17β-Estradiol Enhances the Production of Nerve Growth Factor in THP-1-Derived Macrophages or Peripheral Blood Monocyte-Derived Macrophages

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We examined *in vitro* effects of 17β-estradiol (E2) on nerve growth factor production by macrophages derived from monocytic cell line THP-1-or peripheral blood monocytes. E2 and membrane-impermeable bovine serum albumin-conjugated E2 (E2-BSA) enhanced nerve growth factor secretion and mRNA expression in both types of macrophages E2 enhanced nerve growth factor promotor activity in THP-1-derived macrophages and two activator protein-1 binding sites on the promoter were responsible for the enhancement. E2 and E2-BSA enhanced transcriptional activity and DNA binding of activator protein-1. E2 and E2-BSA shifted the activator protein-1 composition from c-Jun homodimers to c-Fos/c-Jun heterodimers. E2 and E2-BSA transiently induced c-Fos mRNA, which was constitutively undetectable in both types of macrophages. Adenylate cyclase inhibitor SQ22536 suppressed E2-induced nerve growth factor production and c-Fos expression. E2 and E2-BSA increased intracellular cyclic adenosine monophosphate level in both types of macrophages. Antisense oligonucleotide against guanine nucleotide-binding protein-coupled receptor, GPR30 suppressed the E2-induced cyclic adenosine monophosphate signal, c-Fos expression, and nerve growth factor secretion in both types of macrophages. These results suggest that E2 may enhance nerve growth factor production by inducing c-Fos expression via cyclic adenosine monophosphate signal in macrophages. These effects may be mediated via GPR30. *Key words: activator protein-1/cyclic adenosine monophosphate/c-Fos/GPR30. J Invest Dermatol 121:771–780, 2003*

Nerve growth factor (NGF) accelerates skin wound healing (Matsuda *et al*, 1998). NGF potentiates the proliferation of neurons, which may promote reinnervation in the wound (Matsuda *et al*, 1998). NGF also enhances the growth of keratinocytes (Di Marco *et al*, 1993; Pincelli *et al*, 1994), which may promote re-epithelialization in the wound (Matsuda *et al*, 1998). In the inflammatory phase of wound healing, macrophages migrate to the wound site, and produce a variety of growth factors, which stimulate collagen deposition, epithelialization, and angiogenesis, such as platelet-derived growth factor, transforming growth factor-β, or vascular endothelial growth factor (Moulin, 1995). Macrophages in the wound produce neurotrophic factors inducing reinnervation (Batchelor *et al*, 1999) and also secrete NGF (Caroleo *et al*, 2001).

Previous studies suggest that estrogen may promote wound healing by promoting the production of basic fibroblast growth factor (Rider *et al*, 1997) or transforming growth factor-β in the wound (Ashcroft *et al*, 1997). It is thus plausible that estrogen may upregulate NGF production by macrophages in the skin wound. Previous studies reported the modulatory effects of estrogen on NGF production; however, the effects differ with tissue types or experimental conditions; estrogen *in vivo* increased NGF mRNA and protein levels in murine uterus and salivary gland, whereas it did not alter those in the bladder (Bjorling *et al*, 2002). Estrogen *in vitro* decreased NGF protein level but did not alter its mRNA level in murine L929 fibroblasts (Siminoski *et al*, 1987).

It is known that estrogen manifests its effects by two different mechanisms, genomic effects and nongenomic effects. The former is that estrogen-bound nuclear estrogen receptor (ER) α or β upregulates or downregulates gene expression by binding to the estrogen response element (ERE) of the target genes or by interacting with other transcription factors, such as nuclear factor-κB (Beato, 1989; Kanda and Watanabe, 2003). On the other hand, in the latter case, estrogen interacts with cell surface binding sites and rapidly induces a variety of intracellular signals (Kelly and Levin, 2001); estrogen activates adenylate cyclase (AC) (Aronica *et al*, 1994), phospholipase C (Razandi *et al*, 1999), or extracellular signal-regulated kinase (ERK) (Migliaccio *et al*, 1996), or induces Ca^{2+} signal (Benten *et al*, 2001). These nongenomic signaling events by estrogen may also lead to the upregulation or downregulation of certain gene expression. Membrane

Manuscript received February 28, 2003; revised April 8, 2003; accepted for publication April 22, 2003

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Abbreviations: AC, adenylate cyclase; AP-1, activator protein-1; cAMP, cyclic adenosine monophosphate; CRE, cAMP response element; CREB, CRE-binding protein; DHT, dihydrotestosterone; DMEM/F-12, Dulbecco's modified Eagle medium and Ham's F-12 medium (1:1 mixture); E2, 17β-estradiol; E2-BSA, 17β-estradiol 6-(O-carboxymethyl)oxime bovine serum albumin; FITC, fluorescein isothiocyanate; E2-BSA-FITC, FITC-conjugated E2-BSA; EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; ERE, estrogen response element; ERK, extracellular signal-regulated kinase; G-protein, guanine nucleotide-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NGF, nerve growth factor; PDE, cyclic nucleotide phosphodiesterase; PMA, phorbol-12-myristate-13-acetate; SHBG, sex hormone-binding globulin.

estrogen-binding sites do not appear to be a unique molecule; some may be post-translationally modified forms of nuclear ERα or ERβ, whereas others may be structurally different from classical nuclear ER (Papas *et al*, 1995; Razandi *et al*, 1999; Benten *et al*, 2001). It is reported that estrogen can interact with several guanine nucleotide-binding protein (G-protein)-coupled receptors such as the steroid hormone-binding globulin (SHBG) receptor (Fissore *et al*, 1994; Nakla *et al*, 1994), or an orphan receptor GPR30 (Filardo *et al*, 2000).

In a previous study, it was reported that 17β-estradiol (E2) enhanced the production of vascular endothelial growth factor in phorbol-12-myristate-13-acetate (PMA)-differentiated human monocytic THP-1 cells, a suitable model for tissue macrophages (Kanda and Watanabe, 2002). The results indicate that E2 may promote angiogenesis in the wound. In this study, we examined the effects of E2 on NGF production in THP-1-derived macrophages and peripheral blood monocyte-derived macrophages, much closer to tissue macrophages invading wounds. We found that E2 potentiated NGF production in both cell types. We further examined the precise mechanism for this effect focusing on the E2-induced signaling events.

MATERIALS AND METHODS

Reagents E2, 17β-estradiol 6-(O-carboxymethyl)oxime/bovine serum albumin (E2-BSA), fluorescein isothiocyanate (FITC)-conjugated E2-BSA (E2-BSA-FITC), 17α-estradiol, 4-hydroxytamoxifen, progesterone, dihydrotestosterone (DHT), and testosterone were purchased from Sigma (St Louis, Missouri). ICI 182 780 was from Wako Pure Chemical Industries (Osaka, Japan). U73122, PD98059, and SQ22536 were obtained from Calbiochem (La Jolla, California). Antibodies used in electrophoretic mobility shift assay (EMSA) were purchased from Santa Cruz Biotechnology (Santa Cruz, California).

