AMP-ACTIVATED PROTEIN KINASE IS INVOLVED IN HORMONE-INDUCED MOUSE OOCYTE MEIOTIC MATURATION IN VITRO

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Abstract

We have previously shown that AMP-activated protein kinase (AMPK) can induce the resumption of meiosis in mouse oocytes maintained in meiotic arrest in vitro. The present study was carried out to determine whether AMPK activation is involved in hormone-induced maturation. Follicle-stimulating hormone (FSH) and the EGF-like peptide, amphiregulin (AR), are potent inducers of maturation in cumulus cell-enclosed oocytes (CEO). Within 3 h of FSH treatment, phosphoacetyl CoA carboxylase (ACC) levels were increased in germinal vesicle (GV)-stage oocytes when compared to non-stimulated controls and remained elevated throughout 9 h of culture, indicating AMPK activation. A similar response to AR was observed after 6 h of culture. Using PT172 antibody (binds only to activated AMPK), Western analysis demonstrated active AMPK in both FSH- or AR-treated GV-stage oocytes within 6 h. The AMPK inhibitors, compound C and adenine 9-beta-D-arabinofuranoside (araA), blocked FSH- or AR-induced meiotic resumption and ACC phosphorylation, further supporting a causal role for AMPK in hormone-induced meiotic resumption. Immunocytochemistry using anti-PT172-AMPK antibody showed an increased diffuse cytoplasmic staining and more intense punctate staining in the germinal vesicles of oocytes following treatment with the AMPK activator, 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR), or with FSH or AR, and this staining was eliminated by compound C or a blocking peptide for the anti-PT172 antibody. Staining of oocytes from hCG-stimulated mice with the anti-PT172 antibody also showed pronounced label in the germinal vesicles within 1-2 h. Further, in oocytes from all groups, active AMPK was always observed in association with the condensed chromosomes of maturing oocytes. Taken together, these results support a role for AMPK in FSH and AR-induced maturation in vitro and hCG-induced maturation in vivo.

INTRODUCTION

In mammals, the preovulatory gonadotropin surge stimulates meiotic resumption in fully-grown meiotically competent oocytes in vivo. When released from their follicles prior to the gonadotropin surge and cultured under optimal conditions, these oocytes spontaneously resume maturation without hormone stimulation, suggesting an inhibitory environment provided by the follicular somatic compartment. Several candidate molecules produced by cumulus or granulosa cells have been proposed to play this inhibitory role. The purine metabolite, hypoxanthine (HX), is present in the follicular fluid at a concentration sufficient to maintain oocyte meiotic arrest in vitro (Eppig et al., 1985). Another putative factor, termed oocyte meiosis inhibitor (OMI), may also contribute to oocyte meiotic arrest (Tsafriri and Pomerantz, 1986), though the compound has not been biochemically characterized.

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Moreover, oocyte cyclic adenosine monophosphate is a critical negative regulator of meiotic resumption (Conti et al, 2002; Eppig et al, 2004). Agents that increase cAMP levels, cAMP analogs, or factors that prevent degradation of cAMP reversibly maintain oocyte meiotic arrest in vitro. Recent evidence suggests that the oocyte is the principal site of cAMP production that operates under the control of the somatic compartment (Mehlmann et al, 2004; Hinckley et al, 2005; Ledent et al, 2005).

FSH promotes the maturation of cumulus cell-enclosed oocytes (CEO) under meiosis-arresting conditions and enhances the preimplantation developmental competence of oocytes matured in vitro (De La Fuente et al., 1999; Downs et al., 1988). When oocyte-cumulus cell complexes are stimulated with FSH, cumulus cells generate a gap junction-transmitted positive signal that acts on oocytes to induce meiotic resumption (Downs, 2001). It has also been reported that within ~0.5-2 h of FSH treatment, the cumulus cells are stimulated to produce a meiosis-inducing paracrine signal(s) that acts on the oocyte to induce meiotic maturation (Byskov et al., 1997), although the biochemical character of the signal(s) is unclear at present.

In vivo, the physiological stimulus for oocyte meiotic resumption is the luteininizing hormone surge (Stapleton et al., 1996). Distribution of the LH receptor, a G-protein coupled receptor, is restricted to the mural granulosa cells (Peng et al., 1991). The interaction of LH and its receptor leads to factors released by mural granulosa cells, functioning in an autocrine and paracrine manner to transduce the LH effects within follicle. It has been demonstrated that in rodents members of the epidermal growth factor (EGF) family of ligands play a critical role in mediating LH-induced oocyte maturation (Ashkenazi et al., 2005; Park et al., 2004). LH stimulation induces the transient and sequential expression of the EGF family members amphiregulin (AR), epiregulin and beta-cellulin (Park et al., 2004). Oocytes meiotically arrested in vitro can be induced to resume meiosis by treatment with EGF-like peptides in a cumulus cell-dependent manner (Ashkenazi et al., 2005; Downs and Chen, 2007; Park et al., 2004). Mice lacking AR showed delayed hCG-induced maturation and reduced cumulus expansion (Hsieh et al., 2007), indicating the physiological role of AR in regulation of meiotic induction.

By regulating the degradation of cAMP, phosphodiesterase (PDE) plays an essential role in oocyte meiotic resumption. In rodents, oocyte cAMP hydrolysis is thought to be primarily accomplished by PDE3A (Richard et al., 2001; Shitsukawa et al., 2001). Oocytes lacking PDE3A contained increased cAMP levels and failed to undergo spontaneous maturation in vitro (Masciarelli et al., 2004). During oocyte maturation in vitro or in vivo, an increase of PDE activity is observed (Han et al., 2006; Richard et al., 2001; Tsafriri et al., 1996) and mediates a decrease in oocyte cAMP that precedes meiotic resumption.

The activity of AMP-activated protein kinase (AMPK), a cellular energy sensor, is sensitively regulated by AMP, a byproduct of cAMP degradation. This protein Ser/Thr kinase, comprised of a catalytic α subunit and regulatory β and γ subunits, was initially discovered as an inhibitor of acetyl CoA carboxylase and 3-hydroxy-3-methyglutaryl-CoA reductase (Carling et al., 1987). An upstream kinase (AMPKK) activates AMPK through specific phosphorylation on the threonine-172 (Thr172) residue of the α subunit (Hawley et al., 1996). In a previous study, we have shown that activation of AMPK provides a positive stimulus for mouse oocyte meiotic resumption in vitro (Chen et al., 2006; Downs et al., 2002). Although evidence indicates that an increase in oocyte AMPK activity can trigger GVB, the physiological significance of this relationship is not known.

In this study, we show by Western analysis and immunofluorescent staining that AMPK activity is increased in FSH- or AR-stimulated oocytes before GVB and that hormone-

induced maturation is blocked by inhibitors of AMPK activation. AMPK is also activated within-GV-stage oocytes of hCG-treated mice. These results provide compelling evidence for AMPK mediation of hormone-stimulated GVB in mouse oocytes.

MATERIALS AND METHODS

Oocyte isolation and culture conditions

C57BL/6JxSJL/J Fl mice, 19–23 days old, were used for all experiments. Mice were primed with 5 IU equine choronic gonadotropin and killed 48 h later by cervical dislocation. Ovaries were removed and placed in the culture medium, and cumulus cell-enclosed oocytes (CEO) were obtained by puncturing large antral follicles with sterile needles. Oocytes were cultured in Eagle's minimum essential medium with Earle's salts (GIBCO), supplemented with 0.23 mM pyruvate, penicillin, streptomycin sulfate and 3 mg/ml crystallized lyophilized bovine serum albumin (ICN ImmunoBiologicals, Lisle, IL) and buffered with 26 mM bicarbonate. Tubes were gassed with a humidified mixture of 5% CO_2, 5% O_2 and 90% N_2 and placed in a water bath at 37°C for the duration of culture.

