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Domestic Animal Endocrinology 30 (2006) 144–154
DOMESTIC ANIMAL ENDOCRINOLOGY
www.journals.elsevierhealth.com/periodicals/dae

Short communication

# Cellular localization of IL-18 and IL-18 receptor in pig anterior pituitary gland

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Received 22 July 2005; accepted 22 July 2005

**Abstract**

Pro-inflammatory cytokine interleukin 18 (IL-18) has been proposed to have a role in modulating immuno-endocrine functions. Our previous study showed that IL-18 and IL-18 receptor (IL-18R) colocalized in somatotrophs of the bovine anterior pituitary gland, and the possibility that IL-18 acts on somatotrophs as an autocrine factor. In the present study, we investigated the localization of IL-18 and IL-18R in the pig anterior pituitary gland. RT-PCR analysis showed the expression of IL-18 and IL-18R mRNAin the pig anterior pituitary gland. Immunohistochemistry of IL-18 and specific hormones revealed the presence of IL-18 in somatotrophs, mammotrophs, thyrotrophs and gonadotrophs. IL-18R was localized in somatotrophs and thyrotrophs. Furthermore, the somatotrophs immunoreactive for IL-18 did not contain IL-18R. Thus, IL-18R and IL-18 were not colocalized in an identical somatotroph. These findings suggest that the localization of IL-18 in pig somatotrophs is different from that in bovine somatotrophs, although IL-18 closely associates with somatotrophs in the anterior pituitary glands in both species.


*Keywords:* Interleukin-18; Interleukin-18R; Pig anterior pituitary gland; Immuno-endocrine signalling; Paracrine

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doi:10.1016/j.domaniend.2005.07.008

## 1. Introduction

There is increasing evidence to support a role of cytokines in anterior pituitary function. The cytokines that are expressed in the gland include pro-inflammatory cytokines such as interleukin (IL)-1, IL-6 and macrophage-migration inhibitory factor (MIF), IL-11 and leukemia inhibitory factor (LIF) [1,2]. The main source of IL-1, IL-6 and LIF in anterior pituitary gland is folliculo-stellate (FS) cells [3]. In addition, the mRNA of IL-1 receptor, IL-6 receptor, IL-11 receptor and LIF receptor has been expressed in the anterior pituitary gland [4]. Immunohistochemical analysis has revealed that IL-1 receptor (IL-1R) I and II are restricted to somatotrophs [5]. Thus, a number of cytokines have been identified as potential intercellular mediators in the anterior pituitary gland and may serve as paracrine/autocrine factors on the signaling pathway in immune-endocrine interaction.

### *1.1. Interleukin 18*

Interleukin 18 (IL-18) is a pleiotropic cytokine characterized as interferon gamma (IFN-γ)-inducing factor, which is produced by liver Kupffer cells [6]. IL-18, like IL-1β with which it shares structural homology, is produced as a 24 kDa inactive precursor (pro-IL-18) lacking a signal peptide, and is cleaved by endoprotease IL-1β-converting enzyme (ICE; caspase-1) to generate a biologically active and mature 18 kDa moiety [7]. IL-18 is produced not only by immune cells but also by non-immune cells such as dendritic cells, keratinocytes, osteoblasts, adrenal cortex cells, intestinal epithelial cells, microglial cells, habenula and ependymal cells, synovial cells and hypothalamus [8–16]. In addition, our previous study first reported that IL-18 localized in the anterior pituitary cells [17].

The initial event in IL-18 signal transduction pathway is the interaction of IL-18 with its receptor. Like that of IL-1, the IL-18 receptor (IL-18R) complex consists of a heterodimer containing α binding and β signaling peptide chain [18]. IL-18R is expressed on various cells including macrophages, neutrophils, natural killer (NK) cells, endotherial cells and smooth muscle cells [19]. However, little is known about the expression of IL-18R in endocrine cells, except for the bovine anterior pituitary cells in our previous study [17].

