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—Original Article—

Potential role of hCG in apoptosis of human luteinized granulosa cells

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Abstract. The corpus luteum (CL) forms after ovulation and acts as a temporary endocrine gland that produces progesterone (P4), a hormone that is essential for implantation and maintenance of pregnancy in mammals. In pregnant women, human chorionic gonadotropin (hCG) secreted by the conceptus prevents luteolysis. hCG also increases the survival of cultured human luteinized granulosa cells (hLGCs). To clarify the maintenance mechanism of the human CL, we investigated the effects of hCG and P4 receptor antagonists, onapristone (OP) and RU486, on the viability of hLGCs. With the patients' consent, hLGCs were isolated from follicular aspirates for *in vitro* fertilization. The cells were cultured with hCG (0.1, 1, 10, 100 IU/ml), OP (10, 25, 50, 100 μM), RU486 (100 μM), P4 (1, 10, 25, 50 μM) or some combination of the four for 24 h. Cell viability was significantly increased by hCG (100 IU/ml) and significantly decreased by OP (100 μM) compared with the control. Cells treated with hCG and OP together were significantly less viable than the control and OP-treated cells. The combined treatment also significantly increased CASP3 activity and cleaved CASP3 protein expression. Furthermore, P4 addition reversed the reduction in cell viability caused by the combination of hCG and OP treatment. The overall findings suggest that hCG cooperates with P4 to increase survival of hLGCs and to induce apoptosis when P4 action supported by hCG is attenuated in the human CL.

Key words: Human chorionic gonadotropin, Human luteinized granulosa cell, Progesterone, Survival factor

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The corpus luteum (CL) forms after ovulation and acts as a temporary endocrine gland. It produces progesterone (P4), a hormone essential for implantation and maintenance of pregnancy in mammals. In many mammals, a surge of luteinizing hormone (LH) triggers ovulation, induces the luteinization of granulosa/theca cells and stimulates P4 production by luteal cells [1, 2].

In humans, CL function is regulated by LH for 2 weeks. During a non-fertile cycle, the human CL undergoes luteolysis, with loss of functional and structural integrity [3, 4]. Structural luteolysis in primates is thought to be achieved by programmed cell death (apoptosis) [5–7]. In pregnant women, luteolysis is prevented by human chorionic gonadotropin (hCG) secreted by the conceptus [8]. hCG is a glycoprotein with an alpha subunit identical to that of LH and exerts its effects by binding to the LH receptor. hCG inhibits ATP-induced apoptosis [9] and upregulates several intracellular anti-apoptotic factors [7, 10]. The latter three studies show that hCG increases the survival of luteal cells and consequently maintains the CL.

Progesterone also has anti-apoptotic roles in cultured luteal cells derived from cows [11] and rats [12] and in cultured granulosa/luteal cells derived from humans [13, 14]. hCG stimulates P4 production in the human CL [15]. Thus, P4 may be involved in the survival-enhancing effects of hCG on human luteal cells. RU486, P4 and cortisol receptor antagonist were reported to induce apoptosis in human luteinized granulosa cells (hLGCs) [14]. However, it is unclear whether hCG affects the apoptosis induced by RU486.

To clarify the maintenance mechanisms of the human CL, we investigated the effect of hCG on the viability of hLGCs *in vitro* in the presence and absence of P4 activity using P4 receptor antagonists.

Materials and Methods

Patients and luteinized granulosa cell collection

Granulosa-luteal cells were obtained by follicular aspiration from 35 patients undergoing *in vitro* fertilization (IVF). The clinical reasons for IVF in these patients were primarily male-related factors, tubal factors or unexplained infertility factors. Patients with a poor response to ovarian stimulation were excluded from the study. The mean age was 33 years (range, 25–39 years). The experimental procedures were approved by the Okayama Couples Clinical Screening Committee for Research (No. FC-2009-06-03-3), and signed informed consent for the use of hLGCs was obtained from each patient.

Patients received the GnRH analog Nafarelil (Fuji Pharma, Toyama, Japan) starting from the time of the mid-luteal phase of the cycle, and follicular growth was stimulated with 150-300 IU of HMG daily. hCG (10,000 IU; Fuji Pharma) was administered when the second leading follicle reached a mean diameter of 18 mm. Follicular puncture was performed under transvaginal ultrasound guidance 35.5 h after hCG administration.

