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Research Article

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PKA and AMPK Signaling Pathways Differentially Regulate Luteal Steroidogenesis

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Abbreviations: ACC1, acetyl-coenzyme A carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside; AKT, protein kinase B; AMPK, adenosine monophosphate-activated protein kinase; ANOVA, analysis of variance; BSA, bovine serum albumin; CAMKK2, calcium/calmodulin dependent kinase kinase 2; cAMP, cyclic adenosine monophosphate; CYP11A1, cholesterol side chain cleavage enzyme; ELISA, enzyme-linked immunosorbent assay; FSK, forskolin; HSD3B, 3β-hydroxysteroid dehydrogenase; HSL, hormone-sensitive lipase; LKB1, liver kinase B1; LH, luteinizing hormone;PBS, phosphate-buffered saline; PKA, protein kinase A; SEM, standard error of the mean; STAR, steroidogenic acute regulatory protein

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Abstract

Luteinizing hormone (LH) via protein kinase A (PKA) triggers ovulation and formation of the corpus luteum, which arises from the differentiation of follicular granulosa and theca cells into large and small luteal cells, respectively. The small and large luteal cells produce progesterone, a steroid hormone required for establishment and maintenance of pregnancy. We recently reported on the importance of hormone-sensitive lipase (HSL, also known as LIPE) and lipid droplets for appropriate secretory function of the corpus luteum. These lipid-rich intracellular organelles store cholesteryl esters, which can be hydrolyzed by HSL to provide cholesterol, the main substrate necessary for progesterone synthesis. In the present study, we analyzed dynamic posttranslational modifications of HSL mediated by PKA and AMP-activated protein kinase (AMPK) as well as their effects on steroidogenesis in luteal cells. Our results revealed that AMPK acutely inhibits the stimulatory effects of LH/PKA on progesterone production without reducing levels of STAR, CYP11A1, and HSD3B proteins. Exogenous cholesterol reversed the negative effects of AMPK on LH-stimulated steroidogenesis, suggesting that AMPK regulates cholesterol availability in luteal cells. AMPK evoked inhibitory phosphorylation of HSL (Ser565). In contrast, LH/PKA decreased phosphorylation of AMPK at Thr172, a residue required for its activation. Additionally, LH/PKA increased phosphorylation of HSL at Ser563, which is crucial for enzyme activation, and decreased inhibitory phosphorylation of HSL at Ser565. The findings indicate that LH and AMPK exert opposite

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posttranslational modifications of HSL, presumptively regulating cholesterol availability for steroidogenesis.

Key Words: corpus luteum, luteinizing hormone, lipid droplets, hormone-sensitive lipase, protein kinase, progesterone

The corpus luteum is a transient endocrine gland formed after ovulation from the remnants of a ruptured ovarian follicle (1). The main function of the corpus luteum is production of progesterone, a steroid hormone important for embryo development and preparation of the uterus for implantation (2, 3). Therefore, appropriate function of the corpus luteum is crucial for pregnancy establishment and maintenance (4). In the absence of an embryo, the corpus luteum undergoes regression, a process characterized by decreased production of progesterone and structural luteolysis, ultimately resulting in the formation of a fibrotic scar called the corpus albicans (1, 4). Luteolysis is triggered by prostaglandin $F_{2\alpha}$, produced within the gland or by the endometrium in domestic farm animals (5, 6). In contrast, if fertilization occurs, the presence of live embryos in the uterus extends the lifespan of the corpus luteum due to the luteotrophic and antiluteolytic actions of conceptus signals (4).

The main hormone regulating formation and maintenance of the corpus luteum in many domestic animals, primates and humans is luteinizing hormone (LH) (7), a glycoprotein hormone synthesized by the anterior pituitary gland (8). The corpus luteum arises from the differentiation of follicular granulosa and theca cells into large and small luteal cells, respectively. Luteal cells are classified based on their size, morphological and functional differences (9-11). Compared with the large luteal cells, the small luteal cells of the bovine corpus luteum respond to LH with greater increase in progesterone secretion (10). LH binds to its cognate cell-surface G protein-coupled receptor (the LHCGR) and stimulates adenylyl cyclase, resulting in the production of the second messenger cyclic adenosine monophosphate (cAMP) (12) that activates cAMP-dependent protein kinase A (PKA) (13, 14). LH can also activate phospholipase C, leading to an increase in intracellular calcium and activation of mitogen-activated protein kinases (13, 14). The LH/cAMP/PKA pathway appears to be the main signaling pathway for stimulation of transcriptional changes of steroidogenic, angiogenic, and anti-apoptotic genes in the corpus luteum of many mammals (15-18).

Luteal cells produce prodigious amounts of progesterone, via a series of enzymatic reactions occurring within the mitochondria and endoplasmic reticulum (19). A key step for luteal steroidogenesis is availability of cholesterol, the main substrate for progesterone biosynthesis. Sources of cholesterol for progesterone synthesis include endocytosis of cholesterol-rich low- or high-density lipoproteins (LDL and HDL, respectively), as well as de novo synthesis of cholesterol (19). Another source of cholesterol is found in cytoplasmic lipid droplets, which are abundant in luteal cells (20-22). These cytoplasmic organelles are surrounded by a phospholipid monolayer that coats an inner neutral lipid core consisting of cholesterol esters and triglycerides (22, 23). In steroidogenic tissues, hormone-sensitive lipase (HSL, also known as LIPE) interacts with lipid droplets and mediates the hydrolysis of cholesterol esters providing cholesterol substrate for the immediate synthesis of steroid hormones (24, 25). Unlike other intracellular lipases, HSL activity against triacylglycerol and cholesteryl ester substrates is regulated by reversible phosphorylation (12, 26). Phosphorylation of HSL occurs at multiple sites, including Ser660 and Ser563, which stimulate catalytic activity; and Ser565, which inhibits HSL activity. In steroidogenic tissue, PKA-dependent phosphorylation and activation of HSL increase association of HSL with lipid droplets, where it can hydrolyze cholesterol esters, providing cholesterol substrate for steroid synthesis (27). Recently, we reported that LH/PKA-dependent activation of HSL is the preferred pathway to mobilize cholesterol for steroidogenesis in bovine luteal cells (24).

AMP-activated protein kinase (AMPK), a key regulator of cellular lipid metabolism, is a trimeric protein composed of catalytic α subunits, scaffolding β subunits and regulatory γ subunits (28). AMPK is expressed in ovarian cells, including granulosa and theca cells, oocytes and luteal cells (29-32). AMPK is activated by reductions in intracellular ATP levels (elevating the AMP:ADP/ATP ratio), which allows AMP or ADP to directly bind to the γ regulatory subunits, leading to a conformational change that protects the activating phosphorylation of AMPKα at Thr172 (28). The activating phosphorylation of AMPKα at Thr172 is triggered by upstream kinases, such as liver kinase B1 (LKB1) or in response to calcium flux, independently of LKB1, via calcium/calmodulin dependent kinase kinase 2 (CAMKK2) (28, 33). Conversely, AMPKα Thr172 is dephosphorylated by phosphatases (protein phosphatases 1 and 2) leading to its inactivation (34). Additionally, PKA-dependent phosphorylation of AMPKα at Ser485 inhibits phosphorylation at Thr172, which limits AMPK activity (28). AMPK plays an important role in lipid metabolism by phosphorylating and inhibiting acetyl-coenzyme A carboxylase (ACC1), an enzyme that catalyzes the carboxylation of acetyl-CoA to

malonyl-CoA, the rate-limiting step in fatty acid synthesis (35). AMPK also plays a key role in inhibiting lipolysis in adipose tissue by phosphorylating HSL at Ser565, which prevents PKA-dependent phosphorylation and activation of HSL (28, 36).

Previous studies demonstrate that activation of AMPK inhibits steroidogenesis in ovarian granulosa cells primarily by reducing the ability of trophic hormones like follicle-stimulating hormone (FSH) or LH to stimulate the transcription of genes required for steroidogenesis (37, 38). The role of AMPK in the corpus luteum has received little attention, but 2 reports indicate that activation of AMPK in cultures of dispersed bovine luteal cells reduces progesterone secretion (31, 32). However, the mechanisms of AMPK action on steroidogenesis in luteal cells remain undetermined. Previously, we reported that LH inhibits AMPK activity in the corpus luteum by acutely increasing the phosphorylation of AMPKα at Ser485 and reducing phosphorylation of AMPK at Thr172 (32). Thus, the aims of the present study were to determine: (i) the effects of AMPK activation on luteal progesterone production; (ii) the effects of AMPK on phosphorylation HSL; (iii) the role of LH/PKA signaling on the phosphorylation of AMPKα and HSL in large and small luteal cells; and (iv) the effect of AMPK on LH-stimulated phosphorylation of HSL and progesterone secretion.