Differentiation of peripheral blood monocytes and THP-1 cells, and measurement of NGF secretion Peripheral blood mononuclear cells were isolated from the blood of healthy adult volunteers who were informed of the objectives and methods of this study and consented to participate. Monocytes were separated from peripheral blood mononuclear cells as described (Gee *et al*, 2002). Briefly, peripheral blood mononuclear cells were isolated by density-gradient centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and T cells and B cells were depleted using CD2 and CD19 dynabeads (Dynal Biotech, Oslo, Norway). The T cell and B cell depleted fractions were incubated in 35 mm dish at 37°C for 2 h following which nonadherent cells were removed. The adherent cells obtained were less than 1% $CD2^+$ and $CD19^+$, and greater than 90% $CD14^+$ as determined by flow cytometry, and were used as monocytes. Human monocytic THP-1 cells were purchased from Dainippon Pharmaceutical (Osaka, Japan) and were cultured in RPMI 1640 (Invitrogen, Grand Island, New York) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) supplemented with 100 U per mL penicillin G, 100 μg per mL streptomycin, 0.25 μg B per mL amphotericin (Invitrogen). For differentiation, peripheral blood-derived monocytes or THP-1 cells were plated on to 24-well plates at 5×10^5 or 2×10^5 cells, respectively, in 1 mL of the culture medium containing 100 nM PMA and allowed to attach for 48 h as described (Bienkowski *et al*, 1989). The cells were washed, and fed with PMA-free medium and cultured overnight. PMA treatment promoted the adherence and induced flattened macrophage-like morphology in both cell types. To measure NGF secretion, monocyte-derived macrophages or THP-1-derived macrophages above were incubated with phenol red and serum-free Dulbecco's modified Eagle medium and Ham's F-12 medium (1:1 mixture) (DMEM/F-12) (Invitrogen) for 24 h, then washed and incubated with various concentrations of sex hormones in 1 mL of phenol red and serum-free DMEM/F-12 for 24 h. Control cells were incubated with medium alone. The supernatants were assayed for NGF by enzyme-linked immunosorbent assay (Promega, Madison, Wisconsin).

Reverse transcription–polymerase chain reaction (reverse transcription–PCR) Monocyte-derived macrophages or THP-1-derived macrophages were incubated with sex hormones as above for indicated periods, and cellular mRNA was extracted using a mRNA purification kit (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. cDNA was made from mRNA samples as described (Tjandrawinata *et al*, 1997). Primers for amplification and the sizes of respective PCR products were as follows: ERα 5′-AAT-GTGTAGAGGGGC ATGG-3′ and 5′-TGATGTGGGAGAGGATAGG-3′ for 296 bp; ERβ, 5′-TCCCAGCAATGTCACTAAC-3′ and 5′-TCCCCACTAACCTTCCTT T-3′ for 219 bp; GPR30, 5′-TTCCAAGTGCACCTCCAGCC-3′ and 5′-A AGCGTGATTCTCCTTGAAG-3′ for 314 bp; NGF, 5′-CACACTGAGG TGCATAGCGT-3′ and 5′-TGATGACCGCT-TGCTCCTGT-3′ for 352 bp; c-fos 5′-GGCTTCAACGCAGACTA CGAGG-3′ and 5′-CTCCTGTCA TGGTCTTCACAACG-3′ for 340 bp; c-jun, 5′-TTCACCTTCTCTCTAA CTGC-3′ and 5′-TCACTCACTGA GCGCTCTTC-3′ for 580 bp; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5′-GCAGGGGGGA GCCAAAAGGG-3′ and 5′-TGCCAGC-CCCAGCGTCAAAG-3′ for 566 bp (Carmeci *et al*, 1997; Lane *et al*, 1998; Risse-Hackl *et al*, 1998; Vegeto *et al*, 2001; Iannone and de Bari, 2002; Kanda and Watanabe, 2003). PCR was performed by one denaturing cycle of 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 3 min. The PCR products were analyzed by electrophoresis on 2.5% agarose gels and stained with ethidium bromide, viewed by ultraviolet light. Densitometric analysis was performed by scanning the bands into Photoshop and performing densitometry with NIH Image Software (http://rsb.info.nih.gov/nih.image/).

Plasmids and transfections The firefly luciferase reporter plasmids driven by human NGF promoter (–600/+250 bp relative to the transcriptional start site) were constructed by PCR and insertion into pGL3 basic vector (Promega) as described (Cartwright *et al*, 1992; Cowie *et al*, 1994), and was denoted as pNGF luc. Site-specific mutation of the promoter was created by multiple rounds of PCR using primers with altered bases as described (Li and Kolattukudy, 1994). p4 × first activator protein-1 (AP-1)-TATA-luc and p4 × second AP-1-TATA-luc were constructed by inserting four copies of upstream AP-1 (5′-GCGTGTGACTCAGGAGT-3′, consensus sequence underlined) or downstream AP-1 (5′-AGCGGTGAGTCAGGCTG-3′) from human NGF promoter in front of TATA box upstream of firefly luciferase reporter as described (Kanda and Watanabe, 2003). Transient transfections were performed with Effectene (Qiagen, Tokyo, Japan) as described (Zellmer *et al*, 2001). THP-1-derived macrophages grown to 60% confluence in 24-well plates were incubated for 6 h in 0.7 mL serum and phenol red-free DMEM/F-12 with 0.15 μg of pNGF luc or p4 × first AP-1-TATA-luc or p4 × second AP-1-TATA-luc and 0.05 μg of SV40 promoter-linked β-galactosidase reporter vector, pCH110 (Amersham, Arlington Heights, Illinois), premixed with enhancer, transfection buffer, and Effectene. The transfected cells were washed and incubated with 1 mL of serum and phenol red-free DMEM/F-12 for 18 h, then treated with indicated concentrations of sex hormones. After 6 h, cell extracts were prepared and luciferase activities were quantified using the luciferase assay system (Promega). The same cell extracts were assayed for β-galactosidase activity using chemiluminescent Galacto-Light kit (Tropix, Bedford, Massachusetts). All readings were taken using a Lumat 9501 luminometer (Berthold, Wildbach, Germany). The results obtained in each transfection were normalized to β-galactosidase activity and expressed as relative luciferase activity.

EMSA EMSA was performed as described (Cartwright *et al*, 1992). The probes used were ^{32}P-labeled annealed double-stranded DNA containing first AP-1 or second AP-1 from human NGF promoter. The sequence of the first AP-1 probe is 5′-ATTTGGAGCGTGTGACTCAGGAGT-ACGGGAG-3′ (consensus sequence underlined) and that of second AP-1 probe is 5′-GGAGCGCAGCGGTGAGTCAGGCTGCCCCGAG-3′. For EMSA, 2 to 5 μg of nuclear protein extracts were incubated at room temperature for 5 min with a mixture containing 6 mM HEPES (pH 7.9), 0.4 mM ethylenediamine tetraacetic acid, 125 mM KCl, 10% glycerol, 0.05 μg per μL, poly(dI-dC) 1 mM dithiothreitol, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 mM NaF, 50 μg per mL, aprotinin and 50 μg per mL leupeptin. One nanogram of labeled probe was added and the reactions were incubated for another 20 min. In antibody supershift experiments, the nuclear extracts were preincubated with various antibodies for 30 min before the addition of the probe. Reactions were then fractionated on a nondenaturing 5% polyacrylamide gel, and visualized with PhosphorImager (Molecular Dynamics, Sunnyvale, California).

Measurement of cyclic adenosine monophosphate (cAMP) amount Monocyte- or THP-1-derived macrophages in 24-well plates were serum and phenol red deprived as above, and incubated with indicated concentrations of sex hormones for the indicated duration. The medium was discarded, and the cells were lyzed with acetate buffer (pH 5.8)

containing 0.25% dodecyltrimethylammonium bromide. The cAMP amount in the lysate was measured by enzyme-linked immunosorbent assay (Amersham) according to the manufacturer's instructions. The sensitivity of the assay was 12 fmol per assay well. The intracellular cAMP level was presented as pmol per 10^6 cells.

Measurement of AC activity Monocyte-derived macrophages or THP-1-derived macrophages were cultured with sex hormones for 5 min in 24-well plates as described above, and were homogenized in the buffer containing 75 mM Tris–HCl (pH 7.4), 2 mM ethylenediamine tetraacetic acid, 8 mM $MgCl_2$, and centrifuged at 1000 × *g* for 5 min and 40,000 × *g* for 20 min. The pellet was used as a membrane fraction for AC assays as described (Salomon *et al*, 1974; Choi *et al*, 1992) using 1 mM [α-^{32}P] adenosine triphosphate (30 Ci per mmol) (Amersham) as a substrate.

