Original Article

Spike protein of SARS-CoV-2 suppresses gonadotrophin secretion from bovine anterior pituitaries

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Key words: ACE2, anterior pituitary gland, Covid-19, gonadotroph, S1, S2

Running head: Spike protein and gonadotropin secretion

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Abstract

Coronavirus disease (COVID-19), the ongoing global pandemic, is caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Recent evidence shows that the virus utilizes angiotensin-converting enzyme 2 (ACE2) as a spike protein receptor for entry into target host cells. The bovine ACE2 contains key residues for binding to the spike protein receptor-binding domain. This study evaluated the hypothesis that bovine gonadotroph expresses ACE2, and spike protein suppresses luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion from cultured bovine anterior pituitary (AP) cells. ACE2 mRNA expression and ACE2 protein expression were detected in the bovine AP cells using reverse transcription PCR and western blot analysis. Immunofluorescence microscopy analysis with the anti-ACE2 antibody revealed the co-localization of ACE2 and gonadotropin-releasing hormone (GnRH) receptor on the gonadotroph plasma membrane. Approximately 90% of GnRH receptor-positive cells expressed ACE2, and approximately 46% of ACE2-positive cells expressed the GnRH receptor. We cultured bovine AP cells for 3.5 days and treated them with increasing concentrations (0, 0.07, 0.7, or 7 pM) of recombinant spike protein having both S1 and S2 regions. The spike protein (0.07–7 pM) suppressed both basal and GnRH-induced LH secretion ($P < 0.05$). Spike protein (0.7–7 pM) suppressed GnRH-induced ($P < 0.05$), but not basal FSH secretion. In contrast, pre-treatment with ERK 1/2/5 inhibitor (U0126) partially restored the GnRH-induced LH and FSH secretion from the spike protein suppression. Collectively, the results indicate that gonadotrophs express ACE2, a receptor for coronavirus 2 spike protein, which in turn suppresses LH and FSH secretion from AP cells.

Key words: ACE2, anterior pituitary gland, COVID-19, gonadotroph, S1, S2

Introduction

The ongoing global coronavirus disease (COVID-19) pandemic has been caused by the spread of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Recent evidence shows that the virus utilizes angiotensin-converting enzyme 2 (ACE2) as a spike protein receptor to enter target host cells [1]; however, the primary role of ACE2 is to convert angiotensin II to angiotensin-(1–7). Cells expressing ACE2 may thus be a target for the virus. ACE2 is highly expressed in the human testes, ovaries, and other reproductive organs [2]. Indeed, the virus may be an important cause of infertility in men because of its deleterious effects on semen quality and quantity through unclarified mechanisms, as reviewed by Agolli *et al.* [3] and Moshrefi *et al.* [4]. However, it remains unclear whether the virus also causes female infertility, especially in domestic animals.

The anterior pituitary (AP) gland lies outside the blood-brain barrier [5], and may be affected by the virus [6]. Gonadotrophs are important cells located in the AP glands, and secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which regulate the testes, ovaries, and, via gonadal steroids, other reproductive organs in animals [7]. However, to the best of our knowledge, the effect of the virus on gonadotrophs remains unclear. Gu *et al.* [8] reported that human gonadotrophs express ACE2. A recent study clarified that the spike protein of SARS-CoV-2 directly activates the cytoplasmic extracellular signal-regulated kinase (ERK) pathway downstream of ACE2 in human platelets [9]. The ERK pathway is also the cytoplasmic pathway downstream of the membrane estradiol receptor (GPR30), that suppresses LH secretion in bovine gonadotrophs [10]. However, the mechanism by which spike protein-activated ACE2 affects LH and FSH secretion by gonadotrophs in all species remains unclear.

Cattle are important domestic animals for food supply worldwide; thus, infertility in cattle is an important issue. Unlike human AP glands, bovine AP glands can be obtained for primary culture as they can be collected from slaughterhouses. Indeed, using bovine gonadotrophs, we discovered new receptors that control LH and FSH secretion, colocalizing with gonadotropin-releasing hormone

(GnRH) receptor (GnRHR) in the lipid rafts of bovine gonadotrophs [7, 11, 12, 13]. Bovine ACE2 contains most of the key residues for the receptor-binding domain of SARS-CoV-2 [14] (details are shown in Supplementary Fig.1), and SARS-CoV-2 replicates in bovine respiratory tissues [15]. Therefore, we tested the hypothesis that gonadotrophs express the spike protein receptor ACE2, colocalizing with GnRHR on the bovine gonadotroph cell surface, and that the recombinant spike protein suppresses LH and FSH secretion from cultured bovine AP cells. It is also important to clarify the cytoplasmic signaling pathway downstream of ACE2. Therefore, we used an inhibitor to evaluate the contribution of the ERK pathway to the effect of spike protein on gonadotropin secretion from the bovine AP.

Materials and Methods

Anterior pituitary sample collection

All experiments were performed in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences (Physiological Society of Japan). All experiments involving animals were approved by the Animal Ethics Committee of Yamaguchi University (approval number 301).

We obtained AP tissue from post-pubertal (26 months of age) Japanese Black heifers at a local abattoir, using a previously described method [16]. All heifers were in the luteal phase, as determined by macroscopic examination of the ovaries and uterus [11]; the AP gland exhibits the highest LH, FSH, and GnRH receptor levels in this phase [17].

The AP samples for RNA or protein extraction (n = 5) were stored at −80°C. The AP samples for immunohistochemistry (n = 5) were fixed with 4% paraformaldehyde at 4°C for 16 h. The AP samples to be used for cell culture followed by immunocytochemical analysis (n = 5), and those that were to be used for cell culture to evaluate the effect of spike on LH and FSH secretion (n =6) were stored in ice-cold 25 mM HEPES buffer (pH 7.2) containing 10 mM glucose and transported to the laboratory on ice.

RT–PCR, sequencing of amplified products, and homology search in gene databases

Total RNA was extracted using RNAzol RT isolation reagent (Molecular Research Centre, Inc., Cincinnati, OH, USA) and treated with deoxyribonuclease. The concentration and purity of each RNA sample was evaluated using spectrophotometry (acceptable range, 1.8–2.1) and electrophoresis (28S:18S ratios were 2:1). Complementary DNA was synthesized using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA).

We used previously reported RT-PCR methods [11] to detect the mRNA levels of *ACE2* (NCBI reference sequence, NM_001024502). The expected amplicon size of *ACE2* was 470 bp (orange highlighted region in Supplementary Fig.1; nucleotides 1297-1766; forward primer in 9th exon: 5′-CCGCAGCCACACCTCACTAT- 3′; reverse primer in 13th exon: 5′-GGTCCAGGGTTCTGATTTTCC-3′). PCR was performed using 20 ng of cDNA and polymerase (Tks Gflex DNA Polymerase, Takara Bio Inc., Shiga, Japan) under the following thermocycling conditions: 94°C for 1 min for pre-denaturation, followed by 35 cycles of 98°C for 10 s, 60°C for 15 s, and 68°C for 30 s. The PCR products were separated on 1.5% agarose gel using electrophoresis with a molecular marker (Nippon Gene, Tokyo, Japan), stained with Gelstar (Lonza, Allendale, NJ, USA), and observed using a charge-coupled device (CCD) imaging system (GelDoc; Bio-Rad, Hercules, CA, USA). The PCR products were purified using a NucleoSpin Extract II kit (Takara Bio Inc.) and then sequenced using PCR primers and the Dye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The sequences obtained were used as query terms to search the homologous sequences using the basic nucleotide local alignment search tool (BLAST) (available on the NCBI website).

