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Original Article

Over-activation of cAMP signaling increases vulnerability to hydrogen peroxide via actin rearrangement, which is inhibited by prostaglandin E_2 in a mouse granulosa cell line.

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Apoptosis of granulosa cells (GC) contributes to ovarian follicular atresia, and has been implicated to depend on the oxidant status of GC within follicles. Here, we investigated the effects of cAMP and prostaglandin E_2 (PGE_2) on sensitivity to oxidative stress using 4B2 cells with cAMP-dependent, steroidogenic and differentiated properties. This cell line was isolated from mouse GC co-transfected with genes for SV40 large T antigen and Ad4BP/SF1 transcription factor (Kamei *et al*. 2005). Treatment of serum-starved cells with 8-Br-cAMP caused 30 to 40% of the cells to become polygonal within 2 h through actin rearrangement. Interestingly, H_2O_2 treatment showed that these polygonal cells were vulnerable to oxidative stress that led to cell death, which was inhibited by pretreatment with phalloidin, an F-actin stabilizing agent. Although PGE_2 alone had no effect, co-treatment with PGE_2 and 8-Br-cAMP completely inhibited the effects of 8-Br-cAMP on cell shape change and oxidative stress vulnerability through phosphatidylinositol 3-kinase (PI3K)-dependent manner. Notably, PGE_2 and 8-Br-cAMP cooperated additively to increase progesterone secretion. These data suggest that cAMP signaling in GC may enhance oxidative stress risk through actin rearrangement, and PGE_2 may reduce such risk through activating PI3K, while cooperating with cAMP signaling in steroidogenesis.

Key words: granulosa cells, oxidative stress, cAMP, prostaglandin E_2, actin

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Introduction

The vast majority of ovarian antral follicles, which consist of an oocyte surrounded by multiple layers of granulosa cells (GC) and theca-interstitial cells, undergo degeneration in a process called atresia, while only selected follicles develop and release healthy oocytes. Accumulating evidence has demonstrated that a form of physiological cell death, or apoptosis, in GC contributes to follicular atresia. Hence, elucidation of the underlying events that lead GC into apoptosis would help elucidate the mechanism determining the fate of antral follicles, i.e. atresia or further development. The survival of GC in antral follicles is strictly under the control of pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH)[1,2]. In fact, abolishment of circulating FSH or LH causes the apoptotic death of GC and results in massive follicular atresia[3]. The anti-atretic effect of FSH has been demonstrated to be mediated through locally produced regulators such as insulin-like growth factor and interleukin-1[4]. Reactive oxygen species (ROS) have been implicated as a trigger for the initiation of

atresia by causing apoptosis in GC[5]. In fact, the apoptosis of GC in antral follicles, which is prevented by FSH, is also blocked by superoxide dismutase or free radical scavengers such as ascorbic acid and *N*-acetyl-L-cysteine[6]. Thus, the oxidant status appears crucial for FSH-dependent folliculogenesis. It has long been known that prostaglandin E_2 (PGE_2) stimulates progesterone secretion in GC and that this prostanoid is essential for the ovulatory process[7]. Little is known, however, about the role of PGE_2 in the process of atresia.

Previously, it was reported that the over-activation of cAMP signaling by chemical reagents, but not proper activation by gonadotropins, caused apoptosis of GC in a p53-dependent way, as the apoptosis was blocked in a cell line of GC with p53 depressed by SV40 large T antigen[8,9]. This appears paradoxical, given that cAMP signaling, including the protein kinase A (PKA) pathway, plays a central role in folliculogenesis. Here we have investigated whether over-activation of cAMP signaling influences the oxidant status in GC. To address this we used 4B2 cells, an immortalized cell line that was established by transfecting the Ad4BP/SF-1 gene, together with the SV40 large T antigen gene, into primary cultured mouse GC[10]. This cell line retains differentiation potential following activation of cAMP signaling, including progesterone production and differentiated characteristic changes in cell shape, mitochondria structure and gap junction formation. We demonstrated that cAMP induces cell shape changes through actin rearrangement, rendering the cells more susceptible to an H_2O_2 challenge, and that PGE_2 inhibits the cAMP-induced changes via activating the phosphatidylinositol 3-kinase (PI3K) pathway, cooperating with cAMP signaling in progesterone secretion.

Materials and Methods

Reagents

PGE_2 was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Phalloidin, 8-Br-cAMP, propidium iodide (PI) and wortmannin were purchased from Sigma-Aldrich (St Louis, MO, USA). N - [2 - (p - Bromocinnamylamino) ethyl] - 5 - isoquinolinesulfonamide/2HCl (H89) was from BIOMOL (Plymouth, PA, USA). A progesterone assay kit was from Diagnostic Products Co. (Los Angels, CA, USA). Alexa Fluor 488-phalloidin was purchased from Molecular Probes (Eugene, OR, USA). Boc-Asp-(OMe)-CH_2F (ZD) was purchased from Calbiochem (La Jolla, CA, USA).