Measurement of cyclic nucleotide phosphodiesterase (PDE) activity Monocyte-derived macrophages or THP-1-derived macrophages were homogenized as above, then sonicated on ice, and centrifuged at 30,000 × *g* for 15 min. PDE activity of the supernatants was assayed as described (Robicsek *et al*, 1991) using 1 μM [2,8-^{3}H] cAMP (30 Ci per mmol) (Amersham) as a substrate.

Flow cytometry Monocyte-derived macrophages or THP-1-derived macrophages were harvested and labeled with E2-BSA-FITC 15 μM for 1 h at room temperature. BSA-FITC was used as a control. Cells were analyzed in a FACScan (Becton Dickinson, Sunnyvale, California) with a sample size of 10^4 cells gated, and the data were processed using the FACScan software as described (Benten *et al*, 1991).

Treatment with anti-sense oligodeoxynucleotides Anti-sense oligonucleotides were synthesized as described (O'Dowd *et al*, 1998; Lau *et al*, 2000; Taylor *et al*, 2001). The oligonucleotides were ERα, 5′-GACCATGACCATGACCCT-3′; ERβ, 5′-CATCACAGCAGGGCT-ATA-3′; GPR30, 5′-TTGGGAAGTCACATCCAT-3′; random control, 5′-GATCTCAGCACGGCAAAT-3′. For anti-sense experiments, monocyte-derived macrophages or THP-1-derived macrophages were washed twice in phosphate-buffered saline, then transfected with finally 0.2 μM of the indicated oligonucleotides premixed with Oligofectamine (Invitrogen) in serum and phenol red-free DMEM/F-12 for 4 h. The medium was aspirated and fresh medium containing E2 was added.

RESULTS

E2, E2-BSA, and anti-estrogens enhanced NGF secretion in THP-1-derived macrophages or monocyte-derived macrophages THP-1-derived macrophages constitutively secreted a low amount of NGF (mean ± SEM, 21.3 ± 2.6 pg per mL, n = 5), and the secretion was increased by E2; the stimulatory effect of E2 was manifested at 10^{-10} M, increased in a concentration-dependent manner, and was maximized at 10^{-8} M, which enhanced NGF secretion 4.6-fold of controls (**Fig 1*a***). On the other hand, progesterone, DHT (**Fig 1*a***), and testosterone (data not shown) were ineffective. E2 stereoisomer 17α-estradiol did not enhance the NGF secretion (**Fig 1*b***). E2-BSA, membrane-impermeable BSA-conjugated E2, enhanced NGF secretion as well as E2 (**Fig 1*b***), indicating that the effect of E2 may be exerted on the membrane E2-binding sites, and not via intracellular ER. BSA alone did not affect NGF secretion. Anti-estrogens, ICI 182 780 and 4-hydroxytamoxifen did not counteract the effects of E2 but enhanced NGF secretion in the absence of E2 (**Fig 1*b***). When the effect of each agent on NGF secretion was analyzed in peripheral blood monocyte-derived macrophages (**Fig 1*c***), the results were mostly the same as those in THP-1-derived macrophages. Thus E2 enhanced NGF secretion, and the effect appeared to occur at the membrane surface. We then examined if E2 may enhance NGF mRNA expression.

E2 increased NGF mRNA levels At 3 h of incubation, E2 increased NGF mRNA level in THP-1-derived macrophages (**Fig 2*a***) or monocyte-derived macrophages (**Fig 2*b***). E2-BSA, ICI 182 780, and 4-hydroxytamoxifen increased NGF mRNA level as well as E2, whereas progesterone, DHT (**Fig 2**), and testosterone (data not shown) were ineffective. Thus E2 enhanced NGF production at pretranslational level. We then examined if E2 may enhance NGF promoter activity.

E2 enhanced NGF promoter activity It is reported that human NGF gene contained one AP-1-binding site upstream from transcriptional start site (first AP-1; −62/−56 bp) and another AP-1 site in first intron (second AP-1; +35/+41 bp), and these two AP-1-binding sites may act as enhancer elements for NGF transcription (Cartwright *et al*, 1992) (**Fig 3*a***). We transiently transfected NGF promoter (−600/+250 bp) linked to luciferase reporter into THP-1-derived macrophages, and the NGF promoter activity was evaluated by relative luciferase activity of the cell lysates. The attempt to transfect peripheral blood monocyte-derived macrophages was unsuccessful. E2 increased NGF promoter activity by 5.1-fold of controls (**Fig 3*b***). Membrane-impermeable E2-BSA (**Fig 3*b***), ICI 182 780 and 4-hydroxytamoxifen (data not shown) enhanced the NGF promoter activity as well as E2, whereas progesterone, DHT, and testosterone were ineffective (data not shown). The mutation of first or second AP-1 each reduced the basal promoter activity and E2 or E2-BSA induced enhancement of the activity. The mutation of both AP-1 sites completely abrogated the basal and E2 or E2-BSA-induced promoter activities. These results suggest that both AP-1 sites may be required for basal and E2 or E2-BSA-induced NGF promoter activities. We then analyzed if E2 may enhance transcriptional activities dependent on first or second AP-1 sites.

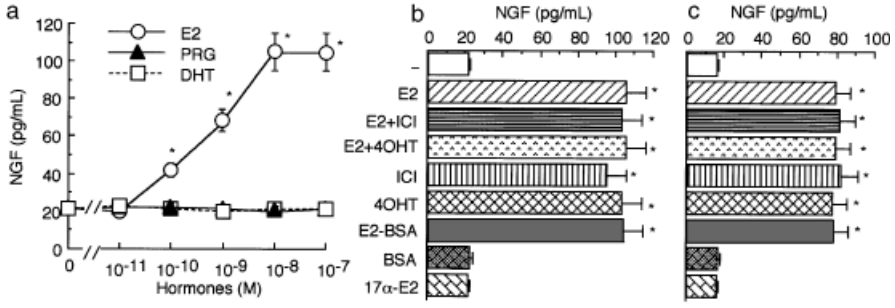

Figure 1. Dose-dependency for the effects of sex hormones on NGF secretion (*a*) and the hormone specificity in the enhancement of the secretion (*b,c*). (*a*) THP-1-derived macrophages were incubated with indicated concentrations of E2, progesterone (PRG), or dihydrotestosterone (DHT) for 24 h. The culture supernatants were assayed for NGF secretion. Values are mean ± SD of triplicate cultures. *$p < 0.05$ *versus* control cultures without hormones, by one-way ANOVA with Dunnett's multiple comparison test. The data shown in the figure are representative of five separate experiments. THP-1-derived macrophages (*b*) or monocyte-derived macrophages (*c*) were incubated with 10^{-8} M E2, 10^{-6} M ICI 182 780 (ICI), 10^{-6} M 4-hydroxytamoxifen (4OHT), 10^{-7} M E2-BSA, 10^{-7} M BSA, or 10^{-8} M 17α-estradiol (17α-E2) alone or in combination for 24 h. Values are mean ± SEM (n = 5). *$p < 0.05$ *versus* control cultures with medium alone, by one-way ANOVA with Scheffe's multiple comparison test.

