Estradiol 17β and Its Metabolites Stimulate Cell Proliferation and Antagonize Ascorbic Acid-Suppressed Cell Proliferation in Human Ovarian Cancer Cells

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Abstract

Estradiol 17β (E$_2$β) and ascorbic acid (AA) have been implicated in cancer progression. However, little is known about the actions of biologically active metabolites of E$_2$β, 2-hydroxyestradiol (2OHE$_2$), 4-hydroxyestradiol (4OHE$_2$), 2-methoxyestradiol (2ME$_2$), and 4-methoxyestradiol (4ME$_2$) synthesized sequentially by cytochrome P450, family 1, subfamily A (CYP1A1) and B (CYP1B1), polypeptide 1, and catechol-O-methyltransferase (COMT) on ovarian cancer. Herein, we examined the expression of CYP1A1, CYP1B1, COMT, and estrogen receptor α (ERα) and β (ERβ) in human ovarian surface epithelial (IOSE-385) and cancer cell lines (OVCAR-3, SKOV-3, and OVCA-432). We also investigated the roles of E$_2$β, 2OHE$_2$, 4OHE$_2$, 2ME$_2$, and 4ME$_2$ in cell proliferation, and their interactive effects with AA on ovarian cells. We found the expression of CYP1A1, CYP1B1, COMT, ERα, and ERβ in most cell lines tested. Treating cells with physiological concentrations of E$_2$β and its metabolites promoted (13%-42% of the control) IOSE-385 and OVCAR-3 proliferation. The ER blockade inhibited IOSE-385 (~76%) and OVCAR-3 (~87%) proliferative response to E$_2$β but not to its metabolites. The ERα blockade inhibited (~85%) E$_2$β-stimulated OVCAR-3 proliferation, whereas ERβ blockade attenuated (~83%) E$_2$β-stimulated IOSE-385 proliferation. The AA at ≥250 μmol/L completely inhibited serum-stimulated cell proliferation in all cell lines tested; however, such inhibition in IOSE-385, OVCAR-3, and OVCA-432 was partially (~10%-20%) countered by E$_2$β and its metabolites. Thus, our findings indicate that E$_2$β and its metabolites promote cell proliferation and antagonize the AA-suppressed cell proliferation in a subset of ovarian cancer cells, suggesting that blocking the actions of E$_2$β and its metabolites may enhance AA's antiovarian cancer activity.

Keywords

E$_2$β, E$_2$β metabolites, ascorbic acid, ovarian cancer cells, growth

Introduction

Ovarian cancer is the most lethal gynecological malignancy, largely because cancer cells acquire a chemoresistant phenotype after initial cytoreductive surgery and chemotherapy in the majority of cases.[1] In addition, another major challenge of the current cancer treatment is that ovarian cancer cells are highly heterogeneous at the cellular and molecular levels.[1] Thus, understanding individual subtypes of ovarian cancer at these levels is critical for developing an efficacious individual therapy.[1]

Estrogen has been considered as a major risk factor for ovarian cancer, particularly since recent epidemiological studies have demonstrated an elevation of ovarian cancer incidence with postmenopausal usage of estrogen.[1] Nonetheless, the exact role of estrogen in ovarian cancer is still controversial,

since estrogen may have stimulatory, inhibitory, or no effect on ovarian cancer cell growth, possibly depending on the individual subtypes of ovarian cancer, doses of estrogen used, and patients' ages (eg, pre- vs postmenopause).[1,2] Additionally,

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although the majority of ovarian cancer cells express estrogen receptor α (ERα) and β (ERβ), these receptors may exert opposite actions on ovarian cancer as ERα promotes ovarian cancer progression, whereas ERβ may antagonize ERα's actions.[1-3] Moreover, recent evidence has also indicated that estrogen-induced growth of ovarian cancer could be independent of ER.[1,3] To further complicate the situation and possibly explain this latter observation, estrogen can be converted into a number of biologically active metabolites. For example, as catalyzed by cytochrome P450, family 1, subfamily A (CYP1A1) polypeptide 1 and subfamily B (CYP1B1) polypeptide 1, estradiol 17β (E$_2\beta$) can be further hydroxylated to form the catecholestradiols 2-hydroxyestradiol (2OHE$_2$) and 4-hydroxyestradiol (4OHE$_2$), which can be subsequently methylated into the methoxyestradiols 2-methoxestradiol (2ME$_2$) and 4-methoxyestradiol (4ME$_2$) by catechol-O-methyltransferase (COMT). [4] Apart from CYP450- and COMT-mediated hydroxylation and methylation, E$_2\beta$ can also be conjugated to glucuronides and sulfates.[5,6] These conjugated E$_2\beta$ themselves have no estrogen activity but may restore such an activity after the release of glucuronides and sulfates.[7]

The physiological plasma concentrations of E$_2\beta$ and its metabolites range from 0.01 to 2.2 nmol/L in premenopausal, nonpregnant women, increase dramatically in pregnant women, and decrease sharply in postmenopausal women.[8-11] These E$_2\beta$ metabolites with their biological activities independent of ER[4] are either pro- or antiovarian cancer.[12-14] Indeed, 4OHE$_2$ at 0.01 to 0.1 nmol/L has been show to stimulate ovarian cancer (OVCAR-3) cell proliferation in vitro,[15] whereas 2ME$_2$ at supraphysiological concentrations (inhibitory concentration 50 [IC50] > 200 nmol/L) inhibits cell proliferation of many ovarian cancer cell lines including OVCAR-3[13,16] and primary ovarian cancer cells.[17]

