BIOLOGY OF REPRODUCTION **64,** 80–89 (2001)

Meiosis-Activating Sterol and the Maturation of Isolated Mouse Oocytes[1]

Stephen M. Downs,[2,4] Benfang Ruan,[5] and George J. Schroepfer, Jr.[3,5]

Biology Department,[4] Marquette University, Milwaukee, Wisconsin 53233
Department of Biochemistry,[5] Rice University, Houston, Texas 77005-1892

ABSTRACT

This study was carried out to examine the effects of the meiosis-activating C_{29} sterol, 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol (FF-MAS), on mouse oocyte maturation in vitro. Cumulus cell-enclosed oocytes (CEO) and denuded oocytes (DO) from hormonally primed, immature mice were cultured 17–18 h in minimum essential medium (MEM) containing 4 mM hypoxanthine plus increasing concentrations of FF-MAS. The sterol induced maturation in DO with an optimal concentration of 3 μg/ml but was without effect in CEO, even at concentrations as high as 10 μg/ml. Some stimulation of maturation in hypoxanthine-arrested CEO was observed when MEM was replaced by MEMα. Interestingly, the sterol suppressed the maturation of hypoxanthine-arrested CEO in MEM upon removal of glucose from the medium. FF-MAS also failed to induce maturation in DO when meiotic arrest was maintained with dibutyryl cAMP (dbcAMP). The rate of maturation in FF-MAS-stimulated, hypoxanthine-arrested DO was slow, as more than 6 h of culture elapsed before significant meiotic induction was observed, and this response required the continued presence of the sterol. Although the oocyte took up radiolabeled lanosterol, such accumulation was restricted by the presence of cumulus cells. In addition, lanosterol failed to augment FSH-induced maturation and was even inhibitory at a high concentration. Moreover, the downstream metabolite, cholesterol, augmented the inhibitory action of dbcAMP on maturation in both CEO and DO. Two inhibitors of 14α-demethylase, ketoconazole, and 14α-ethyl-5α-cholest-7-ene-3β,15α-diol that can suppress FF-MAS production from lanosterol failed to block consistently FSH-induced maturation. These results confirm the stimulatory action of FF-MAS on hypoxanthine-arrested DO but do not support a universal meiosis-inducing function for this sterol.

cumulus cells, gamete biology, meiosis, oocyte development, ovary

INTRODUCTION

Fully grown prophase I-arrested oocytes, competent to resume meiotic maturation, are maintained in meiotic arrest within ovarian follicles by inhibitory factors produced by the follicle. When removed from Graafian follicles and cultured in vitro, oocytes resume meiosis spontaneously and progress to the metaphase II stage before encountering a second meiotic arrest. However, meiotic resumption in vivo under normal circumstances requires gonadotropin stimulation and is mediated by the somatic compartment, which is thought to generate a positive stimulus that triggers germinal vesicle breakdown (GVB) [1].

In 1995, Byskov and colleagues [2] extracted lipophilic material from human follicular fluid and bull testicular tissue and tested HPLC fractions on hypoxanthine-arrested denuded mouse oocytes. Certain fractions proved to be stimulatory to oocyte maturation, and further analysis identified two active C_{29} sterols, a trienol from follicular fluid and a dienol from bull testes, that were termed meiosis-activating sterols, or MAS. The MAS found in follicular fluid (FF-MAS) was shown to be 4,4-dimethyl-5α-cholest-8,14,24-triene-3β-ol, an intermediate in the sterol biosynthetic pathway produced by demethylation of lanosterol by the cytochrome P_{450} enzyme, 14α-demethylase (see Fig. 1). The active component in bull testis (T-MAS) was identified as 4,4-dimethyl-5α-cholest-8,24-diene-3β-ol and is the next intermediate in this pathway produced from FF-MAS. The discovery of these sterols has generated much interest due to their possible importance as natural inducers of meiotic maturation.

Since this initial report, numerous studies using purified or synthetic FF-MAS have been carried out to evaluate its effectiveness in stimulating GVB in meiotically arrested oocytes. Grondahl et al. [3] and Ruan et al. [4] demonstrated the specificity of FF-MAS as an inducer of meiosis when compared to a series of related sterol compounds. In addition, GVB can be stimulated in both denuded and cumulus cell-enclosed oocytes when meiotic arrest is maintained with a variety of inhibitors, including hypoxanthine, isobutylmethylxanthine, and dibutyryl cAMP (dbcAMP) [5], and this effect is dependent upon protein synthesis [6]. Positive effects have also been reported on polar body formation and the developmental potential of FF-MAS-treated oocytes [5, 7]. Based on the meiosis-inducing ability of cocultured cumulus cells [8, 9], it has been proposed that FF-MAS produced by these cells acts directly on the oocyte to stimulate maturation [10].

Blocking downstream metabolism of FF-MAS has also been implicated in meiotic induction. GVB was stimulated in hypoxanthine-arrested cumulus cell-enclosed oocytes (CEO), but not denuded oocytes (DO), by AY9944, an inhibitor that acts at two points in the sterol biosynthetic pathway (Fig. 1). Evidence was presented that suppressing FF-MAS metabolism in the cumulus cells leads to accumulation of this intermediate above a meiosis-inducing threshold and thereby overcomes the meiotic arrest [11].

While the above data are consistent with a physiological role for FF-MAS in meiotic resumption, several lines of evidence cast doubt as to its importance. First, FF-MAS is not as effective in CEO as it is in DO. Because healthy oocytes are always surrounded by the cumulus oophorus in situ, the denuded state is an artifact of experimental manipulation and may not be the most physiological target for assessing FF-MAS activity. A concentration of 1.3 μM FF-MAS has been reported in human follicular fluid [10], but this concentration is relatively ineffective in triggering

[1]This research was supported in part by grants from the National Institutes of Health (HD25291 to S.M.D. and HL49122 to G.J.S.).
[2]Correspondence: Stephen M. Downs, Biology Department, Marquette University, 530 N 15 St., Milwaukee, WI 53233. FAX: 414 288 7357; e-mail: downss@marquette.edu
[3]Deceased 11 December 1998.

Received: 23 May 2000.
First decision: 23 June 2000.
Accepted: 25 July 2000.

ISSN: 0006-3363. http://www.biolreprod.org

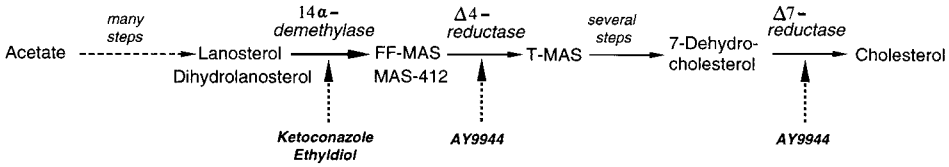

FIG. 1. Relevant portions of sterol biosynthetic pathway. FF-MAS is produced from lanosterol by the action 14α-demethylase (1). MAS-412 is formed from dihydrolanosterol by the same enzyme. FF-MAS is converted to T-MAS by the action of Δ4-reductase (2). The demethylase is inhibited by ketoconazole and 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (ethyldiol). AY9944 blocks the activity of both the Δ4-reductase and Δ7-reductase (3), the latter enzyme catalyzing the synthesis of cholesterol from 7-dehydrocholesterol.

GVB in mouse CEO [2, 3, 11]. The level of FF-MAS required to stimulate maturation in CEO may therefore be supraphysiological, but FF-MAS levels in mouse follicular fluid will need to be measured before this can be determined. Second, eCG priming of immature rats caused a threefold elevation of ovarian cytochrome P_{450} 14α-demethylase activity within 48 h [12]. Although FF-MAS is produced from lanosterol by this enzyme, no induction of GVB normally occurs in the absence of a subsequent ovulatory gonadotropin stimulus; hence, presumptive production of FF-MAS is not associated with meiotic resumption. Third, Tsafriri et al. [13] have demonstrated that an inhibitor of the demethylase, ketoconazole (Fig. 1), blocked hormone-induced progesterone production and ovulation in intact rats but had no effect on meiotic resumption. Similar effects on progesterone production and meiotic induction were obtained when LH-stimulated rat follicles were treated with ketoconazole [13], at concentrations that readily block demethylase activity in ovarian homogenates [12]. Thus, although enzyme activity was effectively suppressed, no loss of meiotic induction was observed, which fails to support the idea that FF-MAS mediates the action of gonadotropin on meiotic resumption.

