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Original Article

Effect of genistein alone and in combination with okadaic acid on the cell cycle resumption of mouse oocytes

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ABSTRACT Our biopharmacological approach suggests that the now well-documented inhibitory effects of genistein on the maturation of mammalian oocytes do not seem to be related to its effect on tyrosine kinases. Indeed, we show that both tyrphostin B46 and Lavendustin A, two selective inhibitors of protein tyrosine kinases, fail to inhibit meiosis reinitiation. According to recent findings, the G2/M arrest induced by genistein could be due to inhibition of the kinase activity of cdc2. We were therefore mainly interested in dissecting the cytological effects of genistein on mouse primary and secondary oocytes. Genistein exerts the same cytological effects as IBMX on primary oocytes: their germinal vesicle is maintained in a central position, the cytoplasmic microtubule network is stabilized, the central GV immobilization is overcome by demecolcine and they complete normal maturation after their transfer to culture medium. The GV-arresting activity of genistein is also bypassed by OA but combination of both drugs results in a dramatic reorganization of the cytoskeleton leading to a huge membrane bulging, which is quite different to apoptotic-related blebbing. MAP Kinase activation is correlated with meiosis reinitiation. When applied after GVBD has taken place, genistein does not inhibit MAPK activation, metaphase spindle formation and metaphase-to-anaphase transition, but prevents the barrel-shaped MI spindle from undergoing its peripheral migration and the oocytes from extruding their first polar body. It may thus be concluded that the checkpoint control for anaphase onset is unaffected by the drug. On the contrary, our results suggest that spindle anaphase A to spindle anaphase B transition, spindle degradation, mid-body formation and cytokinesis are triggered by a genistein-sensitive mechanism that might be a mid-anaphase checkpoint. Finally, we confirm that genistein induces transition to interphase in metaphase II oocytes but never induces cortical granule exocytosis, the cytoplasmic hallmark of activation.

KEY WORDS: *mouse, oocyte, maturation, genistein, okadaic acid.*

Introduction

Resumption of meiosis in mammalian oocytes is physiologically triggered by the preovulatory surge of luteinizing hormone (Channing *et al.*, 1978) maybe through the generation of Meiosis Activating Sterols (Byskov *et al.*, 1997). It can however also be stimulated *in vitro* by releasing the oocyte from the antral follicle into a suitable culture medium (Pincus and Enzmann, 1935; Edwards, 1965). The follicular environment thus provides an inhibitory influence that maintains the oocyte in meiotic late prophase (Downs, 1990). It is indeed well demonstrated that the granulosa cells do actively suppress meiotic progression through junctional coupling with the oocyte cumulus cell complex (Racowsky and Baldwin, 1989; Dekel and Piontkewitz, 1991).

In vitro culture of denuded meiotically competent oocytes remains an invaluable model that may allow for the identification of possible metabolic pathways involved in the negative control of meiotic maturation. It is for instance well established that pharmacological activation of cAMP-dependent protein kinases (PKA) by either cell permeable analogs of cAMP (Cho *et al.,* 1974; Downs and Hunzicker-Dunn, 1995), isobutylmethyl-xanthine (IBMX: Dekel and Beers, 1978, 1980), hypoxanthine or adenosine (Shim *et al.*,

Abbreviations used in this paper: 6-DMAP, 6-dimethylaminopurine; GVBD, Germinal Vesicle Breakdown; MAPK, Mitogen-Activated Protein Kinase; MPF, Maturation Promoting Factor; OA, Okadaic Acid; PKA, Protein Kinase A; PKC, Protein Kinase C; PP1, Protein Phosphatases 1; PP2A, Protein Phosphatases 2A; PTK, Protein Tyrosine Kinase.

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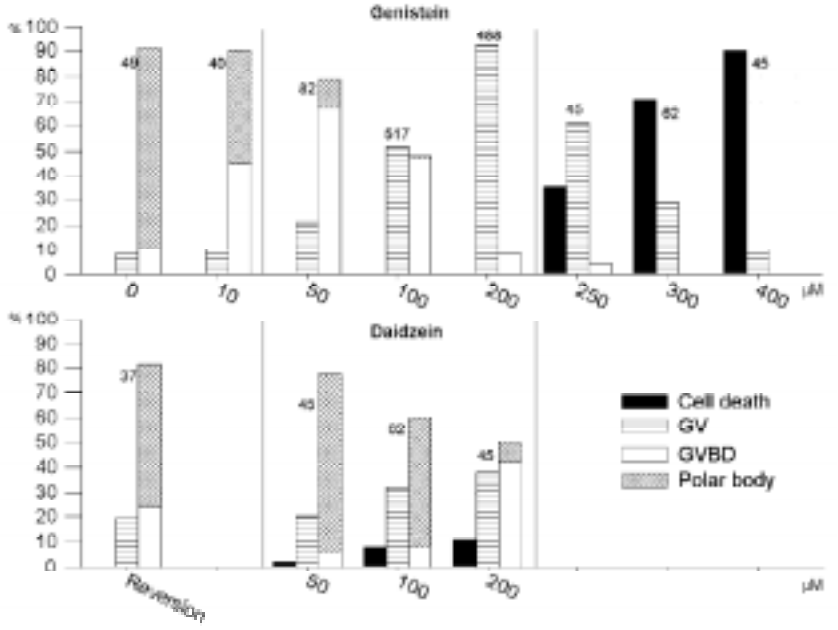

Fig. 1. Effect of increasing concentrations of genistein and daidzein on oocyte viability, GVBD and first polar body extrusion. *Oocytes were incubated or treated for 16 h and scored afterwards. Reversibility of genistein 200 µM was also tested (Reversion). Total numbers of oocytes that had been scored are indicated at the top of each bar diagram.*

1992; Downs, 1999) on the one hand, and of phospholipid-dependent protein kinases (PKC) by phorbol diesters (12-O-tetradecanoyl phorbol-13-acetate, TPA: Bornslaeger *et al.*, 1986) or analogs of diacylglycerols (OAG: Lefevre *et al.,* 1992) on the other hand, suppresses meiotic resumption. Conversely, 6-dimethylaminopurine (6-DMAP), a broad spectrum serine/threonine kinase inhibitor, also prevents mammalian oocytes from undergoing their germinal vesicle breakdown (GVBD) (Rime *et al.*, 1989; Alexandre *et al.*, 1991; Fulka *et al.,* 1991, 1995; Kalous *et al.*, 1993; Lévesque and Sirard, 1995; Avery *et al.,* 1998).

The fact that both activators and inhibitors of Ser/Thr kinases induce such a similar biological effect provides experimental evidence for the involvement of a complex network of interrelated protein phosphorylations (and dephosphorylations) pathways in oocyte meiotic maturation. This fits perfectly with the well-documented biochemistry of the M-phase checkpoint of the cell cycle (Rieder and Khodjakov, 1997). The decision to enter mitosis (or meiosis) is indeed negatively regulated by phosphorylation of Thr14 and Tyr15 of $p34^{cdc2}$, the catalytic subunit of the Maturation Promoting Factor (MPF), and positively by the phosphorylation of Thr161 and rapid dephosphorylation of both Thr14 and Tyr15. Kinases and phosphatases that catalyze these regulatory posttranslational modifications of $p34^{cdc2}$ have been identified: a cyclin-dependent kinase activating kinase (CAK or cdk7-cyclin H) phosphorylates Thr161 (Thuret *et al.*, 1996), WEE phosphorylates Tyr15 (Mc Gowan and Russell, 1995), MYT phosphorylates preferentially Thr14 (Liu *et al.*, 1997) while CDC25 dual phosphatase dephosphorylates both Thr14 and Tyr15 (Millar and Russell, 1992).