Antibodies used in this study

We used a specific anti-ACE2 rabbit polyclonal antibody (HPA000288; Sigma-Aldrich, St. Louis, MO, USA). The antigen sequence that produces the antibody has 86% homology with the corresponding region of bovine ACE2, as shown by the green highlighted region in Supplementary

Fig.1. In particular, ACE2 has a single transmembrane region [18], as indicated by the blue highlighted region in Supplementary Fig.1. There is 91% homology between the extracellular regions of bovine and human ACE2 (the NCBI reference sequences of bovine and human ACE2 are NP_001019673.2 and BAB40370, respectively).

We also used a guinea pig polyclonal antibody that recognizes the N-terminal extracellular domain of the bovine GnRH receptor (anti-GnRHR). The specificity of the anti-GnRHR antibody has been verified previously [16]. We used a mouse monoclonal anti-LH antibody (clone 518-B7) [19] and a mouse monoclonal anti-FSH antibody (clone A3C12) [20] for immunohistochemical analysis of AP tissue and cultured AP cells. These antibodies do not cross-react with other pituitary hormones [20, 21].

Western blot analysis for ACE2

We extracted proteins from AP tissue and performed western blotting using a previously described method [16]. Briefly, total proteins were extracted from frozen stock AP tissue using a tissue protein extraction reagent containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). The extracted protein sample was boiled with Sample Buffer Solution containing Reducing Reagent (6x) for SDS-PAGE (09499-14; Nacalai Tesque, Kyoto, Japan) for 3 min at 100°C. The protein samples (8,000 ng of total protein) were loaded onto a polyacrylamide gel (Any KD Criterion TGX gel, Bio-Rad) along with the whole-cell lysate of human liver-derived HepG2 cells (sc-2227, Santa Cruz, Heidelberg, Germany) as positive controls [22], and a molecular weight marker (Precision Plus Protein All Blue Standards; Bio-Rad). The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V for 90 min. The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Trans-blot turbo PVDF, Bio-Rad) with electroblotting at 2.5 A, 25 V, for 7 min using a Trans-blot Turbo system (Bio-Rad).

A Can Get Signal kit (Toyobo Co. Ltd., Osaka, Japan) was used to block the membrane (1 h at 25°C), primary antibody reaction (1 h at 25°C) with the anti-ACE2 rabbit antibody (1:400,000

dilution with immunoreaction enhancer solution), and secondary antibody reaction (1 h at 25°C) with a goat anti-rabbit IgG horseradish peroxidase-conjugated antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA; 1:400,000 dilution with immunoreaction enhancer solution). The protein bands were visualized using an ECL-Prime chemiluminescence kit (GE Healthcare, Amersham, UK) and a CCD imaging system (LAS-3000 Mini; Fujifilm, Tokyo, Japan). To verify the specificity of the signals, we included several negative controls in which the primary antibodies were omitted, or normal rabbit IgG (Wako Pure Chemicals, Osaka, Japan) antibodies were used instead of the specific primary antibodies. Signal specificity was also confirmed using negative controls in which the primary antibodies were pre-absorbed with 4 nM of the antigen peptide (PrEST Antigen ACE2, APREST74018, Sigma-Aldrich).

The antibodies were removed from the PVDF membrane using a stripping solution (Nacalai Tesque) before blocking and subsequent immunoblotting with an anti-β-actin mouse monoclonal antibody (1:400,000 dilution; Sigma-Aldrich).

Fluorescence immunohistochemistry and confocal microscopy

We followed our previously reported method [16] for the immunofluorescence analysis of AP tissue (n = 5) after storage in 4% paraformaldehyde PBS at 4°C for 16 h, and 30% sucrose PBS until the blocks were infiltrated with sucrose. The blocks were frozen in an embedding medium (Tissue-Tek OCT compound Sakura Finetechnical Co. Ltd., Tokyo, Japan) and maintained at -80°C. Briefly, 15-μm sections were prepared using a cryostat, mounted on slides for treatment with 0.3 % Triton X-100 in PBS for 15 min, and blocked with 10% normal goat serum in PBS for 1 h. Incubation with a cocktail of primary antibodies (anti-ACE2 rabbit antibody, anti-GnRHR guinea pig antibody, and either anti-LH or anti-FSH mouse antibody [all diluted 1:1,000]) for 12 h at 4°C was followed by incubation with secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 546 goat anti-mouse IgG, and Alexa Fluor 647 goat anti-guinea pig IgG [all from Thermo Fisher Scientific and diluted as 1 μg/mL]) for 2 h at room temperature, and counterstaining with 1 μg/mL of 4,

6-diamino-2-phenylindole (DAPI; Wako Pure Chemicals).

The stained sections were observed using a confocal microscope (LSM710; Carl Zeiss, Göttingen, Germany) equipped with diode (405 nm), argon (488 nm), HeNe (533 nm), and HeNe (633 nm) lasers. Images obtained by fluorescence microscopy were scanned with a 40× or 63× oil-immersion objective and recorded using a CCD camera system controlled by ZEN2012 black edition software (Carl Zeiss). To verify the specificity of the signals, we included several negative controls in which the primary antiserum had been omitted or pre-absorbed with 4 nM of the antigen peptide, or in which normal rabbit IgG (Wako Pure Chemicals) was used instead of the primary antibody. Ratios of ACE2 positive gonadotrophs were calculated from 12 representative confocal images per AP gland.

AP cell culture and immunocytochemical analysis of cells

We followed our previously reported method [16] for the enzymatic preparation of AP cells (n = 6) and their culture. Cell viability was confirmed to be greater than 90% by trypan blue exclusion. Total cell yield was 19.8 $\times 10^6 \pm$ 0.8 $\times 10^6$ cells per AP gland. Dispersed cells were then suspended in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) containing 1× nonessential amino acids (Thermo Fisher Scientific), 100 U/mL penicillin, 50 μg/mL streptomycin, 10% horse serum (Thermo Fisher Scientific), and 2.5% fetal bovine serum (Thermo Fisher Scientific). The cells (2.5×10^5 cells/mL, total = 0.15 mL per lane) were cultured in the culture medium at 37°C in 5% CO_2 for 82 h, using a microscopy chamber (μ-Slide VI 0.4, Ibidi, Planegg, Germany). Recombinant human activin A (final concentration, 10 ng/ml; R&D Systems, Minneapolis, MN, USA) was used to stimulate FSH synthesis at 24 h before fixation [11]. FSH secretion from cultured AP cells is weak in ovine and bovine AP cells, and the pre-treatment time and final concentration have already been established in previous studies [23].