The present study was undertaken to evaluate further the action of FF-MAS on mouse oocyte maturation. Stimulatory effects of FF-MAS have been assessed in isolated DO and CEO held in meiotic arrest with hypoxanthine and dbcAMP. The ability of DO, CEO, and oocyte-cumulus cell complexes (OOC) to incorporate radiolabeled precursors of FF-MAS has also been examined as well as the effects of lanosterol and cholesterol on oocyte maturation. Finally, two inhibitors of the 14α-demethylase were tested on FSH-induced maturation of arrested CEO. The results fail to provide compelling support for FF-MAS as an important mediator of hormone-induced meiotic maturation.

MATERIALS AND METHODS

Oocyte Isolation and Culture Conditions

Immature, (C57BL/6J × SJL/J) F_1 mice, 19–23 days old, were used for all experiments. Mice were primed with 5 IU eCG, and 2 days later ovaries were removed and placed in culture medium. Antral follicles were pierced with sterile needles and OCC, comprised of the oocyte and accompanying cumulus oophorus, were collected, washed, and allocated to the appropriate groups. The DO were obtained by repeated pipetting with a Pasteur pipette to remove the cumulus cells. Cumulus cell-enclosed oocytes (CEO) refers to oocytes that were cultured as intact OCC but then assessed for meiotic maturation or sterol accumulation after removal of the cumulus cells.

The culture medium used for most experiments was bicarbonate-buffered minimum essential medium (MEM) with Earle salts, supplemented with antibiotics, 0.23 mM pyruvate, and 3 mg/ml crystallized lyophilized BSA (ICN ImmunoBiologicals, Lisle, IL). For some experiments, the effects of sterol on oocyte maturation was compared in MEM and MEMα. Oocytes were cultured for varying times at 37°C in 1 ml medium in capped plastic or borosilicate glass tubes, after gassing with a humidified mixture of 5% CO_2, 5% O_2, and 90% N_2. Culture times are specified in the legends for each figure.

Uptake of Radiolabeled Lanosterol

The DO or OCC were cultured 6 h in medium containing approximately 10 000 cpm of [^{3}H]lanosterol or [^{3}H]dihydrolanosterol. At the end of the culture period, DO and OCC were washed free of radiolabel and transferred in a small volume to a scintillation vial. A comparable volume of the last wash dish constituted the blank for each treatment group. Cumulus cells were removed from some of the OCC at the end of culture to obtain DO that were then washed and processed for scintillation spectroscopy. These are referred to as CEO, and this treatment measured the effect of the intact cumulus oophorus on uptake by the oocyte. Scintillation fluid was added to each vial, and radioactivity was measured on a scintillation counter. The experiment was carried out three times, with 100 oocytes or complexes assayed per group per experiment.

Sterols and Other Chemicals

FF-MAS and 14α-ethyl-5α-cholest-7-ene-3β,15α-diol were synthesized as previously described [14, 15]. The FF-MAS samples showed no sterol impurities in the 500-MHz NMR spectra (1% detection limit). Culture medium components, hypoxanthine, dbcAMP, ketoconazole, lanosterol, and cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO). For the experiment comparing the effects of MEM and MEMα, both medium kits were purchased from GibcoBRL (Grand Island, NY).

Synthesis of [^{3}H]Lanosterol and [^{3}H]Dihydrolanosterol

(±)-[5-^{3}H]Mevalonolactone (400 μCi, 6.4 nmol; NEN, Boston, MA) was mixed with a 5% bicarbonate solution (500 μl) and incubated with 10 000 × *g* rat liver homogenate (10 ml) under anaerobic condition at 37°C for 3 h. The 10 000 × *g* rat liver homogenate was prepared as described [16] and fortified with 3 mM ATP, 1 mM NADP, 5 mM glucose-6-phosphate, 1 mM NAD, glucose-6-phosphate dehydrogenase (12 U), and 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (7 μg) in ethanol (7 μl). The incubation mixture was saponified with 15% KOH in 95% ethanol (15 ml) at 70°C for 2 h, extracted with hexane (3 × 100 ml), and evaporated to dryness under reduced pressure. The ^{3}H-labeled material was filtered through silica gel (50 mm × 5 mm column; elution with acetone:hexane 3:97), and the eluate was purified on Ag^+-HPLC [14] using a 300-mm × 10-mm column (elution with acetone:hexane 4:96; 3 ml/

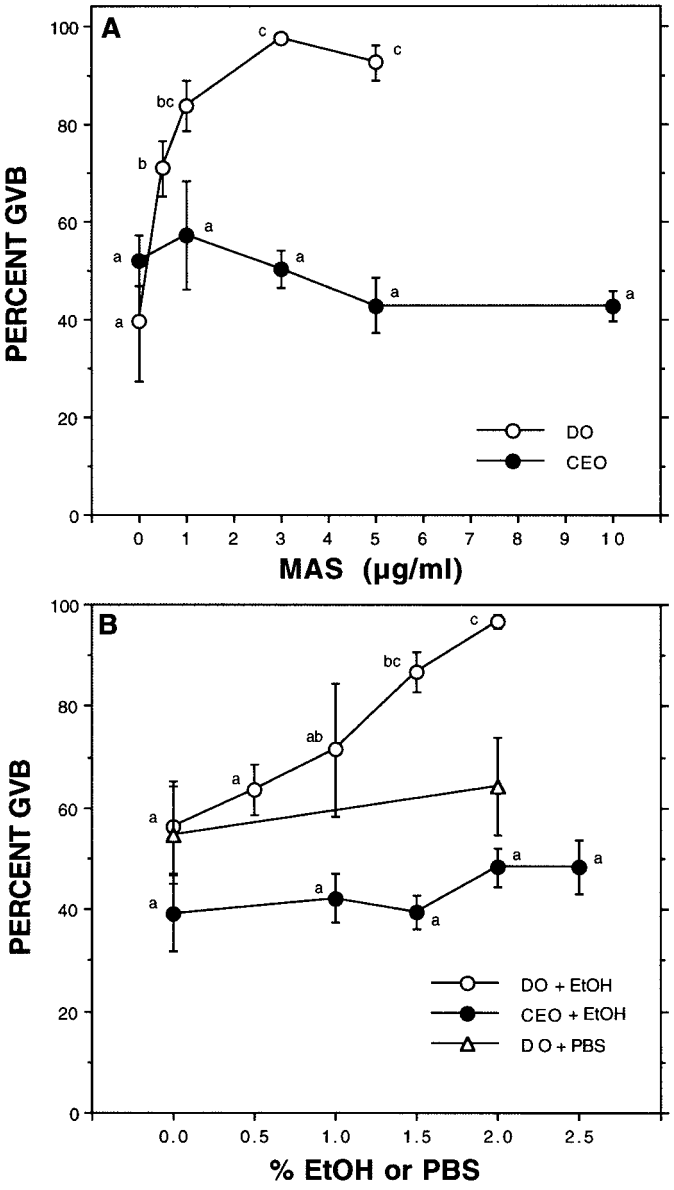

FIG. 2. Dose response of FF-MAS and ethanol on oocyte maturation. CEO and DO were cultured 17–18 h in medium containing 4 mM hypoxanthine plus increasing concentrations of FF-MAS (**A**) or ethanol (**B**). The CEO and DO were analyzed separately, and those groups with no identical letters are significantly different.

min; 3 ml fraction size). Evaporation of fractions 27–30 gave [^{3}H]dihydrolanosterol (0.37 μCi; 10 μCi/μmol); single component by radio-Ag$^+$-HPLC (300 mm × 3.2 mm column) with the same mobility as authentic dihydrolanosterol (t_R 11.3 min). Evaporation of fractions 45–49 gave ^{3}H-labeled lanosterol (0.39 μCi; 10 μCi/μmol); single component by radio-Ag$^+$-HPLC (300 mm × 3.2 mm column) with the same mobility as authentic lanosterol (t_R 20.9 min).

Statistics

Oocyte maturation experiments were carried out a minimum of three times with a minimum of 30 oocytes per treatment group per experiment. Results are expressed as the mean percent GVB ± SEM. Maturation frequencies were subjected to arcsin transformation and analyzed by ANOVA followed by Duncan's multiple range test. Paired groups were compared by Student's *t*-test. A *P* value less than 0.05 was considered significant.

RESULTS

Meiotic Induction in CEO and DO by FF-MAS

The DO or CEO were cultured 17–18 h in medium containing 4 mM hypoxanthine plus increasing concentrations of FF-MAS from 0.5 to 10 μg/ml and then assessed for GVB. As shown in Figure 2A, FF-MAS dose-dependently stimulated maturation in DO. In the hypoxanthine control group, 40% of the DO had undergone GVB, but all concentrations of FF-MAS increased this percentage, with 97% GVB at 3 μg/ml. Interestingly, no significant effect of FF-MAS was observed in CEO, even at concentrations as high as 10 μg/ml.

