Direct non-genomic effects of follicular steroids on maturing human oocytes: oestrogen versus androgen antagonism

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Previously published data have suggested that oestradiol exerts direct beneficial effects on human oocytes during in-vitro maturation and that these effects are at least partly due to a non-genomic action of the steroid at the oocyte surface. Here we provide evidence showing that a non-genomic effect of oestradiol is counteracted by androstenedione. In contrast to these results from in-vitro experiments, in which changes in steroid concentrations are abrupt and the non-genomic responses are rapid, the progressively changing follicular steroid concentrations which occur during in-vivo development may rather have permissive or restrictive effects on the events of spontaneous oocyte cytoplasmic maturation. The oocyte is particularly sensitive at the germinal vesicle stage of development to non-genomic steroid actions. Ovarian stimulation protocols should thus be adjusted so as to avoid androgen predominance at the mid-follicular phase. In patients in whom this condition cannot be met, in-vitro maturation of oocytes may be a solution.

Key words: androgen/human/non-genomic steroid effects/oestrogen/oocyte maturation

Introduction

The final period of ovarian follicular development, during which the newly selected follicles acquire a high degree of responsiveness to gonadotrophins, is very short as compared to the duration of the whole folliculogenetic process. In spite of its brevity, however, this period is marked by a number of important maturational processes taking place in the oocyte, including the resumption of meiosis and its continuation up to the metaphase II block as well as multiple structural and functional changes in the oocyte cytoplasm, globally referred to as oocyte cytoplasmic maturation.

The concentrations of steroid hormones in follicular fluid undergo salient changes in this final phase of follicular development, as a result of changes in the local concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH), the number of steroidogenic cells (theca interna and granulosa cells) in the follicle, the responsiveness of the steroidogenic cells to gonadotrophins, the activity of enzymes involved in steroid biosynthesis in these cells, and an elaborate interplay of follicular autocrine and paracrine regulatory systems (reviewed in Gougeon, 1996). These changes in the intrafollicular steroid concentrations may influence the oocyte indirectly, via granulosa and cumulus cells. However, several lines of evidence suggest that follicular steroids can also act directly upon the oocyte by producing changes in the reactivity of its Ca²⁺ release mechanism supposed to be involved in oocyte cytoplasmic maturation (reviewed in Tesarik and Mendoza, 1995).

In general, steroid effects on cells can employ two different modes of action. The classical (or genomic) mechanism involves a nuclear steroid receptor which, after binding the steroid ligand, acts as a transcription factor (reviewed in Carson-Jurica et al., 1990). The other mechanism has been discovered more recently and does not involve a modification of gene activity; it is thus called non-classical or non-genomic. Both of these mechanisms appear to be active in
human oocytes (Wu et al., 1993; Tesarik and Mendoza, 1995).

This paper will review the non-genomic effects of oestrogens on human oocytes, with particular reference to the physiological, pathophysiological and clinical implications of these new findings. Some new experimental data concerning the effects of androstenedione on the oestradiol-induced Ca\(^{2+}\) response of oocytes are also included. These data were obtained with the same kind of biological material and with the same methodology as described previously (Tesarik and Mendoza, 1995).

Oestrogen effects on oocytes

The present knowledge of oestrogen non-genomic effects on oocytes is essentially based on data reported in one pilot study (Tesarik and Mendoza, 1995). The principal findings of that study were the following: (i) oestradiol (1 \(\mu\)M) induced a rapid (several seconds after hormone addition) increase in the free intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), followed by a series of Ca\(^{2+}\) oscillations, in human germinal vesicle oocytes; (ii) the first oestradiol-induced [Ca\(^{2+}\)]\(_i\) increase was due to Ca\(^{2+}\) influx, whereas Ca\(^{2+}\)-induced Ca\(^{2+}\) release from intracellular stores contributed more significantly to the subsequent Ca\(^{2+}\) oscillations; (iii) a membrane-impermeant oestradiol conjugate (oestradiol covalently linked to bovine serum albumin) was just as effective in inducing a rapid Ca\(^{2+}\) influx and the subsequent Ca\(^{2+}\) oscillations as free oestradiol; (iv) the presence of the membrane-impermeant oestradiol conjugate in oocyte maturation medium did not influence the progression of oocyte meiotic maturation but did improve oocyte cytoplasmic maturation, since oocytes matured in the presence of 1 \(\mu\)M conjugated oestradiol showed better fertilization and cleavage rates when subsequently inseminated in vitro.

