SKF-82958 is a Subtype-Selective Estrogen Receptor-α (ERα) Agonist that Induces Functional Interactions between ERα and AP-1

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SUMMARY

The transcriptional activity of estrogen receptors (ERs) can be regulated by ligands, as well as agents such as dopamine which stimulate intracellular signaling pathways able to communicate with these receptors. We examined the ability of SKF-82958 (SKF), a previously characterized full dopamine D1 receptor agonist, to stimulate the transcriptional activity of ERα and ERβ. Treatment of HeLa cells with SKF-82958 stimulated robust ERα-dependent transcription from an ERE-E1b-CAT reporter in the absence of estrogen and this was accompanied by increased receptor phosphorylation. However, induction of ERβ-directed gene expression under the same conditions was negligible. In our cell model, SKF treatment did not elevate cAMP levels nor enhance transcription from a cAMP-response element (CRE)-linked reporter. Control studies revealed that SKF-82958, but not dopamine, competes with 17β-estradiol (E2) for binding to ERα or ERβ with comparable relative binding affinities. Therefore SKF-82958 is an ERα-selective agonist. Transcriptional activation of ERα by SKF was more potent than expected from its relative binding activity, and further examination revealed that this synthetic compound induced expression of an AP-1 target gene in a TPA response element (TRE)-dependent manner. A putative TRE site upstream of the estrogen response element and the receptor’s amino-terminal domain contributed to, but were not required for, SKF-induced expression of an ERα-dependent reporter gene. Overexpression of the AP-1 protein c-Jun, but not c-Fos, strongly enhanced SKF-induced ERα target gene expression, but only when the TRE was present. These studies provide information on the ability of a ligand which weakly stimulates ERα to yield strong stimulation of ERα-dependent gene expression through crosstalk with other intracellular signaling pathways producing a robust combinatorial response within the cell.
INTRODUCTION

The effects of estrogens are mediated by the products of two separate genes; one for estrogen receptor-α (ERα) and another for ERβ. Both are members of the nuclear receptor superfamily of ligand-activated transcription factors. The mechanisms by which ERs activate target gene expression in response to estrogen signaling have been the subject of intense investigation since their respective cDNAs were cloned (1, 2). Owing to the relatively recent identification of ERβ, the bulk of our knowledge regarding the genomic effects of estrogens is derived from ERα studies. For instance, upon binding to 17β-estradiol (E2), ERα undergoes a series of biochemical alterations including increased phosphorylation and conformational changes as well as the receptor’s homodimerization and binding to its target DNA sequence, the estrogen response element [ERE; refs. (3-5)]. ERβ also undergoes conformational changes in response to ligand binding (6, 7), and is phosphorylated in vivo (8). With respect to DNA binding, ERβ binds to the same consensus ERE that ERα does, although the latter receptor has an ~4-fold higher affinity for this DNA sequence in comparison to ERβ (9, 10).

While many aspects of the regulation of ERα and ERβ transcriptional activity are quite similar (e.g. both bind to EREs and activate transcription in response to E2 binding), a number of differences between these receptors have been noted. For instance, on ERE-containing reporters, ligands such as 4-hydroxytamoxifen exert partial agonist activity on ERα, but act as ERβ antagonists (11). This is likely related to differences in the poorly conserved structure and function of the hormone-independent activation function-1 (AF-1) domain which is located in the amino-termini of these receptors (11-13). The carboxy-terminal AF-2 domain is hormone-dependent, reflecting the ability of agonists to bind to the receptor’s ligand binding domain and induce a conformational change that creates a binding site for coactivators such as steroid receptor coactivator-1 (SRC-1) and its related family members (14, 15). Intriguingly, this domain is only ~60% conserved between ERα and ERβ, and small differences in the affinity of these two receptors for ligands such as genistein and 16α-bromo-17β-estradiol have been demonstrated (16, 17). Although several contexts exist whereby the transcriptional activity of ERα is...
derived predominantly from the AF-1 or AF-2 domains, in most cells the two activation functions work
together to bring about a synergistic activation of transcription (18-20). In contrast, the amino-terminus
of ERβ possesses relatively low transcriptional activity in comparison to ERα, and this region has been
shown to repress the activity of ERβ’s AF-2 domain (11-13).

Estrogen receptors, in addition to their regulation by ligands, can also be activated by extracellular
agents that initiate intracellular signal transduction pathways [reviewed in (21)]. For instance, epidermal
growth factor (EGF) or insulin-like growth factor-1 (IGF-1) treatment of cells results in initiation of a
mitogen-activated protein kinase (MAPK) signal transduction cascade leading to phosphorylation of the
ERα serine^118 phosphorylation site and stimulation of ERα transcriptional activity (22-24). Similarly,
activation of MAPKs by either EGF treatment or by transfection of a dominant active form of ras, induces
ERβ phosphorylation and transcriptional activity (8, 25), and this is accompanied by a phosphorylation-
dependent recruitment of the SRC-1 coactivator (26). In addition to growth factors, insulin, heregulin,
3,3’-diindolylmethane and the neurotransmitter dopamine can also stimulate ERα transcriptional activity
in the apparent absence of ligand (27-31). The latter was among the first agents demonstrated to stimulate
ERα transcriptional activity in a ligand-independent manner (31). There is no information on the ability
of dopamine to stimulate ERβ transcriptional activity. However, dopaminergic activation is not unique to
ERα, since this neurotransmitter also activates the human vitamin D (but not glucocorticoid) and chicken
progesterone receptors (31, 32). Furthermore, in vivo studies have demonstrated that dopamine receptor
agonists administered to the third ventricle of the brain lead to initiation of lordosis behavior, a
progesterone receptor (PR)-dependent biological response in rodents (33-35).

Dopamine receptors are members of the G protein-coupled receptor superfamily and five genes
encoding the D1-D5 subtypes of dopamine receptor have been identified (36). Studies with subtype-
specific synthetic dopamine receptor agonists indicate that it is the D1 and/or D5 dopamine receptors that
stimulate steroid receptor transcriptional activity (33, 34, 37), and this is associated with D1 and D5
dopamine receptor stimulation of intracellular cyclic 3’,5’-adenosine monophosphate (cAMP) production
The mechanisms by which the dopaminergic cell signaling pathway communicates with ERα are not well defined, but it is presumed that increased ERα phosphorylation contributes to this process. In this regard, it is interesting to note that cAMP signaling pathways stimulate ERα transcriptional activity and phosphorylation (38, 39). The chicken PR is also ligand-independently activated by treatment of cells with dopamine or agents that increase intracellular cAMP levels (31, 40). However, cAMP activation of PR-dependent transcription is not accompanied by increased receptor phosphorylation, but rather by an increase in the phosphorylation of the SRC-1 coactivator with which the receptor interacts to stimulate gene expression (41, 42). Taken together, the data support a model in which dopamine and cAMP signaling pathways stimulate gene expression in a receptor-specific manner.

