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Direct Interactions with Gαi and Gβγ Mediate Nongenomic Signaling by Estrogen Receptor α

Premlata Kumar, Qian Wu, Ken L. Chambliss, Ivan S. Yuhanna, Susanne M. Mumby, Chieko Mineo, Gregory G. Tall, and Philip W. Shaul

Departments of Pediatrics (P.K., Q.W., K.L.C., I.S.Y., C.M., P.W.S.) and Pharmacology (S.M.M., G.G.T.), University of Texas Southwestern Medical Center, Dallas, Texas 75390

Estrogen induces G protein-dependent nongenomic signaling in a variety of cell types via the activation of a plasma membrane-associated subpopulation of estrogen receptor α (ERα). Using pull-down experiments with purified recombinant proteins, we now demonstrate that ERα binds directly to Gαi and Gβγ. Mutagenesis and the addition of blocking peptide reveals that this occurs via amino acids 251–260 and 271–595 of ERα, respectively. Studies of ERα complexed with heterotrimeric G proteins further show that estradiol causes the release of both Gαi and Gβγ without stimulating GTP binding to Gαi. Moreover, in COS-7 cells, the disruption of ERα-Gαi interaction by deletion mutagenesis of ERα or expression of blocking peptide, as well as Gβγ sequestration with β-adrenergic receptor kinase C terminus, prevents nongenomic responses to estradiol including src and erk activation. In endothelial cells, the disruption of ERα-Gαi interaction prevents estradiol-induced nitric oxide synthase activation and the resulting attenuation of monocyte adhesion that contributes to estrogen-related cardiovascular protection. Thus, through direct interactions, ERα mediates a novel mechanism of G protein activation that provides greater diversity of function of both the steroid hormone receptor and G proteins. (*Molecular Endocrinology* 21: 1370–1380, 2007)

STEROID HORMONE RECEPTORS (SHRs) function classically in the nucleus as ligand-activated transcription factors. More recently, it has become apparent that steroid hormones also initiate a diverse set of important nongenomic cellular responses via the activation of plasma membrane-associated SHRs (1, 2). In particular, this has been elucidated regarding the nonnuclear actions of estrogen, which modify growth and differentiation, migration, and other processes in cell types as diverse as oocytes, osteoblasts, osteoclasts, neurons, breast cancer cells, adipocytes, and endothelial cells (1–5). The underlying mechanisms are best exemplified by the identification of a caveolae membrane-associated population of the classical estrogen receptor α (ERα) in endothelial cells that activates Src family tyrosine kinases, phosphatidylinositol 3 kinase/Akt kinase, and erk1,2 to stimulate nitric oxide (NO) production by the endothelial isoform of NO synthase (eNOS). These pathways are critically involved in estrogen-related cardiovascular protection (6). Activation of these pathways also stimulates phosphorylation of ERα and its coregulators and *S*-nitrosylation of the receptor to modify nuclear signaling, indicating that there is additional important cross talk between membrane and nuclear SHR function (3, 7).

In previous studies using endothelial cells, we demonstrated that signal initiation by membrane ERα is pertussis toxin (PTX) sensitive and that ERα and Gαi can be coimmunoprecipitated from the plasma membrane (8). These findings and related evidence of heterotrimeric G protein involvement in signaling by ER in other cell types (9) raise the possibility that the most proximal mechanisms underlying membrane SHR actions entail interactions with G proteins. Heterotrimeric G proteins are activated conventionally by members of a family of G protein-coupled receptors (GPCRs), the sequences of which predict structures of seven membrane spans that include binding sites for G proteins. Agonist binding to GPCRs promotes the release of GDP from Gα, thus allowing Gα to bind the more abundant nucleotide in the cell, GTP. A conformational change in Gα accompanies GTP binding, leading to the dissociation of Gα and the high-affinity complex of β and γ subunits from the GPCR. Liberated Gα-GTP and βγ subunits are competent to modulate the activity of downstream effectors (10, 11). In contrast to the in-depth knowledge available regarding G protein and GPCR interactions, the molecular basis of the functional linkage between SHRs such as ERα and G proteins is unknown.