Materials and Methods

Reagents

Antibodies used in this report are summarized in Table 1 (39-51). M199 and fetal bovine serum were obtained from Cambrex (Walkersville, MD, USA). Type II collagenase was obtained from Atlantic Biologicals (Lawrenceville, GA, USA). Forskolin was from EMD Chemicals, Inc. (Gibbstown, NJ, USA). Bovine LH was purchased from Tucker Endocrine Research Institute (Atlanta, GA). 5-Aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR) was from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). H89, metformin, were purchased from Tocris Bioscience (Minneapolis, MN, USA). 22-Hydroxycholesterol was purchased from Avanti (Alabaster, Alabama, USA). Protease and phosphatase inhibitor cocktails and Pierce ECL Western Blotting Substrate were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The Protein Assay kit was purchased from Bio-Rad Laboratory (Richmond, CA, USA). Nitrocellulose membrane was purchased from Millipore (Bedford, MA, USA). Progesterone assay was purchased from Diagnostic Systems Laboratories Inc. (Webster, TX). The adenoviruses (Ad) expressing the endogenous

Table 1. Antibodies Used in this Study

Antibody name	Species specificity	Source	Dilution	Catalog number	Company	RRID	Reference
Total HSL	Mouse	Rabbit pAB	1:1000	4107	Cell Signaling Technology (Beverly, MA, USA)	AB_2296900	(39)
phospho-HSL Ser563	Mouse	Rabbit pAB	1:1000	4139	Cell Signaling Technology (Beverly, MA, USA)	AB_2135495	(40)
phospho-HSL Ser565	Mouse	Rabbit pAB	1:1000	4137	Cell Signaling Technology (Beverly, MA, USA)	AB_2135498	(41)
Total AMPK	Mouse	Rabbit pAB	1:1000	2532	Cell Signaling Technology (Beverly, MA, USA)	AB_330331	(42)
phospho-AMPK Thr172	Mouse	Rabbit pAB	1:1000[a], 1:200[b]	2535	Cell Signaling Technology (Beverly, MA, USA)	AB_331250	(43)
phospho-AMPK Ser485	Mouse	Rabbit pAB	1:1000[a], 1:200[b]	4185	Cell Signaling Technology (Beverly, MA, USA)	AB_2169402	(44)
phospho-ACC1 Ser79	Mouse	Rabbit pAB	1:1000	3661	Cell Signaling Technology (Beverly, MA, USA)	AB_330337	(45)
STAR	Mouse	Rabbit pAB	1:10000	ab96637	Abcam (Cambridge, United Kingdom)	AB_10678397	(46)
CYP11A1	Mouse	Rabbit mAB	1:1000	14217	Cell Signaling Technology (Beverly, MA, USA)	AB_2631970	(47)
ACTB	Bovine	Mouse mAB	1:10000	A5441	Sigma Chemical Co. (St. Louis, MO, USA)	AB_476744	(48)
HRP linked	Anti-rabbit		1:10000	1110335003	Jackson Research Laboratory (West Grove, PA, USA)	AB_2313567	(49)
HRP linked	Anti-mouse		1:10000	1150335205	Jackson Research Laboratory (West Grove, PA, USA)	AB_2338513	(50)
Alexa Fluor 594	Anti-rabbit		1:500	A-11032	Invitrogen (Carlsbad, CA, USA)	AB_2534091	(51)

[a]Dilution used for Western blotting
[b]Dilution used for confocal microscopy
Abbreviations: ACC1, acetyl-CoA carboxylase; ACTB, β-actin; AMPK, 5′ AMP-activated protein kinase; HSL, hormone-sensitive lipase.

inhibitor of PKA (Ad.PKI) and control Ad containing an empty cytomegalovirus promoter have been previously described (38). The adenovirus encoding human wild-type AMPK (Ad.WT.AMPK) and dominant-negative HA-tagged AMPKα1 (Ad.dn.AMPK) with a D159A mutation in the ATP-binding domain were purchased from Eton Bioscience (San Diego, CA, USA). The AMPKα1 mutant lacks the capacity to bind ATP and competes with wild-type AMPK for binding with the β and γ subunits. The AMPK adenoviruses and control adenoviruses containing only the cytomegalovirus promoter (Ad-Empty) or encoding green fluorescent protein (Ad-GFP) were used as previously described (52).

Isolation and Culture of Bovine Luteal Cells

Bovine ovaries of early pregnant cows (fetal crown rump length <12 cm) were collected from a local abattoir and transported to the laboratory on ice in cold M199 supplemented with antibiotics. Isolation and culture of bovine luteal cells was performed as previously described (32). The luteal tissue was dissociated with collagenase (1.03 U/mL) and then small and large steroidogenic luteal cells were enriched using centrifugal elutriation as previously described (32). Enriched bovine steroidogenic small and large luteal cells were cultured overnight on tissue culture plates in basal medium (M199 containing 0.1% bovine serum albumin [BSA] and antibiotics) with 5% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2. On the day of experiment, the medium was removed, and cells were rinsed with warm phosphate-buffered saline (PBS). Cells were equilibrated with fresh serum-free basal medium for 2 hours before applying various treatments as described in the figure legends.

Western Blot Analysis

Western blot analysis was performed as previously described (22, 32). Cells were harvested with ice-cold cell lysis buffer or loading buffer, sonicated for 3 seconds, and centrifuged at 14 000*g* for 5 minutes. The protein content of the supernatant was determined using a Bio-Rad protein assay kit. The cell lysates (30 µg protein per lane) were separated on 10% SDS-PAGE and then transferred (100 V, 2 hours) to nitrocellulose membranes. Membranes were blocked in 5% BSA in Tris-buffered saline with Tween (TBST) at room temperature for 1 hour and then incubated with primary antibodies (Table 1) overnight at 4 °C. Membranes were washed with TBST and incubated with anti-mouse (1:10 000) or anti-rabbit HRP-conjugated IgG (1:10 000) for 1 hour. Intensity of specific bands were quantified with a BioSpectrum Imaging System.

Progesterone Enzyme-Linked Immunosorbent Assay

Luteal cells were cultured in 48-well plates and treated as described in the figure legends. Media samples were collected in order to determine progesterone concentration using the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions.

Immunostaining and Confocal Microscopy

Staining and confocal microscopy was performed as previously described (22, 24). Enriched small luteal cells were seeded onto sterile glass coverslips at 5×10^5 cells/well. The next day, the cells were equilibrated for 2 hours in basal medium and then treated with vehicle, AICAR (1mM) for 1 hour or with LH (10 ng/mL) for 30 minutes. The cells were washed with cold PBS, fixed with 4% paraformaldehyde and incubated at 4 °C for 30 minutes. Cells were rinsed with PBS following fixation and then permeabilized by incubation with 0.1% Triton-X in PBST (0.1% tween-20) at room temperature for 10 minutes. The cells were rinsed with PBS, blocked with 5% BSA for 24 hours at 4 °C. Cells were then rinsed with PBS, incubated with primary antibodies for 1 hours at room temperature, rinsed with PBS and then incubated with appropriate secondary antibodies (Table 1) at room temperature for 1 hour. Afterward, cells were rinsed with PBS and coverslips containing labeled cells were mounted to glass microscope slides using Fluoromount-G (Electron Microscopy Sciences). Images were collected using a Zeiss 800 confocal microscope equipped with a 63× oil immersion objective (1.4 N.A.) and acquisition image size of 1024 × 1024 pixel (101.31 µm × 101.31 µm). Cells were randomly selected from each slide and 30 to 45 z-stacked (0.15 µm) images were generated from bottom to top of each experiment. Images were converted to maximum intensity projections and processed utilizing ImageJ analysis software from the National Institutes of Health. Mean fluorescence intensity was determined as previously described (53).

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism v. 8.0 software (GraphPad Software, Inc., San Diego, CA, USA). All experiments were performed at least 3 times using luteal cells obtained from different animals. The data are presented as the means ± standard error of the mean (SEM) of the averages from multiple experiments. Data were analyzed by repeated measures 1- or 2-way analysis of variance (ANOVA) followed by Bonferroni multiple

comparison tests or Student *t* test. Statistical differences were considered as significant at $P < 0.05$.

Results

The AMPK Activator AICAR, But Not Metformin, Inhibits LH-Stimulated Production of Progesterone by Luteal Cells

The expression of AMPK subunits in bovine luteal cells was confirmed previously by us and others (31, 55). Before starting experiments, we determined the homology of the bovine catalytic subunit α of AMPK (AMPKα) and phospho-sites across different species using BLAST. We observed 100% homology of the catalytic AMPKα subunit between cow (*Bos taurus*; NP_001103272.1), human (*Homo sapiens*; AAD43027.1), and rat (*Rattus norvegicus;* NP_062015.2). Additionally, phosphorylation motifs flanking regulatory phosphorylation sites were conserved, that is, Thr172 specific for upstream kinases activating AMPK (ie, LKB1, CAMKK2) and Ser485 specific for kinases inhibiting AMPK (ie, PKA and AKT [protein kinase B]) (Supplemental Fig. 1; available in an online data repository; (54))

Experiments were performed to evaluate the effect of the AMPK activators AICAR (56) and metformin (57) on the phosphorylation of AMPKα (Thr172) and its downstream target ACC1 (Ser79) (Fig. 1A, 1B). Treatment of luteal cells with AICAR rapidly induced ($P < 0.01$) catalytic phosphorylation of the activation loop of AMPKα (Thr172) in comparison with control cells. Simultaneously, the AMPK substrate ACC1 was phosphorylated in cells treated with AICAR ($P < 0.01$) confirming effective activation of AMPK in luteal cells. The AICAR-induced increase in the AMPKα and ACC1 phosphorylation was sustained throughout 120 minutes of incubation. Of note, treatment with AICAR also stimulated a later increase in phosphorylation of AMPKα at Ser485, an inhibitory site (28). In contrast, incubation of luteal cells with metformin evoked a slight, but not significant, increase in the phosphorylation of AMPKα and ACC1 only after longer periods of incubation (Fig. 1A, 1B). Confocal microscopy confirmed elevated content of AMPKα Thr172 (2.9-fold; $P < 0.01$) and Ser485 (1.8-fold; $P < 0.01$) in luteal cells treated with AICAR (1mM). In contrast, treatment of luteal cells with LH (10 ng/mL) decreased phosphorylation of AMPKα Thr172 (mean ± SEM, relative light units [RLU] × 10^6; control vs LH, 1.73 ± 0.11 vs 1.27 ± 0.06, respectively; $P < 0.01$) and increased phosphorylation of AMPKα Ser485 (mean ± SEM, RLU × 10^6; control vs LH, 1.39 ± 0.07 vs 5.44 ± 0.25, respectively; $P < 0.01$), the residue associated with inhibition of AMPK activity, (Fig. 1C).