### *1.2. IL-18 as an immuno-endocrine modulator*

IL-18 localizes in the neurons of the superior part of the medial habenula and its immunostaining is strongly elevated by acute or chronic resistant stress [14]. IL-18 induces the intracellular expression of IL-1α and pro-IL-1β, and the release of IL-6 from mixed glia in vitro [20]. These studies on the central nervous system (CNS) suggest that IL-18 plays a role in local immune reaction and in modulating stress responses as a neuroimmunomodulator. Several investigators have described the role of IL-18 in response to stress, in which the cytokine is produced in the endocrine cells of the zona reticularis and zona fasciculata of the adrenal cortex [11], and ACTH treatment stimulates the production of IL-18 mRNA and protein in the adrenal cortex [21], indicating that IL-18 production

in the adrenal cortex is regulated through the hypothalamic–pituitary–adrenal (HPA) axis.

Our previous study showed that IL-18 and IL-18R colocalized in identical somatotrophs of the bovine anterior pituitary gland [17]. These data suggest that IL-18 may regulate the function of somatotrophs by the autocrine pathway as an immuno-endocrine mediator. However, the expression of IL-18 and its receptor has not been studied in other animals, including pigs. In the present study, we attempt to define IL-18 and its receptor in pig anterior pituitary cells.

## 2. Materials and methods

### *2.1. Animals and tissues*

In the present experiment, six three-way crossbred female pigs (genotypes: 1/2 Duroc, 1/4 Landrace, 1/4 Large White) were purchased from a local animal farm. All animals were clinically healthy and free of infectious disease. The anterior pituitary glands were removed when the pigs were 3–7 months old. Three months old of the pigs were used for RT-PCR and 7 months old of the pigs were used for immunohistochemistry, respectively. All animals were handled in accordance with the guidelines of the Administrative Panel on Laboratory Animal Care of Tohoku University.

### *2.2. mRNA expression of IL-18 and IL-18R*

Small pieces of the anterior pituitary gland were frozen in liquid nitrogen immediately after removal and stored at −80 °C. Total RNA was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, CA). One micro-gram of total RNA was reverse-transcribed for 60 min at 50 °C in total reaction mixture [200 U Superscript III, 0.5 mM dNTP Mix, 50 μM oligo(dT), 5 mM DTT and 4 μL first strand buffer (Invitrogen)]. RT reaction products were amplified with specific primer (sense: 5′-CGATGAAGACCTGGAATCGG-3′-antisense 5′-CATCATGTCCAGGAACACTTCTCTG-3′, product size 353 bp) for pig IL-18 [22], specific primer (sense: 5′-ATGATTATGTTTTGGAGTTTTG-3′, antisense: 5′-GTAATATTGAAGGTTTTGGTGA-3′, product size 373 bp) [23] for pig IL-18R and pig GAPDH primer (sense: 5′-CATCACTGCCACCCAGAAGAC-3′, antisense: 5′-CAGCCCCAGCATCAAAGGTAG-3′, product size 359 bp) [24] as the house keeping gene. Amplification was performed in 1 cycle for 5 min at 94 °C, 35 cycles of denaturation for 1 min at 94 °C, annealing at 55 °C and extention for 1 min at 72 °C. The last extention step was performed for 7 min at 72 °C. PCR products were run on 2% agarose gels and visualized by UV-transillumination. As a positive control of IL-18 mRNA expression, pig spleen was processed in the same manner.

### *2.3. Localization of IL-18 and IL-18 R*

Tissues from the anterior pituitary glands of three female pigs were immersed in Zamboni fixative overnight at 4 °C and then paraffinized. Mirror sections were cut to 2 μm sections,

deparaffinized and treated with 90% methanol containing 3% $H_2O_2$ for 5 min at room temperature. The sections were washed three times in PBS for 5 min and incubated with 1.5% normal horse, goat or rabbit serum. For an identification of IL-18 immunoreactive cells, one of the mirror sections was incubated with mouse anti-pig IL-18 antibody (5 μg/ml clone no. 2C4 [25], National Institute of Animal Health, Tsukuba, Japan) in PBS overnight at 4 °C and its counterpart was incubated with rabbit anti-bovine GH antiserum diluted 1:8000 (Biogenesis, South Coast, UK), rabbit anti-ovine PRL antiserum diluted 1:5000 (Biogenesis), rabbit anti-pig FSH antiserum diluted 1:1000 (Biogenesis), rabbit anti-rat ACTH antiserum diluted 1:10,000 (kindly provided by Dr. Inoue, Saitama university, Saitama, Japan) or rabbit anti-rat TSH antiserum diluted 1:12,000 (HAC-RT29-01RBP86, Gumma University, Gumma, Japan) in the same manner.

