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A Requirement for Fatty Acid Oxidation in the Hormone-Induced Meiotic Maturation of Mouse Oocytes

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A Requirement for Fatty Acid Oxidation in the Hormone-Induced Meiotic Maturation of Mouse Oocytes[1]

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Abstract: We have previously shown that fatty acid oxidation (FAO) is required for AMP-activated protein kinase (PRKA)-induced maturation in vitro. In the present study, we have further investigated the role of this metabolic pathway in hormone-induced meiotic maturation. Incorporating an assay with ^{3}H-palmitic acid as the substrate, we first examined the effect of PRKA activators on FAO levels. There was a significant stimulation of FAO in cumulus cell-enclosed oocytes (CEO) treated with 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and RSVA405. In denuded oocytes (DO), AICAR stimulated FAO only in the presence of carnitine, the molecule that facilitates fatty acyl CoA entry into the mitochondria. The carnitine palmitoyltransferase 1 activator C75 successfully stimulated FAO in CEO. All

Biology of Reproduction, Vol 89(2), No. 43 (August 1, 2013): pg. 1-9. DOI.

three of these activators trigger germinal vesicle breakdown. Meiotic resumption induced by follicle-stimulating hormone (FSH) or amphiregulin was completely inhibited by the FAO inhibitors etomoxir, mercaptoacetate, and malonyl CoA. Importantly, FAO was increased in CEO stimulated by FSH and epidermal growth factor, and this increase was blocked by FAO inhibitors. Moreover, compound C, a PRKA inhibitor, prevented the FSH-induced increase in FAO. Both carnitine and palmitic acid augmented hormonal induction of maturation. In a more physiological setting, etomoxir eliminated human chorionic gonadotropin (hCG)-induced maturation in follicle-enclosed oocytes. In addition, CEO and DO from hCG-treated mice displayed an etomoxir-sensitive increase in FAO, indicating that this pathway was stimulated during in vivo meiotic resumption. Taken together, our data indicate that hormone-induced maturation in mice requires a PRKA-dependent increase in FAO.

Keywords: AMPK, fatty acid oxidation, meiotic resumption, oocyte maturation, PRKA

Introduction

Mammalian oocytes are maintained in meiotic arrest at prophase-I from the time of birth until puberty. Beginning at puberty, a cohort of ovarian follicles undergoes growth and development to the preovulatory stage, the number varying by species. During this time, the oocytes attain meiotic competence, and, in response to the preovulatory gonadotropin surge, resume meiosis. They complete meiosis-I, extruding a polar body, and then arrest at metaphase-II, at which stage they remain until fertilization. The entire sequence of events from the resumption of meiosis up to metaphase-II arrest is known as oocyte maturation.

Oocyte maturation is regulated by a number of different molecules in the oocyte as well as the cumulus granulosa and mural granulosa cells. The granulosa compartment of the follicle plays a key role in maintaining the oocyte in prophase-I arrest. Thus, oocytes removed from the follicle and cultured in suitable medium resume meiosis spontaneously in the absence of gonadotropins, visually evident by the loss of the nuclear envelope, called germinal vesicle breakdown (GVB). Meiotic arrest is maintained by maintaining elevated cAMP levels in the oocyte, which blocks the activation of maturation promoting factor, responsible for triggering the downstream events of meiosis, including GVB [1]. This cAMP-mediated arrest is overcome by gonadotropin stimulation of the somatic compartment that leads to phosphodiesterase-mediated degradation of

Biology of Reproduction, Vol 89(2), No. 43 (August 1, 2013): pg. 1-9. DOI.

cAMP. AMP, the end product of such degradation, is an activator of AMP-activated protein kinase (AMPK; hereafter referred to as PRKA) that acts as a fuel gauge and responds to a high AMP:ATP ratio by turning off energy-consuming pathways and turning on energy-generating pathways [2, 3].

Activation of PRKA is essential for the resumption of meiotic maturation in mouse oocytes [4–7]. Treatment of meiotically arrested mouse oocytes with PRKA activators 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) [8] or the resveratrol analog RSVA405 [9] stimulates meiotic resumption in vitro [4, 10, 11]. On the other hand, Compound C, a PRKA inhibitor [12], blocks both the activation of PRKA and meiotic induction brought about by treatment with AICAR [4], hormones [5], or stress [6, 7], implicating PRKA as an important player in the mechanism(s) regulating meiotic induction.

Acetyl CoA carboxylase (ACAC), an important substrate of PRKA, converts acetyl CoA to malonyl CoA, which plays two important roles in lipid metabolism: 1) it acts as precursor in the synthesis of long-chain fatty acids, and 2) it inhibits carnitine palmitoyltransferase 1 (CPT1), the mitochondrial outer membrane enzyme that links a carnitine moiety to a fatty acyl group to facilitate transport into mitochondria for beta-oxidation [13] (see Fig. 1). PRKA phosphorylates ACAC at serine-79 (mouse), resulting in its inactivation, and the resulting decrease in malonyl CoA removes the impediment to mitochondrial fatty acid transport and thereby facilitates fatty acid oxidation (FAO) [13]. Thus, ACAC provides an important link between PRKA activation and FAO.