In the present investigation we designed experiments to test the hypothesis that ERα interacts directly with Gαi. Further studies were performed to address the following questions: 1) Are interactions between

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Abbreviations: AR, Androgen receptor; βARK-ct, β-adrenergic receptor kinase C-terminal tail; BAEC, bovine aortic endothelial cell; eNOS, endothelial isoform of nitric oxide synthase; ER, estrogen receptor; GPCR, G protein-coupled receptor; GR, glucocorticoid receptor; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; NTA, nitrilotriacetic acid; PTX, pertussis toxin; SHR, steroid hormone receptor; VDR, vitamin D receptor.

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ERα and Gαi required for nongenomic signaling by the receptor? 2) Do other SHR that mediate membrane-initiated signaling interact directly with Gαi? 3) Does ERα also interact directly with Gβγ? 4) What are the domains of ERα that interact with Gαi and Gβγ? 5) How does the interaction of ERα with G proteins initiate signaling? and 6) Do these mechanisms modify the function of endothelial cells, which have well-recognized nongenomic responses to estrogen of importance to cardiovascular protection?

RESULTS

ERα Interaction with Gαi

To first investigate whether ERα interacts directly with monomeric Gαi, we performed pull-down experiments using purified myristoylated Gαi-GDP that contained a hexahistidine tag inserted at amino acid position 121 to preserve myristoylation and typical receptor interactions with the Gαi C terminus (His_6-Gαi-GDP) (12, 13). Direct protein-protein interactions were evaluated with recombinant ERα protein in the absence or presence of varying concentrations of 17β-estradiol (E_2). In the absence of ligand, ERα bound Gαi and the interaction was enhanced by E_2 in a dose-dependent manner (Fig. 1A). The addition of ICI 182,780 alone blunted the interaction, and it also attenuated the enhancement in interaction prompted by E_2 (supplemental Fig. 1A, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). In contrast to E_2, dexamethasone and dihydrotestosterone had no effect on the ERα-Gαi interaction (data not shown). ERα interacted preferentially with GDP-bound *vs.* GTPγS-bound Gαi (Fig. 1B), and the interaction was enhanced by Gαi myristoylation (Fig. 1C).

To determine whether G protein interaction with ERα mediates nongenomic responses to E_2, we evaluated src activation in COS-7 cells expressing ERα in the presence or absence of PTX. Src activation is an early signaling event in multiple membrane-initiated actions of E_2 and ERα (1–5), numerous known GPCRs control src activation (14, 15), and we have previously shown that PTX prevents ligand-dependent ERα-Gαi coimmunoprecipitation from plasma membranes (8). E_2 (10^{-8} M)-induced src phosphorylation was prevented by PTX treatment (Fig. 1, D and E), indicating that Gαi interaction with ERα is a required proximal process in membrane ERα signaling.

Interactions between Other SHRs and Gαi

Multiple SHRs in addition to ERα initiate rapid responses upon ligand activation that are independent of the modification of gene transcription (1, 2, 16). To investigate whether the direct interaction observed between ERα and Gαi is shared by other SHRs for which there is evidence of nongenomic signaling involving G proteins (1, 2, 17–19), plasma membranes purified from COS-7 cells expressing ERβ, androgen receptor (AR), glucocorticoid receptor (GR), or vitamin D receptor (VDR) were tested in the myristoylated His_6-Gαi-GDP pull-down assay. Membrane-associated ERβ and AR bound Gαi, and binding was enhanced by the relevant SHR ligand (Fig. 2, A and B). In contrast, GR and VDR did not bind to His_6-Gαi-GDP in the absence or presence of ligand (Fig. 2, C and D). To determine whether the membrane-associated ERβ and AR interactions with Gαi signify direct protein-protein binding, additional His_6-Gαi-GDP pull-down experiments were performed with purified recombinant receptor proteins. Recombinant ERβ bound Gαi, and the interaction was enhanced by E_2 (Fig. 2E). Recombinant AR in truncated form (amino acids 606–902) also bound Gαi, and there was increased interaction in the presence of dihydrotestosterone (Fig. 2F). Thus, direct interaction with Gαi is a shared feature of ERα and select SHRs that initiate signaling at the plasma membrane.

