AMP-activated protein kinase activation modulates progesterone secretion in granulosa cells from hen preovulatory follicles

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Abstract

AMP-activated protein kinase (AMPK) is a fuel sensor in glucose, lipid, and cholesterol metabolism. Using RT-PCR and Western blot, AMPK subunits mRNAs (α1/2, β1/2, and γ1/2) and proteins (α1/2 and β1/2) can be found in the hen preovulatory follicles and precisely in both granulosa and theca cells. These preovulatory follicles are organized in a hierarchy according to their size (F5/6 to F1). The smallest number (F1) corresponds to the largest size and the latest mature stage. Phosphorylation of AMPKα on Thr172 and of acetyl-CoA carboxylase on Ser79 are higher in F4 and F3 than in F1 granulosa cells. However, they are not affected in F4–F1 theca cells. Treatment with 1 mM 5-amino-imidazole-4-carboxyamide-1-β-D-ribofuranoside (AICAR), an activator of AMPK, dose dependently increased phosphorylation of AMPKα on Thr172 in primary F3/4 and F1 granulosa cells. In the absence of FSH, AICAR treatment increased progesterone, P450 side chain cleavage and steroidogenic acute regulatory (StAR) production in both F3/4 and F1 granulosa cells. However, in the presence of FSH, AICAR treatment for 36 h increased progesterone secretion, StAR protein levels and reduced extracellular signal-regulated kinase (ERK)1/2 phosphorylation in F3/4 granulosa cells. Opposite data were observed in F1 granulosa cells. Adenovirus-mediated expression of dominant-negative AMPK totally restored the effects of AICAR on FSH-induced progesterone secretion, StAR protein production, and ERK1/2 phosphorylation in F3/4 and F1 granulosa cells. Using a specific inhibitor of ERK1/2 (U0126), we also showed that this kinase is a negative regulator of the FSH-induced progesterone secretion in F3/4 and F1 granulosa cells, suggesting that AICAR-mediated AMPK activation modifies FSH-induced progesterone secretion differently through the ERK1/2 signaling pathway in hen F3/4 and F1 granulosa cells.

Journal of Endocrinology (2006) **190,** 85–97

Introduction

The ovary of the reproductively active hen represents an interesting model for studying follicular development. It consists of small prehierarchical and maturing preovulatory follicles showing a hierarchy according to their size (F5/6 to F1; Etches & Petitte 1990). Depending on hen breed, ovulations may occur almost every day; the largest follicle (F1), filled with yolk, is to be ovulated first; then the second largest (F2) the following day, and so on until a pause interrupts the sequence of ovipositions generating multiple and successive series of ovipositions. These preovulatory follicles grow very quickly; for example, the F1 follicle weight can be six- to eight-fold higher than the F5 follicle weight (cited by Asem & Hertelendy 1985). In the hen layer ovary, as in mammals, theca and granulosa cells can be easily isolated from developing follicles. The granulosa cells from the largest follicles produce large amounts of progesterone, but unlike the mammalian granulosa cells, no estrogen (Hammond *et al.* 1978, Huang *et al.* 1979, Marrone & Hertelendy 1983). Indeed, avian estrogens are synthesized only in theca cells. During the rapid growth phase of chicken preovulatory follicles, DNA synthesis and steroidogenesis in granulosa cells are finely regulated by numerous factors including hormones (luteinizing hormone (LH) and follicle-stimulating hormone (FSH); Bahr & Johnson 1984) and growth factors such as insulin-like growth factor-I (IGF-I; Onagbesan *et al.* 1999). However, the precise mechanisms governing the rapid growth of follicles and their filling up with lipoproteins, according to the hierarchy, are poorly understood. Thus, we investigated the presence of AMP-activated protein kinase (AMPK) in the hen ovary and its potential changes during follicle maturation.

AMPK is involved in the regulation of cellular energy homeostasis; it regulates the expression and activity of enzymes involved in the synthesis of fatty acid, cholesterol, and glycogen (Carling *et al.* 1987). Recent evidence suggests that AMPK is also a link between the sensing of intracellular energy levels and the regulation of protein synthesis (Bolster *et al.* 2002). AMPK is activated by some conditions that

DOI: 10.1677/joe.1.06828
0022–0795/06/0190–085
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deplete cellular ATP (Corton *et al.* 1994). Thus, under extreme nutritional conditions, AMPK responds to increases in the AMP/ATP ratio by switching off ATP-consuming pathways and switching on pathways for ATP generation (Unger 2004). AMPK is a heterotrimer comprising one α-catalytic subunit, one β-regulatory subunit and one γ-regulatory subunit (Woods *et al.* 1996, Stapleton *et al.* 1997). In mammals, there are multiple isoforms of each subunit encoded by separate genes (α1, α2, β1, β2, γ1, γ2, and γ3) (Hardie 2003, Carling 2004). AMPK activity depends upon phosphorylation of the Thr172 residue in the activation loop of the α-subunit by an upstream kinase named LKB1, (Hawley *et al.* 1996) and both phosphorylation and dephosphorylation processes are sensitive to AMP (Davies *et al.* 1995). The adenosine analog, 5-amino-imidazole-4-carboxyamide-1-β-D-ribofuranoside (AICAR), has been largely used as an activator of the AMPK system (Corton *et al.* 1995). In mammals, the metabolic effects of AICAR have been studied extensively in skeletal muscle, adipose tissue, and liver. Recently, the presence of AMPK and the effects of AICAR have been investigated in rodent ovary, both in oocytes (Downs *et al.* 2002) and granulosa cells (Tosca *et al.* 2005). In mouse oocytes, AMPK may play a key role in meiotic induction (Downs *et al.* 2002, Larosa & Downs 2006). In rat granulosa cells, we have demonstrated that AMPK activation reduces basal and FSH- and/or IGF-I-induced progesterone secretion (Tosca *et al.* 2005). The AMPK system has been investigated very recently in chicken. Chicken LKB1 and AMPK subunits have been totally or partially sequenced showing a very high homology at the amino acid level compared with their human homologs (Proszkowiec-Weglarz *et al.* 2006). AMPK subunits are expressed in various chicken tissues (Proszkowiec-Weglarz *et al.* 2006); however, chicken ovary was not included in this study.

The objectives of the present work were to identify and characterize the expression and activity of AMPK *in vivo* in hen preovulatory follicles during their development. We also examined the potential role of AMPK in hen granulosa differentiation in response to FSH by using AICAR and an adenovirus mediated expression of dominant-negative AMPK in cultured F3/4 and F1 granulosa cells.

Materials and Methods

Hormones and reagents

Purified ovine FSH-20 (oFSH) (lot no. AFP-7028D, 4453 IU/mg, FSH activity = 175 times activity of oFSH-S1) was used for culture treatment (a gift from NIDDK, National Hormone Pituitary Program, Bethesda, MD, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were purchased from Invitrogen, and methyl-[^{3}H]thymidine from Perkin Elmer Life and Technological Sciences (Boston, MA, USA). AICAR was obtained from Sigma.