To examine the effect of the FF-MAS vehicle on oocyte maturation, hypoxanthine-arrested DO and CEO were exposed to increasing concentrations of ethanol from 0 to 2.5%. Because FF-MAS dilutions were prepared from a 1-mg/ml stock solution, 1% ethanol corresponds to an FF-MAS concentration of 10 μg/ml. Similar to the effects of FF-MAS, ethanol dose-dependently stimulated maturation in DO (by 40% at 2% ethanol) but had no effect on CEO even at concentrations as high as 2.5% (Fig. 2B). The stimulation of DO was not due to dilution of hypoxanthine, because 2% PBS was without effect. It is important to point out that although higher concentrations of ethanol were able to stimulate GVB significantly in hypoxanthine-arrested DO, the concentrations required were higher than those present in the stimulatory FF-MAS preparations; e.g., 3 μg/ml FF-MAS was most potent in stimulating maturation in DO, but the ethanol present was 0.3%, a concentration that would have no effect alone on GVB. Thus, the action of FF-MAS is specific for this sterol and is not likely a vehicle effect.

We considered whether the lack of effect of FF-MAS on CEO might be due to an inhibitory influence of the cumulus cells that impedes sterol action on the oocyte. Results from a previous study showed that glucose, acting through the glycolysis pathway, augments the inhibitory action of hypoxanthine in CEO in association with elevated ATP levels [17]. An experiment was therefore carried out to determine if glucose removal would allow a stimulatory effect of FF-MAS on CEO to be manifested. The CEO were cultured 17–18 h in hypoxanthine-supplemented MEM in the presence or absence of 5.5 mM glucose, and FF-MAS was added at a concentration of 3 μg/ml. A parallel culture of DO was carried out as a positive control. In the presence of glucose, FF-MAS again had no stimulatory effect on the maturation of CEO (Fig. 3). Elimination of glucose increased the maturation percentage in FF-MAS-free cultures from 14% to 47%, but curiously, under glucose-free conditions FF-MAS was inhibitory, with only 15% GVB observed in its presence, a maturation frequency identical to that in glucose-containing medium. In the denuded oocyte-positive control group, FF-MAS stimulated maturation from 23% to 73% GVB.

The effects of FF-MAS were also tested on dbcAMP-arrested DO. The DO were cultured 17–18 h in medium containing 300 μM dbcAMP alone or dbcAMP plus 3 μg/ml FF-MAS. This experiment was repeated eight times. In the presence of dbcAMP alone, 35% of the oocytes resumed maturation; however, FF-MAS had no effect on maturation (Fig. 4, left panel). Because this result conflicted

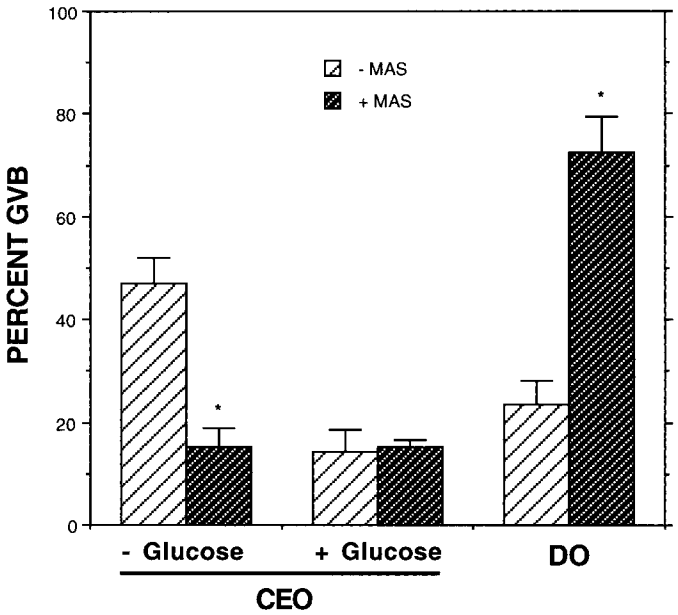

FIG. 3. Effect of glucose on FF-MAS-induced maturation in CEO. CEO or DO were cultured 17–18 h in medium containing 4 mM hypoxanthine ± 3 μg/ml FF-MAS. The CEO were cultured in either glucose-supplemented (5.5 mM) or glucose-free medium, while DO were cultured in the presence of glucose. An asterisk denotes a significant difference from the −FF-MAS control.

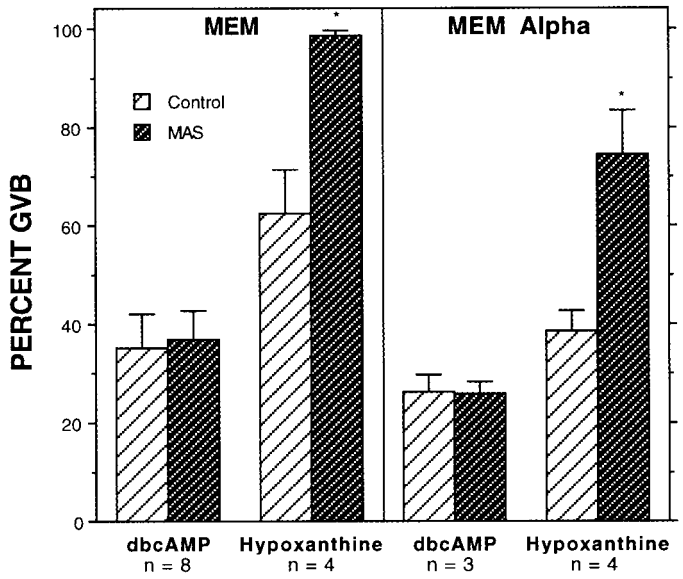

FIG. 4. Effect of FF-MAS on hypoxanthine- and dbcAMP-arrested DO maturation in MEM and MEMα. DO were cultured 17–18 h in MEM (left panel) or MEMα (right panel) containing either 300 μM dbcAMP or 4 mM hypoxanthine ± 3 μg/ml FF-MAS. The number of times each experiment was carried out is shown below the inhibitor. An asterisk denotes a significant difference from the −FF-MAS control.

with a stimulatory effect previously reported by Grondahl et al. [3], a parallel set of hypoxanthine-treated groups was tested as a positive control. Consistent with the reported results, FF-MAS at the same concentration was stimulatory to hypoxanthine-arrested DO (Fig. 4, left panel).

The kinetics of FF-MAS-induced maturation were next examined. The DO were cultured in 4 mM hypoxanthine ± 3 μg/ml FF-MAS, and oocytes were assessed for GVB at 3, 6, and 12 h. At 3 and 6 h, no significant difference in GVB was observed in the FF-MAS treatment groups when compared to the corresponding FF-MAS-free groups (Fig. 5). By 12 h, however, significant stimulation was evident (92% versus 63% GVB in the presence and absence of FF-MAS, respectively). It should be noted that the basal level of maturation in FF-MAS-free medium was higher in this particular experiment than in the other experiments in this study. The reason is not readily apparent, but these frequencies fall within a previously observed range of maturation percentages for hypoxanthine-treated DO.

To establish if a brief FF-MAS treatment would suffice to stimulate GVB or if a more protracted exposure was required, a washout experiment was performed. The DO were cultured 17–18 h in medium containing 4 mM hypoxanthine ± 3 μg/ml FF-MAS. A third group was exposed to FF-MAS for 3 h, and then the oocytes were washed free of FF-MAS and returned to hypoxanthine medium for 14–15 h. Three hours of FF-MAS exposure had no effect on maturation, as 29% GVB was observed in treated oocytes compared to 26% in untreated oocytes (Fig. 6). That a long-term exposure to FF-MAS was required for meiotic induction was demonstrated by the 70% GVB exhibited by DO exposed continuously to FF-MAS.

Does the Type of Culture Medium Influence the Efficacy of FF-MAS as a Meiosis-Inducing Agent?

The above results (Figs. 2–4) are inconsistent with previous reports in that we were unable to demonstrate FF-MAS stimulation of GVB in either hypoxanthine-arrested CEO or dbcAMP-arrested DO. One possible explanation is that the present experiments were carried out with MEM, while MEMα has been used in MAS studies in other laboratories. Notable differences between the two media are the presence in MEMα of ribo- and deoxyribonucleosides as well as a higher concentration of pyruvate (1 mM; we routinely supplement MEM with 0.23 mM pyruvate). To test if differences in the type of culture medium influence the meiosis-inducing capability of FF-MAS, we compared FF-MAS activity in the two media.