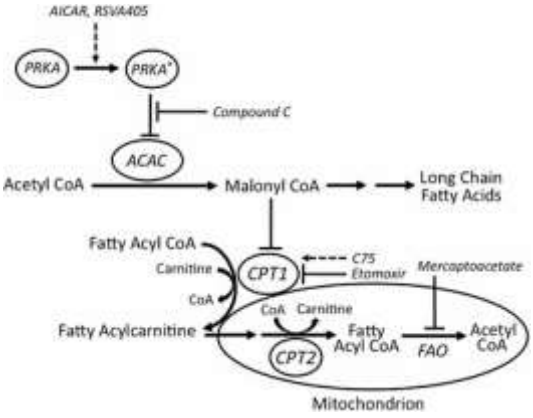

FIG. 1: Schematic of fatty acid oxidation and its regulation. Carnitine palmitoyl transferase 1 (CPT1) catalyzes the rate-limiting step in fatty acid oxidation (FAO):

Biology of Reproduction, Vol 89(2), No. 43 (August 1, 2013): pg. 1-9. DOI.

exchange of the coenzyme A (CoA) moiety of fatty acyl with carnitine, forming fatty acylcarnitine that is then transported into the mitochondrial matrix by a translocase. CPT1 is allosterically inhibited by malonyl CoA, produced by carboxylation of acetyl CoA via acetyl CoA carboxylase (ACAC). Malonyl CoA is also a precursor in the synthesis of long chain fatty acids. ACAC is inactivated through phosphorylation by AMP-activated protein kinase (PRKA; active state designated by asterisk) that results in decreased malonyl CoA production and release from CPT1 inhibition. This facilitates fatty acid entry into mitochondria, subsequent exchange of carnitine for CoA (driven by carnitine palmitoyl transferase 2, or CPT2), and FAO. The various pharmacological modulators used in this study are italicized. Dotted lines represent stimulation. AICAR and RSVA405 are PRKA activators, whereas compound C is a PRKA inhibitor. Etomoxir, like malonyl CoA, inhibits CPT1, while C75 activates this enzyme. Mercaptoacetate blocks the first step in mitochondrial FAO.

Carbohydrates—particularly pyruvate and glucose—have attracted more attention than lipids as potential energy sources for mammalian oocyte maturation, and their utilization has formed the basis for many culture medium formulations. Pyruvate is an important substrate utilized directly by the oocyte [14–16], and, although the oocyte is poorly equipped to metabolize glucose, this substrate is crucial for meiosis and acquisition of developmental competence [17] because the somatic follicular compartment readily metabolizes glucose to pyruvate that is then made available to the oocyte [14, 18, 19]. Dramatic shifts in GVB in vitro can be achieved by manipulating relative carbohydrate levels in the culture medium [20, 21]. Further evidence of the importance of glucose stems from compromised oocyte physiology in diabetic animals [22–25].

With so much support for carbohydrate involvement in oocyte development, there has been less motivation to address lipid metabolism, particularly in rodent oocytes. Rodents have been a popular model system for oocyte maturation studies, but these oocytes have a very low level of lipid when compared to other mammals (e.g., 4 ng/oocyte in the mouse [26] versus 63 ng in the cow, 89 ng in the sheep, and 161 ng in the pig [27]). It is not surprising that lipids represent an important energy source for oocytes from domestic species (reviewed by [28, 29]). One untested speculation attempting to explain the disparity of lipid content between species is the different amounts of time taken by embryos to implant in the uterine wall and thus different needs of energy stores within the oocyte [28]. In a study by Cetica et al. [30], the lipase activity in bovine oocytes was found to increase during meiotic maturation, whereas Ferguson and Leese [31] reported that methyl palmoxirate, a

Biology of Reproduction, Vol 89(2), No. 43 (August 1, 2013): pg. 1-9. DOI.

CPT1 inhibitor, decreased oxygen consumption in maturing bovine oocytes and affected subsequent blastocyst development.

Despite the low levels of lipid in mouse oocytes, PRKA involvement in oocyte maturation and its well-established regulation of FAO led us to examine a possible role for FAO in meiotic induction in mouse oocytes [32]. The results of this earlier study provided compelling evidence that FAO plays an important role in PRKA-induced GVB in mouse oocytes. Among our findings, AICAR-induced in vitro maturation was blocked by inhibitors of FAO that include etomoxir, malonyl CoA, and mercaptoacetate. Etomoxir works in a similar fashion to that of malonyl CoA, by inhibiting CPT1-mediated fatty acid transport into mitochondria [33], whereas mercaptoacetate inhibits long chain acyl CoA dehydrogenase, thereby blocking the first step in the beta-oxidation pathway [34]. On the other hand, stimulation of oocytes with C75, a CPT1 activator [35], increased meiotic resumption in vitro, and this stimulatory effect was not blocked by inhibiting PRKA, suggesting that FAO stimulation can bypass PRKA inhibition.

Since our study, an important series of experiments has been presented by Dunning and associates [36] that further implicates lipid metabolism as an integral component of oocyte maturation in mice. They demonstrated by radioisotopic assay etomoxir-sensitive oxidation of fatty acids by cell-enclosed oocytes (CEO) and denuded oocytes (DO) as well as a direct relationship between the extent of FAO during oocyte maturation and developmental competence (similar findings have been reported for cow [31] and pig [37] oocytes); moreover, carnitine increased the cleavage percentage of one-cell embryos in the absence of external carbohydrate or protein sources, indicating utilization of internal lipid stores [36]. They extended these findings to show carnitine stimulation of FAO associated with increased developmental potential of oocytes grown in follicle culture [38]. Other groups have reported similar beneficial effects of carnitine on oocytes from a variety of species (e.g., [37–42]), which is consistent with increased lipase activity during oocyte maturation [30]. Carnitine has beneficial effects beyond fatty acid utilization, also providing protection against reactive oxygen species [43]; however, results of experiments measuring FAO and effects of FAO inhibitors suggest a significant portion of its influence is through modulation of FAO. Manipulating FAO by providing media supplements such as carnitine holds promise for

improving developmental competence in oocytes matured in vitro [44].

In the present study, we have incorporated a metabolic assay for FAO to extend our work on meiotic induction, with special emphasis on more physiological hormonal stimulation. To this end, we 1) confirm the involvement of FAO in meiotic induction triggered by pharmacological PRKA activation and 2) demonstrate that hormone-induced maturation, whether occurring in vivo or in vitro, also requires FAO.

Materials and Methods

Chemicals

Equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG), and follicle-stimulating hormone (FSH) were purchased from the National Hormone and Peptide Program and Dr. A.F. Parlow, whereas amphiregulin (AR) and epidermal growth factor (EGF) were obtained from R&D Systems. AICAR was from Toronto Research Chemicals, RSVA405 from ChemBridge, and Compound C from Calbiochem (EMD Millipore). Tritiated palmitic acid and water were purchased from PerkinElmer. All the other chemicals, including L-carnitine, were supplied by Sigma-Aldrich.