The immunostaining of IL-18 was validated in additional experiments using other mouse anti-pig IL-18 (clone no. 5F6 and 12C12, National Institute of Animal Health) and mouse anti-human IL-18 (5 μg/ml; MBL, Nagoya, Japan).

For the identification of IL-18R immunoreactive cells, one of the mirror sections was treated in DAKO Cytomation Target Retrieval Solution High pH (Dako Co., Carpinteria, CA) as a target retrieval technique for 5 min at 121 °C, and incubated with goat anti-human IL-18R antiserum (5 μg/ml; R&D Systems, Oxon, UK). The counterpart was incubated with mouse anti-human IL-18 antibody or rabbit anti-pituitary hormone antiserum as described above. After several washings, the sections were then incubated with the secondary antibody, biotinylated horse anti-mouse IgG, goat anti-rabbit IgG or rabbit anti-goat IgG (Vector) matched to the primary antibody in PBS. This was followed by incubation in avidin-biotin peroxidase complex (ABC; Vectastain ABC kit; Vector). The tissue-bound peroxidase was visualized with 0.025% 3,3′-diaminobenzidine and 0.01% $H_2O_2$ in 0.05 M tris buffer (pH 7.6). The sections were counterstained with Mayer's hematoxylin. For specification of the immunostainings of IL-18 and IL18R, negative controls were run in which the primary antibody was omitted or replaced with an irrelevant IgG. The number of immunoreactive cells in hormonal cells were counted in three fields of about 20,000 $\mu m^2$ randomly selected from the sections and proportion of IL-18 immunoreactive cells was described as a percentage of the colocalized cells to all hormone reactive cells as previously reported [26].

### 2.4. Colocalization of IL-18 and IL-18R in somatotrophs

To investigate the localization of IL-18 and IL-18R in somatotrophs, an immunorestaining was performed using IL-18Rα antibody following IL-18 and GH immunostaining. In brief, double-immunofluorescences using mouse anti-IL-18 and rabbit anti-GH antibody as the primary antibodies were first performed and subsequently visualized with the secondary antibodies, FITC-conjugated goat anti-mouse IgG (1:400; Sigma, St. Louis, MO) and Cy5-coujugated goat anti-rabbit IgG (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA), respectively. After being counterstained with propidium iodide and photographed by laser microscope (MRC-1024, Bio Rad, Richmond, CA), the slides were heated for 5 min at 12 °C in DAKO Cytomation Target Retrieval Solution High pH to completely abrogate the antibody reaction. After that, IL-18R was immunostained by the ABC method as described above.

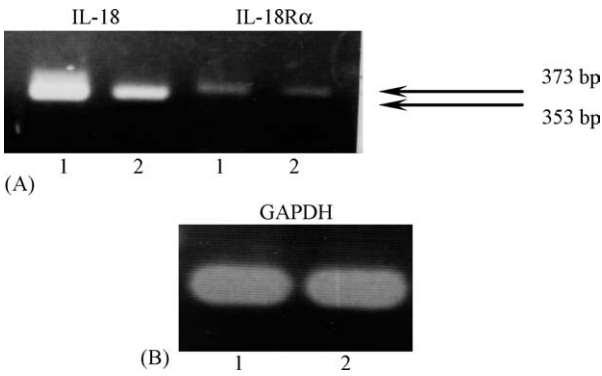


Fig. 1. mRNA expression of IL-18 and IL-18R in the pig anterior pituitary gland. The products (353 and 373 bp band as IL-18 and IL-18R) marked with arrows are detected in the spleen and anterior pituitary, respectively (A). The house-keeping gene, GAPDH, served as the internal standard (B). Lane 1: spleen; lane 2: anterior pituitary gland.

## 3. Results

### *3.1. Expression of IL-18 and IL-18Rα*

The mRNA expression of IL-18 and IL-18R was demonstrated by RT-PCR in the pig anterior pituitary gland (Fig. 1). These mRNA were consistently expressed with an expected size of 353 and 373 bp, respectively. These mRNA were also detected in pig spleen as a positive control and not detected in the same anterior pituitary total RNA without RT reaction (data not shown). The mRNA of both expressions was confirmed by several experiments.