Ascorbic acid (AA; vitamin C) has long been implicated in preventing and treating cancers.[18] However, no conclusive clinic results have been obtained so far. This is thought to be largely attributed to the fact that AA levels in vivo cannot reach concentrations sufficient to induce anticancer actions even with oral supplementation approaching maximally tolerated doses.[19,20] For instance, with the daily oral supplement of AA up to 2500 mg in human, circulating AA levels are likely to be maintained approximately at 80 μmol/L similar to those by the consumption of 5 servings of fruits and vegetables.[19,20] At these physiological levels (<100 μmol/L[19,20]), acute (1-2 hours) treatment of AA does not have any significant impact on the growth of cancer cells.[21,22] Conversely, circulating AA levels can be easily elevated to nontoxic, pharmacologic levels (up to 20-30 mmol/L by intravenous injection[19,20]). At these pharmacologic levels, AA can specifically kill ovarian cancer cells in vitro (with half maximal effective concentration [EC50] < 10 mmol/L) and suppress the progression of ovarian cancer in mice, possibly via locally generating high levels of H$_2$O$_2$.[21-23]

To date, little is known regarding the actions of E$_2\beta$ and its metabolites and prolonged AA treatment on the growth of ovarian cancer cells. Particularly, it is unknown whether E$_2\beta$ and its metabolites within their physiological ranges can affect anticancer actions of AA, which may be part of reason for the failure of AA at physiological concentrations to prevent and treat ovarian cancer. Therefore, we examined the expression of CYP1A1, CYP1B1, and COMT in ovarian cancer cell lines. To test the hypotheses that E$_2\beta$ and its metabolites stimulate ovarian cancer cell proliferation and migration via an ER or ER-independent manner and attenuate the effects of AA on ovarian cancer cell proliferation, we also investigated cell proliferation and/or migration in response to estrogen and its metabolites as well as AA in the presence of ER antagonists using 1 human ovarian surface epithelial (IOSE-385) and 3 ovarian cancer cell lines (OVCAR-3, SKOV-3, and OVCA-432).

Materials and Methods

Cell Lines

A human ovarian surface epithelial cell line IOSE-385 immortalized by SV40 large-T antigen was kindly provided by Dr Nelly Auersperg of the Canadian Ovarian Tissue Bank. Human ovarian adenocarcinoma cell lines, OVCAR-3 and SKOV-3, were obtained from the American Type Culture Collection (Manassas, Virginia), and OVCA-432 was established as described.[24] All these cancer cell lines were isolated from ascites fluid and were classified as cisplatin resistant.[25] However, these cancer cells differ in many other aspects. For example, OVCAR-3 and SKOV-3 are p53 mutant and null, respectively, whereas OVCA-432 expresses wild-type p53.[25] In addition, OVCAR-3, but not SKOV-3, responds to estrogen, although both express ERα and ERβ.[26] Moreover, OVCAR-3 and OVCA-432, but not SKOV-3, express CA125, a major ovarian cancer biomarker.[1] Thus, these cancer cell lines may represent cisplatin-resistant cohorts of patients with cancer cells with either positive or negative expression of p53 and CA125.

The OVCAR-3 cells were expanded in RPMI1640 medium (Gibco-BRL, Gaithersburg, Maryland) containing 10% fetal bovine serum (FBS; Gibco-BRL, 1% penicillin/streptomycin [P/S; HyClone], and 10 μg/mL insulin [Sigma, St Louis, Missouri]). The SKOV-3 cells were expanded in RPMI 1640 medium containing 10% FBS and 1% P/S. The OVCA-432 and IOSE385 cells were expanded in medium 199/105 (Sigma) containing 10% FBS and 1% P/S. All cell lines were maintained in a humidified atmosphere containing 5% CO$_2$ at 37°C. The IOSE-385 at passages 13 to 15, OVCAR-3 at passages 32 to 35, SKOV-3 at passages 29 to 31, and OVCA-432 at passages 18 to 20 were used in the current study.

Western Blotting

To examine the expression of CYP1A1, CYP1B1, COMT, ERα, and ERβ in ovarian cells, Western blotting was performed.[27-29] The cells were lysed by sonication in buffer. After centrifugation, protein concentrations of the supernatant were determined using bovine serum albumin (fraction V; Sigma) as standards. Protein samples (30 μg) were separated on 10%
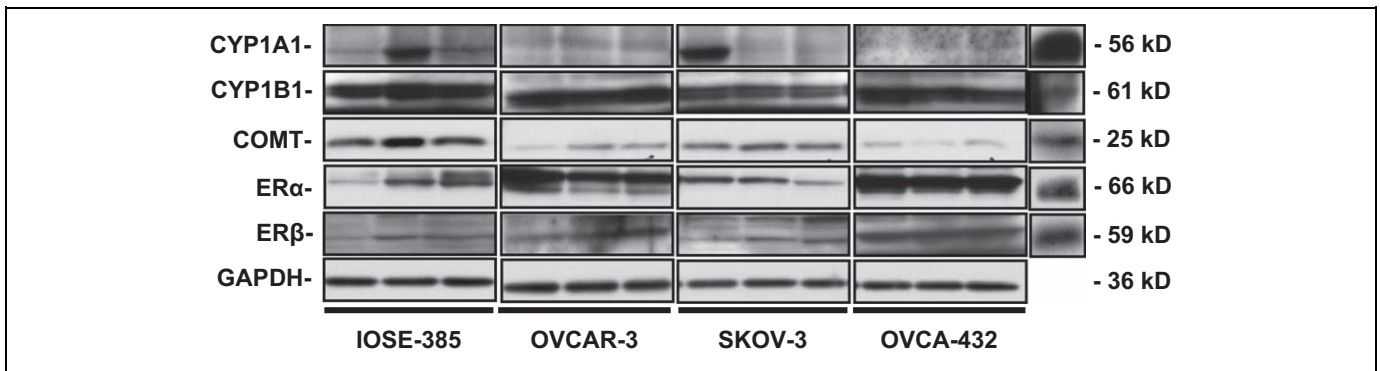
Figure 1. Western bolt analysis of CYP1A1, CYP1B1, COMT, ERα, and ERβ in ovarian cells. Different lanes in each ovarian cell line represent different passages of cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as a loading control. Positive controls (CTRL) were mouse liver (for CYP1A1), human placenta (for CYP1B1 and COMT), and mouse thyroid extracts (for ERα and ERβ). COMT indicates catechol-O-methyltransferase; CYP1A1, cytochrome P450, family 1, subfamily A; CYP1B1, cytochrome P450, family 1, subfamily B; ERα, estrogen receptor α.

sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and electrically transferred to polyvinylidene difluoride membranes (100 V, 60 minutes). Nonspecific binding was blocked with 5% fat-free milk in Tris buffer (50 mmol/L Tris-HCl, pH 7.5, 0.15 mol/L NaCl, and 0.05% Tween-20) for 60 minutes. Membranes were incubated with primary antibodies overnight at 4°C. The CYP1A1, CYP1B1, COMT, ERα, and ERβ proteins were probed using anti-CYP1A1 (1:500), anti-CYP1B1 (1:500), anti-COMT (1:1000), anti-ERα (1:500), or anti-ERβ (1:500) antibody, respectively. Glyceraldehyde 3-phosphate dehydrogenase was utilized as a loading control. After washing, membranes were incubated with corresponding peroxidase-conjugated immunoglobulin G for 60 minutes and detected with enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, New Jersey), followed by exposure to chemiluminescence films.

Cell Proliferation

Cell proliferation assay was performed.[28,29] Cells seeded in 96-well plates (1000-5000 cells/well) were cultured in phenol red-free RPMI1640 medium containing 5% charcoal-stripped FBS and 1% P/S (designated as the complete media). After 24 hours, the cells were treated without or with 0.01 to 100 nmol/L of $E_2\beta$ and its metabolites freshly made in the complete media up to 6 days. Additional cells were treated without or with 65 to 2000 μmol/L of AA in media (diluted from the stock solution at 20 mmol/L; pH in final media ≈ 7.4). The medium was replaced with freshly made hormones or AA every 48 hours to ensure stable bioavailability. After 4 to 6 days of culture, the cell number was determined using the crystal violet staining method.[28,29] After an optimal dose of $E_2\beta$, its metabolites, and AA was determined, additional cells were treated with $E_2\beta$ or its metabolites in the presence of 1 μmol/L of ICI (a nonspecific ER antagonist), methyl-piperidino-pyrazole (MPP; a selective ERα antagonist), or pyrazolo [1,5-a] pyrimidine (PHTPP; a selective ERβ antagonist; 1 hour of pretreatment) to verify the role of ER subtypes in ovarian cell proliferation. To

examine the effects of $E_2\beta$ and its metabolites on the AA's actions, additional cells were pretreated (1 hour) with $E_2\beta$ or its metabolites (0.1 nmol/L) in the presence of 1 μmol/L of ICI, MPP, or PHTPP (added 1 hour before the treatment of $E_2\beta$ or its metabolites) and in the absence or presence of 1 μmol/L of ICI, MPP, or PHTPP (added 1 hour before the treatment of $E_2\beta$ or its metabolites), followed by treatment with 80 μmol/L of AA.[19,20]

Cell Migration

To determine the effects of $E_2\beta$ and its metabolites on ovarian cell migration, the wound healing assay was performed as described.[28] Cells were cultured in 12-well plates in complete growth media until reaching confluence, followed by serum starvation for 16 hours. A sterilized 200-μL pipette tip was used to make a straight scratch. The cells were washed once and then treated without or with 0.1 nmol/L of $E_2\beta$, 2OHE$_2$, 4OHE$_2$, 2ME$_2$, and 4ME$_2$ in the complete media. Two images per scratch were photographed under a 10× objective immediately after scratching and then for every 8 hours up to 40 hours. Sizes of the wound area were calculated using the MetaMorph image analysis software (Molecular Devices, Sunnyvale, California).

Statistical Analysis

Data were analyzed using 1-way analysis of variance (Sigma-Stat; Jandel Co, San Rafael, California). When an *F* test was significant, data were compared with their respective control by the Bonferroni multiple comparison test or Student *t* test. $P \leq .05$ was considered statistically significant.

Results

Expression of CYP1A1, CYP1B1, COMT, ERα, and ERβ

Western blotting revealed the presence of CYP1A1, CYP1B1, COMT, ERα, and ERβ in all cell lines tested except CYP1A1

Table 1. Protein Levels of CYP1A1, CYP1B1, COMT, ERα, and ERβ in IOSE-385, OVCAR-3, SKOV-3, and OVCA-432 Cells.[a]

	IOSE-385	OVCAR-3	SKOV-3	OVCA-432
CYP1A1	0.36 ± 0.19[b]	0.04 ± 0.02[b]	0.30 ± 0.30	ND
CYP1B1	1.10 ± 0.19[b]	1.21 ± 0.09[b]	0.75 ± 0.19	1.07 ± 0.22
COMT	1.17 ± 0.57[c]	0.18 ± 0.06	0.56 ± 0.04	0.07 ± 0.02
ERα	0.35 ± 0.14	0.78 ± 0.08	0.38 ± 0.14	1.96 ± 0.16[c]
ERβ	0.19 ± 0.07	1.13 ± 0.35	0.16 ± 0.10	1.63 ± 0.12[c]

Abbreviations: COMT, catechol-O-methyltransferase; CYP1A1, cytochrome P450, family 1, subfamily A; CYP1B1, cytochrome P450, family 1, subfamily B; ERα, estrogen receptor α; ND, not detectable; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of the mean.
[a] Data normalized to GAPDH are expressed as mean $\pm$ SEM ($P \leq .05$, n = 3).
[b] Differs from each other within each individual cell line.
[c] Differs from other cell lines.

that was undetectable in OVCA-432 (Figure 1). The levels of CYP1A1 and CYP1B1 were similar among IOSE-385, OVCAR-3, and SKOV-3. The levels of COMT in IOSE-385 cells were at least 2-fold greater ($P \leq .05$) than those in other cell lines, and the levels of ERα and ERβ in OVCA-432 cells were higher ($P \leq .05$) than those in other cell lines (Table 1). It is also noted that the CYP1B1 levels were at least 2-fold higher ($P \leq .05$) than the CYP1A1 levels in IOSE-385, OVCAR-3, and OVCA-432, suggesting that CYP1B1 is a predominant member of CYP1 family in these ovarian cells.