Oocyte Isolation and Culture

C57BL/6JxSJL/J F1 mice were used for all the experiments. All the work was carried out with the preapproval of the Marquette University Institutional Animal Care and Use Committee. Nineteen- to twenty-three-day-old females were primed with 5 IU (international units) eCG and killed 48 h later by cervical dislocation. Ovaries were dissected out and placed in a dish containing culture medium, and CEO were isolated by puncturing large antral follicles with sterile 26 gauge needles. The CEO were washed several times and distributed to tubes containing 1 ml of appropriate medium or microwells of a 96-well plate with a final medium volume of 100 µl, depending on the experiment involved. The DO were obtained by repeated pipetting of CEO with a small bore pipet to remove cumulus cells. For culture in tubes, Eagle

minimum essential medium (MEM) containing L-glutamine and supplemented with 0.23 mM sodium pyruvate, 26 mM sodium bicarbonate, penicillin, streptomycin, and 3 mg/ml crystallized lyophilized bovine serum albumin (BSA) was used. Tubes were gassed with a humidified mixture of 5% CO_2/5% O_2/90% N_2, capped and sealed with parafilm, and incubated at 37°C in a water bath. For FAO assay cultures in 96-well plates and for experiments testing the effect of palmitic acid or carnitine on FSH-induced maturation, fatty acid-free BSA was used. For cultures in 96-well plates (both FAO assays and maturation experiments), the above medium was buffered with 12.5 mM HEPES and 15.5 mM sodium bicarbonate.

Follicle Culture

Ovaries from eCG-primed mice were transferred to a dish of Leibovitz L-15 medium supplemented with 3 mg/ml BSA, and antral follicles were dissected free using sterile 27 gauge needles. Follicles were then washed in MEM/5% fetal bovine serum (FBS) and transferred to 1ml of MEM/FBS in a 10 ml stoppered flask, gassed with 95% O_2/5% CO_2, capped, and incubated in a water-jacketed incubator at 37°C with constant, gentle agitation for 3.5 hours.

Solubilization of Palmitic Acid

For experiments testing the effect of palmitic acid doses on FSH-induced resumption of maturation, BSA-bound palmitic acid was used to enhance its solubility and reduce precipitation, and was prepared using a method described by Kane [45]. Five-hundred mg of fatty acid-free BSA was dissolved in 30 ml sterile water to which was added 23 mg palmitic acid in 1 ml of absolute ethanol. This mixture was shaken well till clear, frozen overnight, and lyophilized.

FAO Assay

FAO was measured using a modification of the protocol described by Dunning et al. [36]. All the cultures were carried out in a volume of 100 µl in wells of a 96-well plate and contained 9 µCi 9,10-[^{3}H] palmitate, with cold palmitate added to bring the final concentration to 0.3 mM. Medium also contained 3 mg/ml fatty acid-

free BSA, 12.5 mM HEPES, 15.5 mM sodium bicarbonate, and, when appropriate, stimulators or stimulators plus inhibitors of FAO. A 2× solution of palmitate/BSA was first made by adding 250 µl 12 mg/ml fatty acid-free BSA in sterile water, 36 µl 9,10-[^{3}H] palmitate (specific activity 32.4 Ci/mmol, 5 mCi in 1 ml ethanol), and 25 µl 24 mM cold palmitate in ethanol to a plastic test tube and drying down at 55°C under N_2 gas. It was then resuspended in 1 ml warm BSA-free MEM and maintained at 37°C in a water bath until use; 50 µl of this mixture was placed in each microwell, to which was added 50 µl MEM/fatty acid-free BSA (3mg/ml) containing the appropriate number of CEO or DO and 2× concentration of milrinone, an inhibitor of oocyte-specific phosphodiesterase, and appropriate FAO modulators. The 96-well plates were transferred to a modular incubator, gassed with a humidified mixture of 5% CO_2/5% O_2/90% N_2 and placed in a 37°C water-jacketed incubator. Following the culture, samples were processed as described by Dunning et al. [36], and 0.5 ml of the final supernatant was counted for each sample. Twenty-five CEO or 40 DO per well were used for each experiment. Experiments were carried out at least three times, with duplicate groups per treatment. For each experiment, blanks were included that contained no tissue to determine background counts that were then subtracted from the radioactivity from each sample, and values were corrected for dilution during processing, percent recovery, and dilution with cold palmitate.

Statistical Analysis

All the experiments were repeated at least three times, and those involving oocyte maturation included at least 25 CEO or DO per group per experiment. Percent GVB values were subjected to arcsine transformation followed by ANOVA and Duncan multiple range test to assess statistical significance, with a *P* value less than 0.05 considered significant. Maturation data are plotted as mean percent GVB ± SEM. FAO assay data were calculated as ratio of the treatment group compared to the control milrinone group and were analyzed by ANOVA followed by Duncan's multiple range test.

Biology of Reproduction, Vol 89(2), No. 43 (August 1, 2013): pg. 1-9. DOI.

Results

Pharmacological Activators of PRKA Induce Maturation and Stimulate FAO

We have previously shown that the PRKA activators, AICAR and RSVA405, stimulate meiotic maturation [10, 46] and that AICAR-induced maturation is prevented by agents that block FAO [32]. These results implicated FAO as an essential component of the meiotic induction process. It was therefore important to establish that FAO could indeed be demonstrated during meiotic induction triggered by pharmacological activation of PRKA. To this end, we used an assay reported by Dunning et al. [36] in which oxidation was determined by the production of tritiated water from radiolabeled palmitic acid. Before carrying out the FAO assays, we first established that meiotic induction was observed under the conditions of the assay, that is, when oocytes maintained in meiotic arrest with milrinone in 100 µl cultures within 96-well plates were stimulated with the appropriate meiotic inducer. Under these conditions, AICAR and RSVA405 triggered GVB in CEO by 52% and 25%, respectively (Supplemental Fig. S1, available online at www.biolreprod.org).