The cultured cells were fixed with either 4% paraformaldehyde for 3 min followed by treatment with 0.1% Triton X-100 for 1 min (PFA-Triton method), or with CellCover (Anacyte Laboratories UG, Kuhreder, Hamburg) for 2 min without Triton X-100 treatment (CellCover method), as described by

Kadokawa *et al.* [16]. For the PFA-Triton method, fixed cells were incubated with 0.1 mL of the same cocktail of primary antibodies for 2 h at room temperature. Incubation with Triton X-100 allowed anti-GnRHR and anti-ACE2 antibodies to bind to target proteins in the cytoplasm and at the cell surface. For the CellCover method, the fixed cells were incubated with guinea pig anti-GnRHR and rabbit anti-ACE2 (both 1:1,000) for 2 h at room temperature. As the cells were not treated with Triton X-100, the antibodies could only bind to the extracellular domains of the respective receptors in most cells. For both the PFA-Triton and CellCover methods, cells were incubated with the fluorochrome-conjugated secondary antibody cocktail and DAPI and subjected to confocal microscopy. Signal specificity was confirmed using negative controls in which the primary antibody was omitted or pre-absorbed at 4 nM with the same antigen peptide. Normal rabbit IgG was used as the primary antibody. Eight randomly selected images of cells prepared with the CellCover method were analyzed for co-localization using the ZEN 2012 black edition software (Carl Zeiss) to calculate overlap coefficients [24] for Alexa Fluor 488 and Alexa Fluor 647.

Effects of the recombinant spike protein of SARS-COV-2 on LH and FSH secretion

AP cells derived from six post-pubertal heifers were plated in 48-well cell culture plates (Sumitomo Bakelite, Tokyo, Japan) and incubated at 37°C and 5% CO_2 for 82 h. Recombinant human activin A (final concentration, 10 ng/mL) was added to the plates 24 h before the test to stimulate FSH synthesis.

The medium was replaced with 270 µL of DMEM containing 0.1% BSA (IgG-free Protease-free culture grade, 032-22364, Wako Pure Chemicals) and 10 ng/mL activin A (base medium) and incubated for 2 h to evaluate the effect of spike protein in the absence of GnRH. Treatment was performed by adding 30 µL of base medium alone or 30 µL of base medium with different concentrations of spike protein (final concentrations of 0, 0.07, 0.7, and 7 pM). We used a recombinant spike protein of SARS-CoV-2 containing both the S1 and S2 regions (40589-V08H4; Sino Biological US Inc., Wayne, PA, USA). After incubation for 2 h, the medium from each well was collected for

radioimmunoassay (RIA) analyses of LH and FSH levels.

The old medium was replaced with 240 µL of base medium and incubated at 37°C for 2 h to evaluate the effect of the spike protein in the presence of GnRH. The cells were pre-treated by adding 30 µL of base medium containing different concentrations of the recombinant spiked protein (final concentrations of 0, 0.07, 0.7, and 7 pM). The cells were incubated with gentle shaking for 5 min, and then treated with 30 µL GnRH (Peptide Institute Inc., Osaka, Japan; final concentration of 1 nM [16]) dissolved in the base medium for 2 h. As previously reported [16], gonadotropin secretion was stimulated by increasing the amounts of GnRH, with a peak at 1 nM of GnRH, and reduced secretion at GnRH concentrations higher than 1 nM. Therefore, the final concentration of GnRH used in this study was 1 nM in all treatments, except in the controls. After incubation for 2 h, the medium from each well was collected for LH and FSH RIAs.

Effect of ERK pathway inhibitor on the suppression of secretion

We evaluated the effect of the ERK1/2/5 pathway inhibitor, U0126, on spike-mediated suppression of secretion from bovine AP cells. AP cells obtained from a different set of post-pubertal Japanese Black heifers (n = 8, in the middle of the luteal phase, 26 months of age), were cultured for 82 h in the medium described in the previous section. Each experiment was repeated eight times with each of the eight AP glands, using four wells per treatment. The wells were washed twice with PBS and then incubated with 287 µL of DMEM containing 0.1% BSA and 10 ng/mL activin A for 2 h. Cells were pre-treated with 3 µL of DMEM alone or 3 µL of DMEM containing U0126 (final concentration, 1,000 nM; Enzo Biochem, Inc., New York, USA). After 30 min of incubation, either 5 µL of DMEM alone or 5 µL of DMEM containing spike protein (final concentration of 7 pM, which showed a significant inhibitory effect on gonadotropin secretion) was added to each culture well. The cells were incubated with gentle shaking for 5 min, after which they were incubated for 2 h with 5 µL of DMEM containing GnRH (final concentration, 1 nM) to stimulate gonadotropin secretion. After 2 h of incubation, the medium was collected for RIA. We previously confirmed that pre-treatment with

1,000 nM U0126 alone had no effect on GnRH-induced gonadotropin secretion, but inhibited the ability of estradiol to suppress GnRH-induced gonadotropin secretion from cultured bovine AP cells [10].

RIAs to measure gonadotropin concentration in culture media

The concentration of LH was measured in duplicate samples of culture media using double-antibody RIA using ^{125}I-labeled bLH and anti-oLH-antiserum (AFP11743B and AFP192279, National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, CA, USA). The intra- and inter-assay coefficients of variation (CV) were 3.6% and 6.2%, respectively. The concentration of FSH was measured by double-antibody RIA using bFSH and anti-oFSH antiserum (AFP5318C, AFP5346D, and AFPC5288113, NIDDK). The intra- and inter-assay CVs were 4.3% and 7.1%, respectively.

Statistical analysis

The statistical significance of differences in LH or FSH concentration was analyzed by one-factor ANOVA followed by *post-hoc* comparisons using Fisher's protected least significant difference test in StatView version 5.0, for Windows (SAS Institute, Inc., Cary, NC, USA). The level of significance was set at $P < 0.05$. Data are expressed as the mean ± standard error of the mean (SEM).

Results

Expression of ACE2 in AP of post-pubertal heifers

The expected PCR products were obtained by electrophoresis (Fig. 1A). Homology searching for the amplified product sequences revealed that the best match alignment was bovine *ACE2* (NM_001024502), which was identical (identities were 100% (470/470) with no gaps). No other bovine genes were found to have any homology with the obtained sequences of the amplified products, suggesting that the sequences of the amplified products were identical to the sequence of bovine

ACE2.

Western blotting revealed similar bands for AP and HepG2-cells (Fig. 1B). One difference was the band size of 42 kDa for AP and 50 kDa for HepG2. Another difference was that a 100-kDa band was observed in the AP sample, but not in HepG2. No bands were observed in the negative control membranes, where the primary antiserum was pre-absorbed with the antigen peptide.

Immunofluorescence analysis of ACE2 expression in bovine AP tissue

ACE2 and GnRHR were colocalized in the majority of LH-positive (Fig. 2A) and FSH-positive (Fig. 2B) cells in bovine AP tissue. The percentages of single- and double-labeled ACE2- and GnRHR-positive cells were determined from 15 representative confocal images per AP gland. In each AP gland, there was an average of 54.4 ± 2.4 GnRHR-positive cells, 105.2 ± 1.1 ACE2-positive cells, and 48.6 ± 1.7 double-positive cells; further, 89.5 ± 1.6% of GnRHR-positive cells were ACE2-positive, and 46.3 ± 1.9% of ACE2-positive cells were GnRHR-positive. No immunostaining signals were observed in the negative control tissues, where the primary antiserum was pre-absorbed with the antigen peptide.