Cell culture and treatment

Cells were maintained in basic medium comprised of DMEM, 10 mM HEPES-NaOH (pH 7.4) and 100 IU/ml penicillin, supplemented with 10% fetal bovine serum, at 37C, 5% CO_2-95% air. Cultured cells were plated in 48-well tissue culture plates at approximately 2 x 10^4 cells per well in 300 μl of this basic medium and incubated for approximately 24 h. When the cultures reached 60-70% confluence, cells were washed three times with serum-free basic medium and were serum-starved for 24 h in basic medium supplemented with 0.2% fetal bovine serum. All of the following experiments were conducted using serum-starved 4B2 cells. Cells were treated with 1 mM 8-Br-cAMP in the absence or presence of 1 μg/ml PGE_2, 100 nM wortmannin or 20 μM ZD, which was added to cells 30 min before 8-Br-cAMP addition. H89 at 10 μM was added 3 h after addition of 8-Br-cAMP. After 4 h incubation with 8-Br-cAMP, cells were subjected to an oxidative stress by adding 300 μM H_2O_2. To study the involvement of actin rearrangement, 20 μM phalloidin was added simultaneously with the start point of the serum starvation. Cell viability was determined by permeability of the plasma membrane to PI 4 h after H_2O_2 addition. Images were collected by fluorescence and phase contrast microscopy (IX71, Olympus).

Actin staining

Cells, grown on 35 mm-plates, were treated with vehicle or 1 mM 8-Br-cAMP in the absence or presence of 1 μg/ml PGE_2 for 4 h. The cells were then fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS), washed with PBS, permeabilized with 0.05% (vol/vol) Triton X-100-PBS for 3 min, washed with PBS, and stained with 2 U/ml Alexa Fluor 488-phalloidin for 1 h. Fluorescent images were obtained by laser scanning confocal microscopy (FV1000, Olympus). For detection of fluorescence, 488 nm excitation and 510-530 nm bandpass emission filter settings were used.

Determination of progesterone

Serum-starved cells in 35 mm-plates were stimulated for 4 h by addition of 8-Br-cAMP (1 mM), PGE_2 (1 μg/ml) with or without H89, or 8-Br-cAMP plus PGE_2. Following this, 0.1 ml of medium was recovered for progesterone measurement by RIA. The protocol followed was that furnished in a commercial progesterone assay kit.

Statistics

Cell death experiments were repeated at least three times, and quantitative data represent the mean±SE. Statistical comparisons were made using ANOVA (analysis of variance) followed by Tukey's test, using the program StatView (Abacus Concepts, Berkeley, CA, USA). Differences were considered to be significant if $P < 0.05$.

Results

We investigated effect of 8-Br-cAMP on serum-starved 4B2 cells. Treatment with 1 mM 8-Br-cAMP induced a significant change in cell shape, to a polygonal conformation, in 30 to 40% of the cells within 2 h while the remaining cells maintained a fibroblastic conformation (Fig. 1A, B). This shape change was dose dependent, as 0.3 mM 8-Br-cAMP elicited a cell shape change in about 20% of the cells whereas 0.1 mM 8-Br-cAMP had no significant effect (Fig. 1A, B). The polygonal cells were found to be firmly attached on the plate, since they could not be removed by aspiration. When serum was added to a final concentration of 2% 4 h after 8-Br-cAMP addition, most of the polygonal cells became fibroblastic, similar to the original cell shape (Fig. 1A), indicating that the cell shape change was reversible. Moreover, when H89, an inhibitor of PKA, was added at a concentration of 10 μM 3 h after 8-Br-cAMP addition, it reversed completely the cell shape change caused by 8-Br-cAMP, showing that the cell shape change was dependent on PKA activation (Supplementary Fig. 1). To visualize directly the changes in actin cytoskeleton networks, cells were treated with Alexa Fluor 488-phalloidin. The polygonal cells displayed a breakdown of their actin cytoskeletons, with the appearance of characteristic actin bundles, in sharp contrast to control fibroblastic cells (Fig. 1C). Thus, the cell shape change was due to a rearrangement of actin cytoskeleton networks.

Although 8-Br-cAMP treatment did not induce cell death, due to the suppression of p53 by SV 40 large T antigen in 4B2 cells as previously reported[8,9], we sometimes noticed that polygonal cells all together died spontaneously within 12 h. We hypothesized that these occasional cell deaths may have been due to oxidative stress caused by our frequent microscopic observations outside a CO_2 incubator. Therefore we studied the potential susceptibility of 8-Br-cAMP-treated 4B2 cells to oxidative stress using H_2O_2. After exposure to 300 μM H_2O_2 more than 80% of the polygonal cells, but not the fibroblastic cells, died within 4 h if they had been treated with 8-Br-cAMP, in contrast with a less than 5% death rate for control cells (Fig. 2). The mode of cell death was necrotic, since the cells did not exhibit nuclear fragmentation (Supplementary Fig. 2A) and their death was not prevented by ZD, a general caspase inhibitor (Supplementary Fig. 2B). These findings indicated that 8-Br-cAMP increased their vulnerability to oxidative stress. To determine whether the cell shape changes were associated with oxidative stress vulnerability we studied the effect of an actin stabilizing agent, phalloidin, on cell viability after exposure to H_2O_2. Phalloidin pretreatment greatly reduced the number of polygonal cells (Fig. 3A) and the susceptibility to H_2O_2 caused by 8-Br-cAMP treatment (Fig. 3B, C). This suggested that a cell shape change elicited by actin rearrangement underlies the increased susceptibility to H_2O_2.

