Effects of ketoconazole on ovulatory changes in the rat: implications on the role of a meiosis-activating sterol

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In-vitro studies on mouse oocytes have shown that human follicular fluid and bull testes contain an activity which partially overrides the inhibitory action of hypoxanthine on meiosis. This activity was ascribed to two closely related sterols, subsequently named meiosis-activating sterols (MAS). We have used a potent inhibitor of sterol synthesis, ketoconazole, in order to test in vivo and in vitro whether MAS play a necessary physiological role in the resumption of meiosis in the rat. When administered systemically, ketoconazole (8.3–16.6 mg/rat) suppressed ovulation by 40%. Local unilateral administration of the drug into the ovarian bursa (1.25 mg/bursa) resulted in 75% inhibition of ovulation in comparison with the contralateral ovary. All the ovulated ova in the oviduct were mature. Histological examination of the ketoconazole-treated ovaries revealed mature oocytes trapped in follicles which failed to ovulate. Furthermore, extraction of oocytes from the large follicles of such ovaries revealed that 79% of them were mature. Addition of ketoconazole (0.0001–0.01 mM) to the culture medium did not affect significantly the spontaneous maturation of rat oocytes. However, ketoconazole at a higher concentration (0.1 mM) caused the degeneration of oocytes. Ketoconazole (0.01 mM) did not affect luteinizing hormone (LH)-stimulated oocyte maturation in explanted preovulatory follicles, even though it inhibited follicular progesterone production to levels below the hormone-free control follicles. At higher levels, ketoconazole caused the degeneration of follicles and the enclosed oocytes. In conclusion, using a potent inhibitor of MAS we have failed to confirm the suggested obligatory role of MAS in the resumption of meiosis in the rat both in vivo and in vitro.

Key words: ketoconazole/meiosis-activating sterols/oocyte maturation/ovulation

Introduction

The meiotic process in antral follicles is normally arrested until stimulated by the preovulatory surge of luteinizing hormone (LH) (Ayalon et al., 1972; Tsafriri et al., 1972). Most, if not all, of the ovulatory actions of LH are initiated by activation of follicular adenylyl cyclase and are mediated by cAMP (Lindner et al., 1974). Nevertheless, the cascade initiated by LH includes activation of additional intracellular signal transduction pathways (Tsafriri and Dekel, 1994). Resumption of meiosis could be stimulated in preovulatory follicles by a transitory exposure to permeant cAMP analogues, phosphodiesterase inhibitors and forskolin (Tsafriri et al., 1972, 1996; Dekel and Beers, 1978; Hillensjö et al., 1978; Dekel et al., 1981; Dekel and Sherzily, 1983; Yoshimura et al., 1992a,b). Thus, at present, most of the data available regarding the resumption of meiosis in preovulatory follicles can be related to a temporal increase in follicular and oocyte cAMP levels. The role of cAMP in oocyte maturation has been reviewed in detail (Tsafriri and Dekel, 1994; Wassarman and Albertini, 1994; Downs, 1995).

The induction of ovulatory changes by LH, including the resumption of meiosis, is exerted through its interaction with receptors in the somatic cells of the follicle (Lindner et al., 1974). Gap junctional transport of small molecules between the granulosa cell compartment and the oocyte has been suggested and experimentally confirmed by many studies (Lindner et al., 1974; Dekel and Beers, 1978; Gilula et al., 1978) (for reviews see Tsafriri and Dekel, 1994; Wassarman and Albertini, 1994; Downs, 1995). The transport of cAMP from the somatic compartment to the oocyte seems to be involved in both the maintenance of meiotic arrest, requiring low sustained oocyte cAMP levels and the resumption of meiosis, which is associated with a temporary surge of cAMP (Dekel et al., 1988).