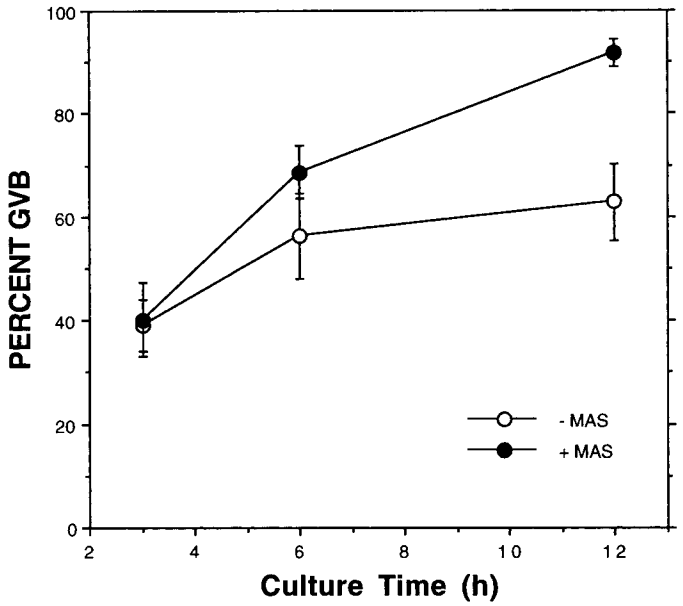

FIG. 5. Kinetics of FF-MAS-induced maturation. DO were cultured for varying periods of time up to 12 h in medium containing 4 mM hypoxanthine ± 3 μg/ml FF-MAS. The FF-MAS-treated group was significantly different only at the 12-h time point.

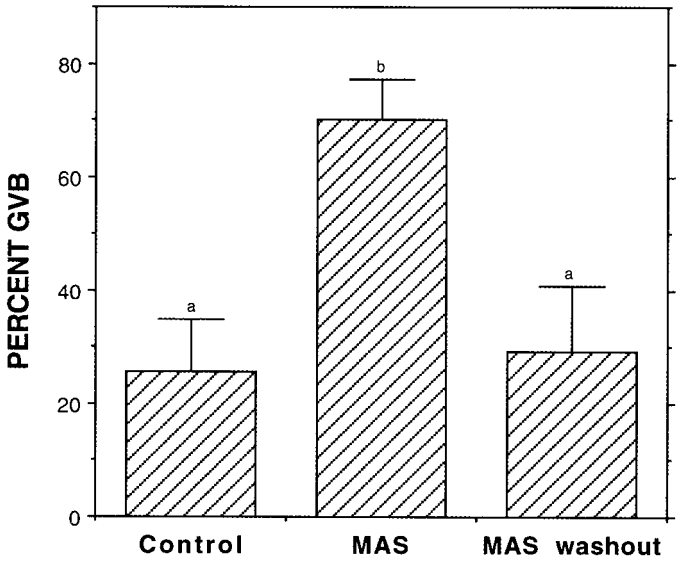

FIG. 6. The effect of FF-MAS washout on meiotic induction. DO were cultured in medium containing 4 mM hypoxanthine ± 3 μg/ml FF-MAS. After 3 h, half of the FF-MAS-treated oocytes were washed free of FF-MAS and returned to control medium minus FF-MAS for 17–18 h. The remaining FF-MAS-treated oocytes remained in FF-MAS-containing medium for the duration of the experiment. A parallel group remained in control, FF-MAS-free medium for the entire time. Bars with a different letter are significantly different.

In the first set of experiments, CEO were maintained in meiotic arrest in 4 mM hypoxanthine in MEMα and were treated with increasing concentrations of FF-MAS or the corresponding concentration of ethanol and after 17–18 h were assessed for GVB. As shown in Figure 7A, ethanol had no significant effect on maturation, but a small dose-dependent stimulation of maturation was observed in FF-MAS-treated CEO, with 10 μg/ml producing an increase in GVB from 6% to 33%. A parallel experiment was carried out in MEM using the highest concentration of FF-MAS (10 μg/ml) and the corresponding concentration of ethanol vehicle (1%). The number of CEO undergoing GVB in hypoxanthine alone was somewhat higher in MEM (23%), but neither FF-MAS nor ethanol vehicle significantly altered the maturation percentage (Fig. 7B).

In the second set of experiments, the meiosis-inducing action of FF-MAS on dbcAMP-arrested DO was tested in MEMα. A separate group of hypoxanthine-arrested DO was used as a positive control. The DO were cultured 17–18 h in medium containing 300 μg/ml dbcAMP or 4 mM hypoxanthine ± 3 μg/ml FF-MAS. As shown in Figure 4 (right panel), the maturation percentage in hypoxanthine-treated DO was significantly increased by FF-MAS (from 39% to 75%); however, consistent with the earlier results in MEM, no stimulation of GVB was observed in the dbcAMP-treated groups. Thus, the type of medium cannot account for our inability to demonstrate FF-MAS-stimulated maturation in dbcAMP-arrested oocytes.

Do Cumulus Cells Influence Sterol Uptake by the Oocyte?

One possible explanation for the different effects of FF-MAS in DO and CEO is that cumulus cells somehow impede accessibility of FF-MAS to the oocyte so that CEO are exposed to lower levels of the sterol. To test this idea, uptake of sterol was compared in OCC, DO, and CEO (cultured with cumulus oophorus intact, followed by cumulus cell removal) by 6-h culture in medium containing [^{3}H]lanosterol or [^{3}H]dihydrolanosterol. These two compounds are substrates for 14α-demethylase that converts lanosterol to FF-MAS and dihydrolanosterol to 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol, another sterol with meiosis-inducing activity [12, 14]. As shown in Figure 8, the greatest amount of label was taken up by OCC, with 41–49% as much sterol in the DO. Interestingly, only 17–20% as much sterol accumulated in CEO as in DO. These results

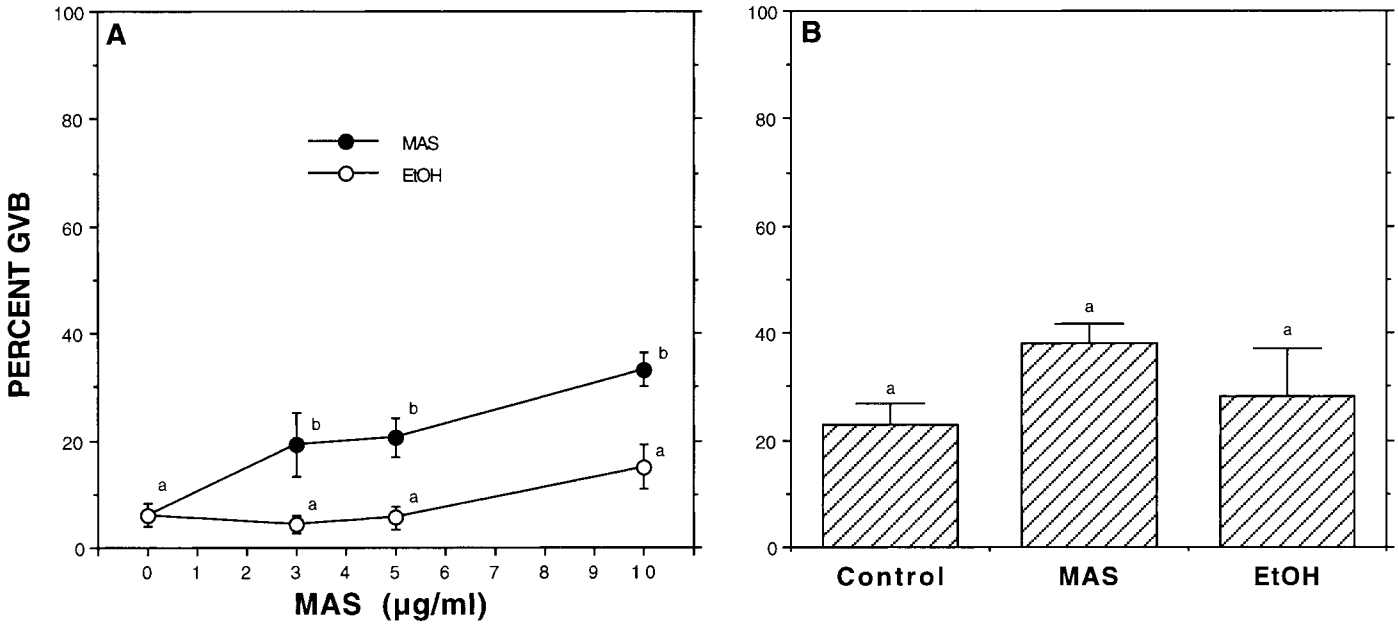

FIG. 7. Effect of the type of culture medium on FF-MAS induction of maturation in CEO. **A**) CEO were cultured 17–18 h in MEMα containing 4 mM hypoxanthine plus increasing concentrations of FF-MAS or the corresponding concentration of ethanol. The FF-MAS- and ethanol-treated groups were analyzed separately, and a different letter denotes a significant difference. **B**) CEO were cultured 17–18 h in MEM containing 4 mM hypoxanthine ± 10 μg/ml FF-MAS or the corresponding concentration of ethanol (1%). No significant difference was detected between any of the three groups.