Alterations in the biology of dopamine and its receptors play an important role in a number of human diseases, such as Parkinson’s disease, as well as contribute to the reward seeking behaviors associated with cocaine abuse (43-45). The molecular mechanisms of dopamine and dopamine receptor action have therefore been extensively studied, and these efforts have been aided by the identification of high affinity and potent ligands for dopamine receptors. One such compound, SKF-82958 (SKF), is a full dopamine D1-subtype selective receptor agonist with greater potency than dopamine (46, 47). SKF has also been shown to stimulate the transcriptional activity of ERα in SK-N-SH neuroblastoma and MCF-7 breast cancer cells (27, 37). We therefore used SKF-82958 to determine the ability of dopaminergic signaling pathways to regulate ERβ transcriptional activity. We observed that this D1 receptor-selective agonist stimulated the transcriptional activity of ERα, but had negligible agonist activity for ERβ. We also found that SKF-82958 stimulates phosphorylation of ERα to an extent similar to that observed for E2. However, SKF-82958 competed with E2 for binding to the receptor, suggesting that it exerts at least some of its effects on ERα transcriptional activity as an ERα agonist. Stimulation of ERα transactivation was greater than that anticipated from its relative binding affinity for ERα, and we therefore examined the ability of SKF-82958 to stimulate intracellular signal transduction pathways. While SKF-82958 did not increase cAMP production, it did stimulate pathways leading to activation of AP-1, a transcription factor.
known to functionally interact with many steroid receptors (3), and we therefore examined the contribution of AP-1 to SKF-induced ERα transcriptional activity. These studies provide novel information on the ability of a compound to simultaneously stimulate the activity of two transcription factors and in so doing produce robust stimulation of gene expression through a combinatorial response within the cell.

**EXPERIMENTAL PROCEDURES**

**Chemicals**

17β-Estradiol (E2), tetradecanoylphorbol-13-acetate (TPA) and poly-L-lysine were obtained from Sigma Chemical Company (St. Louis, MO). The antiestrogens, ICI 182,780 (ICI) and 4-hydroxytamoxifen were gifts from Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK) and D. Salin-Drouin (Laboratoires Besins Iscovesco, Paris, France), respectively. 8-Bromo-cyclic AMP (8Br-cAMP) and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Research Biochemicals International (Natick, MA) as were dopamine and the synthetic D1 receptor agonist, SKF-82958. All other chemicals were reagent grade.

**Plasmids**

The mammalian expression vectors for wild type human ERα (pCMV5-hERα) and its corresponding phosphorylation mutants (S104A/S106A/S118A, S118A and S167A) have been described previously (39) as have the plasmids for human ERβ [pCMV5-hERβ (48)], mouse ERα-Y541A (49), c-Jun [pRSV-jun (50)], c-Fos [pBK-28 (51)] and the pRSV-Not control vector (52). Experiments with deletion mutants of ERα used constructs encoding wild-type ERα (amino acids 1-595), ERα-N282G (amino acids 1-282), ERα-179C (amino acids 179-595), ERα-3x (amino acids 1-595 with three point mutations – D538A/E542A/D545A) and ERα-179C-3x (amino acids 179-595 with the D538A/E542A/D545A
mutations) expressed from the pRST7 vector (20). Plasmids for the SRC-1e, TIF2, RAC3 and CBP coactivators in the pCR3.1 expression vector have been previously described (53). The estrogen-responsive reporter genes, ERE-E1b-CAT (54) and ERE-E1b-LUC (55), have been used in previous studies and both contain nucleotides –331 to –87 of the vitellogenin A2 promoter linked upstream of the adenovirus E1b TATA box. The p-169αCG-CAT and p-100αCG-CAT reporter genes contain portions of the chorionic gonadotropin gene, with or without a cAMP response element (CRE), respectively, upstream of the chloramphenicol acetyltransferase (CAT) reporter gene (56). The coll73-CAT reporter and the coll60-CAT reporters contain portions of the collagenase gene upstream of CAT differing in the inclusion or exclusion of a TRE, respectively (57). An expression vector for β-galactosidase, pCMVβ was obtained from Clontech (Palo Alto, CA).

The mammalian expression vector for Flag-hERα was constructed as follows: the yeast expression vector for human ERα, YEPE2 (58) was digested with TthIII, blunted, and subsequently digested with KpnI. The resulting fragment was cloned into the BamHI (blunted) and KpnI sites of pSelect-1 (Promega). The ER cDNA was removed from the resulting vector with KpnI and SalI restriction enzyme digestion, and subcloned into the mammalian expression vector, pJ3Ω (59) to create pJ3-hER Val400. The amino-terminal Flag epitope was created by utilizing a PCR approach. Briefly, a 5’ primer (5’-GGGGTCGACCATGACTACAAGGACGACGATGACAAGATGACCATGACCCTCCAC) encoding a methionine residue linked to the Flag epitope sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) and the first six amino acids of human ERα and a 3’ primer (5’-GCGCTTGTGTTTCAACATTCTCC) corresponding to nucleotides 1017-1039 were used to amplify a 844 base pair nucleotide fragment of the ERα cDNA using pSVMTwt:ER as template (30). The resulting PCR product was digested with SalI and NotI and substituted for the SalI-NotI fragment of pJ3-hER Val400 to create pJ3-Flag-hERα Val400. To replace valine 400 with cDNA encoding the wild type amino acid (glycine 400), the NotI-SacI fragment of pSVMTwt:ER (corresponding to amino acids 65 to 595) was substituted for the corresponding region of pJ3-Flag-hERα Val400 to create pJ3-Flag-hERα.
Reporter genes lacking the putative TPA response element (TRE) were generated from the parent ERE-E1b-CAT plasmid by deletion or site-directed mutagenesis. In the former case, a 195 base pair fragment of ERE-E1b-CAT was removed by digestion with \textit{NdeI} and \textit{Eco0109I}. The resulting vector was blunt ended with Klenow and religated to yield ERE-E1b-CAT(\textit{\Delta NdeEco}). To remove the putative TRE sequence by site-directed mutagenesis, the \textit{SspI-HindIII} fragment of ERE-E1b-CAT was subcloned into pALTER-1 (Promega). Using the PCR Site Directed Mutagenesis System (Life Technologies) and a mutagenic primer, the putative TRE sequence, TGACACA, was mutated to GGACTCA following the manufacturer’s recommendations. The latter sequence had been demonstrated previously to prevent AP-1 binding (60). Following sequencing to verify appropriate nucleotide substitutions, a \textit{NdeI-Eco0109I} fragment was removed from pALTER-1 and substituted for the comparable region of ERE-E1b-CAT to generate ERE-E1b-CAT(mTRE).

\textbf{Cell Culture, DNA Transfections and Trans-Activation Assays}

HeLa cells were routinely maintained in Dulbecco’s Modified Eagle’s media (DMEM) supplemented with 10% fetal bovine serum (FBS). DNA transfections were performed by either Lipofectin (Life Technologies, Grand Island, NY) or adenovirus-mediated approaches (61). For trans-activation assays, 24 h prior to transfection, 3 x 10^5 HeLa cells were seeded per well of a 6-well multiwell dish in phenol red-free DMEM containing 5% dextran-coated-charcoal stripped serum (sFBS). For Lipofectin transfections, cells were incubated with the indicated DNAs and Lipofectin according to the manufacturer’s guidelines. Six hours later, the DNA/Lipofectin mixture was removed and cells were fed with phenol red-free media containing 5% sFBS and the indicated treatments, and 24 h thereafter the cells were harvested.