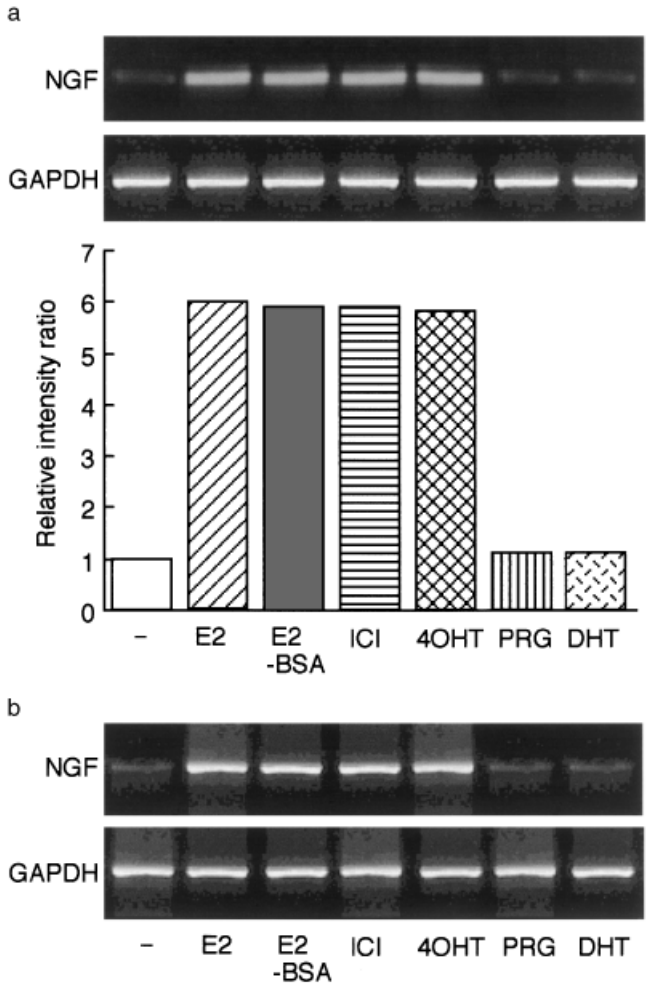

Figure 2. The effects of sex hormones on NGF mRNA expression in THP-1-derived macrophages (*a*) or monocyte-derived macrophages (*b*). The cells were incubated with 10^{-8} M E2, 10^{-7} M E2-BSA, 10^{-6} M ICI 182 780 (ICI), 10^{-6} M 4-hydroxytamoxifen (4OHT), 10^{-8} M progesterone (PRG), or 10^{-8} M DHT for 3 h mRNA was isolated, and reverse transcription–PCR was performed. (*a*) The intensity of the band for NGF was corrected to that for GAPDH. The lower graph shows the corrected intensities relative to that in control cells with medium alone (set as 1.0). The results shown in the figure are representative of five separate experiments in each cell type.

E2 enhanced AP-1 transcriptional activities THP-1-derived macrophages were transiently transfected with luciferase reporter linked to four repeats of first or second AP-1 in front of the TATA box. E2 increased transcriptional activities of first and second AP-1 (**Fig 4**). E2-BSA (**Fig 4**), ICI 182 780, and 4-hydroxytamoxifen (data not shown) enhanced transcriptional activities through both AP-1 sites as well as E2, whereas progesterone, DHT, and testosterone were ineffective (data not shown). We then examined if E2 may enhance the DNA-binding activity of AP-1.

E2 induced c-FOS/c-Jun binding to AP-1 sites THP-1-derived macrophages were incubated with E2 or E2-BSA for 1 h, and nuclear extracts were obtained and used for EMSA. E2, E2-BSA (**Fig 5**, *lanes 3 and 4*), ICI 182 780, and 4-hydroxytamoxifen (data not shown) increased the amount of DNA-protein complex with the first AP-1-containing probe derived from the NGF promoter. Progesterone, DHT, or testosterone did not increase the amount of DNA-protein complex (data not shown). In nuclear extracts from unstimulated cells, anti-c-Jun did but anti-c-Fos antibody did not supershift the complex (**Fig 5**, *lanes 5 and 6*), whereas in the extracts from E2 (**Fig 5**, *lanes 7 and 8*) or E2-BSA, ICI 182 780, or 4-hydroxytamoxifen-stimulated cells (data not shown), both anti-c-Jun and anti-c-Fos antibodies supershifted the complex. Antibodies against the other Fos family (FosB, Fra-1, Fra-2) or Jun family (JunB, JunD) proteins or anti-ERα or anti-ERβ antibodies did not supershift the complexes by nuclear extracts from unstimulated or E2, E2-BSA, or anti-estrogen-stimulated cells (data not shown). The results were similar in monocyte-derived macrophages (data not shown). These results suggest that E2 may shift the AP-1 composition from c-Jun homodimers to c-Fos/c-Jun heterodimers at the first AP-1 site. Similar results were obtained by using the second AP-1-containing probe (data not shown).

E2 induced c-Fos expression in THP-1-derived macrophages or monocyte-derived macrophages We then examined if E2 may enhance c-Fos or c-Jun expression in THP-1-derived macrophages. E2 rapidly and transiently induced c-Fos mRNA. c-Fos mRNA was undetectable in unstimulated cells; however, in E2-stimulated cells, the expression was induced at 15 min, maximized at 30 min, reduced at 1 h, and disappeared at 3 h (**Fig 6*a***). c-Jun mRNA expression was constitutively detectable and was not enhanced by E2. Kinetics of c-Fos and c-Jun mRNA levels in monocyte-derived macrophages (**Fig 6*b***) were mostly the same as those in THP-1-derived macrophages. E2-BSA, ICI 182 780, and 4-hydroxytamoxifen induced c-Fos mRNA expression in a manner similar to that of E2, but did not alter the c-Jun mRNA level, whereas progesterone, DHT, or testosterone did not alter c-Fos or c-Jun mRNA expression in both cell types (data not shown). The results indicate that E2, E2-BSA, or anti-estrogen-induced NGF production may be mainly attributable to the induction of c-Fos expression.

AC activation was involved in E2-induced NGF production and c-Fos expression It is reported that E2 binding to the cell surface activates a variety of signal-transducing enzymes: AC, phospholipase C, or ERK (Migliaccio *et al*, 1996; Razandi *et al*, 1999). We thus aimed to define the signaling enzyme responsible for the NGF production and c-Fos induction by E2. SQ22536, an inhibitor of AC that catalyzes cAMP generation, counteracted the E2-induced enhancement of NGF promoter activity (**Fig 7*a***), NGF secretion (**Fig 7*b***), first AP-1 transcriptional activity (**Fig 7*c***), and c-Fos induction (**Fig 7*d***) in THP-1-derived macrophages, whereas these were not counteracted by phospholipase C inhibitor U73122 or ERK kinase 1 inhibitor PD98059. In monocyte-derived macrophages, SQ22536 did but PD98059 or U73122 did not inhibit the effects of E2 on NGF secretion and c-Fos expression (**Fig 7*e*,*f***). The effects of E2-BSA, ICI 182 780, or 4-hydroxytamoxifen on NGF production, AP-1 activity, and c-Fos induction were similarly counteracted by SQ22536 but not by U73122 or PD98059 in both cell types (data not shown). These results indicate that AC activation and resultant cAMP signal may be responsible for the E2-induced NGF production and c-Fos expression. This is supported by the fact that cAMP analog dibutyryl cAMP enhanced NGF production and AP-1 activity and induced c-Fos expression as well as E2 (**Fig 7**).