Each of these enzymes is however just one of the lasts links in a signaling cascade or most likely convergent metabolic chains. Indeed, our understanding of the way by which meiotic maturation is initiated is still expanding. A Ras-dependent alternative pathway was first demonstrated in amphibians (Birchmeier *et al.*, 1985) and, more recently, protein kinases including MAPK and Mos were shown to be activated during maturation in both amphibians (Jessus *et al.*, 1991; Shibuya *et al.*, 1992, 1996; Shibuya and Ruderman, 1993; Nebreda and Hunt, 1993; Posada *et al.,* 1993; Kosako *et al.*, 1994; Haccard *et al.,* 1995; Yamashita, 1998) and mammals (Verlhac *et al.,* 1993, 1994, 1996: Gebauer *et al.*, 1994; Kalab *et al.,* 1996; Oh *et al.*, 1998; Polanski *et al.*, 1998) but their functions remain a matter of speculation (Sagata, 1997). In this context, it is worth mentioning that, quite interestingly, okadaic acid (OA), a potent inhibitor of protein phosphatases type 1 (PP1) and type 2A (PP2A), has been shown to override the inhibitory effect of all the above-mentioned drugs on both chromatin condensation and GVBD but not on further steps of meiosis resumption (Alexandre and Van Cauwenberge, 1990; Rime and Ozon, 1990; Gavin *et al.,* 1991; Alexandre *et al.*, 1991). It has therefore been concluded that meiosis reinitiation is also negatively regulated by PP1 and/or PP2A activities.

Since the primary purpose of the present work was to more clearly define the relationship between the broad spectrum serine/threonine kinase inhibition induced by 6-DMAP and the meiotic arrest of the mouse oocyte, various selective inhibitors of serine/threonine kinases [PKA, PKC, Ca^{2+}/calmodulin dependent kinases (CaMK) and myosin light chain kinase (MLCK)] were tested. This biopharmacological approach actually failed to assign the inhibitory effect of 6-DMAP to one of these categories of protein kinases (Van Cauwenberge, data not shown). On the other hand, while, to the best of our knowledge, the *c-kit* tyrosine kinase, Kit, contributing to the maintenance of oocyte meiotic arrest (Ismail *et al.*, 1997) and $p59^{c\text{-}fyn}$, playing a role in late events of egg activation, are the only protein tyrosine kinases (PTK) that have been identified in the mammalian eggs (Talmor *et al.*, 1998), indirect experimental evidences suggest that tyrosine phosphorylations are implicated in the positive regulation of mammalian oocyte maturation (Gillet *et al.*, 1992; Jung *et al.*, 1993; Kimura, 1996). GVBD is indeed inhibited by genistein, a broad range tyrosine kinase inhibitor (IC_{50} = 2.6 - 26 µM), which however exerts a trivial effect on PKA and CaMK (IC_{50}> 200 µM) and a more significant one on PKC (IC_{50} = 15-185 µM) (Akiyama and Ogawara, 1991).

In order to assess whether the inhibitory effect of genistein on maturation is attributable to tyrosine kinase inhibition, we compared the effect of genistein with that of tyrphostin B46 and lavendustin A (Anafi *et al.*, 1992; Hsu *et al.*, 1991; Kovalenko *et al.*, 1997), two selective inhibitors which act by binding to the substrate binding site of the enzyme instead of to the ATP binding site. We also used daidzein, a structural analog of genistein that does not block PTK (Akiyama *et al.*, 1987), in order to assess the specificity of genistein as a tyrosine kinase inhibitor. Both drugs actually received increasing attention mainly because of their potentially chemoprotective role against cancer and cardiovascular diseases (Mazur, 1998; Dubey *et al.*, 1999). They are indeed known to inhibit proliferation and to promote differentiation of both normal and cancer cells (Kim *et al.*, 1998a). However, there are presently few data on their effects on meiotically dividing reproductive cells. Therefore we were mainly interested in dissecting the cytological effects of genistein on mouse primary and secondary oocytes, especially since we show that, in contrast to both tyrphostin B46 and lavendustin A (data not shown), genistein reversibly inhibits GVBD with an ED of about 100 μM.

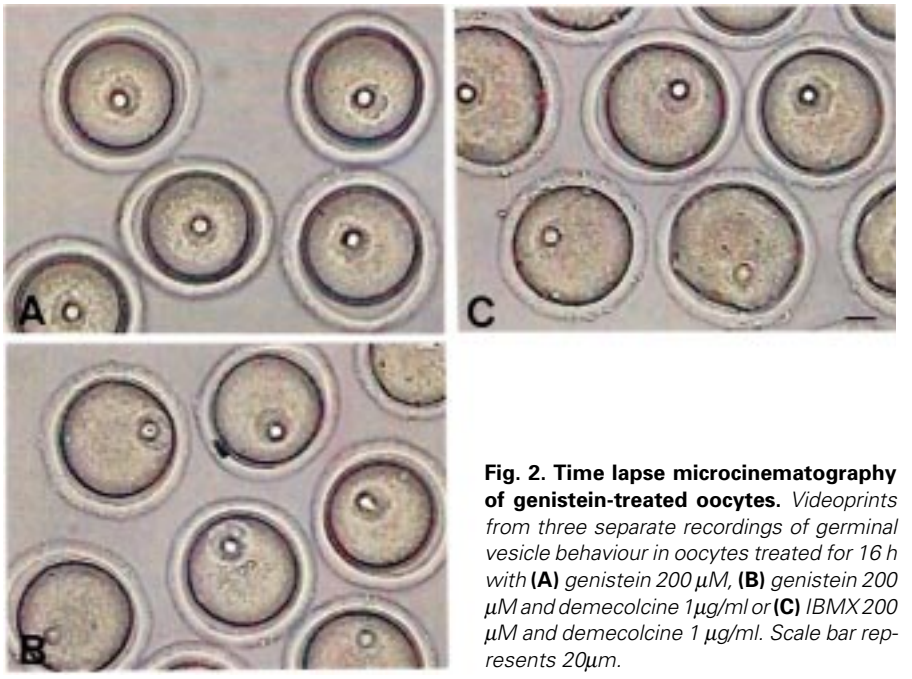

Fig. 2. Time lapse microcinematography of genistein-treated oocytes. *Videoprints from three separate recordings of germinal vesicle behaviour in oocytes treated for 16 h with* **(A)** *genistein 200 μM,* **(B)** *genistein 200 μM and demecolcine 1μg/ml or* **(C)** *IBMX 200 μM and demecolcine 1 μg/ml. Scale bar represents 20μm.*

We used an immunocytological approach in order to analyze both cytoplasmic and nuclear features of the meiotic resumption in treated oocytes, and to compare the cytological effects of genistein to those induced by the few other pharmacological agents displaying the same cytostatic effect (Alexandre and Mulnard, 1988; Alexandre *et al.*, 1989, 1991). In order to know whether inhibition of PP1 and PP2A abolishes the inhibitory effect of genistein on GVBD and chromatin condensation as it does with activators of both PKA and PKC and with 6-DMAP (Alexandre *et al.*, 1991; Gavin *et al.*, 1991), oocytes were treated simultaneously with genistein and okadaic acid, particular attention being paid to MAP Kinase. The activation of the latter, easily revealed by observing its mobility shift in a gel, was indeed demonstrated to be correlated with meiosis reinitiation in the mouse (Verhlac *et al.*, 1993, 1994, 1996; Sun *et al.*, 1999). Genistein exerts the same cytological effects as IBMX on primary oocytes. Its GV-arresting activity is also bypassed by OA but combination of both drugs results in a dramatic cytoskeleton reorganization leading to a extensive membrane bulging. When applied after GVBD has taken place, genistein does not inhibit MAPK activation, metaphase spindle formation and metaphase-to-anaphase transition but prevents the oocytes from extruding their first polar bodies. It is also shown to induce metaphase-to-interphase transition in about 50% of the MII oocytes but neither cortical granules (CG) exocytosis nor second polar body extrusion.