Since PGE_2 is a luteotropic factor, we expected PGE_2 to induce cell shape change and oxidative stress susceptibility. However, treatment with 1 μg/ml PGE_2, unlike 8-Br-cAMP, had no effect on cell shape (Fig. 1C and 4A) or H_2O_2 vulnerability (Fig. 4B). In contrast to our expectations, when cells were treated with PGE_2 simultaneously with 8-Br-cAMP, the effects of 8-Br-cAMP on cell shape and oxidative stress vulnerability were abolished (Fig. 4). These inhibitory effects of PGE_2 could not be accounted for by activation of the cAMP/PKA pathway. PGE_2 is known to activate PI3K[11], so we tested the possibility that PGE_2 executes its inhibitory effects through the PI3K pathway by employing wortmannin, a specific PI3K inhibitor. In the presence of wortmannin, PGE_2 failed to block the cell shape change (data not shown) and oxidative stress susceptibility caused by 8-Br-cAMP (Fig. 4B), confirming that PGE_2 acts through a PI3K pathway.

Progesterone synthesis in GC is mediated by a cAMP/PKA pathway. Inhibition of the 8-Br-cAMP-induced effects by PGE_2, described above, caused us to suspect that PGE_2 might function as a luteotropic factor in steroidogenesis; thus progesterone secretion was assayed. Progesterone secretion of the cells increased three-fold in response to 1 mM 8-Br-cAMP or 1 μg/ml PGE_2, and they showed a five-fold increase after co-stimulation with 8-Br-cAMP and PGE_2 (Fig. 5). The progesterone secretion by PGE_2 was partially inhibited by 10 μM H89, a PKA inhibitor (Supplementary Fig. 3). These results indicated that PGE_2 promoted steroidogenesis in PKA-dependent and independent ways and had a cooperative effect on it when