Antibodies

Rabbit polyclonal antibodies to phospho-extracellular signal-regulated kinase (ERK)1/2 (Thr202/Tyr204), phospho-AMPKα Thr172, and acetyl-CoA carboxylase (ACC) were purchased from New England Biolabs, Inc. (Beverly, MA, USA). Rabbit polyclonal antibodies to ERK2 (C14) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-actin antibodies were obtained from Oncogene Research (Boston, MA, USA). Rabbit polyclonal antibodies to AMPKα1/2 (α-pan AMPK antibody recognizes AMPKα1 and α2 at 63 kDa), AMPKβ (recognizes AMPKβ1 (40 kDa) and β2 (34 kDa)) and phospho-ACC (pACC) Ser79 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Monoclonal antibody to green fluorescent protein (GFP) was obtained from Clontech. Rabbit polyclonal antibodies to P450 side chain cleavage (P450scc), steroidogenic acute regulatory (StAR) protein, and 3β-hydroxysteroid dehydrogenase (HSD3β) were generously provided by Dr Dale Buchanan Hales (University of Illinois, Chicago, IL, USA) and Dr Van Luu-The (CHUL Research Center and Laval University, Quebec City, Quebec, Canada) respectively. All antibodies were used at 1:1000 dilution in Western blotting.

Animals, granulosa cells isolation and culture

Laying breed hens (50–60 weeks old) (ISA Brown, egg layer type; Institut de Selection Animale, Saint Brieuc, France) were housed individually in laying batteries with free access to feed and water and were exposed to a 15 h light:9 h darkness photoperiod, with lights-on at midnight. Individual lay patterns were monitored daily. The average clutch size was about 12 eggs. Hens were sacrificed by an overdose of pentobarbital (Sanofi-santé, Libourne, France) between 10 and 12 h before next oviposition and the ovary was immediately removed and placed in ice-cold sterile 1% NaCl saline solution for immediate use. This pentobarbital treatment did not have any significant effect on basal levels of AMPK phosphorylation. All procedures described herein were approved by the Agricultural Agency and the Scientific Research Agency and conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Granulosa cells from F1 and F3/4 follicles were dispersed in 0·3% collagenase type A (Roche) in F12 medium containing 5% fetal bovine serum (FBS). Cells were recovered by centrifugation, washed with fresh medium, and counted in a hemocytometer. The viability of F3/4 or F1 granulosa cells estimated by Trypan Blue exclusion was about 95%. The culture medium was DMEM supplemented with 100 U/ml penicillin, 100 mg/l streptomycin, 3 mmol/l L-glutamine and 5% FBS. The cells were initially cultured for 24 h with no treatment and incubated in fresh culture medium with or without test reagents for the appropriate time. All cultures

were maintained under a water-saturated atmosphere of 95% air/5% CO_2 at 37 °C.

RNA isolation and reverse transcriptase (RT)-PCR

Total RNA was extracted from tissues (ovary and pectoralis muscle) and fresh cells (theca and granulosa cells removed from different preovulatory follicles, F5/6 to F1) using Trizol reagent, according to the manufacturer's procedure (Invitrogen). RNA samples were quantified by measuring the absorbance at 260 nm and treated with DNase to remove any possible DNA contamination. Samples were stored at −80 °C until use. RT-PCR was performed to test the expression of different *ampk* subunits as previously described (Tosca *et al.* 2005). Specific sets of primer pairs designed to amplify parts of the different subunits of *ampk* are shown in Table 1. PCR amplifications without RNA or without reverse transcriptase were performed in parallel as negative controls. RT-PCR disposables were purchased from Sigma, except Moloney murine leukemia virus reverse transcriptase and RNase inhibitor from Promega. All the PCR products were cloned into pCRII-TOPO vector (TOPO TA Cloning kit, Invitrogen), and then sequenced.

Western blot

Lysates of granulosa and theca cells or ovary were solubilized and centrifuged as previously described (Tosca *et al.* 2005). Cell extracts were then submitted to electrophoresis on 10% (w/v) SDS-PAGE under reducing conditions and electrotransferred as previously described (Tosca *et al.* 2005). The membranes were then incubated overnight at 4 °C with appropriate antibodies (final dilution 1:1000) in Tris-buffered saline (TBS, 2 mM Tris–HCl, pH 8, 15 mM NaCl, pH 7·6) containing 0·1% Tween-20 and 5% nonfat dry milk powder (NFDMP). After washing in TBS–Tween-20 0·1%, nitrocellulose membranes were incubated for 2 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulinG (final dilution 1:10 000; Diagnostic Pasteur, Marnes-la-Coquette, France) in TBS–0·1% Tween–20 NFDMP 5%. After washing in TBS–Tween-20 0·1%, the signal was detected by enhanced chemiluminescence (Amersham). The films were analyzed and signals quantified with MacBas V2·52 software (Fuji PhotoFilm, Inc., New Haven, CT, USA).

Thymidine incorporation into granulosa cells

Granulosa cells were seeded in 24-well dishes (2×10^5 viable cells per 500 µl). After 48 h, cells were serum starved overnight and [^{3}H]thymidine (1·5 µCi/ml) was added in the absence or presence of AICAR (1 mM), IGF-I (10^{-8} M) and FSH (10^{-8} M). After 24 h, excess [^{3}H]thymidine was removed by washing twice with PBS; the cells were fixed with ice-cold trichloroacetic acid (50%) and lysed by NaOH (0·5 M). The radioactivity in the cells was determined after resuspension by scintillation counting in a β-photomultiplier. Furthermore, the protein concentration was determined using a colorimetric assay (Kit BC assay, Uptima Interchim, Montluçon, France) to normalize the thymidine incorporation data.

Adenoviruses and infection of hen granulosa cells

Dominant-negative AMPK adenovirus (α1-DN) was constructed from AMPKα1 carrying the Asp157 to Ala (D157A) mutation as described previously (Woods *et al.* 2000). Recombinant adenovirus (Ad) was propagated in HEK293 cells, purified by cesium chloride density centrifugation, and stored as described previously (Woods *et al.* 2000). Hen granulosa cells from preovulatory follicles (F3/4 and F1) were infected with 10 or 100 pfu/cell adenovirus in

Table 1 Oligonucleotide primer sequences

	Sequence	Accession number	Product size (bp)
Primer			
ampk α1 sense	5′ AGT ATG GTC GCA TCT GTA AC 3′	XM 424772	500
Antisense	5′ AGT GTT GGC ACG TGA TCA TC 3′		
ampk α2 sense	5′ GGA ATA CGT ATC TGG CGG TG 3′	XM 426666	450
Antisense	5′ GTG GCA ACG GAG CGG TTG AG 3′		
ampk β1 sense	5′ CCA ACG GTG TTT CGA TGG AC3′	XM 415278	460
Antisense	5′ TGG GCT CAG GAA GCA AAG CT 3′		
ampk β2 sense	5′ TGG GAA ACA CCA CCA GTG AG 3′	XM 424619	810
Antisense	5′ TGG GCT TAT ACA GCA GCG TG 3′		
ampk γ1 sense	5′ GGT GTA CCT GCA GGA CTC CT 3′	DQ133597	600
Antisense	5′ AGG ATA TCC GAG AGG GAG AC3′		
ampk γ2 sense	5′ ATC GGC ATT ACC TGT TGT GG 3′	DQ 212711	300
Antisense	5′ GTG AGT ACC AAA GCC TGT AG 3′		
ampk γ3 sense	5′ ACG CTG GGC AAT GAG ATC CT 3′	DQ 079814	530
Antisense	5′ ATG GTG GAG CCG AAG ATG TG 3′		
actin sense	5′ ACG GAA CCA CAG TTT ATC ATC 3′	D12816	188
Antisense	5′ GTC CCA GTC TTC AAC TAT ACC 3′		

serum-starved DMEM for 24 h and cultured for another 24 h in the presence or absence of FSH and 1 mM AICAR. Preliminary studies revealed that within 24 h of infection (100 pfu/cell) with a GFP-expressing virus, the majority of granulosa cells (>75%) expressed GFP.