Previous studies demonstrated that activators of AMPK decreased production of progesterone by granulosa, luteal, and Leydig cells (31, 32, 37, 38). In order to determine the effect of AMPK on LH-stimulated production of progesterone, small luteal cells were pretreated with AICAR (1mM) or metformin (25mM) for 1 hour and then incubated with LH (10 ng/mL) for 4 hours. Metformin did not inhibit LH-induced production of progesterone, while pretreatment with AICAR significantly inhibited ($P < 0.05$) the stimulatory effect of LH on progesterone synthesis by luteal cells (Fig. 1D).

To understand the possible reason for the lack of response of metformin in luteal cells, we analyzed the expression of metformin transporters in follicle cells and luteal cells by mining our previous microarray analysis (GSE83524) (55). Metformin is uptaken or secreted by members of the solute carrier (SLC) family superfamily, including SLC22A1-3, SLC29A4, and SLC47A1-2 (56). Additionally, the expression of multidrug-resistance genes like *ABCB1* (ATP-Binding Cassette Subfamily B Member 1) impairs the accumulation of metformin in cells (58). We found low expression of metformin transporters responsible for its uptake (*SLC22A3*) and higher level of transporters (*SLC47A1*) and multidrug-resistance genes (*ABCB1*, also known as MDR1) responsible for preventing metformin accumulation in small and large luteal cells in comparison to granulosa and theca cells (Fig. 1E). Therefore, further experiments were performed using AICAR as an effective activator of AMPK.

Exogenous Cholesterol Reverses Inhibitory Effects of AMPK on LH-Stimulated Production of Progesterone

AMPK affects steroidogenesis in granulosa and Leydig cells by decreasing the content of steroidogenic enzymes such as steroidogenic acute regulatory protein (STAR), cholesterol side chain cleavage enzyme (CYP11A1) and 3β-hydroxysteroid dehydrogenase (HSD3B) (37, 38, 57). Thus, we analyzed the content of these proteins in luteal cells pretreated for 1 hour with AICAR (1mM) and then stimulated with LH (10 ng/mL) for 4 hours. Activation of AMPK did not change the content of steroidogenic enzymes STAR, CYP11A1, and HSD3B in luteal cells (Fig. 2A). Because cholesterol availability is critical for luteal progesterone synthesis (24), we examined whether provision of an exogenous cholesterol substrate, 22-hydroxycholesterol, would restore progesterone production by luteal cells pretreated with the AMPK activator AICAR. In the absence of 22-hydroxycholesterol, AICAR effectively inhibited the stimulatory action of LH (Fig. 2B). As expected, incubation with 22-hydroxycholesterol

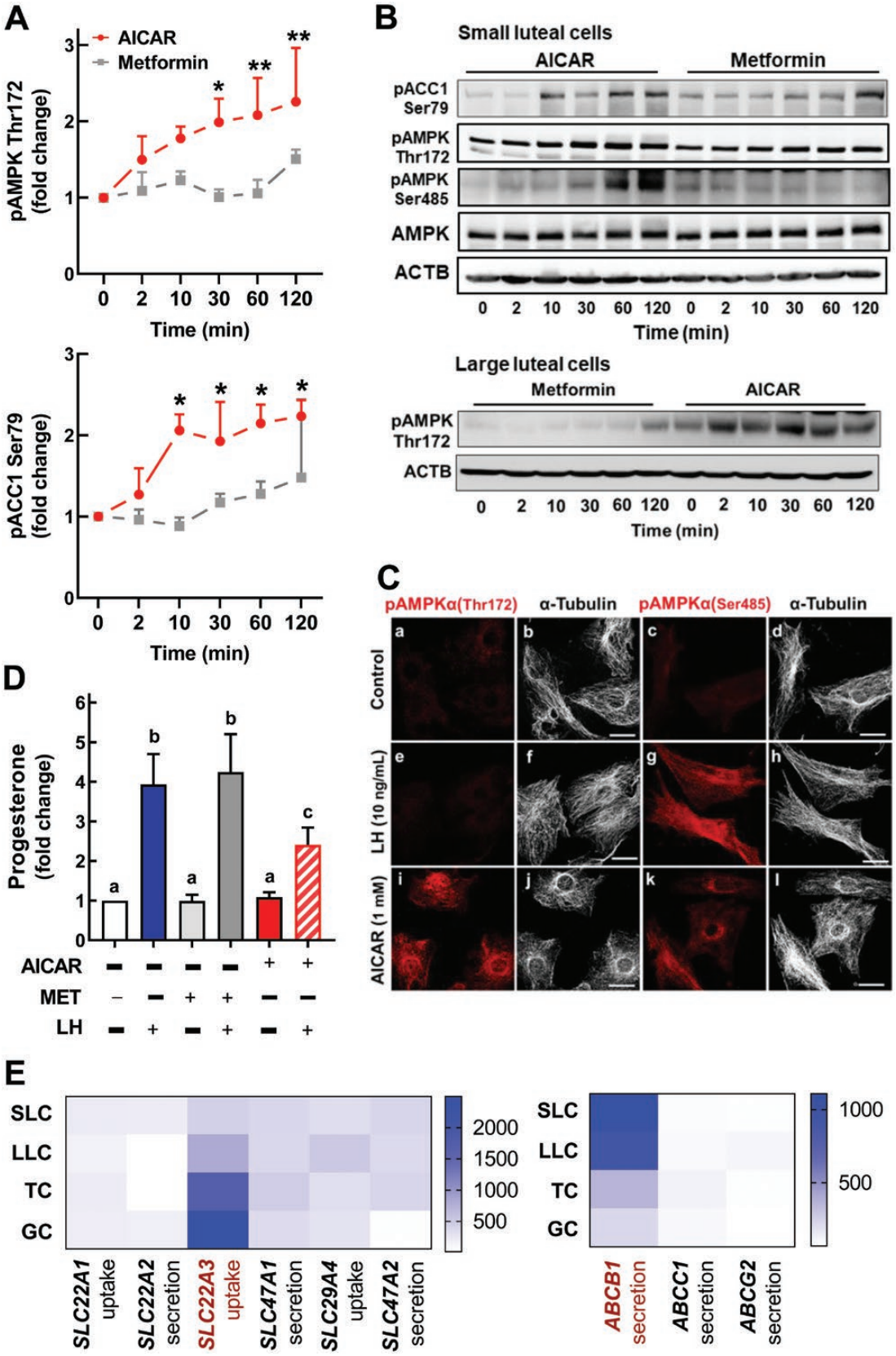

Figure 1. The AMPK activator AICAR, but not metformin, inhibits LH-stimulated production of progesterone by luteal cells. (A-B) Primary cultures of bovine small and large luteal cells were treated with the AMPK activators, AICAR (1mM; ●) or Metformin (25mM; ■) for 2 to 120 minutes. Line graphs (A) and representative immunoblots (B) showing phosphorylation of AMPKα Thr172, AMPKα Ser485, and the AMPK substrate ACC1 Ser79.

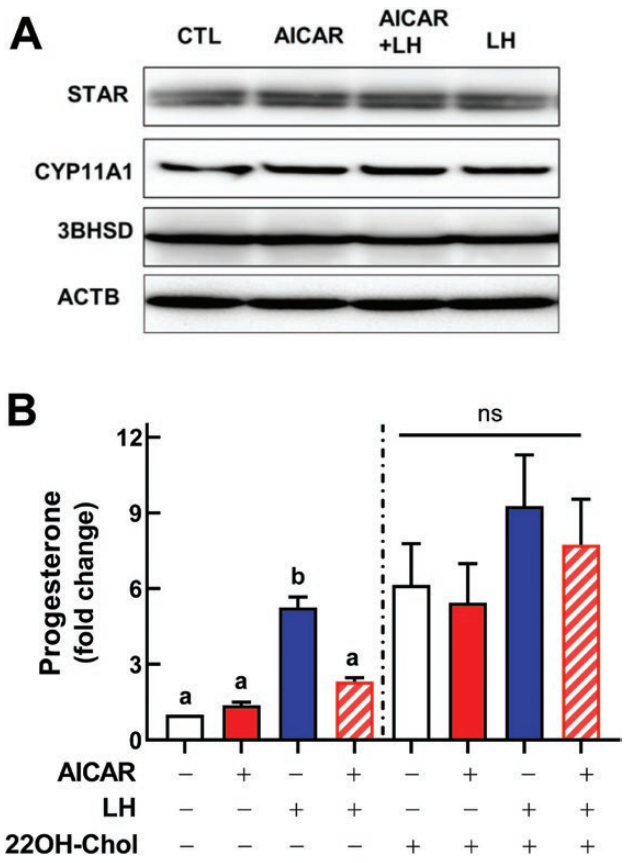

Figure 2. Exogenous cholesterol reverses inhibitory effects of AMPK on LH-stimulated production of progesterone. Panel A: Representative blots showing content of STAR, CYP11A1, and HSD3B in the primary small luteal cells pretreated with AICAR (1mM) for 1 hour and then treated with LH (10 ng/mL) for 4 hours. Panel B: Bovine luteal cells were pretreated with AICAR (1mM) for 1 hour and then treated with or without 22-hydoxycholesterol (22-OH Chol; 15 μM) in the presence or absence of LH (10 ng/mL) for 4 hours. Progesterone measured by ELISA. Data are shown as a fold change and presented as means ± SEM from 4 separate experiments. Statistical analysis was performed using 1-way ANOVA test with post hoc Bonferroni test. Bars with different letters[abc] are significantly different; $P < 0.05$.