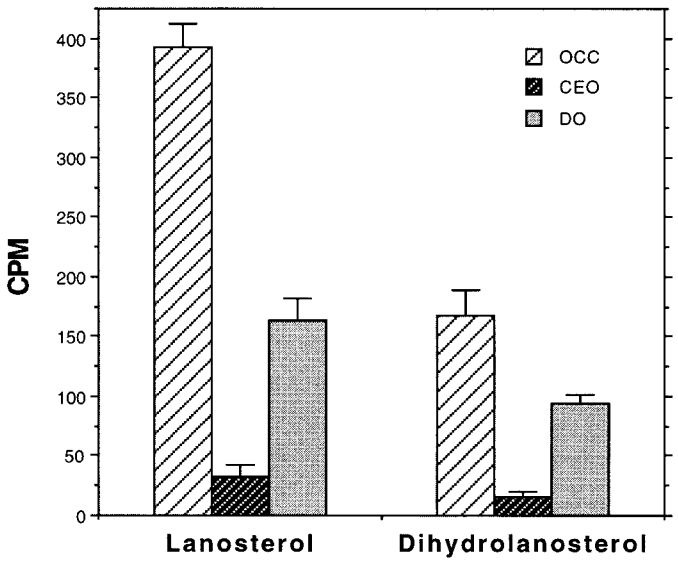

FIG. 8. Lanosterol and dihydrolanosterol uptake by OCC, CEO, and DO. DO or CEO were cultured for 6 h in medium containing radiolabeled lanosterol or dihydrolanosterol. At the end of the culture period, cumulus cells were removed from some of the complexes, and uptake by these CEO as well as by DO and the intact OCC was determined. One hundred oocytes or complexes were assayed per group per experiment, which was repeated three times.

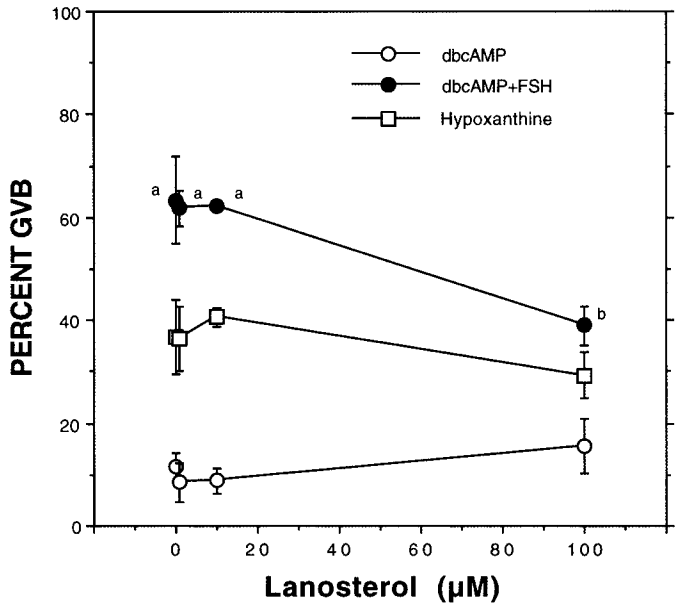

FIG. 9. Effect of lanosterol on oocyte maturation. CEO were cultured 17–18 h in medium containing 4 mM hypoxanthine, 300 μM dbcAMP or dbcAMP plus FSH, and exposed to increasing concentrations of lanosterol from 1 to 100 μM. No differences in maturation were observed in either of the FSH-free groups. In the dbcAMP plus FSH group, a different letter denotes a significant difference.

are consistent with the idea that cumulus cells hinder FF-MAS uptake by the oocyte.

The above experiment demonstrated that OCC take up and accumulate lanosterol from the culture medium. Because it has been proposed that hormone-induced maturation is mediated by increased synthesis of FF-MAS by the somatic compartment [2, 10], it was therefore important to test whether lanosterol, the immediate precursor of FF-MAS, would 1) reverse the meiotic arrest maintained by hypoxanthine or dbcAMP or 2) augment the meiotic resumption induced by FSH in dbcAMP-arrested oocytes. To this end, CEO were cultured 17–18 h in medium containing 4 mM hypoxanthine or 300 μM dbcAMP (±FSH) and subjected to increasing concentrations of lanosterol from 0 to 100 μM. In hypoxanthine or dbcAMP alone, lanosterol had no effect on oocyte maturation (Fig. 9), which agrees with a previous report [2]. Lanosterol was also without effect on FSH-treated oocytes at 1 and 10 μM, but, surprisingly, at 100 μM it reduced the maturation frequency from 63% to 39%.

The reduced maturation frequency at the highest concentration of lanosterol raised the possibility that metabolites downstream from FF-MAS may exert an inhibitory influence on maturation. To test this idea, DO and CEO were cultured 17–18 h in the presence of 150 μM dbcAMP plus increasing concentrations of cholesterol from 1–50 μM. The lower concentration of dbcAMP was used to illustrate better the suppressive action of cholesterol, because preliminary experiments showed it to be inhibitory. As shown in Figure 10, cholesterol dose-dependently lowered the maturation frequency in both groups, with CEO exhibiting greater sensitivity. Maturation in CEO was reduced over 30% by 10 μM cholesterol, while comparable inhibition in DO required 50 μM cholesterol.

Does Treatment with Sterol Synthesis Inhibitors Block FSH-Induced Maturation?

In the last series of experiments, we tested the effects of two inhibitors of the 14α-demethylase (Fig. 1) on FSH-induced maturation in meiotically arrested oocytes. The CEO were cultured 17–18 h in medium containing 300 μM dbcAMP, ± FSH, and increasing concentrations of ketoconazole from 0 to 5 μM. In the absence of FSH, only 17% of the dbcAMP-treated oocytes resumed maturation, and ketoconazole lowered this percentage, with significant inhibition at 5 μM (5% GVB; Fig. 11A). Follicle-stimulating hormone increased the maturation frequency to 72%, but this was dose-dependently suppressed by ketoconazole such that stimulation was eliminated at 5 μM (18% GVB).

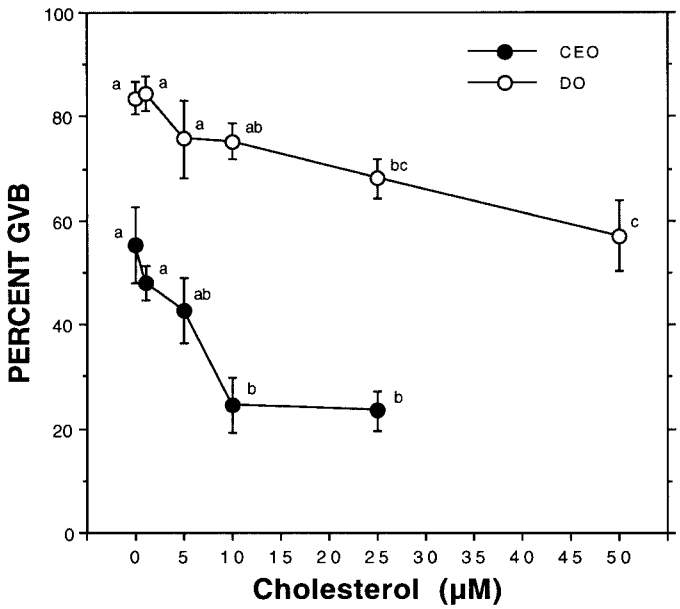

FIG. 10. Effect of cholesterol on oocyte maturation. CEO or DO were cultured 17–18 h in medium containing 150 μM dbcAMP plus increasing concentrations of cholesterol from 1 to 50 μM. Groups with and without FSH were analyzed separately, and those groups with no identical letters are significantly different.

