Naloxone acts as an antagonist of estrogen receptor activity in MCF-7 cells

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Abstract
Estrogen promotes the growth of breast cancer via estrogen receptors (ER). Earlier studies showed that the opioid receptor antagonist naloxone inhibits MCF-7 breast cancer growth in mice. We examined the cellular and molecular mechanism of naloxone antagonism of ERα activity in human MCF-7 cells. Naloxone (100 nmol/L) inhibited 17β-estradiol (E2)–induced (10 nmol/L) MCF-7 cell proliferation by 65% and mitogen-activated protein kinase/extracellular signal-regulated kinase phosphorylation. Naloxone blocked the E2-induced activation of ERα, with 85% inhibition after 5 minutes and 100% recovery after 60 minutes. This assay is based on quantitation of E2-activated nuclear ERα binding to the immobilized coactivator peptide. A significant decrease in E2-induced ERα transactivation was observed in the presence of naloxone in the estrogen response element-luciferase reporter assay (P < 0.05, E2 versus E2 + naloxone). Naloxone also blocked E2-induced down-regulation of ERα mRNA at 30 minutes and 6 hours. Although naloxone inhibits ERα activity directly, it also induces a cross-talk between µ-opioid receptor (MOR) and ERα. Immunoprecipitates with anti-MOR antibody showed the presence of ERα in cells incubated with E2 in the presence of naloxone but not in cells incubated with E2 or naloxone alone. Higher amounts of ERα associated with MOR after 60 minutes compared with 10 minutes of incubation. Naloxone inhibited E2-bovine serum albumin-FITC binding to plasma membrane–associated ERα and also inhibited the direct binding of [3H]E2 to ERα. Thus, naloxone modulates ERα activity directly as well as indirectly via MOR. This study suggests that naloxone-like compounds can be developed as novel therapeutic molecules for breast cancer therapy. [Mol Cancer Ther 2006;5(3):611–20]

Introduction
Breast cancer is the most common malignancy among women in the United States. Steroid hormone estrogen acts as a mitogenic factor and promotes the growth and development of breast cancer (1). The biological activity of estrogen is primarily mediated through estrogen receptor (ER), which belongs to the superfamily of steroid transcription factors. ERα is the major ER in neoplastic breast epithelium, whereas ERβ is the predominant ER in normal breast tissue (2, 3). Two thirds of breast tumors express ERα. In women with metastatic disease, antiestrogen therapy induces an objective response ~60% of the time; this leaves a significant number of women with ERα-expressing tumors that do not respond to hormone therapy (4). Moreover, of those who respond initially, some develop resistance eventually (5). Multiple molecular mechanisms of this process have been described, but the clinical relevance of these mechanisms has not been established (6).

ER directly binds to estrogen response element (ERE) and induces gene expression (7). However, estrogen can exert nongenomic effects on cell biology by interacting with other factors, including growth factors, cell signaling molecules, and cell surface receptors (6, 8, 9). Translocation and activation of classic ER to the plasma membrane or to the cytoplasm is facilitated by growth factor receptors, including insulin growth factor receptor-1 (IGF-1R) and/or epidermal growth factor receptor (EGFR; refs. 6, 10, 11). Phosphorylation of ERα by EGFR and other growth factor receptor pathways plays a mechanistic role in tamoxifen resistance (12). ER can be transactivated by mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), which are downstream components of the ER signaling pathway (13). It is noteworthy that µ-opioid receptor (MOR), a Gαi/Gqi protein-coupled receptor, transactivates EGFR and MAPK/ERK phosphorylation (14, 15). Therefore, MOR can activate ER activity via transactivation of EGFR.

Membrane ERα, as well as chronic activation of opioid receptors, stimulates adenylyl cyclase activity as well as rapid changes in intracellular calcium levels/flux, potassium conductance, and cyclic AMP levels (9, 16). Activation of ER or MOR stimulates cell proliferation, growth, and MAPK/ERK phosphorylation (17, 18). We observed that the MOR agonist morphine promotes angiogenesis-dependent tumor growth in 17β-estradiol
(E2)-dependent human MCF-7 cell tumor xenografts in mice at medically relevant concentrations (17). In contrast, naloxone (an opioid receptor antagonist) inhibits the progression of breast tumor growth (17, 19). These studies suggest that morphine promotes tumor growth by stimulating angiogenesis, but naloxone acts directly on MCF-7 tumor cells and inhibits tumor growth. The mechanism by which naloxone inhibits tumor growth remains unknown.

Structurally, naloxone shares similarities with ERα agonists and antagonists (Fig. 1). The hydroxyl group bearing aromatic ring is present in all ERα ligands as well as naloxone, which facilitates the binding of ligands to ERα. Therefore, naloxone may bind to ERα and modulate its activity directly. At the same time, it may modulate the activity of plasma membrane ERα via MOR. Therapeutic strategies to treat ER-dependent breast cancers are targeted to growth factor receptors, whereas transactivation of growth factor receptor tyrosine kinases by G protein-coupled receptors may continue to activate mitogenic signaling (20). Molecules, like naloxone, which antagonize both growth factor receptor (ERα in this case) and G protein-coupled receptors, provide a new class of compounds for cancer therapy. Therefore, we examined the mechanism of MOR-dependent and MOR-independent antagonism of ERα activity by naloxone in MCF-7 human breast cancer cells. In physiologically relevant concentration, naloxone inhibits E2-induced proliferation, signaling, and molecular activity of ER in estrogen-dependent human MCF-7 breast cancer cells. Blocking the binding of estrogens to ERα using antiestrogenic compounds is one of the critical strategies to impede the growth of breast cancer. Therefore, this study provides an alternative view about an efficacious therapeutic agent to treat breast cancer.