Progesterone RIA

The concentration of progesterone (P4) in the culture medium of granulosa cells was measured after 24 or 36 h of culture as indicated in the legend of the figures by an RIA protocol as previously described (Tosca *et al.* 2005) and adapted to measure steroids in cell culture media. The limit of detection of P4 was 12 pg/tube (60 pg/well) and the intra- and interassay coefficients of variation were less than 10 and 11% respectively. Results were expressed as the amount of steroids secreted for 24 or 36 h (as indicated in the legends of the figures) per 100 μg protein.

Statistical analysis

All experimental data are presented as means±S.E.M. One-way ANOVA was used to test differences. If ANOVA revealed significant effects, the means were compared by Newman's test, with $P<0{\cdot}05$ considered significant. Different letters indicate significant differences.

Results

In vivo *AMPK subunit expression and Thr172 AMPKα-phosphorylation in hen preovulatory follicles*

RT-PCR analysis was first performed on total RNA from hen ovary. This resulted in the amplification of different cDNAs corresponding to specific fragments of *ampk* α1, *ampk* α2, *ampk* β1, *ampk* β2, *ampk* γ1, and *ampk* γ2. As shown in Fig. 1a, all these *ampk* subunits were expressed in both theca and granulosa cells from follicles at different preovulatory stages (F5/6 to F1). In contrast, *ampk* γ3 subunit mRNA was undetectable in ovary but present in muscle. By Western blot, AMPKα1/2 (62 kDa), AMPKβ1 (40 kDa), and β2 (34 kDa) proteins were shown to be present in whole hen ovary and precisely in theca and granulosa cells from different preovulatory follicles (Fig. 1b). Furthermore, quantitative analysis indicated no significant difference in the protein level of AMPKα1/2, AMPKβ1, and AMPKβ2 in granulosa and theca cells along with the various preovulatory follicle categories. However, there is a threefold decrease in the phosphorylation level of the α-subunit of AMPK on the Thr172 residue in granulosa cells when comparing F1 follicle to F3 or F4 follicles (Fig. 1c, left panel). The phosphorylation of one of the downstream targets of AMPK, ACC on the Ser79 residue follows a similar pattern (Fig. 1c, middle panel). Interestingly, no difference in the levels of AMPKα Thr172 (Fig. 1c, right panel) and ACC Ser79 phosphorylation (data not shown) was detected in theca cells from F4 to F1 preovulatory follicles. Thus, AMPK is present and active *in vivo* in granulosa and theca cells from preovulatory follicles. Moreover, AMPKα Thr172 phosphorylation decreases in only granulosa cells during the preovulatory follicle development.

AICAR treatment activates AMPK in granulosa cells of hen preovulatory follicles

We next determined whether AMPK can be activated by AICAR *in vitro* in primary F3/4 and F1 granulosa cells. As shown in Fig. 2, the treatment of granulosa cells from preovulatory follicle F1 elicited a Thr172 phosphorylation of AMPKα in a dose-dependent manner. After a 36 h exposure to AICAR, AMPK was phosphorylated maximally (about fourfold) for a 1 mM concentration. Unless otherwise indicated, this concentration will be used in subsequent studies to determine the biological effects of AICAR in granulosa cells. Furthermore, we have observed that AICAR also increased phosphorylation of ACC at Ser79 in a dose-dependent manner, paralleling the stimulation of Thr172 phosphorylation of AMPK (Fig. 2). Similar results were observed in granulosa cells from F3/4 preovulatory follicles (data not shown). Thus, AMPK is active in cultured granulosa cells from preovulatory hen follicles.

Effects of the AICAR treatment on the progesterone secretion in hen granulosa cells from F3/4 and F1 hen preovulatory follicles

We next investigated the effect of AICAR treatment on progesterone production in F3/4 and F1 granulosa cells. As expected (Etches & Duke 1984), in the absence of FSH, progesterone production was lower in F3/4 ($1{\cdot}5\pm0{\cdot}2$ ng/ml per 100 μg protein) than in F1 granulosa cells (39 ± 2 ng/ml per 100 μg protein; Fig. 3a and b). However, FSH-induced progesterone secretion was higher in F3/4 than in F1 (Fig. 3a and b). Indeed, FSH increased by about 20-fold progesterone secretion in F3/4, whereas it increased it by only 1·5-fold in F1 granulosa cells. In the absence of FSH, a 36-h AICAR treatment increased progesterone production by about twofold in F3/4 granulosa cells and by 1·5-fold in F1 granulosa cells ($P<0{\cdot}05$). In the presence of FSH, AICAR treatment increased progesterone secretion by twofold in F3/4 granulosa cells (Fig. 3a). However, AICAR unexpectedly reduced FSH-induced progesterone secretion by about 30% as compared with the FSH treatment alone in F1 granulosa cells (Fig. 3b).

We next examined whether these effects of AICAR on progesterone production were a result of changes in StAR, a crucial cholesterol carrier, and/or of the two key enzymes of steroidogenesis (P450scc and HSD3β). AICAR treatment (1 mM), in the absence of FSH, increased the level of StAR and P450scc proteins by two- to threefold in both F3/4 and F1 granulosa cells, whereas it had no effect on the production of the HSD3β protein (Fig. 3c and d). Interestingly, AICAR