Western Analysis

After different treatments, oocytes were denuded by repeated pipetting through mouth-operated small bore pipettes in cold culture medium and washed in phosphate-buffered saline (PBS, pH 7.4)/PVP (3 mg/ml) plus protease inhibitors (Protease Inhibitors Cocktail Tablets, 1 mM Na orthovanadate, 2 μg/ml pepstatin, 50 mM beta-Glycerophospate) and then added to an equal volume of 2× Laemmli's buffer containing 20% beta-mercaptoethanol. After heating at 95°C for 5 min, samples were stored frozen at −80°C until used for Western blotting. To detect phospho-ACC levels, proteins were electrophoresed on a 3–8% Tris-Acetate mini Gel (Invitrogen) for 1 h at 150 V and then transferred to nitrocellulose at 100 V for 1 h. For active AMPK (PT172) blots (figure 1B, C), samples were electrophoresed on a 4–12% Bis-Tris SDS mini Gel (Invitrogen) for 50 min at 100 V and then in a semi-dry system transferred to nitrocellulose at 250 mA for 2 h at 4°C. After blocking with 5% nonfat milk for 2 h at room temperature, blots were then incubated with primary antibodies (anti-PT172 or anti-phospho-ACC, 1:250) overnight at 4°C, washed in Tris-buffered saline (TBS pH 7.4) and incubated with HRP-conjugated goat-anti-rabbit IgG (1:1000, in 5% nonfat milk) for 1 h at room temperature. After washing in TBS, detection was performed with Supersignal Western Dura Chemiluminescent Substrate (Pierce) and exposed to film. Blots were stripped and reprobed with ACC antiserum (1:2000) as a loading control.

Immunofluorescent staining

After different treatments, CEO were denuded and fixed with 4% paraformadehyde for 1 h at 4° C, and then permeablized with 0.1% Triton in blocking buffer (0.05% saponin in PBS, PH 7.4, plus 10% sheep serum) for 30 min. Oocytes were then washed free of Triton and continuously blocked for another 90 min at room temperature. Oocytes were incubated with primary antibodies (1:100) overnight at 4° C and then washed in wash buffer (PBS containing 1mg/ml BSA and 0.05% saponin) and incubated with FITC-conjugated sheep-anti-rabbit antibody (1:1000) at room temperature for 1 h. After washing, propidium iodine (PI, 20 μg/ml) was applied to counterstain the chromosomes. Images were observed with a confocal microscope.

Confocal Microscopy

Oocytes were viewed on a laser scanning confocal microscope (Carl Ziess Co.) with a 40× objective. During imaging all settings were kept constant, i.e. laser power, detector gain,

amplifier offset, amplifier gain and pinhole size. Digitally recorded images were exported by LSM Examiner (Carl Ziess Co.).

Chemicals

Saponin, propidium iodide, araA, dbcAMP, sheep serum, and FITC-labeled sheep-anti-rabbit antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Compound C and AICAR were supplied by Toronto research Chemicals, Inc. (North York, Ontario). Anti-pACC antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY), anti-ACC antibodies were provided by Dr. Grahame Hardie, anti- PT172 antibody was from Cell Signaling Technology (Beverly, MA), and anti-PT172-specific blocking peptide came from Santa Cruz Biotech. Inc. (Santa Cruz, CA). HRP-conjugated goat-anti-rabbit antibody and Supersignal western dura chemiluminescent substrate were obtained from Pierce (Rockford, IL) and vectashield mounting medium from Vector Laboratories, Inc. (Burlingame, CA). Highly purified ovine FSH was obtained from the National Hormone and peptide Program (NHPP), NIDDK, and Dr. A.F. Parlow.

Statistical Analysis

Oocyte maturation experiments were repeated at least 3 times with at least 30 oocytes per group per experiment. Data are reported as mean percentage GVB ± SEM. Following arcsin transformation, maturation frequencies were analyzed statistically by ANOVA followed by Duncan's multiple range test. For all statistical analyses, a P value less than 0.05 was considered significant.

RESULTS

AMPK activity is increased in FSH-treated oocytes before GVB

dbcAMP-arrested oocytes treated with FSH initiate meiosis between 4 and 6 h of culture and about 60% are induced to resume maturation after 10 h (Downs and Chen, 2007). If AMPK is involved in FSH-induced maturation, it should be activated before meiotic resumption. Acetyl-CoA carboxylase (ACC), a key enzyme involved in fatty acid metabolism, is an important substrate of active AMPK (Davies et al., 1992). To assess AMPK activity in FSH-treated GV-stage oocytes, a time course of phospho-ACC levels was determined by Western analysis. CEO were cultured in 300 μM dbcAMP with or without FSH for 3, 6 or 9 h. After culture, cumulus cells were removed and GV-stage denuded oocytes from all culture groups or GVB-stage oocytes from the 9 h treatment group were collected and analyzed by Western analysis. At all time points, GV-stage oocytes treated with FSH contained increased phospho-ACC levels compared to the control groups (figure 1A), indicating that FSH stimulated AMPK activation in oocyte before meiotic resumption. Interestingly, the amount of phospho-ACC decreased to the basal level in oocytes that resumed meiosis after 9 h of FSH stimulation.

To confirm AMPK activation and directly detect active AMPK, Western analysis using anti-PT172 antibody was performed. Cellular extracts were collected from GV-stage oocytes cultured for 6 h with or without FSH in dbcAMP-containing medium. Consistent with phospho-ACC levels, PT172-AMPK levels were increased in FSH-stimulated GV-stage oocytes, indicating FSH treatment activates intra-oocyte AMPK before oocyte maturation (figure 1B).

AMPK inhibitors block AMPK activation and FSH-induced maturation

Compound C, a small molecule AMPK inhibitor (Zhou et al., 2001), blocks AICAR-induced maturation by decreasing AMPK activity in oocytes (Chen et al., 2006). To test whether AMPK activation mediates FSH-induced maturation, CEO were preincubated increasing

concentrations of compound C for 0.5 h in 300 μM dbcAMP before addition of 0.1 μg/ml FSH. After 17–18 h of culture, GVB percentage was assessed. FSH increased the maturation percentage from 10% to 70%, and this stimulation was eliminated in a dose-dependent manner by compound C (figure 2A).

araA is the precursor of araATP, which is a competitive inhibitor of AMPK (Henin et al., 1996), and its effects on FSH-induced maturation were also determined. CEO were maintained in meiotic arrest with 300 μM dbcAMP and pretreated with increasing concentrations of araA for 0.5 h before addition of 0.1 μg/ml FSH. Maturation percentages were determined after 17–18 h of culture. araA dose-dependently blocked FSH-induced maturation, with a complete suppression at 1 mM (figure 2B). These data further support the idea that AMPK activation mediates FSH-induced maturation.

To assess the effect of compound C or araA on AMPK activity in these oocytes, phospho-ACC levels were determined in GV-stage oocytes by Western analysis. CEO were cultured in 300 μM dbcAMP and pretreated with 5 μM compound C or 1 mM araA for 0.5 h before addition of FSH. After 6 h of culture, cumulus cells were removed and denuded GV-stage oocytes were processed for Western analysis. Figure 2C shows that FSH treatment increased phospho-ACC levels compared to oocytes cultured in dbcAMP alone, and exposure of FSH-treated oocytes to either compound C or araA eliminated the increase in ACC phosphorylation, indicating suppression of AMPK activity within oocytes.

AMPK activation mediates Amphiregulin-induced maturation

Amphiregulin (AR), an EGF-like peptide, mediates LH-stimulated maturation in vivo (Hsieh et al., 2007) and induces CEO meiotic resumption in vitro (Downs and Chen, 2007; Park et al., 2004). To test whether AMPK activation is involved in AR-induced maturation, after 6 h treatment in the absence or presence of AR, CEO were denuded and extracts from 500 GV-stage oocytes were subjected to Western analysis. As shown in figure 1C, active AMPK and phospho-ACC levels were elevated in AR-treated oocytes before meiotic maturation.

The AMPK inhibitors, compound C and araA, were tested on AR-induced maturation to assess the involvement of AMPK. CEO were pretreated with increasing concentrations of compound C or araA in dbcAMP-containing medium for 0.5 h before addition of 50 ng/ml AR, and cultures were continued for 17–18 h before assessment of GVB. AR increased the maturation percentage in CEO from 10% to 81%, which was dose-dependently blocked by compound C (figure 3A) or araA (figure 3B). Western analysis showed that the AR-stimulated phosphorylation of ACC was suppressed by compound C or araA (figure 3C), demonstrating inactivation of AMPK.