Material and Methods
Chemicals, cell culture reagents, and secondary antibodies were from Sigma Aldrich (St. Louis, MO), Invitrogen Life Technologies (Carlsbad, CA), and Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), respectively, or as indicated.

Cell Culture
MCF-7 cells between p5 and p15 available in the Yee and Gupta laboratories and from American Type Culture Collection (Manassas, VA) were used as described by us earlier (17, 21). Cells were routinely maintained in complete medium containing IMEM supplemented with 10% fetal bovine serum, 0.75 IU/mL human insulin (Eli Lilly & Co., Indianapolis, IN), 50 IU/mL penicillin, and 50 μg/mL streptomycin at 37°C and 5% CO2. Medium was changed twice weekly. Serum-free medium [consisting of phenol-free IMEM with 2 mmol/L l-glutamine, 10 mmol/L HEPES (pH 7.4), 1 μg/mL transferrin, 1 μg/mL fibronectin (BD Biosciences, Palo Alto, CA), trace elements (Biofluids, Rockville, MD), antibiotics; ref. 21] was used for several experiments as indicated.

Tumor Model
Six-week-old female nude mice (National Cancer Institute, Frederick, MD) were implanted with 0.125 mg slow-release E2 pellet (Innovative Research of America, Sarasota, FL). MCF-7 cells (5 × 10⁶) were injected into the mammary fat pad of mice and tumors were dissected out after 36 days as described (17).

Immunofluorescence Staining of the Cells and Tumor Sections
Human dermal microvascular endothelial cells, mouse breast tumor cells (SCK), MCF-7 human breast cancer cells, and MCF10A nontumorigenic cell line (derived from spontaneously immortalized cells of a fibrocystic disease specimen) were grown on chamber slides (Nalge Nunc International, Naperville, IL) for 48 hours in complete medium. Cells were then fixed with 2% paraformaldehyde.
for 10 minutes at room temperature. Tumor cryosections were acetone fixed as described (17). Slides were washed with PBS and blocked with 3% bovine serum albumin (BSA) and incubated with anti-MOR antibody (1:200 dilution, 1 hour at room temperature), which recognizes MOR of mouse as well as human origin (Santa Cruz Biotechnology, Santa Cruz, CA, or Chemicon International, Temecula, CA). This was followed by incubation with rhodamine or FITC-conjugated secondary antibody (1:100 dilution, 30 minutes at room temperature) and nuclear stain 4',6-diamidino-2-phenylindole (15 minutes at 37°C; Molecular Probes, Eugene, OR). Tumor sections were costained with phycoerythrin-conjugated anti-CD31 antibody (PharMingen, San Diego, CA). In parallel, negative controls were incubated with an isotype-matched irrelevant IgG and rhodamine or FITC-conjugated secondary antibody.

Reverse Transcription-PCR

MCF-7 cells grown for 48 hours in complete culture medium were serum depleted for 24 hours followed by incubation with 10 nmol/L E2 and/or 100 nmol/L naloxone for the indicated time period. Cells were harvested and total RNA was isolated with TRIzol reagent. Total RNA (5 μg) was reverse transcribed using the first-strand synthesis system (Invitrogen Life Technologies). PCRs were done by using Taq DNA polymerase (Cepheid, Sunnyvale, CA), or hactinase for 45 minutes at room temperature. Proteins were washed off and the peptide-bound receptor was quantitated using [3H]thymidine incorporation assay. In parallel, cells were also enumerated using a hemocytometer and cell viability was confirmed using trypan blue.

Immunoblotting

Proteins from whole-cell lysates resolved on SDS-PAGE (3–15% gradient gels to detect MAPK/ERK and 7.5% for immunoprecipitation experiments) were transferred to the polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA). Membranes were blocked for 1 hour at room temperature in 5% nonfat dry milk in TBS with 0.1% Tween 20 and incubated with anti-phospho-p42/44 MAPK/ERK (Thr202 and Tyr204) or total MAPK/ERK (both at 1:500 dilution; Cell Signaling, Beverly, MA), anti-ERα (1:1,000 dilution; Upstate Biotechnology, Lake Placid, NY), anti-MOR (1:1,000 dilution; Biosource, Camarillo, CA), or β-actin (1:1,000 dilution; Santa Cruz Biotechnology) at 4°C overnight. Proteins were visualized using secondary antibodies (1:10,000 dilution for rabbit and 1:1,000 dilution for goat) conjugated to alkaline phosphatase for 45 minutes at room temperature. Proteins were visualized with the enhanced chemiluminescence Western blotting system (Amersham Life Sciences, Buckinghamshire, United Kingdom) Chemiluminescent signals were acquired using Storm 860 Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Densitometric analysis of protein bands was done using Molecular Analyst software (Molecular Dynamics; ref. 17).

