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Inhibition of Estradiol Uptake and Transforming Growth Factor α Secretion in Human Breast Cancer Cell Line MCF-7 by an Alkyl-lysophospholipid

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ABSTRACT

We investigated the effect of 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OCH_3), an alkyl-lysophospholipid, on the uptake of estrogen, the secretion of transforming growth factor (TGF) α and the content of progesterone receptors (PRs) in the hormone-dependent breast cancer cell line, MCF-7. The uptake of labeled estradiol by MCF-7 was dose dependently decreased by 12 h pretreatment with 10–25 μg/ml ET-18-OCH_3, and this suppression occurred prior to the onset of the inhibitory action of ET-18-OCH_3 on MCF-7 growth. Scatchard analysis demonstrated that ET-18-OCH_3 reduced the number of estrogen receptors in MCF-7 without affecting their affinity. Both the secretion of TGF-α from MCF-7 into the conditioned medium and the PR content of MCF-7 were decreased by 48 h treatment with 10 μg/ml ET-18-OCH_3. The estradiol uptake, the TGF-α secretion, and the PR content were not affected by platelet-activating factor, lyso-PAF, and palmitoyl-lysophosphatidylcholine, all at 10 μg/ml. These results suggest that the reduction of estrogen receptor level induced by ET-18-OCH_3 resulted in decreases in both the secretion of TGF-α and the content of PR in MCF-7, and these effects are specific to ET-18-OCH_3.

We concluded that these effects of ET-18-OCH_3 may lead, at least partly, to its antitumor action in hormone-dependent breast cancer cell lines.

INTRODUCTION

Breast cancer cells have been demonstrated to be associated with several autocrine growth factors (1, 2). It has also been reported that the proliferation of hormone-dependent breast cancer cells is controlled by autocrine growth factors such as TGF-α[2] (3), TGF-β (4), and insulin-like growth factor type I (2, 5). One class of these factors, TGF-α, has been shown to act on breast cancer cells via an interaction with EGF receptors (6). Induction by estradiol of a TGF-α-like activity was reported in 3 species of hormone-sensitive breast cancer cells (3). Considerable research interests have been focused on the role of autocrine factors in the control of breast cancer cell proliferation.

Recently, we have reported that ET-18-OCH_3 inhibits the growth of MCF-7 and ZR-75-1, hormone-dependent breast cancer cell lines, by blocking EGF internalization and hence by reducing the EGF binding, while it does not affect both the growth of BT-20, a hormone-independent breast cancer cell line, and EGF binding to this cell line (7, 8).

In the present study, we investigated the effect of ET-18-OCH_3 on the estrogen uptake, TGF-α secretion, and PR content in the hormone-dependent breast cancer cell line, MCF-7.

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[2] The abbreviations used are: TGF, transforming growth factor; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; EGF, epidermal growth factor; ER, estrogen receptor; PR, progesterone receptor; lyso-PAF, 1-*O*-octadecyl-2-hydroxy-*sn*-glycero-3-phosphocholine; PAF, 1-*O*-octadecyl-2-acetoyl-*sn*-glycero-3-phosphocholine (platelet activating factor); LPC, palmitoyllysophosphatidylcholine; TPA, phorbol-12-tetradecanoate-13-acetate; FBS, fetal bovine serum; DCC, dextran-coated charcoal; EIA, enzyme immunoassay; PBS, phosphate-buffered saline; PKC, protein kinase C.

MATERIALS AND METHODS

Chemicals. [^{3}H]Estradiol (166 Ci/mmol) was purchased from New England Nuclear (Boston, MA). ET-18-OCH_3, lyso-PAF, and PAF were obtained from BACHEM Feinchemikalien AG (Bubendorf, Switzerland), and LPC (from egg yolk) was from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cell Lines. The human breast cancer cell line, MCF-7, was subcultured in RPMI 1640 (Flow Laboratories, McLean, VA) supplemented with 10% (v/v) FBS (Filtron, Altona, Australia) as described previously (7). For experiments involving estrogen induction, the cells were passaged for at least 1 week in RPMI 1640 supplemented with 10% FBS treated with DCC (9) and contained 10 nM estradiol with a final 0.1% of absolute ethanol in order to keep the estrogenic action constant.

