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

Dimas Arya ABDILLAH1), Onalenna KEREILWE1), Raihana Nasrin FERDOUSY1), Risa SAITO1) and Hiroya KADOKAWA1)

1) Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi 753-8515, Japan

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: Angiotensin-converting enzyme 2 (ACE2), Anterior pituitary gland, Coronavirus disease (COVID-19), Gonadotroph, S1, S2
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′-GGTCCAGGGTTCTGATTTTCC-3′). PCR was performed using 20 ng of cDNA and polymerase (Tks Gflex primer in 13th exon: 5′-GGTCCAGGGTTCTGATTTTCC-3′). PCR products were stored on 1.5% agarose gel electrophoresis with a molecular marker (Nippon Gene, Osaka, Japan), stained with Gelstar (Lonza, Allendale, NJ, USA), and observed using a charge-coupled device (CCD) imaging system (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 Finetechical Co., Ltd., Tokyo, Japan) and maintained at −80°C. Briefly, 15-µm sections were prepared using a cryostat, mounted on slides for treat-
ment 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 2 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 antisera had been omitted or pre-absorbed with 4 nM of the antigen peptide, or in which normal rabbit IgG 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 × 10^6 ± 0.8 × 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 × 10^6 cells/mL, total = 0.15 ml per lane) were cultured in the culture medium at 37°C in 5% CO₂ 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 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 (Anacey 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 µl 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% CO₂ 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 µM). 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 spike protein (final concentrations of 0, 0.07, 0.7, and 7 µM). The cells were incubated with gentle shaking for 5 min, and then treated with 30 µl GnRH (Pepptide 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 µM, 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 125I-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
Figure 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 < 0.05) 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).

Figure 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
Therefore, ACE2 may form a heteromer with GnRHR in gonadotrophs. ACE2 and GnRHR on the cell surface of bovine gonadotrophs. [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-CoV-2-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-CoV-2 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 suppression. In particular, Smad7 pathway suppresses cell function downstream of ACE2 in diabetic nephropathy [36], and inhibits FSHβ 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 dimazene acetate 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 Interests: 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 antisera. 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.

References

1. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell 2020; 181: 271–280.e8. [Medline] [CrossRef]
2. Sharma GT, Chandra V, Maskusky P, Thirupathi Y, Swain DK, Pillai H, Patel SK, Pathak M, Gattula S. Physiological implications of COVID-19 in reproduction: angiotensin-converting enzyme 2 as a key player. Reprod Fertil Dev 2021; 33: 381–391. [Medline] [CrossRef]
3. Aggini L, Yakselen Z, Aggini O, Patel MH, Bhat KP, Concepcion L, Halpern J, Abi S, Abreu R. SARS-CoV-2 effect on male infertility and its possible pathophysiological mechanisms. Discoveries (Croatia) 2021; 4: e131. [Medline] [CrossRef]
4. Meshrefi M, Ghassem-Esmailabad S, Ali J, Findikli N, Mangoli K, Khalili MA. The probable destructive mechanisms behind COVID-19 on male reproduction system and fertility. J Assist Reprod Genet 2021; 38: 1691–1708. [Medline] [CrossRef]
5. Takano H, Suhara T. Imaging blood-brain barrier function by using positron emission tomography to evaluate drug penetration into the brain. Brain Nerve 2015; 65: 137–143 (in Japanese). [Medline]
6. Pal R. COVID-19, hypothalamic-pituitary-adrenal axis and clinical implications. Endocr Pract 2020; 26: 251–252. [Medline] [CrossRef]
7. Kadokawa H. Discovery of new regulators regulating luteinizing hormone and follicle-stimulating hormone secretion by bovine gonadotrophs to explore a new paradigm for regulators modifying regulation. J Reprod Dev 2020; 66: 291–297. [Medline] [CrossRef]
8. Gu WT, Zhou F, Xie WQ, Wang S, Yao H, Liu YT, Gao L, Wu ZB. A potential important impact of SARS-CoV-2 on pituitary glands and pituitary neuroendocrine tumors. Endocrine 2021; 72: 340–348. [Medline] [CrossRef]
9. Zhang S, Liu Y, Wang X, Yang L, Li H, Wang Y, Liu M, Zhao X, Ye Y, Yang Z, Zhang S, Fan Z, Dong J, Yuan Z, Ding Z, Zhang Y, Hu L. SARS-CoV-2 binds platelet ACE2 to enhance thrombin in COVID-19. Hematol Oncol 2020; 13: 120. [Medline] [CrossRef]
10. Rudolf FO, Kadokawa H. Cytoplasmic kinases downstream of GPIR3 suppress gonadotropin-releasing hormone (GnRH)-induced luteinizing hormone secretion from bovine anterior pituitary cells. J Reprod Dev 2016; 62: 55–69. [Medline] [CrossRef]
11. Kereszthe O, Pandey K, Kadokawa H. Influence of brain plasminogen changes on gonadotropin secretion from the cultured bovine anterior pituitary cells. Domest Anim Endocrinol 2018; 64: 77–83. [Medline] [CrossRef]
36. Chen Z, Chen X, Bai Y, Diao Z, Liu W. Angiotensin-converting enzyme-2 improves diabetic nephropathy by targeting Smad7 for ubiquitin degradation. *Mol Med Rep* 2020; 22: 3008–3016. [Medline]

37. Bohaczuk SC, Cassin J, Slaiwa Tl, Thackray VG, Mellon PL. Distal enhancer potentiates activin- and GnRH-induced transcription of FSHB. *Endocrinology* 2021; 162: bqab069. [Medline] [CrossRef]

38. Yang F, Chung ACK, Huang XR, Lan HY. Angiotensin II induces connective tissue growth factor and collagen I expression via transforming growth factor-beta-dependent and -independent Smad pathways: the role of Smad3. *Hypertension* 2009; 54: 877–884. [Medline] [CrossRef]

39. Wang LA, de Kloet AD, Smeltzer MD, Cahill KM, Hiller H, Bruce EB, Pioquinto DJ, Ludin JA, Katovich MJ, Raizada MK, Krause RG. Coupling corticotropin-releasing hormone and angiotensin converting enzyme 2 dampens stress responsiveness in male mice. *Neuropharmacology* 2018; 133: 85–93. [Medline] [CrossRef]