Nuclear ER Activity Assay

Cells were lysed by resuspending in hypotonic buffer [20 mmol/L HEPES (pH 7.5) containing 5 mmol/L NaF, 0.1 mmol/L EDTA, protease inhibitor cocktail (Roche Diagnostics)]. After 15 minutes on ice, 0.5% Nonidet P-40 was added to the cells followed by centrifugation at 2,500 rpm for 5 minutes at 4°C. The nuclear pellet was incubated in complete lysis buffer [20 mmol/L HEPES (pH 7.5), 400 mmol/L NaCl, 10 mmol/L NaF, 0.1 mmol/L EDTA, 1 mmol/L sodium vanadate, 20% glycerol, protease inhibitor cocktail] for 30 minutes at 4°C and centrifuged at 14,000 rpm for 10 minutes to obtain nuclear extracts containing ER. Protein concentration was determined using Bradford reagent (Bio-Rad, Hercules, CA). We used a nuclear receptor ERα ELISA assay kit capable of capturing and quantitating the activated form of the receptor, which recognizes the LXXL coactivator signature motif (Active Motif, Carlsbad, CA). Briefly, nuclear extract (30 μg protein) was incubated with 10 nmol/L E2 and/or 100 nmol/L naloxone for the indicated time period. Stimulated extracts were then added to the wells precoated with ER coactivator binding motif peptide LXXLL. After 1-hour incubation at room temperature, the unbound receptor was washed off and the peptide-bound receptor was quantitated using α-ERα antibody-based colorimetric ELISA at 450 nm.

Immunoprecipitation

MCF-7 cells were grown in complete medium for 24 hours and then serum depleted overnight before the addition of 10 nmol/L E2 and/or 100 nmol/L naloxone...
for the indicated time period. Cells were lysed and harvested in ice-cold lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 10% glycerol, 150 mmol/L NaCl, 1% Nonidet P-40, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, protease inhibitors]. After centrifugation, supernatants containing 500 µg protein were precleared with 25 µL protein A beads for 30 minutes at 4°C. The precleared supernatants were incubated with 4 µL polyclonal α-MOR antibody (Chemicon) or with an isotype-matched control of the same class for 2 hours at 4°C followed by addition of protein A beads for an additional incubation for 2 hours at 4°C and extensive washing with the lysis buffer. Immunoprecipitates as well as whole-cell lysates were resolved on 7.5% SDS-PAGE and immunoblotted using anti-ERα or anti-MOR antibody, respectively.

**Binding of E2-BSA-FITC**

MCF-7 cells were serum and growth factor starved, as described above, for 24 hours. Cells were washed with ice-cold PBS and fixed with 2% paraformaldehyde for 5 minutes at room temperature. Cells were then incubated with 10 nmol/L E2-BSA-FITC for 10 minutes with or without preincubation with 100 nmol/L naloxone for 5 minutes. To study the binding of ligands in permeabilized conditions, cells were first fixed and then permeabilized with ice-cold 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice and washed thrice with PBS. Nuclear colocalization was done with 4,6-diamidino-2-phenylindole (DAPI). Parallel controls were done using BSA-FITC.

**Luciferase Induction Assay**

MVLN cells (which are MCF-7 cells stably transfected with ERE-luciferase reporter plasmid; ref. 22) were serum and growth factor depleted as described above and stimulated with 10 nmol/L E2 and/or 100 nmol/L naloxone for 24 hours. Cells were then lysed and luciferase activity was measured using a luciferase reporter assay kit (Promega, Madison, WI; ref. 6). Induced light was measured with a Berthold luminometer.

**Binding of [3H]E2 to ER In vitro**

COS-1 cells (American Type Culture Collection) were maintained at 37°C with 5% CO2 in medium containing DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin. For the expression of ER, cells were transiently transfected with plasmid pCMV6-XL4-expressing human ERα-full length (Origene, Rockville, MD) using the calcium phosphate precipitation method (23) by growing the cells in Opti-MEM (Invitrogen Life Technologies) without serum. Cells were harvested 48 hours after transfection and cell lysates were prepared and processed for immunoprecipitation with anti-ERα antibody as described earlier. Protein A–associated ERα was incubated for 10 minutes with [3H]E2 (10 nmol/L E2, 1 mCi/mL, Perkin-Elmer, Boston MA) with or without preincubation with 100 nmol/L and 500 nmol/L naloxone for 5 minutes. Protein A agarose beads were washed with coimmunoprecipitation buffer thrice to remove unbound ligands. ERα-associated [3H]E2 was quantitated using a liquid scintillation counter (Beckman, Fullerton, CA).