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Cell preparation and culture

hLGCs were obtained from follicular aspiration via transvaginal ultrasound-guided oocyte retrieval from patients undergoing *in vitro* fertilization and embryo transfer. Follicular fluid samples were centrifuged separately at 200 × *g* for 20 min and resuspended in 3 ml phosphate-buffered saline (PBS). The resulting suspension was layered onto 45% ISolate (99264; Irvine Scientific, Irvine, CA, USA) and centrifuged at 200 × *g* for 20 min. The hLGCs were collected from the interphase, washed with PBS and centrifuged at 200 × *g* for 5 min. The pellet was resuspended in 5 ml Tris-NH_4 buffer for complete removal of red blood cells. The cells were centrifuged immediately and washed three times with 3 ml culture medium (Dulbecco's modified Eagle's medium / Nutrient Mixture F-12 Ham (D/F; 1:1 [v/v]; D-8900; Sigma-Aldrich, St. Louis, MO, USA) containing 5% calf serum (CS; 16170078; Invitrogen, Grand Island, NY, USA), 20 mg/ml gentamicin (15750-060; Invitrogen) and 2 µg/ml amphotericin B (A9528; Sigma-Aldrich). To isolate the cell masses, the suspension was passed through a 24-G needle at least 50 times. Pellets were resuspended in 1 ml culture medium, and cell numbers and viability were assessed by the trypan blue exclusion test using a hemocytometer.

The dispersed hLGCs were seeded at 2 × 10^5 viable cells/ml in 96-well culture plates (130188; Thermo Fisher Scientific, Waltham, MA, USA) for detection of cell viability, in 48-well culture plates (3548; Sigma-Aldrich) for detection of P4 production or in tissue culture flasks (353014; BD Falcon, Franklin Lakes, NJ, USA) for detection of protein expression. The culture plates were coated with 0.01% rat tail collagen at room temperature for 1 h before starting the experiments. The cells were cultured at 37 C in a 5% CO_2 atmosphere with high humidity. After 72 h of culture, the medium was replaced with D/F medium without phenol red and containing 0.1% BSA (15408; Roche Diagnostics, Mannheim, Germany), 5 ng/ml sodium selenite (S5261; Sigma-Aldrich) and 5 µg/ml transferrin (T3400; Sigma-Aldrich), and the cells were exposed to hCG (0.1, 1, 10 or 100 IU/ml), the specific P4 receptor antagonist OP (ZK98299; Schering AG, Berlin, Germany; 10, 25, 50 or 100 µM), RU486 (M8046; Sigma-Aldrich; 100 µM), P4 (Fuji Pharma; 1, 10, 25 or 50 µM) or some combination of the four for 24 h. Each experiment was performed at least three times using different cell preparations to ensure consistent findings.

Cell viability assay

Cell viability was determined by a Dojindo Cell Counting Kit, including WST-1 (no. 345-06463; Dojindo, Kumamoto, Japan). WST-1, a derivative of 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT), is a yellow tetrazolium salt reduced to formazan by live cells containing active mitochondria. For the viability assay, 10 µl assay solution (0.3% WST-1 and 0.2 mM 1-methoxy PMS in PBS, pH 7.4) was added to each well. The cells were then incubated for 1 h at 37 C. The absorbance (A) at 450 nm (A_{450}) was read using a microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA). Cell viability (%) was calculated as 100 × ($A_{test}/A_{control}$), where $A_{control}$ is the mean A of the non-treated wells and A_{test} is the mean A of the hCG-, OP- and P4-treated wells. The mean A of wells in the absence of cells was subtracted from that of all experimental wells. The mean intra- and interassay coefficients of variation were 3.1% and 6.9%, respectively. The relationship between the number of living cells (0.06×10^5 –4.0×10^5 cells) and A_{450} was linear and had a strong correlation coefficient (R^2 = 0.965) (Supplementary Fig. 1: on-line only).

