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Luteinizing Hormone-Induced Caspase Activation in Rat Preovulatory Follicles Is Coupled to Mitochondrial Steroidogenesis

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Atresia and luteolysis are well-documented processes in which most of the growing ovarian follicles and all corpora lutea, respectively, are eliminated by apoptosis. We have previously reported that LH and FSH enhance caspase-3 and -7 activity and apoptosis in the theca-interstitial cells of rat preovulatory follicles in culture. Here we have used cultured follicles to examine whether LH-induced caspase activation is related to the ability of LH to stimulate steroid production. In these studies, we used three inhibitors of enzymes involved in steroid production: aminoglutethimide and ketoconazole, acting on cytochrome P450 side-chain cleavage (P450scc) located at the mitochondria, and epostane, acting on 3β-hydroxysteroid dehydrogenase located at the endoplasmic reticulum. We found that treatment with either aminoglutethimide or ketoconazole, but not with epostane, significantly reduced LH-induced caspase-3 and -7 activation and apoptosis, suggesting the mediation of LH-induced caspase activation by P450scc. Supplementing pregnenolone, the product of P450scc catalysis, to follicles treated with aminoglutethimide did not restore LH-induced caspase activation. On the other hand, treatment with antioxidants inhibited LH-induced caspase activation. Moreover, LH treatment was associated with an increase in reactive oxygen species which was inhibited by aminoglutethimide. Thus, P450scc catalysis results in an increase in reactive oxygen species, which in turn may trigger/facilitate caspase-3 activation. Finally, we found that in rat corpora lutea *in vivo*, an increase in steroidogenesis was accompanied by an increase in caspase activity. Thus, this study reveals a linkage between two seemingly distinct processes in which LH-induced caspase activation in cultured rat preovulatory follicles is coupled to mitochondrial steroidogenesis via P450scc. (*Endocrinology* 148: 1717–1726, 2007)

DURING THE REPRODUCTIVE lifespan, the mammalian ovary comprises follicles in different stages of development as well as corpora lutea (CL). Preovulatory follicles contain an oocyte surrounded by several layers of somatic granulosa cells (GC), a fluid-filled antrum, and outer somatic theca cells separated from the GC by a basement membrane. Growth of follicles and the formation of CL is dependent on multiple factors such as steroids and growth factors and mainly on the gonadotropins FSH and LH (1, 2). Most of the follicles in the ovary will not complete the cycle of development culminating with ovulation and corpus luteum formation but will undergo atretic degeneration due to apoptosis of the somatic GC (3, 4).

Several protein families participate in the regulation and execution of the apoptotic process. B-cell lymphoma protein (BCL)-2 family members are major regulators of apoptosis and comprise of both proapoptotic [*e.g.* BCL-2-associated X protein (BAX) and BCL-2-antagonist/killer (BAK)] as well as antiapoptotic members (*e.g.* BCL-2 and BCL-X_L) (5). Caspases, a family of cysteine proteases, are major executioners of apoptosis. The caspase family is divided into initiator caspases (*e.g.* caspase-8 and -9) and effector caspases (*e.g.* caspase-3 and -7) based on the fact that the first group mediates the activation of the second group (6).

Two major pathways leading to apoptosis exist in cells, the extrinsic and the intrinsic. The extrinsic pathway involves the activation of the TNF/Fas death receptor family. The intrinsic pathway involves the activation of proapoptotic BCL-2 family members, leading to initiation of the mitochondrial apoptotic program. This program includes the release of several intermembrane-space mitochondrial proteins (*e.g.* cytochrome c and Smac/Diablo) that actively promote apoptosis (7). Both pathways ultimately result in activation of effector caspases.

All caspases are expressed as inactive enzymes (zymogens), and their activation involves several cleavage events. In the case of caspase-3, the first cleavage event occurs between the large and small subunits, resulting in the generation of the p20 product (prodomain plus large subunit) and the p12 product (small subunit), which form a heterotetramer complex (8). The second cleavage event occurs between the prodomain and large subunit, resulting in the p17 product (large subunit), and the formation of a fully active enzyme. Inhibitors of apoptosis bind to the p20-p12 heterotetramer and inhibit its processing to the fully active complex. On the other hand, Smac (which is released from the mitochondria during apoptosis) binds to inhibitors of apoptosis to antag-

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Abbreviations: BCL, B-cell lymphoma protein; carboxy-H_2DCFDA, 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescin diacetate; CL, corpora lutea; DCF, 2′,7′-dichlorofluorescein; DEVD-AMC, acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin; eCG, equine chorionic gonadotropin; ER, endoplasmic reticulum; GC, granulosa cells; hCG, human chorionic gonadotropin; 3βHSD, 3β-hydroxysteroid dehydrogenase; GSH, glutathione; MnTBAP, Mn(III)tetrakis(4-benzoic acid)porphyrin chloride; P450scc, P450 side-chain cleavage; ROS, reactive oxygen species; TIC, theca-interstitial cells; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; zVAD-fmk, Z-Val-Ala-Asp-fluoromethylketone.

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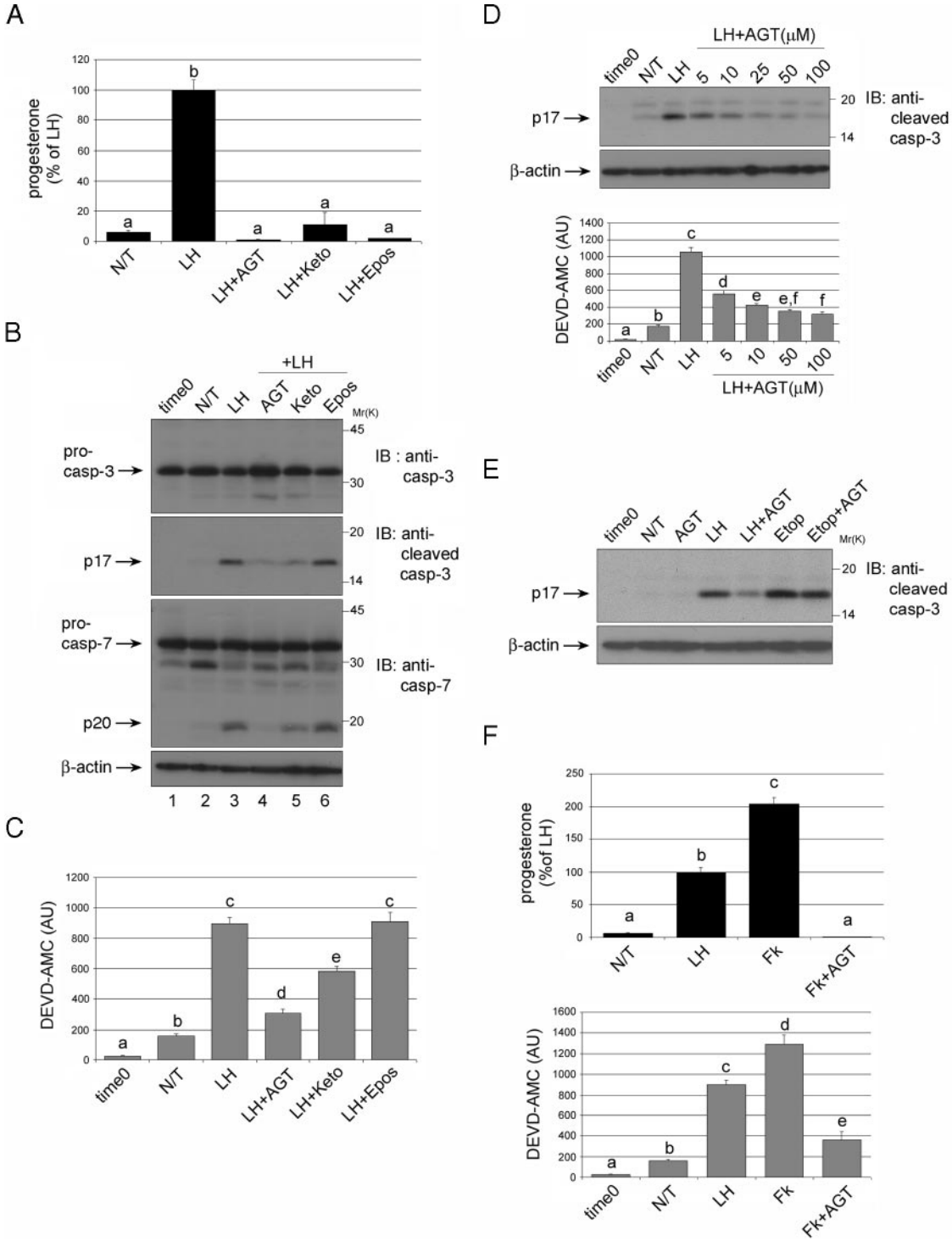