Immunofluorescent localization of active AMPK (PT172-AMPK)

AICAR stimulation—Immunofluorescent staining using anti-PT172 antiserum was performed to directly observe active AMPK in the oocytes following treatment with the AMPK activator, AICAR. After 6 h of culture in medium containing dbcAMP with or without 1 mM AICAR, oocytes were denuded, fixed with 4% paraformaldehyde and stained for immunofluorescence using anti-PT172 antiserum. Counterstaining with propidium iodide (PI) enabled an assessment of AMPK localization in relation to the germinal vesicle in immature oocytes or condensed chromosomes following meiotic resumption. Control groups for nonspecific staining were carried out in which either primary antibody was omitted or a blocking peptide representing the phosphorylated peptide recognition site was added to neutralize the primary antibody. In the control group, in which primary antibody was omitted, a very faint fluorescence was uniformly distributed throughout the oocyte (data

not shown). Fluorescence was never seen in the nucleolus in any of the groups. Oocytes meiotically arrested in dbcAMP-supplemented medium contained a diffuse fluorescence throughout the cell, which was above the background (figure 4A). AICAR stimulation resulted in a stronger diffuse signal in the cytoplasm, but most notable was an intense punctate labeling in the germinal vesicle (figure 4B). In all GVB-stage oocytes, even those spontaneously maturing in dbcAMP alone, a punctate staining pattern was observed associated with the condensed chromosomes (figure 4C). Pretreatment of oocytes with compound C (figure 4D) or pretreatment of antibody with a blocking peptide containing the specific peptide sequence recognized by the antibody (figure 4E) eliminated both the increased cytoplasmic staining as well as the punctate nuclear staining. When quantified, 75% of AICAR-treated GV-stage oocytes exhibited nuclear staining, compared to 11% of controls, and compound C completely prevented the AICAR-stimulated response (figure 5).

FSH stimulation—To test if FSH-treated oocytes exhibited a similar staining pattern, immunocytochemistry was performed on dbcAMP-arrested CEO stimulated with FSH for 6 h. Similar to AICAR-stimulated CEO, GV-stage oocytes treated with FSH exhibited increased diffuse staining in the cytoplasm and intense punctate fluorescence in the nucleus (figure 6B) compared to a faint diffuse staining pattern in untreated controls (figure 6A). When oocytes resumed meiosis, active AMPK was again consistently observed in association with the condensed chromosomes. Compound C, at a concentration that eliminated FSH-induced maturation, also prevented the punctate nuclear staining and reduced the diffuse cytoplasmic staining (figure 6D, 7).

Amphiregulin stimulation—Experiments were next performed to determine the active AMPK staining pattern in AR-treated oocytes. dbcAMP-arrested CEO treated 6 h with AR exhibited the same pattern of staining, with an increase in diffuse cytoplasmic staining and pronounced punctate staining in the germinal vesicle of immature oocytes (figure 8B) and around the condensed chromosomes of oocytes that had resumed maturation (figure 8C). Compound C again prevented this increased staining (figure 8D).

When a dose response experiment was carried out with AR using concentrations of 2.5, 10, and 50 ng/ml, a dose-dependent stimulation of meiotic resumption in dbcAMP-arrested oocytes was observed that was paralleled by corresponding increases in the frequency of nuclear staining in GV-stage oocytes using anti-PT172 antibody (figure 9A). Thus, as the maturation-inducing potency of AR was increased, so was its ability to stimulate AMPK activation within the GV of immature oocytes. Compound C abolished the increased nuclear staining brought about by 50 ng/ml AR (figure 9B).

hCG stimulation in vivo—To determine if AMPK activation within the oocytes occurs prior to meiotic resumption in vivo, eCG-primed mice were treated with 5 IU hCG and oocyte-cumulus cell complexes were isolated at 1, 2 and 3.5 h post-hCG and processed for immunofluorescence using anti-PT172 antibody. A separate set of primed mice was injected with the PBS vehicle as controls. As shown in figure 10, very little label was detected in the 0 h group, but as early as 1 h after hCG injection a slight increase in staining intensity was evident in the germinal vesicle that became very pronounced after 2 h. In two experiments, 41 out of a total of 74 oocytes (55.4 %) isolated 2 h post-hCG displayed this increased nuclear staining. As with oocytes stimulated in vitro, blocking peptide eliminated the increase in nuclear fluorescence (data not shown), confirming the specificity for active AMPK in vivo. Such staining was not observed in oocytes from vehicle-treated mice at any time point. Nearly all of the oocytes (90%) isolated 3.5 h post-hCG had undergone GVB and, of these, 100% showed intense phospho-PT172 staining that colocalized with the condensed chromosomes (figure 10). Although an increase in diffuse cytoplasmic fluorescence was apparent in oocytes that had resumed maturation, this increase was more

subtle in immature oocytes from hCG-stimulated animals. Interestingly, a common finding in oocytes from hCG-treated mice were foci of intense staining in the cytoplasm and sometimes in the germinal vesicle that were usually larger than the aggregations of label in oocytes receiving an inducing signal in vitro.

DISCUSSION

In this study, we have demonstrated the participation of AMPK in hormone-induced maturation of mouse CEO in vitro. FSH triggered AMPK activation in oocytes before meiotic resumption, and the AMPK inhibitors, compound C and araA, blocked both FSH-induced maturation and the FSH-stimulated increase in AMPK activity in GV-stage oocytes. Identical results were observed in AR-stimulated oocytes. By immunofluorescence microscopy using anti-PT172 antibody, increased diffuse cytoplasmic staining and punctate staining in the germinal vesicle were observed in oocytes stimulated with FSH or AR, similar to the staining pattern in AICAR-stimulated oocytes. With each stimulatory protocol, the increase in active AMPK staining was eliminated by compound C. AMPK was also shown to be activated prior to meiotic resumption in oocytes from hCG-stimulated mice. These results demonstrate the essential involvement of AMPK in hormone-induced maturation.

FSH is an effective inducer of meiotic resumption in arrested mouse CEO in vitro. FSH binding to its receptor on the cumulus cells activates a cAMP–regulated pathway (Tayar, 1996) and generates a positive stimulus to induce maturation in a gap junction-dependent manner (Downs, 2001; Downs et al., 1988). The nature of this positive stimulus and the means by which it brings about GVB remain active areas of research. We show that FSH stimulation for 3 h is sufficient to increase ACC phosphorylation in GV-stage mouse oocytes, indicating AMPK activation. In addition, immunoblotting and immunofluorescent staining using anti-PT172 antiserum directly showed an increase of active AMPK in FSH-treated GV-stage oocytes. These observations are consistent with AMPK involvement in FSH-induced meiotic resumption, since the kinase is activated prior to meiotic resumption. Support for this idea was provided by the use of the AMPK inhibitors, compound C (Zhou et al., 2001) and araA (precursor of araATP, (Henin et al., 1996)), which blocked both hormone-induced maturation and AMPK activation.

In a normal reproductive cycle, it is the LH surge that regulates oocyte maturation in vivo. It has been shown that EGF-like peptides, such as amphiregulin (AR), epiregulin, and betacellulin, mediate LH-induced effects on oocyte meiotic maturation in rodents (Ashkenazi et al., 2005; Hsieh et al., 2007; Park et al., 2004). A model system has been proposed in which secretion of EGF-like peptides by the mural granulosa cells in response to gonadotropin stimulation functions in an autocrine and paracrine manner to transduce the hormone effects in the follicle (Park et al., 2004). In cultured CEO, FSH induces AR mRNA expression within 0.5 h by a prostaglandin synthase 2 and progesterone receptor-dependent mechanism (Shimada et al., 2006). In addition, a recent study showed that AR mediates FSH-induced maturation in mouse oocytes in vitro (Downs and Chen, 2007). Therefore, it was important to test whether AMPK is involved in oocyte maturation mediated by EGF-like peptides. Indeed, Western analysis indicated that AR induces AMPK activation in mouse oocytes before GVB, and this was confirmed by immunocytochemistry. Moreover, as in FSH-treated CEO, AR-induced oocyte maturation and AMPK activation were blocked by AMPK inhibitors, further supporting the involvement of AMPK in AR-induced maturation.