Effects of E$_2\beta$ and Its Metabolites on Cell Proliferation

The E$_2\beta$ and its metabolites stimulated ($P \leq .05$) IOSE-385 and OVCAR-3 proliferation (Figure 2) but did not affect SKOV-3 and OVCA-432 proliferation (not shown). The E$_2\beta$ stimulated IOSE-385 and OVCAR-3 proliferation with maximum responses observed at 0.1 nmol/L (1.42 ± 0.05 and 1.33 ± 0.05 fold of the control for IOSE-385 and OVCAR-3, respectively; Figure 2A1 and B1). The 2OHE$_2$, 4OHE$_2$, and 4ME$_2$ at all doses studied also similarly promoted ($P \leq .05$) IOSE-385 proliferation; however, 2ME$_2$ did so only at 0.1 nmol/L (Figure 2). Moreover, 4OHE$_2$, 2ME$_2$, and 4ME$_2$ at all doses studied also significantly promoted ($P \leq .05$) OVCAR-3 proliferation; however, 2OHE$_2$ did so only at 0.1 and 100 nmol/L (Figure 2).

Roles of ERα and ERβ in Cell Proliferation

The ICI alone had no effects on IOSE-385 and OVCAR-3 proliferation; however, ICI partially ($\sim$76% and 87% for IOSE-385 and OVCAR-3) inhibited ($P \leq .05$) cell proliferative response to E$_2\beta$ but not to its metabolites (Figure 3A1 and B1). The ERα blockade with MPP inhibited ($\sim$80%; $P \leq .05$) E$_2\beta$-stimulated OVCAR-3 but not IOSE-385 proliferation (Figure 3A2 and B2). In contrast, ERβ blockade with PHTPP attenuated ($\sim$80%; $P \leq .05$) E$_2\beta$-stimulated IOSE-385 but not OVCAR-3 proliferation (Figure 3A3 and B3). However, neither MPP nor PHTPP affected IOSE-385 and OVCAR-3 proliferative responses to these E$_2\beta$ metabolites, confirming

no participation of ERα and ERβ in these E$_2\beta$ metabolites-stimulated cell proliferations (Figure 3A2 and B2).

Ascorbic Acid Suppresses Cell Proliferation But Not Migration

When compared to the day 4 control (without AA), AA decreased ($P \leq .05$) cell number in all the doses and in all the cell lines tested (Figure 4). Interestingly, when compared to the cell number initially seeded, AA at any dose studied did not cause cell loss in IOSE-385; however, AA at doses $\geq$250, 500, and 125 µmol/L, respectively, for SKOV-3, OVCAR-3, and OVCA-432 caused significant ($P \leq .05$) cell loss (not shown).

When compared to the corresponding time control, treatments of E$_2\beta$ and its metabolites (not shown) did not alter cell migration in all the cell lines tested even up to 40 hours. However, the pattern of cell migration differed among these cells tested. For example, when compared to the time 0 control, E$_2\beta$ and its metabolites did not cause any change in the scratch gaps in OVCAR-3 and OVCA-432 up to 40 hours (not shown). Nonetheless, the scratch gaps in SKOV-3 and IOSE-385 similarly narrowed sharply at 16 hours and were almost healed at 24 hours between the control versus E$_2\beta$ and its metabolites (not shown).

Estradiol$_2\beta$ and Its Metabolites Antagonize AA-Suppressed Cell Proliferation

When compared to the control, AA at 80 µmol/L inhibited ($P \leq .05$) cell proliferation in IOSE-385, OVCAR-3, OVCA-432 (Figure 5), and SKOV-3 (not shown). However, pretreatment of E$_2\beta$ and its metabolites at 0.1 nmol/L slightly but significantly ($P \leq .05$) attenuated the AA-inhibited cell proliferation in IOSE-385, OVCAR-3, and OVCA-432 (Figure 5) but not in SKOV-3 (not shown). Moreover, neither ICI, MPP, nor PHTPP affected the effects of E$_2\beta$ and its 4 metabolites on AA-inhibited cell proliferation (not shown), suggesting no participation of ER in such stimulatory actions induced by E$_2\beta$ and its 4 metabolites.