Domain of ERα Mediating Interaction with Gαi

To identify the domain of ERα involved in direct interaction with Gαi, Flag-tagged wild-type and deletion mutant human ERα proteins (Fig. 3A) were expressed and purified from baculovirus-infected *Sf*9 cells and used in pull-down assays with myristoylated His_6-Gαi-GDP. Wild-type ERα and an N-terminal deletion mutant lacking amino acids 1–175 (ERαΔ1–175) bound Gαi comparably (Fig. 3B). A C-terminal deletion mutant lacking amino acids 271–595 (ERαΔ271–595) also interacted with Gαi, but an internal deletion of amino acids 180–268 (ERαΔ180–268) prevented binding with Gαi (Fig. 3C). In contrast to ERαΔ180–268, a mutant receptor lacking amino acids 185–251 (ERαΔ185–251) was capable of interaction with Gαi (Fig. 3D), implicating amino acids 180–184 and/or 252–268 in binding with the G protein. Further investigation revealed that Gαi interaction does not involve amino acids 261–271, and instead it was found that the region of ERα between amino acids 250 and 260 mediates direct binding to Gαi (Fig. 3E). Moreover, the introduction of a peptide representing amino acids 251–260 of ERα disrupted the interaction between the wild-type receptor and Gαi, whereas scrambled peptide did not (Fig. 3F), confirming the findings made by deletion mutagenesis.

The involvement of Gαi binding to amino acids 250–260 of ERα in nongenomic receptor signaling was then evaluated in studies of src phosphorylation in COS-7 cells. Whereas wild-type ERα promoted src phosphorylation with E_2, no response was evident in cells expressing ERαΔ250–260 (Fig. 4, A and B). In parallel, we found that expression of an HA-tagged peptide consisting of only amino acids 251–260 of ERα mimicked the action of PTX (Fig. 1, D and E) and blocked nongenomic signaling by wild-type ERα to src (Fig. 4, C and D). These collective observations identify amino acids 251–260 of ERα as a Gαi binding domain that is critically involved in nongenomic signaling by the receptor.

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ERα Interaction with Heterotrimeric Gαβγ and Gβγ Dimer

In order for Gαi to interact effectively with classical GPCRs, Gαi is associated with Gβγ as a heterotrimer. In addition, both activated Gαi and Gβγ are capable of modulating the activity of downstream effector molecules (10, 11). Therefore, pull-down experiments were performed using purified components to compare the capacity of ERα to bind monomeric His_6-Gαi-GDP and His_6-Gαi-GDP associated with $G\beta_1\gamma_2$. In the absence of agonist, ERα interaction with Gαi was enhanced by Gβγ (Fig. 5A). Whereas ERα interaction with monomeric Gαi was increased by E_2, the addition of the ligand diminished the interaction between the receptor and Gαi in heterotrimeric form. These observations raised the possibility that ERα also binds Gβγ directly. Pull-down experiments employing Flag-tagged ERα demonstrated that such an interaction occurs, and that it is attenuated by E_2 (Fig. 5B). The addition of ICI 182,780 reversed the E_2-induced decrease in interaction between ERα and Gβγ, and ICI 182,780 alone actually caused an increase in the interaction (supplemental Fig. 1B, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Furthermore, a decline in the ERα-$G\beta_1\gamma_2$ interaction, and also in the ERα-Gαi interaction, occurred with the addition of E_2 to the Flag-tagged ERα pull-down of G protein heterotrimer (Fig. 5C). Moreover, in heterotrimer experiments ICI reversed the loss of ERα-Gαi interaction that