FAO assays were then carried out for CEO cultured in the presence of carnitine, PRKA activator, or the two agents together. Carnitine is a molecule that is bound to fatty acids at the outer mitochondrial membrane by the action of CPT1 and helps facilitate their transport into mitochondria, thereby promoting their oxidation [13]. In the presence of carnitine alone, FAO was stimulated in CEO by nearly 1.7- to 2.2-fold (Fig. 2, A and C). AICAR and RSVA405 also stimulated oxidation, by 1.6- and 1.3-fold, respectively. Although the stimulation by RSVA405 was not significant as determined using ANOVA, it was by the paired *t*-test. The addition of carnitine to AICAR- or RSVA405-containing medium did not result in an additive effect (Fig. 2, A and C). When DO were tested, AICAR stimulated GVB in the microwells (Supplemental Fig. S1); however, neither carnitine nor AICAR alone stimulated FAO, although the two agents together increased FAO by 1.8-fold (Fig. 2B).

Biology of Reproduction, Vol 89(2), No. 43 (August 1, 2013): pg. 1-9. DOI.

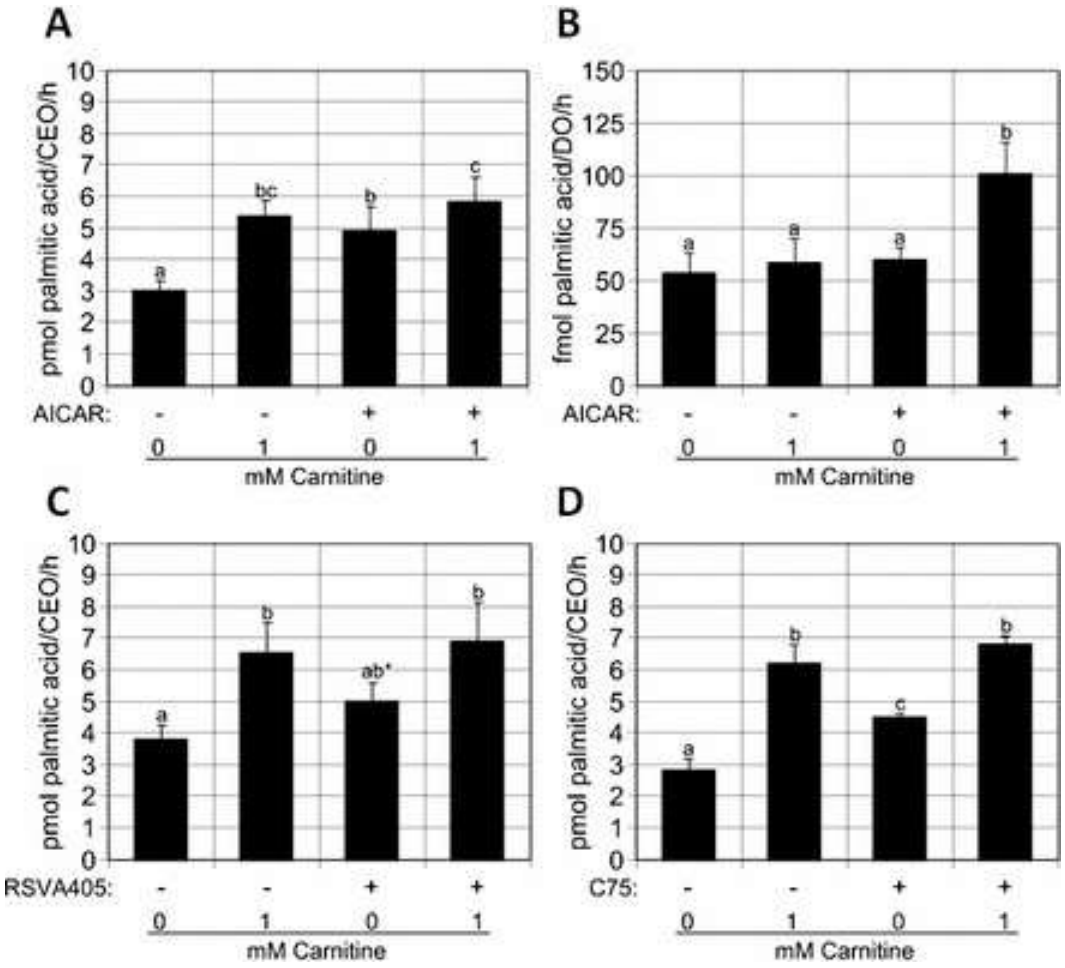

FIG. 2. Pharmacological activators of PRKA and C75, a CPT1 activator, stimulate FAO in oocytes. For the FAO assay, CEO (**A**, **C**) were maintained in meiotic arrest with 2 μM milrinone and cultured with or without 1 mM carnitine and/or 250 μM AICAR (**A**) or 1.5 μM RSVA405 (**C**) for 18 h. For assays with DO (**B**), oocytes were cultured in 1 μM milrinone ± 1 mM carnitine ± 125 μM AICAR for 8 h. In **D**, FAO levels were measured in CEO cultured in medium containing 2 μM milrinone ± 1 mM carnitine ± 25 μM C75. Groups with no common letter are significantly different. The asterisk indicates a significant difference from the control, as determined using the Student paired *t*-test ($P = 0.004$). The number of times each experiment was carried out is given in parentheses: **A** (3); **B** and **C** (5); **D** (4).

Our laboratory has shown that C75, a CPT1 activator [35], induces meiotic resumption [32]; herein, we confirmed that C75 stimulates meiotic resumption in 96-well plates (Supplemental Fig. S1). As shown in Figure 2D, C75 stimulated FAO in CEO by 1.6-fold. In this way, C75 stimulation of maturation was associated with an increase in FAO, and its effect in combination with carnitine was not additive.

Biology of Reproduction, Vol 89(2), No. 43 (August 1, 2013): pg. 1-9. DOI.