To prepare reagents for adenovirus-mediated transfections, replication-deficient adenovirus dl312 was propagated and covalently modified with poly-L-lysine by the method of Cristiano \textit{et al.} (62) modified as described previously (61). CsCl-purified fractions of the modified virus were stored at -80 C until use. Adenovirus-DNA complexes were prepared by adding the lysine-modified adenovirus to
plasmid DNA and subsequently incubating with a 200-fold molar excess of poly-L-lysine (M, 18-20,000). The adenovirus-DNA-lysine complex was then added to the cells at a virus to cell multiplicity of infection of 500:1. After incubation for 2 h, the medium was replaced with phenol-red-free DMEM supplemented with 5% sFBS. Hormones and/or other treatments, as indicated, were added to the cells 4 h later, and the cells were then harvested 24 h thereafter.

Assays of reporter gene expression were performed on cell extracts prepared by lysing cells by rapid freeze-thaw or addition of lysis buffer (Promega). CAT activity was measured by a phase-extraction method utilizing [\(^3\)H]chloramphenicol (NEN, Boston, MA) and butyryl-coenzyme A (Pharmacia) as substrates (30, 63). Luciferase activity was measured using the Luciferase Assay System (Promega). Duplicate samples were measured in each experiment and data are presented as the average ± SEM of at least three experiments normalized to protein content measured by BioRad protein assay reagent or β-galactosidase.

**Relative Binding Affinity Assays**

Relative receptor binding affinities were determined *in vivo* as described previously (64). Briefly, the adenovirus-mediated DNA transfer procedure was used to transfect HeLa cells with 0.25 µg/well of the appropriate expression vector (pCMV\(_5\)-hER\(\alpha\) or pCMV\(_5\)-hER\(\beta\)). Twenty-four hours thereafter, media was aspirated from wells, and replaced with phenol-red-free DMEM containing 5% sFBS, ~1 pmol [\(^3\)H]estradiol (NEN, Boston, MA) and increasing concentrations (ranging from 10\(^{-10}\) to 10\(^{-3}\) M) of either unlabelled E2, SKF-82958 or dopamine. Following 2 h incubation at 37°C, media was aspirated from plates, cells were washed 3 times in cold PBS, and then incubated in 100% ethanol for 15 minutes at room temperature to extract bound steroid. The amount of ER-bound [\(^3\)H]estradiol in the ethanol extract was quantified with a Beckman LS 6500 scintillation counter and Biodegradable Counting Scintillant (Amersham, Arlington Heights, IL).
Western Blot Analyses

To assess ER expression, cells were transfected as described above, and harvested for Western blot analysis 24 h thereafter. Cell pellets were resuspended in 50 mM Tris buffer (pH 8.0) containing 400 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.2% Sarkosyl, 100 µM sodium vanadate, 10 mM sodium molybdate and 20 mM NaF, incubated on ice for 60 minutes, and centrifuged at 12,000 g for 10 minutes at 4 C. The resulting supernatant was mixed with SDS-PAGE loading buffer, and resolved by 7.5% SDS-PAGE and electrotransferred to nitrocellulose. Filters were incubated sequentially with primary antibodies against ERα (H222) or the Flag epitope (M2; Sigma) and the appropriate horseradish peroxidase (HRP)-conjugated antibody. Immunodetection was performed with enhanced chemiluminescence (ECL) reagents as recommended by the manufacturer (Amersham, Piscataway, NJ).

32P Labeling and ERα Immunoprecipitation

Cells were transfected with either pJ3-Flag-hERα or pJ3Ω by the adenovirus method. Eight hours thereafter, media was removed, cells were rinsed with phosphate-free DMEM and re-fed with phosphate-free DMEM containing 5% dialyzed sFCS (HyClone, Logan, UT). Radiolabelled inorganic phosphate (83 µCi/ml media) was added, and cells were incubated for 16 hours. Vehicle (ethanol), 1 nM E2 or 25 µM SKF-82958 was added 90 minutes prior to harvesting cells. Cells were lysed in 50 mM Tris (pH 8.0) containing 5 mM EDTA, 1% Triton X-100, 0.2% Sarkosyl, 400 mM NaCl, 200 µM sodium vanadate, 10 mM sodium molybdate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 µg/ml aprotinin, 3 µg/ml leupeptin, 3 µg/ml pepstatin, 20 mM disodium p-nitrophenylphosphate, 25 mM β-glycerophosphate, 5 mM L-Phe-Ala and 0.15 mM 1,10-phenanthroline for 60 minutes on ice. Lysates were precleared with rabbit anti-rat IgG and protein-A sepharose prior to the sequential addition of 5 µg of H222 antibody, 10 µg rabbit anti-rat IgG and protein A-sepharose. The immunoreactive sepharose complex was washed with 100 mM Tris buffer (pH 9.0) containing 150 mM NaCl, 1% Triton, 1% Tween-20, 20 mM sodium fluoride, 1 mM sodium vanadate and 10 mM sodium molybdate, and eluted.
with 1 M acetic acid. Samples were resolved by 7.5% SDS-PAGE and electroblotted to nitrocellulose
and subjected to autoradiography at –80C. Protein levels were subsequently assessed by subjecting this
same membrane to Western blot analysis using the anti-Flag M2 antibody, followed by a secondary
antibody of horseradish peroxidase-conjugated sheep anti-mouse IgG. Signals were revealed with ECL
methods following the manufacturer’s instructions (Amersham). The 32P signals were quantitated by a
Betagen Betascope 603 Blot Analyzer and normalized to immunoprecipitated protein assessed by
Western blot analysis and quantitated by scanning laser densitometry (Model 620, BioRad Laboratories).

RESULTS

SKF-82958 activation of ERα-dependent gene transcription

As previously reported (37), the dopamine D1-selective agonist SKF-82958 (±-6-chloro-7,8-
dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; see Fig. 1A), like dopamine, can
stimulate ERα transcriptional activity, and this is inhibited by the pure ER antagonist ICI 182,780 (Fig.
1B). Dose response studies indicated that half-maximal induction of ER-directed gene expression by
SKF-82958 occurred at 2 µM (data not shown). In contrast, maximal dopamine induction of ER-directed
gene expression occurs at 100-250 µM (23, 30, 31), suggesting that SKF-82958 is a more potent activator
of this response. However, the potency (Km) and maximum efficacy of SKF-82958 induction of cAMP
are similar to that for dopamine in rat brain striatum after treatment in vivo (46). This discrepancy
suggested that there may be mechanistic differences in the ability of SKF-82958 and dopamine to
stimulate ERα transcriptional activity.