E2-induced cAMP signal We then examined if E2 may generate cAMP signal in THP-1-derived macrophages. E2 rapidly (within 5 min) increased intracellular cAMP concentration up to 4.8-fold of basal level, which was followed by gradual decrease and recovery to the basal level at 3 h (**Fig 8*a***). E2-BSA, ICI 182 780, and 4-hydroxytamoxifen increased cAMP level similarly to E2, whereas progesterone, DHT (**Fig 8*a***), and testosterone (data not shown) had no effects. In monocyte-derived macrophages treated with each agent, the kinetics of cAMP levels were mostly comparable with those in THP-1-derived macrophages (data not shown). The cAMP increases by E2, E2-BSA,

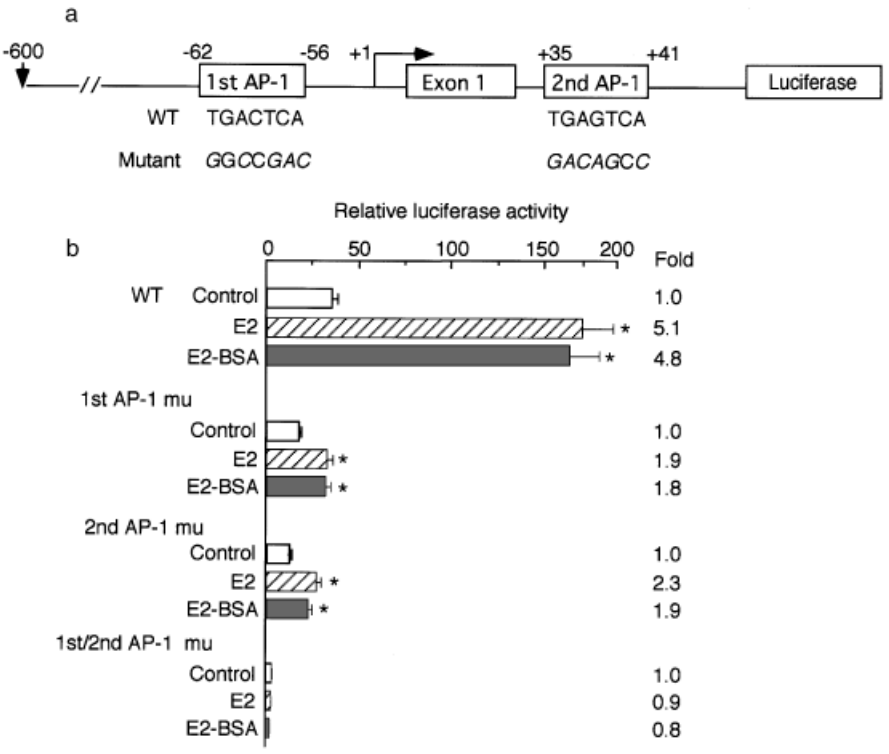

Figure 3. The effects of E2 on wild-type or mutated NGF promoters in THP-1-derived macrophages. (*a*) Schematic representation of human NGF promoter. The locations of two AP-1 sites are shown with their sequences, and substituted bases for mutation are indicated in italics. The nucleotide positions are relative to the transcriptional start site. (*b*) THP-1-derived macrophages were transiently transfected with wild-type (WT) or mutated pNGF luc together with pCH110, and incubated with medium alone, or with 10^{-8} M E2 or 10^{-7} M E2-BSA for 6 h. Relative luciferase activities normalized to β-galactosidase activities were shown. The data are mean ± SEM (n = 4). Values at right indicate the fold induction *versus* basal promoter activity. $^{*}p < 0.05$ *versus* control values, by one-way ANOVA with Scheffe's multiple comparison test.

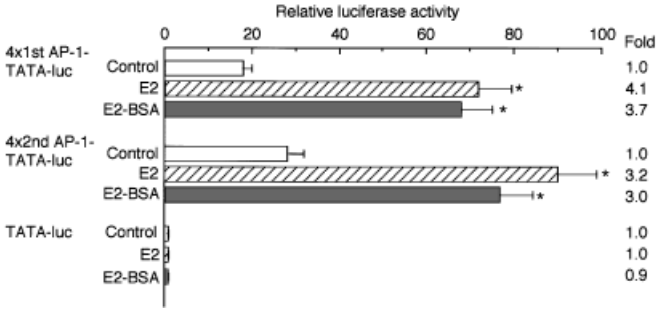

Figure 4. The effect of E2 on transcriptional activities through two AP-1 sites. THP-1-derived macrophages were transiently transfected with p4 × first or second AP-1-TATA-luc or enhancerless pTATA-luc together with pCH110. The cells were incubated with medium alone, or with 10^{-8} M E2 or 10^{-7} M E2-BSA for 6 h. The results are shown as relative luciferase activities normalized to β-galactosidase activities, and represent mean ± SEM (n = 4). Values at right indicate the fold induction *versus* basal activity. $^{*}p < 0.05$ *versus* control values, by one-way ANOVA with Scheffe's multiple comparison test.

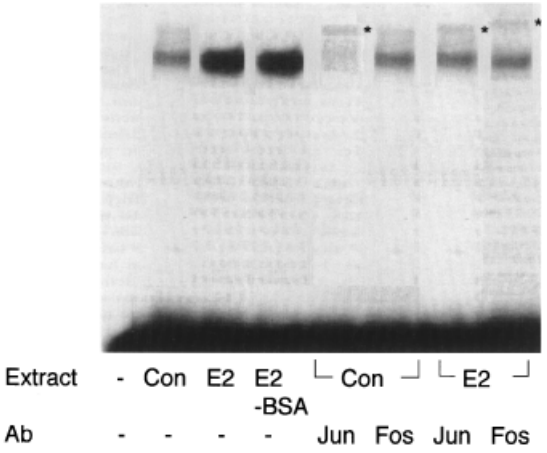

Figure 5. The effect of E2 or E2-BSA on transcription factor binding to AP-1 sites. THP-1-derived macrophages were incubated with medium alone, or with 10^{-8} M E2 or 10^{-7} M E2-BSA for 1 h. Nuclear extracts were prepared, and incubated with ^{32}P-labeled oligonucleotides containing the first AP-1 element. In supershift assays, nuclear extracts were incubated with antibodies against c-Fos or c-Jun for 30 min before the addition of the probe. The asterisks indicate the supershifted complexes. The results shown in the figure are representative of four separate experiments.

and anti-estrogens were associated with increased activities of cAMP-generating AC in both cell types (**Fig 8*b,d***). These agents did not alter the activity of cAMP-hydrolyzing PDE in THP-1-derived macrophages (**Fig 8*c***) and monocyte-derived macrophages (data not shown). These results suggest that E2 may activate AC and generate cAMP in both cell types.

Anti-sense GPR30 suppressed E2-induced cAMP signal, NGF secretion, and c-Fos expression As membrane-impermeable E2-BSA generated cAMP as well as E2, E2 may act on the cell surface of THP-1-derived macrophages or monocyte-derived macrophages. To test the presence of membrane estrogen-binding sites, we analyzed the binding of E2-BSA-FITC to THP-1-derived macrophages. FACScan analysis showed that the binding of E2-BSA-FITC occurred on 64% of the cells (**Fig 9*a***). This binding was specific to E2, as BSA-FITC without conjugated E2 did not adhere to THP-1-derived macrophages. Similar results were obtained in monocyte-derived macrophages (data not shown). It is reported that E2-induced cAMP signal is mediated via membrane-localized ERα, ERβ (Razandi *et al*, 1999), or GPR30 (Filardo *et al*, 2002). Reverse transcription–PCR detected ERα, ERβ, and GPR30 mRNA expression in THP-1-derived macrophages (**Fig 9*b***) and monocyte-derived macrophages (data not shown), indicating that these receptors localized at the membrane may mediate the E2-induced AC

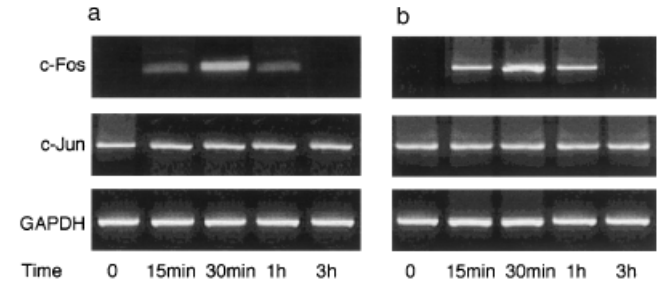

Figure 6. The effects of E2 on c-Fos or c-Jun mRNA expression in THP-1-derived macrophages (*a*) or monocyte-derived macrophages (*b*). The cells were incubated with 10^{-8} M E2. At indicated time-points, mRNA was isolated, and reverse transcription–PCR was performed. The results shown in the figure are representative of five separate experiments in each cell type.