By immunofluorescent staining, we showed that a considerable portion of active AMPK α subunit accumulated in the germinal vesicle after AICAR, FSH or AR stimulation in vitro or hCG stimulation in vivo. Evidence for the active AMPK specificity of this staining stems

from its elimination by treatment of oocytes with compound C or treatment of antibody with a specific blocking peptide. In *Drosophila melanogaster*, the AMPK homologue, DmAMPK, has also been shown to be associated with the nucleus when it was activated by oligomycin (Pan and Hardie, 2002). Moreover, in yeast, the AMPK homologue, SNF1, translocalizes from cytoplasm to the nucleus in response to alkaline pH but not sodium ion stress (Hong and Carlson, 2007). It is tempting to speculate that active AMPK within the germinal vesicle is important for its meiosis-inducing action. However, it should be noted that an increased diffuse cytoplasmic staining was also observed in stimulated CEO, through it was of lesser intensity.

In mammals, the AMPK catalytic subunit contains two isoforms, α1 and α2, which are encoded by different genes. In somatic cells, α1-AMPK is only present in the cytoplasm, whereas α2 is mainly localized in the nucleus (Salt et al., 1998; Stapleton et al., 1996; Turnley et al., 1999). A recent study showed that in a mouse myoblast cell line, α2 and α1 isoforms are both localized in the cytoplasm, while activation of AMPK results in α2, but not α1, translocation into the nucleus (Suzuki et al., 2007). Interestingly, we have observed increased α1 AMPK accumulation in or around the germinal vesicle of AICAR-stimulated oocytes (unpublished data), suggesting a movement of this subunit from the cytoplasm into the GV prior to meiotic resumption. Thus, different active AMPK isoform dynamics may be manifest in mouse oocytes when compared to somatic cells, but clarification awaits further study.

Immunofluorescent staining of oocytes from hCG-stimulated animals also revealed that AMPK was activated prior to GVB in vivo. An increase in fluorescence was observed 1 h after hCG injection that became significantly more intense by 2 h post-hCG. Moreover, as was observed in oocytes stimulated in vitro by AICAR, FSH or AR, active AMPK was always observed in association with the condensed chromosomes of maturing oocytes. Taken together, our data support a physiological role for AMPK in hormone-induced maturation both in vitro and in vivo. However, an interesting difference in staining pattern was observed in that oocytes in vitro tended to have smaller, punctate staining, while those maturing in vivo had fewer, but larger, concretions of fluorescence. The significance of these differences is not known and they provide a challenge for future experimentation.

The observation that active AMPK accumulated around the periphery of condensed chromosomes in all maturing oocytes suggests that AMPK participates in subsequent events leading to the completion of meiosis I, such as the segregation of chromosomes. Indeed, we have reported increased frequencies of metaphase II oocytes following in vitro culture in the presence of AICAR (Downs and Chen, 2006). A study in *Saccharomyces cerevisiae* has shown that the AMPK homologue, SNF1, is required for both early (DNA replication and meiotic recombination) and late (meiotic chromosome segregation and spore formation) stages of meiosis through different mechanisms (Honigberg and Lee, 1998). It will be interesting to determine the pattern of AMPK activation throughout maturation and fertilization in the mouse and its involvement in these later developmental stages.

Data presented herein and in previous studies have demonstrated a meiosis-inducing action of AMPK in mouse oocytes. The present study is important because it implicates AMPK in hormone-induced oocyte maturation in the mouse, thereby verifying a physiological role in meiotic regulation. Curiously, in oocytes from two other species, cow and pig, AMPK has the opposite action on oocyte maturation (Bilodeau-Goeseels et al., 2007; Mayes et al., 2007; Tosca et al., 2007). In a recent study, Mayes et al. (2007) showed that activation of AMPK transiently suppressed the spontaneous maturation of pig oocytes, and that this effect was mediated by the cumulus cells. A similar cumulus cell-dependent inhibitory effect was reported by Tosca et al (2007) when bovine oocytes were treated with the AMPK activator,

metformin. Bilodeau-Goeseels et al (2007) also reported suppression of bovine maturation with AICAR and metformin, but these agents were effective in both CEO and DO. These findings are striking not only because the response to AMPK activation is one of meiotic suppression, but also because the site of action may vary between germ and somatic compartments. Thus, fundamental differences exist in meiotic regulation of mammalian oocytes that serve to emphasize the difficulty in extrapolating experimental findings to another species. As a further example of this, in preliminary experiments we have been unable to stimulate GVB in rat oocytes with AICAR or a brief heat shock, both of which are effective meiotic inducers in mice (Downs et al., 2002; LaRosa and Downs, 2007). It is apparent that the mouse possesses unique physiological characteristics associated with the control of meiotic resumption. A future challenge will be the delineation of similarities as well as differences between mammalian species in how oocyte maturation is regulated.

