Eicosatetraynoic and eicosatriynoic acids, lipoxygenase inhibitors, block meiosis via antioxidant action

M. Takami, S. L. Preston, and H. R. Behrman

Reproductive Biology Section, Departments of Obstetrics and Gynecology and Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06520-8063

Eicosatetraynoic and eicosatriynoic acids, lipoxygenase inhibitors, block meiosis via antioxidant action. Am J Physiol Cell Physiol 278: C646–C650, 2000.—We previously showed that nordihydroguaiaretic acid (NDGA) and other antioxidants inhibit the resumption of meiosis in oocyte-cumulus complexes (OCC) and denuded oocytes (DO). Because NDGA is well known to be an inhibitor of lipoxygenases (LOX), we assessed whether other LOX inhibitors influence spontaneous germinal vesicle breakdown (GVBD) in OCC and DO. Spontaneous GVBD in rat OCC obtained from preovulatory follicles was significantly and reversibly inhibited by the minimum effective doses of 80 and 100 µM 5,8,11,14-eicosatetraynoic acid (ETYA) and 5,8,11-eicosatriynoic acid (ETI), respectively. In DO, GVBD was significantly inhibited by 100 µM ETYA or ETI. The minimum effective concentrations of ETYA and ETI for inhibition of GVBD in either OCC or DO are ∼30- to 50-fold higher than the concentrations necessary to inhibit LOX activity by 50% in intact cells. Because we previously showed that NDGA and other antioxidants inhibit the spontaneous resumption of meiosis, we assessed whether ETYA and ETI may act similarly as scavengers of reactive oxygen species (ROS). Luminal-amplified chemiluminescence showed that 50 µM of either ETYA or ETI markedly and significantly reduced ROS generated with 10 mM 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH). Moreover, incubation of DO with 30 mM AAPH reversed the inhibition of GVBD produced by 100 µM ETYA or ETI. These findings support the conclusion that ETYA and ETI inhibit oocyte maturation by acting as antioxidants rather than by inhibiting LOX.

MATERIALS AND METHODS

Hormones, drugs, and reagents. 5,8,11,14-Eicosatetraynoic acid (ETYA) and 5,8,11-eicosatriynoic acid (ETI) were purchased from BIOMOL (Plymouth Meeting, PA). 2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) was purchased from Wako Chemicals (Richmond, VA). 3-Isobutyl-1-methylxanthine (IBMX) was purchased from Sigma Chemical (St. Louis, MO) and was dissolved in 50% ethanol-water. Stock solutions (100 mM) of ETYA and ETI were prepared in 95% ethanol. The final concentration of ethanol was <0.05%, and parallel controls were run to assess the effect of ethanol alone.

Animals. Follicle development was induced in immature (25- to 27-day-old) female rats (Sprague-Dawley strain; Taconic Farms, Germantown, NY) by subcutaneous injection of 10 IU pregnant mare serum gonadotropin (PMSG; Gestyl; Organon Pharmaceuticals, West Orange, NJ). Animals were housed and cared for in the fully accredited facilities operated by the Animal Resource Center (Yale University School of Medicine, New Haven, CT). All treatments and procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and in accordance with a protocol approved by the Yale University Animal Care Committee.

Isolation and incubation of oocyte-cumulus complexes. The animals were killed 44–48 h after PMSG treatment. Ovaries were removed and placed in Earle’s minimal essential medium (MEM-2360; Gibco, Grand Island, NY) containing BSA (1 mg/ml), glutamine (0.29 mg/ml), and IBMX (100 µM). After the fat was trimmed from the ovaries, preovulatory follicles were bluntly dissected from the ovaries under a stereomicroscope. Oocyte-cumulus complexes (OCC) from
large preovulatory follicles were expelled by puncturing antral follicles with a stainless steel needle. Isolated OCC were pooled, washed three times in fresh medium without IBMX, and allotted to treatment groups. The isolation procedure from the time of puncture of the follicle to the allocation to treatment groups took ~30–45 min. OCC were incubated for 2 h under a humidified atmosphere of 5% CO₂-95% air at 37°C in 1 ml of medium (MEM-2360) containing BSA (1 mg/ml), glutamine (0.29 mg/ml), and ETYA or ETI to be tested in multiwell plates (Falcon 3047; Becton-Dickinson, Lincoln Park, NJ).