FIG. 1. Inhibitors of P450scc, AGT and ketoconazole, suppress LH-induced caspase-3 and -7 cleavage/activation. A, AGT, epostane, and ketoconazole inhibit LH-induced progesterone production. Follicles were cultured in the absence or presence of LH, LH plus AGT (100 μM), LH plus ketoconazole (Keto; 1 μM), or LH plus epostane (Epos; 100 μM), and the levels of progesterone in the medium were determined by RIA. The data represent mean ± SEM. *Columns with different superscripts* are significantly different ($P < 0.05$). B, AGT and ketoconazole, but not epostane, inhibit LH-induced caspase-3 and -7 cleavage. Follicles were freshly isolated from ovaries (time 0) or cultured as in A, lysed, separated by SDS-PAGE, and analyzed by Western blot using an anti-caspase-3 antibody (*top*), an anti-cleaved caspase-3 antibody (*middle upper panel*) and an anti-caspase-7 antibody (*middle lower panel*). Representative immunoblots of a typical experiment of three are shown together with β-actin as an internal standard (*bottom*). C, AGT and ketoconazole, but not epostane, inhibit LH-induced caspase-3 and -7 activity. Follicles were cultured according to the indicated conditions, and caspase-3/-7 activity was assessed using the fluorogenic peptide substrate DEVD-AMC. The results are presented in arbitrary units (AU) as mean ± SEM. *Columns with different superscripts* are significantly different ($P < 0.05$). D, AGT inhibits LH-induced caspase-3 cleavage/activity in a dose-dependent manner. *Top*, Follicles were cultured according to the indicated conditions, lysed, separated by SDS-PAGE, and analyzed by Western blot using an anti-cleaved caspase-3 antibody. A representative immu-

onize their inhibitory action (9). Caspase-3 is the most characterized effector caspase, and its activation leads to the cleavage of many death substrates, which eventually result in cell death (10).

The LH surge triggers in the preovulatory follicle ovulatory changes, including among others the resumption of meiosis by the oocyte, luteinization of the granulosa and theca cells (which later form the corpus luteum), rupture of the follicle wall, and release of a fertilizable ovum. The luteinization of follicle cells includes stimulation of overall steroid production and a change of its profile from secretion of estrogen to progesterone (11). Steroid production is initiated at the mitochondria and includes uptake of cholesterol into the mitochondria mediated by the steroid acute regulatory (StAR) protein and its conversion into pregnenolone by cytochrome P450 side-chain cleavage (P450scc). Pregnenolone is further processed into progesterone by 3β-hydroxysteroid dehydrogenase (3βHSD) at the endoplasmic reticulum (ER).

We have previously reported that LH and FSH enhance caspase activity and apoptosis in the theca-interstitial cells (TIC) of rat preovulatory follicles in culture (12). Mitochondria play a key role in both steroidogenesis and the execution of apoptosis, and therefore we suspected that steroid production that is triggered by LH and is initiated at the mitochondria might have an effect on caspase activation and apoptosis. In this study we examined this possibility by using inhibitors to enzymes involved in steroidogenesis. Using these inhibitors we demonstrated that the LH-induced caspase activation is coupled to steroid production via stimulation of mitochondrial P450scc and production of reactive oxygen species (ROS) in cultured rat preovulatory follicles.

Materials and Methods

Reagents

Equine chorionic gonadotropin (eCG) was purchased from Vertimex (Bladel, The Netherlands). Ovine LH (LH-S-26; 2300 IU/mg) was generously provided by Dr. A. F. Parlow and the National Hormone and Pituitary Distribution Program, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (Bethesda, MD). Human chorionic gonadotropin (hCG) was purchased from Organon (Pregnyl, The Netherlands). Aminoglutethimide was obtained from Ciba-Geigy (Basel, Switzerland), and epostane was a gift from Sterling-Winthrop Research Institute (Rensselaer, NY). Ketoconazole, forskolin, pregnenolone, glutathione and etoposide were purchased from Sigma Chemical Co. (St. Louis, MO). The broad caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) was purchased from Calbiochem (Darmstadt, Germany). 5-(and-6)-Carboxy-2′,7′-dichlorodihydrofluorescin diacetate (carboxy-H_2DCFDA) was purchased from Molecular Probes (Eugene, OR). The caspase-3 fluorogenic substrate, acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (DEVD-AMC) was purchased from Alexis (San Diego, CA). Cleaved caspase-3 blocking peptide was purchased from Cell Signaling (Danvers, MA).

Follicle cultures

All animal experiments were approved by the Institutional Animal Care and Use Committee. Follicle cultures were performed as previously described (13). Briefly, immature female Wistar rats at the age of 23–25 d were injected with 12 IU eCG in PBS (Life Technologies, Inc., Grand Island, NY) to enhance follicular growth. After 48 h, rats were killed by CO_2, the ovaries were placed in Leibovitz medium (L-15; Life Technologies) containing 2 mM L-glutamine, 0.1% BSA (Fraction V; Sigma), and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Life Technologies) and immediately dissected. The largest preovulatory follicles (>800 μm in diameter) were isolated from the ovaries and cleaned from adherent tissue using fine forceps. Ten intact follicles of similar size were transferred into an organ culture dish (Falcon, NJ) and cultured on stainless steel grids. Cultures were carried out at 37 C under 50% O_2/50% air and sufficient CO_2 to give pH 7.2. Follicles were cultured for 24 h in the absence or presence of LH (100 ng/ml) or forskolin (10 μM), LH or forskolin plus aminoglutethimide (AGT) (100 μM or as indicated), LH plus epostane (100 μM), or LH plus ketoconazole (1 μM; a low concentration of ketoconazole was used because higher concentrations showed toxic effects). In the experiments in which CL were collected, rats were injected with 12 IU eCG and 48 h later were injected with 10 IU hCG. CL were dissected from ovaries 2, 3, 4, 8, or 14 d after hCG injection.

