activities in pigs, e.g., improving the feed efficiency, increasing the growth rate, and changing the carcass composition [7-9]. It has been demonstrated that the porcine liver is an important target of porcine growth hormone (pGH), which endogenously expresses pGHR, and studies have demonstrated that porcine hepatocytes are an important model for studying the interactions between pGH and pGHR.

However, thus far, there is no report of the nuclear localization of pGHR in domestic animals *in vivo*. In addition, it remains unclear whether pGH could induce nuclear localization of pGHR *in vivo*, although the phenomenon of GH-induced GHR nuclear localization has been reported *in vitro*. There are many differences *in vitro* and *in vivo*, and the *in vivo* environments are more complicated compared to the *in vitro* environments. In the present study, we selected the pig as an animal model to study pGHR nuclear translocation under pGH treatment *in vivo*. We demonstrated that the *in vivo* treatment of pGH could induce nuclear localization of pGHR.

MATERIALS AND METHODS

Antibodies and reagents

pGH was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-pGHR antibody was obtained from Abcam (Cambridge, England). Nuclear/Cytoplasmic Extraction Reagent Kits were obtained from Pierce (Rockford, IL, USA). Bovine serum albumin (BSA), non-fat milk, tissue lysis buffer and enhanced chemiluminescence (ECL) were purchased from Beyotime (Shanghai, China). Glutaraldehyde and paraformaldehyde were obtained from Hua-Yi Biotechnology (Changchun, China). Polyvinylidene fluoride (PVDF) membranes were obtained from Millipore (Bedford, MA, USA). The colloidal gold-conjugated secondary antibody was purchased from Abcam (England). Second antibodies were purchased from Kai-Ji Biotechnology (Nanjing, China).

pGH treatment *in vivo*

Pigs (Landrace, average ~15 kg body weights) were used following experimental treatment, and the study was approved by the Animal Ethical Committee of Jilin Agricultural University. The animals were housed three per cage at room temperature. Pigs were randomly assigned to experimental and control groups, and the experimental group was treated with pGH (0.5 mg/kg). The pGH dose that was used was the same as that from a previous study, and the control group was treated with saline. The pigs were deeply anaesthetized using

pentobarbital (100 mg/kg). The abdominal cavity was opened, the inferior vena cava was exposed, and pGH was injected into the inferior vena cava. After 30 min, the livers were isolated, and subsequent experiments were performed as described below.

Immunoelectron microscopy

After the pGH treatment described above, the left liver lobes were isolated. The liver samples (size 1 mm³) were subsequently sectioned. The samples were fixed with 4% glutaraldehyde for 2 h at 4°C. After washing, the tissue samples were post-fixed in 2% osmic acid solution for 3 h at 4°C, dehydrated in graded ethanol solutions and further infiltrated and embedded in araldite. Ultrathin sections (40 to 50 nm) of tissue samples were cut using a Leica Ultracut UCT ultramicrotome (Leica Company, Solms, Germany). After washing three times, the sections were incubated with 5% normal goat serum for 30 min to block non-specific binding. The sections were subsequently rinsed and incubated with the primary antibody (anti-GHR antibody) for 1 h. After washing, the sections were incubated with a secondary antibody (goat anti-mouse) conjugated to 10-nm colloidal gold particle for 30 min, followed by three washes. After staining with uranyl acetate for 10 min, the sections were examined using transmission electron microscopy (EM) (HITACHI H-7650, Hitachi Limited, Tokyo, Japan).

Immunoprecipitation and Western-blotting

After pGH treatment, the liver tissues were sampled. The Nuclear/Cytoplasmic Extraction Reagent Kit (Pierce, USA) was used to isolate nuclear and cytosol fractions of the liver tissue according to the manufacturer's instructions. For subsequent immunoprecipitation, the cytosolic or nuclear fractions were incubated with the anti-pGHR antibody or an irrelevant isotype-matched antibody overnight at 4°C. The immunoprecipitated proteins were subsequently subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-pGHR antibodies as previously described [10].

Indirect immunofluorescence assay

The indirect immunofluorescence assay (IFA) experiments were carried out according to our previous procedure [11]. In brief, after pGH injections, the porcine hepatocytes were rapidly isolated and fixed with 4% paraformaldehyde for 20 min, then permeabilized for 5 min in 0.5% Triton X-100. After washing with phosphate-buffered saline (PBS), the cells were then treated with 2% BSA for 1 h, after which, the porcine hepatocytes were incubated with anti-pGHR or isotype control monoclonal antibody (negative control) for 1 h. The cells were washed again with PBS and incubated with fluorescein isothiocyanate-labelled goat anti-mouse immunoglobulin G for

1 h in the dark. In addition, the propidium iodide was used to stain the cell nucleus. After finishing three washes, the cells were analysed using confocal laser scanning microscopy (Olympus FV1000, Olympus, Tokyo, Japan).