elevated basal and LH-stimulated production of progesterone by 6- and 9-fold, respectively. Additionally, treatment with 22-hydroxycholesterol abrogated the inhibitory effect of AICAR on LH-stimulated progesterone production (Fig. 2B). These findings are consistent with an ability of AMPK to inhibit cholesterol availability in luteal cells, thus compromising steroidogenesis. Studies in adipocytes and macrophages indicate that AMPK phosphorylates and inhibits HSL, the enzyme capable of hydrolyzing triglycerides and cholesteryl esters stored in lipid droplets, thus limiting availability of cholesterol and fatty acids (28, 36, 59). Because HSL and cholesterol availability are rate limiting for steroidogenesis (24), further experiments aimed to clarify relationships among PKA, AMPK, and HSL.

AMPK Affects Posttranslational Modification of Enzyme Involved in Lipolysis

Among targets of AMPK are proteins and enzymes that regulate de novo lipogenesis and lipolysis (28, 36). For example, AMPK phosphorylates HSL at Ser565, leading to inactivation of lipolysis (36). BLAST analysis showed that the residues in bovine HSL (*Bos Taurus*; NP_001073689.1) subject to phosphorylation by AMPK and PKA are highly conserved with the HSL sequences of other species (99%-100%) including human (*Homo sapiens*; NP_005348.2), rat (*Rattus norvegicus*; NP_036991.1) and mouse (*Mus musculus*; NP_001034596.1) (Supplemental Fig. 2A; available in an online data repository; (54)).

To confirm whether AMPK triggers inhibitory phosphorylation of HSL, luteal cells were incubated with AICAR (1mM) for 2 to 60 minutes. We observed that treatment with AICAR stimulated time-dependent increases in the phosphorylation of AMPKα (Thr172), ACC1 (Ser79), and HSL (Ser565) (Fig. 3A). AICAR treatment did not alter total AMPK or HSL. The increase in phosphorylation of HSL Ser565 was rapid and persisted for over 60 minutes in small (Fig. 3A) and large luteal cells (Supplemental Fig. 2B; available in an online data repository; (54)).

In mammals, there are 2 genes encoding the AMPKα catalytic subunit (α1 and α2), 2 β genes (β1 and β2), and 3 γ subunit genes (γ1, γ2, and γ3) (28). Our previous microarray data showed the higher expression of AMPKα1 (*PRKAA1*) than AMPKα2 (*PRKAA2*) in small and large luteal cells (55) (Supplemental Fig. 2C; available in an online data repository; (54)). Therefore, further experiments evaluated the role of AMPKα1. Small and large luteal cells were infected for 48 hours with

Figure 1: continued

(C) Representative confocal micrographs of phosphorylated AMPKαThr172 and Ser485 in primary bovine small luteal cells following treatment with control media (panels a-d), LH (10 ng/mL) for 30 minutes (panels e-h), or AICAR (1mM) for 1 hour (panels i-l). **(D)** Small luteal cell cultures were pretreated with AICAR (1mM) or metformin (25mM) for 1 hour and then treated with LH (10 ng/mL) for 4 hours. Progesterone in conditioned media was measured by ELISA. Data are shown as a fold change and presented as means ± SEM from 4 to 7 separate experiments. Statistical analysis was performed using 1-way ANOVA test with post hoc Bonferroni test. **(E)** Heatmaps showing expression of metformin transporters in small luteal cells (SLC), large luteal cells (LLC), granulosa cells (GC), and theca cells (TC). Data were obtained from previous studies (55). Abbreviations: SLC22A1-3 (Solute Carrier Family 22 Member 1-3); SLC29A4 (Solute Carrier Family 29 Member 4; SLC47A1 (Solute Carrier Family 47 Member 1); ABCB1 (ATP Binding Cassette Subfamily B Member 1); ABCC1 (ATP Binding Cassette Subfamily C Member 1); ABCG2 (ATP Binding Cassette Subfamily G Member 2). **Panel A:** Data were normalized to β-actin (ACTB). Significant differences from zero time control are indicated with asterisks; * and ** represent $P < 0.05$ and $P < 0.01$, respectively. **Panel D:** Bars with different letters[abc] are significantly different; $P < 0.05$.

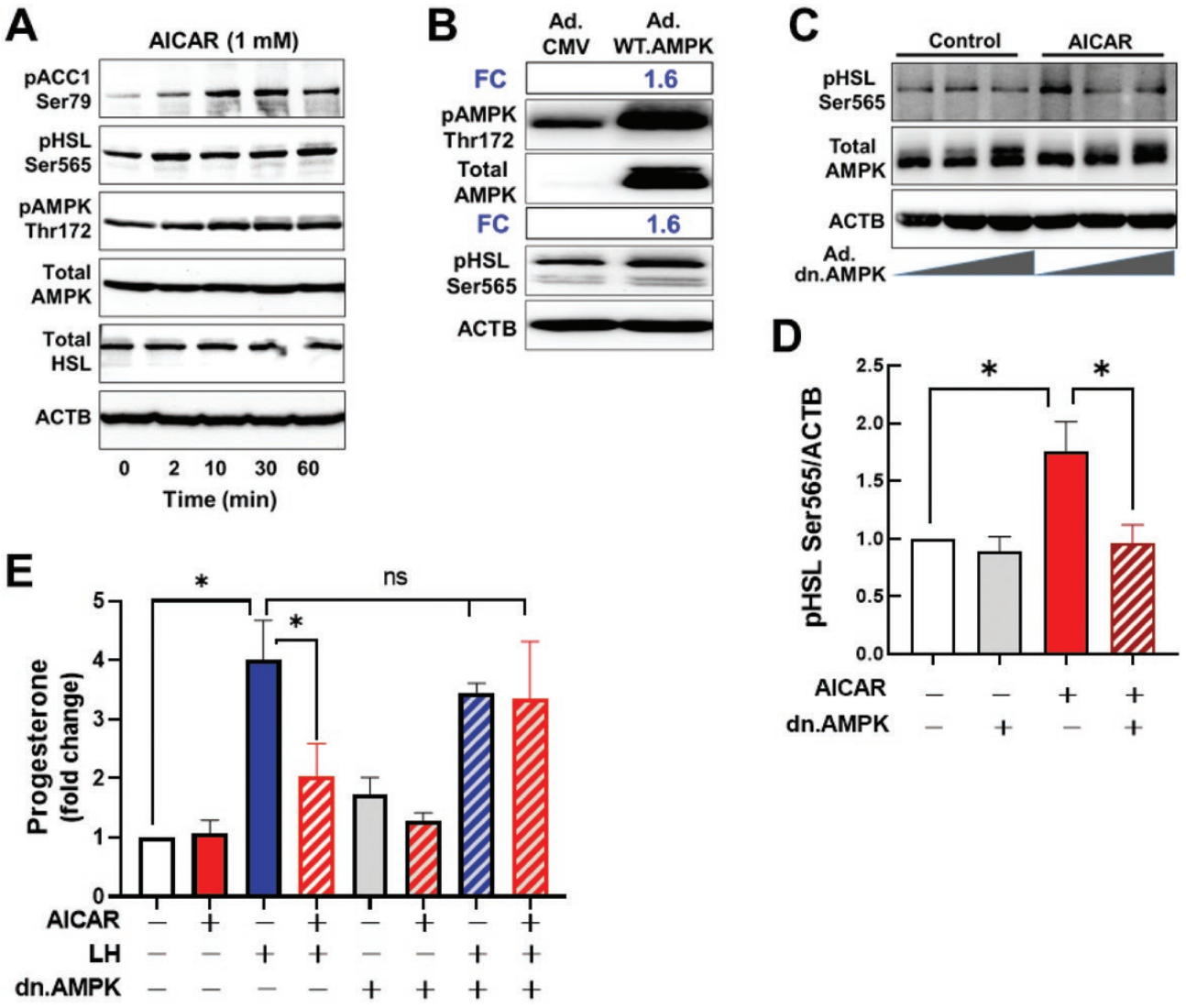

Figure 3. AMPK affects posttranslational modification of enzyme involved in lipolysis. (A) Small luteal cells were treated with AICAR (1mM) for 2, 10, 30, and 60 minutes. Representative Western blots of phosphorylation of AMPKα and downstream targets HSL Ser565 and ACC1 Ser79. Total AMPK, HSL, and β-actin (ACTB) are shown. **(B)** Small luteal cells were infected with control adenovirus Ad.CMV or adenovirus expressing wild-type AMPKα (Ad.WT.AMPK). Representative blots showing levels of phospho-AMPKα (Thr172), phospho-HSL (Ser565), and total AMPKα. **(C-D)** Small luteal cells were infected with control adenovirus (Ad.CMV) or adenovirus expressing dominant negative AMPKα1 (Ad.dn.AMPK) for 48 hours and then treated with AICAR (1mM) for 15 minutes. Representative blots (Panel C) and bar graph (Panel D) showing levels of phospho-HSL Ser565 and total AMPKα. Significant differences between treatments are indicated with asterisks * reflecting $P < 0.05$, n = 4. **(E)** Small luteal cells were infected with Ad.dn.AMPK and then pretreated with AICAR (1mM) for 1 hour followed by incubation with LH (10 ng/mL) for 4 hours. Progesterone was measured by ELISA. **Panels D and E:** Data are shown as a fold change and presented as means ± SEM from 3 to 4 separate experiments. Statistical analysis was performed using 1-way ANOVA test with post hoc Bonferroni test. * $P < 0.05$.