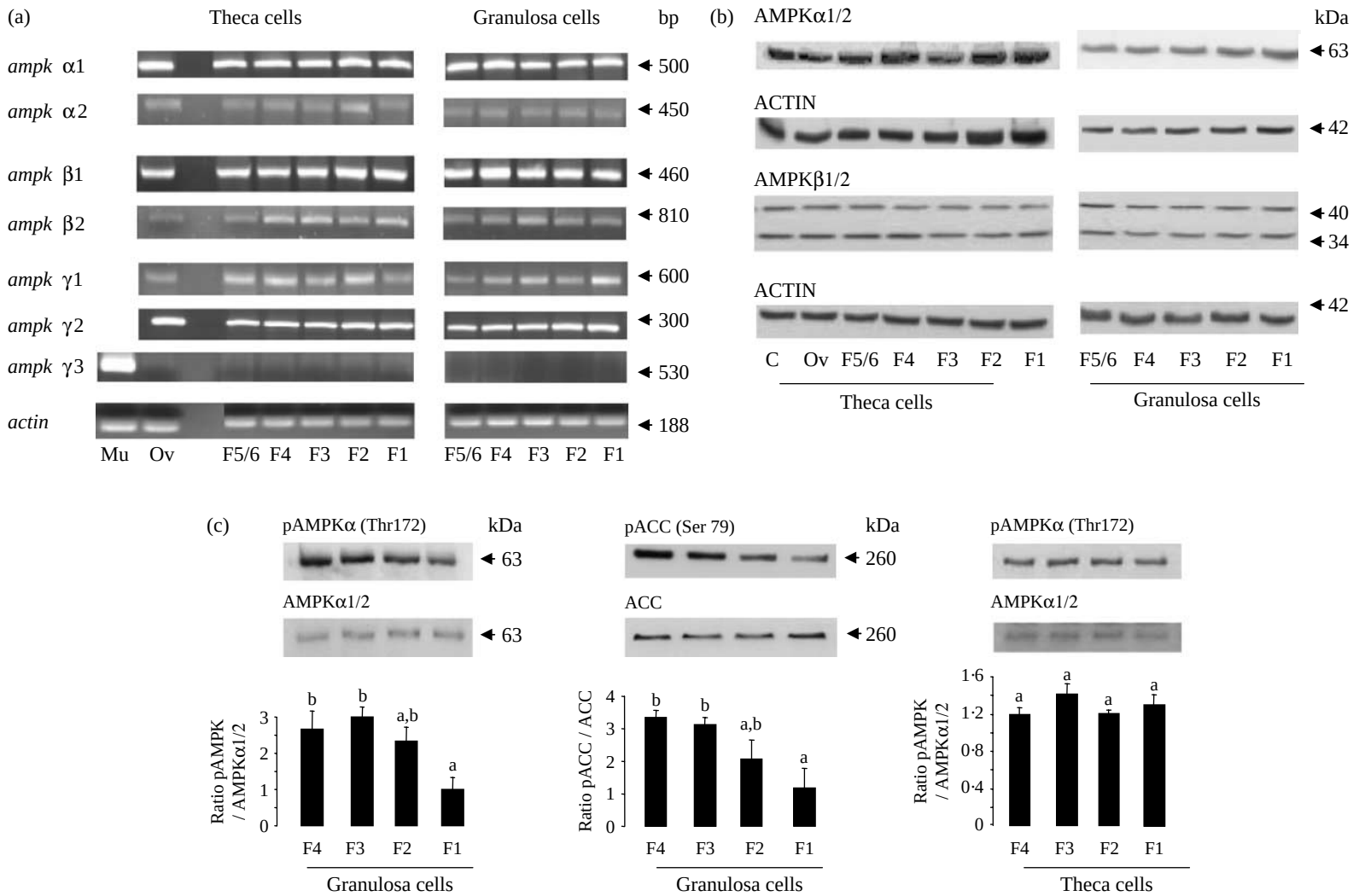

Figure 1 AMPK characterization in hen preovulatory follicles. (a) RT-PCR analysis of the *ampk* regulatory (β1/β2 and γ1/γ2/γ3) and catalytic (α1/α2) mRNA subunits in whole hen ovary (Ov), in theca and granulosa cells from F5/6 to F1 hen preovulatory follicles and in pectoralis muscle (Mu) as a positive control for AMPKγ3. (b) Detection of the AMPKα1/2 and AMPKβ1/2 proteins by immunoblotting in whole hen ovary (Ov), in theca and granulosa cells from F1 to F5/6 hen preovulatory follicles. Actin is used as a loading control ($n=3$). C is a rat ovary sample used as a positive control. (c) AMPKα and ACC phosphorylation in granulosa and theca cells from F4 to F1 preovulatory follicles. Lysates from F4 to F1 granulosa and theca cells were prepared and subjected to Western blotting using anti-phospho-AMPKα-Thr172, anti-AMPKα1/2 (α-pan AMPK antibodies recognize both AMPKα1 and α2 isoforms at 63 kDa), anti-phospho-ACC (pACC) and ACC antibodies. The results are expressed as means ± S.E.M. of the pAMPK/AMPK and pACC/ACC ratios. Different letters indicate significant differences at $P<0{\cdot}05$, $n=6$.

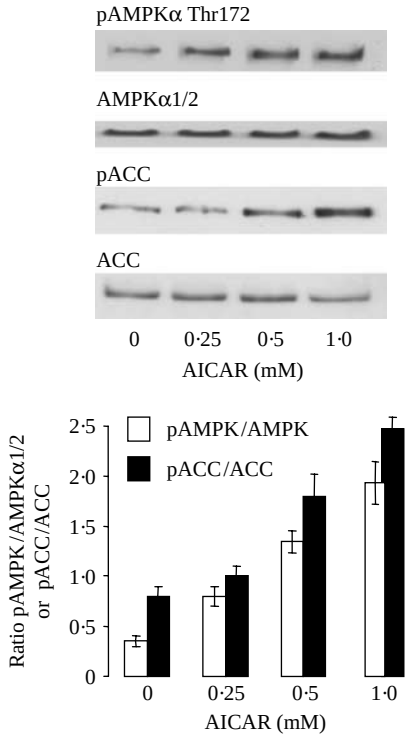

Figure 2 Effect of the AICAR treatment on AMPKα and ACC phosphorylation *in vitro* in cultured F1 granulosa cells. Granulosa cells from hen F1 preovulatory follicles were incubated with the indicated concentrations of AICAR for 36 h. Lysates were then prepared and subjected to Western blotting using pACC-AMPKα-Thr172 and anti-AMPKα1/2 as a protein loading control, and anti-pACC-Ser79 and anti-ACC as a protein loading control. The results are expressed as means ± S.E.M. of the pAMPK/AMPK or pACC/ACC ratio. These results are representative of three independent experiments.

treatment in the presence of FSH increased by about threefold, the content in StAR protein in F3/4 granulosa cells, whereas it decreased by twofold those produced in F1 granulosa as compared with FSH alone (Fig. 3c and d). AICAR treatment increased by almost twofold FSH-induced P450scc production in F1 granulosa cells (Fig. 3d), whereas it had no effect in F3/4 granulosa cells (Fig. 3c). AICAR did not modify the FSH-induced HSD3β protein level in both F3/4 and F1 granulosa cells. Thus, AICAR treatment has parallel effects on progesterone and StAR expressions, but intriguingly, the direction of these effects is opposite when considering F3/4 and F1 granulosa cells.

Overexpression of a dominant-negative AMPKα opposes AICAR effects on FSH-induced progesterone production and STAR protein level in F3/4 and F1 granulosa cells

Although AICAR is widely used as a pharmacological activator of AMPK, AICAR has many different cellular effects (Corton *et al.* 1995, Kemp *et al.* 1999). Therefore, we tested whether the differential effects of AICAR on the response to FSH observed between F3/4 and F1 granulosa cells for progesterone and StAR production were indeed mediated by AMPK. F3/4 and F1 granulosa cells were infected for 24 h with a dominant-negative (α1-DN) form of AMPK or GFP as a control using Ad vectors. α1-DN and GFP proteins were detected in granulosa cells infected with Ad α1-DN or Ad GFP (10 and 100 pfu/cell) for 24 h (data not shown). Furthermore, α1-DN (100 pfu) significantly attenuated basal AMPKα Thr172 phosphorylation in both F1 and F3/4 granulosa cells (data not shown). Infection of granulosa cells with Ad GFP had no effect on AMPKα1/2 expression or AMPKα Thr172 phosphorylation. Expression of α1-DN abolished AICAR-induced increase in the production of both progesterone and StAR protein in response to FSH (24 h) in F3/4 granulosa cells (Fig. 4a). Furthermore, it also abolished AICAR-induced decrease in the production of both progesterone and StAR protein in response to FSH (24 h) in F1 granulosa cells (Fig. 4b). Thus, these data demonstrate that AMPK is involved in the AICAR effects (Fig. 4a and b). Furthermore, we did not observe any significant effect on progesterone production and StAR protein level in response to the α1-DN treatment alone or in combination with FSH (data not shown). Infection of cells with a control GFP virus for 24 h had no effect (data not shown). As revealed by the Trypan Blue staining assay, we did not detect any difference in the viability between cells not infected and cells infected with 100 pfu α1-DN or GFP (data not shown).