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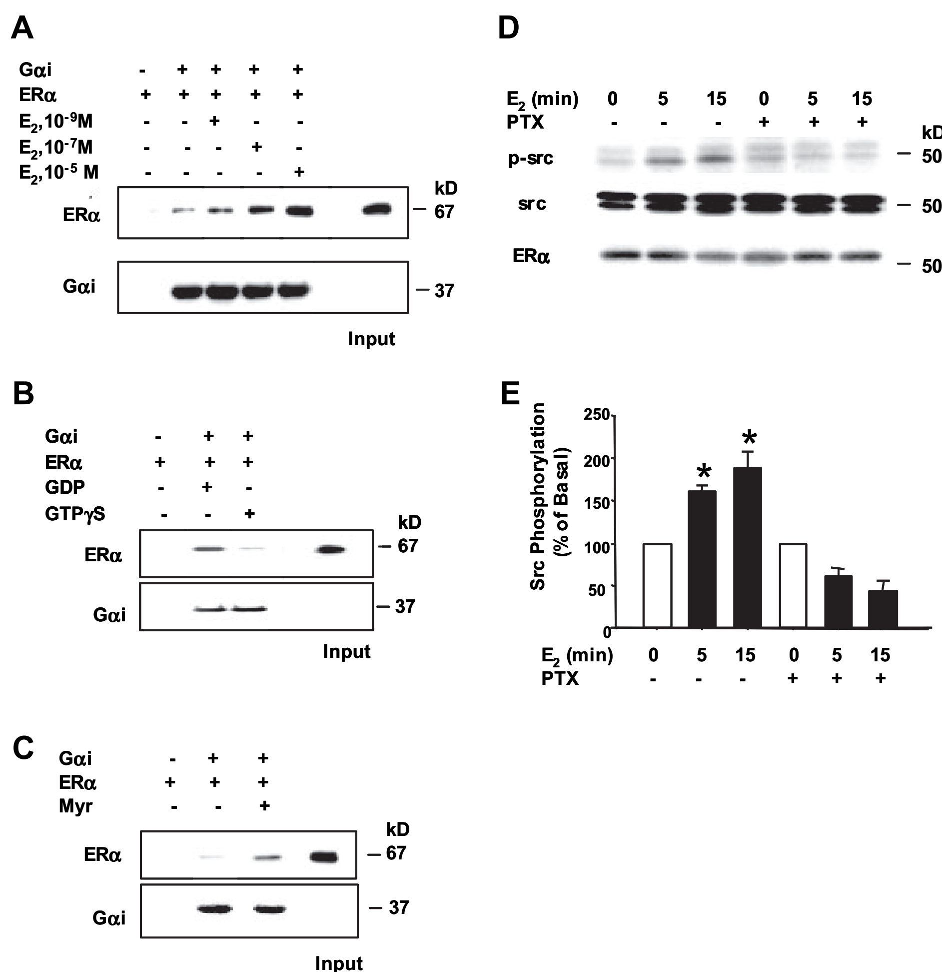

Fig. 1. ERα Interacts Directly with Gαi, and the Interaction Is Required for Signaling to src

A, Pull-down experiments were performed with myristoylated His_6-Gαi-GDP and recombinant ERα in the absence or presence of E_2 at the indicated concentrations. Immunoblot analyses were performed for ERα and Gαi. In all pull-down experiments, the input lanes represent 20% of the amount of protein used and the sample lanes contain 50% of the pull-downs. Results shown for all pull-downs are representative of three or more independent studies. B, Pull-downs were performed with myristoylated GDP- *vs.* GTPγS-bound His_6-Gαi. C, Pull-downs were performed with nonmyristoylated *vs.* myristoylated His_6-Gαi-GDP. D, COS-7 cells expressing ERα were pretreated with vehicle or PTX (100 ng/ml for 120 min) and incubated with 10^{-8} M E_2 for 0–15 min, and src activation was evaluated by immunoblot analyses of cell lysates using anti-phospho-src (p-src) polyclonal and anti-src monoclonal antibodies. E, Cumulative results for src phosphorylation expressed as percentage in nontreated cells (basal) for three independent studies (mean ± SEM; *, $P < 0.05$ *vs.* basal).