Statistical analysis

The data are presented as the mean values \pm standard error of triplicate samples.

RESULTS

Characterization of pGHR expression

We first established pGHR expression in porcine liver tissue (Landrace, ~15 kg) by western-blot using an anti-pGHR antibody and, as indicated in Figure 1, a pGHR band (~120 kDa) was detected in liver tissue, and the control antibody showed no effects (Figure 1).

GHR's localization with or without pGH stimulation

After the pGH injections, as described in the Materials and Methods section, the cytoplasmic and nuclear fractions from the liver tissue were isolated using the Nuclear/Cytoplasmic Extraction Reagent Kit (Pierce, USA). The isolated Nuclear/Cytoplasmic proteins were subjected to immunoprecipitation and Western-blot experiments. As shown in Figure 2A, after pGH treatment, pGHR was detected in the nuclear extract and was also detected in the cytoplasm. pGHR from liver tissue after saline treatment was detected in the cytoplasm but not in the nucleus.

Next, IFA assays were used to further confirm these observations. As illustrated in Figure 2B and 2C, after pGH treatment, pGHR was primarily localized in the cytoplasm and

nucleus. By contrast, pGHR in liver tissue after saline treatment was primarily localized in the cytoplasm, and little or no pGHR signalling was detected in the cell nucleus. By the co-localization analysis of Confocal laser scanning microscope (yellow signal), it can be found that pGH and pGHR may form a dimer in the cytoplasm and nuclei (Figure 2D). In this section, we did not perform immunohistochemistry experiments to detect and localize pGHR, this is because that the anti-pGHR antibody applied in the current experiments is not suitable to be used in the immunohistochemistry assay.

In addition, we also performed EM experiments to observe pGHR nuclear localization, and as shown in Figure 2E (see indicated arrows), pGHR was detected in cell nuclei and the nuclear membrane.

pGHR's nuclear localization is pGH-dependent

To determine whether pGHR nuclear localization is specifically induced by pGH, a pGHR-specific antibody and control antibody (B32 developed in a previous study and demonstrated to compete with pGH for GHR binding [11]) were pre-injected in the inferior vena cava for 30 min prior to pGH injection. As indicated in Figure 3, pGHR was not detected in the cell nucleus in liver tissue pre-injected with B32. To further evaluate if the pGHR's nuclear localization is GH-dependent, we also performed the corresponding experiments by the injection of human growth hormone (hGH) or bovine growth hormone (bGH), we found that hGH and bGH also could induce pGHR's nuclear localization. These observations are similar with that of the *in vitro* experiments. These findings suggest that the nuclear localization of pGHR may be GH-dependent.

DISCUSSION

In the present study, to our knowledge, we demonstrated the first exploration of pGHR nuclear translocation induced by pGH *in vivo* in domestic animals. The results showed that pGHR was primarily localized to the cytoplasm without pGH stimulation. By contrast, pGHR was strongly localized to cell nuclei after pGH treatment, suggesting a new scientific study point, namely, the potential functions of nuclear-pGHR in porcine liver tissue, which deserve further study.

pGH plays important roles in the growth and development of pigs [12]. Almost all tissues express pGHR, suggesting that pGH has potential effects on all tissues. The traditional paradigm of the GH/GHR interaction is that after GH binding to membrane-GHR, JAK2 and GHR are activated through phosphorylation, followed by activation of down-stream signalling molecules [13]. These signal molecules transport from the cytoplasm to the nucleus, where they regulate target gene transcription. However, a number of studies have demonstrated the phenomenon of GHR nuclear localization, although the

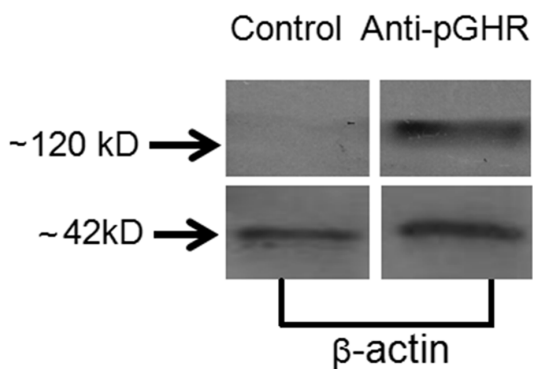


Figure 1. Western-blot analysis of pGHR (porcine growth hormone receptor) expression in porcine liver tissue. The liver tissue samples were collected, and subsequently, the extracted protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were incubated with 3% bovine serum albumin to block non-specific binding. After washing, the membranes were incubated with primary antibody (anti-pGHR), followed by a secondary antibody, for 1 h each. Enhanced chemiluminescence (ECL) was used to detect the immune-reactive bands.