P4 production

The P4 concentrations in the culture medium were assayed using a direct enzyme immunoassay (EIA) as described previously [16]. Briefly, 20 µl aliquots of standards or medium samples (diluted 1:200) were incubated in the dark at room temperature for 18–24 h with 100 µl P4 antiserum (final dilution 1:100 000) and 100 µl P4-horseradish peroxidase (HRP; final dilution 1:150 000) in duplicate in 96-well ELISA plates (442404; Thermo Fisher Scientific) coated with ovine anti-rabbit secondary antibody. After discarding the reagent, we washed the plates four times with 300 µl Tween 80 (0.05%), and 150 µl substrate buffer containing 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) was added to each well. The plates were then incubated at 36 C for 40 min in the dark. The reaction was stopped by the addition of 50 µl 2 M H_2SO_4 to each well. The A_{450} was measured using a plate reader (Bio-Rad Laboratories).

Caspase-3 activity

Caspase-3 (CASP3) activity was measured using the commercially available SensoLyte Homogeneous AMC Caspase-3/7 Assay Kit (71118; AnaSpec, Fremont, CA, USA) according to the manufacturer's instructions. Cells were cultured in 96-well plates and combined with 50 µl substrate reaction buffer containing the CASP3 colorimetric substrate acetyl-DEVD-amido-4-methylcoumarin (Ac-DEVD-AMC). This mixture was incubated for 1 h at 37 C. Finally, the fluorescence intensity was recorded by excitation at 354 nm and emission at 442 nm using an Ultramark microplate reader (Bio-Rad Laboratories). CASP3 activity is expressed as a percentage of the activity measured in corresponding unexposed cells in three separate experiments.

Cleaved CASP3 protein analysis

CASP3 in cultured human luteinizing granulosa cells was detected by Western blotting analysis. The cultured cells were lysed in 200 µl lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 10% glycerol [G7757; Sigma-Aldrich] and Complete protease inhibitor cocktail [11697498001; Roche Diagnostics], pH 7.4). Protein concentrations in the lysates were determined by the method of Osnes *et al.* [17] using BSA as a standard. The proteins were then solubilized in SDS gel loading buffer (50 mM Tris-HCl, 2% SDS [31607-94; Nacalai Tesque, Kyoto, Japan], 13% glycerol and 1% β-mercaptoethanol [137-06862; Wako Pure Chemical Industries, Osaka, Japan], pH 6.8) and heated at 95 C for 10 min. Samples (30 µg protein) were subjected to electrophoresis on a 10% SDS-PAGE gel for 1 h at 200 V.

The separated proteins were transferred electrophoretically to a 0.2-µm nitrocellulose membrane (LC2000; Invitrogen) at 250 mA for 3 h in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3). The membrane was washed in TBS-T (0.1% Tween 20 in TBS [25 mM Tris-HCl, 137 mM NaCl, pH 7.5]) and incubated in blocking buffer (PVDF Blocking Reagent for Can Get Signal; Toyobo, Osaka, Japan) for 3 h at room temperature. After blocking, the membrane was cut into two pieces: one piece was used

for detecting cleaved CASP3 protein (17, 19 kDa), and the other was used for detecting β-actin (internal standard; 45 kDa). The membranes were then incubated separately with a primary antibody specific for either cleaved CASP3 (9661; Cell Signaling Technology, Beverly, MA, USA; diluted 1:1,000 in Can Get Signal [Toyobo]) or β-actin (A2228; Sigma-Aldrich; diluted 1:4,000 in TBS-T) overnight at room temperature. Next, the membranes were washed three times for 10 min in TBS-T at room temperature and incubated for 1.5 h with a secondary antibody to detect cleaved CASP3 (anti-rabbit HRP-linked whole antibody produced in goat [diluted 1:10,000 in TBS-T], Assay Designs, Ann Arbor, MI, USA) or β-actin (anti-mouse HRP-linked whole antibody produced in sheep [diluted 1:40,000 in TBS-T], NA931, Amersham Biosciences, Piscataway, NJ, USA). They were then washed three times in TBS for 10 min at room temperature. The signal was detected using an ECL Western Blotting Detection System (RPN2109; Amersham Biosciences).

Statistical analysis

All experimental data are shown as means ± SEM. Results were analyzed by analysis of variance (ANOVA), followed by Williams' multiple comparison test, Tukey's multiple comparison test or the Student's *t*-test, as appropriate for the experimental design. Differences were considered significant at $P<0.05$.