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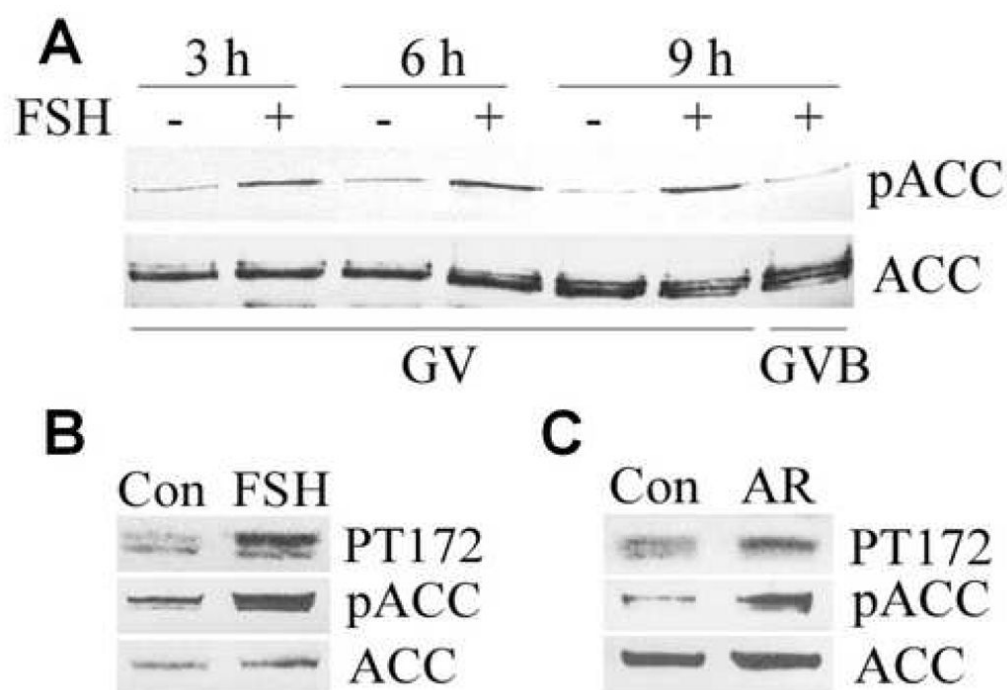
Figure 1.
AMPK activation in FSH-or AR-treated GV-stage oocytes. (A) Western analysis of phospho-ACC in FSH-stimulated oocytes. CEO were denuded and extracted after culture with or without FSH in dbcAMP-supplemented medium for the indicated times. All oocytes were at the GV-stage, except a group of GVB-stage oocytes were collected after 9 h FSH treatment. Western analysis of PT172-AMPK in GV-stage oocytes after 6 h culture in the absence (Con) or presence of 0.1 μl/ml FSH (B) or 50 ng/ml AR (C) in dbcAMP-containing medium.
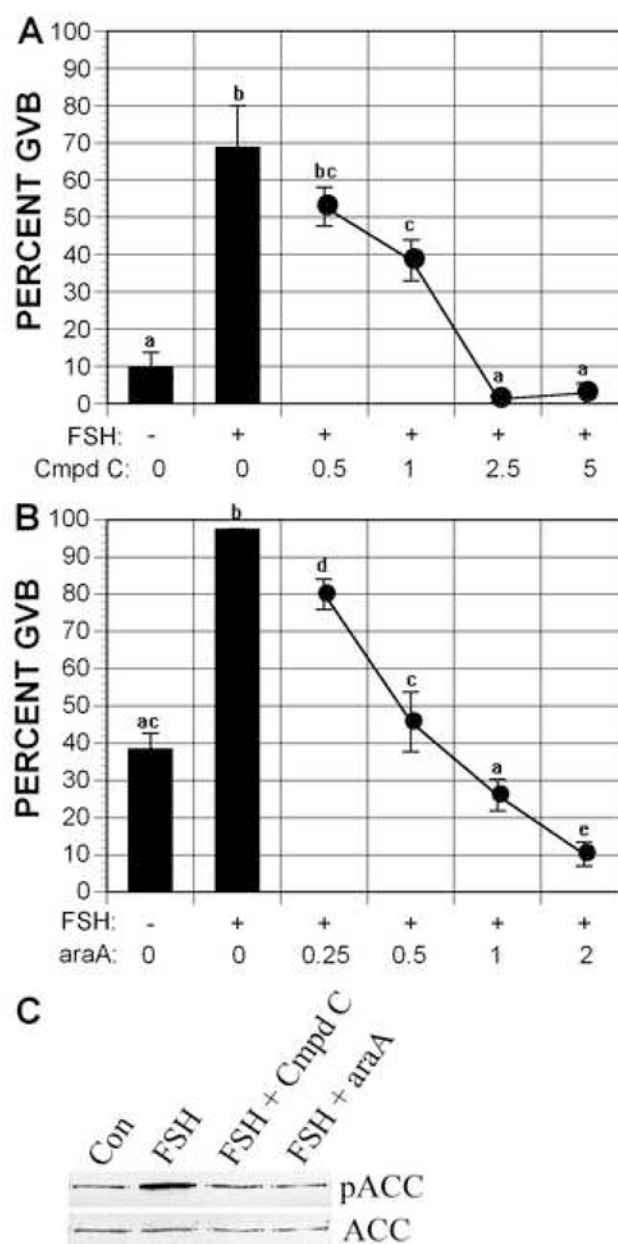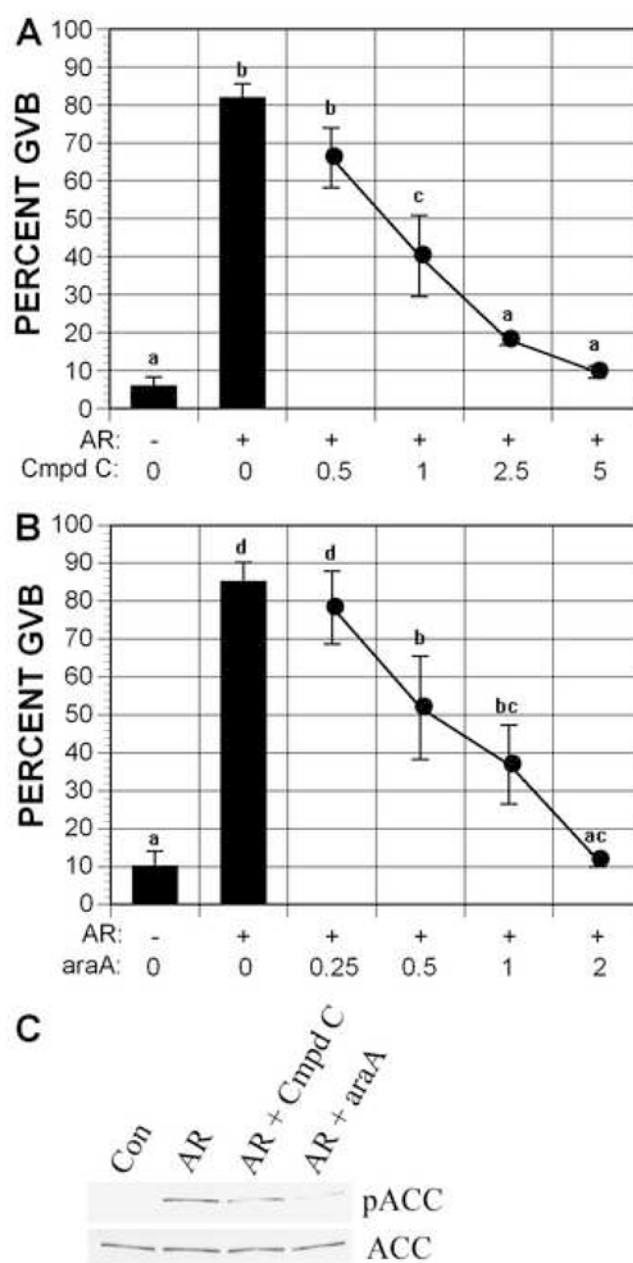
Figure 2.
Effects of compound C and araA on FSH-induced maturation and ACC phosphorylation.
CEO were precultured for 0.5 h with increasing doses of compound C (A) or araA (B) in
300 μM dbcAMP. FSH (0.1 μg/ml) was then added and cultures were continued for 17–18 h
before assessment of GVB percentage. Groups with no common letters are significantly
different. (C) Western analysis of phospho-ACC in compound C or araA-treated GV-stage
oocytes. CEO were preincubated 0.5 h with 5 μM compound C or 1 mM araA in 300 μM
dbcAMP before addition of FSH. After 6 h of culture, GV-stage oocytes were prepared for
the Western analysis.

Figure 3.
Effects of compound C and araA on AR-induced maturation and ACC phosphorylation.
CEO were pretreated with increasing concentrations of compound C (A) or araA (B) in 300
μM dbcAMP for 0.5 h. AR (50ng/ml) was then added and cultures were continued for 17–18
h before assessment of GVB. Groups with no common letters are significantly different. (C)
Western analysis of phospho-ACC in compound C or araA-treated oocytes. CEO were
preincubated 0.5 h with 5 μM compound C or 2 mM araA in 300 μM dbcAMP before
addition of AR. CEO were denuded after 6 h of culture and GV-stage oocytes were collected
and processed for Western analysis.