Hormone-Induced Resumption of Maturation Is Accompanied by an Increase in FAO Levels and Blocked by Inhibitors of CPT1 and FAO

We examined whether inhibitors of CPT1 and FAO affect FSH-induced maturation. CEO were cultured in tubes and maintained in meiotic arrest in medium containing dibutyryl cAMP (dbcAMP), a cAMP-mimicking agent [47], and FSH was added to induce maturation. To these FSH-treated cultures were added increasing doses of etomoxir, a pharmacological inhibitor of CPT1 [48], malonyl CoA, a physiological inhibitor of CPT1 [49], and mercaptoacetate, a direct inhibitor of FAO [34]. In control medium, only 13%–20% of the oocytes underwent GVB, whereas FSH stimulated maturation by 44%–64%, and this effect was reduced to control levels by all three inhibitors in a dose-dependent fashion (Fig. 3, A–C).

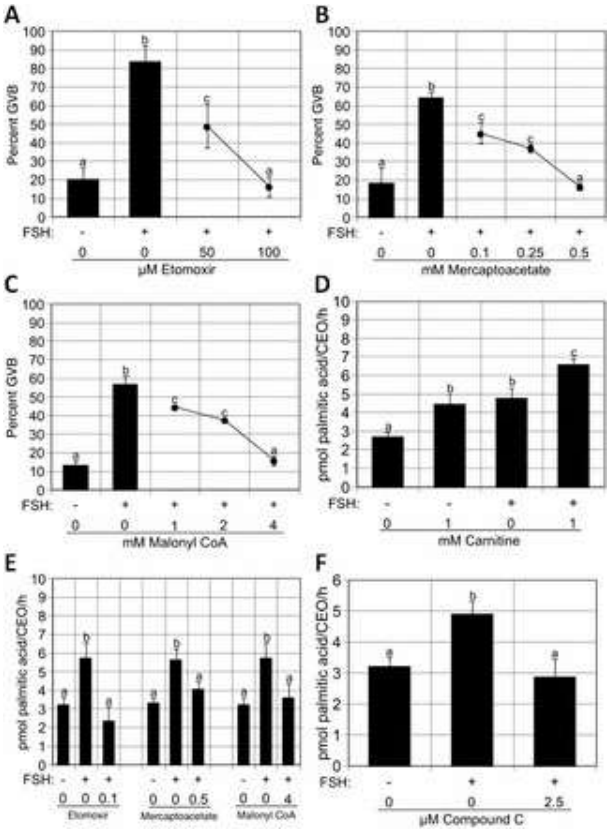

FIG. 3. FSH-induced maturation is blocked by inhibitors of FAO and accompanied by increased FAO. In **A**–**C**, CEO were cultured in medium containing 300 μM dbcAMP ±0.1 μg/ml FSH and exposed to different doses of etomoxir (**A**), mercaptoacetate (**B**), or

Biology of Reproduction, Vol 89(2), No. 43 (August 1, 2013): pg. 1-9. DOI.

malonyl CoA (**C**) for 17–18 h and assessed for GVB. FAO levels were measured in CEO cultured in 2 μM milrinone ± 0.1 μg/ml FSH for 18 h in the presence or absence of 1 mM carnitine (**D**), an FAO inhibitor (etomoxir, mercaptoacetate, or malonyl CoA) (**E**), or 2.5 μM compound C, a PRKA inhibitor (**F**). In **E**, concentrations of FAO inhibitors are in millimolar. Groups with no common letter are significantly different. The number of times each experiment was carried out is given in parentheses: **A**–**D** (3); **E** (6); **F** (4).

We also carried out assays to investigate the effect of FSH on FAO. As shown in Figure 3D, FSH alone stimulated FAO by 1.8-fold, whereas carnitine and FSH together had an additive effect (FAO increased by 2.6-fold). On the other hand, the three FAO inhibitors each prevented the FSH-induced increase in FAO (Fig. 3E). Also, FSH-stimulated FAO was blocked by Compound C (Fig. 3F), a PRKA inhibitor [12] that blocks FSH-induced meiotic resumption [5].

EGF has also been shown to induce GVB in cultured mouse CEO [50], and the maturation-inducing effect of FSH on the oocyte via cumulus cells is mediated by EGF-like peptides such as AR [51–53]. Thus, we tested if the three FAO inhibitors suppress AR-induced GVB. CEO were again cultured in tubes, maintained in meiotic arrest with dbcAMP, and AR was added to stimulate maturation. As shown in Figure 4, A–C, each inhibitor prevented AR-induced GVB. We also tested if AR stimulated meiotic resumption in microwells (Supplemental Fig. S1) but were unable to achieve meiotic induction even at a concentration 4-fold higher than those used in the tubes (data not shown). Thus, for FAO assays in microwells, EGF was used in place of AR because EGF successfully stimulated maturation in the wells (Supplemental Fig. S1). The EGF stimulation increased FAO by 1.6-fold, whereas together with carnitine, it had a greater stimulatory effect (2.1-fold increase; Fig. 4D) that was not significantly different from carnitine alone. The EGF-induced stimulatory effect was completely blocked by etomoxir (Fig. 4E). To determine if oocyte maturation was also affected, the action of etomoxir on EGF-stimulated, dbcAMP-arrested CEO was tested in tubes. Like its effect on other meiotic inducers, etomoxir significantly suppressed GVB in EGF-treated oocytes at the concentration used in the FAO assay (Fig. 4F).

Biology of Reproduction, Vol 89(2), No. 43 (August 1, 2013): pg. 1-9. DOI.