Results

Effect of hCG on P4 secretion in hLGCs

The P4 concentration in cells left untreated for 24 h was 11.1 ± 3.1 μM (min = 6.7 μM, max = 17.5 μM), and hCG increased P4 secretion ($P<0.05$; Fig. 1), indicating that hLGCs responded to hCG.

Effects of hCG, OP and P4 on cell viability

hCG (100 IU/ml) significantly increased cell viability ($P<0.05$; Fig. 2A). On the other hand, OP (100 μM) significantly decreased cell viability ($P<0.05$; Fig. 2B) by inhibiting P4 action. However, P4 did not increase cell viability at any of the concentrations used (Fig. 2C).

Combined effect of hCG and OP on cell viability

Cell viability was significantly lower in cultures treated with hCG plus OP than in those treated with OP alone ($P<0.05$; Fig. 3A). The decline in cell viability was dependent on the OP concentration but not on the hCG concentration.

hCG caused the extended cells on the culture plate to shrink in size, whereas OP did not (Fig. 3B). Many degenerated cells were observed after treatment with hCG plus OP (Fig. 3B).

Combined effect of hCG and OP on P4 secretion

hCG, alone or in combination with OP, significantly increased P4 production compared with the control ($P<0.05$; Fig. 4), indicating that OP did not affect P4 production by hLGCs.

Combined effect of hCG and OP on CASP3 activity and expression

In combination with OP, hCG increased cleaved CASP3 protein expression and CASP3 activity (Fig. 5A and 5B).

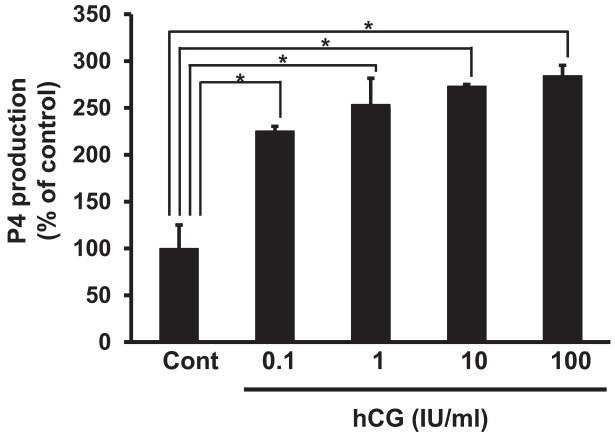

Fig. 1. Effects of hCG on P4 production by hLGCs. Cultured cells were exposed to hCG (0.1, 1, 10, 100 IU) for 24 h. The P4 concentration in the control was 11.1 ± 3.1 μM (min = 6.7 μM, max = 17.5 μM). Results are presented as the mean ± SEM of four independent experiments, each done in triplicate and expressed as the percentage of the control, which was set as 100. * $P<0.05$ *vs.* control by ANOVA followed by Williams' multiple comparison test.

Effect of P4 on the reduction in cell viability caused by hCG plus OP or RU486

P4 reversed the reduction in cell viability caused by the combination of hCG and OP treatments (Fig. 6A). Another PR antagonist, RU486, when combined with hCG, tended to reduce cell viability ($P = 0.083$) (Fig. 6B).

Discussion

The present study demonstrated that hCG induced the death of hLGCs *in vitro* when P4 binding was inhibited. This cell death response was reversed by exogenous P4 treatment, suggesting that P4 stimulated by hCG increases cell survival and that hCG cooperates with P4 to regulate cell survival. Attenuating P4 action may result in homeostatic disruption, ultimately causing cell death.

The viability of hLGCs was increased significantly at 100 IU/ml hCG after 24 h of culture (Fig. 2A). However, hCG at less than 10 IU/ml did not improve cell viability. Our results are consistent with those of a previous report, in which hCG at 20 IU/ml did not enhance the viability of hLGCs after 24 h of culture [18]. In that study, cell viability decreased gradually over 5 days of *in vitro* culture, and hCG actually suppressed the decline of viability. Furthermore, the authors of that study demonstrated that hCG enhanced the viability of hLGCs through anti-apoptosis but not proliferation. In the present study, because the viability of hLGCs was only slightly decreased after 24 h of culture due to the suppression of cell death by the high level of P4, it was difficult to determine the effect of hCG on cell survival. Therefore, differences in the relative number of live cells may be observable only under high concentrations of hCG, which enhance cell viability to a greater extent. In contrast, P4 production reached a plateau at low concentrations of hCG (0.1 IU/ml) (Fig. 1). Hence, hCG may increase cell survival not only via P4 but also