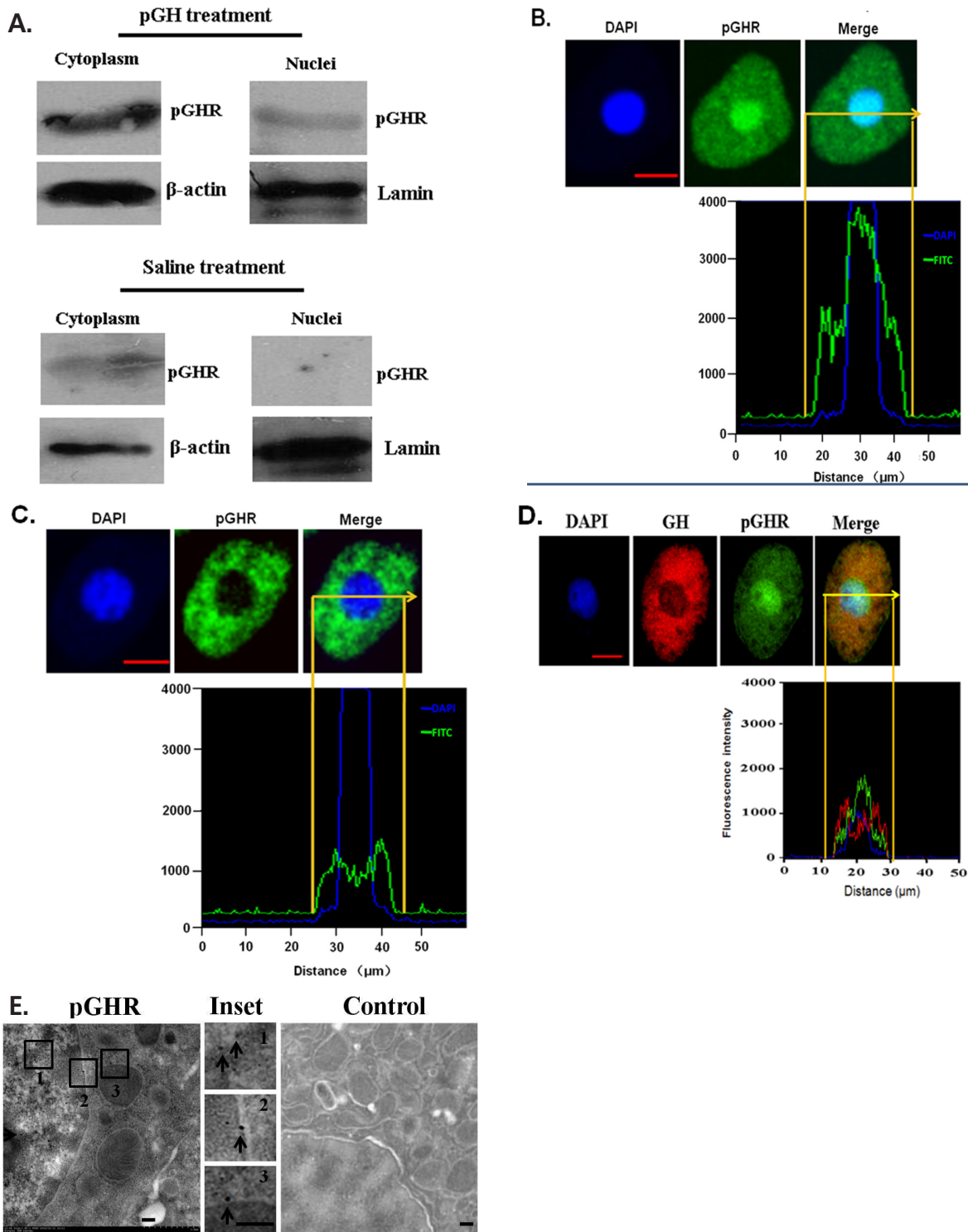


Figure 2. (A) Analysis of porcine growth hormone receptor (pGHR) nuclear localization by Western-blotting. The Nuclear/Cytoplasmic Extraction Reagent Kit (Pierce, Rockford, IL, USA) was used to isolate nuclear and cytosol fractions of the liver tissue. Subsequently, for immunoprecipitation, the cytosolic or nuclear fractions were incubated with the anti-pGHR antibody or an irrelevant isotype-matched antibody overnight at 4°C. The immunoprecipitated proteins were subsequently subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by immunoblotting with anti-pGHR antibodies. (B), (C) Analysis of pGHR localization by immunofluorescence assay (IFA). After porcine growth hormone (pGH) or saline treatment, the liver tissue samples were collected. The following IFA experiments were performed as described as in the Materials and Methods section. The corresponding histograms show the co-localization of pGHR (green signal) and the cell nuclei (blue signal), which were achieved by scanning across the one cell. (D) The co-localization analysis of GH and pGHR. (E) Immunoelectron microscopy of pGHR’s nuclear localization. After pGH treatment, liver tissues were collected. Ultrathin sections were prepared and treated as described in the Materials and Methods section. The sections were examined using transmission electron microscopy (HITACHI H-7650, Tokyo, Japan). Arrow points to colloidal gold particles. N, nuclei; C, cytoplasm. Bar: 200 nM. The figure represents three independent experiments.

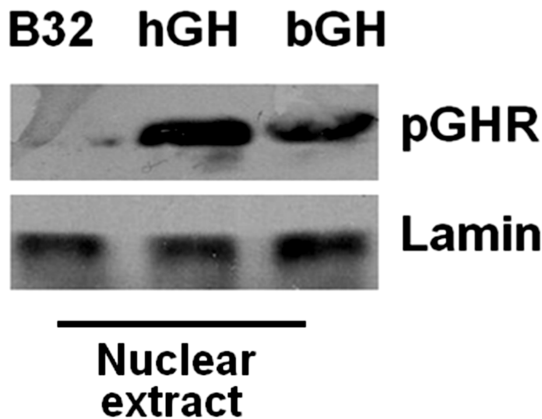


Figure 3. Analysis of porcine growth hormone receptor (pGHR) nuclear localization by Western-blotting. After treatments with the indicated ligands, the Nuclear Extraction Reagent Kit (Pierce, Rockford, IL, USA) was used to isolate nuclear fractions of the liver tissue. Subsequently, the nuclear fractions were subsequently subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by immunoblotting with anti-pGHR antibodies.