Western blotting

Proteins were size-fractionated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Bio-Rad, Hercules, CA). Antibodies included polyclonal anti-caspase-3 (H-277; Santa Cruz Biotechnology, Santa Cruz, CA; dilution of 1:1000; this antibody recognizes full-length caspase-3 and weakly recognizes the p20 and p17 cleavage products), polyclonal anti-cleaved caspase-3 (Cell Signaling; dilution of 1:300; this antibody recognizes the p20 and p17 cleavage products of caspase-3 but not full-length caspase-3), monoclonal anti-caspase-7 (a gift from Dr. Y. Lazebnik, Cold Spring Harbor, NY; dilution of 1:1000; this antibody recognizes the full-length and p20 cleavage product of caspase-7), and monoclonal anti-β-actin (Sigma; dilution of 1:5000). Western blots were developed by use of the enhanced chemiluminescence reagent (Amersham, Little Chalfont, UK).

Caspase-3/-7 activity assays

Caspase-3/-7 activity assays were preformed as previously described (12). Briefly, follicles (10 per sample) were homogenized twice in lysis buffer [20 mM HEPES (pH 7.3), 5 mM EGTA, 5 mM EDTA, 10 μM digitonin, 2 mM dithiothreitol]. The lysates were clarified by centrifugation, and the supernatants were used for the assays. Protein concentrations were determined by Bradford (Bio-Rad). Enzymatic reactions were carried out in lysis buffer containing 20 μg protein and 50 μM DEVD-AMC to assess caspase-3/-7 activity. Each sample was divided into three parts, one of which included in addition to the extract and substrate 50 μM zVAD-fmk to inhibit caspase activity and two replicates that included extract and substrate without inhibitor. The reaction mixtures were incubated at 37 C, and fluorescent AMC formation was measured at excitation 380 nm and emission 460 nm using a Wallac Victor2 1420 Multilabel Counter (PerkinElmer, Wellesley, MA). Specific activity was calculated for each sample as the mean of the duplicate sample minus the value obtained for the sample containing zVAD-fmk.

noblot of a typical experiment of three is shown together with β-actin as an internal standard. *Bottom*, Follicles were cultured as above, and caspase-3/-7 activity was assessed using the fluorogenic peptide substrate DEVD-AMC. The results are presented in arbitrary units (AU) as mean ± SEM. *Columns with different superscripts* are significantly different ($P < 0.05$). E, AGT does not directly inhibit caspase-3. Follicles were cultured in the absence or presence of AGT, LH, LH plus AGT, etoposide (Etop; 100 μM), or Etop plus AGT, lysed, separated by SDS-PAGE, and analyzed by Western blot using an anti-cleaved caspase-3 antibody. A representative immunoblot of a typical experiment of three is shown together with β-actin as an internal standard. F, Forskolin induces caspase-3 and -7 activation, which is inhibited by AGT. Follicles were cultured in the absence or presence of LH, forskolin (Fk; 10 μM), or forskolin plus AGT. The levels of progesterone in the medium were determined by RIA (*top*), and caspase-3/-7 activity was assessed using the fluorogenic peptide substrate DEVD-AMC (*bottom*). The data represent mean ± SEM. *Columns with different superscripts* are significantly different ($P < 0.05$).

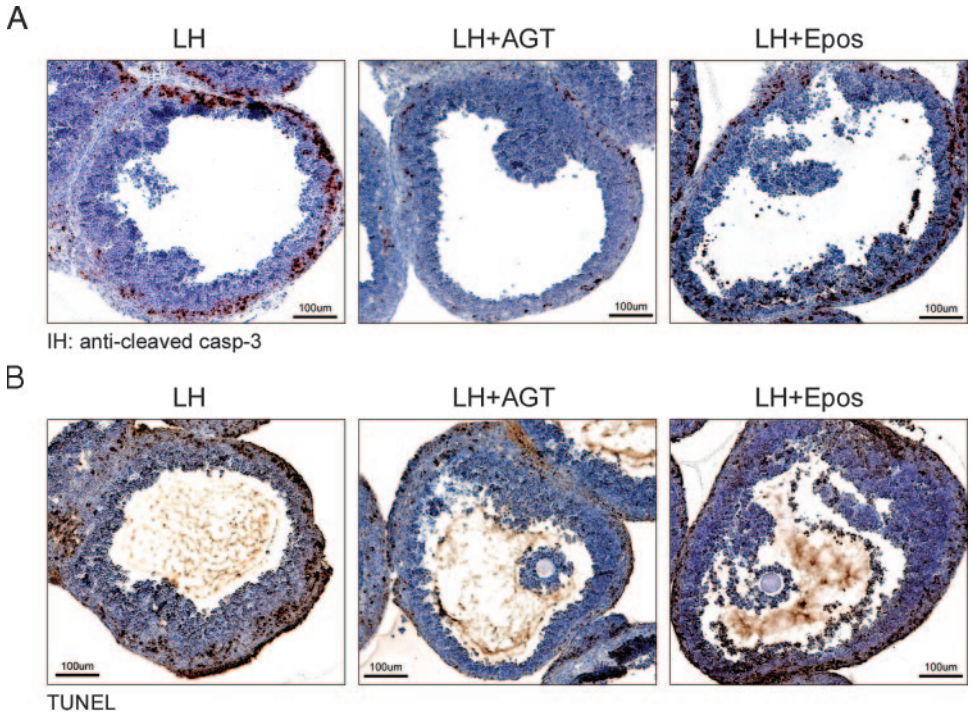

FIG. 2. Inhibition of P450scc suppresses LH-induced caspase-3 activation and apoptosis in TIC. A, Inhibition of P450scc blocks LH-induced caspase-3 cleavage in TIC. Immunohistochemical analysis of cleaved caspase-3, using an anti-cleaved caspase-3 antibody, was performed in sections taken from paraffin-embedded follicles. Sections were taken from follicles cultured in the presence of LH, LH plus AGT, or LH plus epostane (Epos). B, Inhibition of P450scc blocks LH-induced apoptosis in TIC. TUNEL staining of follicle sections treated as in A (in sequential sections). Original magnification, ×100 for all panels. The slides shown are representative of slides obtained from three independent experiments.

Progesterone assay

Levels of progesterone released by the follicles into the culture medium (10 follicles per culture dish) or serum progesterone levels were measured using a commercial kit (COAT-A-COUNT Progesterone RIA; Diagnostics Products Corp., Los Angeles, CA) according to the manufacturers' instructions with slight modifications. A progesterone standard curve was produced using progesterone (Sigma) in PBS containing 0.1% BSA. For measurement of serum progesterone, serum samples were extracted first with petroleum ether and reconstituted with PBS containing 0.1% BSA. Assay sensitivity was 0.02 ng/ml. Within- and between-assay coefficients of variation were 7.3 and 16.6%, respectively.