To investigate this further relative to the mechanisms of SKF-82958 activation of ERα-dependent
gene expression, SKF stimulation of cAMP production in HeLa cells was examined by
radioimmunoassay and compared to the ability of SKF to activate ER-dependent gene expression. No
correlation was found, as micromolar doses of SKF-82958 failed to significantly elevate cAMP levels
To more closely mimic conditions under which our trans-activation assays are performed, the ability of SKF-82958 to stimulate CRE-dependent transcription was assessed. The -169αCG-CAT gene is composed of a fragment of the human chorionic gonadotropin gene promoter containing a CRE element, linked upstream of the CAT reporter gene and is activated by cAMP stimulation of the CREB transcription factor (56). The -100αCG-CAT reporter gene which lacks the CRE was used as a negative control. As shown in Fig. 1C, CRE-dependent transcription was stimulated by 8Br-cAMP and, more modestly, by dopamine. However, there was no stimulation of CRE-dependent transcription by E2 or SKF-82958. These results suggest that SKF-82958 is not acting through stimulation of cAMP-dependent dopaminergic signaling in this system. This result led to a consideration of whether this compound activated ER-dependent gene expression through direct binding to ERα. This question is further underscored by the ring structure of this synthetic D1 receptor agonist (Fig. 1A) which is reminiscent of the structures of some ER agonists and antagonists (65).

**SKF-82958 binds to ERα and ERβ, but preferentially activates ERα**

In order to determine whether SKF-82958 could bind to ERs, whole cell competitive hormone binding assays were performed in HeLa cells transfected with expression vectors for either ERα or ERβ. Cells were incubated with [3H]estradiol and increasing amounts of unlabeled E2, SKF-82958 or dopamine. The displacement curves for ERα and ERβ indicate that SKF-82958 can compete weakly with estradiol for binding to both forms of ER but that dopamine is unable to do so (Fig. 2, A & B). The average relative binding affinities of SKF-82958 in comparison to E2 (100) for ERα (0.077 ± 0.018; n=4) and ERβ (0.069 ± 0.009; n=3) are similar, and are comparable to those measured by other investigators for low affinity ER agonists such as bisphenol A (16). This result suggests that activation of ER-dependent gene expression may arise through SKF-82958 binding to ERs and serving as a weak receptor agonist, and we therefore wanted to determine whether SKF-82958 could activate both subtypes of ER.

HeLa cells were transfected with expression vectors for human ERα or ERβ and the ERE-E1b-Luc reporter gene, which consists of the estrogen response element (ERE) from the vitellogenin A2 promoter
linked to the TATA box sequence of the adenovirus E1b gene and luciferase reporter gene. SKF-82958 was not able to significantly activate ERβ-dependent gene expression in comparison to the ability of this compound to stimulate ERα transcriptional activity as shown in Fig. 3A or in dose response studies (data not shown). SKF therefore appears to be an ERα-preferential agonist. To ensure that SKF-82958 induction of ERα-dependent gene expression was not due to ligand stabilization of ERα expression, Western blot analysis of ERα expression in cells treated with vehicle, E2 and SKF-82958 was performed, and like E2 and dopamine (30, 53), SKF was found to down-regulate the expression of ERα in HeLa cells (Fig. 3B). The ability of SRC family and CBP coactivators to enhance SKF-induced ERα transactivation was also examined. Each was able to significantly enhance the transcriptional activity of ERα (Fig. 3C), suggesting that SKF-82958 binding to the receptor allows coactivator-ERα functional interactions.

**Characterization of Flag-tagged ERα**

Activation of human ERα by E2 is accompanied by increased receptor phosphorylation (39, 66). In order to determine if SKF-82958 alters the biochemical properties of ERα, the phosphorylation status of the receptor was assessed in HeLa cells treated with SKF-82958 versus E2. First, an expression vector was constructed for Flag-ERα so that distinct antibodies could be used for immunoprecipitation (anti-ERα) and for receptor quantitation by Western blot analysis (anti-Flag). To demonstrate that the M2 antibody against the Flag epitope reacted with only Flag-ERα, cell lysates were prepared from HeLa cells transfected with either pJ3-Flag-hERα or empty parent vector (pJ3Ω), and subjected to Western blot analysis. The M2 antibody detected an appropriately sized band in HeLa cells transfected with either pJ3-Flag-hERα or empty parent vector (pJ3Ω), and subjected to Western blot analysis. The M2 antibody detected an appropriately sized band in HeLa cells transfected with pJ3-Flag-hERα, but not in mock-transfected cells (Fig. 4A). In a separate experiment, the hERα antibody, H222, was used to ensure that the protein encoded by the pJ3-Flag-ERα expression vector was immunoreactive with ERα antibodies. As expected, Western blot analysis demonstrated that the Flag-ERα migrated with a slightly lower mobility than wild type ERα (Fig. 4B).
The transcriptional activity of Flag-ERα was compared to wild type ERα in transient transfection experiments to ensure that the fusion of the Flag epitope to the N-terminus of ERα did not adversely affect the relative ability of the chimeric receptor to activate expression of a synthetic target gene. HeLa cells were transfected with the ERE-E1b-Luc reporter gene, as well as an expression vector for β-galactosidase (pCMVβ), and increasing amounts (0 → 1000 ng) of expression vectors for wild type or Flag-tagged ERα. In cells treated with 1 nM E2 both receptors exhibited comparable transcriptional activities in the linear portion of the dose response curve (Fig. 4C). Only when very high levels (≥ 500 ng) of the expression vectors were introduced into cells was a modest reduction in activity observed for Flag-ERα relative to wild type ERα. Equivalent amounts of vectors for wild type and epitope-tagged ERα were then transfected into HeLa cells and the ability of each receptor to activate transcription following SKF-treatment was determined. Both receptors were activated to an equivalent extent by SKF-82958 (Fig. 4D). Taken together these data indicate that the transcriptional activity of Flag-ERα stimulated by either the natural ligand (E2) or the weakly estrogenic SKF-82958 is comparable to untagged ERα, and Flag-ERα was used therefore for analysis in subsequent phosphorylation studies.

**SKF-82958 induces phosphorylation of Flag-ERα**

In order to determine if activation of ERα-dependent gene expression by SKF-82958 is accompanied by alterations in the biochemical properties of the receptor, Flag-ERα was expressed in HeLa cells using the adenovirus transfection method. Cells were subsequently radiolabeled with [32P]orthophosphate, and treated with vehicle, 1 nM E2 or 25µM SKF-82958 for 90 minutes. Flag-ERα was immunopurified with the H222 anti-ERα antibody, resolved by 7.5% SDS-PAGE, and electrotransferred to nitrocellulose. The resulting blot was subjected to autoradiography to visualize the relative amount of phosphate incorporated into receptor, and was subsequently subjected to Western blot analysis with an anti-Flag antibody (M2) to quantitate relative receptor expression levels. A representative blot indicates that SKF-82958 significantly increased the overall phosphorylation level of ERα relative to 32P incorporation observed in
cells treated with vehicle alone (Fig. 5A). As expected, the phosphorylation level of Flag-ERα isolated from cells treated with E2 was also significantly increased in comparison to basal levels. When protein levels were taken into account, the data averaged from 4 experiments indicate that E2 increased ER phosphorylation by $1.7 \pm 0.4$-fold, while SKF treatment increased ER phosphorylation by $2.2 \pm 0.4$-fold (Fig. 5B).

To determine if any of the known ER serine phosphorylation sites are critical for activation of the receptor by the SKF signal transduction pathway(s), the ability of this putative ligand to stimulate the activity of ER phosphorylation site mutants was assessed (39, 66, 67). Although SKF-82958 was able to stimulate the transcriptional activity of each amino-terminal phosphorylation mutant, activation of S118A (2.7 ± 0.3-fold) and S104A/S106A/S118A ERα (2.5 ± 0.5-fold) ERα mutants was decreased relative to the ability of this compound to activate either wild type (4.5 ± 0.3-fold) or the S167A (4.7 ± 0.2-fold) mutant. These data are consistent with the effects of these mutations on E2-dependent activity (39, 66 and our data), and suggest that serines 118 and possibly 104/106 may contribute to, but are not required for, activation of ERα in response to SKF-82958 treatment.