adenoviruses containing (i) control adenovirus (Ad.CMV); (ii) adenovirus expressing wild-type AMPKα (Ad.WT.AMPK) or, (iii) adenovirus expressing a dominant negative AMPKα1 (Ad.dn.AMPK). Afterward, luteal cells were treated with AICAR (1mM) for 15 minutes. The expression of AMPK was confirmed by Western blot for total AMPKα (Fig. 3B, 3C). Levels of phosphorylated AMPKα Thr172 and HSL Ser565 were increased by 1.6-fold in cells transfected with Ad.WT.AMPK in comparison with cells transfected with control plasmid (Fig. 3B). Treatment with Ad.dn.AMPK abolished ($P < 0.05$) the stimulatory effects of AICAR on phosphorylation of HSL Ser565 (Fig. 3C, 3D).

Next, we examined the role of AMPK in the inhibitory effects of AICAR on LH-stimulated production of progesterone in small luteal cells (Fig. 3E). Luteal cells infected with Ad.dn.AMPK had a slight increase in basal progesterone production. Importantly, treatment with Ad.dn.AMPK prevented the inhibitory effect of AICAR on LH-stimulated production of progesterone. These findings

demonstrate that AICAR via AMPK reduces LH-stimulated progesterone synthesis in luteal cells.

LH Affects Phosphorylation of AMPKα and HSL

Because both AMPK and HSL are substrates for PKA, we evaluated LH-dependent phosphorylation of AMPK and HSL in small and large steroidogenic luteal cells. LH provoked concentration-dependent increases in the phosphorylation of HSL on Ser563 in small luteal cells, which were maximal ($P < 0.01$) at 10 ng/mL LH (Fig. 4A). Simultaneously, LH decreased ($P < 0.01$) phosphorylation of HSL at Ser565 in small luteal cells, showing a significant 60% decrease at 3 ng/mL LH ($P < 0.01$). The lowest concentration of LH, 1 ng/mL, significantly increased (3-fold increase, $P < 0.01$) phosphorylation of AMPKα at Ser485, while decreasing ($P < 0.01$) phosphorylation of AMPKα on Thr172 (70% reduction; Fig. 4A). In large luteal cells (Fig. 4B), LH enhanced ($P < 0.05$) phosphorylation of HSL at Ser563 and inhibited ($P < 0.05$) phosphorylation of HSL Ser565 by 70% (Fig. 4B). Concomitantly, the levels of phosphorylated AMPKα Thr172 decreased by 50% (vs. control) in large luteal cells in response to incubation with 1 to 100 ng/mL LH, while levels of AMPKα Ser485 phosphorylation were unchanged (Fig. 4B).

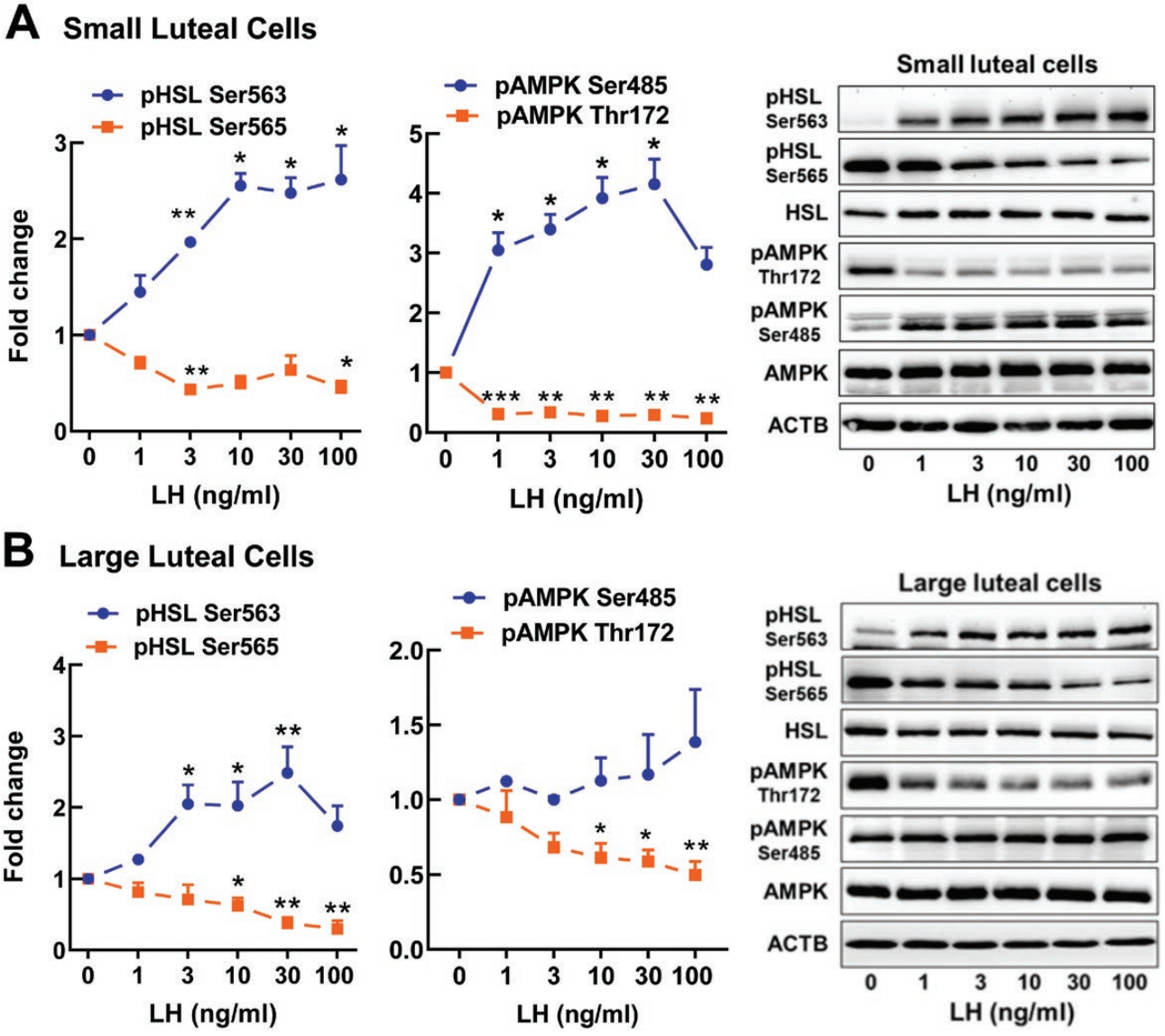

Figure 4. LH affects phosphorylation of AMPKα and HSL. Primary cultures of small luteal cells (**A**) and large luteal cells (**B**) were treated with increasing concentrations of LH (0-100 ng/mL) for 30 minutes. Line graphs and representative blots showing phosphorylation of HSL (Ser563 and 565) and AMPKα (Thr172 and Ser485). **Panels A and B:** Data are shown as the average fold changes ± SEM from 3 to 5 separate experiments. Data were normalized to β-actin (ACTB). Statistical analysis was performed using 1-way ANOVA with post hoc Bonferroni tests. Significant differences between LH concentrations and control are indicated with asterisks *, **, *** reflecting $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.

Activation of the Adenylyl Cyclase/PKA Pathway Mimics the Action of LH on AMPKα and HSL Phosphorylation

Forskolin (FSK), an activator of adenylyl cyclase, was used to determine whether cAMP signaling can trigger phosphorylation of AMPKα and HSL in small and large luteal cells (Fig. 5). Treatment with FSK induced ($P < 0.05$) HSL phosphorylation at Ser563 and reduced ($P < 0.01$) HSL phosphorylation at Ser565, the AMPK specific site. In both luteal cell types, incubation with FSK elevated ($P < 0.01$) the phosphorylation of AMPKα at Ser485 and decreased ($P < 0.01$) phosphorylation of AMPKα Thr172 (Fig. 5A, 5B; Supplemental Fig. 3A, 3B, available in an online data repository; (54)).