ACE2 and GnRHR on the cell surface

Among the AP cells prepared using the PFA-Triton method, we observed ACE2 expression in LH-positive (Fig. 3A) and FSH-positive cells (Fig. 3B).

The AP cells prepared using the cell cover method showed that ACE2 was colocalized on the surface of GnRHR-positive cells (Fig. 4). The overlap coefficient of the cultured AP cell surface between ACE2 and GnRHR was 0.73 ± 0.01.

Effects of spike protein on gonadotropin secretion from cultured AP cells

Fig. 5 shows the effect of various concentrations of spike protein on LH or FSH secretion from AP cells derived from post-pubertal heifers cultured in the absence (A, C) or presence (B, D) of GnRH. In

the absence of GnRH (Fig. 5A), 0.07 pM ($P < 0.05$), 0.7 pM ($P < 0.05$), and 7 pM ($P < 0.01$) of spike protein suppressed LH secretion compared to the controls. Moreover, 0.07 pM ($P < 0.05$), 0.7 pM ($P < 0.01$), and 7 pM ($P < 0.01$) of spike protein suppressed GnRH-induced LH secretion (Fig. 5B).

In the absence of GnRH (Fig. 5C), none of the tested concentrations of spike protein was found to suppress FSH secretion compared to the controls. However, 0.7 pM ($P < 0.01$) and 7 pM ($P < 0.01$), but not 0.07 pM the spike protein suppressed GnRH-induced FSH secretion (Fig. 5D).

Fig. 6 shows that 7 pM of spike protein suppressed GnRH-induced LH secretion, and that pre-treatment with U0126 partially recovered GnRH-induced LH and FSH secretion.

Discussion

Cultured bovine gonadotroph cells express ACE2, and addition of recombinant spike protein to the culture medium suppressed the secretion of LH and FSH, providing clear evidence that the spike protein, containing both S1 (attachment to ACE2) and S2 (fusion with host membrane) regions, affects the cytoplasmic ERK pathways [9] that play important roles in the control of LH and FSH secretion [10]. ACE2 colocalizes with GnRHR on the lipid rafts of gonadotrophs [7], suggesting that the S2 region suppresses LH and FSH secretion by fusing with the lipid rafts [25] to affect heteromer receptors.

We found that approximately 90% of gonadotroph cells in bovine AP were ACE2-positive. Similar to other GPCRs [26], GnRHR forms functionally active homomers and heteromers with different receptors [7, 27]. We obtained a strong positive overlap coefficient between ACE2 and GnRHR on the cell surface of bovine gonadotrophs. Therefore, ACE2 may form a heteromer with GnRHR in gonadotrophs.

However, the results of this study must be interpreted with some caution. Gu *et al.* [8] reported ACE2 expression in the human AP gland, but found no significant difference in blood LH and FSH concentrations between and SARS-CoV2-infected and uninfected patients. However, in another

study, male patients with COVID-19 showed lower blood concentrations of testosterone and higher blood concentrations of LH and prolactin [28]. ACE2 is expressed at higher levels in the human testis and ovary than in the AP gland [29]. Therefore, one possible reason is that gonadal ACE2 may bind spike proteins rather than ACE2, and the direct and indirect suppression of steroidogenesis by the virus and immune system, respectively, may then induce hypogonadism, which reduces the negative feedback of steroid hormones to gonadotrophs [30]. Another possible reason could be that the measurements were performed on a one-point sample and not repeated to measure the parameter of pulsatile secretion of LH and FSH.

Bovine ACE2 can bind the spike protein of SARS-CoV-2 [14] and SARS-CoV-2 replicates in bovine respiratory tissues [15]. Intratracheal and intravenous inoculation with SARS-CoV2 resulted in only minor replication in colostrum-deprived Holstein bull calves [31]. However, white-tailed deer (*Odocoileus virginianus*), a ruminant species, are highly susceptible to infection [32]. Therefore, caution is needed against the spillover of SARS-CoV-2, especially of new variants, among humans, domestic animals, and wild animals.

The primary role of ACE2 is to convert angiotensin II to angiotensin-(1–7). Although gonadotrophs are the likely site of angiotensin II production in rat AP glands [33], little is known about the roles of ACE2, angiotensin II, and angiotensin-(1–7) in gonadotrophs. Therefore, further studies are required to clarify these roles.

There are no previous reports of bovine ACE2 protein size using western blotting. As determined by western blotting, the bovine ACE2 protein was approximately 100 kDa. However, this was larger than the 91 kDa predicted from the amino acid sequence. Additionally, human ACE2 shows a band at approximately 100 kDa along with a 50-kDa band [34]. In a previous study [19], HepG2 cells showed an approximately 100-kDa band using the same anti-ACE2 antibody, but showed a 50-kDa band in this study. This difference in band size may be due to experimental conditions in the culture or sample preparation for western blot. This is because band sizes in western blotting often differ from expected sizes in the case of membrane-bound proteins such as cell-surface receptors,

owing to their complex three-dimensional structures, which can include hydrophilic and lipophilic regions that form extracellular, transmembrane, and cytoplasmic domains [35].

Primary AP cell culture was used to evaluate rapidly activated (within 30 min) pathways that altered LH and FSH secretion from bovine gonadotrophs, without alterations in the mRNA expression of LHα, LHβ, or FSHβ subunits. Thus, we did not perform RT-qPCR for LH and FSH genes. We previously found that the small interfering RNA method could not be used in this system because secretion was suppressed even in control RNA (unpublished data). Therefore, we evaluated the contribution of ERK pathway using an inhibitor. We could not exclude any other pathway as U0126 partially recovered gonadotropin secretion. In particular, Smad7 pathway suppresses cell function downstream of ACE2 in diabetic nephropathy [36], and inhibits *FSHB* gene expression in mouse gonadotrope-derived LβT2 cells [37]. However, to the best of our knowledge, a SMAD7-specific inhibitor has not yet been developed. Therefore, further studies are required in the future to investigate this aspect.

Angiotensin II activates the type 1 receptor (AT1R) and ERK pathways in rat tubular epithelial cells [38]. Thus, if ACE2 is inhibited by the spike protein, angiotensin II may be increased and stimulate the AT1R and ERK pathways. However, to the best of our knowledge, there are no reports on the expression of AT1R in gonadotrophs. In addition, the culture medium did not contain angiotensin II. Therefore, it is unlikely that the angiotensin II-AT1R system contributed to the observed suppression by spike protein.

Moreover, non-gonadotroph cells were also ACE2-positive. Mice that ubiquitously overexpress ACE2 have reduced the expression of proopiomelanocortin and plasma corticosterone in the AP [39]. ACE2 activation by diminazene aceturate increases ACTH secretion from AtT-20 cells but not prolactin secretion from MMQ and GH3 cells [8]. Therefore, ACE2-positive non-gonadotroph cells may be corticotrophs.