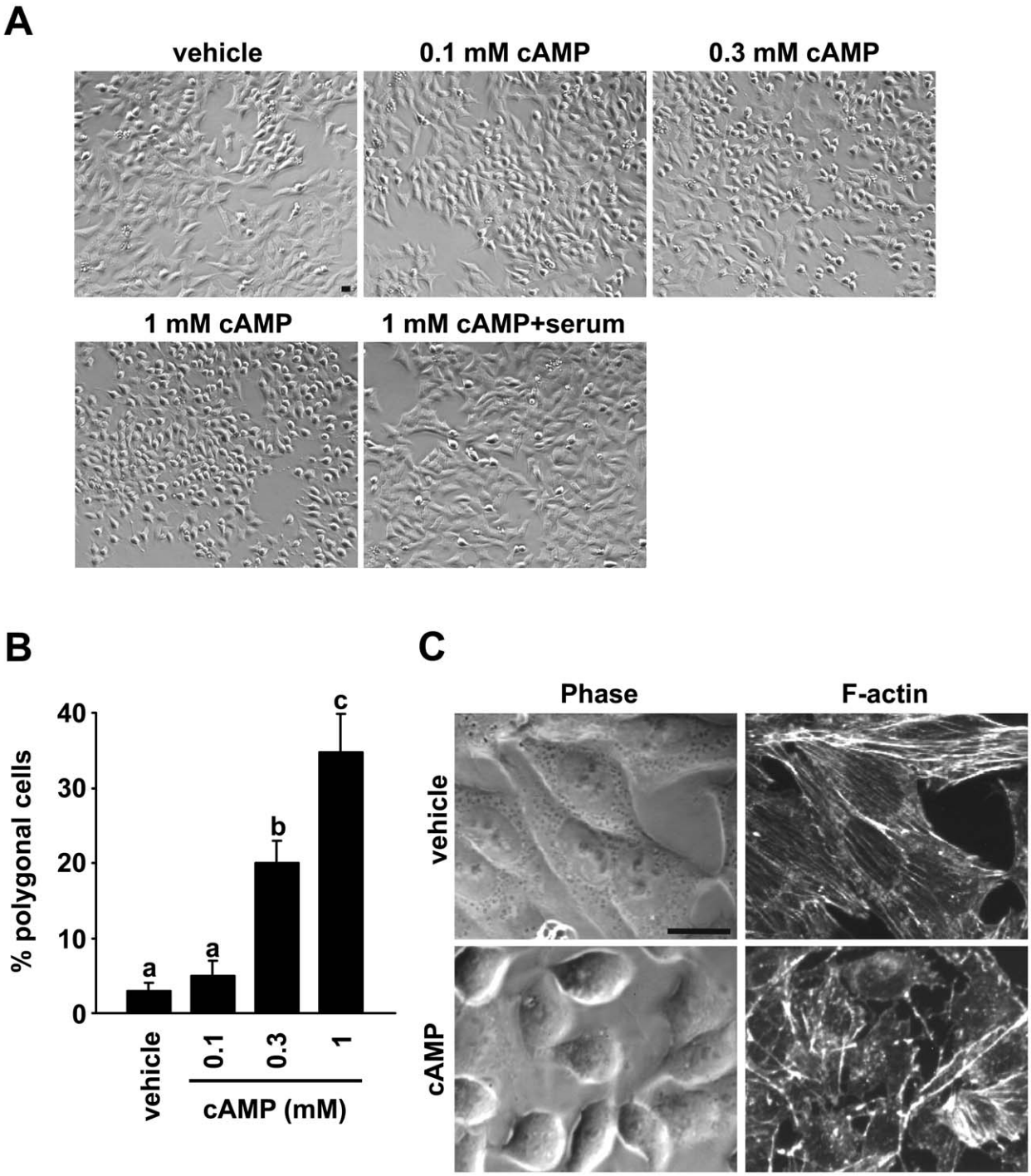

Fig. 1. Effects of 8-Br-cAMP (cAMP) on cell shape and F-actin organization in 4B2 cells. A, Dose-dependent effect of 8-Br-cAMP. After serum starvation for 24 h, cells were treated for 4 h with vehicle or 8-Br-cAMP (0.1, 0.3 or 1 mM cAMP). To determine the reversibility of cell shape changes, serum was added 4 h after 8-Br-cAMP (1 mM cAMP + serum). Cell morphology assessed by phase contrast microscopy. A bar: 10 μm. B, Quantitative analysis of the data obtained as in A. Each data represents the mean$\pm$SE of three independent experiments. a is different from b and c ($P < 0.01$). b is different from c ($P < 0.05$). C, Cells were treated with vehicle or 1 mM 8-Br-cAMP for 4 h. Phase contrast microscopy (*left*) and fluorescent microscopy with Alexa Fluor 488-phalloidin indicating F-actin (*right*) of the same fields. Note the appearance of cell shape change and actin rearrangement in cells treated with 0.3 or 1 mM 8-Br-cAMP. A bar: 10 μm.

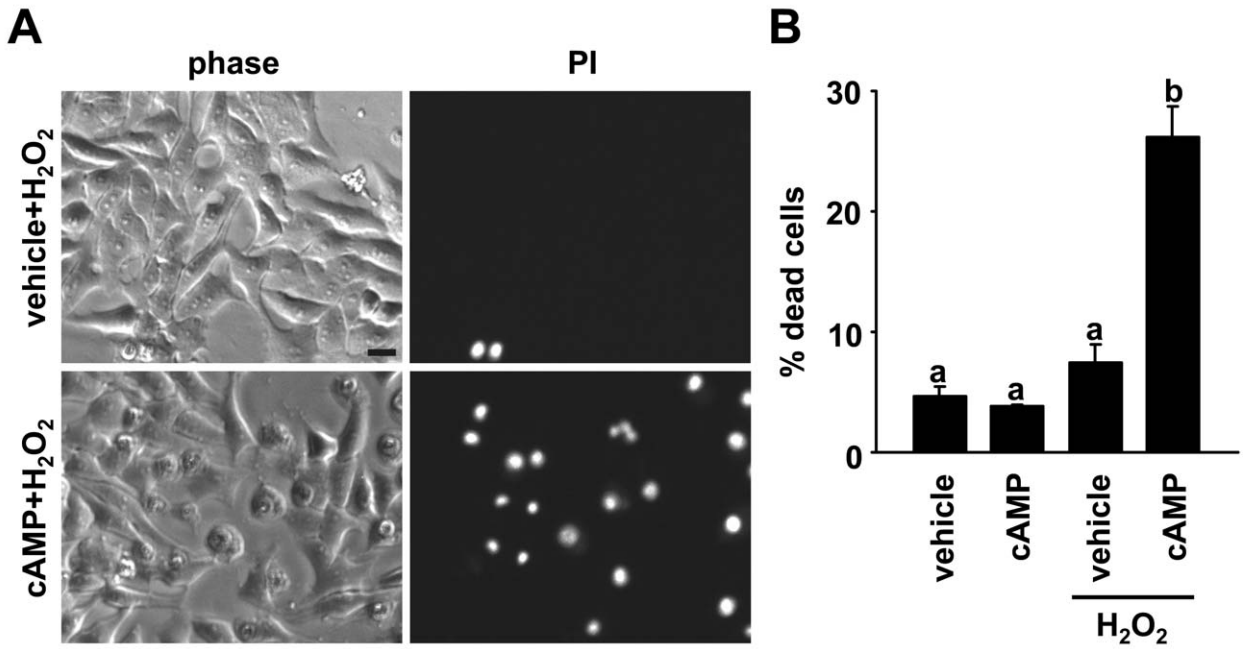

Fig. 2. Effect of oxidative stress with H_2O_2 on cell viability in 4B2 cells treated with 8-Br-cAMP (cAMP). Cells were treated with vehicle or 1 mM 8-Br-cAMP for 4 h under 0.2% serum condition and then were challenged with 300 μM H_2O_2. A, Cells were stained with PI 4 h after H_2O_2 addition and observed by fluorescence (*right*) and phase contrast (*left*) microscopy in the same fields. A bar: 10 μm. B, Quantitative analysis of the data obtained as in A. Note the increased vulnerability to H_2O_2 in cells treated with 8-Br-cAMP. Each data represents the mean±SE of three independent experiments. a is different from b ($P < 0.01$).

added to cells with 8-Br-cAMP.

Discussion

Folliculogenesis, which is controlled by FSH, is also regulated elaborately by a variety of local growth factors such as insulin-like growth factor and death receptor ligands such as Fas, which act in an autocrine/paracrine way[4]. Without adequate support by these regulatory pathways, ovarian follicles have been demonstrated *in vivo* and *in vitro* to undergo atresia due to apoptosis of follicular GC. However, the molecular mechanisms regulating apoptotic responses during atresia have not been precisely clarified. Several lines of evidence have suggested a role for antioxidant and oxidant imbalance in exacerbation of the GC death process[5]. We have shown here for the first time that cAMP promotes actin rearrangement in 4B2 cells, leading to shape changes that increase vulnerability of cells to oxidative stress followed by cell death. We have also shown that PGE_2, another luteotropic factor, blocks these alterations of cell shape and consequently prevents the oxidative stress vulnerability. Since 4B2 cells share important differentiation properties with primary cultured GC[10], it seems that the present findings may help provide a better understanding of cellular mechanisms of GC death during follicular atresia.