The rapidity of the oocyte response to oestradiol, together with the fact that the steroid need not cross the oocyte plasma membrane to exert its biological effect, clearly shows the non-genomic character of this steroid action. A similarly rapid Ca\(^{2+}\) response to oestradiol has been observed in chicken and pig granulosa cells (Morley et al., 1992). The observation that the exposure of oocytes to the membrane-impermeant oestradiol conjugate during in-vitro maturation improves fertilization and the post-fertilization developmental potential, without any obvious effect on nuclear maturation, suggests that oestradiol influences oocyte cytoplasmic maturation by acting at the oocyte surface.

Androgen effects on oocytes

Because the developmental potential of human oocytes appears to be better related to the oestrogen-to-androgen ratio in follicular fluid than to absolute concentrations of follicular oestrogen (Yding Andersen, 1993), and oestradiol has been shown to exert a beneficial effect on oocyte cytoplasmic maturation, presumably through a non-genomic, Ca\(^{2+}\)-mediated effect (Tesarik and Mendoza, 1995), we performed experiments in which we examined whether and how androstenedione, the main follicular androgen, influences the Ca\(^{2+}\) response of human oocytes to oestradiol. These experiments were performed with the use of the same techniques and instrumentation as described previously (Tesarik and Mendoza, 1995).

The addition of 1 \(\mu\)M oestradiol to control germinal vesicle oocytes (\(n=10\)) induced a rapid transient increase in [Ca\(^{2+}\)]\(_i\) which was followed by a series of secondary Ca\(^{2+}\) oscillations that lasted from 1 to 6 h (data not shown). The frequency of the Ca\(^{2+}\) oscillations differed from oocyte to oocyte but did not change significantly during the whole oscillation period in each oocyte. This response was quite similar to that described previously in human germinal vesicle oocytes after the addition of oestradiol or its membrane-impermeant conjugate (Tesarik and Mendoza, 1995).

In eight other oocytes, germinal vesicle breakdown had occurred during preparation, and those oocytes did not show any Ca\(^{2+}\) response to oestradiol. When the addition of oestradiol to germinal vesicle oocytes (\(n=15\)) was followed by that of androstenedione, a rapid change of the oestradiol-induced Ca\(^{2+}\) oscillations occurred. This change
Figure 2. Persistence of the inhibitory effect of androstenedione on the oestradiol-induced Ca\(^{2+}\) oscillations in a germinal vesicle oocyte after the removal of free androstenedione from the oocyte incubation medium. Relative changes in [Ca\(^{2+}\)]\(i\) are expressed as changes in fluo-3 fluorescence intensity. Ca\(^{2+}\) oscillations were induced by adding 1 µM oestradiol (E2) and then interrupted by subsequent addition of 1 µM androstenedione (A). The oocyte was then washed thoroughly in excess medium (corresponding to the gap in the record between the 8-min and the 12-min time points) followed by a new addition of 1 µM oestradiol. No Ca\(^{2+}\) response was induced by this second oestradiol addition. This response is representative of those observed in a total of 10 oocytes.

was characterized by an immediate increase in the oscillation frequency and a progressive decrease in the oscillation amplitude that led to the disappearance of the Ca\(^{2+}\) oscillations within a few min following the androstenedione addition (Figure 1).

The effect of androstenedione on the oestradiol-induced Ca\(^{2+}\) oscillations was maintained after removal of androstenedione from the oocyte incubation medium. In fact, oocytes (\(n = 10\)) in which the oestradiol-induced Ca\(^{2+}\) oscillations had been arrested with androstenedione were not able to resume the oscillations when washed from androstenedione 4 min after its addition and exposed to the same concentration of oestradiol again (Figure 2).

When oocytes were first exposed to androstenedione, no significant changes in [Ca\(^{2+}\)]\(i\) were observed, but the subsequent addition of oestradiol failed to produce the typical Ca\(^{2+}\) oscillations response in most of these oocytes. Out of 38 oocytes included in this experiment, only five (13%) developed regular Ca\(^{2+}\) oscillations (Figure 3A). Moreover, these oscillations never lasted for >30 min. A further 14 (37%) of the androstenedione-primed oocytes developed a short series of several irregular [Ca\(^{2+}\)]\(i\) increases (Figure 3B), whereas seven (18%) of the oocytes showed a single non-oscillatory [Ca\(^{2+}\)]\(i\) increase (Figure 3C) and 12 (32%) of the oocytes did not respond to oestradiol at all.