In conclusion, ACE2 is expressed in gonadotrophs, and the SARS-CoV-2 spike protein significantly suppresses LH and FSH secretion *via* the ERK pathway. This effect may constitute a

cause of infertility in cows; however, further long-term *in vivo* studies are required to validate these results.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgments

The authors thank Prof. Vitaliano Borromeo (Università degli Studi di Milano) for the anti-FSH antibody. The authors thank Dr. A. F. Parlow (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA, USA) for hormones and antiserum. This research was partly supported by a Grant-in-Aid for Scientific Research from The Research Institute for Time Studies, Yamaguchi University (Yamaguchi, Japan) to H. Kadokawa.

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Figure Legends

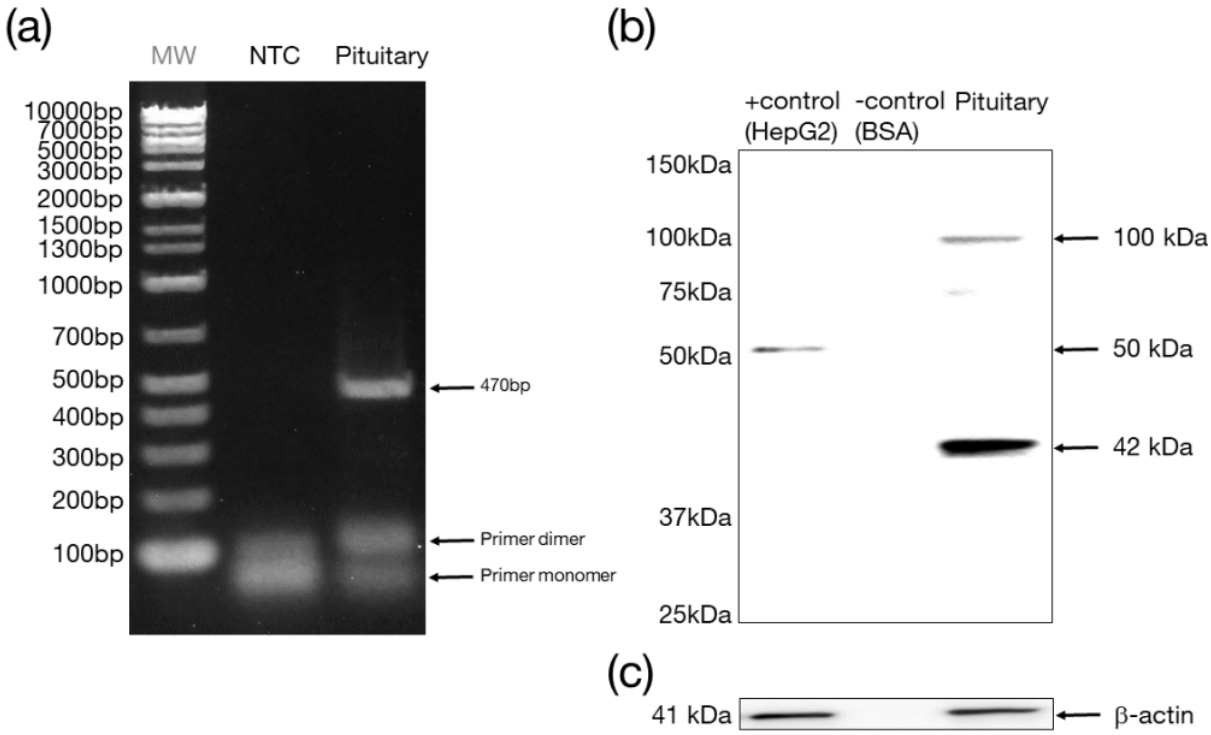
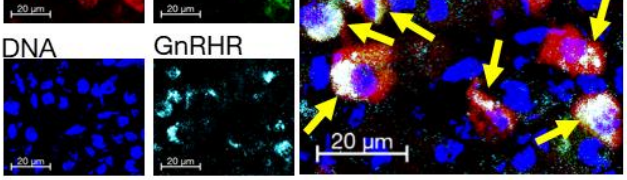


Fig. 1. Expression of *Angiotensin-converting enzyme 2 (ACE2)* detected using RT-PCR and western blotting. Electrophoresis of PCR-amplified DNA products using primers for bovine *ACE2* and cDNA from bovine anterior pituitary (AP) glands; the band was 470 bp (A). Two bands (100 kDa and 42 kDa) appeared on the AP sample, whereas a 50-kDa band was observed in HepG2 cells, which were used as the positive control (B). The relative band of β-actin (41 kDa) was used as a control for both HepG2 and AP (C).