H_2O_2 is known to be toxic to many cells, but cellular damage is not due to a direct effect of the H_2O_2. Rather, it is due to the highly toxic hydroxyl radical that is produced from cleavage of H_2O_2 by the iron-mediated Fenton reaction. This was true for the cell death of 4B2 cells by H_2O_2, which could be completely prevented by deferoxamine, an iron ion chelator (data not shown). Presently, it is not clear why cell shape changes, especially due to actin rearrangement, are associated with increased vulnerability to the hydroxyl radical. Oxidative stress vulnerability was reduced when the cell shape change was inhibited by the actin stabilizer phalloidin; this suggested that the actin rearrangement itself may be responsible for increased cell vulnerability to oxidative injury. It has been reported that actin cytoskeleton rearrangement affects nuclear translocation of Nrf2, a ROS-dependent and PI3K-activated transcription factor for cytoprotective detoxifying enzymes[12]. Therefore, the 8-Br-cAMP-induced actin rearrangement in 4B2 cells may have blocked the

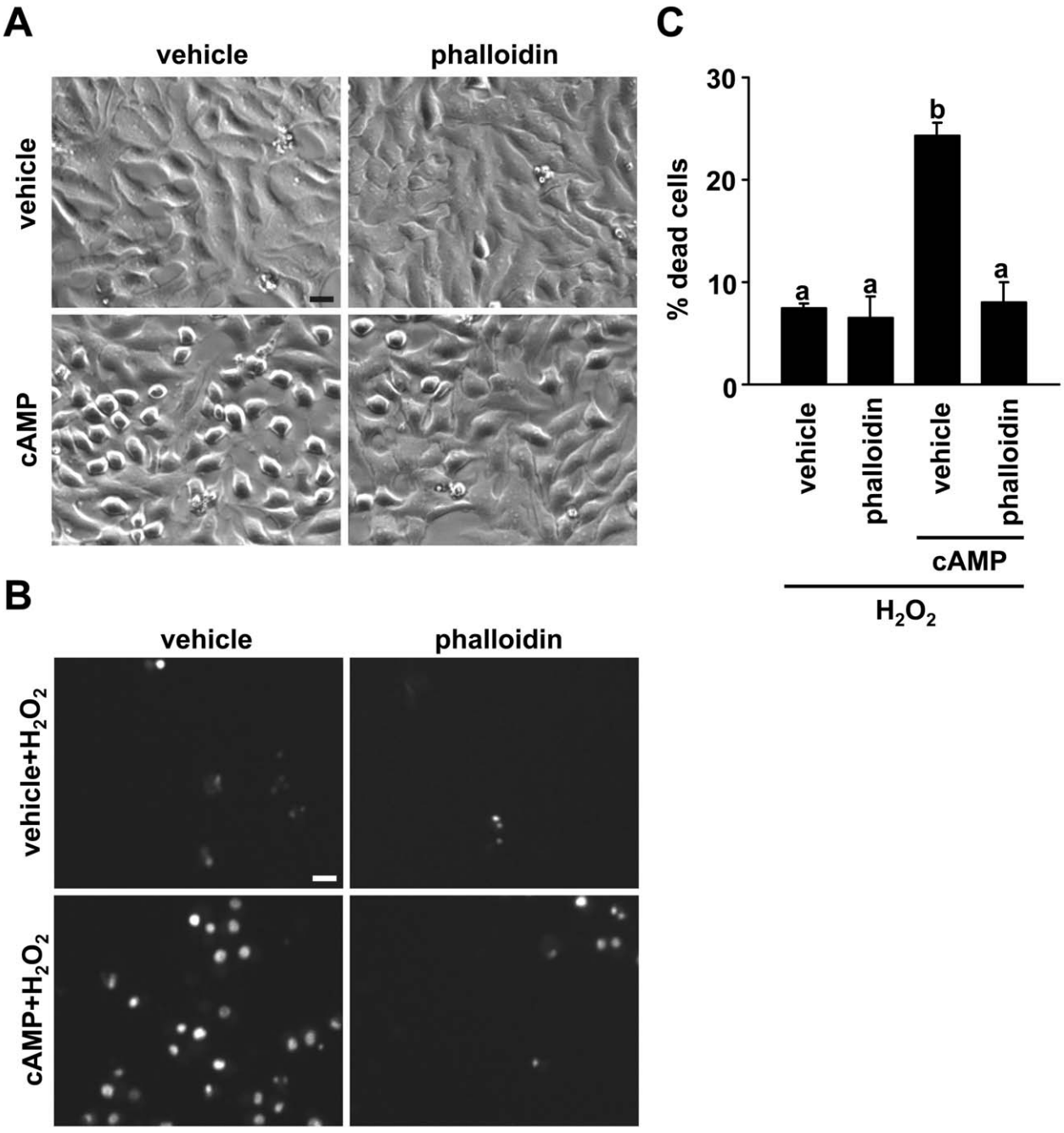

Fig. 3. Effect of phalloidin on 8-Br-cAMP (cAMP)-induced cell shape change in 4B2 cells. A, Cells were pretreated with vehicle or 20 μM phalloidin, an actin stabilizer, for 24 h under 0.2% serum condition, and then treated with vehicle or 1 mM 8-Br-cAMP for 4 h. Cell morphology assessed by phase contrast microscopy. A bar: 10 μm. B, After cells were stimulated for 4 h with 1 mM 8-Br-cAMP in the presence or absence of 20 μM phalloidin, they were challenged with 300 μM H_2O_2. Cells were stained with PI and observed by fluorescent microscopy. A bar: 10 μm. C, Quantitative analysis of the data obtained as in B. Note the inhibition by phalloidin of the 8-Br-cAMP-induced H_2O_2 vulnerability. Each data represents the mean±SE of three independent experiments. a is different from b ($P < 0.01$).