Discussion

Herein, we have demonstrated for the first time that physiological concentrations of 2OHE$_2$, 2ME$_2$, and 4ME$_2$ stimulate IOSE-385 and OVCAR-3 proliferation. We have also shown that ERβ and ERα predominantly mediate the E$_2\beta$-stimulated proliferation of IOSE-385 and OVCAR-3, respectively; however, such E$_2\beta$ metabolite-stimulated cell proliferation is independent of ER. Our current observation that physiological concentrations of AA can inhibit ovarian cancer cell proliferation is important, since it suggests that maintaining stable AA levels at physiological concentrations may have protective benefits against ovarian cancer.[2,30] Interestingly, we found that AA at its pharmacological levels easily reached by intravenous injection[19,20] can specifically cause loss of ovarian cancer cells but not surface epithelial cells in vitro (Figure 4), supporting
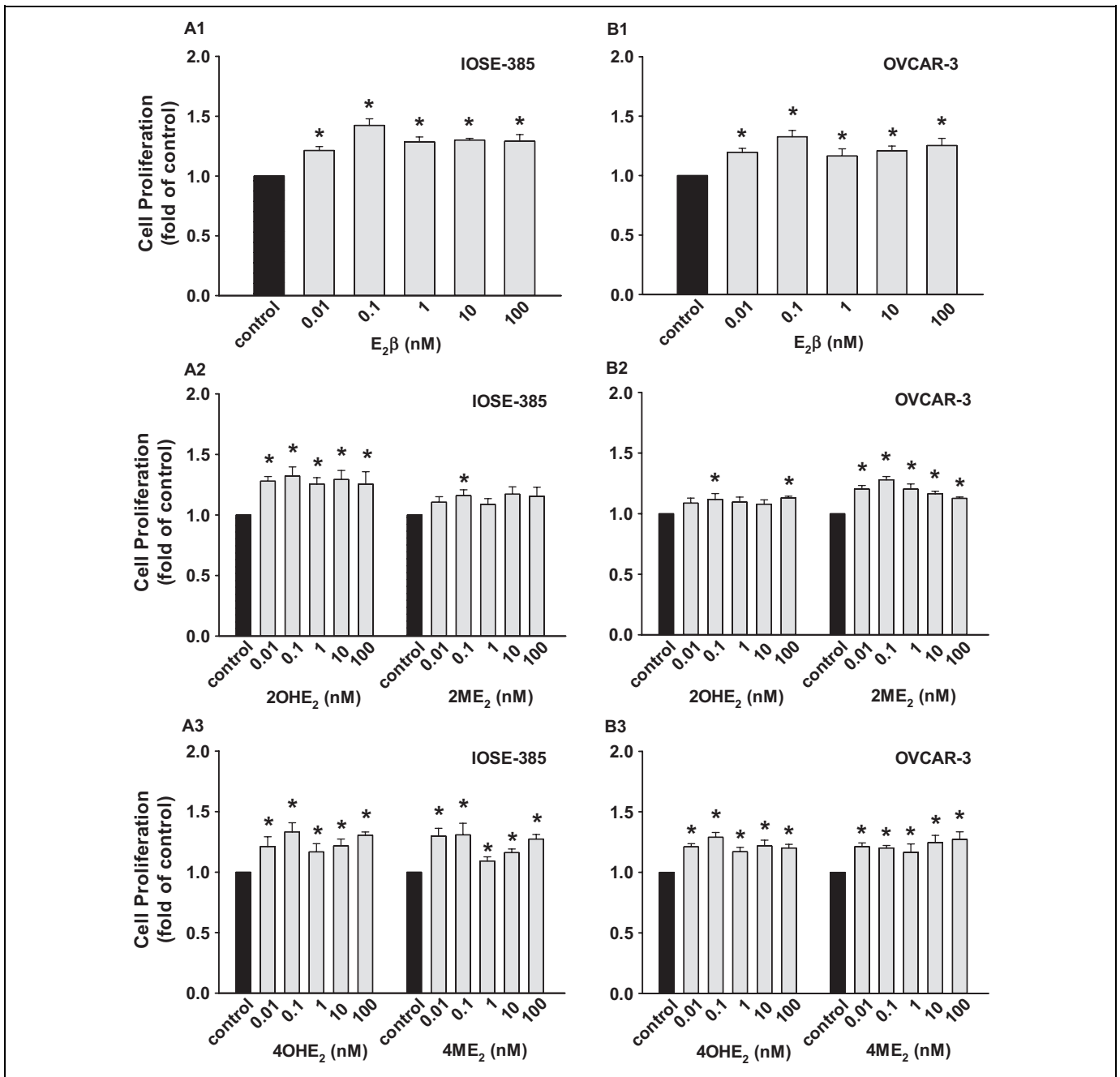
Figure 2. Effects of $E_2\beta$, $2OHE_2$, $4OHE_2$, $2ME_2$, and $4ME_2$ on (A) IOSE-385 and (B) OVCAR-3 proliferation. Cells seeded (1000 and 5000 cells/well for IOSE-385 and OVCAR-3, respectively) were treated with $E_2\beta$ and its metabolites for 6 days. Data are expressed as mean $\pm$ SEM fold of the vehicle control (n = 4). *Differs from the vehicle control ($P \leq .05$). $E_2\beta$ indicates estradiol 17β; SEM, standard error of the mean; $2ME_2$, 2-methoxyestradiol; $4ME_2$, 4-methoxyestradiol; $2OHE_2$, 2-hydroxyestradiol; $4OHE_2$, 4-hydroxyestradiol.

the therapeutic effects of AA on ovarian cancer. More importantly, our finding that $E_2\beta$ and its metabolites slightly but significantly inhibit AA-suppressed ovarian cell cancer proliferation implies that $E_2\beta$ and its metabolites can potentially decrease the AA's antiovarian cancer activity in vivo. These data indicate that in addition to their actions in promoting ovarian cancer cell proliferation, prolonged presence of physiological concentrations of estrogen and its metabolites

in vivo may antagonize the AA suppression on ovarian cancer cell growth.