	PT172	PI	Merged
A Con			
B AICAR			
C AICAR			
D AICAR + Blocking pep			
E AICAR + Cmpd C			
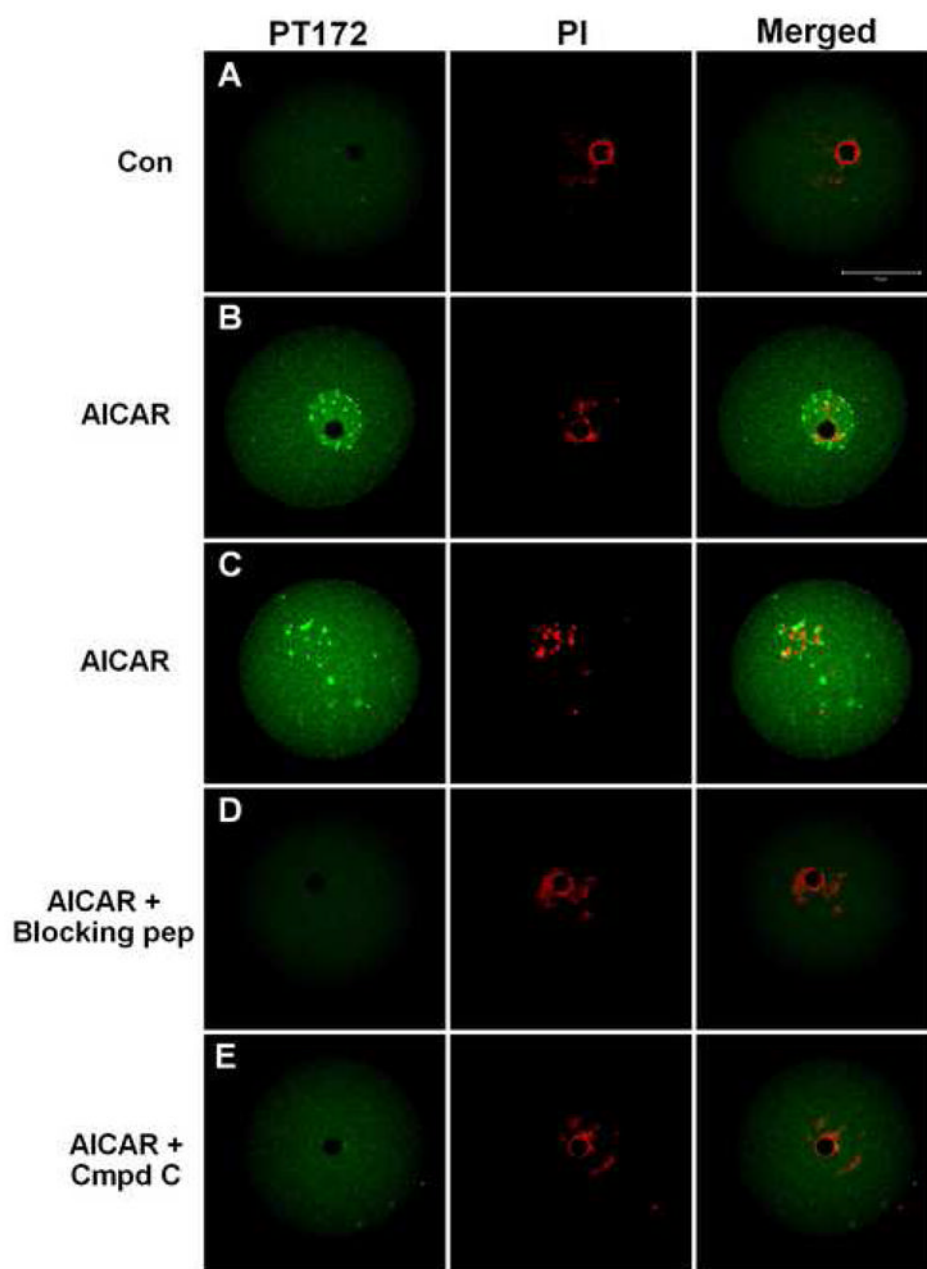
Figure 4.
Localization of active AMPK in AICAR-stimulated CEO. After 6 h of culture, cumulus cells were removed and DO were fixed and stained with rabbit anti-PT172 AMPK antiserum followed by FITC-labeled sheep anti-rabbit antiserum. (A) dbcAMP alone (control), GV-stage; (B) AICAR, GV-stage; (C) AICAR, GVB-stage; (D) AICAR, GV-stage; primary antibody was neutralized with a specific blocking peptide; (E) AICAR plus compound C, GV-stage; CEO were preincubated with 5 μM compound C in 300 μM dbcAMP before the addition of 1 mM AICAR. Oocytes were stained with propidium iodide (PI) for detection of chromatin. The bar represents 50 μm.
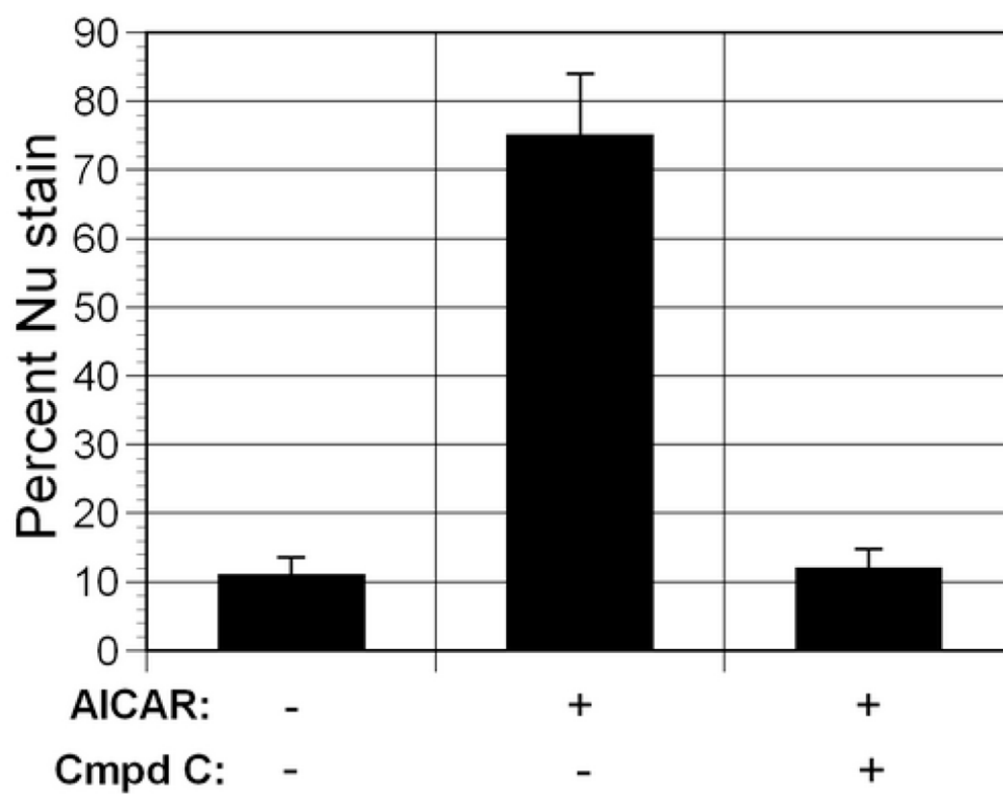
Figure 5.
Effect of compound C on the percentage of GV-stage oocytes that contained nuclear PT172 staining after 6 h of culture in 1 mM AICAR.
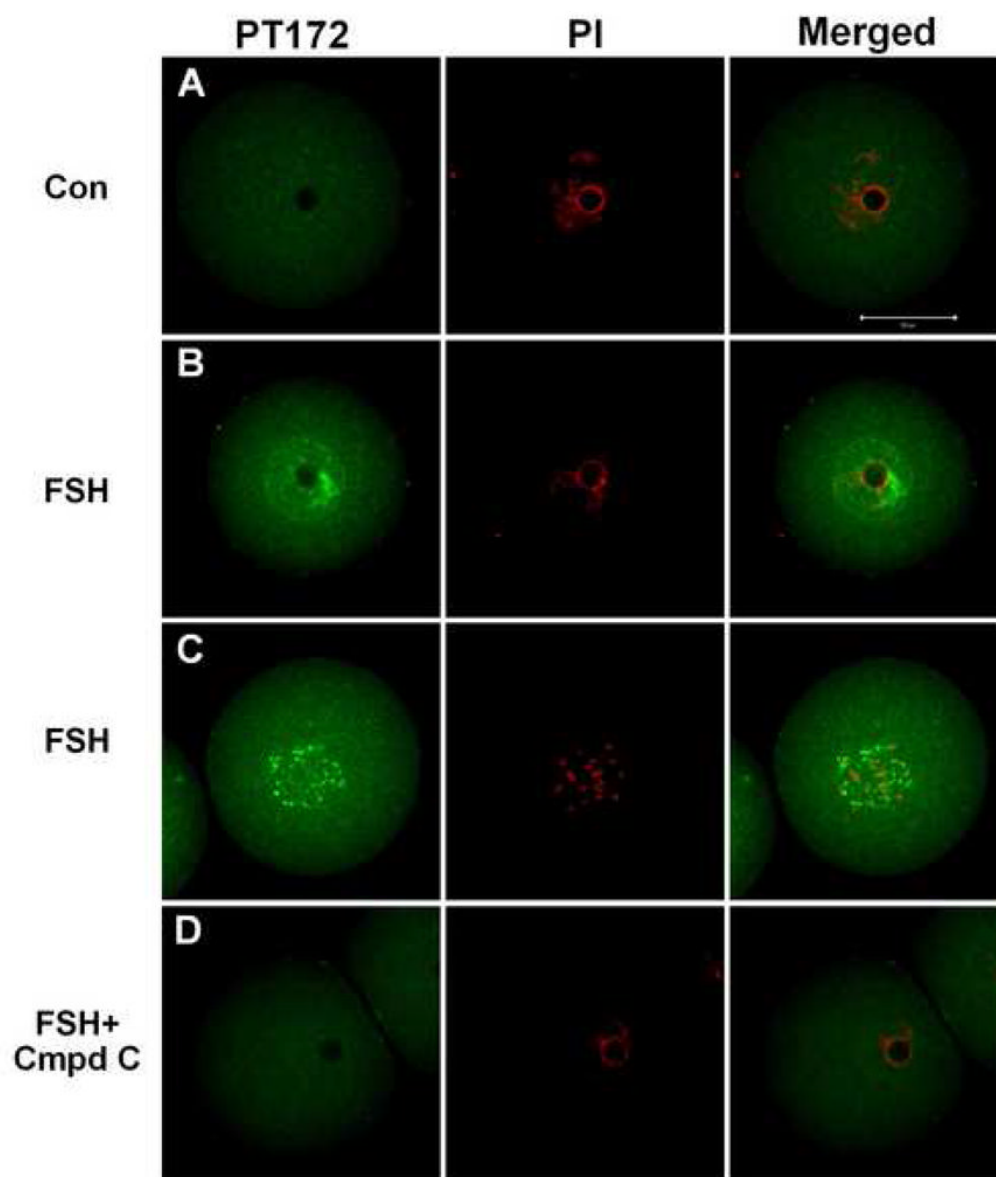
Figure 6.