Results

Concentration dependence, reversibility and specificity of genistein-induced effects on oocyte maturation

The effects of tyrosine kinase inhibitors were evaluated at increasing concentrations on at least 80 oocytes. This allowed us to determine the highest non-toxic concentrations which were the following for genistein: 200 μM, for lavendustin A: 100 μM and for tyrphostin B46: 10 μM. Genistein was the only of these inhibitors that prevented GVBD, making it worth to analyze further its effects.

Since it has been reported that some DMSO-soluble agents may partition to the oil overlay covering the oocytes-containing medium (O'Sullivan *et al.*, 1993), we did preliminary experiments comparing the effect on maturation of different concentrations of genistein and daidzein, its inactive analogue, in the absence of paraffin oil overlay. These demonstrated that the presence of oil does not modify their biological effects. All experiments were thus performed under oil in order to avoid evaporation.

As shown on Fig. 1, genistein inhibited germinal vesicle breakdown (GVBD) as well as the first polar body extrusion in a dose dependent manner, with a highest GVBD inhibitory concentration of 200 μM, which exerted no cell toxicity. Cell mortality indeed appeared from 250 μM. Moreover, the inhibitory effect of genistein at 200 μM was reversible: oocytes treated for 16 h with genistein 200 μM were subsequently put in control medium and scored 16 h later. Both GVBD and polar body extrusion rates, while being slightly lower than in the control group, were high when compared with the status of oocytes after 16 h of genistein treatment. This concentration was therefore applied to all the experiments carried out in this work. Quite unexpectedly, while being significantly less inhibitory on both GVBD and polar body extrusion than genistein, daidzein exerted a more cytotoxic effect on oocytes.

Effect of genistein on microtubule dependent germinal vesicle positioning

In previous time-lapse microcinematography studies (Alexandre and Mulnard, 1988; 1991; Alexandre *et al.*, 1989), we showed that in phorbol diesters-treated oocytes, GV undergoes a displacement towards the cortex according to the same biological clock that triggers the programmed translocation of the spindle in control oocytes. By contrast, in both IBMX- and 6-DMAP-treated oocytes,

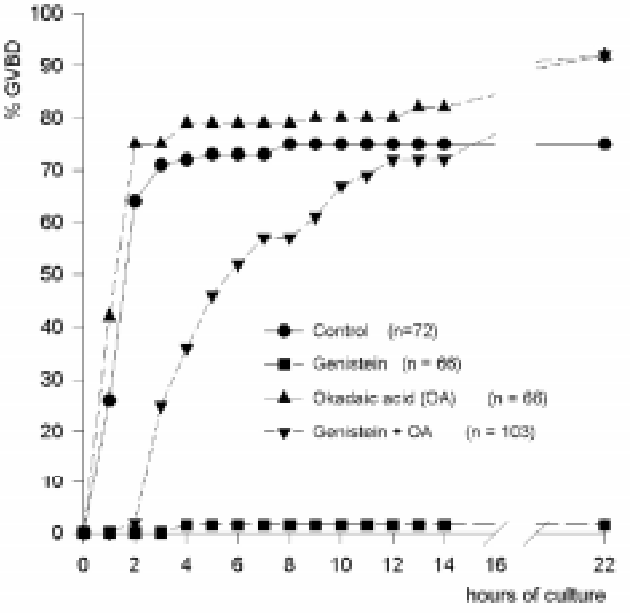

Fig. 3. Effect of okadaic acid on the GVBD kinetics of both control and genistein-arrested oocytes.

GV remains in a central position but the cytoplasmic "centrifugal displacement property" (CDP) is restored when microtubules are depolymerized by demecolcine. In order to determine whether genistein was inhibitory of this 'CDP', we carried out a time-lapse microcinematography study allowing us to follow the behaviour of GV in oocytes cultured for 16 h in the presence of genistein alone or in combination with demecolcine. GV obviously remained in a central position in genistein-treated oocytes (Fig. 2A) as that observed in IBMX-treated oocytes, demecolcine inducing their cortical translocation in both situations (Fig. 2B, C respectively).

Okadaic acid bypasses the inhibitory effect of genistein on GVBD

Oocytes were cultured in control medium, in the presence of OA 1μM alone, genistein 200 μM alone, or both drugs in combination, and scored hourly for evidence of GVBD. According to the well documented time-course of maturation in the mouse, most of the oocyte population underwent GVBD within 2 hours in both control and OA containing medium. While OA 1 μM alone slightly accelerated GVBD, it induced GVBD in genistein-arrested oocytes with a delay of about 3 hours as compared to the control situation (Fig. 3). A difference between control and OA-treated oocytes was detectable, irrespective of the presence of genistein, after 22 hours of culture when 95% of oocytes in both OA groups had undergone GVBD in contrast to 75% in the control group**,** a quite low percentage maybe due to the fact that it was removed hourly from the incubator to be scored. OA might therefore also bypass suboptimal culture conditions. The first polar body was however never extruded in the presence of OA.

The cytoskeleton of IBMX- and genistein-treated oocytes is differentially reorganized by OA

An indirect immunofluorescence study was carried out in order to visualize the effects of genistein applied alone or in combination with other drugs on microtubule organization. Oocytes that were fixed and processed for immunofluorescence immediately after their removal from their follicle (0 h) displayed a typical cytoplasmic network of microtubules centered on a microtubule organizing center (MTOC) generally localized near the germinal vesicle (Fig. 4A), while oocytes cultured for 16 hours in normal culture medium had extruded their first polar body and displayed a typical metaphase II spindle near the cortex with chromosomes aligned on an equatorial plate (Fig. 4B). In genistein-treated oocytes (Fig. 4C) as well as in IBMX-treated oocytes (Fig. 4D), GV never broke down. In addition, the interphase microtubular organization remained until the end of the treatment (16 hours) in both experimental conditions.

We confirm that spindle never forms in OA-treated oocytes (Fig. 4E). A high background was visible together with an array of microtubules radiating from an unstained central area. Chromatin was found segregated into a few separated pycnotic masses closely associated with the microtubule radiating array. The same distribution of both microtubules and chromatin was observed when OA was used to bypass the inhibitory effect of IBMX (Fig. 4F). When OA was used in combination with genistein, chromatin condensation also took place but the immunofluorescence background was higher and a single aster of microtubules sometimes formed (Fig. 4G). The most striking effect was however a dramatic cytoplasmic rearrangement that took place in about 60% of the treated oocytes, where a huge quantity of cytoplasm apparently free of organelles was confined within a single large bulge. The remaining granular cytoplasm containing the pycnotic mass(es) of chromatin was limited by a microtubule cage (Fig. 4H). A time-lapse microcinematography analysis was carried out in order to determine the timing of this cytoplasmic segregation. It showed that the membrane deformation takes place about 8 hours after the beginning of the treatment (Fig. 5A, B). The large hyaloplasmic bulge then starts to move around the remaining part of the oocyte like a large lobopode. This lobopodal movement lasts 6 to 10 hours and culminates with cell death. These oocytes were checked for the presence of F-actin with FITC-Phallacidin. Microfilaments were observed in a relatively uniform area in the cell cortex of both hyaloplasmic bulge and granuloplasm (Fig. 5D).