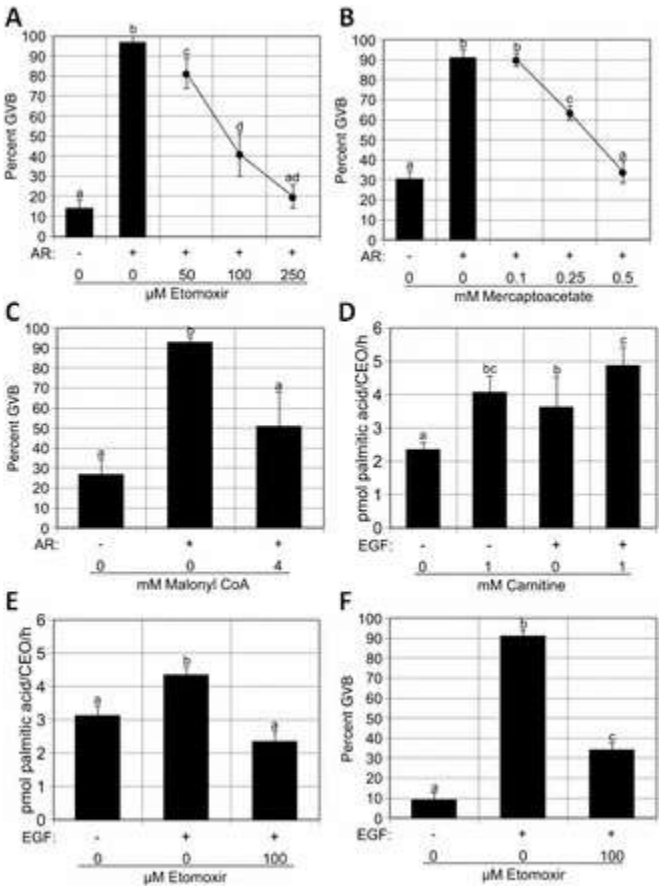

FIG. 4. Meiotic induction by EGF-like peptides is associated with an increase in FAO and blocked by inhibitors of FAO. In **A**–**C**, CEO cultured in medium containing 300 μM dbcAMP were stimulated with 50 ng/ml amphiregulin (AR), and exposed to different doses of etomoxir (**A**), mercaptoacetate (**B**), or malonyl CoA (**C**). In **F**, dbcAMP-arrested CEO were stimulated with 10 ng/ml EGF and treated with 100 μM etomoxir. All groups were assessed for GVB 17–18 h later. FAO was measured in CEO cultured in 2 μM milrinone ± 10 ng/ml EGF in the presence or absence of 1 mM carnitine (**D**) or 100 μM etomoxir (**E**) for 18 h. Groups with no common letter are significantly different. The number of times each experiment was carried out is given in parentheses: **A**–**C**, **E**, and **F** (3); **D** (5).

Carnitine and Palmitic Acid Enhance Hormone-Induced Meiotic Resumption

Because PRKA activation-induced meiotic resumption was augmented by either carnitine or palmitic acid [32], we tested whether the agents also promoted maturation in hormone-stimulated CEO. These experiments were carried out in plastic tubes. The CEO were maintained in meiotic arrest with dbcAMP, and different doses of carnitine or palmitic acid were added in the presence or absence of

FSH or AR. Both carnitine and palmitic acid significantly enhanced meiotic resumption at 1 mM and 0.5 mM, respectively, in the presence of FSH and AR, whereas they had no significant effect on GVB in the absence of hormone (Fig. 5, A and B).

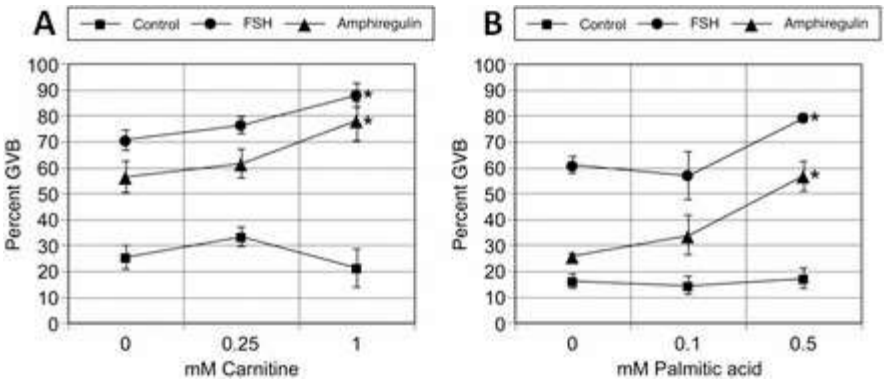

FIG. 5. Carnitine and palmitic acid enhance hormone-induced maturation. The CEO were cultured in medium containing 300 μM dbcAMP ±10 ng/ml FSH or 1 ng/ml AR, and treated with increasing concentrations of carnitine (**A**) or palmitic acid (**B**) and scored for percent GVB 17–18 h later. An asterisk denotes a significant difference from controls. The number of times each experiment was carried out is given in parentheses: **A** (3); **B** (4).

Human Chorionic Gonadotropin-Induced Meiotic Resumption and Stimulation of FAO Is Blocked by Etomoxir

Meiotic arrest in follicle-enclosed oocytes in culture is maintained by the granulosa cells, and meiotic resumption requires gonadotropin stimulation [54]. Therefore, follicle culture more closely mimics the physiological system of oocyte maturation than isolated oocyte-cumulus complexes, making it crucial to test the effect of FAO inhibitors on oocyte maturation in follicle-enclosed, hCG-stimulated oocytes. Follicles were cultured in inhibitor-free medium ± hCG, and the effect of etomoxir was tested. After 3.5 h of culture, 13% of control oocytes had undergone GVB, and hCG increased this number to 90% (Fig. 6A). The addition of etomoxir completely blocked hCG-stimulated maturation (14% GVB).

Biology of Reproduction, Vol 89(2), No. 43 (August 1, 2013): pg. 1-9. DOI.