Localization of active AMPK in FSH-stimulated CEO. After 6 h of culture, cumulus cells were removed and DO were fixed and stained with rabbit anti-PT172 AMPK antiserum followed by FITC-labeled sheep anti-rabbit antiserum. (A) dbcAMP alone (control), GV-stage; (B) FSH (0.1μg/ml), GV-stage; (C) FSH (0.1μg/ml), GVB-stage; (D) FSH (0.5 μg/ml) plus compound C, GV-stage; CEO were preincubated with 5 μM compound C in 300 μM dbcAMP before the addition of FSH. Oocytes were stained with propidium iodide (PI) for detection of chromatin. The bar represents 50 μm.
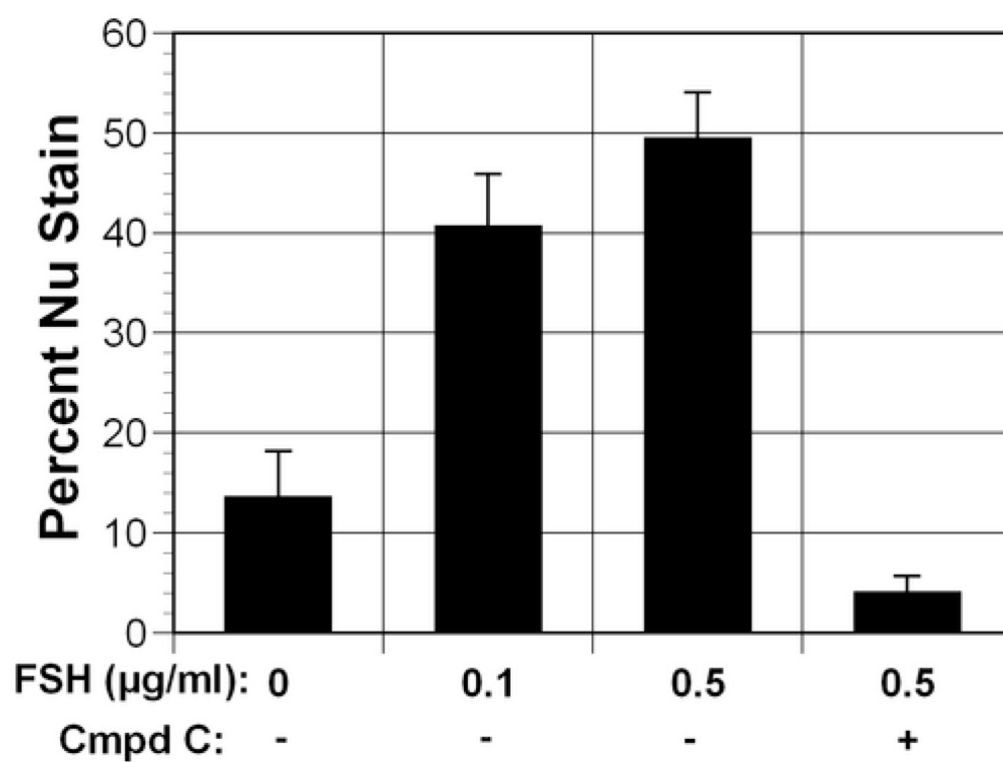
Figure 7.
Effect of compound C on the percentage of GV-stage oocytes that contained nuclear PT172-AMPK staining after 6 h of culture in FSH.
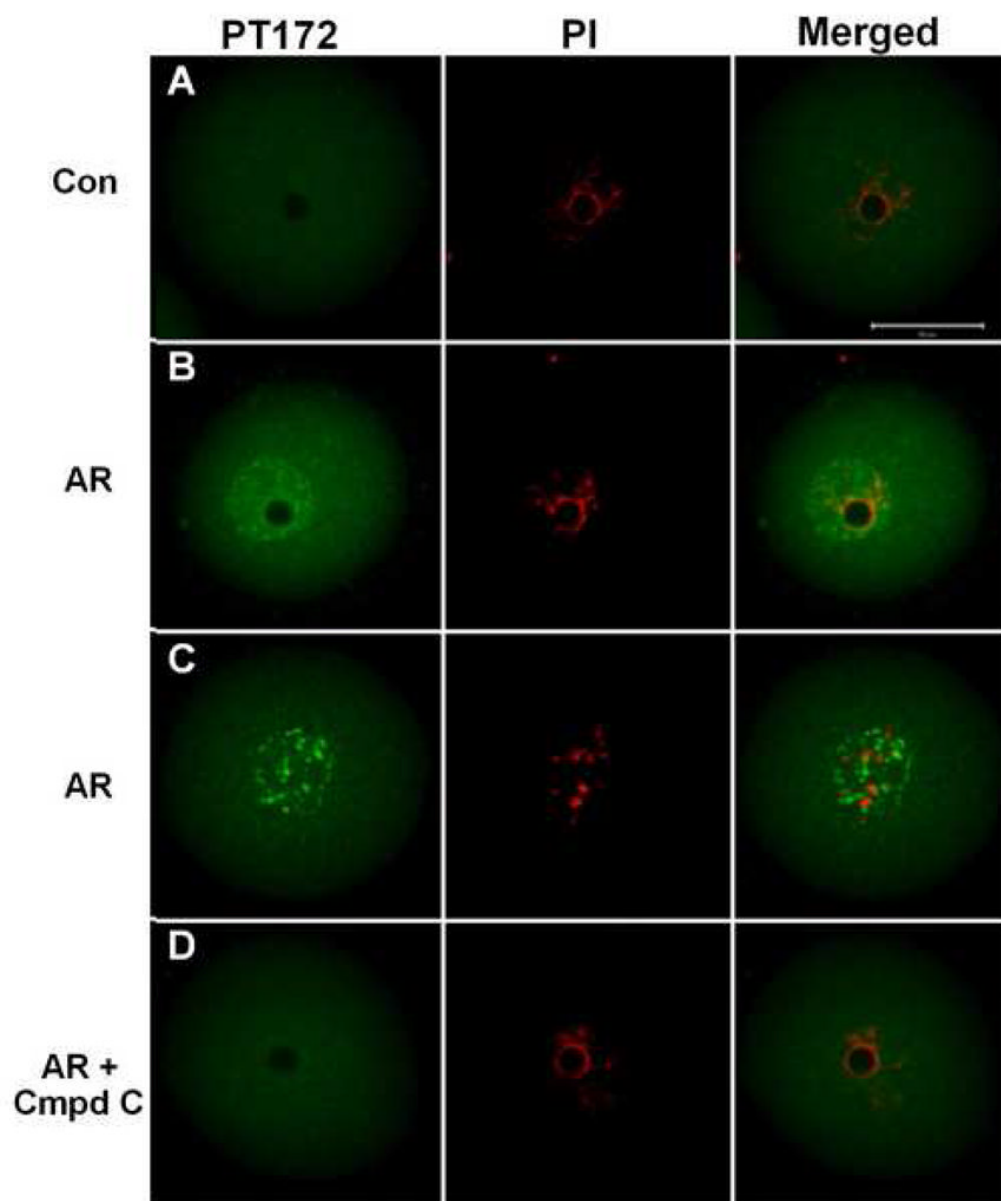
Figure 8.
Localization of active AMPK in AR-stimulated CEO. After 6 h of culture, cumulus cells were removed and DO were fixed and stained with rabbit anti-PT172 AMPK antiserum followed by FITC-labeled sheep anti-rabbit antiserum. (A) dbcAMP alone (control), GV-stage; (B) AR, GV-stage; (C) AR, GVB-stage; (D) AR plus compound C, GV-stage; CEO were preincubated with 5 μM compound C in 300 μM dbcAMP before the addition of AR. Oocytes were stained with propidium iodide (PI) for detection of chromatin. The bar represents 50 μm.