**Statistical Analysis**

All data are expressed as mean ± SD. For the proliferation assay, a multivariate ANOVA was conducted for comparisons between different treatment groups. Dunnett’s method was used to determine whether any treatment differed from control. A factorial two-way ANOVA model was used to analyze the nuclear ER activity. Pairwise comparisons within time strata were done using Tukey honestly significantly different tests. The significance level of the Tukey honestly significantly different tests was set at 1.25% using a Bonferroni correction to adjust for the four time strata, thus maintaining the probability of any false positive at <5%. A factorial three-way ANOVA model was used to analyze the Luciferase assay data. Pairwise comparison of combinations of factors were done using Tukey honestly significantly different tests to control the probability of any false positive at 5%.

**Results**

**Expression of MOR in MCF-7 Cells In vitro and In vivo**

Direct ligand-binding studies suggest the presence of both low and high affinity MOR on MCF-7 cells (24–26). We confirmed the physical presence of MOR on MCF-7 cells. We observed mRNA expression in the expected 480-bp region of MOR in MCF-7 cells by reverse transcription-PCR (Fig. 2A). Sequence analysis of the amplified fragment showed 99% homology to the known human MOR sequence (between 907 and 1,385 bp, Genbank accession no. NM_000914). Expression of MOR protein was also observed using Western immunoblotting. However, the immunofluorescent staining of MCF-7 cells (using two different antibodies from Chemicon and Santa Cruz Biotechnology) as well as mouse breast cancer cells (SCK) did not show the presence of MOR (Fig. 2B). In contrast, human dermal microvascular endothelial cells and human nontumorigenic cells showed the presence of MOR as red fluorescent staining in all the cells. Sections of human MCF-7 tumor xenographs in nude mice showed the presence of MOR in green but only on the endothelium, stained with red using a specific endothelial cell marker α-Cd31 phycoerythrin (Fig. 2C). Merged image on the right shows a yellow staining for endothelium-associated MOR, whereas the remaining blue nuclei of tumor cells do not show any expression of MOR. It seems that MOR is expressed weakly on these cells and immunofluorescent staining may not be sensitive enough to detect it. To the best of our knowledge, there are no existing data on the presence of MOR using immunofluorescence microscopy on MCF-7 cells in culture or tumors.

**Naloxone Inhibits E2-Induced MCF-7 Cell Proliferation In vitro**

In our previous study with E2-dependent MCF-7 breast tumor xenografts in mice, we observed the inhibition of tumor growth by clinically relevant dose (0.36 mg/kg for first 2 weeks followed by 0.7 mg/kg subsequently) of naloxone and promotion of tumor growth by 0.7 mg/kg (for 2 weeks followed by 1.43 mg/kg) morphine (17).
These doses are equivalent to 50 and 100 mg morphine/70 kg patient daily used clinically for analgesia, respectively. We also observed that doses >1 mmol/L morphine were cytotoxic to endothelial cells in vitro. Because the plasma concentration of morphine in multiple studies of several hundred patients is between 2 nmol/L and 3.5 mmol/L (27, 28), results of studies using much higher dose/concentration of morphine may be reflective of cytotoxicity and may be physiologically irrelevant. Tegeder et al. showed decreased MCF-7 tumor growth with 10 to 30 mg/kg morphine or naloxone with increasing time up to 22 days and inhibition of MCF-7 cell proliferation with 250 nmol/L morphine or naloxone (19). Using physiologically relevant concentration of 100 nmol/L naloxone, we observed a 40% decrease (P < 0.05 versus unstimulated) in MCF-7 cell proliferation (Fig. 3A). E2 induced a 2.5-fold increase (P < 0.05 versus unstimulated) in proliferation, which was inhibited significantly by naloxone (by 65%; P < 0.02 versus E2). Morphine at 1 µmol/L did not inhibit the basal E2-induced cell proliferation significantly (P > 0.05 morphine versus unstimulated or E2). These data suggest that naloxone may antagonize estrogen activity in MCF-7 cells.

**Naloxone Inhibits E2-Induced MAPK/ERK Phosphorylation**

E2 stimulates cell proliferation via MAPK/ERK signaling pathway (29). We examined the effect of naloxone on E2-induced MAPK/ERK phosphorylation at 1, 5, and 10 minutes of incubation. E2-induced phosphorylation of MAPK at 1, 5, and 10 minutes (Fig. 3B, top, lanes 3, 5, and 7) compared with the unstimulated condition (lane 2). Naloxone inhibited E2-induced MAPK phosphorylation at all three time points (lanes 4, 6, and 8). Naloxone by itself weekly stimulated MAPK phosphorylation at 1, 5, and 10 minutes (lanes 9–11). These results suggest that the inhibitory effect of naloxone on E2-induced MCF-7 cell proliferation may be mediated by inhibition of MAPK/ERK signaling pathway. Total MAPK protein (Fig. 3B, bottom) was similar in all lanes irrespective of treatment, which shows that only the phosphorylation is modulated and also confers to equal protein loading per lane. These data further support our observations above that naloxone antagonizes E2-induced proliferation.