These data suggest that androgens can influence adversely the oocyte developmental potential by counteracting the non-genomic, Ca\(^{2+}\)-mediated action of oestrogens on germinal vesicle-stage oocytes. However, androgens alone do not appear to act directly upon the oocyte Ca\(^{2+}\) homeostasis. This kind of observation concerning non-genomic effects of steroids on cells is not exceptional, since Blackmore et al. (1994) reported a similar phenomenon in human spermatozoa in which a specific non-genomic effect of one steroid (progesterone-induced...
Ca\textsuperscript{2+} influx) could be precluded by several other steroids (testosterone derivatives) which were, by themselves, poor stimulators of Ca\textsuperscript{2+} influx. Occupation of cell-surface receptors by a steroid lacking biological activity but sharing affinity for the receptors with the natural ligand, and thus acting as an antagonist at the receptor level, is one possible explanation.

We have shown previously that oestradiol may exert two independent effects on oocytes, the one responsible for the Ca\textsuperscript{2+} influx and the other sensitizing an intracellular Ca\textsuperscript{2+} release mechanism (Tesarik and Mendoza, 1995). Androstenedione may rather affect the latter than the former because oestradiol usually induced Ca\textsuperscript{2+} influx even in the presence of androstenedione, but the form of the secondary [Ca\textsuperscript{2+}] changes was altered in most cases (Figure 3). The nature of the hypothetical steroid receptor molecule involved in these effects has not yet been determined, and this step will obviously be decisive for the explanation of the observed steroid effect interaction.

A variety of steroid hormones, including oestrogens and androgens, have been reported to potentiate the FSH-induced inhibition of meiotic resumption in cumulus-enclosed mouse oocytes (Eppig et al., 1983; Schultz et al., 1983). This effect could be mimicked in denuded oocytes provided that oocyte cAMP levels were elevated by cAMP analogs or forskolin (Eppig et al., 1983). It remains to be elucidated whether these observations have some relationship with those described in this study.

(Patho)physiological implications

The demonstrations of rapid effects of oestradiol and its membrane-impermeant conjugate on Ca\textsuperscript{2+} homeostasis of maturing human oocytes (Tesarik and Mendoza, 1995) and of an antagonistic effect of androstenedione on this oestradiol action (this study) provide unequivocal evidence for the existence of a non-genomic steroid response mechanism in the oocytes. However, the experimental conditions under which this mechanism was revealed were quite artificial and did not reflect the physiological situation. In fact, the oocyte is exposed to changing concentrations of follicular oestrogens and androgens during in-vivo maturation, but these changes are far from being as abrupt as those used in vitro to induce or inhibit Ca\textsuperscript{2+} oscillations (Tesarik and Mendoza, 1995; this study).

Interestingly, Ca\textsuperscript{2+} oscillations similar to those inducible in human germinal vesicle oocytes by in-vitro exposure to oestradiol have been observed to occur spontaneously in mouse germinal vesicle oocytes (Carroll and Swann, 1992). Under in-vivo conditions, oestradiol may thus play a permissive role in the development of ‘spontaneous’ Ca\textsuperscript{2+} oscillations in oocytes rather than act as an immediate trigger of these oscillations. To test this hypothesis, we treated human germinal vesicle oocytes with a subthreshold concentration of oestradiol (100 nM) which did not produce any immediate Ca\textsuperscript{2+} response. In spite of the lack of an immediate response, however, 61% (11/18) of oocytes incubated in the continuous presence of 100 nM oestradiol developed a delayed series of Ca\textsuperscript{2+} oscillations which began 1–3 h after hormone addition and lasted for 2–5 h. The temporal characteristics of these Ca\textsuperscript{2+} oscillations were quite similar to those characterizing the immediate oocyte response to the addition of 1 \mu M oestradiol (Tesarik and Mendoza, 1995). A deeper analysis of the spatial characteristics of these Ca\textsuperscript{2+} oscillations was not performed because such a study would require a high image-sampling frequency over extended periods of observation, which is poorly compatible with the preservation of oocyte viability. In contrast to the oocytes incubated with 100 nM oestradiol, no Ca\textsuperscript{2+} oscillations developed in any of 10 germinal vesicle oocytes that were incubated simultaneously with 100 nM oestradiol and 100 nM androstenedione. These results suggest that, similar to the immediate Ca\textsuperscript{2+} response to high concentrations of oestradiol, the delayed response of human germinal vesicle oocytes to lower oestradiol concentrations is also inhibited by androstenedione.