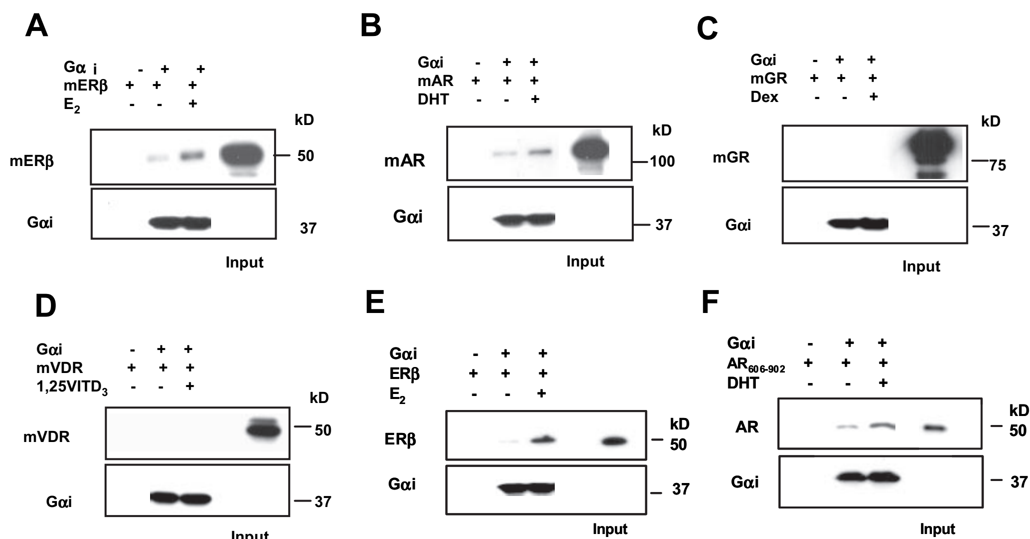

Fig. 2. Gαi Interacts with a Distinct Subset of SHRs

Purified myristoylated His_6-Gαi-GDP was used to pull down plasma membrane-associated (designated "m") ERβ (A), AR (B), GR (C), and VDR (D) in the absence or presence of vehicle or 10^{-5} M E_2, dihydrotestosterone, dexamethasone, or 1,25-dihydroxy vitamin D_3, respectively. Proteins were eluted from the Ni-NTA resin with sample buffer and immunoblot analyses were performed for ERβ, AR, GR, VDR, and Gαi. In all experiments, the input lanes represent 20% of the amount of protein employed in the pull-down, and the sample lanes contain 50% of the pull-down. E and F, Pull-down experiments were performed with myristoylated His_6-Gαi-GDP and either recombinant ERβ, in the absence or presence of 10^{-5} M E_2 (E), or recombinant $AR_{606–902}$ in the absence or presence of 10^{-5} M dihydrotestosterone (DHT) (F). All results shown are representative of three independent studies.

occurs with E_2, but not the decrease in ERα-Gβγ interaction with E_2 (supplemental Fig. 1C, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org); in fact, ICI 182,780 alone acted similar to E_2 alone and caused a decline in ERα-Gβγ interaction. Thus, the modulation of ERα-G protein interactions by the ERα ligands E_2 and ICI 182,780 differs whether or not the G proteins are in heterotrimeric form.

To identify the domain(s) of ERα involved in direct Gβγ binding, experiments were performed using Flag-tagged wild-type ERα and deletion mutants of ERα to pull down Gβγ. Whereas wild-type ERα and the deletion mutant lacking amino acids 180–268 (ERαΔ180–268) displayed comparable binding with Gβγ, the mutant lacking amino acids 271–595 (ERαΔ271–595) did not interact with Gβγ (Fig. 5D). Thus, Gβγ interacts directly with ERα via a receptor domain(s) that is distinct from the Gαi binding domain. Moreover, the interaction with Gβγ promotes receptor interaction with Gαi, and the complex formed by the receptor and heterotrimeric Gαβγ is disrupted upon agonist binding to the receptor.