activation. As specific antibody against GPR30 cannot be obtained, we analyzed if ERα, ERβ, or GPR30 anti-sense oligonucleotide may inhibit E2-induced cAMP signal. GPR30 anti-sense did but ERα or ERβ anti-sense did not suppress the E2-induced cAMP signal (**Fig 10*a,d***), NGF secretion (**Fig 10*b,e***), or c-Fos expression (**Fig 10*c,f***) in THP-1-derived macrophages or monocyte-derived macrophages. The effects of E2-BSA, ICI 182 780, or 4-hydroxytamoxifen on cAMP signal, c-Fos expression, and NGF secretion were also suppressed by GPR30 anti-sense but not by ERα or ERβ anti-sense (data not shown). These results suggest that cell surface GPR30 may mediate the E2, E2-BSA, and anti-estrogen-induced cAMP signal and resultant c-Fos expression and NGF production.

DISCUSSION

In this study, E2 enhanced NGF production in monocyte-derived macrophages or THP-1-derived macrophages. E2 stimulated NGF promoter activity by enhancing AP-1 transcriptional activity via c-Fos induction in THP-1-derived macrophages. The induced c-Fos may heterodimerize with c-Jun and the heterodimers may bind to two AP-1 sites on the NGF promoter and drive NGF transcription. On the other hand, c-Jun homodimers appeared to bind to the AP-1 sites in unstimulated cells. It is reported that c-Fos/c-Jun heterodimers much more avidly bind to DNA and have much higher transcriptional activity than c-Jun homodimers (Allegretto *et al*, 1990; Suzuki *et al*, 1991). Thus E2 may enhance AP-1 binding and transcriptional activity by inducing c-Fos expression and thus shifting AP-1 composition from c-Jun homodimers to c-Fos/c-Jun heterodimers. Though the effects of E2 on NGF promoter or AP-1 transcriptional activities could not be examined in monocyte-derived macrophages, rapid c-Fos induction by E2 occurred in these cells, indicating that the mechanism for NGF potentiation by E2 in monocyte-derived macrophages may be common to that in THP-1-derived macrophages.

E2-induced c-Fos expression was mediated by cAMP signal. Human c-*fos* promoter contains cAMP response element (CRE) at −62 bp, where CRE-binding protein (CREB) binds and drives c-*fos* transcription (Fisch *et al*, 1989; Janknecht *et al*, 1995). The transcriptional activity of CREB is promoted by serine

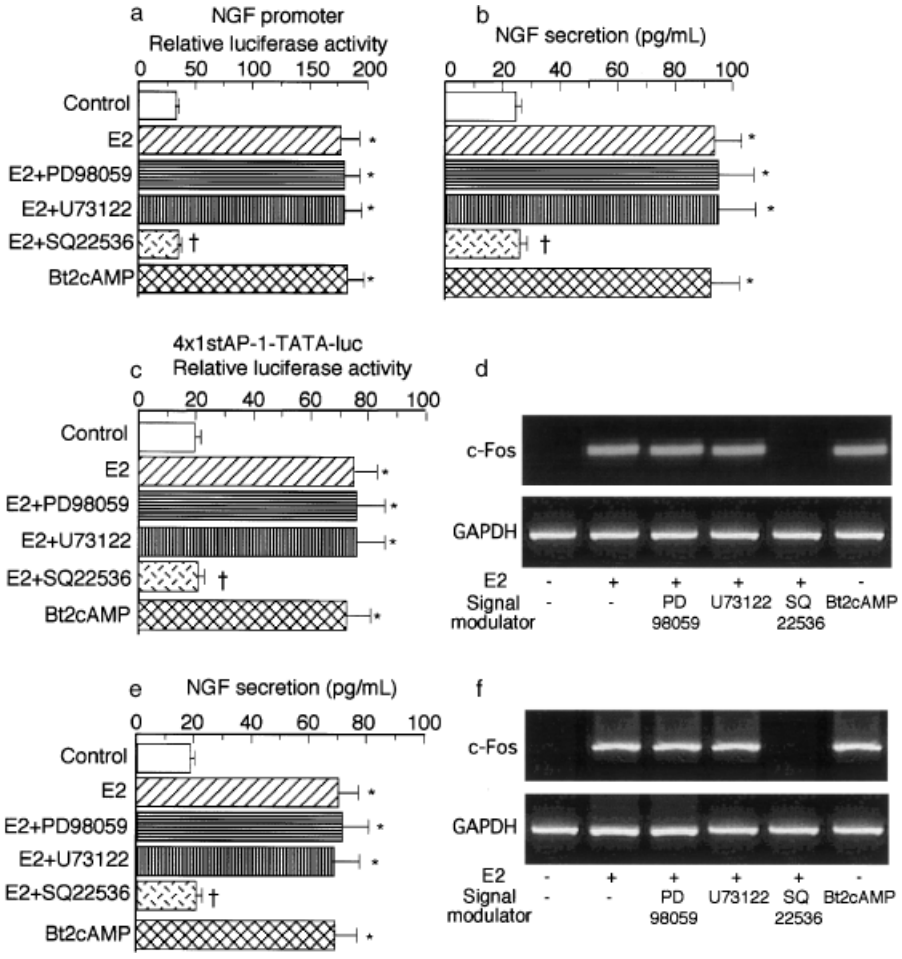

Figure 7. The inhibition by several signal modulators on E2-induced NGF promoter activity (*a*), NGF secretion (*b,e*), AP-1 transcriptional activity (*c*), and c-Fos mRNA expression (*d,f*). THP-1-derived macrophages were transiently transfected with pNGF luc (*a*) or p4 × first AP-1-TATA-luc (*c*), together with pCH110, then preincubated with 200 μM SQ22536, 1 μM U73122, or 20 μM PD98059 for 10 min, then incubated with 10^{-8} M E2 or with 1 mM dibutyryl cAMP (Bt2cAMP). After 6 h, luciferase activities of the cell lysates were analyzed and were normalized to β-galactosidase activities. THP-1-derived macrophages (*b,d*) or monocyte-derived macrophages (*e,f*) were preincubated with inhibitors, then incubated with E2 or dibutyryl cAMP as above. Culture supernatants were harvested after 24 h (*b,e*), whereas mRNA was extracted after 30 min (*d,f*). In (*a,b,c,e*), values are mean ± SEM of five separate experiments in each cell type. $^{*}p < 0.05$ *versus* control values, †$p < 0.05$ *versus* values with E2 alone, by one-way ANOVA with Scheffe's multiple comparison test. The results shown in (*d,f*) are representative of five separate experiments in each cell type.