Collection of Conditioned Media. MCF-7 cells were cultured for 3 days to a density of 4–6 × 10^6 cells/flask in the growth medium, RPMI 1640, supplemented with 10% DCC-FBS containing 10 nM estradiol in T-150 flasks (Falcon No. 3024; Becton-Dickinson, Lincoln Park, NJ). The growth medium was removed, and the cells were rinsed twice with RPMI 1640 and then incubated for 48 h in RPMI 1640 containing ET-18-OCH_3 or one of its analogues, LPC, lyso-PAF, and PAF. [The stock solution (0.5 mg/ml) of ET-18-OCH_3 or each of the analogues was added to RPMI 1640 to obtain desired concentrations.] The medium was harvested and centrifuged at 3500 rpm for 10 min, the supernatant was dialyzed for 2 days in a SpectraPor 3 membrane (M_r 3500 cutoff) (Fisher Scientific, Los Angeles, CA) against distilled water. Finally, the dialysate was lyophilized, and the obtained powder was dissolved in 0.1 volume of distilled water.

Preparation of Cytosols. All the following procedures were carried out at 0–4°C unless otherwise specified. After collection of conditioned media, monolayer cells were harvested with 0.25% trypsin/0.5% EDTA solution for PR assays using an EIA kit. The cells were washed twice with PBS and added to the homogenization buffer (10 mM Tris-1 mM EDTA-3 mM NaN_3-12 mM thioglycerol-10% glycerol, pH 7.4) to be 5 × 10^6 cells/ml. This cell suspension was gently homogenized by a Potter-type homogenizer, and the homogenate was centrifuged at 800 × *g* for 10 min. The supernatant was removed and saved, and the pellet was resuspended in 5 volumes of the homogenization buffer. After centrifugation at 800 × *g* for 10 min, the supernatant was collected and combined with the former supernatant, and the total supernatant was centrifuged at 105,000 × *g* for 1 h. The resultant supernatant was obtained as the cytosol.

Uptake of [^{3}H]Estradiol by Breast Cancer Cell Line MCF-7. Measurement of [^{3}H]estradiol uptake and Scatchard analysis were carried out as described previously (10). MCF-7 cells were plated to be 1 × 10^5 cells/well in a 12-well culture dish (No. 3512; Coster, Cambridge, MA) and cultured for 3 days in the RPMI 1640 containing 10% DCC-FBS. ET-18-OCH_3 was then added and cultured for 12 h, and the medium was removed and replaced with 0.5 ml RPMI 1640 containing 0.1% bovine serum albumin and 6 nM [^{3}H]estradiol with or without 100-fold molar excess of unlabeled estradiol. The cells were incubated at 37°C for 1 h after the addition of the radiolabeled compound. The medium was removed and replaced with 1.0 ml of the PBS containing 0.5% bovine serum albumin (5 mM sodium phosphate-0.25 M sucrose-10% glycerol, pH 7.4), and the cells were allowed to stand for 30 min at room temperature. The cells were then rinsed twice each with 1.0 ml cold PBS, and 1 ml ethanol was added to each well. After 30 min incubation at room temperature, the ethanol was transferred to a scintillation vial, and the ^{3}H radioactivity in the ethanol was counted

in a liquid scintillation counter (LSC-3500; Aloka, Tokyo, Japan).

Enzyme Immunoassay of PR. The EIA kit for PR was purchased from Dainabot Laboratories (Tokyo, Japan). All procedures for PR-EIA were carried out according to the method described previously by Noguchi *et al.* (11). The intraassay coefficient of variation of this assay was <5.2%.

Enzyme Immunoassay of TGF-α. TGF-α was measured by the sandwich type enzyme-linked immunosorbent with rabbit antibody (affinity purified) and mouse monoclonal antibody ATG-2S as described by Inagaki *et al.* (12).

Growth Experiment. Cell proliferation was monitored at various stages by measuring DNA as described previously (7). Total protein was measured by the method of Lowry *et al.* (13).

RESULTS

As shown in Fig. 1, the uptake of [^{3}H]estradiol by MCF-7 was dose dependently decreased by 12 h pretreatment with 10–25 μg/ml of ET-18-OCH_3.