To determine whether PKA was involved in the response to LH, small and large luteal cells were infected with a control adenovirus (Ad.CMV) or adenovirus encoding the endogenous inhibitor of PKA (Ad.PKI) (52) and then treated with LH (10 ng/mL) (Fig. 6A; Supplementary Fig. 4A, 4B, available in an online data repository; (54)). Additionally, experiments were performed using a well-studied chemical inhibitor of PKA, H89 (Fig. 6B-6E; Supplementary Fig. 4C-4G, available in an online data repository; (54)). As previously noted, treatment with LH increased ($P < 0.01$) phosphorylation of HSL Ser563 and decreased ($P < 0.001$) phosphorylation of AMPKα at Thr172 in small and large luteal cells (Fig. 6A-6D). LH also stimulated ($P < 0.05$) phosphorylation of AMPKα Ser485 and inhibited ($P < 0.05$) phosphorylation of HSL Ser565 (Fig. 6B, 6C). Treatment with either Ad.PKI or H89 abrogated effects of LH on phosphorylation of HSL (Ser563 and 565) and AMPKα (Thr172 and Ser485) in both types of luteal cells, indicating that the action of LH on the phosphorylation and dephosphorylation of AMPK and HSL is mediated via PKA (Fig. 6A-6D; Supplemental Fig. 4A-4D, available in an online data repository; (54)).

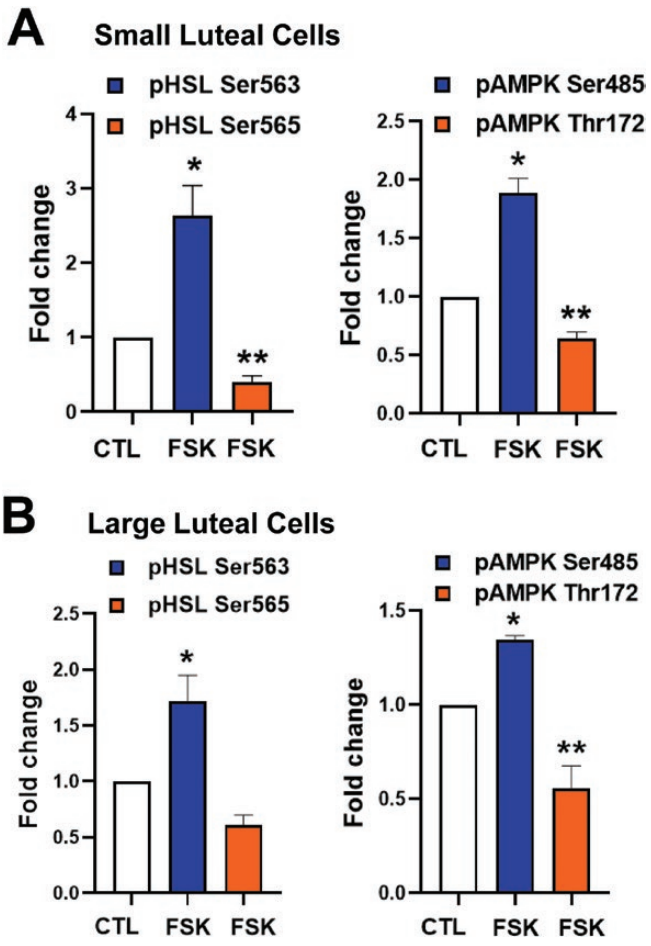

Figure 5. Activation of the adenylyl cyclase/PKA pathway mimics the action of LH on AMPKα and HSL phosphorylation. Cultures of small **(A)** and large luteal cells **(B)** were treated with control medium (CTL) or forskolin (FSK; 10μM) for 30 minutes. Western blot analysis was performed to evaluate phosphorylation of HSL (Ser563 and Ser565) and AMPKα (Thr1712 and Ser485). Data were normalized to β-actin. Bar graphs show average fold changes ± SEM from 3-5 separate experiments. Statistical analysis was performed using 1-way ANOVA test for repeated measurements and Bonferroni post hoc test. Significant differences are indicated with asterisks * and ** representing $P < 0.05$ and $P < 0.01$, respectively.

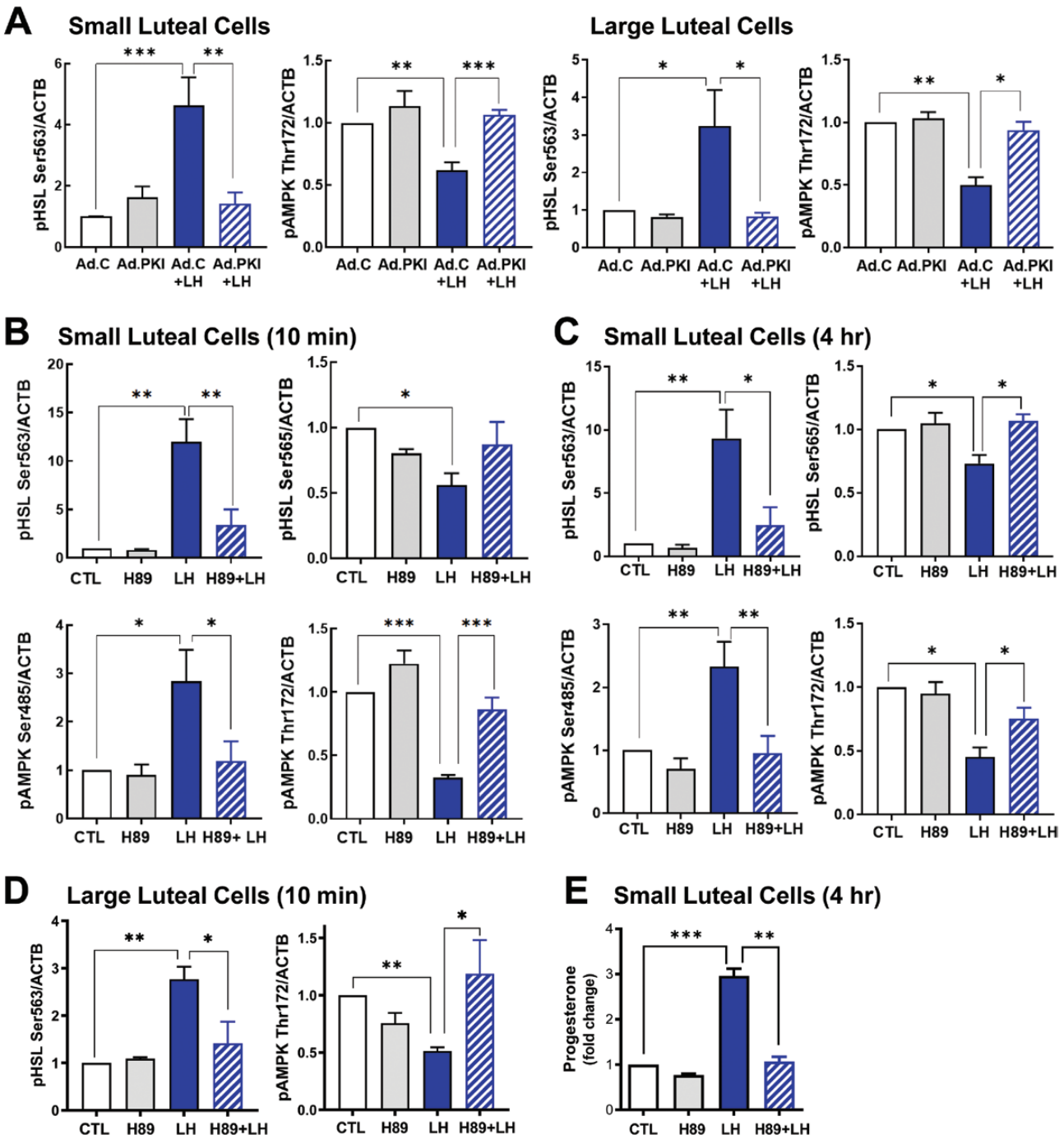

Figure 6. The effect of LH on posttranslational modifications of HSL and AMPK is mediated by protein kinase A. (A) Primary cultures of small and large luteal cells were infected with a control adenovirus (Ad.C) or adenovirus expressing the endogenous inhibitor of PKA (Ad.PKI). After 48 hours, cells were treated with LH (10 ng/mL) for 30 minutes. **(B)** Small luteal cells were pretreated with the PKA inhibitor H89 (30 μM) for 1 hour and then treated with LH (10 ng/mL) for 10 minutes. **(C)** Small luteal cells were pretreated with H89 (30μM) for 1 hour and treated with LH (10 ng/mL) for 4 hours. **(D)** Large luteal cells were pretreated with the PKA inhibitor H89 (30 μM) for 1 hour and then treated with LH (10 ng/mL) for 10 minutes. **(E)** Small luteal cells were pretreated with H89 (30μM) for 1 hour and treated with LH (10 ng/mL) for 4 hours. Media samples were collected for analysis of progesterone using ELISA. Bar graphs represent average fold changes ± SEM from 3-5 separate experiments. Statistical analysis was done using 1-way ANOVA with Bonferroni post hoc test. Significant differences between groups are indicated with asterisks *, **, *** reflecting $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.

LH Stimulates Synthesis of Progesterone Along With Changes in the Phosphorylation of AMPKα and HSL, but Without Influencing the Content of Key Steroidogenic Enzymes

In order to determine whether LH/PKA-mediated effects on progesterone production are related to changes in phosphorylation of HSL and AMPKα, cells were pretreated with H89 for 1 hour and then incubated in the presence of LH (10 ng/mL) for 4 hours. The media were collected to determine concentration of progesterone, and cells were harvested for Western blot analysis. LH stimulated ($P < 0.001$) progesterone synthesis by small and large luteal cells, and pretreatment with H89 blocked ($P < 0.01$) the stimulatory effects of LH on steroidogenesis in both small (Fig. 6E) and large luteal cells (Supplemental Fig. 4E; available in an online data repository; (54)). The inhibition of progesterone was not associated with significant changes in steroidogenic proteins like STAR, HSD3B, and CYP11A1 or levels of HSL or AMPK (Supplemental Fig. 4F-4G; available in an online data repository; (54)). However, at this 4-hour time point, the levels of phosphorylated HSL Ser563 and AMPKα Ser485 remained elevated in response to LH ($P < 0.01$) and levels of phosphorylated AMPKα Thr172 and HSL Ser565 remained suppressed in small luteal cells (Fig. 6C; Supplemental Fig. 4G). Similar effects of LH on HSL Ser563 and AMPKα Thr172 were observed after 4 hours in large luteal cells. These findings support the idea that acute posttranslational changes induced by LH/PKA enhance steroidogenesis in luteal cells.