Immunohistochemistry

Immunohistochemistry of follicles was performed as previously described (12). Follicles (five to six per treatment in duplicates) were fixed in 3% paraformaldehyde and paraffin embedded, and 6-μm sections were analyzed by immunohistochemistry using the Histostain-SP Kit (Zymed Laboratories, South San Francisco, CA) according to the manufacturers' instructions. Briefly, after deparaffinization, dehydration, and blocking of endogenous peroxidases with 3% H_2O_2 in methanol, sections were incubated with 10% goat serum for 1 h, followed by incubation with the anti-cleaved caspase-3 antibody (Cell Signaling; dilution of 1:50) to detect the cleavage products of caspase-3. To demonstrate the specificity of this antibody, the antibody was preincubated with the peptide used to generate it (Cell Signaling; dilution of 1:1 antibody/peptide). The sections were then washed and incubated with the biotinylated secondary antibody, followed by incubation with horseradish streptavidin-peroxidase conjugate. After washing, sections were incubated with the chromogen substrate and counterstained with hematoxylin. In the experiments in which whole ovaries were immunostained with the anti-cleaved caspase-3 antibody, free-floating sections (16 μm thick) were cut with a sliding microtome (Leica SM 2000R; Leica, Nussloch, Germany) and collected in PBS. The sections were incubated with the primary antibody in a blocking solution containing 0.5% Triton X-100 and 3% normal goat serum followed by incubation with a biotinylated secondary antibody (biotinylated antirabbit IgG; Vector Laboratories, Burlingame, CA) and detection with streptavidin-conjugated Cy2 (Jackson ImmunoResearch, West Grove, PA). Stained sections were examined and photographed by a fluorescence microscope (E600; Nikon, Tokyo, Japan) equipped with Plan Fluor objectives connected to a CCD camera (DMX1200F; Nikon).

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay

TUNEL assays were performed as previously described (12). Briefly, apoptosis was assessed by *in situ* 3′-end labeling of DNA fragments using the TUNEL assay. TUNEL was performed using the ApopTag *in situ* apoptosis detection kit (Intergen, Purchase, NY), according to the manufacturer's instructions. Follicles were fixed in 3% paraformaldehyde solution, dehydrated, embedded in paraffin, and serially cut at 6 μm. After deparaffinization, hydrated sections were incubated in 0.5% Triton X-100 for 5 min, and endogenous peroxidases were neutralized by incubation with 3% H_2O_2. The sections were then incubated with terminal deoxynucleotidyl transferase for 1 h at 37 C, followed by incubation with anti-digoxigenin-peroxidase antibodies. Sections were stained with diaminobenzidine solution and counterstained with hematoxylin. Slides were analyzed and photographed as described above.

Assessment of ROS levels in intact follicles

To assess the levels of ROS in intact follicles, we used a protocol similar to the one used by Tsai-Turton and Luderer (14) with some modifications. Preovulatory follicles were cultured for 24 h in the absence (untreated group) or presence of LH, LH plus AGT, and LH plus zVAD-fmk. Another group of follicles treated with 5% H_2O_2 (during 0.5 h) was added to provide a positive control group for oxidative stress. After culture, the medium was removed and the follicles were transferred into PBS containing 100 μM of the ROS indicator carboxy-H_2DCFDA for 30 min, washed several time with PBS, and cultured with L-15 medium for an additional 30 min. Carboxy-H_2DCFDA is a cell-permeable nonfluorescent probe for ROS. Within the cell, the acetate groups are removed by intracellular esterases and upon oxidation the highly fluorescent 2′,7′-dichlorofluorescein (DCF) is produced. Fluorescence was viewed using an Axiovert 100 TV confocal microscope (Zeiss, Oberkochen, Germany) attached to the Bio-Rad Radiance 2000 laser scanning system and operated by LaserSharp software. Each follicle was scanned and divided into 11 equally spaced layers from the bottom to the top. The mean fluorescence intensity was calculated for each follicle after subtraction of background fluorescence values, using

the NIH ImageJ software (W. Rasband, ImageJ version 1.36b, http://rsb.info.nih.gov/ij/). Results were pooled from three independent experiments with five to eight follicles measured in each treatment group.

Statistical analysis

Data regarding caspase activity assays, progesterone assays, and fluorescence intensity from the confocal experiments are expressed as the mean ± SEM of pooled results obtained from at least three independent experiments with at least three repeats in each experiment. In the progesterone assays performed *in vitro*, the mean result of the LH treatment was set as 100%, and the other treatment values are expressed as percentage of the LH treatment. In the confocal fluorescence experiments, the untreated follicle group was set as 100%, and the other treatment values are expressed as a percentage of the untreated follicle group. Statistical analysis was performed by one-way ANOVA test followed by Fisher's protected least significant difference post-test for multiple comparisons using the StatView Program (Abacus Concepts, Berkeley, CA). Significance level was considered as $P < 0.05$.

Results

Inhibitors of P450scc, AGT, and ketoconazole suppress LH-induced caspase-3 and -7 cleavage/activation

Mitochondria play a key role in both steroidogenesis and the execution of apoptosis, and therefore we suspected that steroid production that is triggered by LH and is initiated at the mitochondria might have an effect on caspase activation and apoptosis. To examine this possibility, we used three inhibitors of steroidogenesis: AGT and ketoconazole, acting on P450scc (15, 16), and epostane, acting on 3βHSD (17). Follicles were cultured in a serum-free medium for 24 h in the absence or presence of LH, LH plus AGT, LH plus ketoconazole, or LH plus epostane. We first confirmed that addition of these inhibitors to cultured follicles treated with LH indeed inhibited LH-induced progesterone production (Fig. 1A). To determine the effect of steroidogenesis inhibition on caspase activation, follicles were treated as above, and effector caspase-3 and -7 cleavage/activation was examined by Western blot analysis and by activity assays. As we previously described, addition of LH resulted in a significant increase in caspase-3 and -7 cleavage/activity (Fig. 1B, lane 3, Fig. 1C, and Ref. 12). Strikingly, addition of LH together with AGT significantly reduced the cleavage/activity of caspase-3 and -7 (Fig. 1B, lane 4, and Fig. 1C). Similarly, albeit less potently, addition of LH together with ketoconazole significantly reduced the cleavage/activity of caspase-3 and -7 (Fig. 1B, lane 5, and Fig. 1C). This effect was specific to these inhibitors because addition of LH together with epostane did not inhibit LH-induced caspase-3 and -7 cleavage/activity (Fig. 1B, lane 6, and Fig. 1C). A dose-response analysis revealed that AGT inhibited LH-induced caspase-3 cleavage and activity in a dose-dependent manner (Fig. 1D). Thus, inhibiting P450scc but not 3βHSD blocks LH-induced effector caspase activation.