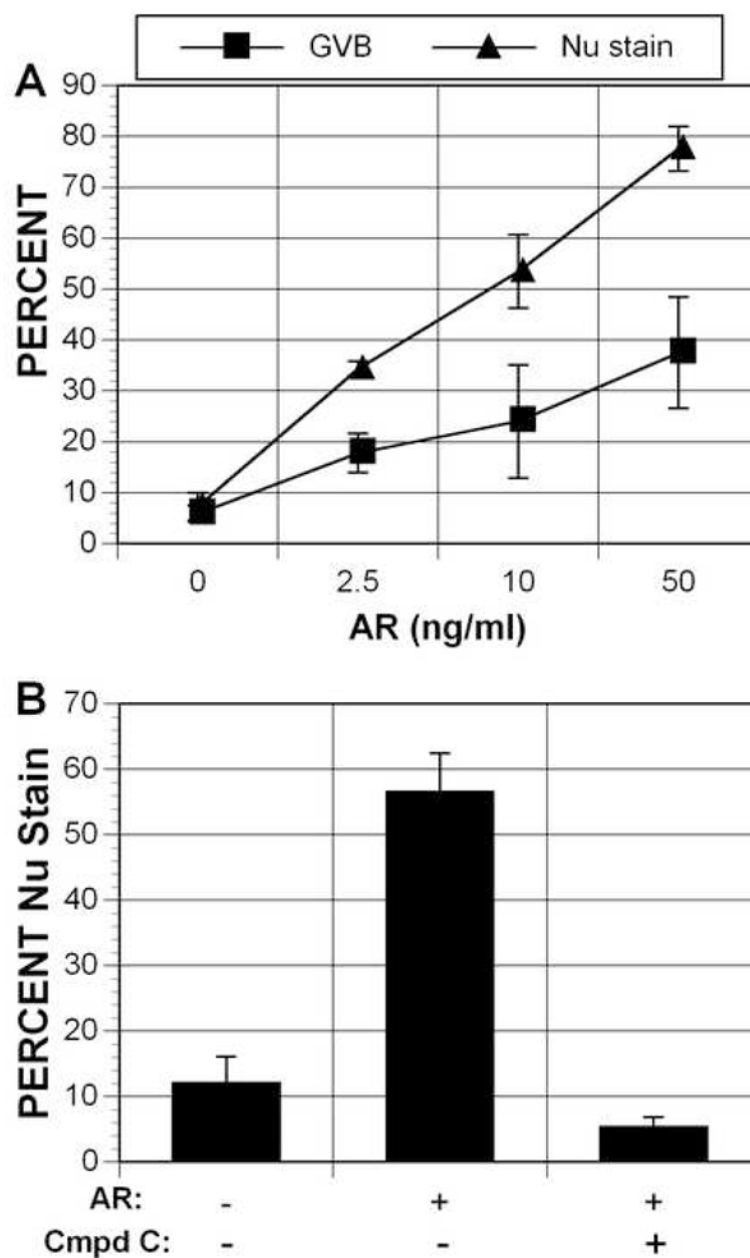
Figure 9.
Effects of AR and compound C on nuclear PT172-AMPK staining. (A) Effect of AR dose
on oocyte maturation and nuclear staining. CEO were cultured 6 h in medium containing
300 μM dbcAMP plus increasing dose of AR and the frequencies of both GVB and nuclear
staining in GV-stage oocytes were determined. (B) Inhibitory effect of compound C on the
percentage of GV-stage oocytes that contained nuclear PT172-AMPK staining after 6 h of
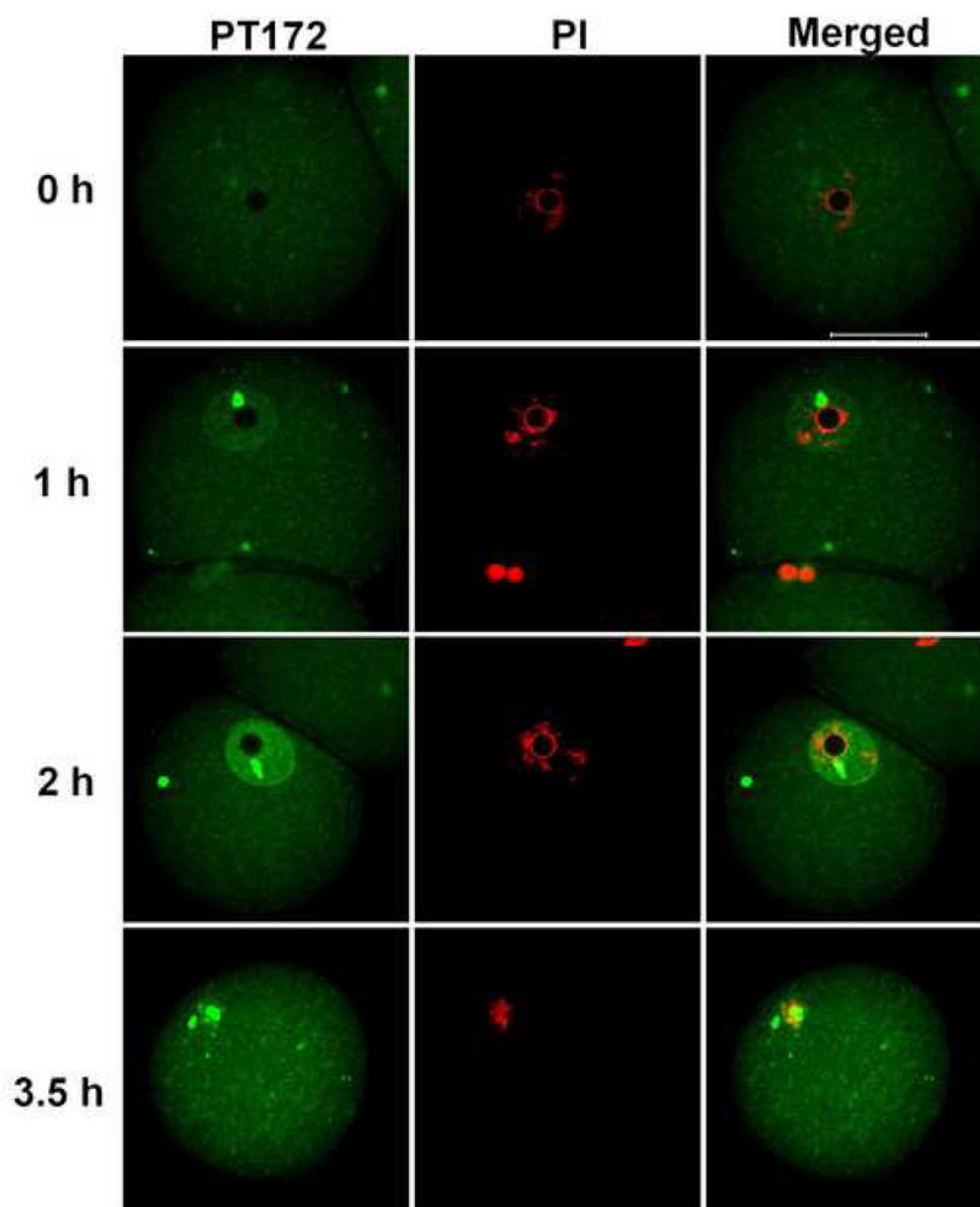culture in AR.

Figure 10.
Localization of active AMPK in oocytes from hCG-stimualted mice. eCG-primed mice were injected with 5 IU hCG and 0, 1, 2 or 3.5 h later oocytes were isolated, fixed and stained with rabbit anti-PT172 AMPK antiserum followed by FITC-labeled sheep anti-rabbit antiserum. Oocytes were stained with propidium iodide (PI) for detection of chromatin. Note that two cumulus cell nuclei stain intensely with propidium iodide on the bottom portion of the micrograph of the 1 h group. The bar represents 50 μm.