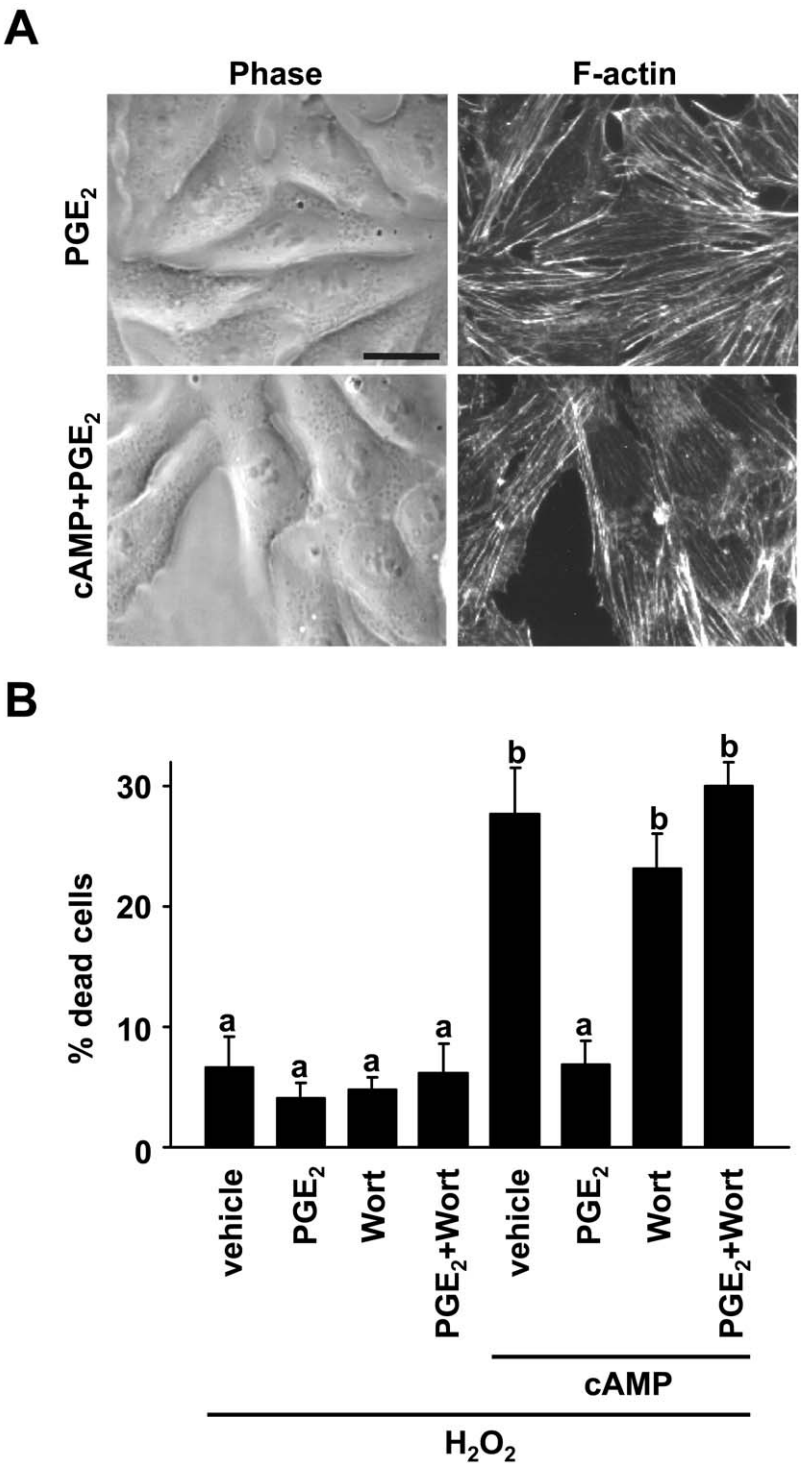

Fig. 4. Effects of PGE_2 or wortmannin (Wort) on the 8-Br-cAMP (cAMP)-induced cell shape change (A) and oxidative stress vulnerability (B) in 4B2 cells. A, Cells were pretreated with vehicle or 1 μg/ml PGE_2 for 30 min, and then treated with vehicle or 1 mM 8-Br-cAMP for 4 h under 0.2% serum condition. Phase contrast micrographs of cells (*left*) and fluorescent signals indicating F-actin from Alexa Fluor 488-phalloidin (*right*). A bar: 10 μm. B, Cells were pretreated with vehicle, 100 nM wortmannin, a PI3K inhibitor, and/or 1 μg/ml PGE_2 for 30 min, and then treated with vehicle or 1 mM 8-Br-cAMP under 0.2% serum condition. After 4 h, cells were subjected to an oxidative stress by adding 300 μM H_2O_2. Cell viability was evaluated by staining of PI. Each data represents the mean$\pm$SE of three independent experiments. a is different from b ($P < 0.01$).