specific and exact functions of nuclear-GHR in cell nuclei remain unclear. Walters et al showed that nuclear-GHR associated with cell proliferation and that abnormal nuclear localization of GHR may induce tumour generation [4]. In the present study, we observed the phenomenon of the nuclear localization of pGHR induced by pGH *in vivo*. However, the functions of nuclear-localized pGHR remain unclear and deserve further study.

Indeed, the mechanism(s) of pGHR nuclear translocation remains to be fully understood. In a previous study, we showed that IMP α/β is involved in pGHR nuclear translocation in porcine hepatocytes [6]. In general, although nuclear translocation of cell membrane proteins can be divided into three basic steps, including protein internalization, cytoplasmic transport and nuclear localization, nuclear translocation is a complicated molecular procedure involving many molecules and cellular organelles. For example, epidermal growth factor receptor (EGFR) nuclear translocation is involved in many cytoplasmic molecules and organelles, involving multiple nuclear localization pathways for EGFR [14–17]. Currently, studies on the mechanism(s) of GHR nuclear transport remain limited, and these studies were only performed *in vitro*. The present study establishes a model for the study of GHR nuclear localization *in vivo*.

Conway-Campbell et al showed that GHR prolongs STAT5 activation based on its nuclear localization. In addition, Graichen et al showed that nuclear-growth hormone binding protein (GHBP) enhances STAT5-mediated transcription [18]. However, until recently, the molecular mechanisms through which GHBP and GHR display these physiological activities remain unclear. In addition, we cannot exclude that, similar to EGFR, nuclear-GHR can be used as a transcription factor.

Taken together, the results of the present study provide the

first evidence that pGH can induce pGHR nuclear translocation *in vivo*, indicating that pGHR not only functions in the cell membrane but also in cell nuclei.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation-Young investigator grant program (Grant number 31602022). This project was partially supported by the Scientific Research projects of the Thirteenth Five-Year plan of Jilin Province Department of Education (Grant number 2016178).

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