Effects of the AICAR treatment on hen granulosa cell number

We also investigated whether the dose of AICAR used affected the number and/or the viability of F3/4 and F1 granulosa cells in culture. [^{3}H]thymidine incorporation by granulosa cells treated with 1 mM AICAR was tested after 24 h in culture in the presence or in the absence of FSH or IGF-I (as a positive control). FSH treatment did not affect the level of [^{3}H]thymidine incorporation, whereas IGF-I treatment used as a positive control did (data not shown). Furthermore, AICAR treatment had no effect either in the absence or in the presence of FSH in both F1 and F3/4 granulosa cells (data not shown). As revealed by the Trypan Blue staining assay, we did not detect any difference in the viability of cells between controls and cells treated with 1 mM AICAR for 36 h (data not shown). Thus, AICAR treatment (1 mM) for 36 h did not affect cell number or viability in F3/4 and F1 granulosa cells.

Role of the ERK1/2 MAPK in the differential effects of AICAR mediated by AMPK on FSH-induced progesterone production in F3/4 and F1 granulosa cells

It has been shown that AICAR can modulate the ERK1/2 signaling pathways in different cell types (Kim *et al.* 2001, Chen *et al.* 2002). In the hen, it has been established that this latter signaling pathway can negatively regulate FSH-induced granulosa cell differentiation *in vitro* (Johnson *et al.* 2001, 2002, Woods *et al.* 2005). Thus, we first determined the

Figure 3 Effect of AICAR treatment on progesterone secretion and StAR, P450scc and HSD3B protein levels in granulosa cells from hen F3/4 and F1 preovulatory follicles. Granulosa cells from hen (a) F3/4 or (b) F1 preovulatory follicles were cultured for 36 h in serum-free medium in the presence or absence of FSH (10^{-8}) and 1 mM AICAR. The culture medium was then collected and analyzed for progesterone content by RIA. Results are means ± S.E.M. of three independent groups of granulosa cells. Different letters indicate significant differences at $P<0{\cdot}05$. Protein extracts of granulosa cells from hen (c) F3/4 or (d) F1 preovulatory follicles were cultured for 36 h in serum-free medium in the presence or absence of FSH (10^{-8}) and 1 mM AICAR and were subjected to SDS-PAGE as described in Materials and Methods. The membranes were incubated with antibodies raised against StAR, P450scc and HSD3B proteins. Equal protein loading was verified by reprobing the membrane with an anti-actin antibody. Results are representative of three independent experiments. Blots were quantified, and the StAR, P450scc, and HSD3B/actin ratios are shown. The results are expressed as means ± S.E.M. Different letters indicate significant differences at $P<0{\cdot}05$.

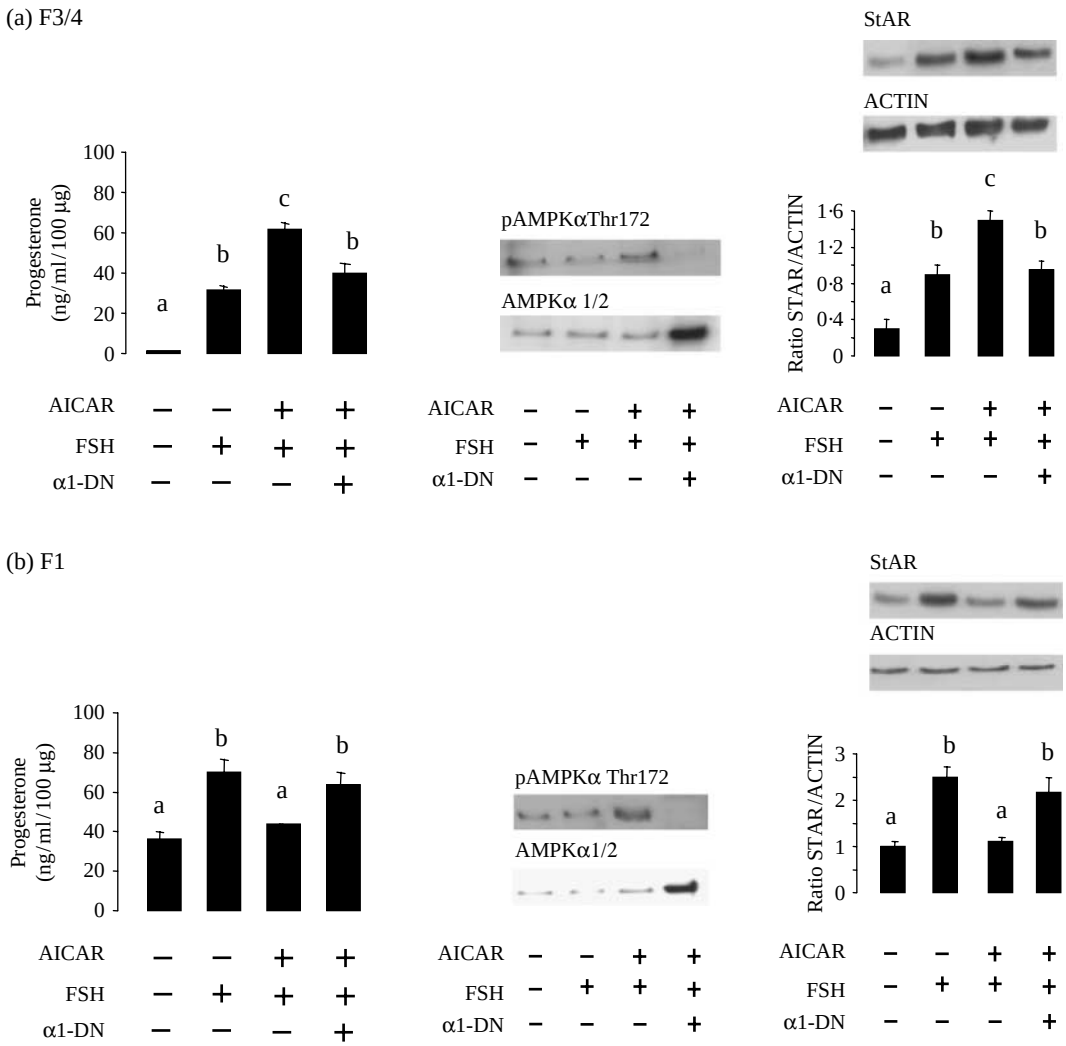

Figure 4 Progesterone secretion and StAR protein level in granulosa cells from hen (a) F3/4 and (b) F1 preovulatory follicles expressing α1-DN in the presence or absence of AICAR and FSH. Granulosa cells from hen (a) F3/4 or (b) F1 preovulatory follicles were infected or not with 100 pfu/cell of α1-DN virus for 24 h and then cultured in the presence or absence of FSH (10^{-8} M) and 1 mM AICAR for another 24 h. The culture medium was then collected and analyzed for progesterone content by RIA, and protein extracts were prepared from cells for immunoblotting with an anti-StAR, anti-actin (as loading control), anti-phospho-AMPKα-Thr172, and anti-AMPKα1/2 antibodies. The data shown represent means ± S.E.M. from three independent experiments of the production of progesterone and the induction of the StAR protein obtained from the ratio StAR/actin. Different letters indicate significant differences at $P<0{\cdot}05$.