Our current findings that CYP1A1, CYP1B1, and COMT were expressed in human ovarian surface epithelial cells and in most cancer cells studied[31,32] suggest that in these cells, $E_2\beta$ derived either from an endogenous or an exogenous source can be potentially converted into $2OHE_2$, $4OHE_2$, $2ME_2$, and $4ME_2$, acting on these ovarian cells. This is supported by the
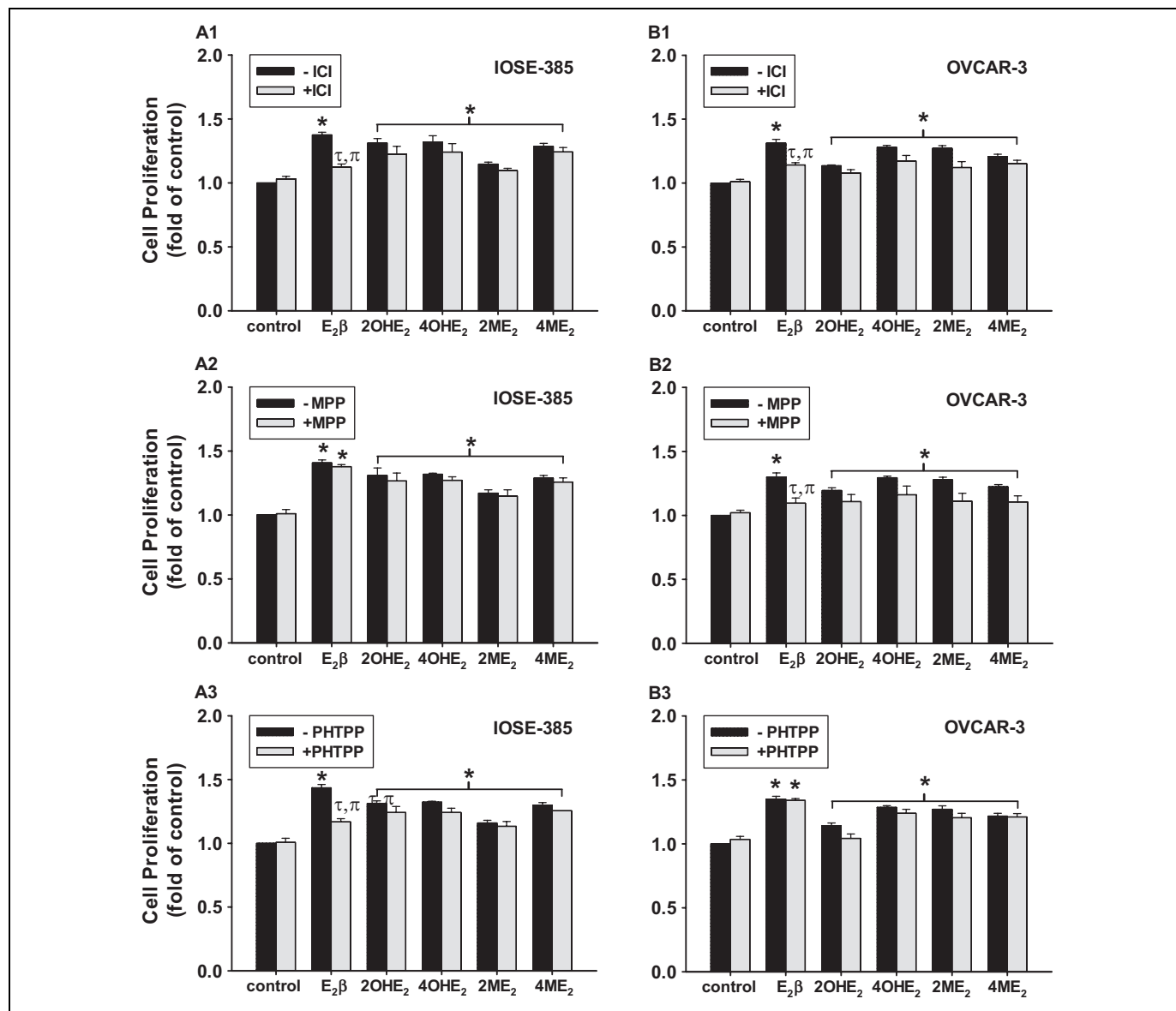
Figure 3. Effects of ICI, MPP, and PHTPP on (A) IOSE-385 and (B) OVCAR-3 proliferative responses to $E_2\beta$, $2OHE_2$, $4OHE_2$, $2ME_2$, and $4ME_2$. The cells were treated with 0.1 nmol/L $E_2\beta$ and its metabolites in the presence of 1 µmol/L of ICI, MPP, or PHTPP for 6 days. Data are expressed as mean $\pm$ SEM fold of the vehicle control (n = 4). *Differs from the vehicle control ($-$ICI; $P \leq .05$). ${}^\tau$Differ from the $E_2\beta$ treatment ($P \leq .05$). ${}^\pi$Differs from the ICI control ($P \leq .05$). $E_2\beta$ indicates estradiol 17β; MPP, methyl-piperidino-pyrazole; PHTPP, pyrazolo [1,5-a] pyrimidine; SEM, standard error of the mean; $2ME_2$, 2-methoxyestradiol; $4ME_2$, 4-methoxyestradiol; $2OHE_2$, 2-hydroxyestradiol; $4OHE_2$, 4-hydroxyestradiol.

observation that only ICI partially ($\sim 76\%$ and $\sim 87\%$, respectively) inhibited $E_2\beta$-stimulated cell proliferation in IOSE-385 and OVCAR-3, as the remaining part of proliferation may be stimulated by $E_2\beta$ metabolites via an ER-independent fashion (Figure 3).[4]

The $E_2\beta$ within its physiological plasma concentrations[8] has been shown to stimulate proliferation, metastasis, and/or anti-poptotic activity of ovarian surface epithelial and cancer cells.[33-37] Nonetheless, $E_2\beta$ at relatively high concentrations (~ 100 µmol/L, the peak concentrations inside ovarian tissues right before ovulation)[38,39] inhibits proliferation of these

ovarian cells.[40-42] In the current study, of 3 cancer cell lines tested, only OVCAR-3 exhibited proliferative response to $E_2\beta$. The unresponsiveness of SKOV-3 to $E_2\beta$ is not surprising, since ER in SKOV-3 is not functional.[26,43] However, it is unclear whether similar mechanisms render unresponsiveness of OVCA-432 to $E_2\beta$.