AMPK Suppresses the Effects of LH on Posttranslational Modifications of HSL

Because activation of AMPK with AICAR suppresses the ability of LH to stimulate progesterone, we examined whether AICAR would suppress LH induced HSL (Ser563) phosphorylation in cultures of small luteal cells. To accomplish this, luteal cells were pretreated in the absence or presence of AICAR and then treated with LH for up to 2 hours. In the absence of AICAR treatment, LH stimulated time-dependent increases in phosphorylation of HSL Ser563, and decreased the phosphorylation of HSL Ser565 and AMPKα Thr172 (Fig. 7; Supplemental Fig. 5; available in an online data repository, (54)). Maximal phosphorylation of HSL (Ser563) in response to LH was observed within 10 minutes and persisted for 120 minutes (Fig. 7). Pretreatment with AICAR abolished the stimulatory effect of LH on HSL phosphorylation and enhanced ($P < 0.01$) phosphorylation of HSL at Ser565 in comparison with cells treated with LH alone. Treatment with AICAR reversed the inhibitory effect of LH on phosphorylation of AMPKα Thr172 ($P < 0.01$). Similar results were observed for large luteal cells (Supplemental Fig. 5; available in an online data repository; (54)).

Discussion

The fundamental role of the corpus luteum is production of progesterone, a steroid hormone required for establishment and maintenance of pregnancy (1-4). The present study provides new insight into the mechanism of action of LH and a potential role of the metabolic rheostat AMPK

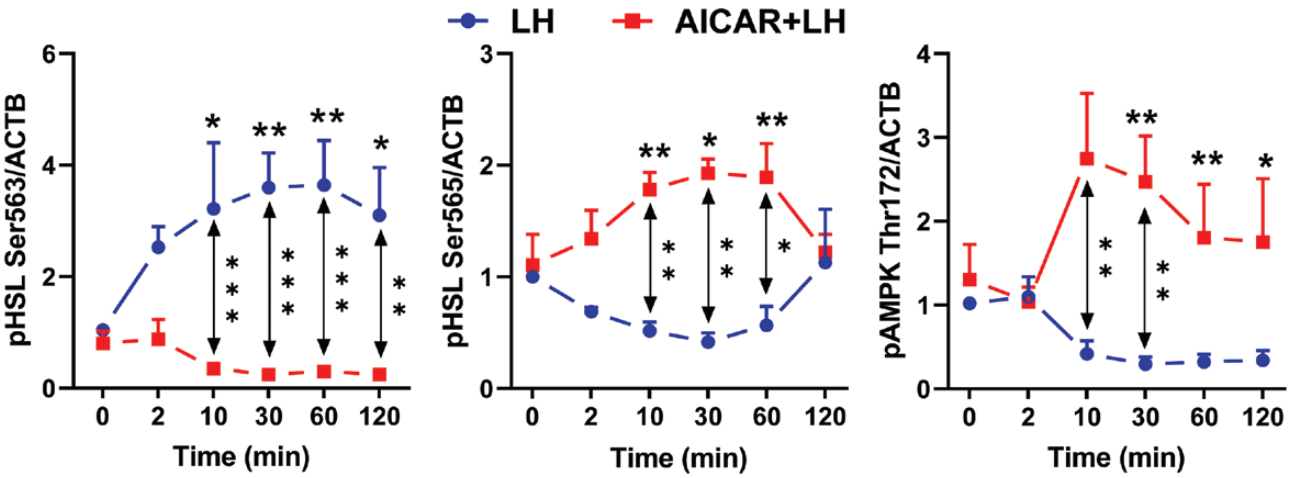

Figure 7. AICAR suppresses the effects of LH on posttranslational modifications of HSL and AMPK. Cultures of small luteal cells were pretreated without (●) or with AICAR (1mM, ■) for 1 hour and then treated with LH (10 ng/mL) for up to 120 minutes. Line graphs showing phosphorylation of HSL Ser563, HSL 565, and AMPKα Thr172 are shown as average fold changes ± SEM from 3 to 5 separate experiments. Statistical analysis was performed using 1-way (LH versus control) or 2-way ANOVA (↔, LH versus LH + AICAR) with post hoc Bonferroni test. Significant differences are indicated with asterisks *, **, and *** as $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

in controlling luteal steroidogenesis. Studies using primary cultures of LH-responsive bovine luteal cells demonstrate that activation of AMPK suppresses the ability of LH to stimulate progesterone synthesis without suppressing levels of STAR, CYP11A1, or HSD3B. The negative effects of AMPK on progesterone production were ameliorated by treatment with 22-hydroxycholesterol, suggesting that AMPK acutely suppresses cholesterol availability, which is rate limiting for luteal progesterone synthesis. Furthermore, the present findings support a role for acute posttranslational regulation of the cholesterol-ester hydrolase HSL in the regulation of luteal progesterone synthesis. Activation of AMPK induces inhibitory phosphorylation of HSL, and LH, via PKA signaling, opposes AMPK by rapidly suppressing activating phosphorylation of AMPK, reducing inhibitory phosphorylation of HSL and inducing HSL phosphorylation on sites associated with HSL activation. This study reveals that LH/PKA signaling and AMPK signaling exert opposing actions on HSL, a key regulator of cholesterol availability for luteal steroidogenesis (24).

In this study, we employed 2 well-known activators of AMPK, AICAR, and metformin. AICAR is taken into cells by adenosine transporters, phosphorylated by the cytosolic enzyme adenosine kinase and then metabolized to form AICAR monophosphate (ZMP), which mimics AMP and activates AMPK (60, 61). The bioavailability of metformin is controlled by transporters belonging to the solute carrier family superfamily and multidrug-resistant transporters of the superfamily of ATP-binding cassette (ABC) transporters (56). Metformin is thought to activate AMPK secondary to the inhibition of complex I of the electron transport chain, leading to reductions in ATP production (56, 58). Our studies demonstrate that AICAR is an effective activator of AMPK in large and small luteal cells as evidenced by elevations in the phosphorylation of AMPKα on Thr172, and increased phosphorylation of the AMPK substrates, ACC1 (Ser79) and HSL (Ser565). In contrast, when compared with AICAR, metformin had little effect on the activation of AMPK in luteal cells. Furthermore, AICAR, but not metformin, inhibited progesterone production in response to LH. The lack of effect of metformin on progesterone production in this study contrasts with previous studies in rat and chicken granulosa cells demonstrating that metformin effectively activated AMPK and reduced the content of main steroidogenic proteins in granulosa cells (37, 57). However, studies conducted with human luteinized granulosa cells also found metformin to be an ineffective activator of AMPK. Accordingly, microarray data indicate that when compared to follicular cells, small and large luteal cells express reduced levels of transporters responsible for metformin uptake (*SLC22A3*) and more transporters responsible for metformin secretion (*ABCB1*) (55). Therefore, it seems that luteinization of ovarian follicle cells to the luteal phenotype may result in ineffective accumulation and action of metformin on AMPK in luteal cells.

Activation of AMPK with AICAR acutely reduced progesterone synthesis in response to LH that was not explained by reductions in STAR, CYP11A1, or HSD3B, the main components required for progesterone synthesis. The findings herein contrast to previous studies using cultures of rat and chicken ovarian granulosa cells showing that AMPK activators reduced gonadotropin-induced expression of genes that control progesterone production (37, 57). Undifferentiated granulosa cells require acute upregulation of transcripts for STAR, CYP11A1, and HSD3B to produce progesterone. The corpus luteum uniquely expresses constitutively high levels of these vital transcripts; and therefore, delivery of cholesterol is rate-limiting for progesterone synthesis. Since the AICAR-induced reduction in progesterone was rapid and occurred without influencing expression of steroidogenic enzymes, we determined whether provision of exogenous cholesterol could overcome this AMPK-induced defect. Indeed, treatment with 22-hydroxycholesterol, a cell-permeable cholesterol analogue, was able to restore the progesterone production by luteal cells. This finding is consistent with the possibility that AICAR-induced AMPK activity limits progesterone production by limiting the ability of LH to regulate cholesterol availability in luteal cells.

Cholesterol for steroidogenesis can be derived from a number of sources, namely, lipoprotein-delivered cholesteryl esters, de novo synthesis of cellular cholesterol, and hydrolysis of cholesteryl esters stored in lipid droplets (19). AMPK can mediate acute posttranslational modifications of proteins that limit synthesis and hydrolysis of lipids (28, 32, 36, 37). Treatment with AICAR increased phosphorylation of AMPKα on its activation site, Thr172, and also stimulated the phosphorylation of 2 AMPK substrates involved in luteal lipid metabolism, ACC1-Ser79 and HSL-Ser565, which inhibit fatty acid synthesis and lipolysis, respectively. Additionally, overexpression of AMPKα enhanced phosphorylation of HSL at Ser565, while transfection with adenovirus inhibiting AMPK prevented the stimulatory effects of AICAR on phosphorylation of HSL Ser565, indicating that HSL is a target of AMPK in luteal cells. In adipocytes, AMPK-mediated phosphorylation of HSL at Ser565 precludes the ability of PKA to phosphorylate HSL on Ser563, a residue required for HSL activation (28, 36). Our findings indicate a similar mechanism may be operational in luteal cells that impacts hydrolysis of lipid droplet cholesteryl esters instead of the triglycerides stored in adipocyte lipid droplets.