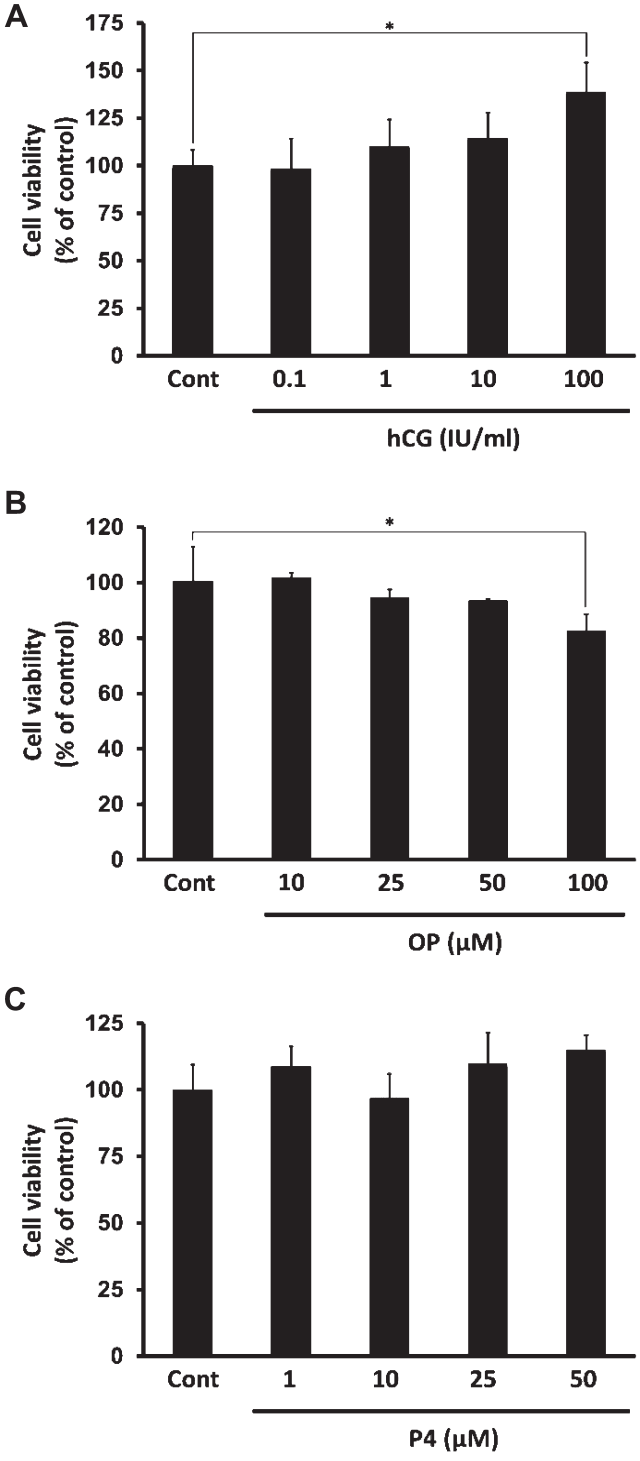

Fig. 2. Effect of hCG, OP and P4 on cell viability. hLGCs were exposed to hCG (0.1, 1, 10, 100 IU/ml) (A), OP (0, 10, 25, 50, 100 μM) (B) or P4 (1, 10, 25, 50 μM) for 24 h. After 24 h of culture, cell viability was determined at 450 nm in a WST-1 assay. Each value represents the mean ± SEM of four independent experiments, each done in triplicate. Results are expressed as the percentage of the control, which was set as 100. * P<0.05 *vs.* control by ANOVA followed by Williams' multiple comparison test.