Although the underlying mechanisms remain not fully understood, the roles of $E_2\beta$ metabolites in ovarian cancer have been proposed. For example, $2OHE_2$ and $4OHE_2$ are carcinogenic in ovarian cancer,[16,44] whereas $2ME_2$, especially at high concentrations, may be anticarcinogenic in many cancers
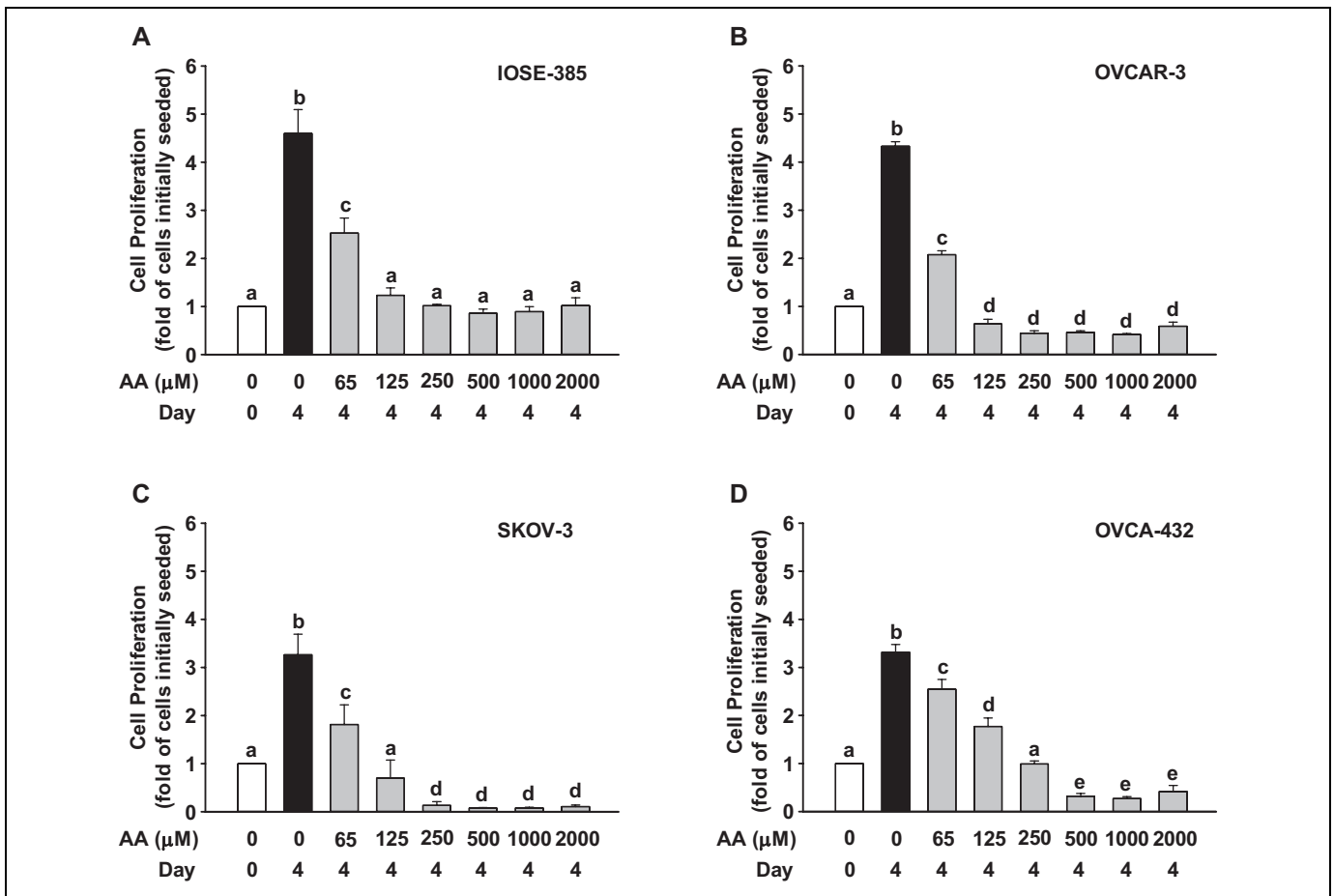
Figure 4. Effects of AA on (A) IOSE-385, (B) OVCAR-3, (C) SKOV-3, and (D) OVCA-432 proliferation. Cells initially seeded at 3000 cells/well for IOSE-385 and 5000 cells/well for OVCAR-3, SKOV-3, and OVCA-432 were treated with AA in the complete media for 4 days. Data are expressed as mean $\pm$ SEM fold of the cells initially seeded (n = 4).[a-e]Different letters differ from each other ($P \leq .05$). AA indicates ascorbic acid; SEM, standard error of the mean.

including ovarian cancer.[14,45] Our current data confirmed the similar pro-proliferative activity of $2OHE_2$ and $4OHE_2$ in ovarian epithelial (IOSE-385) and in an ovarian cancer cell line (OVCAR-3 cells).[15] However, in contrast to previous reports on OVCAR-3[16] and other types of ovarian cancer cells,[17] we found that physiological concentrations of $2ME_2$ also stimulated the OVCAR-3 cell proliferation. This discrepancy may be raised from high supraphysiological dosages (IC50 > 0.2 µmol/L) of $2ME_2$ used in those previous studies.[16,17] Moreover, our current data are the first, as far as we are aware, to report the stimulatory effects of $4ME_2$ on normal and malignant ovarian epithelial cells.

Our observation that ICI inhibited $E_2\beta$-stimulated IOSE-385 and OVCAR-3 proliferation clearly indicates a predominant role of ER in these $E_2\beta$-stimulatory effects. More interestingly, using selective $ER\alpha$ and $ER\beta$ antagonists, we further revealed different roles of $ER\alpha$ and $ER\beta$ in $E_2\beta$-mediated cell proliferation between normal and malignant ovarian epithelial cells. These findings support the concept that $ER\alpha$ is the major form of ER responsive for the growth of malignant cancer cells, whereas $ER\beta$ is the dominant form in normal epithelial cells

or benign tumors.[2] Thus, specifically blocking activation of $ER\alpha$ should be considered as a therapeutic approach for a subset of $E_2\beta$-sensitive ovarian cancer cells as suggested in other cancers. However, blockade of $ER\alpha$ alone may not be sufficient to suppress ovarian cancer growth, since $E_2\beta$ metabolites can similarly stimulate ovarian cancer growth independent of $ER\alpha$ and $ER\beta$ (Figure 3). To date, it remains elusive what receptor mediates this $E_2\beta$ metabolites-stimulated cell proliferation; however, 1 candidate might be G protein-coupled ER 30 (GPR30), a potent membrane bound ER, as it is expressed and functional in ovarian cancer cells.[46,47]