**Figure 2.** Expression of MOR in MCF-7 cells. A, reverse transcription-PCR for MOR (480 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 460 bp) expression. B, immunofluorescent staining using anti-MOR antibody (red) and nuclear stain 4', 6-diamidino-2-phenylindole (blue). Human dermal microvascular endothelial cells and human nontumorigenic cells show the presence of MOR (red), whereas both mouse SCK and human MCF-7 tumor cells do not. Original magnification, ×600. C, immunofluorescent staining for MOR and endothelial specific marker CD31 in acetone-fixed sections of human MCF-7 tumor xenografts in mice. MOR (green, middle) colocalizes with endothelial-specific α-CD31 staining (red, left). Large blue nuclei in the merged image (right) show the presence of tumor cells in the field without expression of MOR. Original magnification, ×600. Representative of three separate and reproducible experiments.

**Figure 3.** Naloxone inhibits E2-induced MCF-7 cell proliferation and MAPK/ERK phosphorylation. A, effect of 10 nmol/L E2 (dotted), 100 nmol/L naloxone (Nal; solid), or both (checkered), 1 µmol/L morphine (MS; horizontal bars), or morphine + E2 (vertical bars) cell proliferation in MCF-7 cells after 48 h of incubation in serum-depleted conditions. Data were normalized from three different experiments and each experiment was done in triplicate. Columns, mean; bars, SE. *P < 0.05 versus unstimulated; †, P < 0.02 versus E2. B, cells were serum depleted for 24 h and incubated with E2 and/or naloxone for different time periods as indicated. MAPK/ERK phosphorylation (top) was detected in whole-cell lysates by Western immunoblotting. Bottom, total MAPK, which remained unaltered. Lane 1, loaded with color markers. Numbers below the bands show densitometric units. Representative of three different and reproducible experiments.
Naloxone Down-Regulates the Nuclear ER Activity

Because the binding to the coactivator plays a critical role in subsequent ER-induced transcription, we used a highly sensitive assay that detects ER bound to the coactivator. The ERe activity in the nuclear extracts of MCF-7 cells stimulated by E2 for 5 minutes was reduced to 85 ± 0.018% when the nuclear extracts were preincubated with naloxone ($P < 0.05$ versus E2), suggesting that naloxone antagonized the E2-induced activation of the receptor. This antagonistic activity of naloxone seems to be time dependent, because longer E2 incubation (60 minutes) resulted in 100% recovery of the ERe activity (Fig. 4A).

To further corroborate the above antagonism of naloxone, we used MVLN cells (MCF-7 cells expressing the firefly luciferase gene Luc, inserted in front of the herpes simplex virus promoter of thymidine kinase). Binding of ER to E2 leads to its association as a dimer to ERE site and regulates transcription of luciferase gene driven by thymidine kinase promoter (22). Incubation with 10 nmol/L E2 for 1, 6, and 24 hours was accompanied by a gradual increase in ER transactivation function compared with unstimulated cells in the ERE-luciferase reporter system (Fig. 4B). After 24 hours of E2 stimulation, a 1.9-fold increase ($P < 0.02$ versus E2 at 1 hour) in luciferase activity was observed compared with 1-hour stimulation. These data suggest the binding of E2 to ERe leading to the recruitment of a coactivator and “turning on” of the transcription resulting in increased luciferase activity (Fig. 4B). However, preincubation of cells with 100 nmol/L naloxone for 10 minutes before stimulation with E2 decreased the ERe-mediated transactivation to 73% after 24 hours compared with E2 alone ($P < 0.02$). Inhibition by naloxone alone versus unstimulated was not significant. This transcriptional repression of E2-induced ERe activity by naloxone suggests the possibility of a conformational change in the receptor that may either facilitate the recruitment of the corepressor and/or inhibition of coactivator recruitment leading to impaired transcription.

E2 exerts a feedback regulation of ERe gene by down-regulating ERe after activation (30). Consistent with the data above, naloxone antagonized this negative regulation of ERe gene expression by E2 (Fig. 4C). Reverse transcription-PCR analysis showed the down-regulation of ERe mRNA after 30 minutes and 6 hours of incubation with E2 (Fig. 4C, lanes 1 and 4, respectively) compared with unstimulated (lane 7). This E2-induced down-regulation was significantly inhibited by preincubation with 100 nmol/L naloxone at both 30 minutes and 6 hours (lanes 3 and 6). Naloxone by itself did not change the basal ERe mRNA expression (lanes 2 and 5). Expression of glyceraldehyde-3-phosphate dehydrogenase remained unaltered. DNA fragments of ERe and glyceraldehyde-3-phosphate dehydrogenase amplified by PCR were confirmed by sequencing and found homologous to human sequences for ERe and glyceraldehyde-3-phosphate dehydrogenase [Genbank accession nos. X03635 (1,649–1,962 bp) and M33197 (261–718 bp), respectively] on sequence analysis.