The culture of mouse isolated oocytes with inhibitors of the spontaneous resumption of meiosis, such as hypoxanthine, suggested the activity of a positive regulator produced by somatic cells and transported to the oocyte (Eppig and Downs, 1987; Downs et al., 1988; Guoliang et al., 1994; Byskov et al., 1997). Using the same model system, Byskov et al. (1995), identified meiosis-activating sterols (MAS) in human follicular fluid and bull testicular tissue. These two sterols, trienol (4,4-dimethyl-5α-cholest-8,14,24,-triene-3α-ol) from follicular fluid and dienol (4,4-dimethyl-5α-cholest-8,24,-diene-3β-ol) from bull testes, as well as two closely related synthetic C29 sterols modestly increased the proportion of maturing mouse oocytes cultured with the inhibitor hypoxanthine. A key enzyme in cholesterol biosynthesis is sterol 14α-
demethylase (P450 14DM) which is encoded by the CYP51 gene, a member of the cytochrome P450 (CYP) gene superfamily involved in sterol synthesis in fungi, plants and animals (Aoyama et al., 1996). The human and rat P450 14DM enzymes are 93% identical (Stromstedt et al., 1996). Trienol is formed by P450 14DM-dependent demethylation of lanosterol and converted to dienol by a sterol 14-reductase (Aoyama and Yoshida, 1986). The enzyme P450 14DM has been recently shown to be stimulated in immature rat ovaries by equine chorionic gonadotrophin (CG), resulting in a 6-fold increase within 48 h. The activity of ovarian and testicular P450 14DM was inhibited in vitro by ketoconazole (Yoshida et al., 1996).

Here we have tested the actions of the potent P450 14DM inhibitor, ketoconazole, on ovulation in vivo and on the resumption of oocyte maturation both in vivo and in vitro using well-characterized rat models. It was assumed that if the described C29 sterols play an essential and physiological role in the resumption of meiosis, and thus function as MAS, ketoconazole should block or attenuate the spontaneous maturation of isolated oocytes and of follicle-enclosed oocytes stimulated to mature by LH.

Materials and methods

Animals

Rats from the Department of Hormone Research Wistar-derived colony were provided with water and rat chow ad libitum and housed in air-conditioned rooms illuminated for 14 h/day. The experiments were carried out in accordance with the principles and guidelines for the use of laboratory animals and approved by the research animal committee of the Weizmann Institute of Science, Rehovot, Israel. Immature rats were injected with equine CG (10 IU) at 09:00–09:30 on day 25–26 of age in order to enhance multiple follicular development. For testing ovulation in vivo, the animals were stimulated to ovulate by human chorionic gonadotrophin (HCG, 4 IU) 53 h later (afternoon of proestrus). For exploiting cumulus–oocyte complexes for culture the animals were killed 48–50 h after equine CG by cervical dislocation. For explantation of follicles, only mature proestrous rats that had shown at least two 4-day cycles, as determined by daily vaginal smears immediately before the start of the experiment, were used.

The ovulatory response in vivo

Two days after equine CG treatment, immature rats were administered with the indicated doses of ketoconazole or vehicle (0.6 ml) i.p. or into their peri-ovarian sac (bursa) at 09:30–10:30. For intrabursal injection the animals were anesthetized by a cocktail of ketamine (40–60 mg/kg) and diazepam (2–3 mg/kg) and one of the ovaries was exteriorized via a small lumbosacral incision. A 29-gauge needle was threaded into the ovarian bursa via the adjoining fat pad. Before transfer to the test medium the ovaries were washed twice in plain medium. Oocytes (n = 25–50), within their attached cumulus cell mass (‘isolated oocytes’), were cultured in organ culture dishes (Falcon, Cockeysville, MD, USA) for 6 or 24 h in 1 ml of control or test media, as indicated in the Results section. Oocytes from three rats were pooled and distributed into at least three different treatment dishes. Each treatment was tested in at least two replicate cultures repeated in two separate experiments.

Follicle cultures

Immature Wistar-derived rats treated with equine CG (10 IU) or mature rats, that have shown two consecutive 4-day cycles immediately before the experiment, were killed on the morning of the day of proestrus by cervical dislocation. Preovulatory follicles were excised under a dissecting microscope as previously described (Tsafir et al., 1972). The follicles, 5–10 per dish, were cultured for 24 h alone or with LH (5 µg/ml) or in combination of LH and the indicated concentration of ketoconazole.