Finally, DNA fragmentation was tested using the terminal transferase-mediated DNA end-labeling (TUNEL) assay. Both OA- and OA + genistein-treated oocytes were TUNEL negative (not shown), suggesting that OA does not induce apoptosis.

Effect of genistein on spindle formation, metaphase-anaphase transition and polar body extrusion when added after GVBD

Oocytes were allowed to undergo spontaneous maturation, fixed at different times of incubation (2, 4, 6, 10 and 16 hours) and processed for both chromatin and tubulin staining. After 2 hours of incubation, the oocytes had undergone GVBD and reached the very first step in spindle formation, as demonstrated by Fig. 6A which shows an array of short microtubules surrounding the condensing chromosomes. By 4 hr of incubation, a well-defined spindle was visible in a central position, with bivalents aligning at its equator (Fig. 6C). A typical metaphase I spindle with chromosomes aligned on it was always found in oocytes incubated for 6 hr in normal culture medium (Fig. 6E). By 10 hr, metaphase I spindle had moved to the cortex, while, by 16 hr, the first polar body had been extruded and the metaphase II spindle was oriented parallel to the cortex (Fig. 6I).

In another experiment, samples of oocytes incubated in control medium for 2, 4, 6 and 10 hours were not fixed immediately but were transferred into genistein- or daidzein- containing medium for additional periods of 14, 12, 10 and 6 hours, respectively, before fixation and staining of chromatin and tubulin. All preparations were thus incubated for a total period of 16 hours, similar to oocytes allowed to mature in culture medium from the beginning of the experiment.

Oocytes having undergone GVBD after 2 hr of incubation and treated afterwards with genistein for 14 hr, did organize a barrel-shaped spindle which reached the anaphase stage (Fig. 6B). In oocytes incubated for 4 or 6 hours before exposure to genistein, spindles had reached telophase without elongation of the fibers, thus retaining a metaphase configuration. Microtubules were also found on the poles and in the cytoplasm, radiating from microtubule organizing centers (MTOC) (Fig. 6 D, F). As could be expected from its effect on microtubule dependent germinal vesicle positioning (Fig. 2), genistein prevented the spindle from translocating towards the cortex when applied before it started to move (Fig. 6C, D versus Fig. 6E, F). In sharp contrast to these three situations in which cytokinesis never took place, when treated with genistein at the anaphase I stage (oocytes incubated for 10 hours: Fig. 6G), oocytes did extrude their first polar body quite normally (Fig. 6H).

Oocytes having undergone GVBD after 2 hours of incubation and treated afterwards with daidzein instead of genistein complete their maturation normally (data not shown).

Differential effect of genistein on MAP kinase activity when added before and after GVBD

Samples of 25 maturing oocytes were collected at different times of incubation (0, 2, 4, 6 and 16 hr) and immunoblotted using an antiserum raised against a peptide present in both ERK-1 and ERK-2, in order to detect the slight increase of the *Mr* of MAP kinase due to its activation by phosphorylation. Only GVBD-stage oocytes were analyzed after 2, 4, 6 and 16 hours of culture. The two proteins of approximate *Mr* 44 and 42 kDa, respectively, were detected. After 2 hr of incubation, the *Mr* of both ERK-1 and –2 remained unchanged (Fig. 7: control 0 h, control 2 h) while by 4 h of incubation, the *Mr* had increased slightly and, once the slow-migrating forms of both proteins had been generated, they remained throughout maturation to metaphase II (Fig. 7: control 16 h).

The mobility shift did not occur when oocytes were incubated in the presence of genistein from the GV stage onwards (Fig. 7: genistein 16 h). By contrast, it occurred when oocytes preincubated for 2 hr, thus having undergone GVBD, were treated for an additional 14 hr (Fig. 7: del. treat. 2 h). Finally, once generated, slow migrating forms of ERK-1 and ERK-2 remained in oocytes treated with genistein for 10 and 12 hr after preincubations of 6 and 4 hr, respectively.

Effect of genistein on metaphase II oocytes

Oocytes cultured for 16 hours in normal culture medium and which had extruded their first polar body (Fig. 8B) were treated for an additional 24 hr with 200 μM genistein. This treatment resulted in the appearance of a readily identifiable pronucleus-like structure without chromosome segregation in about 50% of them, as revealed by both interference contrast (Fig. 8D) and immunofluorescence (Fig. 8E) microscopy. A similar metaphase-interphase transition has not been observed in MII oocytes cultured for an additional 24 hr in genistein-free medium. Immunofluorescence microscopy also showed that an interphase-like network of microtubules organizes in the cytoplasm (Fig. 8E). A time-lapse microcinematography analysis clearly confirmed that the second polar body was never extruded (not shown). Finally, these oocytes

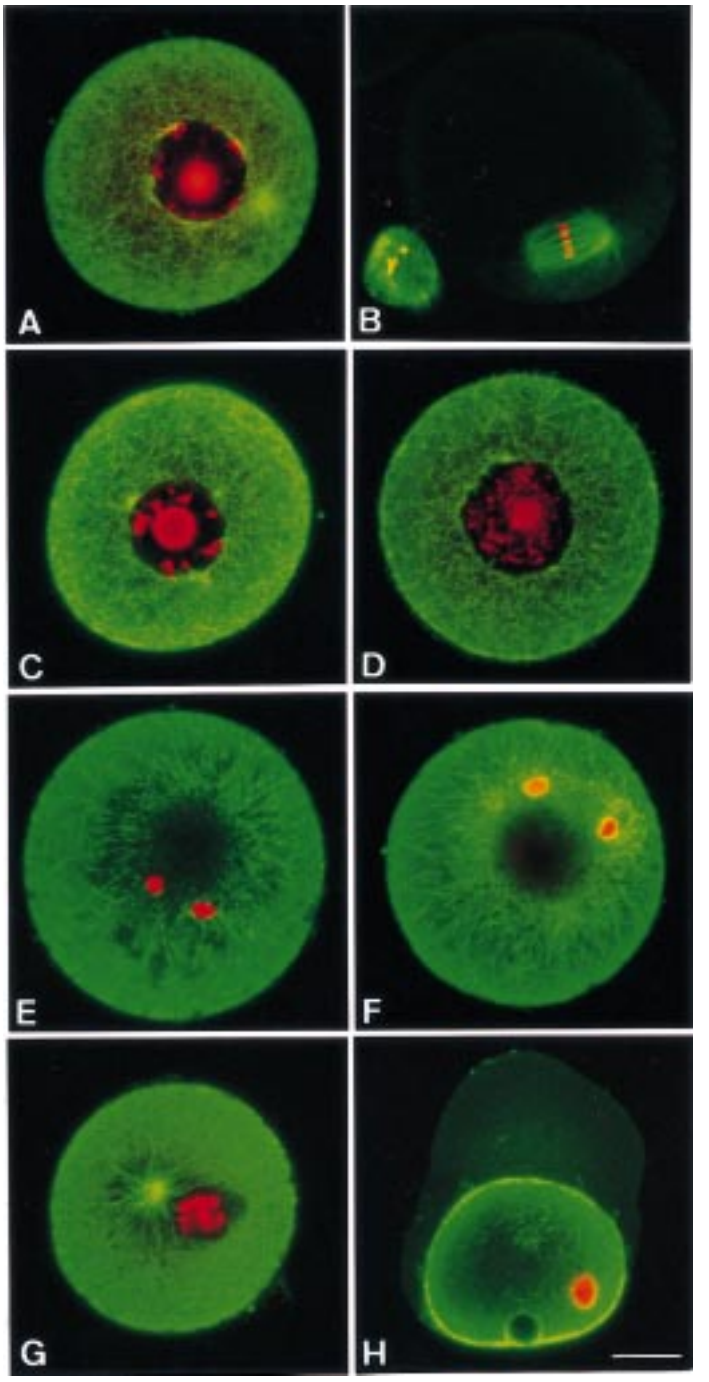

Fig. 4. Effect of OA on microtubules and chromatin organization in pharmacologically - arrested primary oocytes. *Immunolocalization of microtubules (green) and chromatin staining (red) were performed on primary oocytes* **(A)** *removed from the follicle, or incubated for 16 hr in* **(B)** *control medium, in the presence of* **(C)** *genistein,* **(D)** *IBMX,* **(E)** *OA and a combination of* **(F)** *OA and IBMX or* **(G, H)** *OA and genistein. All pictures are single confocal sections. Scale bar represents 20μm.*