**Functional domains of ERα required for SKF-82958 activation**

To more generally test the regions of ER required for SKF activation, the ability of this compound to stimulate the transcriptional activity of a series of ER mutants (Fig. 6A) was tested. Mutation of the AF-2 domain (D538A/E542A/D545A) in the ERα-3x mutant reduced the ability of E2 and SKF to stimulate ER activity by ~64% and ~78%, respectively, suggesting that the C-terminal AF-2 domain contributes to both mechanisms of activation (Fig. 6B). An ER mutant lacking the ligand binding and F domains (N282G) was not activated by SKF-82958 or E2 treatment, and this is in agreement with previous studies in SK-N-SH neuroblastoma cells in which the C-terminus of ERα was required for SKF-82958 activation of target gene expression (37). Deletion of the N-terminal AF-1 domain reduced E2-dependent transcriptional activity by ~62%, and SKF-dependent gene expression by ~70% in the ERα-179C mutant
in comparison to wild type receptor, while deletion of the A/B domain in conjunction with the 3x mutation yielded an ER mutant (ERα-179C-3x) unable to activate gene expression in comparison to the empty parent vector. Taken together these data suggest that the N- and C-terminal domains of ERα both contribute to receptor activity stimulated by SKF-82958.

**SKF-82958 activates gene expression from a TPA response element-containing promoter**

A growing body of evidence indicates that most receptors, whether membrane or nuclear, activate and/or interact with numerous signaling pathways. The dual actions of SKF-82958 in activating dopamine D1 receptors and ERα provided an opportunity to explore the impact of multiple signaling mechanisms induced by a multifunctional activator on nuclear receptor-induced transcription. Although SKF-82958 did not appear to appreciably increase cAMP levels in HeLa cells, activation of dopamine D1 receptors has also been shown to stimulate the activity of PKC (68, 69). We therefore examined whether SKF treatment of cells could stimulate the activity of a sequence-specific transcription factor, AP-1, which is a downstream target of the PKC pathway (70). AP-1 is composed of either homo- or heterodimers within the Jun family (c-Jun, JunB and JunD) or between members of the Jun and Fos (c-Fos, FosB, Fra1 and Fra2) families (71). HeLa cells were transfected with a coll73-CAT reporter, which contains a TRE to which the AP-1 proteins c-Jun and c-Fos bind, or coll60-CAT reporter plasmid lacking the TRE (Fig. 7A) and treated with ethanol (vehicle), 1 nM E2, 100 nM TPA, 10 µM SKF-82958 or 100 nM 4-hydroxytamoxifen. TPA strongly induced TRE-dependent gene expression from coll73-CAT, whereas neither E2 nor 4-hydroxytamoxifen resulted in transcriptional activation (Fig. 7B). In contrast, treatment with SKF-82958 resulted in weaker, but significant (p<0.05), stimulation of TRE-dependent transcriptional activity. None of the treatments increased transcription from a reporter gene (coll60-CAT) lacking the TRE enhancer.
**Enhanced SKF-82958 stimulation of ERα-dependent gene transcription by an upstream TRE**

Since SKF weakly stimulated TRE-dependent gene expression and the ERE-E1b-CAT reporter gene contains a putative TRE site in the vector backbone ∼255 base pairs upstream of the ERE, we examined the contribution of TRE binding factors to SKF induction of ERα-dependent gene expression. Thus, SKF-82958 or E2-induced CAT expression were compared in the intact ERE-E1b-CAT reporter versus constructs in which the putative TRE site was eliminated by deletion (ΔNde-Eco) or point (mTRE) mutagenesis (Fig. 8A). The latter point mutant was examined to rule out the possibility that the deletion mutant introduced structural perturbations or removed other cryptic DNA sequences from the reporter that might alter transcriptional responses. As shown in Fig. 8B, significant CAT expression was induced by treatment with E2 or SKF-82958 from either the intact or mutant forms of the ERE-E1b-CAT reporter gene. Moreover, the fold-induction by E2 was similar for the three reporter genes, whereas SKF-82958 induction of CAT gene expression was diminished by ∼23% and ∼28%, when the TRE was deleted or mutated, respectively. The similarity in SKF-82958 effect on gene expression between the reporters generated by deletion versus site directed-mutagenesis is consistent with the interpretation that it is the upstream TRE element, rather than some other element or structural alteration, that contributes to the magnitude of SKF-82958-induced ERα transactivation under these conditions. Moreover, in experiments in which a ClaI to BglII linear fragment of the ERE-E1b-Luc plasmid encompassing just the ERE, E1b and luciferase sequences was transfected into HeLa cells with an ERα expression plasmid, SKF stimulation of ERα activity relative to E2 was 50% the level seen for unaltered (circular) target gene (data not shown). Taken together, these results support the hypothesis that TRE elements in reporter plasmids may enhance, but are not required for, induction of ERα-dependent gene transcription by multifunctional ligands such as SKF-82958.

Since TRE-dependent activity significantly enhanced SKF activation of ERα-dependent gene expression, and since c-Jun has been shown to bind to the amino-terminus of ERα (57), we wanted to ensure that the LBD and AF-2 could support SKF activation of gene expression in the absence of AF-1.
We therefore examined the ability of ERα-179C to be activated by SKF-82958 in the absence of the upstream TRE. As shown in Fig. 8C, loss of the reporter’s TRE site and the receptor’s AF-1 domain significantly compromises the ability of SKF-82959 to activate ERα-dependent gene expression, consistent with the interpretation that both the AF-1 and TRE contribute to SKF-induced transcriptional activity. Taken together, these data suggest that SKF-82958 on its own is a weak ERα agonist and that the robust activation seen with full-length receptor is a result of the synergistic activation of ERα and cellular factors, such as c-Jun or c-Fos, that can bind to the TRE (60).

Effect of AP-1 overexpression on ERα transactivation by SKF-82958

The above observations suggest that transcription factors able to interact with the TRE binding site can contribute to ERα-mediated gene expression stimulated by SKF-82958. Protein-protein interactions have been reported between the AP-1 protein c-Jun and ER, but not between c-Fos and ER, and occur principally through the N-terminal AF-1 domain of the ER protein (57). In order to further investigate the ability of SKF to synergistically activate ER/AP-1-dependent transcription, we tested the hypothesis that increased Jun/Fos expression would enhance SKF-82958 activation of ERα-dependent transcription. HeLa cells were co-transfected with expression plasmids for c-Jun, c-Fos, or equivalent levels of c-Jun+c-Fos (12.5-100 ng/well), with total DNA/well maintained constant by altering the levels of co-transfected empty plasmid. Jun overexpression resulted in strong and significant increases in basal, E2 and SKF-82958 induced transcription from ERE-E1b-CAT but not from reporter genes lacking the TRE (ΔNde-Eco), suggesting that c-Jun activated transcription was primarily dependent on the intact reporter’s TRE and not through binding to ERα (Fig. 9A). Fos overexpression resulted in only very modest increases in the effects of E2 and SKF-82958, with no significant effect on basal activity (Fig. 9B). The result from the combination of c-Jun with c-Fos was similar to that of c-Jun alone (Fig. 9C). In all experiments performed with the ERE-E1b-CAT(ΔNde-Eco) reporter construct lacking the TRE, no significant
increases in transcriptional activation were induced by AP-1 overexpression (Figs. 9, A-C), suggesting that the TRE binding site was required for strong AP-1 effects.