Examination of oocytes

At the end of the 6 or 24 h incubation, isolated oocytes were collected for microscopic examination. Follicle-enclosed oocytes were released and collected after 24 h of culture by making a small incision in the follicle. Some of the ovulated oocytes from the bursae as well as oocytes retained in unruptured large follicles were examined for their meiotic status by Nomarski interference microscopy (Zeiss, Oberkochen, Germany). Oocytes showing a clear nuclear membrane (germinal vesicle; GV) or only an intact nucleolus were classified as immature. Oocytes that did not show any nuclear structures because they have undergone germinal vesicle breakdown (GVBD) were classified as mature (Tsafir et al., 1989).

Statistical analysis

Analysis of variance (ANOVA) and t-test was performed for the ovulation data. Values of oocyte maturation, SE and statistical significance were calculated according to Ott (1988). P < 0.05 was considered to be significant.

Progesterone radioimmunoassay

The accumulation of progesterone in medium from follicular cultures was measured by a previously described radioimmunoassay procedure (Lindner and Bauminger, 1974).
Ketoconazole and ovulatory changes

Figure 1. Effect of systemic injection of ketoconazole on ovulation in the rat. The drug was injected s.c. 4 h before human chorionic gonadotrophin (HCG) administration to immature rats treated with equine CG 48 h earlier. The number of rats is indicated on the columns.

Figure 2. Effect of intrabursal injection of ketoconazole. The number of ovulated ova is given separately for the ketoconazole- and vehicle-treated oviduct and for the untreated contralateral one. For other details, see Figure 1.

Results

Effects of ketoconazole on follicle rupture and ovum maturation in vivo

The administration of ketoconazole s.c. (8.3–16.6 mg/rat) into proestrous equine CG-treated immature rats, 4 h before the stimulation of ovulation by HCG, attenuated ovarian follicle rupture by ~40% (P < 0.05) (Figure 1). Local administration of the drug into the periovarian sac (i.b.) (1.25 mg/bursa) resulted in an even more pronounced inhibition of ovulation, 75% in comparison with the untreated contralateral ovary (P < 0.0001) and 70% when compared with vehicle-administered control ovaries (P < 0.001) (Figure 2). All oviductal oocytes in both, s.c.- and i.b.-administered rats were mature and had expanded cumuli (Figure 3). Histological examination of i.b.-treated ovaries revealed many large follicles which failed to ovulate and that contained entrapped mature oocytes (Figure 4). Extraction of oocytes from the large follicles of the ketoconazole-treated ovaries revealed that 79% of them were mature (Figure 3) and that the cumuli had expanded. Some of the 21% of oocytes which were not mature (in fact six with GV and three degenerated) may have been released from smaller follicles or inadvertently during the handling of the ovaries.

Ketoconazole and oocyte maturation in vitro

Addition of ketoconazole (0.0001–0.01 mM) to the culture medium did not affect the spontaneous maturation of isolated rat follicles (Table I), although the highest dose tested (0.1 mM) caused degenerative changes in the oocytes. This detrimental effect of ketoconazole on oocytes was even more pronounced when the culture was extended to 24 h. This resulted in advanced degeneration of the oocytes, many of them showing concentration of coarse granules in the centre and the cortical areas of the oocytes (Figure 5). To test the effect of ketoconazole on LH-induced maturation, the drug (0.01–1.0 mM) was added to the medium of follicle-enclosed oocytes. The two higher concentrations caused degeneration of the follicles during the culture with complete destruction of the oocytes, precluding the assessment of their meiotic status. Ketoconazole (0.01 mM) did not affect the resumption of oocyte maturation induced by LH (Figure 6A) nor cumulus expansion, although this dose suppressed follicular progesterone production to levels below the untreated control follicles (Figure 6B). This agrees with the previously reported ability of 1 µM ketoconazole to inhibit rat ovarian P₄₅₀₁₄DM in vitro (Yoshida et al., 1996).

Discussion

Ketoconazole, an inhibitor of P₄₅₀₁₄DM, at doses which partially inhibited follicle rupture in intact equine CG–HCG...
Figure 4. Histology of ovaries treated locally with ketoconazole by intrabursal injection. (A) Immature germinal vesicle (GV) oocyte from non-responding antral follicle (arrow shows position of GV). (B, C) Mature oocytes entrapped within follicles which failed to rupture. (B) Arrow shows position of spindle and metaphase plate; (C) arrow shows position of polar body. Bar = 50 µm.