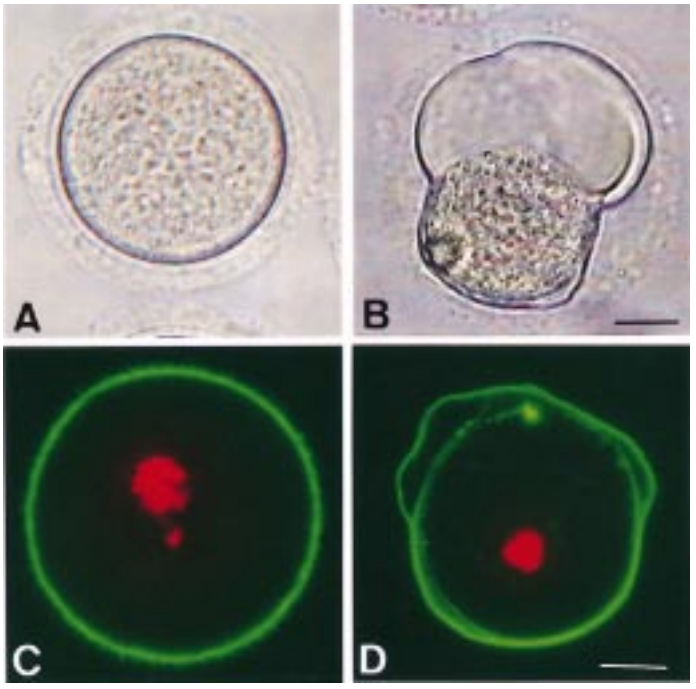

Fig. 5. Effect of a combination of OA and genistein on the cell cortex. *About half of the oocytes treated for 16 h display a huge cytoplasmic bulge as shown on one single videoprint from our time-lapse recording* **(A***: no bulging;* **B***: bulging***)**. *Cortical F-actin and chromatin of similar oocytes were stained with FITC-Phalloidin (green) and TOTO-3 (red) respectively. The single optical sections clearly show that the cortical layer of microfilaments is continuous in oocytes displaying* **(C)** *no bulge and* **(D)** *split in one cortical and one internal leaflet where the bleb is formed. Scale bar represents 20μm.*

did not undergo the loss of their cortical granules since they displayed the same CG density and distribution as untreated oocytes (Fig. 8C, F).

Discussion

The inhibitory effects of genistein on the maturation of mammalian oocytes is now well documented (Gillet *et al.*, 1992; Jung *et al.*, 1993; Kimura, 1996) but it seems from the present work that it is not due to its effect on tyrosine kinases. The comparison between the effects of genistein and daidzein, two structurally related isoflavones, has often been used to make sure that the effect of genistein is attributable to inhibition of tyrosine kinases (Neye and Verspohl, 1998). The fact that daidzein does exert a less inhibitory effect on meiosis reinitiation than genistein and no effect on GVBD-stage oocytes does not however allow one to conclude that the effects on maturation that we observed are a consequence of tyrosine kinase inhibition. We show indeed that both tyrphostin B46 and lavendustin A, two selective inhibitors acting by binding to the substrate binding site of PTK (Hsu *et al.*, 1991; Anafi *et al.*, 1992; Kovalenko *et al.*, 1997) fail to inhibit meiosis reinitiation.

It is now well established that genistein may affect cells by mechanisms other than inhibition of PTK activity (Peterson, 1995; Kim *et al.,* 1998a), some of which being relevant to its inhibitory activity on meiosis reinitiation in mouse oocytes. G2/M arrest induced by genistein in mammalian cell lines has indeed been reported as a unique effect among flavonoids (Matsukawa *et al.*, 1993; Balabhadrapathruni *et al.*, 2000) and was suggested to be in part due to an inhibition of kinase activities of cdc2 and cdk2, and a decrease in cyclin B1 expression (Choi *et al.*, 1998). Genistein was also shown to inhibit mammalian type II topoisomerase and to stabilize the topoisomerase-DNA cleaved complex (Yamashita *et al.*, 1990; Kaufmann, 1998), raising the possibility that it induces G2 delay by triggering the G2 checkpoint response (Lock and Ross, 1990). Moreover, Nakajo *et al.,* (1999) recently succeeded in identifying a G2 checkpoint kinase, XChk1, involved in physiological prophase I arrest of *Xenopus* oocytes. Chk1 was shown to phosphorylate serine-216 of the mammalian dual-specificity protein phosphatase Cdc25C, creating a binding site for 14-3-3 protein which inhibits the function of the phosphatase. This prevents activation of the Cdc2-cyclin B complex and mitotic entry (Sanchez *et al.*, 1997; Peng *et al.*, 1997). A similar pathway was demonstrated in *Xenopus* eggs (Kumagai *et al.*, 1998a,b). According to these findings, it may be suggested that in mammalian oocytes, genistein indirectly keeps MPF in its inactive state via Chk1 activation, as does IBMX via PKA activation. This hypothesis, which obviously needs biochemical confirmation, might explain why genistein- and IBMX-treated prophase I oocytes (Alexandre and Mulnard, 1988; Alexandre *et al.*, 1989; Alexandre *et al.*, 1991) behave quite similarly, i.e. their germinal vesicle is maintained in a central position, the cytoplasmic microtubule network is stabilized, the central GV immobilization is overcome by demecolcine and they resume and complete normal maturation after their transfer to culture medium.

Confocal microscopy used in the present study allowed us to demonstrate that GV immobilization by both IBMX and genistein is correlated with the stabilization of the perinuclear microtubule network centered on a microtubule organizer center (MTOC). As in many cells, MTOC is closely associated with the nucleus (GV). This, together with the fact that demecolcine overcome the GV immobilization and allows its cortical translocation, strongly suggests that the well documented central nuclear positioning in rodents primary oocytes (Szöllösi, 1993) is of the predominant "MTOC-dependent' type according to the Reinsch and Gönczy classification (Reinsch and Gönczy, 1998). It was reported that, at least in rodents and carnivores, the peripheral migration of the GV represents the first morphological sign of initiation of oocyte maturation *in vivo* (Szöllösi, 1993). The use of pharmacological modulators of protein kinases might thus help us to understand the molecular basis of this early step potentially controlled by microtubule polymerization and dynamics, motor proteins, Microtubule Associated Proteins (MAP) and/or cortical anchors (Reinsch and Gönczy, 1998). It may for instance be suggested that central nuclear positioning is positively controlled by PKA that might be indirectly activated by genistein. It has also been suggested that microfilaments, which seem to move the chromatin to the proper position after GVBD in mouse (Longo and Chen, 1985; Maro *et al.*, 1986; Van Blerkom and Bell, 1986), pig (Kim *et al.*, 1996) and human oocytes (Kim *et al.*, 1998b), are also instrumental in the *in vitro* induced centrifugal migration of the GV (Alexandre *et al.*, 1989). We concluded from these results that centrifugal translocation of either GV or metaphase I spindle is triggered by the same process mediated by both microtubules and microfilaments (Alexandre *et al.*, 1989). This conclusion now is strengthened by the fact that in genistein-treated oocytes both GV and the barrel-

shaped MI spindle are prevented from undergoing their peripheral migration, respectively, from the beginning of incubation and just after GVBD took place (2 hr of incubation).