ability of AICAR treatment to modulate the activation of the ERK1/2 (Fig. 5a) and, secondly, examined whether this kinase may be involved in mediating the differential effects of AICAR treatment on FSH-induced progesterone production in F3/4 and F1 granulosa cells (Fig. 5b and c). As shown in Fig. 5a, AICAR treatment for 24 h caused a dose-dependent increase in the phosphorylations of ERK1/2 in F1 granulosa cells. Similar results were obtained in F3/4 granulosa cells (data not shown). Consistent with previous reports using chicken cells (Soler *et al.* 1999, Johnson *et al.* 2001), phosphorylated ERK1/2 appeared as a single band (approximately 42 kDa), likely corresponding to ERK2.

AICAR treatment (1 mM) for 24 h also strongly increased ERK1/2 phosphorylation in the basal state (without FSH) in F3/4 and F1 granulosa cells (Fig. 5b and c, left panel). In the presence of FSH, AICAR treatment decreased ERK1/2 phosphorylation in F3/4 granulosa cells, whereas it increased ERK1/2 phosphorylation in F1 granulosa cells. The differential effects of AICAR on ERK1/2 phosphorylation in F3/4 and F1 granulosa cells were abolished by expression of the α1-DN Ad (Fig. 5b and c, right panel), whereas a control GFP virus had no effect (data not shown). Furthermore, the α1-DN treatment alone or in combination with FSH did not have any effects on ERK1/2 phosphorylation (data not shown). The inverse pattern

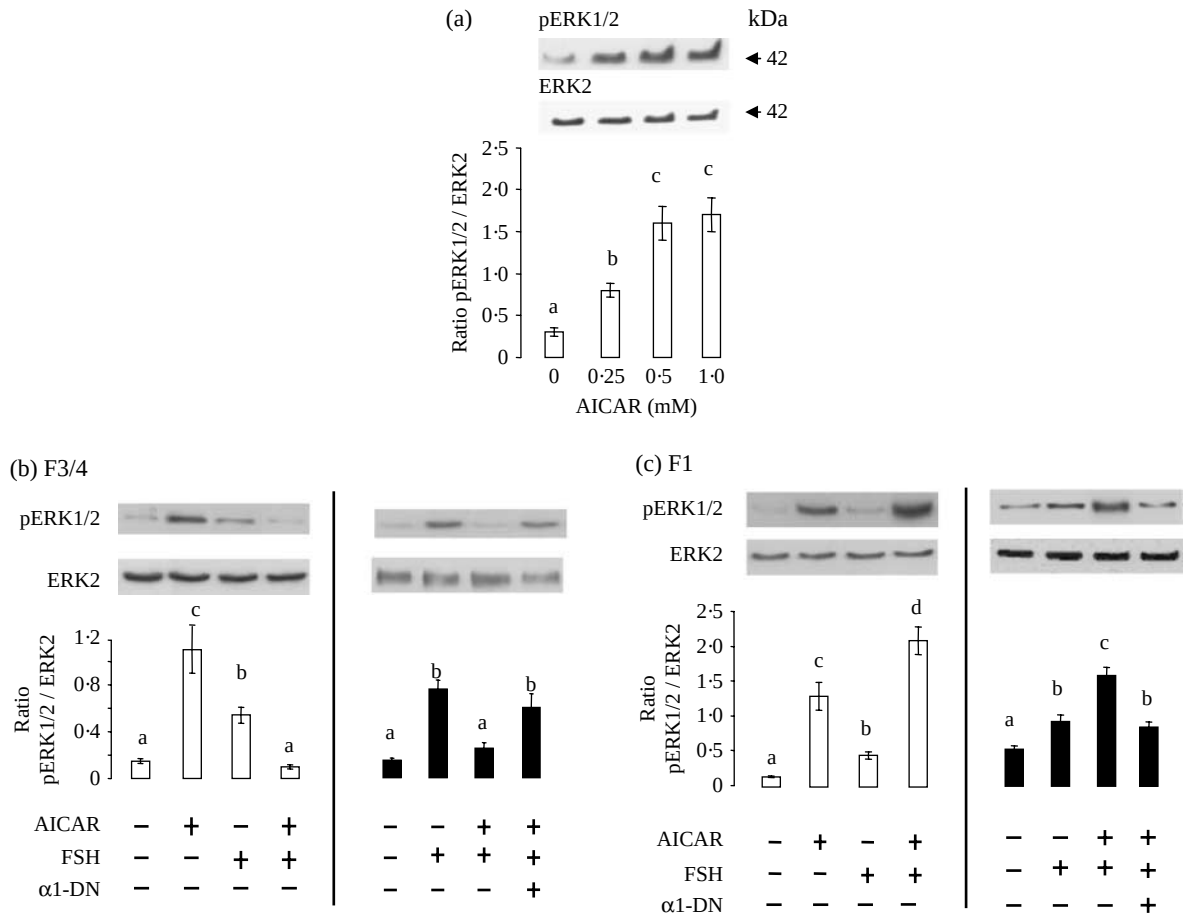

Figure 5 Effect of AICAR treatment (a) alone or in response to FSH on the ERK1/2 phosphorylation in (b) F3/4 and (c) F1 granulosa cells. (a) F1 granulosa cells were incubated with different doses of AICAR for 24 h. (b) F3/4 and (c) F1 granulosa cells were infected or not with 100 pfu/cell of the α1-DN virus and incubated or not with AICAR 1 mM in the presence or in the absence of FSH (10^{-8} M) as indicated in Materials and Methods. Cells were then prepared and the lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-phospho-ERK1/2 antibodies. The blots were then stripped and reprobed with anti-ERK2 antibodies. Representative immunoblots are shown, and the experiment was repeated with similar results on three different samples of lysates. The dividing line between the two halves of each part (b and c) indicates that the results are obtained from two separate immunoblots. Blots were quantified and the ratio phospho-ERK1/2/ERK2 was determined. Results are representative of at least three independent experiments. They are expressed as means ± S.E.M. Different letters indicate significant differences at $P<0{\cdot}05$.

of progesterone secretion and ERK1/2 phosphorylation in our experiments suggests that the former could be regulated by the latter. This was confirmed by showing that the MEK1/2-specific inhibitor U0126, which totally inhibits ERK1/2 phosphorylation (Fig. 6a and b) without altering cell viability increased FSH-induced progesterone secretion by about 3- and 1·3-fold in F3/4 and F1 granulosa cells, respectively (Fig. 6a and b).

Discussion

In this work, we have shown that AMPK is expressed in hen ovary and more precisely in both granulosa and theca cells from the preovulatory follicles (F5/6 to F1). Furthermore, the activation of AMPK as determined by AMPKα Thr172 and ACC Ser79 phosphorylation decreases in granulosa cells, but not in theca cells, during follicular development (F4–F1). AMPK activation decreases FSH-induced progesterone secretion through the ERK1/2 MAPK in primary F1 hen granulosa cells, whereas opposite data are observed in granulosa cells from mixed F3 and F4 preovulatory follicles. This is the first report, to our knowledge, showing that AMPK is expressed in the avian ovary and involved in the regulation of ovarian function in this species.