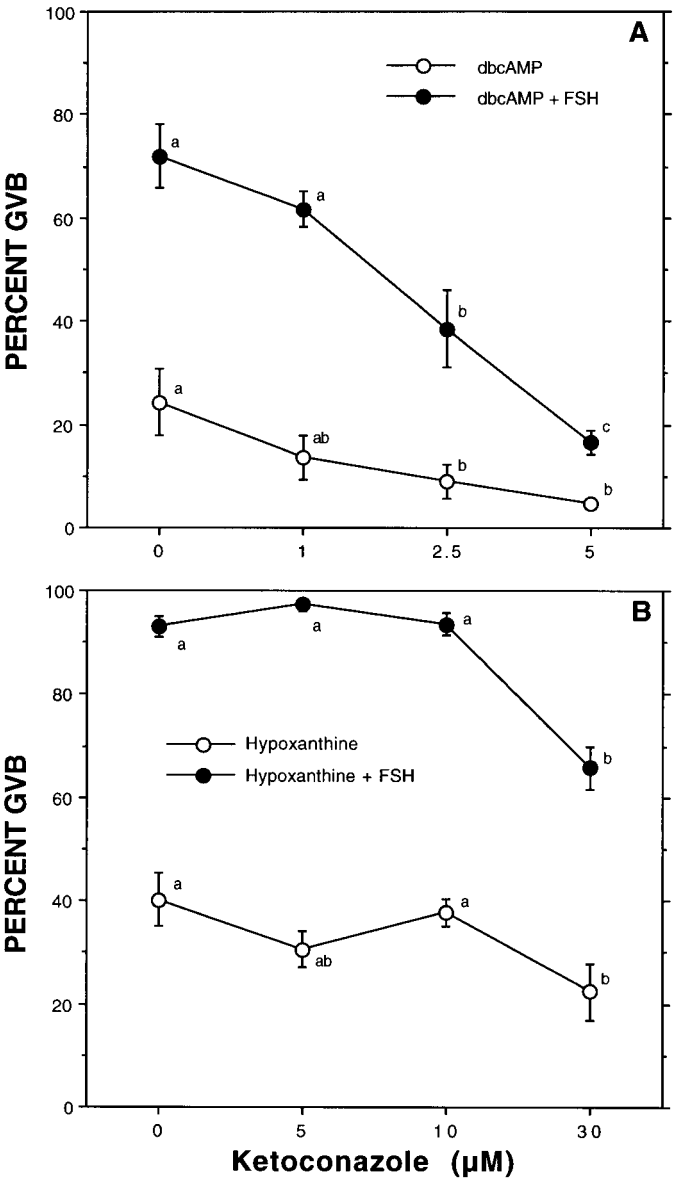

FIG. 11. Effect of ketoconazole on meiotic maturation. CEO were cultured 17–18 h in medium containing 300 μM dbcAMP ± FSH (**A**) or 4 mM hypoxanthine ± FSH (**B**). Ketoconazole was added in increasing concentrations from 1 to 30 μM. The CEO and DO were analyzed separately, and those groups with no identical letter are significantly different.

This experiment was repeated but with meiotic arrest maintained with 4 mM hypoxanthine. Ketoconazole again had a slight suppressive effect on maturation in the absence of FSH, but higher levels of the drug were required (22% GVB at 30 μM compared to 40% in controls; Fig. 11B). Follicle-stimulating hormone increased the maturation frequency to 95%, but very little inhibitory action of ketoconazole was observed: GVB was not suppressed at 5 or 10 μM and was reduced only to 66% at 30 μM. Note that a sixfold higher concentration of the inhibitor produced a much less effective inhibition in hypoxanthine-treated oocytes than in dbcAMP-treated oocytes.

The last experiment tested the effect of a second inhibitor of 14α-demethylase, 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (ethyldiol) [18], on FSH-induced maturation in dbcAMP-arrested oocytes. CEO were again cultured 17–18 h in medium containing 300 μM dbcAMP ± FSH and increasing concentrations of ethyldiol from 0 to 10 μM. As

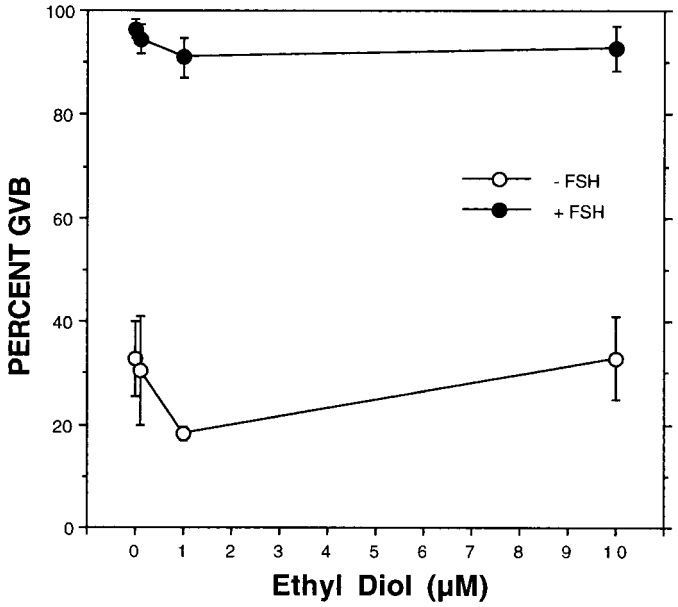

FIG. 12. Effects of ethyldiol on oocyte maturation. CEO were cultured 17–18 h in medium containing 300 μM dbcAMP ± FSH and increasing concentrations of ethyldiol from 0.1 to 10 μM. Groups with and without FSH were analyzed separately, and no significant differences were detected between any of the groups.

shown in Figure 12, the inhibitor had no effect on maturation in either the presence or absence of FSH.

DISCUSSION

This study has addressed the potential role of meiosis-activating sterols on mouse oocyte maturation in vitro. Our results confirm a stimulatory effect of FF-MAS on oocyte maturation in hypoxanthine-arrested denuded mouse oocytes. Meiosis was not induced by FF-MAS in hypoxanthine-arrested CEO when cultured in MEM, although modest induction occurred in MEMα. The reduced effectiveness of the sterol in CEO compared to DO is apparently due in part to its sequestration by cumulus cells. The FF-MAS became inhibitory to maturation in CEO when glucose was removed from the medium and failed to stimulate maturation in dbcAMP-arrested DO in either MEM or MEMα. Long-term exposure to the sterol was required for meiotic induction that was characterized by slow kinetics. Although taken up by complexes, the FF-MAS precursor lanosterol failed to augment meiotic resumption induced by FSH and at a high concentration became inhibitory. Moreover, cholesterol, a downstream product of sterol biosynthesis, proved inhibitory to both CEO and DO maintained in meiotic arrest with dbcAMP. Finally, when tested on FSH-induced maturation, two inhibitors of 14α-demethylase had inconsistent effects on meiotic resumption. Collectively, these data fail to support a physiological role for FF-MAS in meiotic induction in isolated mouse oocytes.

If FF-MAS is an important regulator of meiosis in situ, one would expect effective stimulation of meiotic resumption in CEO as well as DO. However, in MEM, FF-MAS proved ineffective in CEO at concentrations up to 10 μg/ml that corresponds to 24.4 μM. These results conflict with those of others who have demonstrated modest stimulation in CEO at FF-MAS concentrations of 0.7–7 μM [2, 3, 11]. Yet, in the present study, the optimal concentration of FF-MAS for meiotic induction of DO, 3 μg/ml (7.3 μM), compares well with the 7 μM used by Grondahl et al. [3] to

achieve similar results, thus verifying a potent, direct stimulatory action of FF-MAS on the oocyte. The reason for our discrepant results with CEO is unclear but may be due to the choice of culture medium: we cultured oocytes in MEM, whereas other groups have used MEMα (although note that one laboratory has used MEMα with ribonucleosides removed [3]). When the effects of FF-MAS on hypoxanthine-arrested CEO were compared in these two media, stimulation of GVB was achieved in MEMα but not in MEM. However, FF-MAS did little more than negate an increased inhibition brought about by MEMα. Hypoxanthine was more inhibitory in MEMα than MEM, probably due to the presence of ribo- and deoxyribonucleosides in the former medium, but the percentage of maturation in the two groups treated with 10 μg/ml FF-MAS was not different (33–38% GVB).

It might also be argued that differences in macromolecular supplementation, such as the source and concentration of albumin [3, 5], could explain the inability of FF-MAS to trigger GVB in CEO in our experiments. However, under the culture conditions employed in this study, FSH successfully induced meiotic resumption in a high percentage of CEO. If FF-MAS mediates FSH action, one would have expected FF-MAS to be as effective as FSH under these same conditions. Such results raise the possibility that the positive action of FF-MAS on CEO is contingent on culture conditions. In support of this idea, when glucose was removed from the medium, FF-MAS actually became inhibitory to GVB in hypoxanthine-arrested CEO. It is possible that this change in the culture medium altered sterol processing by the cumulus cells and thereby its influence on meiosis.