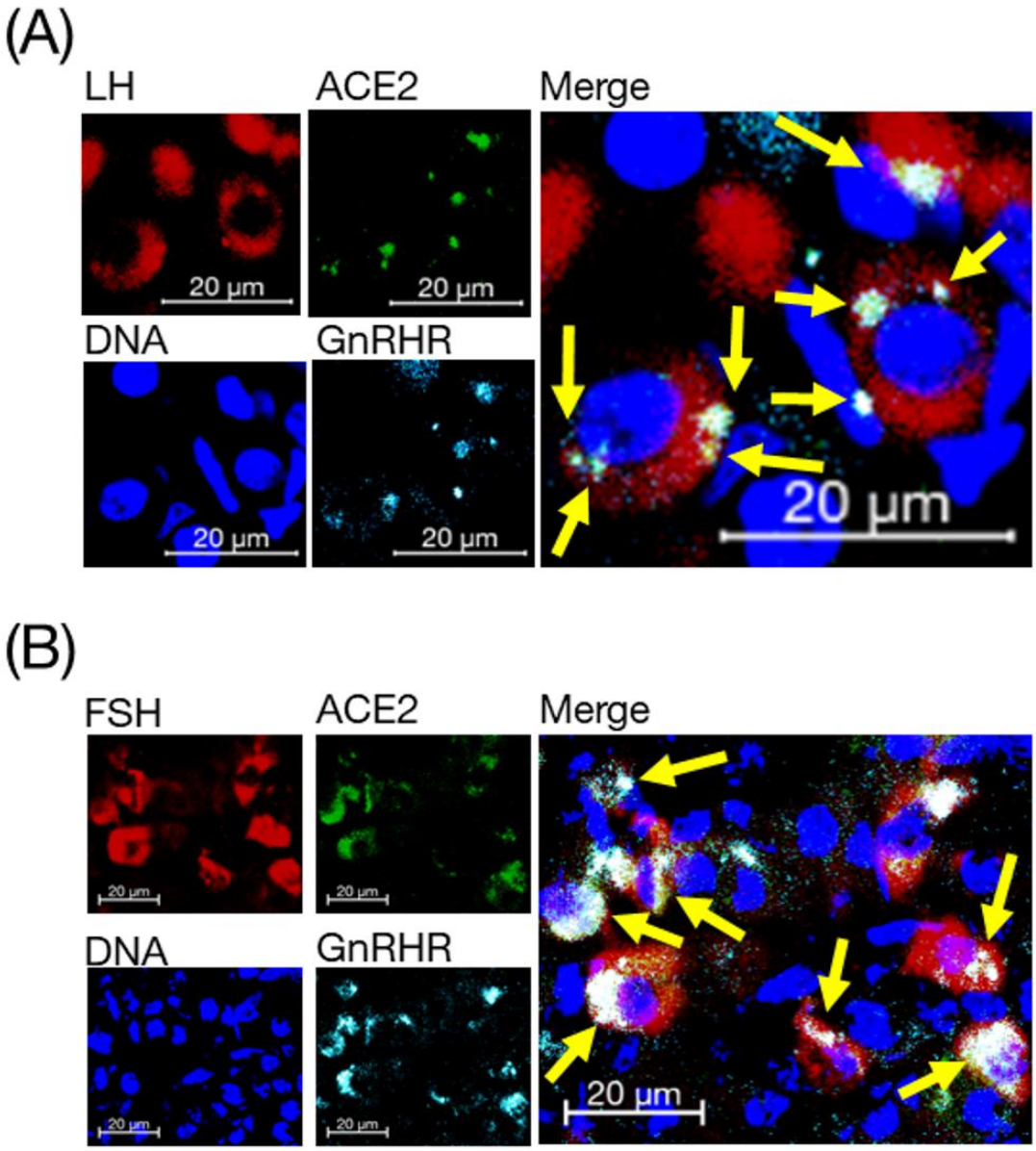


Fig. 2. Triple-fluorescence immunohistochemistry of bovine AP tissue for ACE2, gonadotropin-releasing hormone receptor (GnRHR), and either luteinizing hormone (LH) (A) or follicle stimulating hormone (FSH) (B). Images were captured using laser confocal microscopy for LH or FSH (red), ACE2 (green), and GnRHR (light blue) with counter-staining using DAPI (dark blue). The yellow arrows indicate the colocalisation of ACE2 with GnRHR. Scale bars are 20 µm.

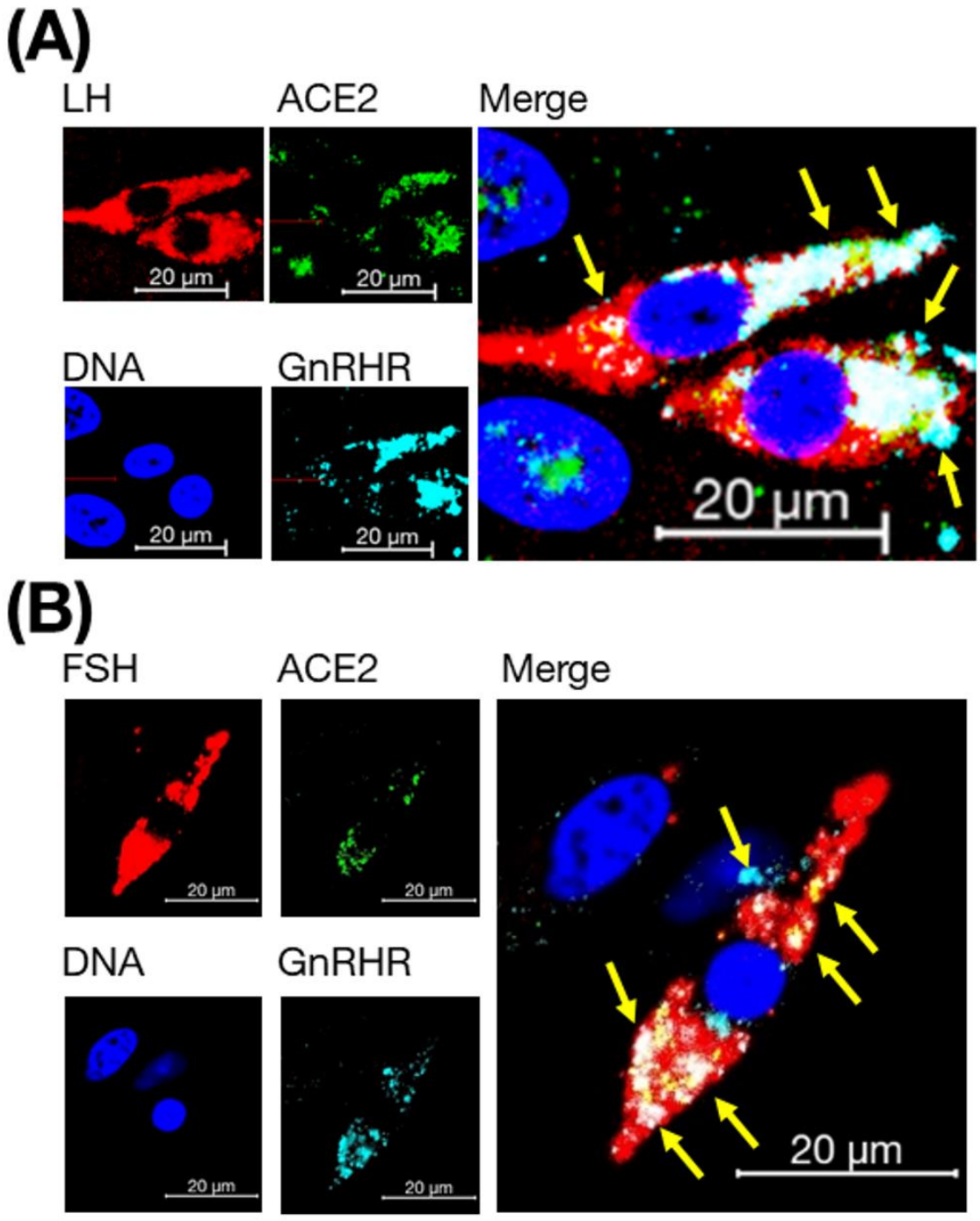


Fig. 3. Triple-fluorescence immunocytochemistry of cultured AP cells (prepared using PFA-Triton method) of post-pubertal heifers for ACE2, GnRHR, and either LH (A) or FSH (B). Images were captured using laser confocal microscopy for LH or FSH (red), ACE2 (green), and GnRHR (light blue) with counter-staining using DAPI (dark blue). The yellow arrows indicate the colocalisation of ACE2 with GnRHR. Scale bars are 20 µm.