Fig. 2 shows the relation between the time of pretreatment with 10 μg/ml ET-18-OCH_3 and the uptake of labeled estradiol by MCF-7. The specific uptake of labeled estradiol was decreased significantly ($P < 0.05$) by 12 h pretreatment with ET-18-OCH_3 and further decreased to 61.5 ± 7.3% (SD) ($n = 3$) of control at 24 h ($P < 0.001$) and to 62.4 ± 7.6% ($n = 3$) at 48 h ($P < 0.001$).

The results of Scatchard analysis of the specific uptake of labeled estradiol by MCF-7 are shown in Fig. 3. By treatment with 10 μg/ml ET-18-OCH_3 for 12 h, the maximum uptake of labeled estradiol was decreased from 7.4 fmol/μg DNA (6.1×10^4 sites/cell) to 4.8 fmol/μg DNA (4.0×10^4 sites/cell), while the uptake rate was unaltered (0.44 nM for control and 0.45 nM in the presence of 10 μg/ml ET-18-OCH_3).

The effects of pretreatment with ET-18-OCH_3 and its analogues on the uptake of labeled estradiol by MCF-7 are shown in Table 1. The uptake of [^{3}H]estradiol by MCF-7 was significantly ($P < 0.05$) decreased by 12 h pretreatment of MCF-7 with 10 μg/ml ET-18-OCH_3, while no such decrease was observed after 12 h pretreatment with 10 μg/ml of any one of LPC, lyso-PAF, and PAF.

The effects of pretreatment with ET-18-OCH_3 and its analogues on the amount of TGF-α secreted from MCF-7 into the conditioned medium and on the content of PR in MCF-7 are shown in Table 2. The secretion of TGF-α from MCF-7 was significantly ($P < 0.01$) suppressed by 48 h pretreatment with 10 μg/ml ET-18-OCH_3, namely from 4.29 ± 0.07 ng/10^7 cells to 2.77 ± 0.50 ng/10^7 cells or from 1.43 ± 0.02 ng/mg total

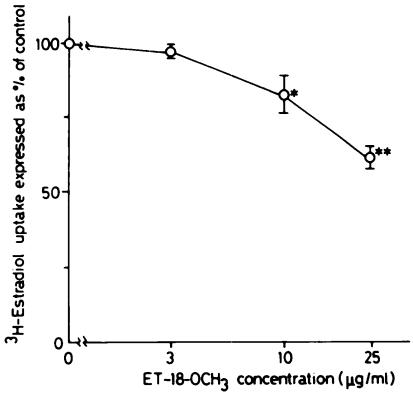

Fig. 1. Dose dependence of the inhibitory effect of ET-18-OCH_3 pretreatment on the specific uptake of [^{3}H]estradiol by human breast cancer cell line MCF-7. The cells were preincubated for 12 h in the presence of 3–25 μg/ml ET-18-OCH_3. The values plotted are the mean ± SD (*bars*) of triplicate determinations in one typical experiment and normalized to the control (352.0 ± 23.0 cpm/μg DNA as 100%) measured in the absence of ET-18-OCH_3. The level of significance as compared to the control (Student's *t* test): *, $P < 0.05$; **, $P < 0.01$.

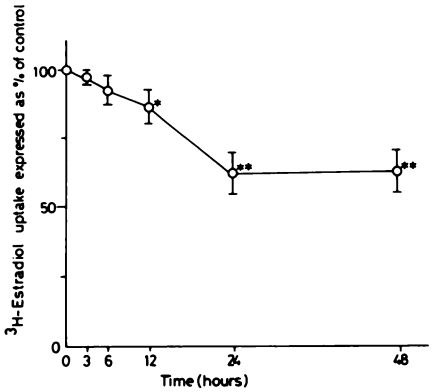

Fig. 2. Time dependence of the inhibitory effect of ET-18-OCH_3 pretreatment on the specific uptake of [^{3}H]estradiol by MCF-7. The cells were pretreated for 3–48 h at 37°C with 10 μg/ml ET-18-OCH_3. After washing, the specific uptake of [^{3}H]estradiol was measured by whole cell uptake method (10). The values plotted are the mean ± SD (*bars*) of triplicate determinations in one typical experiment and normalized to the control (334.0 ± 8.9 cpm/μg DNA as 100%) measured in the absence of ET-18-OCH_3. The level of significance as compared to the control (Student's *t* test): *, $P < 0.05$; **, $P < 0.001$.