Different *ampk* subunits (α1, α2, β1, β2, γ1, and γ2) mRNA were detected in both granulosa and theca cells of hen follicles. However, the ampk γ3 subunit mRNA was not found in hen ovary, whereas it was strongly expressed in hen pectoralis muscle. AMPKα1/2 and AMPKβ1 and β2 protein levels were similar in both granulosa and theca cells from follicles at

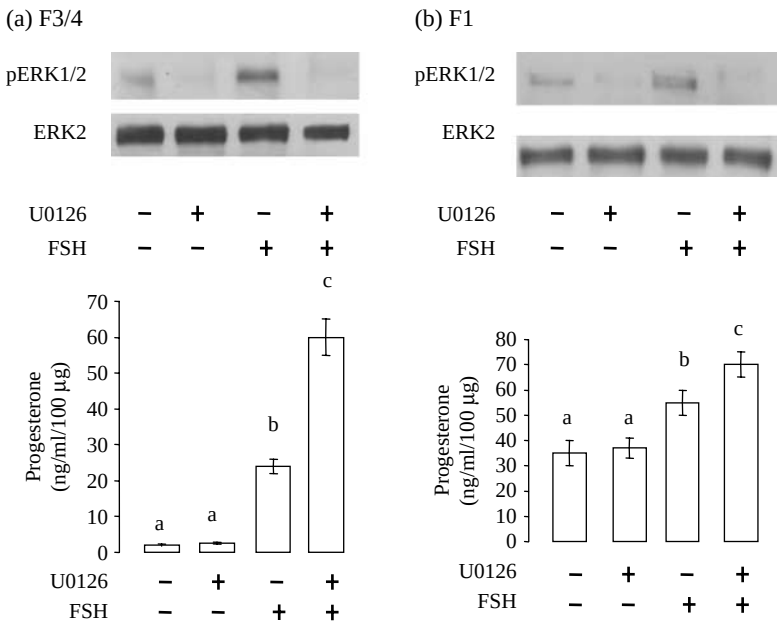

Figure 6 Involvement of the ERK1/2 MAPK on FSH-induced progesterone secretion in granulosa cells from hen (a) F3/4 and (b) F1 preovulatory follicles. (a) F3/4 and (b) F1 granulosa cells were cultured in DMEM medium without serum in the presence or absence of U0126 (50 μM) and in the presence or absence of FSH (10^{-8}) for 24 h. Cells were then prepared and the lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-phospho-ERK1/2 antibodies. The blots were then stripped and reprobed with anti-ERK2 antibodies. The culture medium was then collected and analyzed for progesterone content by RIA. Results are means ± S.E.M. of three groups of F1 and F3/4 granulosa cells. Different letters indicate significant differences at $P<0{\cdot}05$.

different development stages (F4–F1). The fact that AMPKγ3 is specifically expressed in muscle is in agreement with some results recently observed in chicken (Proszkowiec-Weglarz *et al.* 2006) and also in other species, such as human (Cheung *et al.* 2000) and pig (Milan *et al.* 2000). In our study, we demonstrated that the activation of AMPK changes during follicle development in granulosa cells but not in theca cells. Indeed, the levels of AMPKα Thr172 phosphorylation and those of ACC Ser79 phosphorylation decreased in granulosa cells during maturation of yellow preovulatory follicles (F4–F1). AMPK phosphorylates ACC, a rate-limiting enzyme controlling the conversion of acetyl-CoA to malonyl-CoA; this phosphorylation inhibits the activity of ACC and consequently increases the fatty acid oxidation (Merrill *et al.* 1997). Thus, we can speculate that the higher phosphorylation of AMPK Thr172 and ACC Ser79 in F4 and F3 than in F1 granulosa cells can lead to higher fatty acid oxidation and consequently higher energy production in these cells. This energy could contribute to rapid development of chicken ovarian follicles. Indeed, hen follicles contain large amount of lipid as yolk. The lower level of both AMPK and ACC phosphorylation and consequently the higher ACC activity in the help F1 follicle could explain the important lipid storage in the highly mature follicle. However, the avian ovary does not synthesize a lot of lipids, all the lipid components of the yolk are derived from plasma precursors, mainly very low density lipoprotein and vitellogenin produced in the liver (Chan 1983). However, granulosa cells express molecules like apolipoprotein A-I (Apo-AI; Hermann *et al.* 1998) and sterol carrier protein 2 (SCP2; Pfeifer *et al.* 1993) suggesting a role of these cells in the lipid transport. Furthermore, a flow cytometric analysis of steroidogenic organelles in differentiating hen granulosa cells from F6 to F1 suggests a recruitment of lipids into droplets during the differentiation of granulosa cells (Dive *et al.* 1992). Thus, AMPK activation could modulate the local lipid transport from granulosa cells either to theca cells or to the growing oocytes. In mammals, AMPK is regulated by different stimuli (cellular stresses, anti-diabetic drugs, and exercise) and hormones including leptin (Kahn *et al.* 2005). Leptin plays a pivotal role in regulation of energy expenditure (Kahn *et al.* 2005). In mammals, AMPK activation appears to control leptin's effects on fatty acid oxidation in muscle (Minokoshi *et al.* 2002). Thus, some leptin actions on chicken ovarian cells could be mediated through AMPK activation.

Interestingly, we did not detect any variation of AMPK Thr172 and ACC Ser79 phosphorylation in theca cells during follicle development. This differential regulation of AMPK activation between granulosa and theca cells might be partly

due to the specificity of their function. Indeed, unlike mammals, progesterone in chickens is synthesized and secreted mainly by granulosa cells, whereas theca cells generate estradiol (Huang *et al.* 1979). Progesterone produced by granulosa cells from mature follicles provides the positive feedback necessary to stimulate a preovulatory surge of LH (Robinson & Etches 1986). In rat granulosa cells, we have recently shown that AMPK decreases progesterone secretion without affecting estradiol secretion (Tosca *et al.* 2005). Thus, in hen, a variation of AMPK activation in granulosa cells during the follicular hierarchy might also affect ovarian steroidogenesis. That is why we studied the effects of a potent AMPK activator, AICAR, on progesterone secretion and cell growth in both F3/4 and F1 granulosa cells. We pooled granulosa cells from F3 to F4 to isolate enough cells for a culture. As expected, we have observed that in the basal state (no FSH treatment), F3/4 granulosa cells produced less progesterone than F1 granulosa cells. However, the FSH-induced progesterone secretion was higher in F3/4 granulosa cells. These latter data are in good agreement with the literature, since this decline of FSH-stimulated steroidogenesis during follicle maturation is associated with a decrease in the number of FSH receptors (Ritzhaupt & Bahr 1987) and also the adenylate cyclase activity (Asem & Hertelendy 1985). The basal release of progesterone in F1 cells is, however, higher. In our study, we have demonstrated that AICAR treatment alone increases progesterone production and this was associated with an increase in the levels of StAR and P450scc in both F3/4 and F1 granulosa cells (Fig. 7). StAR is an indispensable component of steroidogenesis within preovulatory follicles (Stocco 2001). This protein facilitates the transfer of cholesterol to the inner mitochondrial membrane, where it undergoes conversion to pregnenolone by the P450scc. As in mammals, the conversion of cholesterol to pregnenolone is the rate-limiting enzymatic step within granulosa cells of the hen ovary (Tilly *et al.* 1991). In cultured immature granulosa cells from the diethylstilbestrol-primed rat, AICAR treatment reduces progesterone secretion, probably due to a reduction in the level of the HSD3β protein (Tosca *et al.* 2005). These different AICAR effects observed between rat and hen granulosa cells on progesterone secretion might be explained by the differentiation state of the cells (rat immature vs hen granulosa cells from preovulatory follicle) or by some species specificities.