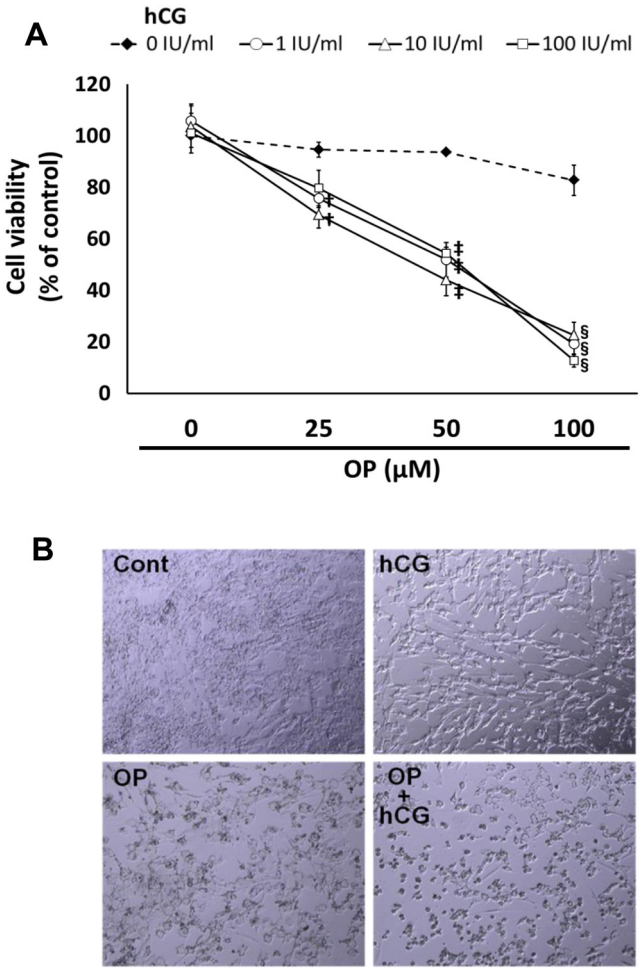

Fig. 3. Combined effect of hCG and OP on cell viability and morphological change. hLGCs were exposed to hCG (1, 10, 100 IU/ml) and/or OP (25, 50, 100 μM) for 24 h. (A) Cell viability was determined at 450 nm in a WST-1 assay. (B) Morphological changes were observed under a microscope (Olympus IX70) equipped with relief contrast objectives (10×). Cont, untreated (control); hCG, treatment with hCG (100 IU/ml); OP, treatment with OP (100 μM) and OP+hCG, treatment with hCG in combination with OP. Each value represents the mean ± SEM of three independent experiments, each done in triplicate. † P<0.05 *vs.* OP alone (25 μM); ‡ P<0.05 *vs.* OP alone (50 μM); §P<0.05 *vs.* OP alone (100 μM) (Student's *t*-test).

through other mechanisms. hCG has been shown to maintain the viability of hLGCs by inducing myeloid cell leukemia-1 expression through the following signal pathway: LH/hCG receptor, adenylate cyclase, protein kinase A and cAMP response element–binding protein [18]. However, it remains unclear whether P4 stimulates this signal pathway. hCG also increases the synthesis of cholesterol side-chain cleavage enzyme and promotes P4 production in luteal cells [19]. P4 promotes de novo cholesterol synthesis and reduces the expression of apoptotic genes [20, 21]. Furthermore, P4 regulates the viability of rat granulosa cells in a protein kinase G–dependent manner [22]. One target for the direct hCG regulation of cell viability is hyaluronan

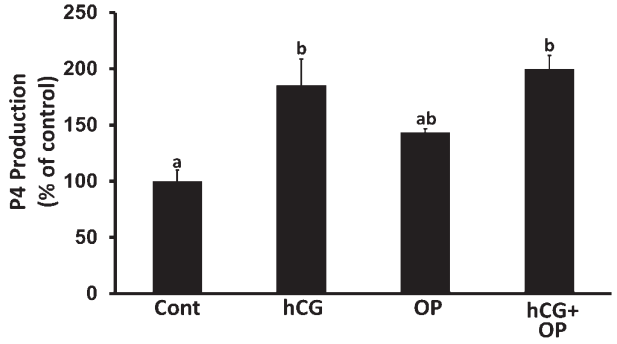

Fig. 4. Effects of hCG and/or OP on P4 production by hLGCs. Cultured cells were exposed to hCG (100 IU) and/or OP (100 μM) for 24 h. Each value represents the mean ± SEM of four independent experiments, each done in triplicate. Results are expressed as the percentage of the control, which was set as 100. Different letters indicate significant differences (P<0.05) as determined by ANOVA followed by Tukey's multiple comparison test.