### *3.2. Cellular localization of IL-18 in the pig anterior pituitary gland*

IL-18 immunoreactive cells were widely distributed throughout the pig anterior pituitary gland. Mirror sections, in which one section was immunostained with anti-IL-18 antibody and the other with a specific anti-pituitary hormone antiserum, revealed that IL-18 was localized in hormone producing cells, including somatotrophs, mammotrophs, thyrotrophs and gonadotrophs (Fig. 2). However, corticotrophs did not show any positive staining. The cells immunoreactive for IL-18 were about 78.3, 40.7, 27.4 and 24.7% of mammotrophs, gonadotrophs, thyrotrophs and somatotrophs, respectively. Furthermore, IL-18 antibodies of different origins (clone no. 5F6 and 12C12, and anti-human IL-18) yielded the same localization. Negative controls, in which the primary antibody was omitted, failed to show any specific staining (data not shown).

### *3.3. Cellular localization of IL-18R*

IL-18R was immunostained in the pig anterior pituitary gland to understand IL-18 signaling among the anterior pituitary cells. Mirror sections of IL-18R and pituitary hormones

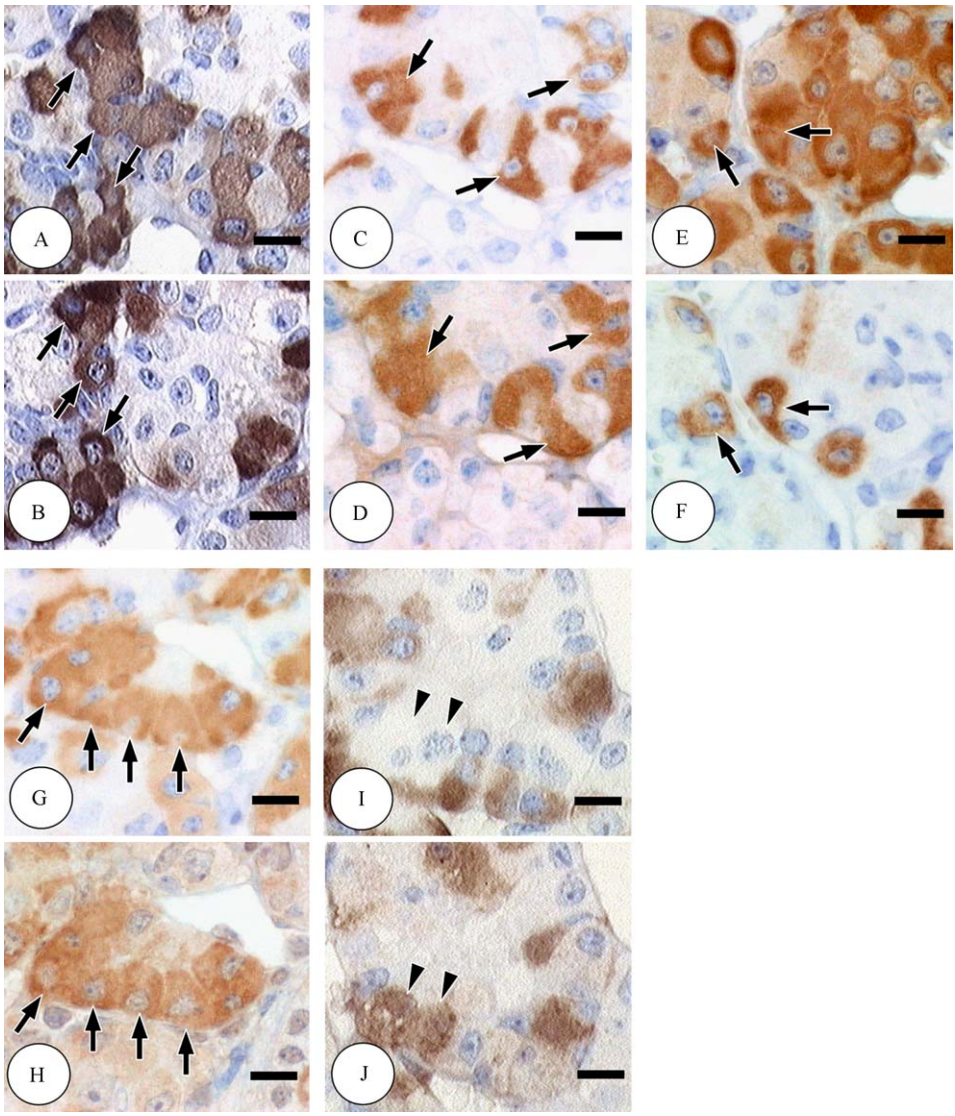


Fig. 2. Cellular localization of IL-18 in the pig anterior pituitary gland. Mirror sections shows the localization of IL-18 in somatotrophs (A and B), mammotrophs (C and D), thyrotrophs (E and F), gonadotrophs (G and H), but dose not show the localization in corticotrophs (I and J). The arrows show the colocalization of IL-18 and pituitary hormone in the identical cells and the arrowheads show the cells without IL-18. A, C, E, G and I were immunostained by anti-IL-18 antibody and B, D, F, H and J were immunostained by anti-GH, anti-PRL, anti-TSH, anti-FSH and anti-ACTH antiserum, respectively. Bars show 10 μm.