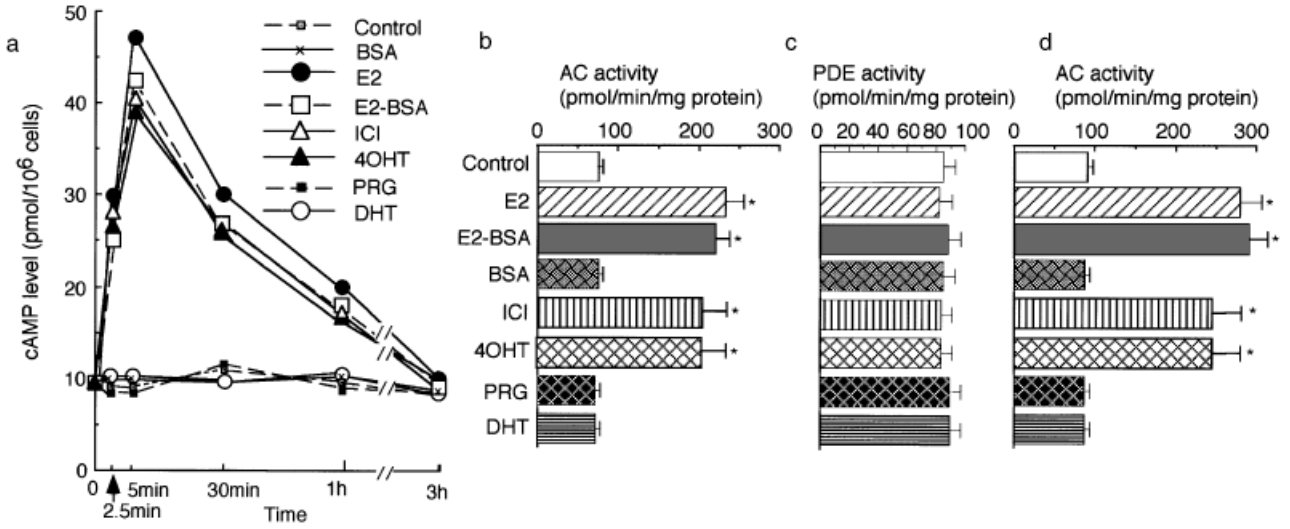

Figure 8. The effects of sex hormones on the intracellular cAMP level (*a*), AC (*b,d*), and PDE activities (*c*). (*a*) THP-1-derived macrophages were incubated with medium alone, E2 (10^{-8} M), E2-BSA (10^{-7} M), BSA (10^{-7} M), ICI 182 780 (ICI; 10^{-6} M), 4-hydroxytamoxifen (4OHT; 10^{-6} M), progesterone (PRG; 10^{-8} M), or DHT (10^{-8} M). The intracellular cAMP level was analyzed at the indicated time points. The mean of triplicate cultures is shown; the SD were $< 10\%$ of the means. The data are representative of four separate experiments. THP-1-derived macrophages (*b,c*) or monocyte-derived macrophages (*d*) were incubated as above. After 5 min, AC (*b,d*) or PDE activities (*c*) were analyzed. The data represent the mean $\pm$ SEM of four separate experiments in each cell type. $^{*}p < 0.05$ *versus* control values with medium alone, by one-way ANOVA with Scheffe's multiple comparison test.

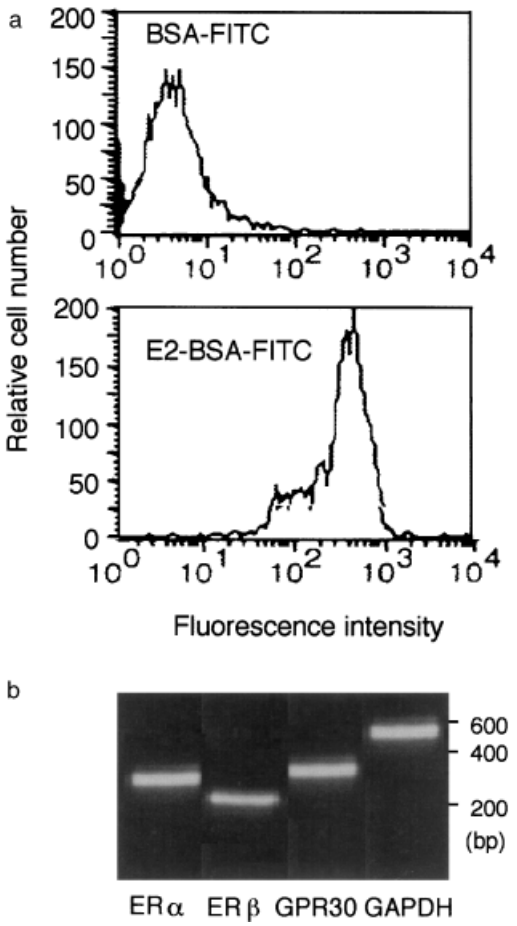

Figure 9. Flow cytometry of THP-1-derived macrophages labeled with E2-BSA-FITC (*a*) and ERα, ERβ, and GPR30 mRNA expression (*b*). (*a*) The cells were labeled with E2-BSA-FITC and were analyzed by FACScan (*lower panel*). Controls in the upper panel shows cells incubated with BSA-FITC. (*b*) Reverse transcription–PCR was performed with cDNA from THP-1-derived macrophages. The results shown in the figure are representative of five separate experiments.

phosphorylation via protein kinase A, which is activated by cAMP (Lalli and Sassone-Corsi, 1994). It is also reported that E2-induced cAMP signal induced CREB phosphorylation and CRE-dependent expression of neurotensin/neuromedin gene in human neuroblastoma SK-N-SH cells (Watters and Dorsa, 1998). In THP-1-derived macrophages or monocyte-derived macrophages, G-protein-coupled receptor, GPR30 appeared to act as a membrane E2-binding site linked to AC, which is consistent with the study performed in human breast cancer cells (Filardo *et al*, 2002). GPR30 is a 375 amino acid G-protein-coupled receptor with seven transmembrane domains (Carmeci *et al*, 1997). GPR30 mRNA is ubiquitously expressed in brain, lung, liver, prostate, or colon (Owman *et al*, 1996). The ligand for GPR30 has not been identified yet; however, it may be a certain peptide or glycoprotein (Carmeci *et al*, 1997). To date, GPR30-coupling G-protein isotypes are unknown; however, GPR30-mediated activation of AC indicates its linkage to G_s. It is hypothesized that the binding of E2 may activate GPR30 and induce the dissociation of the receptor-coupled G-protein heterotrimers, possibly $G_s\alpha\beta\gamma$, into $G_s\alpha$ and $G\beta\gamma$, the former of which may stimulate AC catalytic activity.

Razandi *et al* (1999) showed that membrane ER arises from the same transcript as that of nuclear ER, and that the stimulation of membrane ERα or ERβ by E2 generated the cAMP signal in Chinese hamster ovary cells transfected with ERα or ERβ, respectively. In THP-1-derived macrophages or monocyte-derived macrophages, however, the contribution of membrane ERα or ERβ to the cAMP signal may be little if any as anti-sense ERα or ERβ did not inhibit the E2-induced cAMP signal (**Fig 10**). Another possible membrane E2-binding site linked to AC is the SHBG receptor (Fissore *et al*, 1994; Nakhla *et al*, 1994). In prostate cells or breast cancer MCF-7, E2 interacts with SHBG prebound to membrane SHBG receptor, and generates the cAMP signal (Fissore *et al*, 1994; Nakhla *et al*, 1994). As THP-1-derived macrophages or monocytederived macrophages were not preincubated with exogenous SHBG prior to E2 incubation in this study, the involvement of SHBG receptor in E2-induced AC activation is rather unlikely in these cells.