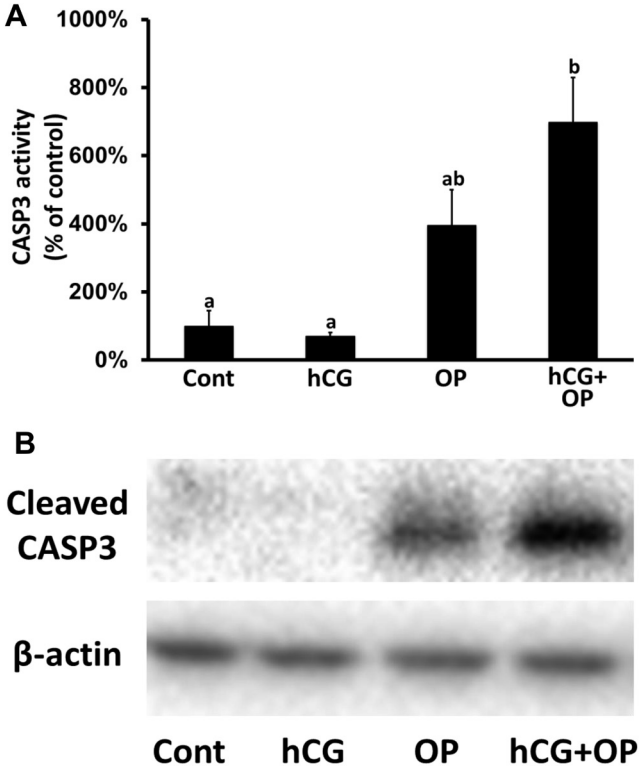

Fig. 5. Effects of hCG and/or OP on CASP3 activity and expression in cultured hLGCs. Cultured cells were exposed to hCG (100 IU) and/or OP (100 μM) for 24 h. (A) CASP3 activity was measured using the CASP3-specific substrate DEVD-AMC. Each value represents the mean ± SEM of three independent experiments, each done in triplicate. Results are expressed as the percentage of the control, which was set as 100. (B) Protein expression of cleaved CASP3 was determined by western blot analysis of cleaved CASP3 (17 and 19 kDa) and β-actin (45 kDa). Representative examples for cleaved CASP3 and β-actin are shown (n = 3). Different letters indicate significant differences (P<0.05) as determined by ANOVA followed by Tukey's multiple comparison test.

and proteoglycan link protein 1 (HAPLN1), a component of the follicular matrix. In a previous study, HAPLN1 increased the viability of cultured rat granulosa cells, and *Hapln1* mRNA expression was upregulated *in vitro* by hCG action, which was not mediated by P4 [23]. Because a high level of immunoreactive HAPLN1 is present in the newly formed CL [24], hCG action that is not mediated by P4 may maintain the viability of hLGCs by stimulating the expression of HAPLN1. However, P4 alone did not affect cell viability at any of the concentrations used in the present study (Fig. 2C). The P4 concentration produced by untreated cells was 11.1 μM (Fig. 1); this endogenous P4 level may thus be sufficient for cell survival. Because the combined action of hCG and P4 is involved in a number of signaling pathways for the maintenance of luteal cells, further study is necessary to determine its mechanism.