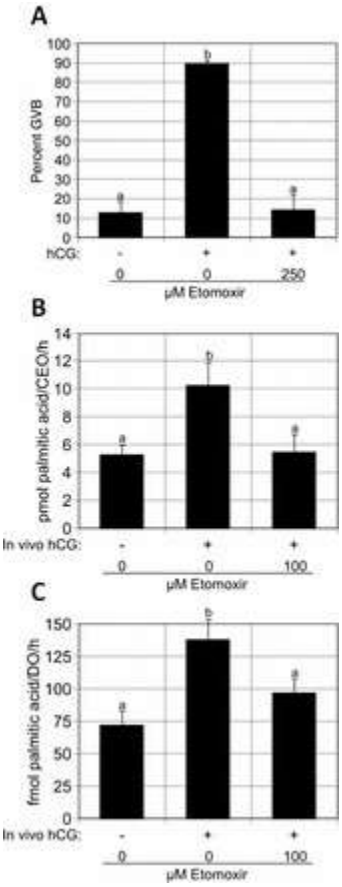

FIG. 6. Human chorionic gonadotropin-induced meiotic resumption and stimulation of FAO are blocked by etomoxir. In **A**, antral follicles from eCG-primed mice were cultured in the presence or absence of 5 IU/ml hCG ± 250 μM etomoxir, and GVB was assessed 3.5 h later. For **B** and **C**, eCG-primed mice were injected with 5 IU hCG, and CEO (**B**) or DO (**C**) were isolated 2 h post-hCG injection. FAO was measured after 16 h culture in the presence of 2 μM (**B**) or 1 μM (**C**) milrinone ±100 μM etomoxir. Oocytes from eCG-primed mice not injected with hCG were cultured as controls. Groups with no common letter are significantly different. Each experiment was carried out four times.

In addition, we measured FAO in oocytes isolated 2 h after hCG injection to eCG-primed mice. Both CEO and DO retrieved from hCG-injected mice displayed higher levels of FAO compared to control oocytes from primed mice not receiving hCG (1.9-fold increase), and this response was blocked by etomoxir (Fig. 6, B and C).

Discussion

In a previous study [32], the use of PRKA and FAO activators and inhibitors implicated FAO in the pharmacological stimulation of

meiotic resumption in vitro. Herein, we have confirmed this finding by carrying out FAO assays under similar experimental conditions. In addition, we extended these experiments by examining the role of FAO in hormone-induced GVB in both cumulus cell- and follicle-enclosed oocytes. Whether maturation was stimulated by FSH, EGF-related peptides, or hCG, two consistent findings were obtained: 1) meiotic induction was blocked by FAO inhibitors, and(2) the hormones stimulated FAO that was also sensitive to these inhibitors. Because the resumption of maturation requires the action of PRKA, these results provide compelling evidence that hormone-induced maturation in mouse oocytes requires a chain of events involving PRKA stimulation of FAO.

The PRKA activators AICAR and RSVA405 [8, 9] and the CPT1 activator C75 [35] all stimulate oocyte maturation. We have used a radioisotopic FAO assay to show that this meiotic resumption is associated with increased FAO, thereby confirming what was inferred by the use of FAO-modulating agents in our earlier study [32]. Treatment of CEO with these activators led to stimulation of FAO; however, in DO, AICAR stimulated FAO only when carnitine was present. Carnitine, a crucial compound for shuttling long chain fatty acids into mitochondria [13], also stimulated FAO by itself, but only in CEO. The lack of its effect in DO is not due to an inability of the oocyte to take up this compound because AICAR stimulation of FAO in DO was carnitine-dependent.

Baseline levels of palmitate oxidation in control CEO and DO maintained in meiotic arrest with milrinone were, on the average, 3.52 ± 0.12 pmol/CEO/h (n = 99) and 0.06 ± 0.01 pmol/DO/h (n = 9), respectively. These results indicate that the majority of FAO in CEO resides within the cumulus oophorus, confirming the results of Dunning et al. [36], and suggest that such cumulus cell activity may play a role in meiotic regulation. Nevertheless, the direct effects of FAO-modulating agents on FAO and meiotic maturation in DO ([32] and data herein) demonstrate that stimulation of FAO within the oocyte is essential for successful meiotic induction. It should be noted that our levels of FAO are considerably higher than those reported by Dunning et al. [36]. The reason for this is not clear but may be influenced by type of culture medium, strain of mouse, or the degree of palmitate solubilization.

Biology of Reproduction, Vol 89(2), No. 43 (August 1, 2013): pg. 1-9. DOI.

To further investigate the role of FAO in meiotic resumption, we examined its participation in hormone-induced maturation. Etomoxir, malonyl CoA, and mercaptoacetate were used to test the effect of FAO inhibition on FSH- or AR-induced GVB. All three inhibitors completely blocked induction of oocyte maturation by either hormone. Furthermore, FSH and EGF caused a 1.7- and ~1.5-fold stimulation of FAO, respectively, that was reduced to control levels or below by FAO inhibitors. The inability of AR to stimulate maturation in microwells was unexpected but may be due to interaction of the hormone with the plastic lining the microwells. Nevertheless, EGF effectively stimulated GVB and FAO, and its effects were blocked by etomoxir. The addition of carnitine to FSH-treated, but not EGF-treated, CEO produced an additive effect on FAO. In this way, we demonstrated that hormonal induction of maturation, like that achieved with PRKA activators, is accompanied by increased FAO, whereas inhibition of FAO blocks meiotic resumption. That carnitine and palmitic acid each significantly enhanced FSH- and AR-induced maturation provides further support for the participation of the FAO pathway in the mechanism(s) controlling hormone-induced GVB in isolated CEO in vitro. Moreover, because FSH-induced GVB requires activation of PRKA [5] and one of the major effects of PRKA activation in cells is increased FAO [55], the suppression of FSH-induced FAO by compound C indicates that active PRKA was crucial for the increase in FAO by FSH.

While carnitine alone stimulates FAO in CEO, it does not induce meiotic resumption, though it augments the meiosis-inducing action of PRKA [32] and hormone stimulation. This suggests that FAO is required, but by itself not sufficient, to induce GVB without stimulation of PRKA or some other physiological response. Yet C75 alone stimulates FAO to a similar extent but also triggers meiotic resumption; furthermore, the fatty acid derivatives palmitoyl carnitine and stearoyl carnitine also stimulate maturation, presumably by feeding directly into the FAO pathway [32]. The reason for these discrepancies is not clear, but possibilities include differing kinetics of FAO activation by various modulating agents or limitations in our assay in discriminating between subtle differences in FAO levels.