Within developing follicles, healthy oocytes are enclosed by the cumulus oophorus that plays a pivotal role in oogenesis. These somatic cells serve to filter and/or mediate the effects of gonadotropins, growth factors, steroids, and other factors that enter from the bloodstream or are locally produced. They also contribute to the syncytial nature of the follicle wherein a direct cell-cell coupling pathway facilitates the transfer of important signals between the germ and somatic cell compartments. While the removal of cumulus cells simplifies interpretation, it also disrupts this syncytial condition and alters the metabolic characteristics of the oocyte. Consequently, oocytes may behave differently in the denuded state than in the cumulus cell- or follicle-enclosed state. Such differences in behavior were manifested in the present study. Grondahl et al. [3] acknowledged the lower efficiency of FF-MAS in CEO when compared to DO and speculated that this might be due either to release by the cumulus cells of inhibitory factors that negate the stimulatory action of FF-MAS or to a loss of sterol accessibility to the oocyte. The present study sheds little light on the former possibility, although results indicate that the presence of glucose does not impede a positive action of the sterol. The second possibility is supported by uptake data. Cumulus cells appear to suppress uptake of sterol intermediates by the oocyte, as oocyte accumulation of radiolabeled lanosterol and dihydrolanosterol was dramatically reduced by the presence of cumulus cells. It is likely that a similar relationship exists for FF-MAS uptake and might account for its reduced efficacy in CEO. Leonardsen et al. [11] proposed that FF-MAS may be ineffective in CEO because cumulus cells metabolize FF-MAS and T-MAS to inactive sterols or steroids; consequently, meiotic induction by MAS in situ would require downstream metabolism to be suppressed by gonadotropin, thereby leading to accumulation of FF-MAS. However, inconsistent with this idea is the finding that FSH, which is a potent inducer of GVB in CEO, has been shown to augment cholesterol synthesis in granulosa cells [19].

It is important to focus on the stimulation of maturation in cumulus cell- or follicle-enclosed oocytes, and physiological concentrations of FF-MAS within the follicle need to be determined at the time GVB is being stimulated. For example, Byskov et al. [10] have reported FF-MAS concentrations of 1.3 μM in follicular fluid obtained in a human in vitro fertilization (IVF) program, but this is low when compared to the levels required to trigger GVB in cultured mouse CEO. If intrafollicular concentrations are less than those required to stimulate maturation in vitro, it brings into question the importance of this molecule as a natural inducer of meiotic resumption. The same group has also demonstrated an increase in FF-MAS in mouse ovarian homogenates 5 h after administering an ovulatory dose of hCG to hormonally primed animals [10], but the significance of such a finding is uncertain, because this time point is several hours after GVB has taken place (see below). It is also important to point out that detection of FF-MAS within the ovarian follicle does not presuppose a regulatory function. Yoshida et al. [12] have reported increased levels of 14α-demethylase activity in rat ovarian homogenates in response to hormonal priming, but the presumptive augmentation in FF-MAS production does not affect the meiotic status of the oocytes.

An important issue in understanding the potential action of FF-MAS or T-MAS in the follicle is how the sterol reaches the oocyte. Byskov's group has demonstrated a paracrine effect of cumulus granulosa cells on cocultured mouse DO and has proposed that secretion of MAS accounts for this activity [8, 9]. It is doubtful that extracellular MAS would have a significant impact on the meiotic status of the oocyte in situ, as the somatic compartment would likely interfere with a direct action on the oocyte. In a later paper, the same group has presented a model for meiotic induction in which LH stimulates metabolic uncoupling between the germ and somatic cell compartments and thereby terminates transfer of an inhibitor (cAMP) to the oocyte; at the same time, FSH triggers the synthesis by cumulus cells of MAS that may reach the oocyte through the gap junction pathway to bring about meiotic resumption. Thus, meiotic induction is achieved by the combined removal of inhibition and generation of MAS [11]. However, what is not clear in this model is how uncoupling can occur to a great enough extent to restrict the passage of inhibitor but still allow the transfer of MAS to the oocyte. Work from our laboratory indicates that coupling between the two compartments is essential for meiotic induction [17, 20], and a recent study suggests that a positive paracrine action of cumulus cells on oocyte maturation only occurs when the coupling pathway is abrogated [21]. While MAS is small enough to pass through gap junctions, such a phenomenon has not yet been demonstrated. The lanosterol uptake data certainly suggest that it does not, because much less sterol accumulated in CEO compared to DO.

The kinetics of meiotic maturation are an additional consideration. The assay protocol for stimulation of GVB in DO by FF-MAS in other publications has typically involved culture times of 20–24 h. This is quite an extended period to test for meiotic induction. In the superovulated mouse model, GVB is initiated between 1.5–2 h post-hCG [22]. The actual kinetics of meiotic induction within the follicle are surely even more rapid, because a lag period

inevitably exists between the time of hCG injection or preovulatory gonadotropin surge and the generation of a direct meiosis-activating stimulus. If, as has been proposed [10, 23], gonadotropin-induced maturation is mediated by FF-MAS produced by the somatic compartment, a similar rapid induction of GVB by FF-MAS in DO would be expected in vitro. We have shown herein that more than 6 h of culture is required before significant stimulation of maturation by FF-MAS and that this requires continual exposure to the sterol. A similar time frame was reported by Hegele-Hartung et al. [5]. Thus, if FF-MAS is the direct activator of maturation, these slow in vitro kinetics are inconsistent with such a role. It is, of course, possible that in vitro conditions, including the choice of meiotic inhibitor and absence of certain follicular constituents, could alter the normal rate of maturation.

To solubilize FF-MAS in culture medium, stock solutions of 1–3 mg/ml in ethanol are typically prepared. Consistent with previous reports [3–5], concentrations of ethanol present in the active FF-MAS preparations (0.05–0.5%) had no significant impact on meiotic maturation, but we report that higher concentrations stimulated GVB in hypoxanthine-arrested DO. Comparable levels of related sterols have previously been shown to have negligible meiosis-inducing potency [3, 4]. Thus, the meiosis-inducing action of MAS is a specific one and is not likely due to an ethanol effect. Nevertheless, recent studies with bovine oocytes have indicated that ethanol can influence both nuclear and cytoplasmic maturation [24, 25]. It is therefore important that caution be exercised when using ethanol as a vehicle in oocyte maturation experiments, particularly those involving DO. An alternative method for solubilization of MAS in studies from Byskov's laboratory has involved several 1-min sonication pulses [2, 11]. It remains to be determined what effects, if any, the sonication procedure might have on the maturation medium.

Follicle-stimulating hormone induces GVB in CEO when meiotic arrest is maintained with one of a number of different inhibitors, including hypoxanthine, dbcAMP, guanosine, and isobutylmethylxanthine [1]. If FF-MAS mediates this action of FSH, then it should also stimulate maturation under a similar array of inhibitory conditions. However, we found that while FF-MAS was effective in hypoxanthine-arrested DO, it failed to trigger GVB in dbcAMP-arrested DO, whether MEM or MEMα was utilized for culture. This conflicts with the study by Grondahl et al. [3] in which FF-MAS stimulated GVB in DO arrested with hypoxanthine, dbcAMP, or isobutylmethylxanthine. Although the reason for this inconsistency is unclear, it is important to note that in the latter study FF-MAS was added at the higher concentration of 20 μM that included ethanol at 0.8%.

Having shown that OCC can take up lanosterol, it was important to test its effects on oocyte maturation. We reasoned that if FSH triggers maturation by stimulating cumulus cell synthesis of FF-MAS, then the uptake of exogenous lanosterol should help drive FF-MAS production and stimulate GVB. However, consistent with previous results [2], we failed to achieve reversal of meiotic arrest with this sterol. In addition, lanosterol had no beneficial effect on dbcAMP-arrested CEO treated with FSH but, rather, proved inhibitory at the highest concentration tested. Although the inhibitory dose was quite high (100 μM), this result suggests that augmenting the metabolic flux from lanosterol to FF-MAS in cumulus cells does not necessarily trigger GVB and may even be inhibitory, perhaps due to downstream metabolites. Indeed, cholesterol was shown to augment the inhibitory effect of dbcAMP in both CEO and DO in dose-dependent fashion. Such a mechanism might explain the inhibitory effect of FF-MAS on hypoxanthine-arrested CEO in glucose-free medium. Furthermore, these findings raise the possibility that induction of meiotic maturation by inhibitors of enzymes situated downstream of FF-MAS in the sterol biosynthetic pathway, such as AY9944 [11], may be acting by preventing accumulation of inhibitory downstream metabolites. This is a plausible consideration, because AY9944 has an additional site of action on Δ7-reductase that produces cholesterol from 7-dehydrocholesterol (Fig. 1).