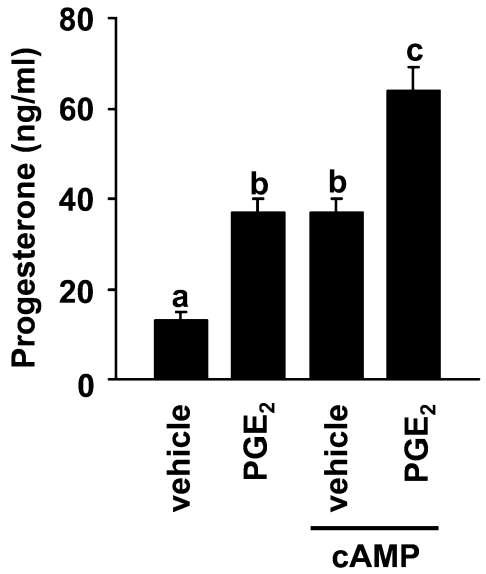

Fig. 5. Effect of 8-Br-cAMP (cAMP) and PGE_2 on progesterone secretion in 4B2 cells. Cells were stimulated with or without 1 mM 8-Br-cAMP, 1 μg/ml PGE_2 or both for 4 h. Each data represents the mean±SE of three independent experiments. a is different from b. b is different from c. ($P < 0.05$) a is different from c ($P < 0.01$).

action of Nrf2, thus reducing the levels of antioxidant enzymes and antioxidants and increasing the oxidative stress vulnerability of the cells. Alternatively, abnormal activation of cAMP signaling, rather actin rearrangement, might exhaust physiological oxidative stress responses that must be promptly induced to maintain cellular survival. It should be noted that the dose of 8-Br-cAMP used here was 1 mM and this dose was shown unequivocally to overactivate the cAMP signaling in GC[8]. Under this condition, ROS may be generated in excess as byproducts of abnormally high steroidogenesis[5], and these could exhaust antioxidant enzymes and cellular antioxidants.

Although PGE_2 alone had no demonstrable effect on the cell structure, it prevented the 8-Br-cAMP-triggered cell shape change and promoted resistance to subsequent oxidative stress through activating the PI3K pathway. At the same time, PGE_2 promoted steroidogenesis by activating the PKA dependent and independent pathways in concert with 8-Br-cAMP. Activation of the PKA and PI3K pathways by PGE_2 may occur following its binding to specific receptor subtypes. In fact, it has been reported that PGE_2 activates these two pathways through EP2 and EP4 receptor subtypes in HEK cells[11] and that these receptor subtypes were present in human ovarian granulosa-luteal cells[13]. The rearrangement of actin cytoskeleton by cAMP is regulated by small GTPases of the Rho family including Cdc42, Rac, and Rho[14]. PKA inhibits Rho function by causing its direct phosphorylation, resulting in the disassembly of stress fibers[15]. Moreover, Rho is known to be activated by PI3K[16]. Thus, differential phosphorylation of Rho by PKA and PI3K may be correlated with the actin-mediated cell shape change caused by 8-Br-cAMP and with the restoration of cell shape by PGE_2 in 4B2 cells. However, a different scenario has been described for the cell shape changes of astrocytes, in which cAMP inhibits PI3K and induces actin rearrangement independently of PKA[17]. It should be noted that the cell shape change in 4B2 cells mediated by 8-Br-cAMP depended on PKA activation, as shown by the inhibitory effect of H89. PGE_2 inhibited cell death through PI3K-mediated inhibition of Bax or Bad[18,19]. However, unlike the present study, the cytoprotective effect of PGE_2 was independent of actin-cytoskeleton rearrangement.

When cells are stimulated with PGE_2 in the presence of the PI3K inhibitor wortmannin, only the PKA pathway should be activated; accordingly, cell shape change should occur. However, this cell shape change did not take place in the present study. This may be because PGE_2 did not produce high enough levels of cAMP in the cells to induce actin rearrangement. In fact, our previous data indicate that secreted cAMP levels were about 2 μM after treatment of 4B2 cells with 1 μg/ml PGE_2[10], which was a much lower level than the 1 mM 8-Br-cAMP treatment used here.

Considering the fact that cAMP is the central regulator of GC, extensive analysis of cAMP-mediated morphological changes leading to oxidative stress vulnerability will lead to a better understanding of GC death during atresia. The GC inside a given antral follicle are heterogeneous, comprised of three populations distinguished by their functions and positions. It is frequently observed that death initially is focused in the GC facing the antral cavity[20]. Interestingly, the cAMP content in the GC is higher in the large antral follicles[21]. This suggests a possible mechanism in which the antral cavity-facing GC may be over-activated by cAMP, conferring vulnerability to oxidative stress leading to cell death. Finally, it is tempting to speculate that GC reduce their risk of having cAMP-dependent oxidative insults through PI3K-mediated actin stabilization by PGE_2.