Figure 5. Oocytes cultured in vitro for 24 h with ketoconazole. Nomarski interference contrast microscopy. (A) Immature oocyte; arrow shows position of germinal vesicle (GV) and nucleolus. (B) Mature oocyte; arrow shows position of polar body. (C) Degenerated oocyte cultured for 24 h with ketoconazole (0.1 mM). Arrow shows position of GV(?), note the aggregation of coarse granules around the GV and the oolemma. Bar = 50 µm.

Table I. Effect of ketoconazole on spontaneous maturation of cumulus-enclosed oocytes. Oocytes were cultured for 6 or 24 h in the presence of the indicated concentrations of ketoconazole or the vehicle (control)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ova examined</th>
<th>GV (%)</th>
<th>GVBD (%)</th>
<th>Deg (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6 h culture</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>225</td>
<td>28 (12.4)</td>
<td>18.8 (83.5)</td>
<td>9 (4.0)</td>
</tr>
<tr>
<td>Ketoconazole (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0001</td>
<td>92</td>
<td>7 (7.6)</td>
<td>83 (90.2)</td>
<td>2 (2.1)</td>
</tr>
<tr>
<td>0.001</td>
<td>212</td>
<td>23 (10.8)</td>
<td>187 (88.2)</td>
<td>2 (1.0)</td>
</tr>
<tr>
<td>0.01</td>
<td>186</td>
<td>32 (17.2)</td>
<td>149 (80.1)</td>
<td>5 (2.7)</td>
</tr>
<tr>
<td>0.1</td>
<td>206</td>
<td>103* (50.0)</td>
<td>65* (31.5)</td>
<td>38 (18.5)</td>
</tr>
<tr>
<td><strong>24 h culture</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>139</td>
<td>12 (8.6)</td>
<td>123 (88.5)</td>
<td>4 (2.9)</td>
</tr>
<tr>
<td>Ketoconazole (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001</td>
<td>157</td>
<td>11 (7.0)</td>
<td>142 (90.5)</td>
<td>4 (2.5)</td>
</tr>
<tr>
<td>0.01</td>
<td>182</td>
<td>26 (14.3)</td>
<td>149 (81.9)</td>
<td>7 (3.8)</td>
</tr>
<tr>
<td>0.1</td>
<td>176</td>
<td></td>
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</tr>
</tbody>
</table>

GV = immature oocytes with intact germinal vesicle; GVBD = germinal vesicle breakdown, oocytes with no nuclear structures (nuclear membrane or nucleolus) visible by interference contrast microscopy; Deg = degenerated oocytes.

*Most of the oocytes had clear degenerative changes, but could be classified as immature or mature.
show that the resumption of meiosis in the rat is contingent on undisturbed sterol synthesis. Therefore, a role for MAS as an essential mediator of the gonadotrophic stimulus triggering the resumption of oocyte maturation or the spontaneous maturation of isolated oocytes is doubtful.

Female gamete production is subject to several stop/go controls (Tsafirri and Dekel, 1994; Wasserman and Albertini, 1994; Downs, 1995). The oocyte embarks on the first meiotic division during embryonic development. Using a fetal gonad co-culture assay it was suggested that the ovary produces an inducer of meiosis, initially termed meiosis inducing substance (MIS) and later renamed as meiosis activating substance (MAS), and that the testis produces a meiosis preventing substance (MPS) (Byskov and Saxen, 1976). Human follicular fluid was shown to exert MAS activity as detected by the fetal testis assay (Westergaard et al. 1984, 1985). By stimulating the resumption of meiosis in oocytes cultured in the presence of hypoxanthine by forskolin and dibutylryl cAMP (Guoliang et al., 1994) or follicle stimulating hormone (FSH) (Byskov et al., 1997) evidence for an inducer of resumption of meiosis was presented. This activity was also referred to as MAS and identified as a sterol (Byskov et al., 1995). Clearly, the initiation of meiosis in fetal gonads and the resumption of the first meiotic prophase by dictyotene oocytes are distinct processes in terms of meiotic stage and the developmental stage of the oocyte. Thus the fetal oocyte is prior to its major growth and synthetic phase and only after completing it do the oocytes gain the competence to resume the meiotic process. As far as we are aware, no compelling evidence has been presented that the two activities (i.e. stimulation to initiate meiosis in fetal gonads and to resume the process in competent oocytes) are exerted by the same signal molecule. While there are ample examples that the same molecule may be regulating different processes at various stages of cellular differentiation, attribution of stimulation of these two biological activities to the same molecule is subject to experimental confirmation.