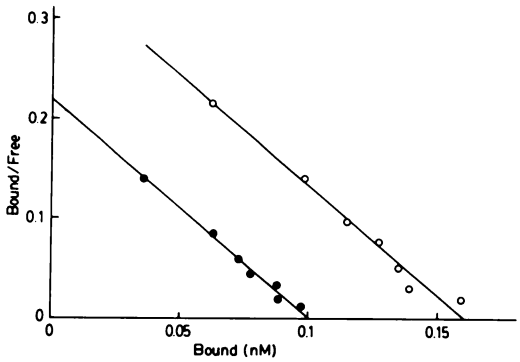

Fig. 3. Scatchard analysis of the specific uptake of [^{3}H]estradiol by MCF-7 incubated in the presence and absence of ET-18-OCH_3. The cells were pretreated for 12 h at 37°C with (●) or without (○) 10 μg/ml ET-18-OCH_3. The data shown are the mean of triplicate determinations.

Table 1 *Effects of ET-18-OCH_3 and its analogues on the uptake of [^{3}H]estradiol by MCF-7*

Drug (10 μg/ml)	[^{3}H]Estradiol uptake cpm/μg DNA	% of control
Control	349.2 ± 16.6[a]	100
LPC	365.0 ± 24.0	105
Lyso-PAF	339.6 ± 18.6	97
PAF	356.3 ± 20.8	102
ET-18-OCH_3	275.2 ± 25.8[b]	79

[a] Mean ± SD of triplicate determinations.
[b] $P < 0.05$ compared to the control value (Student's *t* test).

Table 2 *Amount of TGF-α secreted from MCF-7 into the conditioned medium and progesterone receptor content of MCF-7 cultured for 48 h with 10 μg/ml ET-18-OCH_3 and its analogues*

Drug	TGF-α ng/10^7 cells	TGF-α % of control	Progesterone receptor fmol/10^6 cells	Progesterone receptor % of control
Control	4.29 ± 0.07[a]	100	74.5 ± 4.3	100
LPC	4.55 ± 0.22	106	78.2 ± 7.6	105
Lyso-PAF	4.24 ± 0.39	99	74.8 ± 2.7	100
PAF	4.09 ± 0.53	95	70.2 ± 5.1	94
ET-18-OCH_3	2.77 ± 0.50[b]	65	55.8 ± 5.1[c]	75

[a] Mean ± SD of triplicate observations.
[b] $P < 0.01$ compared to the control value (Student's *t* test).
[c] $P < 0.05$ compared to the control value (Student's *t* test).

protein to 0.83 ± 0.15 ng/mg total protein (58% of control), the total protein being 3.0 mg/10^7 cells and 3.3 mg/10^7 cells for control and 10 μg/ml ET-18-OCH_3-treated MCF-7 cells, respectively. On the other hand, pretreatment for 48 h with 10 μg/ml of LPC, lyso-PAF, or PAF showed no significant effect on the secretion of TGF-α. Similarly, the PR content of MCF-7 was also significantly ($P < 0.05$) decreased by 48 h pretreatment with 10 μg/ml ET-18-OCH_3, while no such effect was noted after 48 h pretreatment with 10 μg/ml of LPC, lyso-PAF, or PAF.

DISCUSSION

We have reported previously that ET-18-OCH_3 inhibits the growth of hormone-dependent breast cancer cell lines, MCF-7 and ZR-75-1, by blocking EGF internalization and hence by reducing the EGF binding, while it does not affect both the growth of and the EGF binding to the hormone-independent breast cancer cell line, BT-20 (7, 8). In the present study, we further investigated the effect of ET-18-OCH_3 on the uptake of labeled estradiol, the secretion of TGF-α in conditioned medium, and the PR content in the hormone-dependent breast cancer cell line, MCF-7.

Since the ER is generally said to be located inside the cell, cytoplasm, and/or nucleus (14), the uptake of labeled estradiol measured here is assumed to represent the binding of labeled estradiol to ERs in the following discussion.