Figure 4. Effect of naloxone on E2-induced ER activity. A, ERe activity was assayed in MCF-7 cell nuclear extracts incubated with 10 nmol/L E2 in the presence or absence of 100 nmol/L naloxone for the indicated time period. Stimulated extracts were then added to wells coated with receptor-coactivator binding motif (LXXLL) peptide. Peptide-bound (active) form of the receptor was quantitated colorimetrically using anti-ERs antibody. Points, mean ($n = 3$); bars, SE. Receptor activity in presence of E2 ($\bullet$), naloxone (■), preincubated with naloxone before E2 stimulation ($\ast$), and unstimulated receptor (●) is plotted as a function of time. *, $P < 0.05$ E2 + naloxone versus E2. B, effect of vehicle (hollow), 10 nmol/L E2 (dotted), 100 nmol/L naloxone (solid), or both (checkered) on ERE-luciferase activity. MVLN cells were incubated with E2 in the presence and absence of naloxone and luciferase activity was measured after the indicated time period using a luminometer. Columns, mean ($n = 4$); bars, SE. *, $P < 0.02$ versus E2 at 1 h; #, $P < 0.02$ versus E2 at 24 h. C, effect of naloxone on E2-induced ERe gene expression. Serum-starved (overnight) cells were stimulated with vehicle (lane 7), E2 alone for 0.5 and 6 h (lanes 1 and 4, respectively), naloxone (lanes 2 and 5), or E2 after preincubation with naloxone (lanes 3 and 6). Reverse transcription-PCR was done on total RNA using ERe-specific primers. E2 down-regulated ERe expression at 30 min and 6 h (lanes 1 and 4). Densitometric values are shown below the bands. Representative of three separate and reproducible experiments.
ER and MOR Cross-talk

MOR has been suggested to modulate insulin receptor-1 (IRS-1) and EGFR-induced MAPK/ERK phosphorylation by a cross-talk with these receptors (14, 15, 31). Thus, it is likely that naloxone-bound MOR cross-reacts with activated ERα. E2 rapidly stimulates IGF-1R phosphorylation and induces formation of a ternary protein complex between Shc, ERα, and IGF-1R, resulting in translocation of the ERα to the plasma membrane and facilitates ERα-mediated rapid E2 action. Adaptor protein Shc plays a crucial role in the formation of this complex, which is activated by IRS-1 as well as IGF-1R. Morphine via MOR desensitizes IRS-1 signaling to Akt and ERK cascades (31). It does so by disrupting the complex formation between IRS-1, Shc, and Grb2 via a cross-talk between the downstream signaling pathways of MOR and IRS-1. Because naloxone significantly inhibits E2-induced MCF-7 cell proliferation and MAPK/ERK (19), we reasoned that naloxone-induced MOR antagonism may modulate ERα translocation via Shc. We immunoprecipitated MOR-associated protein complexes from MCF-7 cells stimulated with E2 in the presence or absence of naloxone for 10 and 60 minutes. Western blot analysis using anti-ERα antibody (Fig. 5A, row I) showed that ERα associates with MOR only when cells were preincubated with naloxone before stimulation with E2 for both 10 and 60 minutes (Fig. 5A, row I, lanes 3 and 6, respectively). Appreciably higher amount of ERα seems to be associated with MOR after 60 minutes compared with 10 minutes of incubation (Fig. 5A, row I, lane 6 versus lane 3). No ERα association with MOR could be seen in immunoprecipitated proteins from cells treated with E2 alone (Fig. 5A, row I, lanes 1 and 4) or naloxone alone (Fig. 5A, lanes 2 and 5) or in unstimulated cells (Fig. 5A, row I, lane 7). Under similar conditions, no ERα precipitation was observed with an isotype-matched control (Fig. 5A, row IV). Thus, naloxone induces sustained association of MOR with E2-stimulated ERα. This also supports our observations above that naloxone impairs the E2-induced negative feedback gene regulation of ERα. Expression of ERα (Fig. 5A, row II) and MOR (Fig. 5A, row III) in whole-cell lysates were not altered.

Association of MOR and ERα may disrupt the formation of the ternary complex between IGF-1R, Shc, and ER, blocking the translocation of E2-induced ERα to the plasma membrane (11). We examined the binding of FITC-labeled E2-BSA to the intact and permeabilized MCF-7 cells in the presence and absence of naloxone. We observed that E2-BSA-FITC binds to the cell membrane receptor after 10 minutes of incubation with E2 (top) in both nonpermeabilized and permeabilized cells (Fig. 5B). Preincubation of cells with 100 nmol/L naloxone for 5 minutes inhibited the binding of E2-BSA-FITC to surface ERα under both conditions. Counterstaining of cells with the nuclear stain 4′,6-diamidino-2-phenylindole (blue) is suggestive of the presence of cells in the field. Previous reports have shown that plasma membrane–impermeable E2-BSA-FITC exclusively localized on the cell surface initially within 5 seconds followed by the emergence of fluorescence inside the cells, suggesting the binding of E2-BSA-FITC to the cell surface receptors followed by sequestration (9, 32). Plasma membrane-impermeable E2-BSA was shown to bind to surface ERα followed by stimulation of the ERE-luciferase activity in MVLN cells (22). It is likely that naloxone inhibits the binding of E2-BSA-FITC followed by the inhibition of the E2 induced luciferase activity observed in Fig. 4B.

Naloxone Inhibits the Binding of [3H]E2 to ERα

Data above suggest that naloxone possibly inhibits E2 for binding to ERα. Therefore, we next confirmed the direct inhibition of [3H]E2 binding to ERα by naloxone. ERα was expressed in COS-1 cells and protein expression was confirmed by Western blotting (Fig. 5C).