To assess whether AGT may inhibit the caspases themselves, we determined its effect on caspase-3 cleavage when added to the follicle culture alone or when added in the presence of etoposide, a specific inhibitor of topoisomerase II, which is a potent inducer of caspase activation and apoptosis. Treatment of follicles with AGT alone did not alter caspase-3 cleavage compared with untreated follicles (Fig. 1E). As expected, addition of etoposide to cultured follicles resulted in a significant increase in caspase-3 cleavage, and the addition of etoposide together with AGT had no effect on this increase (Fig. 1E). Thus, the inhibitory effect of AGT on LH-induced caspase-3 activation is not due to a nonspecific inhibition of caspases.

To further establish the connection between steroid production and caspase activation, we used forskolin, a potent activator of adenylate cyclase, that acts downstream of LH receptor and upstream of P450scc to induce steroid production. As expected, addition of forskolin to cultured follicles stimulated progesterone production, and AGT inhibited this production (Fig. 1F, *top*). Similar to LH, forskolin treatment

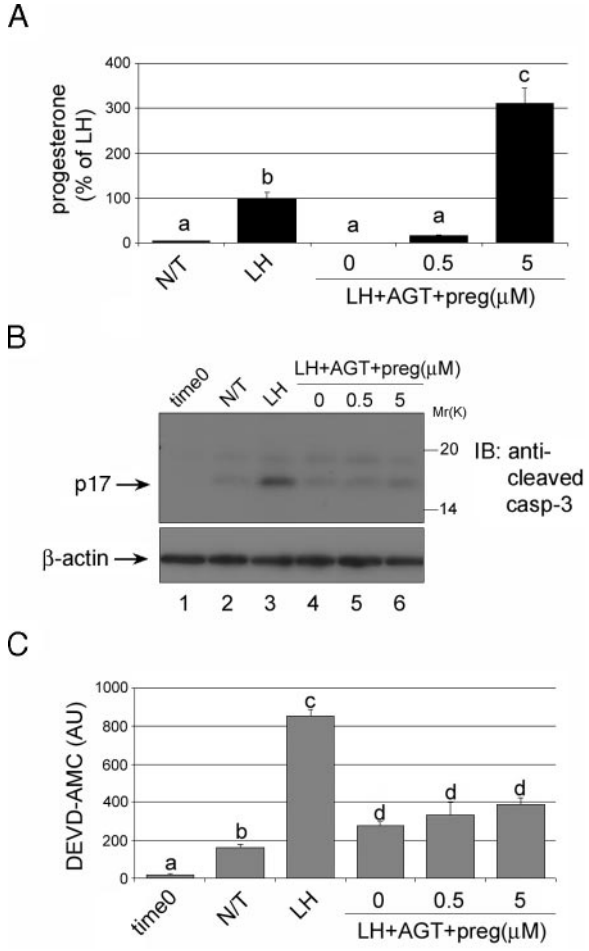

FIG. 3. Pregnenolone restores LH-induced progesterone production but not caspase-3 and -7 activation. A, Addition of pregnenolone to AGT-treated follicles restores LH-induced progesterone production. Follicles were cultured in the absence or presence of LH, LH plus AGT, or LH plus AGT plus pregnenolone (preg; 0.5 or 5 μM), and the levels of progesterone in the medium were determined by RIA. The data represent mean ± SEM. *Columns with different superscripts* are significantly different ($P < 0.05$). B, Addition of pregnenolone to AGT-treated follicles does not restore LH-induced caspase-3 cleavage. Follicles were cultured as indicated and analyzed by Western blot using an anti-cleaved caspase-3 antibody (*top*). Representative immunoblots of a typical experiment of three are shown together with β-actin as an internal standard (*bottom*). C, Addition of pregnenolone to AGT-treated follicles does not restore LH-induced caspase activity. Follicles were cultured as indicated, and caspase-3/-7 activity was assessed using the fluorogenic peptide substrate DEVD-AMC. The results are presented in arbitrary units (AU) as mean ± SEM. *Columns with different superscripts* are significantly different ($P < 0.05$).

resulted in a significant increase in caspase-3 and -7 activity, and addition of forskolin together with AGT significantly reduced this increase (Fig. 1F, *bottom*). Thus, a LH-independent stimulator of steroidogenesis induces caspase activation, and this activation is also P450scc dependent.

Inhibition of P450scc suppresses LH-induced caspase-3 activation and apoptosis in TIC

LH-induced caspase-3 cleavage/activation and apoptosis occur mainly in TIC of the rat preovulatory follicle (12). Thus, we next determined the effect of AGT and epostane on LH-induced caspase-3 cleavage and apoptosis in TIC. To determine the effect on caspase-3 cleavage, sections of rat follicles cultured in the presence of LH with or without AGT or epostane were immunostained with the anti-cleaved caspase-3 antibody. As previously reported, LH treatment resulted in a marked increase in positive staining for cleaved caspase-3 in TIC (Fig. 2A and Ref. 12). As expected, addition of LH together with AGT, but not with epostane, decreased the positive staining for cleaved caspase-3 (Fig. 2A). Next, we determined the effect of AGT and epostane on LH-induced apoptosis in TIC. As previously reported, LH treatment resulted in a marked increase in positive TUNEL/apoptosis staining in TIC (Fig. 2B and Ref. 12). Importantly, addition of LH together with AGT, but not with epostane, largely inhibited apoptosis (Fig. 2B). Thus, the inhibitory effect of AGT on LH-induced caspase cleavage/activation is accompanied by inhibition of apoptosis.

Pregnenolone restores LH-induced progesterone production but not caspase-3 and -7 activation

The results above demonstrate that P450scc catalysis is important for LH-induced caspase activation and apoptosis. Therefore, we next examined whether adding back pregnenolone, the product of P450scc catalysis, would restore LH-induced caspase activation in follicles treated with AGT. A concentration of pregnenolone (5 μM) that was more than sufficient to restore LH-induced progesterone production in the presence of AGT (Fig. 3A) did not restore LH-induced caspase-3 and -7 activation (Fig. 3B, lane 6, and Fig. 3C). Thus, mitochondrial P450scc catalysis, but not its product pregnenolone, is involved in LH-induced caspase activation.