The mechanism underlying the effects of $E_2\beta$ and its metabolites on AA-suppressed ovarian cancer cell proliferation remains unclear. The GPA30, but not classical ER, may mediate such effects. Moreover, given that AA can inhibit cancer growth and progress via elevating local H_2O_2 levels[21-23] and estrogen is a potent scavenger of H_2O_2,[48,49] $E_2\beta$ and its metabolites may decrease local H_2O_2 levels, attenuating AA-suppressed ovarian cell growth. Thus, although future studies are needed to confirm the in vivo effects of $E_2\beta$ and its metabolites on growth of ovarian cancer, based on the current data,
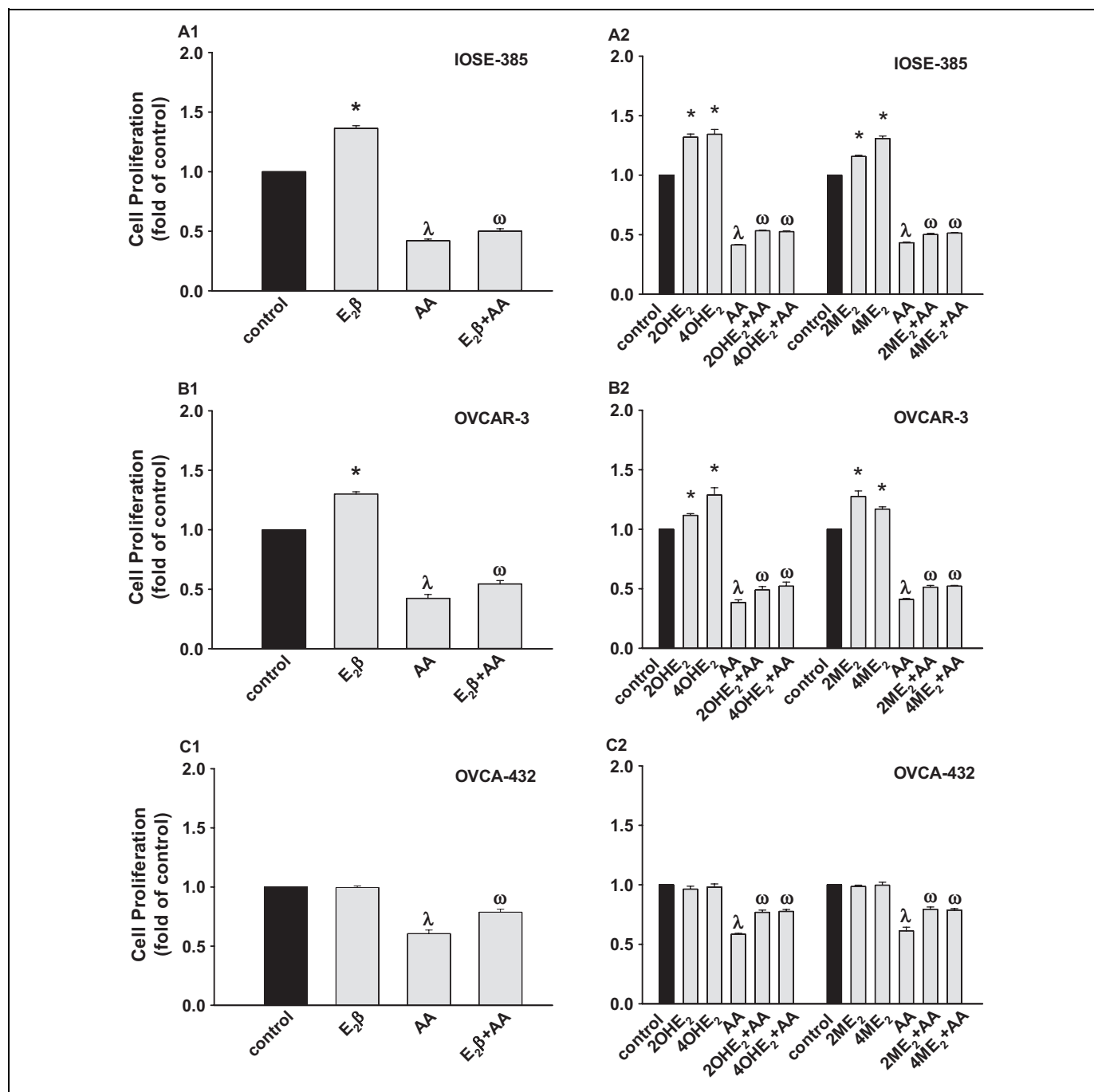
Figure 5. Effects of $E_2\beta$, $2OHE_2$, $4OHE_2$, $2ME_2$, and $4ME_2$ on AA-inhibited (A) IOSE-385, (B) OVCAR-3, and (C) OVCA-432 proliferation. Cells seeded at 5000 cells/well were treated with 80 μmol/L of AA in the presence of 0.1 nmol/L of $E_2\beta$ and its metabolites (1 hour pretreatment) for 4 days. Data are expressed as mean $\pm$ SEM fold of the vehicle control (n = 4). *,λDiffers from the vehicle control ($P \leq .05$). ωDiffers from the AA ($P \leq .05$). AA indicates ascorbic acid; $E_2\beta$, estradiol 17β; SEM, standard error of the mean; $2ME_2$, 2-methoxyestradiol; $4ME_2$, 4-methoxyestradiol; $2OHE_2$, 2-hydroxyestradiol; $4OHE_2$, 4-hydroxyestradiol.

we propose that when using AA as a therapeutic drug for interfering ovarian cancer growth, blocking the actions of $E_2\beta$ and its metabolites should be considered as such blockade may substantially promote AA's antiovarian cancer activity.

Authors' Note

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Declaration of Conflicting Interests

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