We show here for the first time that genistein-treated prophase I oocytes are induced to undergo chromatin condensation and GVBD by okadaic acid (OA). This effect is similar to PKA-, PKC- or 6-DMAP-arrested oocytes treated with OA (Rime and Ozon, 1990; Gavin *et al.*, 1991; Alexandre *et al.*, 1991). OA, a specific inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A), with IC_{50} values of about 10 and 0.1 nM, respectively for these targets (Cohen *et al.*, 1990), is actually known to induce meiosis reinitiation in starfish (Picard *et al.*, 1989; Pondaven *et al.*, 1990), *Xenopus* (Goris *et al.*, 1989) and physiologically incompetent mouse oocytes (Chesnel *et al.*, 1994; Chesnel and Eppig, 1995a,b; de Vantery *et al.*, 1997). It also overcomes pharmacologically induced meiotic block in mouse (Rime and Ozon, 1990; Alexandre *et al.*, 1991; Gavin *et al.*, 1991; Gavin *et al.*, 1992), pig (Kalous *et al.,* 1993) cattle (Kalous *et al.,* 1993; Levesque and Sirard, 1995) and macaque competent oocytes (Smith *et al.*, 1998b). These latter results together with the present work lead to the conclusion that inhibition of critical phosphatase(s), operating downstream from all the enzymatic activities whose pharmacological activation or inhibition prevents GVBD, triggers entry into M phase as a result of MPF activation.

However, we confirm that exposure to OA is deleterious to the later events of meiotic maturation since metaphase spindle never forms whether this inhibitor is used alone or in combination with either IBMX or genistein. This of course raises the question of OA actually inducing early meiotic events by activating MPF, a process well known to trigger spindle formation. It has been suggested that an alternative pathway depending on MAP kinase instead of MPF activation may lead to GVBD in OA-treated oocytes (Gavin *et al.*, 1994). The present study correlates the course of activation of MAP kinase and germinal vesicle breakdown independently of the presence of genistein. According to previous reports (Sobajima *et al.*, 1993; Verlhac *et al.*, 1993; Verlhac *et al.*, 1994; Gavin *et al.*, 1994; Kalab *et al.*, 1996; Polanski *et al.*, 1998), a mobility shift of $p42^{MAPK}$ and $p44^{MAPK}$ on the blot is indeed observed when oocytes have undergone spontaneous or OA-induced GVBD, which suggested that MAP kinase is activated after GVBD. Moreover, MAP kinase is activated in oocytes treated with genistein when they have just finished GVBD, at 2 hr postincubation. This allows one to conclude that genistein is indeed inhibitory of an upstream step of the metabolic pathway leading to MAP kinase activation and not of MAP kinase activation itself. Quite interestingly, using a very sensitive method for detecting MAP kinase activity, Sun *et al.,* (1999) recently confirmed that MAP kinase is activated after GVBD and concluded that it is not the activation of MAP kinase itself, but the activation of some upstream molecule(s) that regulates MAP kinase activity that is required for the resumption of meiosis. Since MPF might be such an upstream activator (Verhlac *et al.*, 1994), it is more likely that OA activates MPF. It might actually overcome GV block by inhibiting a nuclear phosphatase since it has been reported that both MPF (Mitra and Schultz, 1996) and PP1α (Smith *et al.*, 1998a) are highly concentrated in the GV of competent oocytes. Thus, it has been suggested by Smith *et al.,* (1998a) that MPF and PP1α may act antagonistically in the regulation of specific phosphoacceptor sites on nuclear lamins known to become phosphorylated before nuclear envelope matrix disassembly. It may thus be hypothesized that, despite its positive effect on chromatin condensation and GVBD, probably through mechanisms activating MPF, OA is inhibitory of another maybe downstream pathway playing an important role in bipolar spindle formation. Fulka *et al.,*

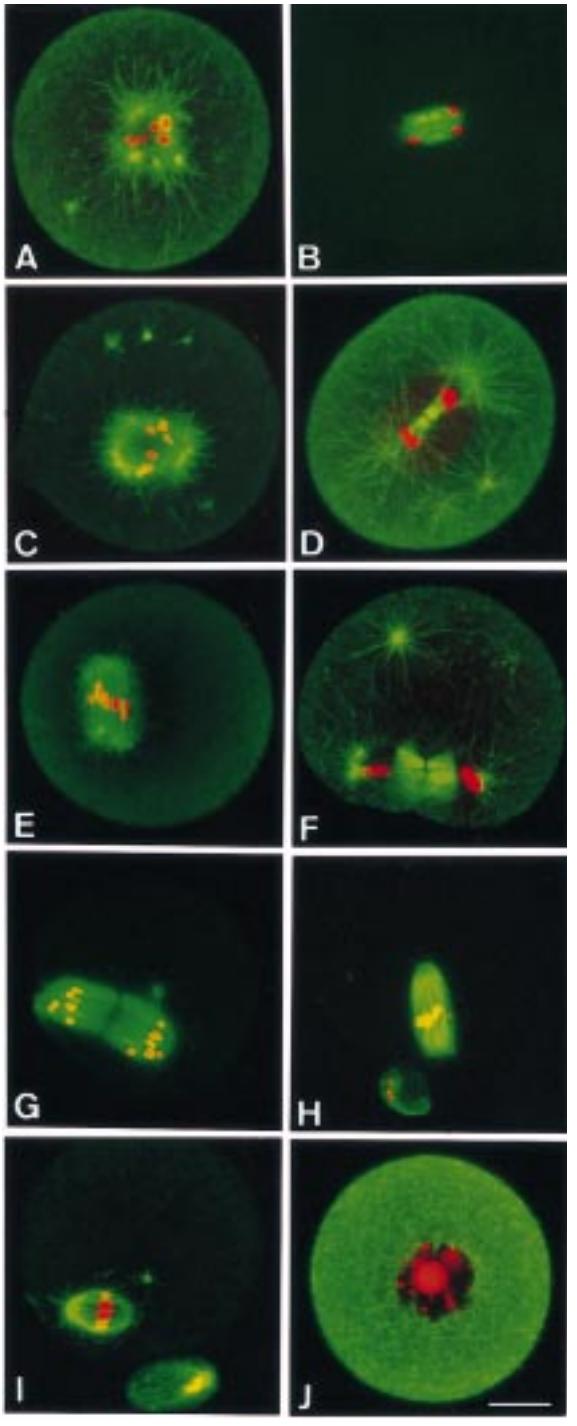

Fig. 6. Microtubules and chromatin organization in control and genistein-treated oocytes. *Maturing oocytes were either fixed at different times of incubation* **(A***: 2 hr;* **C***: 4 hr;* **E***: 6 hr;* **G***: 10 hr;* **I***: 16 hr) or incubated for 2, 4, 6 and 10 hr and treated with genistein for an additional period of* **(B)** *14 hr,* **(D)** *12 hr,* **(F)** *10 hr and* **(H)** *6 hr, respectively.* **(J)***: oocyte treated with genistein for 16 hr from the GV stage on. Oocytes were double stained with FITC for microtubules and TO-PRO 3 for chromatin and chromosomes. Scale bar represents 20μm.*