Mechanism Underlying Signal Initiation by ERα Complexed with Gαβγ

Having observed that the ERα interaction with Gαi is required for signal transduction and that the receptor also complexes Gαβγ directly, the potential ability of ERα to activate G protein heterotrimers by acting as a guanine nucleotide exchange catalyst was explored. Membranes prepared from *Sf*9 cells coexpressing heterotrimeric Gαβγ and either ERα or the M2 muscarinic receptor were incubated with [^{35}S]GTPγS to determine the kinetics of Gαi nucleotide binding. In the absence of ligand, the M2 muscarinic receptor did not alter the rate of Gαi-GTP production appreciably (Fig. 6A); in contrast, expression of ERα appeared to stimulate a slow kinetic exchange of guanine nucleotide (Fig. 6B). However, whereas carbachol stimulation of the M2 muscarinic receptor promoted more rapid Gαi [^{35}S]GTPγS binding (Fig. 6A), E_2 stimulation of ERα did not result in an increase in [^{35}S]GTPγS binding (Fig. 6B), and ICI 182,780 also did not affect guanine nucleotide exchange (data not shown). These results suggest that the mechanism of E_2-induced activation of ERα and G proteins is more complex than simple regulation of the Gαi guanine nucleotide switch, thus differing significantly from GPCR-induced signaling.

Because we demonstrated that Gβγ interacts dynamically with ERα and free Gβγ can mediate subsequent cellular responses in diverse paradigms (10, 11), the possibility that liberated Gβγ modulates downstream signaling by ERα was investigated. COS-7 cells expressing ERα were transfected with either empty plasmid or plasmid encoding the β-adrenergic receptor kinase C-terminal tail (βARK-ct). Under control conditions, E_2 stimulated the phosphorylation of src (Fig. 6, C and D). In contrast, in cells expressing βARK-ct, there was an abrogation of E_2-induced src activation. To evaluate an-

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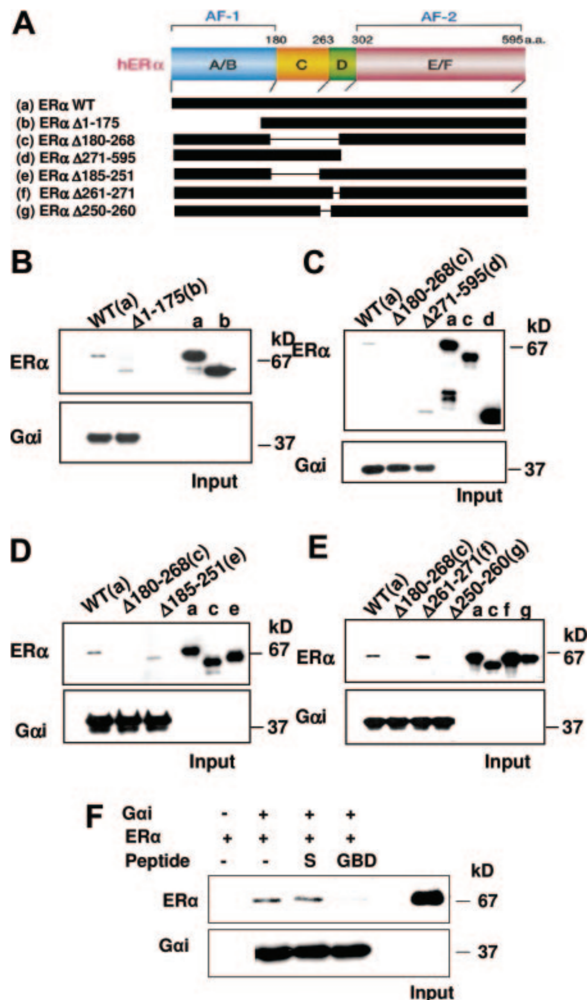