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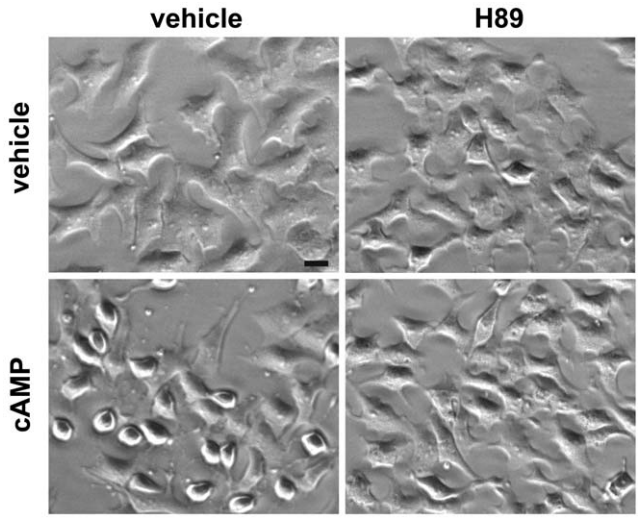
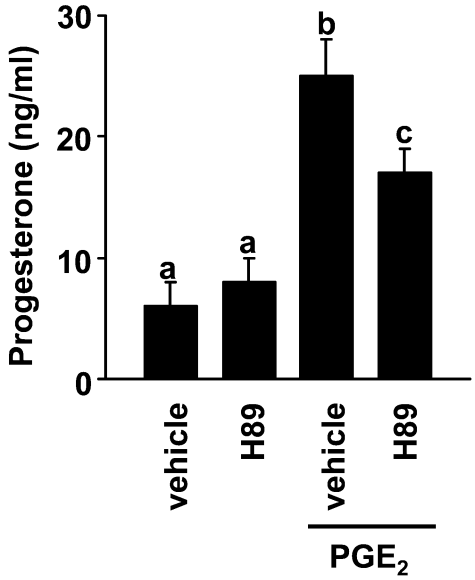
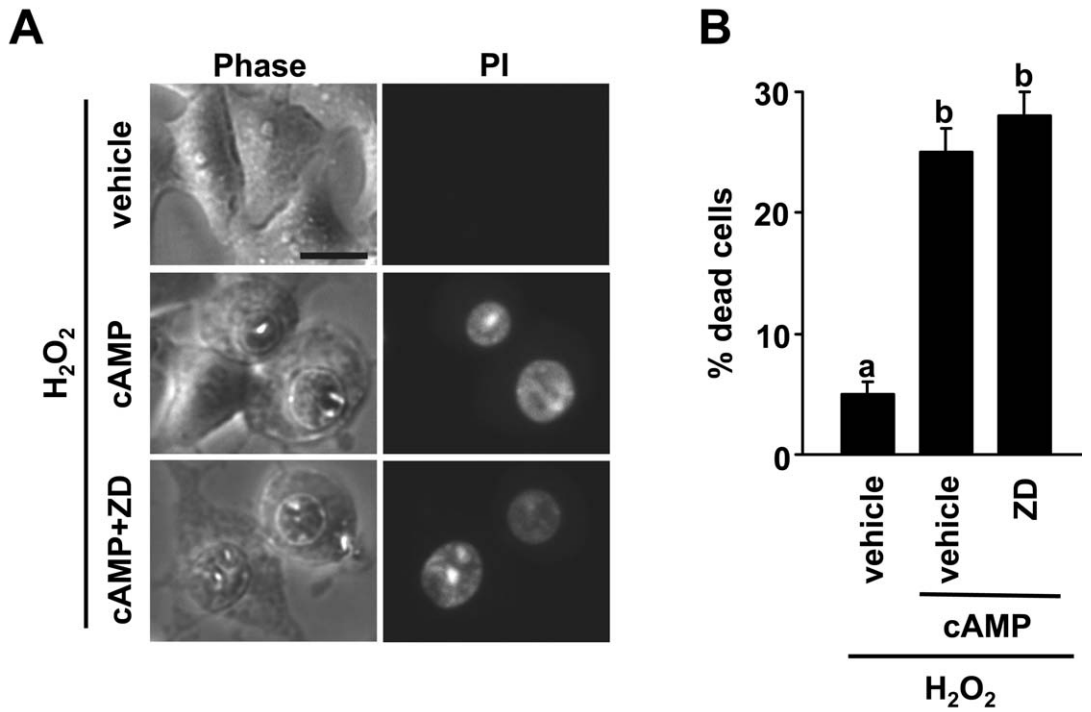


Supplemental fig. 1. Effect of H89 on 8-Br-cAMP (cAMP)-induced cell shape change in 4B2 cells. Cells were treated with vehicle or 1 mM 8-Br-cAMP, after 3 h, cells were treated with vehicle or 10 μM H89, a PKA inhibitor. Cell morphology assessed by phase contrast microscopy. A bar: 10 μm.



Supplemental fig. 3. Effect of H89 on PGE_2-induced progesterone secretion in 4B2 cells. Cells were stimulated with or without 1 μg/ml PGE_2 in the presence or absence of 10 μM H89. Each data represents the mean±SE of three independent experiments. a is different from b ($P < 0.01$). a is different from c. b is different from c. ($P < 0.05$)



Supplemental fig. 2. Effect of ZD on cell vulnerability to H_2O_2 induced by 8-Br-cAMP (cAMP). After cells were stimulated for 4 h with 1 mM 8-Br-cAMP in the presence or absence of 20 μM ZD, a general caspase inhibitor, they were challenged with 300 μM H_2O_2. A, Cells were observed by fluorescence (*right*) and phase contrast (*left*) microscopy in the same fields. A bar: 10 μm. B, Quantitative analysis of the data obtained as in A. Each data represents the mean±SE of three independent experiments. a is different from b ($P < 0.01$).