After incubation, OCC were scored for oocyte maturation by using a squashing technique that enables visualization of the oocyte with its surrounding investment of cumulus cells, as described earlier (13). Briefly, the OCC were placed on slides pretreated with Sigmacote (Sigma Chemical) that were rinsed and dried. Spots of silicone lubricant were mixed with a small amount of Sephadex LH 20 placed on the edges of the slides over which a coverslip was placed. Oocytes that retained a germinal vesicle and/or nucleolus were considered to show inhibition of maturation. Oocyte maturation was expressed as percentage of germinal vesicle breakdown (GVBD).

Isolation and incubation of denuded oocytes. OCC were denuded of their cumulus corona investments by exposure to media composed of a 1:1 ratio of 0.7% sodium citrate in distilled water and MEM-2360 containing 100 µM IBMX followed by repeated pipetting through a narrow-bore glass pipette. Denudation was carried out immediately after isolation of the OCC.

Before incubation, denuded oocytes (DO) were washed three times with fresh medium composed of MEM-2360 supplemented with BSA (1 mg/ml), glutamine (0.29 mg/ml), and sodium pyruvate (1 mM). The oocytes were incubated in 100 µl of medium in a four-well glass slide that was placed on moistened filter paper within a covered petri dish to guard against evaporation in the absence and presence of ETYA or ETI. After 60–360 min of incubation, DO were visualized under Nomarski optics, and DO that retained a germinal vesicle and/or nucleolus were considered to show inhibition of maturation.

Luminol-amplified chemiluminescence assay. Spontaneous production of ROS by AAPH and the effect of ETYA or ETI on the levels of reactive oxygen species (ROS) were measured by luminol-amplified chemiluminescence (LCL), as described earlier (19, 23). The assay is based on the principle that luminol, in the presence of one-electron oxidants, forms an excited aminophthalate ion that emits a photon when it returns to its ground state (19). For the assay of chemiluminescence, AAPH was dissolved in 0.5 ml Dulbecco's PBS (containing Ca²⁺, Mg²⁺, and glucose; Gibco) supplemented with BSA (1 mg/ml) and 4.5 µM luminol (Aldrich, Milwaukee, WI; see Ref. 19). Luminol was detected using a luminometer (Turner Designs, Sunnyvale, CA) during 1-min intervals with 5-s delays, which was calibrated against superoxide as we described earlier (2). ETYA and ETI were prepared in 95% ethanol. The final concentration of ethanol was 0.01%, and parallel controls of ethanol alone showed no effect on LCL.

Reversibility of inhibition of oocyte maturation by ETYA and ETI by AAPH. DO were preincubated for 15 min in 90 µl of medium (MEM-2360) with BSA (1 mg/ml), glutamine (0.29 mg/ml), sodium pyruvate (1 mM), and either ETYA or ETI (100 µM) in a four-well glass slide that was placed on moistened filter paper within a covered petri dish. After preincubation, AAPH was added in 10 µl to each well to achieve a final concentration of 10, 30, and 50 mM. Oocyte maturation was scored as described above.

Statistical analysis. The effect of treatment on oocyte maturation was evaluated by chi square analysis of the total number of oocytes. Each experiment was independently repeated at least three times. Significant differences in the luminometry studies were determined by repeated-measures ANOVA with a level of significance of P < 0.05.

RESULTS

The dose-response effect of ETYA and ETI on oocyte maturation in OCC is shown in Fig. 1. At a concentration of 10 µM, ETYA had no effect on oocyte maturation, whereas 40 and 80 µM significantly (P < 0.005) inhibited oocyte maturation compared with control OCC. No effect of ETI on GVBD was seen at 10 or 30 µM, whereas at 100 µM ETI significantly (P < 0.05) inhibited oocyte maturation. In other studies, OCC were incubated for 2 h with 100 µM ETYA or ETI and either washed or incubated continuously for an additional 3 h with the agents, at which time GVBD was scored. washing significantly (P < 0.005) reduced the inhibition of GVBD by either agent. OCC with continuous incubation of ETYA and ETI showed GVBD of 30 and 50%, respectively, whereas OCC after the agents were washed out showed GVBD of 83.3 and 70%, respectively.