(1995) indeed demonstrated that while molecules or activities required for chromatin condensation are present throughout metaphase, those involved in spindle formation are absent in early M-phase. Relevant to this discussion is the recent identification of some downstream effectors of $p34^{cdc2}$-cyclin B1 such as the polo like kinases (reviewed in Ohi and Gould, 1999) and a kinesin-related motor protein Eg5 (Blangy *et al.*, 1995; Nigg *et al.*, 1996; Roghi *et al.*, 1998) that both colocalize with a portion of $p34^{cdc2}$-cyclin B1 to centrosomes. In addition, the direct involvement of PP2A in spindle assembly is strongly suggested by immunofluorescence microscopy studies that revealed its association with interphasic microtubules, centrosomes and mitotic spindles (Sontag *et al.*, 1995).

In sharp contrast to their identical effect on G2 checkpoint, genistein and IBMX exert quite different effects on further steps of maturation. While metaphase spindle assembly and chromosomes segregation are unaffected by either drug when added after GVBD, only genistein prevents oocytes from undergoing completion of anaphase-telophase I and cytokinesis. Since successful chromosome segregation took place even when genistein was added just after GVBD (2 hr of incubation), it may however be concluded that the checkpoint control for anaphase onset is also unaffected by both drugs. It is worth noting that, in contrast to somatic cells where this checkpoint is known to monitor both spindle architecture and kinetochore attachment (reviewed in Rieder and Khodjakov, 1997), it only monitors the former in mammalian oocytes (LeMaire-Adkins *et al.*, 1997). Genistein thus blocks the exit from the first meiotic division through effects on events taking place after chromosome segregation, that is to say, spindle elongation in anaphase B, spindle degradation, mid-body formation and cytokinesis. In mitotically dividing cells, these events are governed by the anaphase-promoting complex (APC) which triggers the ubiquitin-dependent proteolysis of key regulatory proteins such as the anaphase inhibitor Pds1 and mitotic cyclins (Morgan, 1999). Proteolysis of Pds1 actually triggers sister chromatids separation while destruction of mitotic cyclins together with inactivation of Cdk1 are required for mitotic exit, both steps being however coordinated by the regulatory factor APC^{Cdc20} (Shirayama *et al.*, 1998). It is however likely that Pds1-mediated anaphase onset does not apply to meiotic anaphase I since homologues instead of sister chromatids are segregated to opposite poles of the spindle; this indeed requires preservation of sister cohesion at centromeres until the second meiotic division (Nasmyth, 1999). Because of its action restricted to the first meiotic division exit, genistein might be a suitable tool for the study of APC function during meiosis, the latter being still poorly understood. Our results

Fig. 7. MAP kinase activity. *Oocytes were treated with genistein from the GV stage on (genistein 16h) or at different times after GVBD (del. treat. 2h, 4h, 6h: oocytes incubated for 2, 4 and 6 hr and treated with genistein for an additional period of 14, 12 and 10 hr respectively). Lysates of oocytes were analyzed by immunoblotting using an anti-ERK antiserum and changes in electrophoretic mobility of ERK-1 and ERK-2 were observed.*

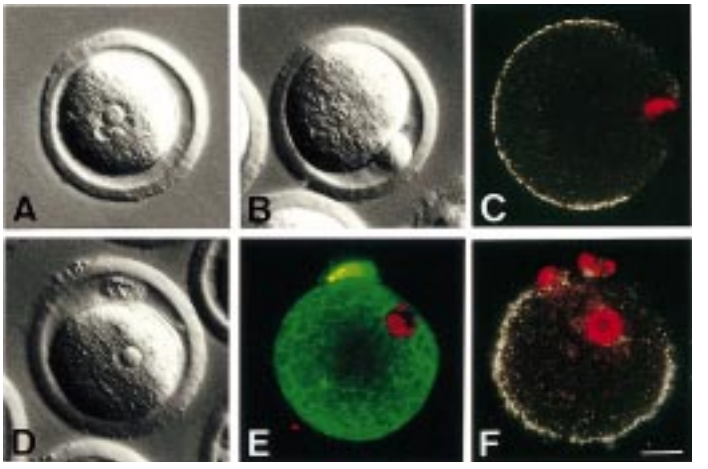

Fig. 8. Metaphase-interphase transition observed in MII oocytes treated with genistein. (A) *An oocyte incubated for 16 hr in genistein;* **(B, C)** *control oocytes incubated for 16 hr and having extruded their first polar body.* **(D, E and F)** *MII oocytes treated with genistein for 20 hr, having retained their first polar body and displaying a pronucleus-like structure. A, B and D: bright-field micrographs made with an inverted Leitz microscope equipped with Hoffmann modulation contrast optics. E: One optical section of an oocyte double stained with FITC for microtubules (green) and TO-PRO 3 (red) for chromatin. C and F: single optical sections of oocytes double stained for cortical granules with FITC (grey) and chromosomes with TOTO-3 (red). Scale bar represents 20μm.*

indeed suggest that spindle anaphase B, spindle degradation, mid-body formation and cytokinesis are triggered by a genistein-sensitive mechanism that might be a mid-anaphase checkpoint monitoring chromosome integrity before cytokinesis, similar to the one recently identified in budding yeast (Yang *et al.*, 1997). As a topoisomerase II poison, genistein might be a clastogen inducing chromosome aberrations (Kaufmann, 1998).

Genistein has also often been reported to induce apoptosis in cultured mammalian cells (reviewed in Balabhadrapathruni *et al.*, 2000). However, oocytes treated with genistein never displayed any of the morphological features of apoptosis, and the reversibility of the treatment demonstrates that they are not even committed to the death program. This might be somewhat surprising since mouse oocytes are known to be competent to undergo apoptosis (Perez at al., 1999; Morita and Tilly, 1999). In contrast, OA might induce several features of apoptosis among such as extensive nuclear condensation (pyknosis) and membrane deformation (Gavin *et al.*, 1991; Alexandre *et al.*, 1991), the latter being dramatically enhanced by genistein. Indeed, the most striking effect of the combination of OA and genistein is a huge surface bulge formation which is by no means a blebbing-like phenomenon. Blebbing is one of the hallmark morphologic features of the extranuclear execution phase of apoptosis (Mills *et al.*, 1999). In most cells, blebbing follows the cell rounding up that occurs during the so-called "release phase" which of course does not apply to oocytes. In addition, while apoptotic blebbing results from microtubule disassembly and focal weakening of the membrane-actin linkage (Mills *et al.*, 1999), the hyaloplasmic bulge that we observe is correlated with a local separation of the cortical microtubule and actin cytoskeleton systems, reminiscent of the separation of cortical hyaline plasma from central granular plasma induced by phalloidin in

amoebas (Stockem *et al.*, 1978). Our observations which are far too preliminary to allow any satisfactory molecular explanation for this unusual phenomenon show however that subtle manipulations of protein kinases and/or protein phosphatases may dramatically modify the cortical architecture of a spherical cell which then display some protoplasmic streaming pattern and locomotory activity.