P450scc catalysis results in an increase in ROS, which in turn may trigger/facilitate caspase-3 cleavage/activation

Our findings above suggest that P450scc catalysis results in a by-product that triggers/facilitates caspase activation. Previous studies have demonstrated that mitochondrial P450 systems may function as a source of ROS (18, 19), which may trigger/facilitate apoptosis in some cell lines (20). Therefore, we initially wished to explore whether ROS were involved in LH-induced caspase-3 activation by examining the effect of antioxidants. For these studies, we used two antioxidants: the tripeptide glutathione (γ-Glu-Cys-Gly; GSH), an antioxidant that provides protection against autooxidation and that was recently shown to be important for protection from apoptosis in the preovulatory follicle (14), and MnTBAP, a superoxide dismutase mimetic with a broad range of anti-

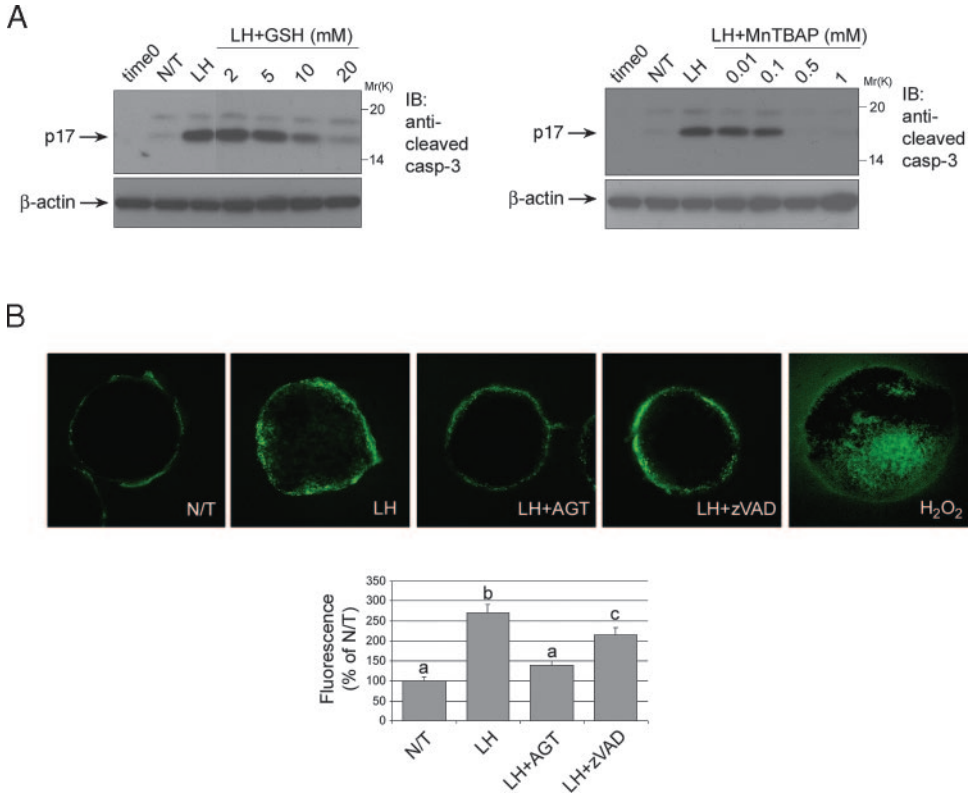

FIG. 4. P450scc catalysis results in an increase in ROS, which in turn may trigger/facilitate caspase-3 cleavage/activation. A, Antioxidants suppress LH-induced caspase-3 cleavage in a dose-dependent manner. Follicles were cultured in the absence or presence of LH, LH plus GSH (using the indicated doses; *left*), or LH plus MnTBAP (*right*), lysed, separated by SDS-PAGE, and analyzed by Western blot using an anti-cleaved caspase-3 antibody. Representative immunoblots of a typical experiment of three are shown together with β-actin as an internal standard. B, P450scc catalysis causes an increase in ROS. *Top*, Follicles were cultured for 24 h in the absence (N/T) or presence of LH, LH plus AGT (100 μM), LH plus zVAD (50 μM), or 5% H_2O_2 (added for 0.5 h). After culture, follicles were incubated with carboxy-H_2DCFDA and assessed for the levels of ROS by DCF fluorescence using a laser scanning confocal microscope as described in *Materials and Methods*. Representative images of medial sections taken from follicles from three independent experiments are shown. *Bottom*, DCF fluorescence intensity was measured as described in *Materials and Methods*. The N/T follicle group was set as 100%, and the other treatment groups were expressed as percentage of the N/T group. Results are presented as mean ± SEM. *Columns with different superscripts* are significantly different ($P < 0.05$).

oxidant activities. Figure 4A shows that both GSH and MnTBAP inhibit LH-induced caspase-3 cleavage in a dose-dependent manner. Thus, ROS are involved in LH-induced caspase-3 cleavage/activation.

Next, we assessed whether ROS are a by-product of P450scc catalysis in cultured follicles. For these experiments, we used the ROS indicator carboxy-H_2DCFDA, which upon oxidation becomes highly fluorescent (21). Follicles were cultured in the absence or presence of LH, LH plus AGT, LH plus zVAD-fmk (a broad caspase inhibitor), or 5% H_2O_2 (a positive control group). The results show that the most prominent fluorescence was localized to the TIC of the follicle in all treatment groups except for the H_2O_2 group in which prominent fluorescence also appeared in granulosa cells (Fig. 4B, *top*). Addition of LH to cultured follicles resulted in an increase in fluorescence intensity, whereas addition of LH together with AGT significantly inhibited this increase (Fig. 4B, *top* and *bottom*). Thus, P450scc catalysis results in an increase in ROS.

Mitochondrial regulation of apoptosis seems to be intimately linked to generation of ROS (22), and therefore the LH-induced increase in ROS may be the result of caspase activation/apoptosis. To examine this possibility, we used the broad caspase inhibitor zVAD-fmk and found that addition of LH together with zVAD-fmk also reduced the LH-induced increase in ROS levels, albeit to a lesser extent than AGT (Fig. 4B). Thus, the LH-induced increase in ROS is partly due to caspase activity/apoptosis.

Taken together, these results suggest that the LH-induced increase in P450scc catalysis also results in an increase in ROS, which in turn may trigger/facilitate caspase-3 activation because antioxidants inhibit LH-induced caspase-3 cleavage/activity.

Correlation between caspase-3 and -7 activation and progesterone production in CL

The results above demonstrate that LH-induced caspase activation is coupled to steroid production in cultured follicles. To assess whether a similar link exists *in vivo*, we determined the activation state of caspase-3 and -7 in rats injected with eCG plus hCG. Immature rats were injected with eCG to induce follicular growth and 2 d later were either left untreated or injected with hCG to induce ovulation and luteinization. Preovulatory follicles dissected from ovaries after eCG injection and CL dissected from ovaries after eCG plus hCG treatment were collected, and caspase-3 and -7 cleavage/activity was determined. Based on our results in cultured follicles (activation of caspases 24 h after LH administration), we anticipated a similar time course of activation *in vivo*. Western blot analysis and activity assays indicated that caspase-3 and -7 were cleaved/activated only 3 d after hCG injection (Fig. 5A, lane 3, and Fig. 5B; note in Fig. 5A the approximately p20 band that appears in the anti-cleaved caspase-3 panel, which is also increased after hCG and represents a partial cleavage product of caspase-3) (8). Serum progesterone levels revealed a typical elevation in progesterone after hCG treatment (Fig. 5C), which correlated with the increase in caspase activity. Thus, the increase in