Figure 2 shows the time course for inhibition of oocyte maturation by ETYA in DO. In these studies, DO were incubated with the indicated concentrations of ETYA, and GVBD was scored at various time intervals up to 6 h of incubation. Although 10 µM ETYA did not inhibit oocyte maturation, both 50 and 100 µM ETYA significantly (P < 0.05) inhibited GVBD up to 6 h of incubation. No evidence of cytotoxicity assessed by granulation or shrinkage of oocytes was seen. The time course for inhibition of oocyte maturation by ETI in DO is shown in Fig. 3. Neither 10 nor 50 µM ETI inhibited oocyte maturation in DO, whereas 100 µM ETI significantly (P < 0.05) inhibited oocyte maturation in DO up...
No evidence of cytotoxicity was visible.

To assess antioxidant properties of ETYA and ETI, AAPH was dissolved in 0.5 ml Dulbecco’s PBS (containing Ca\(^{2+}\), Mg\(^{2+}\), and glucose) supplemented with BSA (1 mg/ml) in the absence and presence of ETYA or ETI (Fig. 4). Upon addition of AAPH, there was an immediate production of ROS that reached a steady state within 10 min. The chemiluminescence produced by AAPH was completely suppressed by a combination of superoxide dismutase (1,700 U/ml) and catalase (2,000 U/ml; data not shown). The addition of ETYA or ETI (50 µM) significantly inhibited chemiluminescence by \(\pm 60\%\) \((P < 0.05)\).

To assess whether AAPH may override the inhibition of oocyte maturation produced by ETYA or ETI, DO were preincubated for 15 min with ETYA or ETI (100 µM) followed by the addition of AAPH. A highly significant inhibition of oocyte maturation was seen with ETYA treatment. Incubation of DO with AAPH (30 or 50 mM) significantly \((P < 0.005)\) reversed the inhibition of GVBD produced by ETYA (Fig. 5). Some evidence of cytotoxicity at 6 h of incubation was seen with treatment of AAPH. Incubation of DO with AAPH (30 or 50 mM) significantly \((P < 0.005)\) reversed the inhibition of GVBD produced by ETI (Fig. 6). Some
indomethacin had no effect on oocyte maturation in intact cells are 8 (3, 15, 18) and 50 (12, 15, 18) µM, respectively. This finding further indicates that PGs are not necessary for spontaneous GVBD. Moreover, we previously showed that NDGA and a host of other antioxidants inhibit oocyte maturation (16). It is well known that NDGA is an antioxidant (8) and that some antioxidants inhibit LOX (22); hence, we were prompted to examine whether ETYA and ETI have antioxidant properties against ROS that are known to be generated by AAPH at a known and constant rate in aqueous media (10). The finding that both ETYA and ETI scavenged ROS produced by AAPH at concentrations equivalent to that necessary to inhibit oocyte maturation indicates that both ETYA and ETI inhibited GVBD via their antioxidant properties. The slight differences in the antioxidant potency and potency for GVBD of the two drugs may be related to cell permeability or other physicochemical properties. Further evidence that ETYA and ETI inhibit oocyte maturation by acting as antioxidants rather than as LOX inhibitors is the finding that AAPH reversed the inhibition of oocyte maturation produced by ETYA or ETI.

In conclusion, the LOX inhibitors ETYA and ETI inhibited the spontaneous resumption of meiosis in OCC and DO, and these drugs were found to be antioxidants. Although the antioxidant and GVBD potencies of the drugs were similar, there is a large discrepancy between the low levels necessary to inhibit LOX compared with the levels necessary to inhibit GVBD. Because we found that a host of antioxidants inhibit oocyte maturation (16) and because of the present findings that ETYA and ETI are antioxidants with similar potency, we conclude that ETYA and ETI inhibit GVBD because of their antioxidant properties independently of their ability to inhibit LOX. This conclusion is supported by the additional finding that AAPH reversed inhibition of GVBD produced by ETYA and ETI. These findings further buttress the possibility that ROS serve a role in the initiation of spontaneous oocyte maturation.

Address for reprint requests and other correspondence: H. R. Behrman, Yale University School of Medicine, Dept. of Obstetrics and Gynecology, 333 Cedar St., PO Box 208063, New Haven, CT 06520-8063 (E-mail: Harold.Behrman@yale.edu).

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