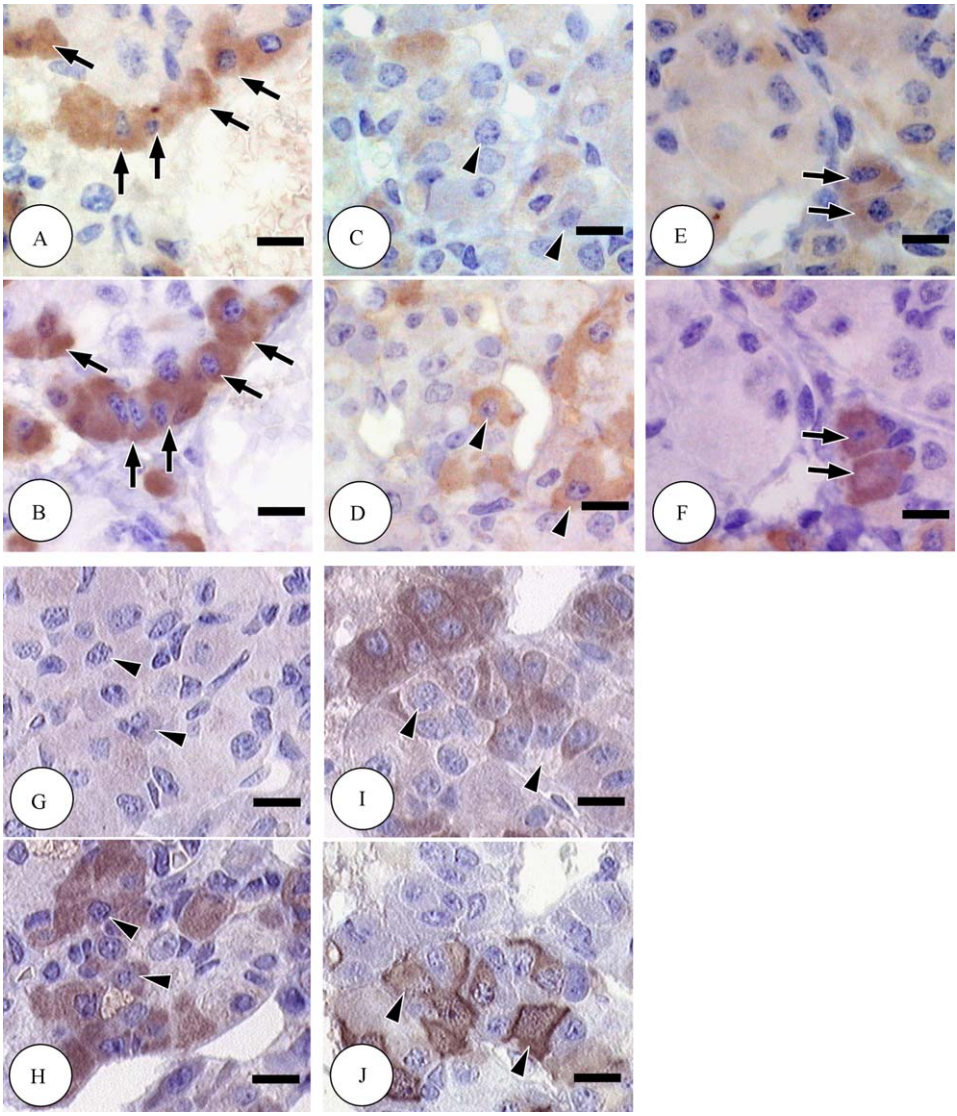


Fig. 3. Cellular localization of IL-18R in the pig anterior pituitary gland. Mirror sections show the localization of IL-18R in somatotrophs (A and B) and thyrotrophs (E and F) but does not show the localization in mammotrophs (C and D), gonadotrophs (G and H) and corticotrophs (I and J). The arrows show the colocalization of IL-18R and pituitary hormone in the identical cells and the arrowheads show the cells without IL-18R A, C, E, G and I were immunostained by anti-IL-18R antibody and B, D, F, H and J were immunostained by anti-GH, anti-PRL, anti-TSH, anti-FSH and anti-ACTH antiserum, respectively. Bars show 10 μm.

revealed that IL-18R was localized in somatotrophs and thyrotrophs, but not mammotrophs, gonadotrophs and corticotrophs (Fig. 3). Negative controls, in which primary antibody IL-18R was substituted for normal goat serum, failed to show any specific staining (data not shown).

Cellular localization of IL-18 and IL-18R in pig somatotrophs was detailed by immuno-restaining of IL-18R (Fig. 4) in order to compare with findings in bovine somatotrophs. IL-18Rα was localized in somatotrophs. The somatotrophs immunoreactive for IL-18 did