The ability of hormones to activate HSL is a primary mechanism to supply cholesterol for steroidogenesis in the adrenal, testis, and ovary (24, 62). HSL hydrolyzes cholesteryl esters stored in lipid droplets, which are prominently expressed in the corpus luteum (22, 24). Similar to the mechanism proposed for adipocytes (36), LH via PKA activates HSL phosphorylation at Ser563 and Ser660 in luteal cells (24). In the present study, we show that LH and forskolin rapidly stimulate phosphorylation of HSL at Ser563, while decreasing phosphorylation on Ser565, an AMPK target for HSL inactivation (28, 36). The reduction in HSL Ser565 phosphorylation is likely due to the suppressive effect of LH on basal AMPK activity (phosphorylation of AMPKα on Thr172) in luteal cells (32). Furthermore, we show that inhibition of PKA eliminates LH-mediated effects on HSL on both residues (Ser563 and Ser565) indicating that LH regulates the phosphorylation of HSL *via* PKA in luteal cells. The effects of LH and PKA on phosphorylation of HSL correlate with requirement for PKA in luteal progesterone, further supporting the role for HSL as an important regulator of luteal steroidogenesis. This idea is supported by our recent studies showing that LH-stimulated progesterone synthesis in bovine small luteal cells is abrogated by a selective small molecule inhibitor of HSL or siRNA knockdown of HSL (24). Results from studies in other steroidogenic cells (26) demonstrate that HSL plays an important role in gonadotropin-stimulated production of progesterone. Further support for a role of HSL in progesterone synthesis is derived from our observations that AICAR-induced activation of AMPK abrogated LH-mediated effects on phosphorylation of HSL and progesterone synthesis. Taken together, these findings suggest that HSL plays a fundamental role in the availability of intracellular cholesterol, which is crucial for steroidogenesis in luteal cells. HSL cleaves cholesteryl esters to produce both cholesterol and fatty acids, and it is likely that fatty acids also contribute to luteal progesterone synthesis via their role in mitochondrial metabolism or de novo lipogenesis. Further studies are required to address these possibilities.

In the present study, we examined the effects of LH and AMPK signaling on progesterone production by small and large steroidogenic luteal cells of the corpus luteum. The bovine luteal cell types are classified based on their size, morphological, and functional differences (10, 11). The main functional differences between small and large luteal cells are that large luteal cells produce 10- to 30-fold more progesterone than small luteal cells, whereas small luteal cells respond better

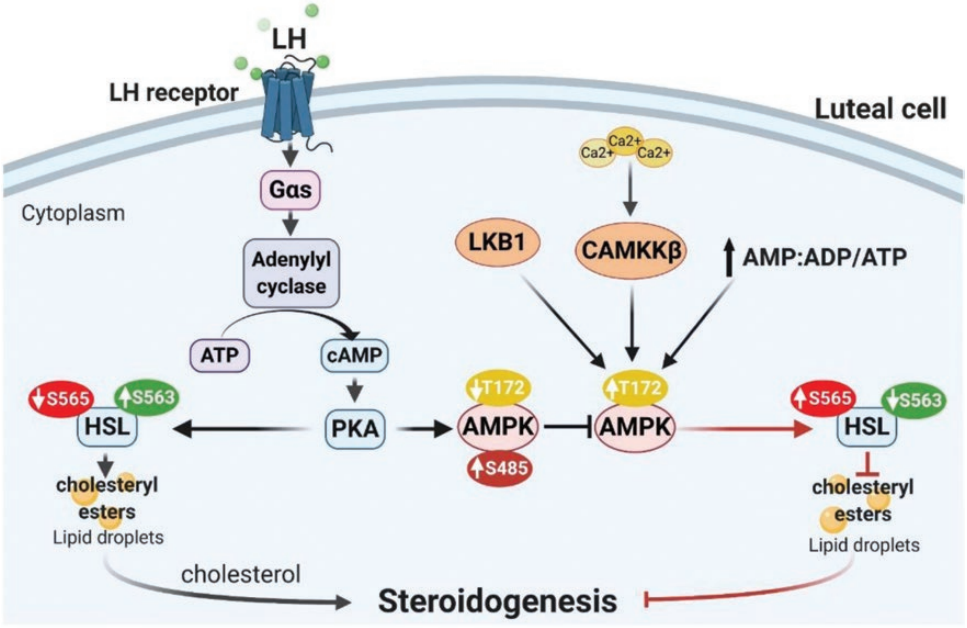

Figure 8. Proposed model of LH action in small and large luteal cells. LH binds to G-protein coupled receptors present on the surface of luteal cells leading to increased activity of protein kinase A (PKA). LH/PKA increases the activity of HSL by triggering phosphorylation of HSL at Ser563 (S563) and reducing phosphorylation at Ser565 (S565). Active HSL translocates to lipid droplets and cleaves cholesteryl esters to release cholesterol for progesterone production by luteal cells. Simultaneously, LH reduces phosphorylation of AMPKα at Thr172 and phosphorylates AMPKα at Ser485 (S485) preventing its phosphorylation at Thr172 (T172) by upstream kinases, ie, calcium/calmodulin-dependent protein kinase kinase 2 (CAMKKβ), liver kinase b1 (LKB1), or increased AMP/ATP ratio. In case of luteolysis, upstream kinases (CAMKK, LKB1) or increased AMP: ATP ratio activate AMPK by triggering its phosphorylation at Thr172. Active AMPK phosphorylates HSL at Ser565, which inhibits phosphorylation at Ser563 leading to inactivation of HSL and preventing hydrolysis of cholesteryl esters stored in lipid droplets and limiting cholesterol availability. Consequently, the elevated activity of AMPK inhibits steroidogenic capacity of luteal cells.

to treatment with LH and PKA activators (9). Herein, we employed large and small luteal cells enriched via centrifugal elutriation (32). We observed that LH and the PKA activator forskolin increased phosphorylation of HSL (Ser563) and decreased phosphorylation of AMPKα (Thr172) in both small and large luteal cells, indicating that the posttranslational modifications are similar in both cell types. In keeping with previous studies (9, 11), the responses to LH and PKA activators were more robust in small luteal cells than large cells. The AMPK activator AICAR also provoked similar responses in both small and large luteal cells, characterized by increased phosphorylation of AMPKα (Thr172) and HSL (Ser565), and reduced progesterone synthesis. Thus, LH/PKA signaling and AMPKα signaling affect opposite posttranslational modifications of HSL and in the small and large luteal cells (Fig. 8).

Elevated phosphorylation of Thr172 on AMPKα subunit directly correlates with enhanced AMPK activity (33). In contrast, elevated phosphorylation of AMPKα at Ser485, triggered by PKA or by AKT, correlates with reduced AMPK activity (28, 32). Mutation of AMPK Ser485 by substitution with aspartate, which mimics constitutive AMPK Ser485 phosphorylation, decreased AMPK activity vascular smooth muscle cells (63). In the present study we observed that LH and forskolin simultaneously increase phosphorylation of AMPK at Ser485 in small and large luteal cells and decrease AMPKα phosphorylation at Thr172, responses that were mediated by PKA. We also observed that AICAR induced phosphorylation of AMPK at Ser485; this response to AICAR has been described as necessary but insufficient for AMPK inhibition or as an autophosphorylation response to limit the activation of AMPK (64). The exact mechanism by which LH via PKA reduces phosphorylation of AMPK Thr172 is unclear but could involve the phosphorylation and activation of a protein phosphatase. Such a mechanism has been reported in rat granulosa cells in which PKA activates protein phosphatase 1 and stimulates the dephosphorylation of serine residues on key regulatory molecules (65). LH via PKA could also inactivate protein kinases, like CaMKK2, upstream of AMPK (66), thus limiting activating phosphorylation of AMPK on Thr172. The exact mechanism by which LH and PKA limit AMPK requires further investigation.

In summary, the results show that LH mediates dynamic posttranslational modifications of key enzymes that control metabolic processes involved in the process of steroidogenesis (Fig. 8). Acute activation of AMPK, a key regulator of cellular metabolism, rapidly induces posttranslational changes in HSL and reduces LH-stimulated progesterone synthesis. Conversely, LH via cAMP/PKA signaling reduces activating posttranslational modifications of AMPK, while stimulating phosphorylation of HSL on residues responsible for activating HSL in small and large steroidogenic cells of the bovine corpus luteum. These findings extend current knowledge about metabolic pathways regulating steroidogenesis in the corpus luteum, but also add information on the role of AMPK and HSL in steroidogenic cells abundant in lipid droplets. Considering the high homology of AMPK and HSL and the phospho-sites triggered by PKA and AMPK in HSL, it is possible that these findings may have translational impact with the advent of small molecules and metabolites that could positively or negatively control progesterone at critical junctures in the reproductive cycle in domestic animals and humans.

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Additional Information

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Data Availability: Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in References. All supplementary materials and figures are located in a digital research materials repository (54).

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