What is the possible physiological significance of the Ca\textsuperscript{2+} oscillations occurring during oocyte maturation? Experiments performed with mouse oocytes have shown that blocking Ca\textsuperscript{2+} oscillations, by chelating intracellular free Ca\textsuperscript{2+} with 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid (BAPTA), does not affect the occurrence of germinal vesicle breakdown (Carroll and Swann, 1992). Similarly, germinal vesicle breakdown in human oocytes incubated in vitro occurred spontaneously, irrespective of whether the incubation medium was supplemented with oestradiol, whereas only oestradiol-exposed oocytes exhibited Ca\textsuperscript{2+} oscillations (Tesarik and Mendoza, 1995). Moreover, the progression of oocyte maturation to metaphase I and metaphase II was also unaffected by the presence of oestradiol (Tesarik and Mendoza, 1995). Hence, the Ca\textsuperscript{2+} oscillations occurring in maturing oocytes may be important for the cytoplasmic rather than meiotic maturation.

Developmental changes in the oocyte’s Ca\textsuperscript{2+}-release mechanism represent an important constituent of oocyte cytoplasmic maturation. In particular, it has been shown that hamster (Fujiwara et al., 1993) and mouse oocytes (Mehlmann and Kline, 1994) increase the sensitivity of the inositol trisphosphate-induced Ca\textsuperscript{2+} release mechanism as...
maturation proceeds. This maturational process is probably required for the oocyte to be able to produce the typical \(\text{Ca}^{2+}\) oscillations in response to spermatozoa at fertilization (Fujiwara et al., 1993). Cytoplasmic changes globally referred to as cytoplasmic maturation are usually, but not necessarily, linked to meiotic maturation (Eppig et al., 1994).

In the light of the present data, suggesting that oocytes need to be primed with oestrogen in order to develop \(\text{Ca}^{2+}\) oscillations during maturation and that this action of oestrogen can be counteracted by androgen, it is tempting to speculate that \textit{in vivo} exposure of oocytes to inadequate oestrogen-to-androgen ratios may cause an abnormal \(\text{Ca}^{2+}\) response to spermatozoa at fertilization and thus be responsible for impaired developmental potential of the resulting embryo. The corollary of this hypothesis is that oocytes are particularly sensitive to steroids while still at the germinal vesicle stage, in small antral follicles, whereas later changes in the intrafollicular steroid concentrations may be less relevant to the oocyte quality.

**Clinical implications**

If the permissive role of oestrogens in oocyte cytoplasmic maturation is counteracted by androgen, the more androgen is present in the small antral follicle, the more oestrogen will be needed to override its adverse effect. Conversely, when the intrafollicular concentration of androgens is low, relatively low concentrations of oestrogens may still be sufficient to allow normal cytoplasmic maturation. This reasoning is supported by case reports of apparently normal fertilization and cleavage achieved in a woman with congenital adrenal hyperplasia due to 17\(\alpha\)-hydroxylase deficiency (Rabinovici et al., 1989) and in another woman suffering from 17,20-desmolase deficiency syndrome (Pellicer et al., 1991). In either case, the intrafollicular concentration of both oestrogens and androgens was extremely low. The demonstration that oocytes from patients with anovulatory polycystic ovary syndrome, in whom follicular androgen concentrations are high, can be rescued for normal development by removing them from small antral follicles and maturing them \textit{in vitro} in the presence of oestradiol (Trounson et al., 1994) also suggests that even high androgen exposures can be overridden by oestrogen provided that it is applied sufficiently early to coincide with the period of oocyte steroid sensitivity.

With regard to the current ovarian stimulation protocols, the data discussed in this paper argue in favour of a reduction of LH activity at mid-follicular phase, since overproduction of androgens by LH-stimulated theca interna cells may outreach the aromatizing capacity of granulosa cells, although this may not be the only mechanism by which excess LH can impair oocyte quality (Shoham et al., 1993; Yding Andersen, 1995). In fact, comparative multicentre studies (Out et al., 1996) show that ovarian stimulation with recombinant FSH (devoid of any LH activity) yields better results in terms of embryo quality and pregnancy rates as compared to urinary FSH preparations (containing LH contamination). However, this point needs further clarification because another study demonstrates better fertilizability of oocytes obtained from a hypogonadal patient after stimulation with human menopausal gonadotrophin (HMG) as compared to recombinant FSH (Balasch et al., 1995). It is thus possible that, once sufficient aromatizing capacity of granulosa cells has been attained, LH may be useful for optimal oestrogen production by stimulating the synthesis of aromatizable substrate by the theca cells. In addition to specific modifications of ovarian stimulation protocols, patients at risk of follicular hyperandrogenism may also benefit from early ovarian aspiration with subsequent \textit{in vitro} maturation of germinal vesicle oocytes or from intrafollicular injection of oestrogen or FSH with subsequent \textit{in vivo} maturation of oocytes obtained from premature or FSH with subsequent \textit{in vivo} maturation of oocytes obtained from premature

**References**


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