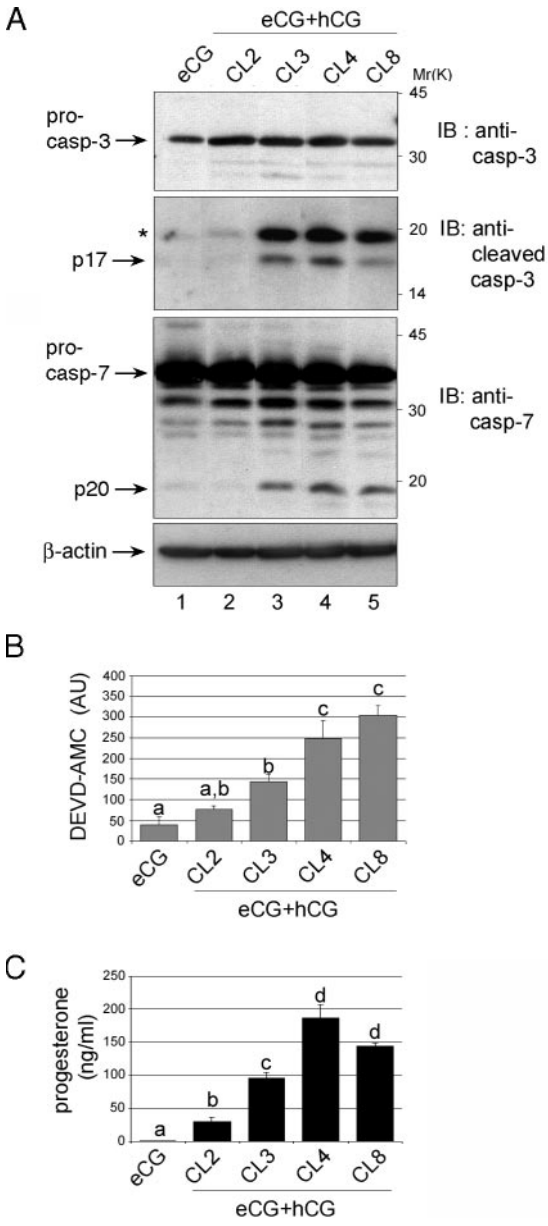

FIG. 5. Correlation between caspase-3 and -7 activation and progesterone production in CL. A, Caspase-3 and -7 are cleaved in functional progesterone-secreting CL. Preovulatory follicles from ovaries of eCG-treated rats or CL from ovaries of eCG-plus-hCG-treated rats [2, 3, 4, or 8 d after hCG injection (CL2, CL3, CL4, and CL8, respectively)] were collected. Follicles or CL were lysed, separated by SDS-PAGE, and analyzed by Western blot using an anti-caspase-3 antibody (*top*), an anti-cleaved caspase-3 antibody (*middle upper panel*), and an anti-caspase-7 antibody (*middle lower panel*). Representative immunoblots of a typical experiment of three are shown together with β-actin as an internal standard (*bottom*). The *asterisk* marks an approximately 20-kDa immunoreactive band, which represents an intermediate cleavage product of caspase-3. B, Caspase-3 and -7 are activated in functional progesterone-secreting CL. Follicles and CL were collected as in A, and caspase-3/-7 activity was assessed using the fluorogenic peptide substrate DEVD-AMC. The results are presented in arbitrary units (AU) as mean ± SEM. *Columns with different superscripts* are significantly different ($P < 0.05$). C, hCG injection induces a typical elevation in progesterone levels. Serum from rats after eCG or eCG plus hCG injection was collected, and progesterone levels were determined by RIA. The data represent mean ± SEM. *Columns with different superscripts* are significantly different ($P < 0.05$).

hCG-induced progesterone production in CL is accompanied by an increase in caspase-3 and -7 cleavage/activity.

The early increase in caspase-3 and -7 cleavage/activity in CL is not accompanied by apoptosis

To examine whether the increase in caspase-3 and -7 cleavage/activity after hCG injection is accompanied by apoptosis, ovaries from rats left untreated or treated with either eCG or eCG plus hCG were immunostained with the anti-cleaved caspase-3 antibody and assessed for apoptosis by TUNEL staining. The results show that positive staining for cleaved caspase-3 is apparent in TIC of follicles and in CL of all stages (Fig. 6A). This positive staining was specific for cleaved caspase-3 because it was completely abolished by preincubation of the anti-cleaved caspase-3 antibody with the peptide used to generate this antibody. Of note, the majority of the positive staining most likely represents the p20 partially cleaved product of caspase-3 because the p20 band is the most abundant band detected in Western blots (see Fig. 5A). Most interestingly, despite the abundance of cleaved caspase-3, TUNEL staining did not detect apoptotic cells in the CL even 8 d after hCG injection (Fig. 6B). To further explore whether the increase in caspase-3 cleavage/activity is ultimately associated with apoptosis in the CL, we examined ovaries taken 14 d after hCG injection. In these ovaries, we detected TUNEL-positive cells in CL and a significant increase in the p17 cleaved/active product of caspase-3 (Fig. 6C, *left* and *right*, respectively). Thus, the increase in caspase-3 cleavage/activity in CL eventually results in apoptosis.

Discussion

The well-balanced response of the ovarian follicle to the combined effect of survival and death factors determines its ultimate fate: death by apoptosis or ovulation. In a previous study, we have discovered that LH, previously shown to be an antiapoptotic factor in follicles, induces caspase-3 and -7 activation that results in apoptosis of TIC but not of GC. In this study, we have investigated the connection between LH-induced caspase activation and steroid production. Using inhibitors of steroidogenesis, AGT and ketoconazole, which act on P450scc at the mitochondria, and epostane, which acts on 3βHSD at the ER, we demonstrated that inhibition of steroidogenesis at the mitochondria, but not at the ER, suppresses LH-induced caspase-3 and -7 activation and apoptosis. The two inhibitors of cytochrome P450-dependent enzymes used, AGT and ketoconazole, are rather efficient but broad acting due to the multiplicity of P450-dependent enzymes (23, 24). Thus, although their effects on inhibition of mitochondrial P450scc have been clearly established, other effects of the inhibitors cannot be ruled out.

We also found that forskolin, a LH-independent stimulator of steroidogenesis induces caspase activation and this activation was inhibited by AGT. Adding back pregnenolone, the product of P450scc catalysis, to follicles treated with AGT did not restore LH-induced caspase activation, suggesting that pregnenolone is not involved in caspase activation. Treatment with antioxidants inhibited LH-induced caspase activation, suggesting that ROS are involved in this process. Examination of ROS levels in follicles indeed revealed that LH treatment elevated ROS levels in TIC of the