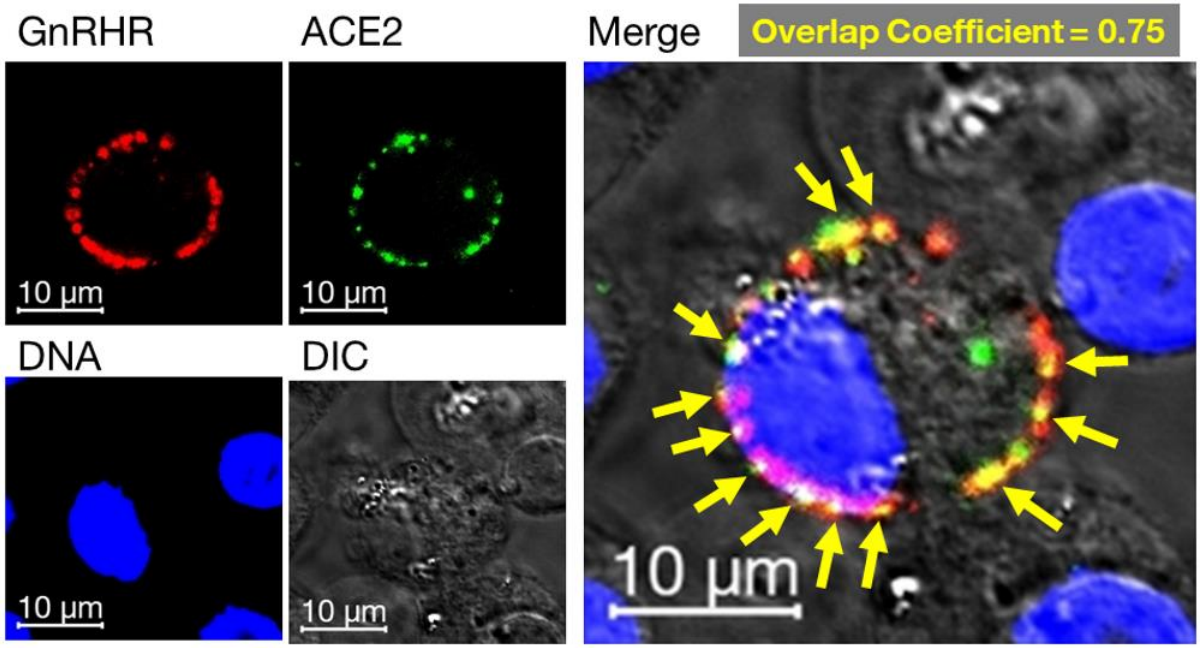
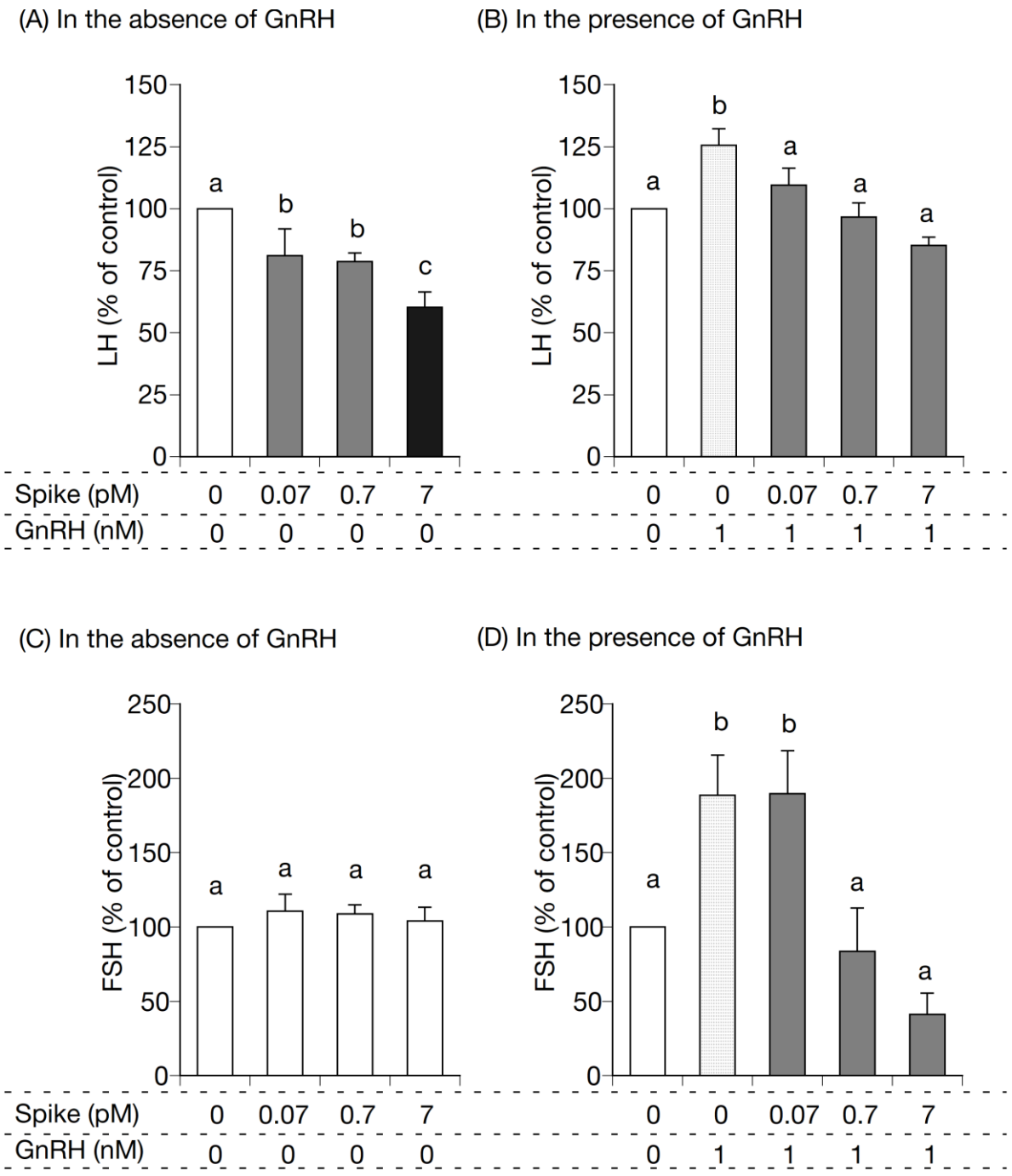


Fig. 4. Fluorescence immunocytochemistry was used to confirm the colocalisation (yellow in the merge panel) of ACE2 and GnRHR on the surface of cultured AP cells (prepared using the Cell Cover method) of post-pubertal heifers. Images were captured using a laser confocal microscope for GnRHR (red), ACE2 (green), DNA (dark blue), and differential interference contrast (DIC) on cultured AP cells which did not receive Triton X-100 treatment for antibody penetration. Thus, the antibody could only bind ACE2 and GnRHR on the surface of gonadotrophs. The arrows indicate the colocalisation of ACE2 with GnRHR. Note that cells prepared using the Cell Cover method are thicker than those prepared using the PFA-Triton method. Scale bars are 10 µm.



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560 **Fig. 5.** Comparison of the effects of various concentrations of spike protein in media without (A, C)
561 and with (B, D) 1 nM GnRH on LH or FSH secretion from cultured AP cells of post-pubertal heifers.
562 All cultured cells were pre-treated with activin. The concentrations of LH or FSH in control cells
563 (cultured in medium alone without spike protein and GnRH) were averaged and set at 100%; the mean
564 LH or FSH concentration for each treatment group is expressed as a percentage of the control value.
565 Different letters indicate statistically significant differences ($P < 0.05$).