To increase the physiological relevance of our findings, we tested follicle cultures and oocytes retrieved 2 h after hCG administration to primed animals. We have previously shown that

compound C blocks hCG-stimulated maturation in cultured mouse follicles [56], thereby implicating PRKA. Thus, if PRKA also promotes maturation in vivo via stimulation of FAO, hCG-induced intrafollicular meiotic resumption should be sensitive to etomoxir treatment. Indeed, the increase in maturation of follicle-enclosed oocytes brought about by hCG was completely abrogated by etomoxir. And, as predicted, DO and CEO from hCG-injected mice displayed 1.8- to 1.9-fold increases in FAO that were inhibited by etomoxir. This finding is important because the FAO increase occurred in response to an in vivo hormonal stimulus and supports a role for FAO within the oocyte during intrafollicular meiosis reinitiation.

In an important series of experiments, Dunning et al. [36, 38] demonstrated etomoxir-sensitive hormonal regulation of FAO in mouse CEO and DO and augmentation of this effect with carnitine. In addition, they reported that modulation of FAO during follicular development and oocyte maturation has a profound effect on developmental competence. Interestingly, stimulation of FAO by carnitine in the absence of exogenous energy sources provided sufficient energy to drive embryo development. We have extended these findings to show that FAO is an essential feature of meiotic induction in mouse oocytes, whether it occurs in response to pharmacological stimulation in vitro or is driven by hormones in vitro or in vivo. Taken together, these data show a role for FAO throughout oocyte maturation, beginning at the earliest stage of GVB and extending to its completion at metaphase II. This conclusion parallels a similar protracted role we have presented for PRKA during mouse oocyte maturation [4, 5, 46, 54]. Based on the well-established regulatory role for PRKA in FAO and our confirmation of this relationship in the mouse oocyte, it is reasonable to propose that a significant portion of PRKA influence on mouse oocyte physiology during this period is mediated by FAO.

In conclusion, we have demonstrated an essential role for FAO in the meiotic induction process in mouse oocytes. The fact that mouse oocytes have very limited lipid reserves does not discount their potential importance because only a small amount of energy-rich lipid would be required to produce considerable energy via FAO to drive meiotic resumption [28, 36]. Meiotic maturation in the mouse oocyte requires PRKA activation [4] that leads to increased FAO ([32] and

Biology of Reproduction, Vol 89(2), No. 43 (August 1, 2013): pg. 1-9. DOI.

data herein) through the inactivation of ACAC located at the mitochondrial outer membrane ([36]; see *Introduction*). This would necessitate close proximity of lipid substrate to the mitochondria, and, consistent with this idea, mitochondria/lipid droplet colocalization has been demonstrated in the porcine oocyte [57]. It is possible that FAO is necessary during meiotic induction because it supplies energy and/or reducing equivalents at discrete locations within the oocyte that are not well served by carbohydrate metabolizing pathways. FAO may also remove fatty acids that would prove harmful to the oocyte or suppress maturation if allowed to reach threshold levels (e.g., through long-chain acetyl CoA synthetase-mediated palmitoylation reactions [58]). The increased evidence for lipid participation in oocyte development indicates that energy dynamics in this system are more complex than previously thought. Future studies will hopefully shed important light on the interplay between the different metabolic pathways involved in energy dynamics during gametogenesis.

Footnotes

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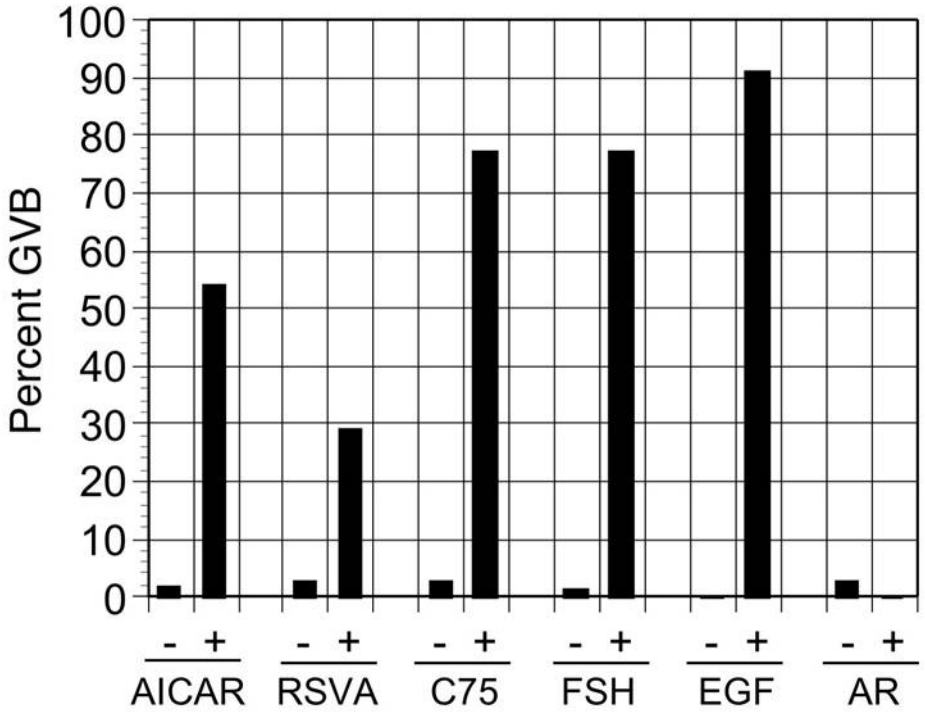
Supplementary Material



Supplemental Figure S1. Effects of pharmacological and physiological stimulators on meiotic maturation in the microwell culture system used for FAO assays. CEO were cultured 17-18 h in 2 μM milrinone ± 250 μM AICAR, 1.5 μM RSVA405, 25 μM C75, 0.1 μg/ml FSH, 10 ng/ml EGF or 50 ng/ml amphiregulin and scored for GVB 18 h later. AICAR stimulation was carried out twice, while all other treatments were tested three times and the means for each treatment are presented.