**DISCUSSION**

The relatively high concentration of SKF-82958 required to achieve ERα transcriptional activity in comparison to dopamine D1 receptor activation suggested that this compound was an ERα agonist, and our relative binding affinity analyses demonstrated that SKF competed with E2 for binding to either ERα or ERβ. However, the results obtained in this study demonstrate that SKF-82958 stimulates the transcriptional activity of ERα, but not ERβ, and therefore SKF-82958 is an ERα selective agonist. Intriguingly, our studies also demonstrated that SKF stimulates the transcriptional activity of AP-1, and provides evidence that in the appropriate promoter context activation of target gene expression by SKF is the combinatorial result of AP-1 and ERα activation. Understanding the role of AP-1 in SKF-dependent ERα transactivation is particularly important given the ability of SKF to activate both transcription factors. In so doing, the results of these studies provide an example of how other transcription factors can seemingly enhance the potency of weak ER ligands.

Although SKF-82958 is a full agonist of dopamine D1 receptors, it failed to stimulate increases in intracellular cAMP, nor was it able to stimulate CRE-dependent gene expression in our HeLa cells. We had previously shown that dopamine treatment of HeLa cells increased cAMP levels in a dose-dependent manner *in vitro* (30), and the inability of SKF to do so here was unexpected. Although SKF-induction of cAMP in SK-N-SH cells had not been characterized, the protein kinase A inhibitor, H89, partially blocked ERα transactivation by SKF-82958 (37), suggesting that a cAMP signaling transduction pathway was playing a role in these cells. Similarly, H89 reduced SKF induction of ER transcription activity in MCF-7 cells (27). These reports are consistent with the ability of SKF to stimulate adenylate cyclase and cAMP production via the dopamine D1 receptor (46, 47), and it is possible that in these cell models ERα
transactivation by SKF is at least partially cAMP/protein kinase A dependent and/or that H89 is inhibiting the activity of other signaling pathways able to crosstalk with ERα or AP-1. Indeed, while H89 effectively inhibits protein kinase A, it also blocks the activity of other kinases including protein kinase B (Akt) and mitogen-and stress-activated protein kinase-1 (72).

The inability of SKF to stimulate ERβ transcriptional activity is unlikely to be due to the minor differences in the relative binding affinities of this compound for ERα and ERβ. A large number of naturally-occurring substances, as well as pharmacological and environmental agents, bind to ERs (16, 17). The crystal structures of receptors complexed with E2, diethylstilbestrol, raloxifene or 4-hydroxytamoxifen, and molecular modeling studies suggest that binding of a phenolic group to the A-ring binding pocket of the receptors’ ligand binding domains is a common feature (14, 73-75). While SKF-82958 does not possess a simple phenolic ring characteristic of many ER ligands (Fig. 1A), it does have a hydroxy-phenolic ring with a large, bulky chlorine substituent. Based on the ability of C(2) substituted derivatives of E2 and estrone (2-hydroxyestradiol and 2-hydroxyestrone, respectively) to have severely reduced relative binding affinities for ERs (16, 17) and the chlorine atom on SKF-82958, it was unexpected that SKF would inhibit E2 occupancy of the ligand binding pocket. This result is perhaps even more surprising in view of the inability of dopamine to bind to ERα or ERβ, since dopamine also possesses a hydroxy-phenol ring. However, it is possible that the remainder of the dopamine molecule is of insufficient size to interact with other regions of the ligand binding pocket required for high affinity binding. It will be interesting to determine whether chemical derivatives of SKF-82958 can be generated with increased receptor affinity.

There is significant interest in identifying ER subtype selective agonists and antagonists, and several investigators have made progress in identifying and characterizing such compounds. These include a cis-diethyl-substituted tetrahydrochrysene which has a 4-fold preferential binding affinity for ERβ and is an ERα agonist and complete ERβ antagonist (76), and a methoxychlor metabolite that inhibits estrogen-induced ERβ activity, yet stimulates the transcriptional activity of ERα (77). Potency selective agonists
have also been identified such as pyrazole, which has a 120-fold greater potency for stimulating ERα activity in comparison to ERβ (76), and A-ring reduced metabolites of the 19-nor synthetic progestins, norethisterone and Gestodene, which have at least a 100-fold greater potency for ERα in comparison to ERβ transcriptional activity (78). In addition to these compounds, differences in the ability of steroidal derivatives and non-steroidal phytoestrogens to bind to ERα and ERβ have also been reported (16, 17). Moreover, the differences in the relative agonist and antagonistic activity of several of these novel compounds has been found to correlate with changes in the conformation of the receptors and their ability to bind to SRC family coactivators (79). For instance, the ERα agonist propyl pyrazole triol induces an agonistic conformational change in ERα and promotes interaction of this receptor with SRC-1, GRIP1 and ACTR, but does not promote interaction of ERβ with these coactivators. We have demonstrated that SRC family coactivators as well as the general coactivator CBP can enhance SKF-induced ERα transactivation and this is consistent with SKF inducing a conformational change able to promote ERα-coactivator interactions.

Having established that SKF-82958 is an ERα-selective agonist, we examined the mechanism(s) by which it stimulated ERα-dependent gene expression. Deletion of the amino-terminal A/B domain of ERα indicates that the AF-1 domain is not required for SKF-82958 activation of ERα-dependent gene expression, nor is a fully functional AF-2 as demonstrated by data from the ERα-3x mutant. However, both these mutations reduce the relative ability of ERα to activate gene expression, and the AF-1 and AF-2 regions are therefore required to yield a full response to SKF stimulation as has been shown in other contexts for E2 and SKF (20, 37). Deletion of the entire LBD confirms that SKF-induced ERα transcriptional activity involves the receptor’s carboxy terminus. As noted above, mutations of the core domain of AF2 reduced, but did not block, the ability of SKF-mediated signaling pathways to activate gene expression, except when combined with deletions of the receptor’s A/B domain. This supports the supposition that SKF activation of ERα transcriptional activity requires the cooperative effects of both the
amino- and carboxy-terminal domains. The inability of SKF to stimulate ERβ transcriptional activity is interesting in view of the contributions of ERα’s AF-1 domain to this response and differences in the structure and relative transcriptional activity of the AF-1 domains of the two ER subtypes (11-13). It should also be noted that the lack of ERβ transactivation by SKF is not due to an inability of ERβ to functionally interact with AP-1, as we have observed this mechanism in the context of cAMP signaling pathways (K.M. Coleman & C.L. Smith, unpublished data).