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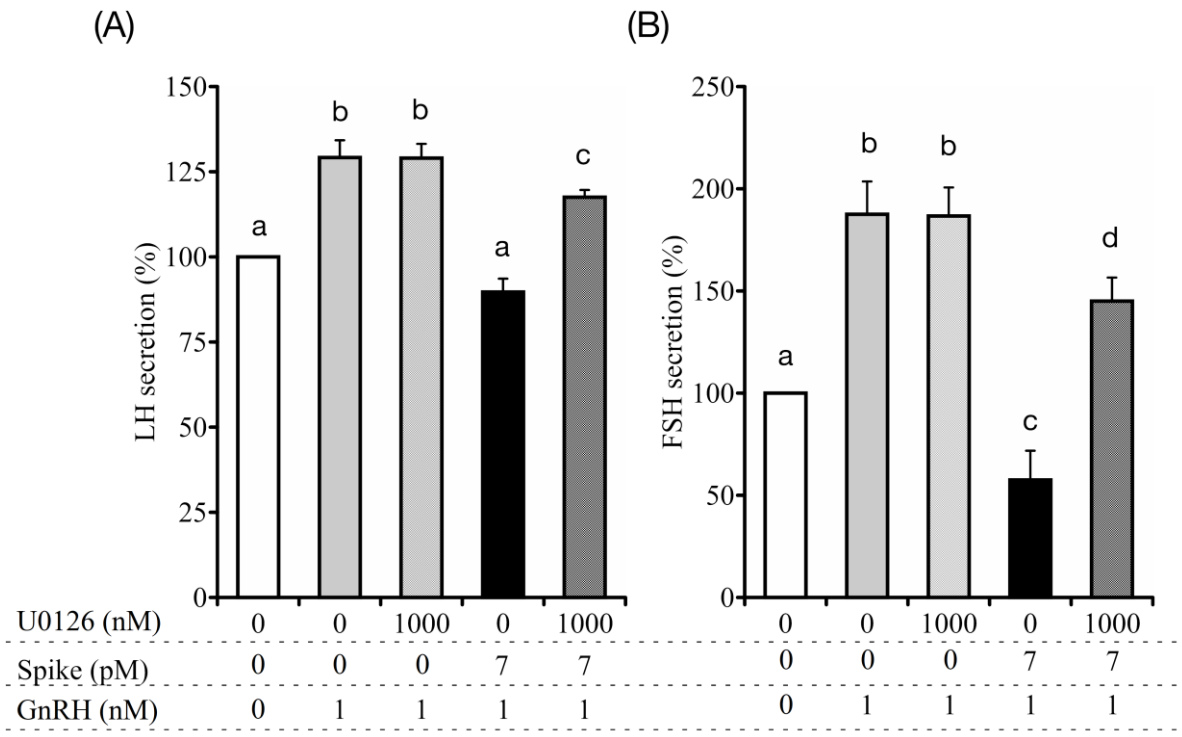
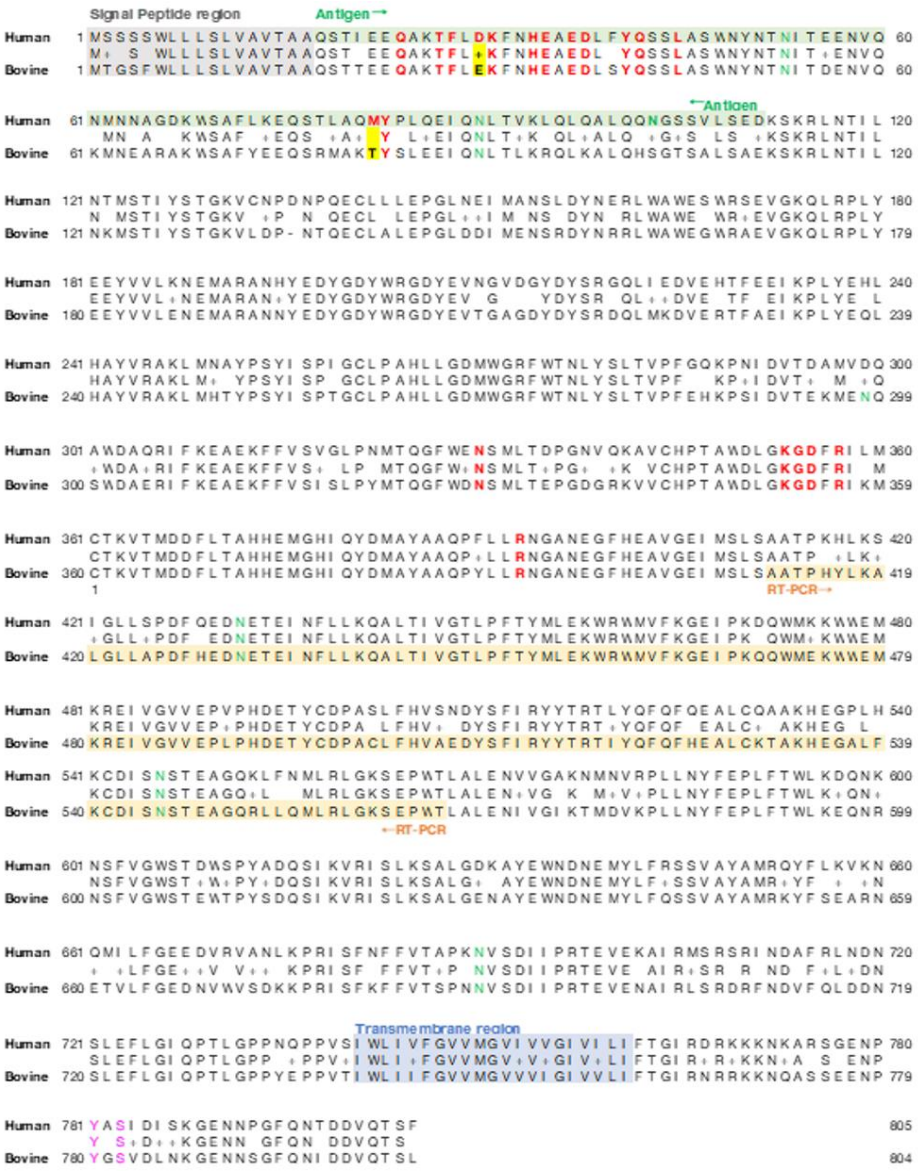


Fig. 6. Effect of the ERK pathway inhibitor, U0126, on spike protein-mediated suppression of GnRH-induced secretion of LH (A) and FSH (B) from cultured bovine AP cells. All cultured cells were pre-treated with activin. The mean LH or FSH concentration for each treatment group is expressed as a percentage of the control value. Different letters indicate statistically significant differences ($P < 0.05$).



Supplementary Fig. 1. Alignment of human (BAB40370) and bovine ACE2 (NP_001019673.2) amino acid sequences using the basic local alignment search tool available on the NCBI website. Red fonts indicate the key amino acids for severe acute respiratory syndrome coronavirus-2 binding reported by Luan *et al.* [14]. Green fonts indicate N-linked glycosylation sites. Pink fonts indicate the phosphorylation sites according to the annotation of each protein. The grey region indicates the signal peptide. The green region indicates the antigen peptide immunized to create the used anti-human ACE2 antibody, where the homology between human and bovine ACE2 is 86% (79/92) and gap is 0% (79/92). The orange region indicates the target of RT-PCR. The blue region indicates the transmembrane region.