If increasing FF-MAS production is expected to promote meiotic resumption, then blocking 14α-demethylase with putative inhibitors should prevent FF-MAS synthesis and have an opposite, inhibitory action on maturation. Consistent with this idea, ketoconazole completely eliminated FSH-induced maturation in dbcAMP-arrested CEO. Yet only minor suppression of meiotic induction occurred in hypoxanthine-arrested CEO, and considerably higher concentrations of the drug were required for this effect. These results are surprising, considering that FF-MAS triggered meiotic resumption in hypoxanthine-arrested, but not dbcAMP-arrested, oocytes. It follows that hypoxanthine-arrested oocytes should be more sensitive to changes in FF-MAS production, but just the opposite was observed. At concentrations similar to ours, Tsafriri et al. [13] reported a total suppression of LH-induced progesterone synthesis by ketoconazole in cultured rat follicles but no effect on meiotic resumption. Unlike ketoconazole, the more specific inhibitor, 14α-ethyl-5α-cholest-7-ene-3β,15α-diol, did not block meiotic induction in dbcAMP-arrested CEO. Although the activity of the drug was not assayed, the concentrations used were well within the range previously shown to block sterol synthesis in Chinese hamster ovary-K1 cells [18]. Consequently, our results with these two metabolic blockers fail to support a role for FF-MAS in meiotic induction and raise the possibility that the effect of ketoconazole on meiotic maturation is unrelated to FF-MAS levels.

To summarize, FF-MAS has a definite stimulatory action on hypoxanthine-arrested DO. Its high specificity among closely related sterols in eliciting meiotic resumption makes it an attractive candidate for elucidating substrates and pathways involved in meiotic regulation. However, the lack of effect in dbcAMP-arrested DO, the suppressive effect of cumulus cells and downstream metabolites, the slow maturation kinetics, and the limited influence of enzyme inhibitors raise questions as to its physiological significance. If not physiological, it could act by a parallel pathway(s) that converges downstream on the cascade of events activated by gonadotropin. Until further information is obtained concerning the temporal relationship between follicular FF-MAS levels and meiotic resumption as well as the effects of gonadotropin stimulation and enzyme inhibitors on the sterol biosynthetic pathway, a role for FF-MAS in meiotic regulation remains inconclusive.

ACKNOWLEDGMENTS

I am indebted to Ann Utecht for dedicated technical assistance. Thanks to Drs. John Eppig and Bill Wilson for insightful comments and suggestions concerning the manuscript.

REFERENCES

1. Downs SM, Daniel S, Bornslaeger EA, Hoppe PC, Eppig JJ. Induction of maturation in cumulus cell-enclosed mouse oocytes by follicle stimulating hormone and epidermal growth factor: evidence for a positive stimulus of somatic cell origin. J Exp Zool 1988; 245:86–96.
2. Byskov AG, Andersen CY, Nordholm L, Thogersen H, Guoliang X, Wassmann O, Andersen JV, Guddal E, Roed T. Chemical structure of sterols that activate oocyte meiosis. Nature 1995; 374:559–562.
3. Grondahl C, Ottesen JL, Lessl M, Faarup P, Murray A, Gronvald FC, Hegele-Hartung C, Ahnfelt-Ronne I. Meiosis-activating sterol promotes resumption of meiosis in mouse oocytes cultured in vitro in contrast to related oxysterols. Biol Reprod 1998; 58:1297–1302.
4. Ruan B, Watanabe S, Eppig JJ, Kwoh C, Dzidic N, Pang J, Wilson WK, Schroepfer GJ Jr. Sterols affecting meiosis: novel chemical syntheses and biological activities and spectral properties of the synthetic sterols. J Lipid Res 1998; 39:2005–2020.
5. Hegele-Hartung C, Kuhnke J, Lessl M, Grondahl C, Ottesen J, Beier HM, Eisner S, Eichenlaub-Ritter U. Nuclear and cytoplasmic maturation of mouse oocytes after treatment with synthetic meiosis-activating sterol in vitro. Biol Reprod 1999; 61:1362–1372.
6. Grondahl C, Lessl M, Faerge I, Hegele-Hartung C, Wassermann K, JL Ottesen. Meiosis-activating sterol-mediated resumption of meiosis in mouse oocytes in vitro is influenced by protein synthesis inhibition and cholera toxin. Biol Reprod 2000; 62:775–780.
7. Hegele-Hartung C, Lessl M, Ottesen JL, C Grondahl. Oocyte maturation can be induced by a synthetic meiosis activating sterol (MAS) leading to an improvement of IVF rate in mice. Hum Reprod 1998; 13:98.
8. Guoliang X, Byskov AG, Andersen CY. Cumulus cells secrete a meiosis-inducing substance by stimulation with forskolin and dibutyric cyclic adenosine monophosphate. Mol Reprod Dev 1994; 39:17–24.
9. Byskov AG, Andersen CY, Hossaini A, Guoliang X. Cumulus cells of oocyte-cumulus complexes secrete a meiosis-activating substance when stimulated with FSH. Mol Reprod Dev 1997; 46:296–305.
10. Byskov AG, Andersen CY, Leonardsen L, Baltsen M. Meiosis activating sterols (FF-MAS) and fertility in mammals and man. J Exp Zool 1999; 285:237–242.
11. Leonardsen L, Stromstedt M, Jacobsen D, Kristensen KS, Baltsen M, Andersen CY, Byskov AG. Effect of inhibition of sterol Δ14-reductase on accumulation of meiosis-activating sterol and meiotic resumption in cumulus-enclosed mouse oocytes *in vitro.* J Reprod Fertil 2000; 118:171–179.
12. Yoshida Y, Hamashita C, Noshiro M, Fukuda M, Aoyama Y. Sterol 14-demethylase P450 activity expressed in rat gonads: contribution to the formation of mammalian meiosis-activating sterol. Biochem Biophys Res Commun 1996; 223:534–538.
13. Tsafriri A, Popliker M, Nahum R. Beyth Y. Effects of ketoconazole on ovulatory changes in the rat: implications on the role of a meiosis-activating sterol. Mol Hum Reprod 1998; 4:483–489.
14. Ruan B, Shey J, Gerst N, Wilson WK, GJ Schroepfer Jr. Silver ion high pressure liquid chromatography provides unprecedented separation of sterols: applicaton to the enzymatic formation of cholesta-5,8-dien-3β-ol. Proc Natl Acad Sci U S A 1996; 93:11603–11608.
15. Schroepfer GJ Jr, Parish EJ, Kandutsch AA. 14α-Ethyl-5α-cholest-7-ene-3β,15α-diol, an extraordinarily potent inhibitor of sterol biosynthesis in animal cells. J Am Chem Soc 1977; 99:5494–5496.
16. Paliokas AM, Schroepfer GJ Jr. Stereospecificity in the enzymatic conversion of Δ7-cholesten-3β-ol to 7-dehydrocholesterol. J Biol Chem 1968; 243:453–464.
17. Downs SM. The influence of glucose, cumulus cells, and metabolic coupling on ATP levels and meiotic control in the isolated mouse oocyte. Dev Biol 1995; 167:502–512.
18. Pinkerton FD, Izumi A, Anderson CM, Miller LR, Kisic A, Schroepfer GJ Jr. 14α-Ethyl-5α-cholest-7-ene-3β,15α-diol, a potent inhibitor of sterol biosynthesis, has two sites of action in cultured mammalian cells. J Biol Chem 1982; 257:1929–1936.
19. Baranao JL, Hammond JM. FSH increases the synthesis and stores of cholesterol in porcine granulosa cells. Mol Cell Endocrinol 1986; 44: 227–236.
20. Fagbohun CF, Downs SM. Metabolic coupling and ligand-stimulated meiotic maturation in the mouse oocyte-cumulus cell complex. Biol Reprod 1991; 45:851–859.
21. Downs SM. A gap junction-mediated signal, rather than an external paracrine factor, predominates during meiotic induction in isolated mouse oocytes. Zygote 2000; (in press).
22. Eppig JJ, Downs SM. Gonadotropin-induced murine oocyte maturation *in vivo* is not associated with decreased cyclic adenosine monophosphate in the oocyte-cumulus cell complex. Gamete Res 1987; 20: 125–131.
23. Anderson CY, Baltsen M, Byskov AG. Gonadotropin-induced resumption of oocyte meiosis and meiosis-activating sterols. Curr Top Dev Biol 1999; 41:163–185.
24. Avery AM, Faerge I, Grondahl C, Ottesen J. Nuclear maturation and embryo development of bovine oocytes matured in a semi-defined medium supplemented with meiosis-activating sterol (MAS). Theriogenology 1999; 51:367 (abstract).
25. Avery B, Greve T. Effects of ethanol and dimethylsulphoxide on nuclear and cytoplasmic maturation of bovine cumulus-oocyte complexes. Mol Reprod Dev 2000; 55:438–445.