Preincubation with naloxone at 100 and 500 nmol/L concentration inhibited the binding of [3H]E2 to ERα by 66% (P < 0.05 versus E2) and 46% (P < 0.05 versus E2), respectively (columns 2 and 3). Lysates from untransfected COS-1 cells showed a low basal binding of [3H]E2 to the endogenous ERα seen as weak band on Western immunoblotting (Fig. 5C and D).

Discussion

The presence of opioid receptors has been suggested on human cancer cells, including MCF-7 cells for more than two decades based on opioid peptide immunoreactivity or binding of ligands (24–26, 32–34), but the physical presence has not been shown. In addition, it was speculated that opioid ligands could be binding to non-opioid receptor-binding sites. We show the physical presence of MOR on MCF-7 cells obtained from three different sources. Our data corroborate the binding studies by showing the presence of mRNA and MOR protein on MCF-7 cells. However, we could not detect the presence of MOR by immunofluorescent staining on human MCF-7 cells or mouse SCK tumor cells in vitro or MCF-7 cells in vivo in tumor sections, which could be due to a conformational change in the opioid receptor presentation on the intact tumor cells in vitro and in vivo. To the best of our knowledge, there are no studies showing the expression of MOR on MCF-7 cells in vitro or in vivo by immunofluorescent staining. Regardless of the physical presence of MOR on MCF-7 cells, naloxone (100 nmol/L) inhibits MCF-7 cell proliferation in the presence and absence of E2. This is consistent with earlier studies showing the inhibition of MCF-7 cell proliferation by naloxone (17, 19). Conversely, the proliferative or anti-proliferative effects of opioids on cancer cells/tumor growth were not antagonized by naloxone (17, 19, 35, 36). Unconventional and partially opioid receptor-mediated activity has been suggested for MOR agonists in cancer cells (18, 35, 36). Our results on antagonism of ER activity by naloxone suggest that this is the case.

Growth and survival promoting signaling induced by opioids has recently been reviewed (18, 37). At physiologically relevant concentration, opioid agonists have been shown to activate MAPK/ERK, Akt, and p53 signaling,
resulting in proliferation and survival of endothelial, neuronal, and Chinese hamster ovary cells transfected with opioid receptors (17, 38–40). Conversely, the growth inhibitory effects of opioid receptor agonist/morphine have been shown for MCF-7 cells and MCF-7 xenografts (17, 24). In general, most of the studies showing anti-proliferative or apoptotic effect have used extremely high concentration of opioids, which are physiologically irrelevant and even toxic (19, 41). We observed earlier that morphine induced proliferation at physiologically relevant concentration (1–100 µmol/L) but was cytototoxic at ≥1 mmol/L (17). Plasma concentration of opioids in patients receiving low to extremely high doses of opioid analgesics are between 2 nmol/L and 3.5 µmol/L (17). There seems to be a lack of attention toward the dose used and more attention toward the effects seen in experimental studies. For example, a more recent study (19) shows that 30 mg/kg morphine (plasma concentration of 60 µmol/L after 10–15 minutes of i.p. administration) inhibits MCF-7 tumor growth significantly up to 20 days (last day of observation). In the same figure, naloxone inhibits tumor growth significantly versus vehicle treated after 20 days (last day of measurement), which has been neglected and not been discussed in the text. On the other hand, naloxone has been shown to clearly inhibit dimethylbenzanthracene-induced mammary tumors in rats and cause complete regression of mammary tumor growth in mice (35, 42). Therefore, the results of our study rationalize the inhibitory effects of physiologically relevant doses of naloxone on tumor growth observed by several investigators using physiologically relevant doses.

Our data suggest that naloxone plausibly antagonizes E2-induced MAPK/ERK phosphorylation. Indeed, a cross-talk between ERα and EGFR/IGF-1R has been strongly linked to the synergistic induction of cancer cell proliferation via activation of MAPK/ERK (6, 11, 42). Because MOR can transactivate EGFR, which in turn can modulate MAPK/ERK phosphorylation (14), naloxone being a MOR antagonist probably has an inactivating effect. Indeed, the immunoprecipitation of ERα with MOR in the presence of both E2 and naloxone confirms the cross-talk between the two receptors. This is further confirmed by the reduced binding of E2-BSA to ERα on the plasma membrane in the

Figure 5. A, naloxone induces association of MOR with E2-induced ER. Lysates of MCF-7 cells incubated with vehicle or 10 nmol/L E2 in the presence and absence of 100 nmol/L naloxone were either analyzed by Western immunoblotting for the presence of ERα (row II) and MOR (row III) or immunoprecipitated with anti-MOR antibody (row I) or nonspecific IgG (row IV). MOR-associated ERα was detected by Western immunoblotting in the immunoprecipitates. Top, bands for ERα (67 kDa) above the IgG bands. Densitometric values are shown above the bands. Representative of three separate and reproducible experiments.