Stimulation of ERα transcriptional activity by SKF is accompanied by increases in the levels of receptor phosphorylation which are similar to those induced in parallel experiments by E2. However, the enzyme(s) responsible for this post-translational modification and the residue(s) within ERα that are phosphorylated following SKF treatment remain undefined. The similarity of SKF- and E2-induced phosphorylation of ERα does not correlate with the relative ability of these two compounds to activate this receptor’s transcriptional activity, and this suggests that SKF-induced phosphorylation of ERα may not be important for this process. Although E2 and growth factor signaling pathways able to stimulate ERα activity induce receptor phosphorylation (4, 21), so do the ERα antagonists, ICI 164,384 and 4-hydroxytamoxifen (39, 66). Taken together, these data suggest that the role of receptor phosphorylation in ligand-induced ERα function may be quite complex, and possibly ligand-specific. Alternatively, it is possible that signal transduction pathways initiated by SKF-82958 (see below) could affect receptor-dependent gene expression by phosphorylating coactivators and altering their intrinsic transcriptional activity. For instance, 8Br-cAMP treatment of COS-1 cells phosphorylates SRC-1 and stimulates its intrinsic transcriptional activity (42). Similarly, growth factor signaling pathways increase the transcriptional activity of the GRIP1 and AIB1 coactivators (80, 81) and cAMP and MAPK signaling pathways increase CBP activity (82, 83). Thus, SKF-induced, ERα-dependent gene expression may also be influenced by SKF-induced alterations in coactivator function.
The ability of SKF to stimulate AP-1 activity contributes to the ability of this compound to stimulate ERα dependent gene expression on the ERE-E1b-CAT reporter gene. Activation of AP-1, however, is insufficient to stimulate CAT activity from this reporter in cells lacking ERα (see Fig. 6B). Several lines of evidence indicate that the TRE site contributes to the magnitude of target gene expression by ERα and SKF-82958. First, this synthetic dopamine receptor agonist did activate transcription from a TRE-dependent reporter in the absence of co-transfected ER. Moreover, eliminating a functional AP-1 element ~255 bp upstream from the ERE-E1b-CAT reporter sequence, either by deletion or site-directed mutagenesis, significantly reduced the ability of SKF to stimulate ER transactivation. Interactions between ERα and c-Jun are mediated via the amino-terminus of ERα (57), and eliminating both the upstream AP-1 binding site from ERE-E1b-CAT and the ERα’s AF-1 domain severely compromised the ability of SKF to activate ERα-dependent gene expression, suggesting that the A/B domain contributes to this activity through its ability to interact with AP-1 and/or accessory transcription factors which link AP-1 and ERα function.

Although steroid receptors can activate the transcription of target genes containing only their response elements and minimal promoters such as TATA boxes, natural target gene promoters are significantly more complex and contain binding sites for many different transcription factors. Regulation of target gene expression is therefore a result of the coordinate regulation of the activity of all transcription factors that can bind to a target promoter, and for this reason, it is important to examine the interaction between AP-1 and ERα. The mechanisms by which SKF enhanced activation of the TRE (coll73-CAT) reporter gene are not defined, but could be mediated by increased expression of AP-1 transcription factors and/or their activation by signal transduction pathway-induced post-translational modifications [e.g. phosphorylation (71, 84)]. However, we demonstrated that the magnitude of SKF-dependent ERα transactivation paralleled the relative levels of c-Jun expression (i.e. enhanced when c-Jun was overexpressed) confirming that SKF effects dependent on the TRE site are mediated by AP-1. There seems to be a preferential role for c-Jun in this system, since its overexpression resulted in a substantial
enhancement of overall transcriptional activity, while c-Fos overexpression only modestly enhanced ERα-dependent transactivation. Alternatively, it is possible that other Fos family members may better stimulate ERα activity, analogous to the situation where the ability of E2 to stimulate or repress AP-1 activity appears to correlate with the relative expression of the Fos family member Fra-1 (85).

These effects of either c-Jun or c-Fos were greatly diminished on ERE-E1b-CAT reporters lacking the upstream TRE site. This is important since it suggests that AP-1 interaction with ERα in the absence of TRE DNA binding site makes very modest contributions to ERα-dependent gene expression. These relationships were particularly well demonstrated when SKF-dependent ERα transactivation of the ERE-E1b-CAT TRE site mutants was compared in the presence of wild type ER versus the ER mutant lacking the AF-1 domain (Fig. 8). Under these conditions, which limit the contribution of AP-1 both through its DNA binding site and through protein-protein interactions with ERα, E2- and SKF-82958-induced ERα transactivation were substantially diminished. Collectively, these observations are consistent with the hypothesis that AP-1 enhances SKF-dependent ER transactivation both by AP-1/TRE interaction and by protein/protein interaction between the ER and AP-1 proteins. Whether this latter interaction is direct or is indirectly mediated through other proteins such as coactivators is presently unknown.

The interactions between ERs and AP-1 are complex, and using reporters containing only AP-1 binding sites, other investigators have demonstrated two pathways for ER activation of AP-1-dependent gene expression [reviewed in ref. (3)]. There appears to be an activation function-dependent pathway that estrogen- or antiestrogen-liganded ERα utilizes, while ERβ stimulates AP-1 activity in an activation function-independent manner (57, 86). The results of our study suggest that AP-1 can stimulate the activity of ERα activated by a weak agonist such as SKF-82958 or as expected with the full agonist, E2 (57), indicating that these two classes of transcription factors have the ability to regulate each other’s transcriptional activity. This also suggests that the ability of any given ER ligand to activate receptor-dependent gene expression may vary depending on the presence of DNA binding sites for other transcription factors that can functionally interact with the ER and/or that the ligand may regulate. Since
ERs have been reported to functionally interact with AP-1 (discussed above), as well as Sp1, NF-Y and USF (87, 88), many possible regulatory combinations would seem to be possible, leading to complex regulation of ER-dependent gene expression. Taken together, these results suggest that the ability of pharmacological and environmental compounds to exert estrogen-like effects may need to take into account the activities from other transcription factors able to functionally interact with ERα.
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REFERENCES


FOOTNOTES

1The abbreviations used are: AF, activation function; cAMP, cyclic 3’-5’ adenosine monophosphate; CAT, chloramphenicol acetyltransferase; E2, 17β-estradiol; EGF, epidermal growth factor; ERE, estrogen response element; IBMX, 3-isobutyl-1-methylxanthine; IGF-1, insulin-like growth factor-1; MAPK, mitogen-activated protein kinase; PR, progesterone receptor; SKF, SKF-82958; SRC, steroid receptor coactivator; TRE, TPA responsive element; TPA, tetradecanoylphorbol-13-acetate.
FIGURE LEGENDS

Figure 1: SKF-82958 activates ERα-dependent gene expression.  (A) Chemical structures of the compounds used to regulate ERα activity in this study.  (B) Activation of ERE-E1b-Luc target gene expression by SKF-82958 is ER dependent.  HeLa cells were transfected with expression vectors for ERα (pCMV5-hERβ) and β-galactosidase (pCMVβ), and the ERE-E1b-Luc reporter gene and subsequently treated with ethanol (vehicle), 1 nM E2 or 10 µM SKF in the absence or presence of 100 nM ICI 182,780.  Data represent the average of three independent experiments ± SEM.  (C) SKF-82958 does not stimulate CRE-dependent transcriptional activity.  HeLa cells were transfected with either a CRE-containing (p-169αCG-CAT) or CRE-minus (p-100αCG-CAT) reporter gene and subsequently treated with ethanol (Vehicle), 1 nM E2, 25 µM SKF, 1 mM 8Br-cAMP and 100 µM IBMX (cAMP) or 200 µM dopamine (DA).  Activation data represent the average ± SEM of three independent experiments.