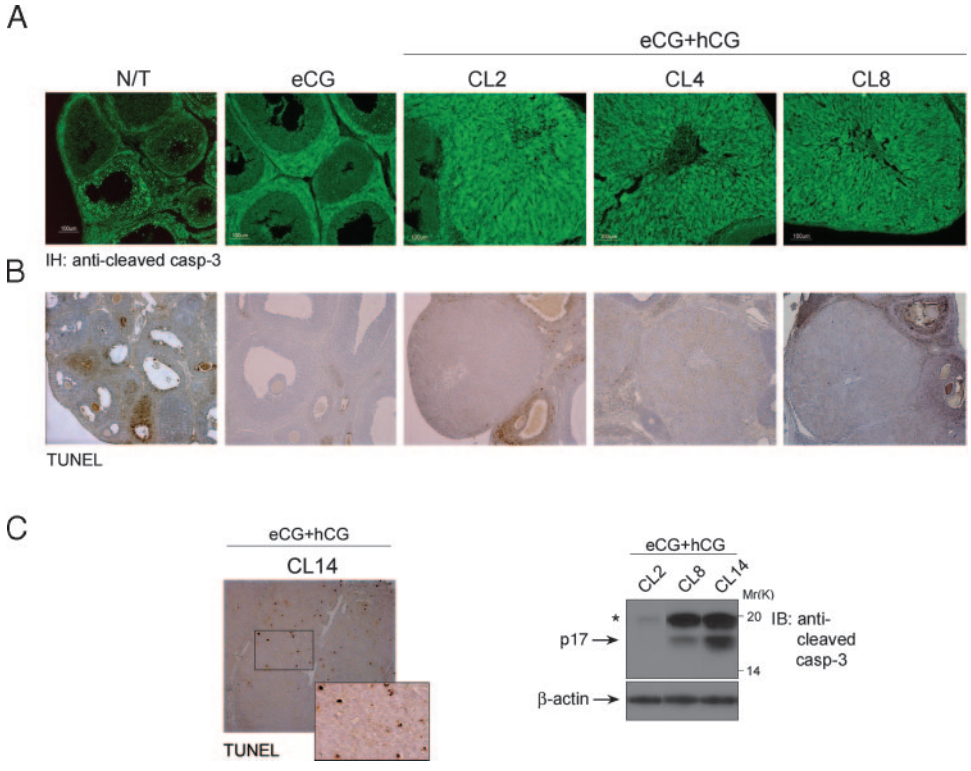

FIG. 6. The early increase in caspase-3 and -7 cleavage/activity in CL is not accompanied by apoptosis. A, Cleaved caspase-3 is apparent in TIC of follicles and in CL of all stages. Immunohistochemical analysis of cleaved caspase-3, using an anti-cleaved caspase-3 antibody, in floating sections of ovaries taken from untreated rats (N/T; note that these ovaries contain apoptotic follicles that are positively stained for cleaved caspase-3 in GC), rats after eCG injection, or rats after eCG plus hCG injections [2, 4, or 8 d after hCG injection (CL2, CL4, and CL8, respectively)]. B, Cleaved caspase-3 staining in TIC and CL is not accompanied by positive TUNEL staining. TUNEL staining was done on ovarian sections taken from rats treated as in A. Original magnification, ×100 for all panels. The slides shown are representative of slides obtained from at least three independent experiments. C, TUNEL-positive cells are detected in CL of ovaries taken 14 d after hCG injection. *Left*, TUNEL staining of an ovarian section taken 14 d after eCG plus hCG injection (CL14; the area marked by a *black square* is enlarged). *Right*, Western blot analysis of CL from ovaries of eCG-plus-hCG-treated rats [2, 8, or 14 d after hCG injection (CL2, CL8, and CL14, respectively)], using an anti-cleaved caspase-3 antibody together with β-actin as an internal standard. The *asterisk* marks the 20-kDa-intermediate cleavage product of caspase-3.

follicle, and this elevation was suppressed by addition of AGT. Thus, P450scc catalysis results in an increase in ROS in TIC, which in turn may trigger/facilitate caspase-3 activation. We have previously shown that both LH and FSH inhibit apoptosis in GC of preovulatory follicles (12), suggesting that gonadotropins may inhibit ROS production in GC. Indeed, a recent study by Tsai-Turton and Luderer (14) has shown production of ROS in GC of cultured follicles, which is suppressed by the addition of FSH via an increase in GSH levels.

Previous reports have demonstrated that apoptosis in rat GC *in vivo* or in culture is accompanied by an increase in the production of progesterone (25, 26). On the other hand, studies using progesterone receptor antagonists demonstrated that progesterone inhibits caspase activation and apoptosis in luteinized rat and human GC (27–29). Thus, P450scc activity may result in a dual effect: an antiapoptotic action through progesterone that serves as a survival factor and a proapoptotic action through production of ROS and subsequently stimulation of caspase activity (Fig. 7). The inhibitory effect of progesterone on caspase activation might limit the caspase-stimulating effect of P450scc catalysis, and the balance between these two actions may ultimately determine the cellular fate.

Our *in vitro* studies show that the LH treatment induces elevation in preovulatory follicles' progesterone production and caspase activity within 24 h. In CL, caspases were activated much later, and apoptosis appeared even later. A likely explanation for the differences between the preovulatory follicles and CL may be related to the changes in the timing of steroid production between the two systems. Indeed, examining the timing of progesterone production in CL showed a substantial elevation in progesterone production only 3 d after hCG injection. This increase was correlated with an increase in caspase activation. The fact that we did not detect apoptotic cells in CL up to 8 d after hCG injection suggests that this increase in caspase activity was not robust enough to induce apoptosis. Moreover, in these CL, the p20 partially cleaved product of caspase-3 is much more abundant than the fully processed/active p17 product. The accumulation of this partially cleaved product suggests that its further processing into the p17 product is under strict regulation.

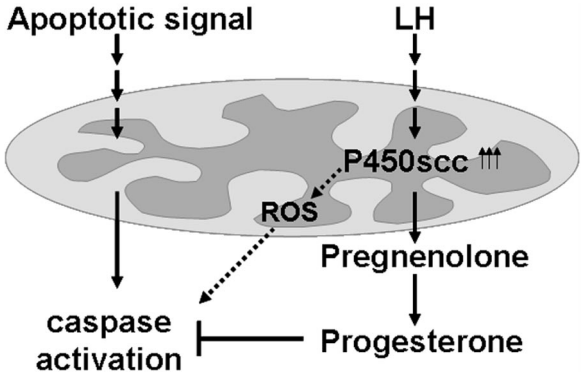

FIG. 7. Schematic representation of a linkage between steroid production and caspase activation via P450scc at the mitochondria. Mitochondria are the site at which LH action on the cell stimulates progesterone production via P450scc and the site at which an apoptotic signal leads to caspase activation. We now show a linkage between these two pathways in which P450scc catalysis causes an increase in ROS, which in turn may trigger/facilitate effector caspase activation (*dotted line*). The inhibitory effect of progesterone on caspase activation might limit the caspase-stimulating effect of P450scc catalysis.

The partial activation of caspases in CL to levels that do not result in apoptosis suggests that there is a mechanism preventing untimely apoptosis/luteolysis. In the ovary, the increase in steroidogenic capacity during the luteal phase is correlated with increased activity of the antioxidant machinery (30). In contrast, luteal regression is associated with accumulation of ROS and a decrease in the levels of antioxidants (30, 31), events that may eventually contribute to a further increase in caspase activity resulting in apoptosis.

In summary, we have demonstrated that LH-induced caspase activation is coupled to steroid production via P450scc catalysis in the rat preovulatory follicle in culture. The fact that hCG-induced progesterone production in CL is accompanied by an increase in caspase activity suggests that the caspase-steroidogenesis link has a functional role *in vivo*.

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