B, naloxone competes with E2-BSA-FITC for binding to plasma membrane ERα in MCF-7 cells: serum-depleted cells (nonpermeabilized or permeabilized) were incubated with E2-BSA-FITC in the presence or absence of 100 nmol/L naloxone. Nuclei were costained with 4′,6-diamidino-2-phenylindole. Green staining for E2-BSA-FITC is seen on the cell membrane but not in the nuclei of the cells, which is inhibited in the presence of naloxone. Green staining for E2-BSA-FITC is seen on the cell membrane but not in the nuclei of the cells, which is inhibited in the presence of naloxone. Green staining for E2-BSA-FITC is seen on the cell membrane but not in the nuclei of the cells, which is inhibited in the presence of naloxone. Green staining for E2-BSA-FITC is seen on the cell membrane but not in the nuclei of the cells, which is inhibited in the presence of naloxone. Green staining for E2-BSA-FITC is seen on the cell membrane but not in the nuclei of the cells, which is inhibited in the presence of naloxone.

C, transient transfection of ERα in COS-1 cells: COS-1 cells were transiently transfected with pCMV6-XL4 expression vector for human ERα. Western immunoblotting of cell lysates of transfected and untransfected resolved on 3% to 15% SDS-PAGE and probed with anti-ERα antibody. D, naloxone inhibits the [3H]E2 binding to ERα in transfected and untransfected COS-1 lysates with ERα and immunoprecipitated with anti-ERα. Immunoprecipitates from transfected (filled) and untransfected (hollow) cells were incubated with [3H]E2 alone for 10 min or after preincubation with 100 and 500 nmol/L naloxone for 5 min. Beads were washed to remove unbound radioactive counts were measured in scintillation counter. Representative of three separate and reproducible experiments. *, P < 0.05 versus E2 bound to transfected ERα.
presence of naloxone. It is also suggestive of the decreased translocation of ER to the membrane/cytoplasm, perhaps another effect of naloxone on regulation of ERα activity. It is becoming increasingly well known that transmembrane receptors are an integral component of the growth response to E2. Pretreatment of IC-21 macrophages with pertussis toxin results in a decrease of E2-BSA-FITC binding (9). Because opioid receptors act via a pertussis toxin–dependent Gt/Gt protein-coupled receptors, it is likely that opioid receptors mediate the translocation of ERα to the membrane. Recent studies suggest that ERα/Shc/IGF-1R complex is required to activate MAPK/ERK and also for the translocation of ER to the cytoplasmic membrane (11). This is particularly important because morphine via MOR caused serine phosphorylation of the IRS-1 and impaired the formation of the signaling complex among the IRS-1, Shc, and Grb2 (31). We speculate that the association of MOR with ERα disrupts the translocation of ERα to the plasma membrane and impairs ERα/Shc/IGF-1R assembly resulting in the blockade of MAPK/ERK phosphorylation. MOR-induced modulation of MAPK/ERK activation through EGFR (14) and IRS-1 via Shc (31) could be a stimulatory factor in the translocation of ERα. Therefore, inhibition of E2-induced MAPK/ERK may be involved in the naloxone-induced blockade of E2 binding to transmembrane ER.

It seems that due to the presence of the phenolic hydroxyl-bearing aromatic ring, naloxone is capable of binding to the ERs. The N-allylic substitution on naloxone occupies the same region as D ring, which may be responsible for its antagonistic activity (43, 44). Indeed, antagonistic activity of naloxone is corroborated by our observations that naloxone inhibits E2-induced activation of nuclear ERα directly in the nuclear fraction of the cells devoid of any membrane receptors. Because this assay is based on the binding of activated ERs to the coactivator, it is likely that binding of naloxone to ERα inhibits its binding to the coactivator and impairs the receptor activity. This thesis is further supported by the inhibition of E2-mediated transactivation of the ERE-luciferase reporter system and inhibition of direct [3H]E2 binding to immortalized ERα by naloxone.

Our observations on the cross-talk between ERs and opioid antagonist naloxone are consistent with earlier studies suggesting an interaction between estrogen, ER(s), and opioids/opioid receptors (45–47). Estrogen-induced translocation of MOR from plasma membrane to the intracellular compartment in cell groups of limbic system and hypothalamus was blocked by naltrexone an opioid receptor antagonist (45). More recent studies using ERα knockout mice confirmed the involvement of ERα in the E2-induced internalization of MOR (46). Furthermore, expression of ERβ gene is regulated by morphine in human arterial tissue (47). Our data further strengthen the relationship between ER and MOR.

Naloxone therefore acts via MOR and also directly on ERα and inhibits E2 activity in MCF-7 cells. We envisage that this inhibitory activity of naloxone may have implications in the treatment of breast cancer. This is especially important in hormone-dependent breast cancer, where women invariably experience tumor regrowth after receiving tamoxifen long-term (48). Studies in mouse xenograft models show that regrowth is associated with the development of enhanced sensitivity to the estrogenic properties of tamoxifen involving MAPK/ERK phosphorylation (49, 50). Considering that naloxone inhibits E2-induced MAPK/ERK phosphorylation, it is likely that it may block the agonist activity induced by tamoxifen via MAPK/ERK. We therefore speculate that, in addition to inhibiting ER activity independently, low-dose naloxone may block the estrogenic activity of tamoxifen and prolong the therapeutic benefit.

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