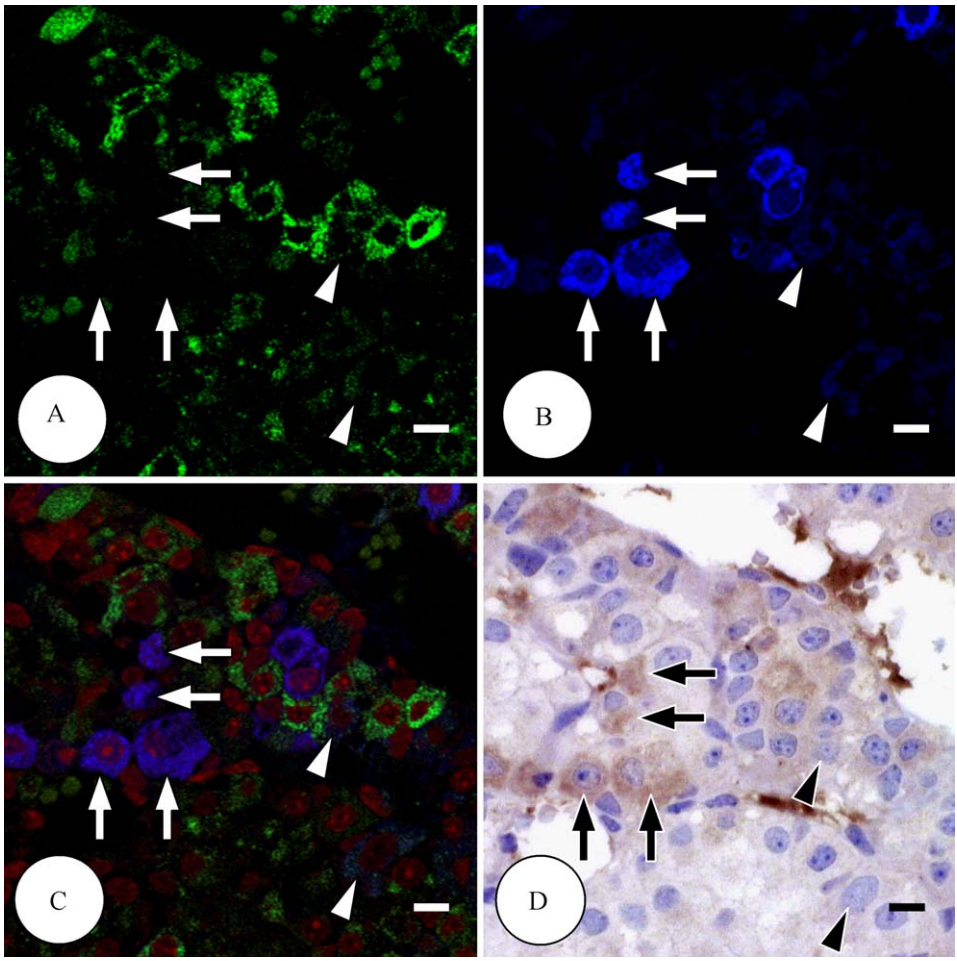


Fig. 4. Colocalization of IL-18 and IL-18R in the somatotroph. Double-immunofluorescence method (A–C) for IL-18 (green in A) and GH (blue in B) show somatotrophs with IL-18 (A and B: arrowheads) and without IL-18 (A and B: arrows). (C) Merged image. IL-18R immuno-restaining by ABC method (D) shows that somatotrophs immunoreactive for anti-IL-18R antibody (D: arrows) do not contain IL-18(A: arrows) and somatotrophs containing IL-18 (A: arrowheads) are not positive for anti-IL-18R antibody (D: arrowheads). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

not present IL-18R immunostainings, indicating that IL-18Rα and IL-18 did not colocalize in an identical somatotroph, unlike bovine somatotrophs.

## 4. Discussion

In the present study, we confirmed mRNA expression of IL-18 and IL-18R by RT-PCR in the pig anterior pituitary gland. Our previous study showed that the bovine anterior pituitary gland expressed the mRNA of IL-18 and IL-18R [17]. These results suggest that IL-18 has a role in modulating anterior pituitary function in pigs as it does in cattle. IL-18 and IL-18R protein were also immunostained in pig anterior pituitary cells. Mirror section techniques demonstrated that IL-18 was colocalized in somatotrophs, mammotrophs, thyrotrophs and gonadotrophs in the pig anterior pituitary gland. In the bovine anterior pituitary gland, more than 90% of IL-18 positive cells are somatotrophs [17]. However, it requires further investigation to determine whether differential proportions of pituitary cell types for IL-18 immunostaining vary according to species or not.

In the pig anterior pituitary gland, IL-18R was localized in somatotrophs and thyrotrophs. Unexpectedly, IL-18R was not colocalized with IL-18 in pig somatotrophs. These results suggest that IL-18 may play a role in modulating somatotroph function through a paracrine pathway. In the bovine anterior pituitary gland, IL-18R was colocalized with IL-18 in somatotrophs, suggesting that bovine IL-18 acts mainly through an autocrine pathway in the somatotrophs. Studies from several groups have demonstrated that there are many differences in response to endotoxin