Finally, we confirm that genistein induces transition to interphase in about 50% of metaphase II oocytes. This however took place without emission of the second polar body, in contrast to the recent data of Sun *et al.,* (1998) who also demonstrated that this transition is induced by inactivating MAPK. The difference in concentrations used may be the reason for this discrepancy. On the other hand, we show here that genistein never induces cortical granules (CG) exocytosis, the cytoplasmic hallmark of activation (Ducibella *et al.*, 1990, 1993; Ducibella and Buetow, 1994). This allows us to conclude that genistein induces a metaphase-interphase transition instead of a true activation.

Material and Methods

Oocytes collection, culture and treatment

Fully grown mouse oocytes were collected by puncture of ovaries from hormonally unprimed 6- to 8- week old NMRI mice (IFFA Credo Belgium) in a Krebs-Ringer-Bicarbonate culture medium (KRB-4, Mulnard and Puissant, 1984) supplemented with 4 mg/ml bovine serum albumin (BSA, Sigma) containing 45 μg/ml 3-isobutyl-1-methylxanthine (Sigma). Most of the oocytes were already denuded at the time they were released from the follicles. They were cultured for 16 hours in 0,8 ml of control or experimental culture medium under 0,4 ml paraffin oil (Sigma) at 37°C in a humidified atmosphere of 5% CO2 in air, using four-well multidishes (Nunc) as culture vessels.

Drugs were diluted in KRB-4 medium from the following stock solutions: genistein (Calbiochem) 100 mM/DMSO; 12-O-tetradecanoyl-13 phorbol acetate (TPA, Sigma) 1 mg/ml DMSO; okadaic acid (OA, Boehringer Mannheim) 1 mM/DMSO, 3-isobutyl-1-methylxanthine (IBMX, Sigma) 200 mM/DMSO; tyrphostin B46 (Calbiochem) 100 mM/DMSO; lavendustin A (Sigma) 10 mM/DMSO; daidzein (Sigma) 100 mM/DMSO; demecolcine 1 mg/ml KRB-4. For the dose dependent study, the following concentrations were used for genistein: 10, 50, 100, 200, 250, 300 and 400 μM, for daidzein: 50, 100 and 200μM, for tyrphostin B46: 0.01, 0.1, 1, 10 and 20μM and for lavendustin A: 10, 100 nM and 1, 10, 100 and 200 μM.

Immunofluorescence

Oocytes were freed from their *zona pellucida* in acid Tyrode solution (Nicolson *et al.*, 1975) and allowed to recover for 10 min. in culture medium at 37°C. They were then attached onto glass coverslips within specially prepared chambers treated with concanavalin A (0,1 mg/ml PBS) as described by Maro (Maro *et al.*, 1984) and gently centrifuged for 5 min. at 1000 t/min. Oocytes were then simultaneously fixed and permeabilized in PBS containing 0,5 % glutaraldehyde (Sigma) and 1% Triton X-100 (Sigma). Formaldehyde 0,35% was added since it has been observed that this treatment highly improves results obtained from DNA staining with propidium iodide (5 μg/ml in PBS, Calbiochem). Oocytes were post-permeabilized for 20 min. in PBS with 2 % Triton X-100. They were neutralized for 3 x 10 min with a solution of 2 mg/ml $NaBH_4$ in PBS (extemporaneous), rinsed in PBS – 1% Tween 20 (Sigma) and incubated overnight at 37°C with a rat monoclonal antibody YL specific for tyrosinated tubulin α (ProBio, England) diluted 1:2000 in PBS – 1% Tween 20 – 3% BSA. They were rinsed three times in PBS - 1% Tween and incubated with fluorescein-labeled goat anti-rat IgG (Sigma) diluted 1:50 in PBS – 1% Tween 20 – 3% BSA for 1 hour at 37 °C. After three rinses in PBS - 1% Tween, coverslips were mounted in Vectashield medium (Vector), and sealed with nail polish. For actin staining, oocytes were fixed and permeabilized as described before, incubated with Phalloidin-FITC (Sigma) 0.4 μg/ml - 1% Tween20 – 3% BSA in PBS, rinsed three times and mounted. Observations were made on a TCS 4D confocal laser scanning microscope (LEICA) equipped with an Ar/Kr laser source. The precise stage of meiotic maturation was additionally determined by the chromatin configuration of oocytes revealed by costaining with TO-PRO 3 (Molecular Probes) 1:1000 in PBS or propidium iodide (Calbiochem) 5 μg/ml in PBS after the tubulin staining.

Immunocytochemical detection of apoptosis

Programmed cell death or apoptosis was detected using of *In Situ* Cell Death Detection kit (Boehringer) based on an enzymatic *in situ* labeling of apoptotic DNA strand breaks with fluorescein labeled nucleotides (TUNEL method: Terminal deoxynucleotidyl transferase -mediated dUTP Nick End Labeling). Briefly, oocytes were freed from their *zona pellucida*, fixed with paraformaldehyde 4% in PBS for 10 min. at room temperature, permeabilized in citrate buffer 0,1% with 0,1% Triton X-100 for 2 min. on ice (4°C), rinsed with PBS, incubated with TUNEL reaction mixture for 35 min at 37°C, rinsed three times with PBS and mounted in Vectashield medium (Vector). Positive controls were made by incubating fixed and permeabilized oocytes with DNaseI (Sigma, 1 mg/ml, 10 min. at room temperature) to induce DNA strand breaks. Negative controls were obtained by incubating oocytes in TUNEL reaction mixture without terminal transferase. Observations were made under an epifluorescence Leica DMRB microscope.

Staining of cortical granules (CGs)

Oocytes were fixed with 1.7% paraformaldehyde in PBS for 10 min. and CGs were stained with *Lens culinaris* agglutinin (LCA) coupled to biotin (10 μg/ml PBS, Sigma) and Streptavidin-Fluoresceine Isothiocyanate (FITC) (10 μg/ml PBS, Sigma) according to Ducibella *et al.,* (1988). The chromatin configuration was determined by costaining with 1μM TOTO-3 (Molecular Probes) for 10 min. after the lectin staining procedure. Both CGs and chromatin were visualized by fluorescence microscopy using a TCS 4D confocal laser scanning microscope (Leica).

Immunoblotting

Groups of 25 experimentally treated oocytes were depellucidated, transferred in 2x sample buffer (Laemmli, 1970) and heated to 100 °C for 5 min. Proteins were then separated by electrophoresis on 15 % SDS-polyacrylamide mini-gels (Mini -Protean II, BioRad) and transferred to nitrocellulose membranes (Semi-dry transfer cell, BioRad). Membranes were blocked in PBS containing 4% BSA and 0,2% Triton X-100 for at least 4 hours and then incubated overnight at room temperature with anti-ERK 1 antibody (K-23, Santa Cruz Biotechnology) diluted 1:500 in PBS containing 2% BSA and 0,2% Triton X-100. After four washes of 15 min. each in the same buffer, the membranes were incubated at room temperature for 1 hour in HRP-conjugated anti-rabbit Ig (Amersham Life Science) diluted 1:1000 in the antibody dilution buffer. After 4 washes of 15 min., membranes were processed using the ECL detection system (Amersham Life Science).

Time-lapse microcinematography

Oocytes were introduced into one well cut out of a four-well multidish (Nunc) within a sealed Plexiglas chamber filled with a humidified atmosphere of 5% CO2 in air. The assembly was placed on the stage of a Leica DM IRB inverted microscope equipped with a Plexiglas thermostatic climate box maintained at 37°C. Pictures were taken at a rate of 3/min by a JVC color camera mounted on the microscope and were recorded on a Panasonic AG-6730 time-lapse video recorder. Documents were printed with a Sony Mavigraph video printer.

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