Figure 2: SKF-82958 binds to ERα and ERβ.  In vivo hormone binding assays of (A) ERα or (B) ERβ were performed to assess the relative binding affinity of E2, SKF or dopamine (DA) with respect to competition for [3H]estradiol binding to receptor.  Total [3H]estradiol binding in the absence of competitor (♦) is shown for cells treated with ethanol (Veh).  Values represent the average of duplicate samples from a representative experiment.  Similar results were obtained in n=3-4 independent experiments.

Figure 3: SKF-82958 is an ERα selective activator of transcription.  (A) HeLa cells were transfected with expression vectors for ERα (pCMV5-hERα) or ERβ (pCMV5-hERβ) along with ERE-E1b-Luc and pCMVβ, and subsequently treated with ethanol (Veh), 1 nM E2 or 10 µM SKF.  Data represent the average ± SEM of four independent experiments.  (B) Downregulation of ERα expression by SKF.  Western blot analysis of cell extracts prepared from HeLa cells transfected with an ERα expression vector and subsequently treated with ethanol (Veh), 1 nM E2 or 25 µM SKF.  Signals were detected with H222.
antibody. (C) HeLa cells were transfected with ERE-Elb-Luc and expression vectors for ERα and β-galactosidase along with plasmids for SRC-1e, TIF2, RAC3, CBP or the corresponding parental (empty) vector, pCR3.1. Cells were subsequently treated with ethanol (Veh), 1 nM E2 or 10 µM SKF. Values represent results from an experiment performed in duplicate and repeated at least three times.

Figure 4: Comparison of wild type and Flag-tagged ERα. (A) Western blot analysis of extracts prepared from cells transfected with pJ3-Flag-ERα or pJ3Ω (mock). Blot was probed with anti-Flag (M2) antibody. (B) Western blot of wild type and Flag-ERα expressed in HeLa cells. Blot was probed with anti-hERα (H222) antibody. (C) Dose response curves for wild type (wt; □) and Flag-tagged (○) ERα in HeLa cells. Cells were transfected with increasing amounts of expression vectors for wild type or Flag-tagged ERα along with ERE-E1b-Luc and CMVβgal, and subsequently treated with 1 nM E2. Data are standardized to the activity of cell lysates prepared from cells transfected with 250 ng wild type ERα, and represent the mean ± SEM of three independent experiments. (D) HeLa cells were transfected with 250 ng of the expression vector for each of the indicated receptor forms along with ERE-E1b-Luc and CMVβgal. Cells were treated with ethanol (Veh), 1 nM E2 or 10 µM SKF. Results are standardized to E2 values and represented the average ± SEM of three independent experiments.

Figure 5: SKF-82958 induces ERα phosphorylation. (A) HeLa cells transfected with expression vector for Flag-ERα (lanes 1-3) or empty vector (pJ3Ω; lane 4) were radiolabelled with [32P]orthophosphate and treated with ethanol (Veh), 1 nM E2 or 25 µM SKF. Receptors were immunoprecipitated with H222 antibody, resolved by SDS-PAGE, transferred to nitrocellulose and exposed for autoradiography (top) and subsequently subjected to Western blot analysis with an anti-Flag (M2) antibody (bottom). (B) Values represent the average ± SEM of relative ERα phosphorylation determined in 4 independent experiments.
Figure 6: The AF1 and AF2 domains of ERα are required for optimal activation of transcription by SKF-82958. (A) Schematic of ER mutants used in experiments shown in panel B. The location of the D538A/E542A/D545A amino acid mutations are indicated by ‘.’. (B) HeLa cells were transfected with pRST7 (empty plasmid) or pRST7 expression vectors for wild type ERα (wt), ERα-3x (3x), ERα-N282G (N282G), ERα-179C (179C) or ERα-179C-3x (179C-3x) along with ERE-E1b-Luc and pCMVβ. Data are presented as the average ± SEM of 3 experiments. Cells were treated with ethanol (Veh), 1 nM E2 or 10 µM SKF. The activity of wild type ERα in the presence of 1 nM E2 was defined as 100.

Figure 7: SKF-82958 modestly activates TRE-dependent gene expression. (A) Schematic representation of the coll73-CAT and coll60-CAT reporter genes used in these experiments. (B) HeLa cells were plated at a low density (2 x 10^5 cells/well), switched to media containing 0.5% sFBS and transfected with coll73-CAT or coll60-CAT reporter plasmid. Cells were treated with ethanol (vehicle), 1 nM E2, 100 nM TPA, 10 µM SKF or 100 nM 4-hydroxytamoxifen (4HT). Values represent mean ± SEM for n=4-5 experiments and are expressed as fold induction relative to vehicle-treated cells transfected with coll73-CAT.

Figure 8. An upstream TRE enhances SKF-82958 activation of ERα-dependent gene expression. (A) Schematic representation of reporter genes used in these experiments. HeLa cells were transfected with expression vectors for (B) wild type ER (pSVMT-wtER) or (C) ERα-179C (pRST7-hERα-179C) along with ERE-E1b-CAT reporter genes encoding a putative AP-1 responsive element (TRE-ERE), or lacking this site through deletion (ΔNde-Eco-ERE) or mutation (mTRE-ERE). Cells were treated with the ethanol (Veh), 1 nM E2 or 10 µM SKF. Bars represent mean ± SEM for n=4-6 independent experiments and values are expressed relative to the CAT activity induced by E2 treatment from the intact TRE-ERE-E1b-CAT reporter in each experiment.
Figure 9. Overexpression of c-Jun enhances ER activity stimulated by E2 or SKF-82958. HeLa cells were co-transfected with increasing concentrations of expression plasmid for (A) c-Jun (B) c-Fos or (C) equivalent amounts of c-Jun and c-Fos along with pSVMT-wtER and ERE-E1b-CAT reporter genes with (TRE-ERE) or without (∆Nde-Eco-ERE) a TRE. Total DNA levels were normalized in each group by co-transfecting appropriate levels of the empty plasmid pRSV-Not. Transfections were done 6h prior to addition of the indicated agonists, with harvest following 18h thereafter. Cells were treated with ethanol (vehicle), 1 nM E2 or 10 μM SKF. Bars represent mean ± SEM for n=3 independent experiments and values are expressed relative to the CAT activity (100) induced by E2 treatment from ERE-E1b-CAT in each experiment. ANOVA indicated that (a) c-Jun overexpression, both in the presence and absence of co-transfected c-Fos, significantly elevated basal (p<0.001), and E2- (p<0.01) and SKF-induced (p<0.001) transcriptional activation from ERE-E1b-CAT, but not from the TRE deletion mutant and (b) c-Fos overexpression resulted in modestly significant (p<0.05) increases in E2- and SKF-induced transcriptional activity from the intact reporter.
Figure 1
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SKF-82958 is a subtype-selective estrogen receptor-(α) (ERα) agonist that induces functional interactions between ERα and AP-1
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