and cytokines produced by different species [27]. In pigs, lipopolysaccaride (LPS) injection induces increased plasma GH concentration [28]. However, TNFα, which induces several pathological states such as the endotoxic shock after injection of LPS, inhibits GH secretion in vivo and in vitro in cattle [29,30]. The difference between IL-18 and IL-18R localization in pigs and in cattle may reflect different responses to cytokines. GH has a role in modulating immune function, and enhances IL-1α, IL-6 and TNFα production by monocytes in whole blood [31]. In addition, mononuclear leukocytes synthesize GH after exposure to endotoxin [32]. Also lymphoid cells express GH and pit-1 in cattle [33]. In sheep, CD14, the LPS receptor, is localized in somatotrophs [34]. Our studies showed that IL-18R localize in somatotrophs in pigs and cattle, suggesting that somatotrophs interact with the endocrine regulation of immune responses.

The mRNA of several cytokines such as IL-1, IL-6, IL-11, LIF and MIF are expressed in the anterior pituitary gland, and IL-6, LIF and MIF are produced by folliculo-stellate cells immunoreactive for S-100 protein [2,3]. IL-1 induces GH secretion in pituitary cell cultures [35]. In rats, IL-1 stimulates GH release following intracerebroventricular (ICV) injection [36] IL-18 is a pro-inflammatory cytokine like IL-1, IL-6 and MIF, and induces IL-1 and IL-6 [20]. In addition, our studies showed that IL-18 and IL-18R mRNA were also expressed in the pig and bovine anterior pituitary gland. These findings indicate that IL-18 is closely associated with endocrine function, including the anterior pituitary gland.

In conclusion, we found the expression of IL-18 and IL-18R in the pig anterior pituitary gland. The study also showed that IL-18 and IL-18R were not colocalized in pig soma-

totrophs. Although IL-18 was localized in somatotrophs in pigs and cattle, the appearance in pig somatotrophs was different from that in bovine somatotrophs.

## Acknowledgment

This work was supported by a Grant-in-Aid for Scientific Research (B) (No. 13460122) from the Ministry of Education, Science and Culture of Japan.

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