The addition of ketoconazole to rat LH-stimulated follicles blocked follicular steroidogenesis, as revealed by the suppression of progesterone accumulation, without affecting the resumption of meiosis. Thus, the use of ketoconazole confirms and extends previous studies showing that the inhibition of follicular steroidogenesis does not affect the resumption of meiosis in mammals (Lieberman et al., 1976; Moor and Trounson, 1977), in contrast to the situation in fish (Nagahama, 1994) and amphibia (Maller, 1990). In spite of some conflicting results, the bulk of evidence favors the view that steroids play only a marginal modulatory role in mammalian oocyte maturation (see detailed reviews by Tsafirri and Dekel, 1994; Wassarman and Albertini, 1994). Follicular development in mammals and most other vertebrates differs markedly, and this was suggested to explain why steroids could not serve as the stimulus for the resumption of meiosis in these species (Tsafirri and Dekel, 1994). Large crops of follicles and oocytes growing simultaneously in most lower vertebrates allows the utilization of follicular steroids as a signal between the somatic and germ cell compartments of the follicle. In contrast, mammalian cyclic ovulations and hierarchical follicular development (which span several reproductive cycles), result in the

**Figure 6.** The effect of ketoconazole on luteinizing hormone (LH)-stimulated maturation of follicle-enclosed oocytes and follicular progesterone production. (A) Oocyte maturation. The number of oocytes examined is indicated on the columns. (B) Progesterone accumulation. The numbers on columns are the number of replicate cultures assayed, each with five follicles. *Degenerated oocytes.
coexistence of follicles and oocytes at different stages of development and the exposure of oocytes to differing levels of steroids. Such cyclic changes in ovarian steroid levels make the distinction between signal and noise in individual oocytes/follicles very difficult. The same logic may apply to precursors of cholesterol, the steroids. Furthermore, the finding that equine CG treatment of immature mice resulted in an increase in ovarian MAS sterol levels within 48 h (Yoshida et al., 1996), a time at which oocyte maturation (GVB) does not yet occur and is waiting for additional stimulus (exposure to endogenous or exogenous LH/HCG stimulation), argues against the function of the sterol as MAS. If it was the physiological MAS, it should have stimulated the resumption of meiosis.

In summary, the use of ketoconazole, a potent inhibitor of 
P45014DM and of MAS synthesis, failed to confirm the suggested obligatory role of MAS in the stimulation of the resumption of oocyte meiosis in vivo or in vitro. The suggested physiological role of MAS in the regulation of oocyte maturation should await confirmation by other approaches. Conversely, the attenuation of follicle rupture by a single injection of ketoconazole confirms and extends the study demonstrating inhibition of ovulation and expression of 
P450scc when the drug is given at an early stage of equine CG-stimulated ovarian follicular development (Gal et al., 1997). Furthermore, these results confirm the essential role of follicular steroids, most probably progesterone, in follicle rupture (for reviews see Espey and Lipner, 1994; Tsafiriri and Chun, 1996). Thus in rodents, ablation of steroid synthesis inhibits follicle rupture, which is re-established by the administration of progesterone (Lipner and Wendelken, 1971; Snyder et al., 1984). Likewise, midcycle administration of triolostane, a progesterone synthesis inhibitor, prevents follicle rupture, but not oocyte maturation, in macaques. Only pregrains, but not andrograins, replacement restored ovulation (Hibbert et al., 1996). Finally, the antiprogestin RU486 inhibits follicle rupture in rodents and the rabbit (Tsafiriri et al., 1987; Loutradis et al., 1991; Iwamasa et al., 1992; Kanayama et al., 1994, 1996), suggesting a paracrine receptor-mediated role of progesterone in the mediation of gonadotrophin-induced follicle rupture.

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