It was found in the present study that the uptake of labeled estradiol by MCF-7 was significantly ($P < 0.05$) reduced, when the cells were preincubated with more than 10 μg/ml ET-18-OCH_3 for 12 h or longer. In 12 h preincubation, 10 μg/ml ET-18-OCH_3 halved the maximum binding of labeled EGF to MCF-7 in the previous study (7) and significantly decreased the uptake of labeled E_2 by MCF-7 in the present study. Further, the growth of MCF-7 was little affected by preincubation with 10 μg/ml ET-18-OCH_3 for 12 h monitored by DNA content and [^{3}H]thymidine uptake (7). Therefore, we used here an incubation time of 12 h and an ET-18-OCH_3 concentration of 10 μg/ml.

Scatchard plots showed that ET-18-OCH_3 caused an apparent reduction in the maximum binding of labeled estradiol without affecting the binding affinity. It has been reported recently by Murphy *et al.* (15) that the ^{125}I-EGF binding is not induced by estradiol, and this provides strong evidence that estradiol is not involved in the steroidal regulation of the expression of EGF receptors in hormone-dependent breast cancer cell lines. On the other hand, Berthois *et al.* (16) have found that a short-term (24–48 h) treatment of MCF-7 with estradiol leads to a decrease in the number of EGF receptors. Moreover, Cormier *et al.* (17) have demonstrated that the treatment of MCF-7 with EGF can inhibit estradiol-stimulated PR synthesis, and EGF appears to down-regulate the ER by approximately 50% in MCF-7.

Based on these previous studies (15–17) of the relationship between the EGF receptor and estradiol and also on the data shown here in Figs. 1–3, we tentatively speculate at present that ET-18-OCH_3 might directly decrease the number of ERs, and this effect is specific to ET-18-OCH_3. How ET-18-OCH_3 decreases the number of ERs in MCF-7 is unclear at present and must be a subject for future studies.

TGF-α, which is induced by estradiol, may be one of important regulators in hormone-dependent breast cancer cell lines (1, 2). Moreover, PRs are said to be induced by the effect of estradiol on ERs (18). Therefore, both TGF-α and PR are regulated by the effect of estradiol on ERs. In our findings, the secretion of TGF-α from MCF-7 into the conditioned medium was inhibited and the PR content of MCF-7 was decreased by 48 h pretreatment with 10 μg/ml ET-18-OCH_3, while LPC, lyso-PAF, and PAF, all at 10 μg/ml, did not affect both the TGF-α secretion and the PR content. It is conceivable from these findings that the reduction in ER level induced by ET-18-OCH_3 resulted in decreases in the secretion of TGF-α from MCF-7 and the PR content of this cell line. It is likely that the suppression of TGF-α precedes the inhibition of cell growth induced by 10 μg/ml ET-18-OCH_3, because the growth of MCF-7 was little affected by pretreatment with 10 μg/ml ET-18-OCH_3 for 12 h (7) and also because that a significant reduction in ER occurred only after MCF-7 was treated with ET-18-OCH_3 for more than 12 h.

Recently, Roos *et al.* (19) have preliminarily demonstrated that TPA decreases not only the EGF binding but also the level of PR, but not that of ER, in hormone-dependent breast cancer cells. ET-18-OCH_3, on the other hand, reduced both the uptake of labeled estradiol and the PR content in MCF-7 as reported here and also decreased the EGF binding to MCF-7 and ZR-75-1 as reported previously (7). These findings indicate a difference in the mode of action between ET-18-OCH_3 and TPA.

According to the report of Ghosh-Dastidar *et al.* (20), PR is a high affinity substrate for phosphorylation by the EGF receptor kinase, and the level of PRs is said to be controlled by TPA through this phosphorylation. The down-regulation of the EGF receptor mediated by PKC may also change the state of phosphorylation of PRs, resulting in a final reduction in the receptor concentration (19). TPA is a PKC activator (21), while ET-18-OCH_3 is a PKC inhibitor (22). This functional difference may account for the difference in the mode of steroid receptor regulation between TPA and ET-18-OCH_3, although further investigation are required on this point.

From these results together with our previous findings (7, 8), we conclude that ET-18-OCH_3 reduces not only the EGF binding probably by inhibiting EGF internalization but also the secretion of TGF-α by reducing ER sites. These effects of ET-18-OCH_3 may lead to its antitumor action in hormone-dependent breast cancer cell lines. If this concept could be extended to hormone-dependent breast cancers in general, the modulation of EGF binding and the inhibition of TGF-α secretion by ET-18-OCH_3 may become useful probes for treatment of breast cancer.

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