In this and previous studies (Kanda and Watanabe, 2002), anti-estrogens, ICI compounds, or tamoxifen derivatives, did not antagonize E2-induced AC activation. ICI 182 780 and 4-hydroxytamoxifen rather activated AC as well as E2. The results are

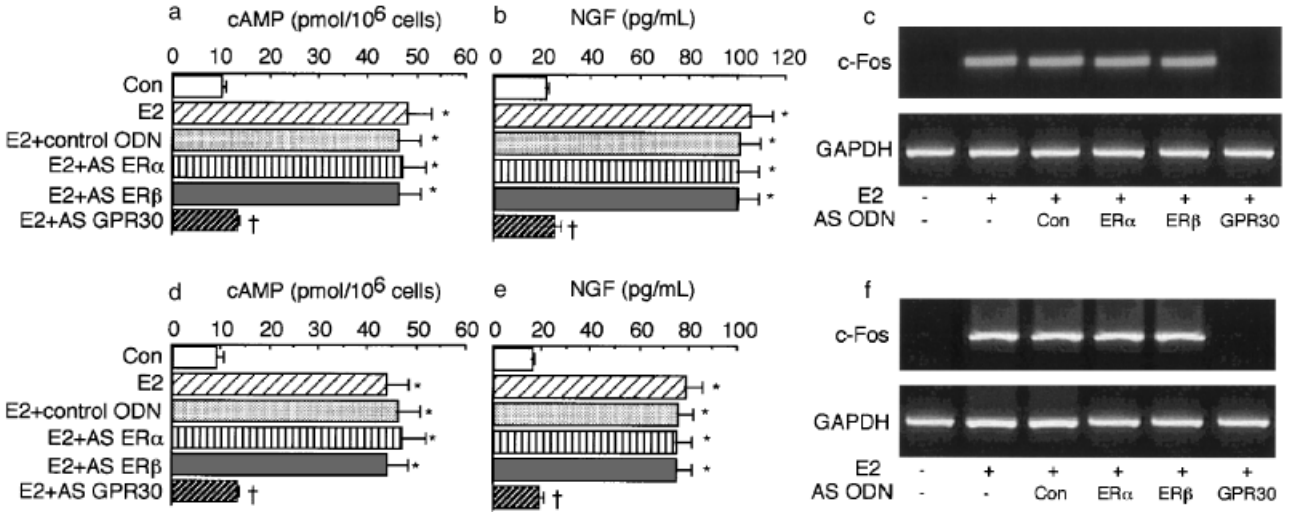

Figure 10. The inhibition by GPR30 anti-sense oligonucleotide of E2-induced cAMP signal (*a,d*), NGF secretion (*b,e*), and c-Fos expression (*c,f*). THP-1-derived macrophages (*a–c*) or monocyte-derived macrophages (*d–f*) were pretreated with 0.2 μM of indicated anti-sense (AS) or control scrambled oligonucleotides (ODN) for 4 h. The medium was aspirated and fresh medium containing E2 10^{-8} M was added. The intracellular cAMP level was analyzed at 5 min (*a,d*), NGF secretion was analyzed at 24 h (*b,e*), and c-Fos mRNA expression was analyzed at 30 min (*c,f*). In (*a,b,d,e*), values are mean ± SEM of five separate experiments in each cell type. *$p < 0.05$ *versus* control values, †$p < 0.05$ *versus* values with E2 alone, by one-way ANOVA with Scheffe's multiple comparison test. The results shown in (*c,f*) are representative of five separate experiments in each cell type.

consistent with previous studies (Aronica *et al*, 1994; Farhat *et al*, 1996; Watters and Dorsa, 1998; Razandi *et al*, 1999; Filardo *et al*, 2002). Though tamoxifen derivatives or ICI compounds can act as antagonists for nuclear ER (Berry *et al*, 1990), these anti-estrogens may act as agonists for membrane GPR30.

It is reported that E2 induces human c-*fos* gene expression in cells other than macrophages. The mechanisms for the induction differ with cell types, however; in ERα-transfected HeLa cells, ERα drives c-*fos* gene expression by binding to 5′-flanking imperfect ERE (CGGCAGCGTGACC, underlined sequences matching to consensus ERE) at −1212 bp (Weisz and Rosales, 1990). In MCF-7 cells, ERα interacts with transcription factor Sp1 and enhances its DNA binding and c-*fos* transcription through the GC-rich motif at −1168 bp (Duan *et al*, 1998). These modes of c-*fos* transcription are mediated by nuclear ERα, and which mechanism works in individual cell types may depend on the amounts or subtypes (α or β) of nuclear ER and on the amounts or activities of other transcriptional activators. On the other hand, membrane-impermeable E2-BSA induced c-*fos* expression in SK-N-SH cells by activating ERK, which may be mediated via membrane E2-binding sites (Watters *et al*, 1997). c-*fos* promoter contains serum response element at −317 bp where ternary complex factors such as Elk1 are binding (Gilman *et al*, 1986; Janknecht *et al*, 1995; Soh *et al*, 1999). It is known that ERK-mediated phosphorylation of Elk1 promotes its transcriptional activity (Marais *et al*, 1993). It is thus anticipated that Elk1 phosphorylation by ERK may be involved in E2-BSA-induced c-*fos* expression in SK-N-SH cells. E2 is reported to transactivate epidermal growth factor receptor-Ras-Raf-1-ERK kinase 1-ERK signaling pathway via GPR30 by releasing cell surface heparan-bound epidermal growth factor in MCF-7 (Filardo *et al*, 2000). Thus a similar manner of ERK activation may occur in THP-1-derived macrophages or monocyte-derived macrophages containing GPR30; however, ERK was dispensable for E2-induced c-*fos* expression in these cells (**Fig 7**). Possibly in THP-1-derived macrophages or monocyte-derived macrophages, cAMP-activated protein kinase A may attenuate ERK activation by inhibiting the activity of Raf-1, which phosphorylates and activates ERK kinase 1, catalyzing ERK phosphorylation (Filardo *et al*, 2002).

In contrast to c-Fos, c-Jun mRNA level was not increased by E2 (**Fig 6**). It is reported that E2 increased c-*jun* expression in rat uterine myometrium (Nephew *et al*, 1994). The coding sequence of the rat c-*jun* gene contains an imperfect ERE (Hyder *et al*, 1995) and a similar element is also conserved in the human gene (ACAGAGCATGACC) (Hattori *et al*, 1988); however, ER binding affinity and E2-induced transcriptional activity of such imperfect ERE were much lower than those of consensus ERE (GGTCAnnnTGACC). In THP-1-derived macrophages or monocyte-derived macrophages, this imperfect ERE in c-*jun* gene may not function as an E2-inducible enhancer element.

E2 stimulated NGF production not only in THP-1-derived macrophages but also in peripheral blood monocyte-derived macrophages, which are closer to macrophages invading wounds. This suggests that topical application of E2 to wounds may enhance NGF production by invading macrophages. NGF induction by E2 in macrophages may accelerate wound repair in an autocrine and paracrine manner. As NGF promotes the survival, differentiation, and phagocytosis of macrophages (Caroleo *et al*, 2001), macrophage-derived NGF may maintain the number of macrophages and enhance their elimination of bacteria or cell debris, which is required for rapid wound repair. The application of E2 to wound site may promote the proliferation and migration of keratinocytes and neural cells via NGF induction in macrophages, and thus accelerate re-epithelialization and reinnervation required for wound healing. NGF also induces neurons to synthesize a neuropeptide substance P, which stimulates the proliferation of keratinocytes, fibroblasts, and endothelial cells and thus promotes fibroplasia, re-epithelialization, and angiogenesis at the wound site (Linsay and Harmar, 1989; Ansel *et al*, 1996). Skin wound healing is impaired in sensory nerve-deficient conditions, such as diabetes, leprosy, or aging (Khalil *et al*, 1994; Ansel *et al*, 1996; Khalil and Merhi, 2000), and in such states, skin NGF content is reduced (Anand *et al*, 1994, 1996). Thus E2 may be therapeutically useful in the impaired wound healing associated with sensory nerve deficits by inducing NGF. It is also reported that E2 enhances high-affinity NGF receptor trkA expression in neural cells (Sohrabji *et al*, 1994), indicating that E2 may promote responsiveness to NGF in addition to NGF production. As NGF is produced by many cell types other than macrophages, such as keratinocytes, fibroblasts, or mast cells (Kinkelin *et al*, 2000), further studies should elucidate if E2 may enhance NGF production in these cells by mechanisms similar to or different from that in macrophages.

This work was supported in part by an aid from Foundation for Total Health Promotion.

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