Our results are similar to those of previous studies, which have

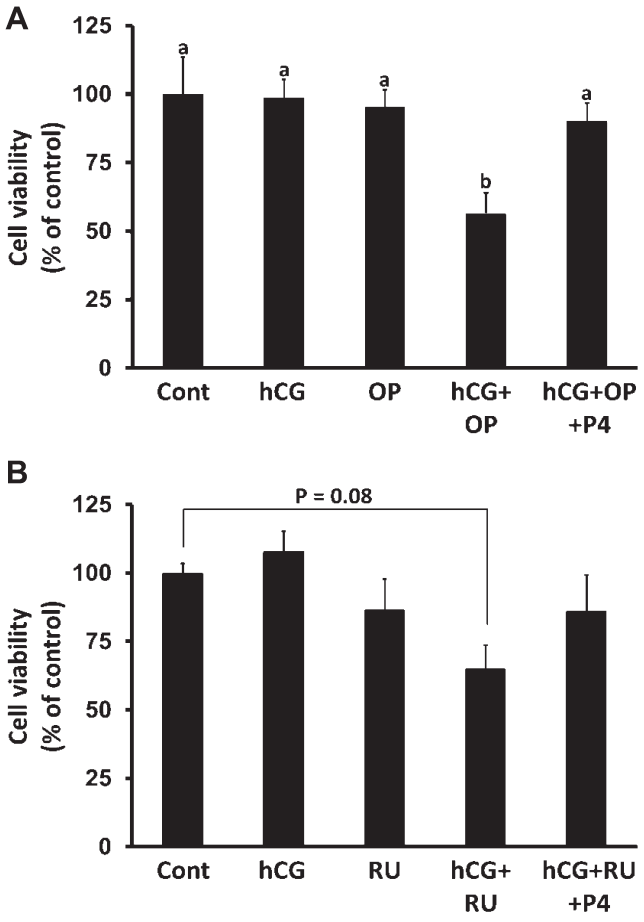

Fig. 6. Effect of P4 on the reduction in cell viability caused by hCG plus P4 receptor antagonists. Cultured cells were exposed to hCG (50 IU/ml), OP (50 μM), RU486 (50 μM) and P4 (50 μM) each or together for 24 h. Cell viability was determined at 450 nm in a WST-1 assay. Each value represents the mean ± SEM of three or more independent experiments, each done in triplicate. Results are expressed as the percentage of the control, which was set as 100. Different letters indicate significant differences (P<0.05) as determined by ANOVA followed by Tukey's multiple comparison test.

shown that the combination of RU486 and hCG induces cell death in hLGCs [14, 25]. The morphological change was consistent with the results observed for WST-1; many degenerated cells were observed after treatment with hCG and OP (Fig. 3B). Interestingly, cell death was induced by P4 inhibition at the time of hCG action in the present study (Fig. 3A). The two progesterone receptor (PR) antagonists used for the inhibition of P4 binding, OP and RU486, yielded similar results, whereas the combination of hCG and RU486 tended to reduce cell viability (Figs. 6A and 6B). OP is a type I selective PR modulator, displaying purely antagonistic properties, while RU486 is a type II modulator, displaying agonistic potential upon protein kinase A stimulation *in vitro* [26, 27]. By inhibiting P4 action as a pure antagonist, OP caused significant cell death (Fig. 6A). Although it was not clear how OP and RU486 affected the signal pathway of cell viability in detail, exogenous P4 treatment reversed the cell death induced by hCG in combination with OP. This result confirms that cell death occurred due to the inhibition of P4 binding by PR antagonists but not through toxicity.

Programmed cell death occurs in mainly three forms: apoptosis, autophagy and necroptosis [28]. In humans, luteolysis occurs mainly through the apoptosis of luteal cells [5]. Caspases are regulatory proteins that participate in transmission of the apoptotic signal from the cytoplasm to the nucleus [29]. CASP3 is a typical effector caspase, and its activity is essential for the detection of apoptosis [30]. In the present study, we assessed the cleavage of CASP3 as an indicator of its activity. Treatment with hCG alone reduced CASP3 activity to 68% of the control level. However, treatment with hCG in combination with OP increased CASP3 activity (Fig. 5A) and cleaved CASP3 protein expression (Fig. 5B). These results support the finding that hCG plus OP decreased cell viability (Fig. 3A) and indicate that hCG-induced cell death under the inhibition of P4 binding in hLGCs is due to apoptosis.

hCG has been reported to stimulate the production of reactive oxygen species (ROS) [31], and ROS are known to induce apoptosis in luteal cells [32–34]. In contrast, P4 has been reported to increase levels of superoxide dismutase (SOD), an antioxidant enzyme [35, 36]. Therefore, the P4-induced reduction of SOD may be responsible for the excess ROS stimulated by hCG. This seems to be the reason for the apoptosis induced by hCG in combination with OP (Fig. 3A). Further studies are needed to clarify whether hCG regulates the balance between ROS and SOD via P4 and whether hCG consequently regulates cell survival and cell death.

In conclusion, our results suggest that hCG cooperates with P4 to increase survival of hLGCs and to induce apoptosis when P4 action is attenuated in the human CL. This relationship between hCG and P4 might help to explain some early miscarriages caused by luteal insufficiency.

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