Similar to the situation in mammals, FSH is proposed to be essential not only for maintaining hen prehierarchical follicular viability (Johnson & Brooks 1996), but also for the initiation of granulosa cell differentiation following follicle selection (Johnson & Bridgham 2001). In our study, we have shown that the AICAR effects on FSH-induced progesterone secretion are opposite between F3/4 and F1 granulosa cells (Fig. 7). Moreover, these results were confirmed using an AMPKα dominant-negative Ad, suggesting that these different effects of AICAR between F3/4 and F1 granulosa cells on progesterone secretion were mediated by AMPK activation. Using a specific MEK1/2 inhibitor, we have also demonstrated that inhibition of the ERK1/2 MAPK leads to an increase in the FSH-induced progesterone secretion in both F3/4 and F1 granulosa cells. This ability of MAP kinase signaling via ERK1/2 to inhibit gonadotropin-stimulated steroidogenesis has been reported in human granulosa-derived cells (Seger

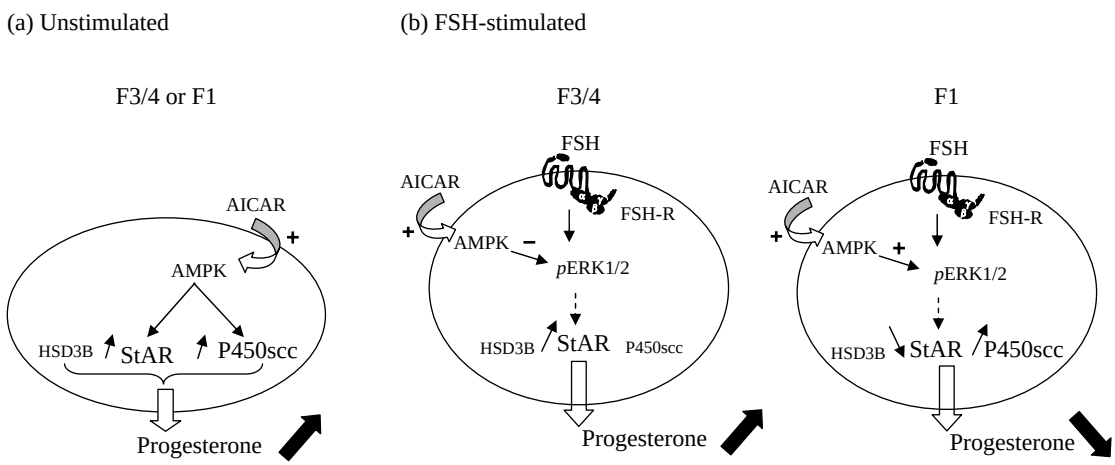

Figure 7 Schematic representation of AMPKα-mediated AICAR effect and the mechanism involved in the (a) basal and (b) FSH-induced progesterone production in hen F3/4 and F1 granulosa cells. (a) In the absence of FSH, AICAR treatment leads to an increase in the StAR and P450scc protein levels and progesterone production in both F3/4 and F1 hen granulosa cells. (b) In the presence of FSH, StAR protein levels, progesterone secretion, and phosphorylation of ERK1/2 are increased in both F3/4 and F1 granulosa cells. AMPK activation induced by AICAR treatment (1 mM) for 24 h increases FSH-induced progesterone secretion by increasing StAR protein levels and reducing phosphorylation of ERK1/2 in F3/4 granulosa cells. Opposite results are observed in F1 granulosa cells. The diagonal directed upward arrows signify increased production/expression. The molecules involved in the progesterone synthesis (P450scc, StAR, and 3βHSD) that are regulated by the AICAR are represented with a larger font. The dotted arrows between pERK1/2 and StAR suggest an effect of the variation of the ERK1/2 phosphorylation in response to AICAR in the presence of FSH on the StAR protein expression.

et al. 2001, Tai *et al.* 2001) and also in cultured prehierarchical follicle hen granulosa cells (Johnson & Bridgham 2001). However, we have recently shown that in rat primary granulosa cells, the U0126 MEK1/2 inhibitor strongly reduced progesterone production (Tosca *et al.* 2005). Thus, the ERK1/2 MAPK appears to be a positive or negative regulator of progesterone secretion in granulosa cells according to the species and/or the differentiation state of the granulosa cells. In the present study, we showed that in F1 granulosa cells, AICAR treatment reduces progesterone secretion and StAR protein levels, but increases ERK1/2 phosphorylation in response to FSH. Furthermore, we have observed opposite results in F3/4 granulosa cells. This opposite regulation of AICAR-mediated AMPK activation between follicles in response to FSH is striking. However, it is well known that granulosa cells have different protein composition during follicular maturation. For example, the number of FSH receptors is decreased in F1 granulosa cells as compared with F3/4 granulosa cells (Ritzhaupt & Bahr *et al.* 1987). Thus, we can speculate that AICAR-induced AMPK activation leads to activation or inhibition of molecules that inhibit or activate the ERK1/2 MAPK and consequently modulates progesterone secretion in response to FSH. Interestingly, we observed that α1-DN treatment alone or in combination with FSH did not have significant effects on progesterone secretion, StAR protein levels and ERK1/2 phosphorylation levels *in vitro* in both F3/4 and F1 granulosa cells. Thus, it appears that *in vitro* in the basal state, AMPK phosphorylation does not strongly affect steroidogenesis. However, these data do not exclude a role of AMPK *in vivo* in granulosa cells.

In conclusion, AMPK is expressed and active in granulosa and theca cells from preovulatory hen follicles. Furthermore, we have shown that AMPK activation *in vivo* is reduced in F1 as compared with F3/4 granulosa cells, suggesting a role of this kinase in hen ovarian steroidogenesis. In the basal state, we have demonstrated that AICAR treatment increases *in vitro* progesterone secretion in both F3/4 and F1 primary granulosa cells. Furthermore, in response to FSH, AICAR-mediated AMPK activation increases progesterone production and StAR protein levels, whereas it decreases ERK1/2 phosphorylation in F3/4 granulosa cells. Opposite results are observed in F1 granulosa cells. Since FSH is a key hormone in follicular maturation, we can speculate that the activation of AMPK in response to different stresses, such as a variation in the nutritional status, might lead to a modification of steroidogenesis and consequently a dysfunction of the follicular hierarchy in the hen.

Acknowledgements

The authors thank to M Peloille for the sequencing, and J Simon for helpful discussions and for reading the manuscript. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 5 April 2006
Accepted 6 April 2006
Made available online as an Accepted Preprint
26 April 2006