Fig. 3. Amino Acids 251–260 of ERα Interact Directly with Gαi

A, Schematic of Flag-tagged wild-type ERα (WT) and ERα deletion mutants used in myristoylated His_6-Gαi-GDP pull-down experiments. The identities of the constructs are designated by lowercase letters on the schematic (A) and in the results shown (B–E). Comparisons were made in simultaneous pull-downs with WT ERα *vs.* ERαΔ1–175 (B), WT ERα *vs.* ERαΔ180–268 or ERαΔ271–595 (C), WT ERα *vs.* ERαΔ180–268 or ERαΔ185–251 (D), or with WT ERα *vs.* ERαΔ180–268, ERαΔ261–271, and ERαΔ250–260 (E). The interaction between WT ERα and Gαi was also tested in the absence or presence of a peptide representing the Gαi binding domain (GBD) comprised of amino acids 251–260 of the receptor or scrambled peptide (S) (F). Results shown are representative of three or more independent studies.

other downstream target of activated Gβγ, erk phosphorylation was also examined (Fig. 6, C and E). Paralleling the findings for src, E_2-stimulated erk activation was also blunted by βARK-ct. These cumulative findings show that E_2 activation of ERα releases both Gαi and Gβγ without stimulating GTP binding to Gαi, and that Gβγ in turn activates the immediate downstream signaling targets src and erk, which are of known importance to multiple nongenomic ERα actions (1–5).

ERα-G Protein Interactions and E_2 Modulation of Endothelial Cell Function

The importance of direct ERα-G protein interactions to cell function was then addressed in the context of G protein-dependent, plasma membrane-associated ERα activation of eNOS (8). In bovine aortic endothelial cells (BAEC), stimulation of eNOS by E_2 was prevented by ICI 182,780 (Fig. 7A). In further experiments, BAEC were transfected with empty vector or plasmid encoding the ERα mutant ERαΔ250–260, which displayed an inability to interact with Gαi (Fig. 3E) and an inability to promote E_2 activation of src (Fig. 4, A and B). Of note, in this model system ERα and Gαi are present at endogenous levels, and in previous studies we have shown that the overexpression of wild-type ERα in endothelial cells enhances eNOS activation by E_2 (20). Whereas control cells displayed eNOS activation by E_2, the response was absent in cells expressing ERαΔ250–260 (Fig. 7B). In contrast, eNOS activation by vascular endothelial growth factor or acetylcholine was not altered by ERαΔ250–260 expression and E_2-mediated gene transcription assessed using an estrogen response element-luciferase promoter-reporter construct was also not affected (data not shown), indicating that the mutant has a selective dominant-negative action on nongenomic ER function. Similarly, in cells expressing an ERα peptide consisting of amino acids 251–260, E_2 stimulation of eNOS was fully impaired (Fig. 7C) but E_2-mediated gene transcription was unchanged (data not shown). To test the requirement for ERα-G protein interactions in the modulation of an endothelial cell phenotype of relevance to E_2-related cardioprotection, the impact of the dominant-negative mutant ERαΔ250–260 on E_2-induced attenuation of monocyte adhesion was evaluated (Fig. 7, D and E). In BAEC transfected with empty vector, the marked increase in monocyte adhesion caused by lipopolysaccharide (LPS) was fully prevented by E_2. The effect of E_2 was due to nongenomic activation of eNOS because it was abrogated by nitric oxide synthase (NOS) antagonism with *N*-nitro-L-arginine methyl ester and the hormone did not alter eNOS enzyme abundance (Fig. 7E, *inset*). In contrast, in cells expressing the dominant-negative mutant ERα (ERαΔ250–260), the E_2-related, NO-dependent decrease in monocyte adhesion was absent. Thus, the direct interactions between ERα and G proteins are required for E_2-induced nongenomic actions in endothelial cells of significance to vascular health and disease.

DISCUSSION

SHRs including ERα, ERβ, AR, GR, and VDR mediate a variety of nongenomic responses that govern the behaviors of multiple cell types, and evidence has accumulated that in many contexts these processes

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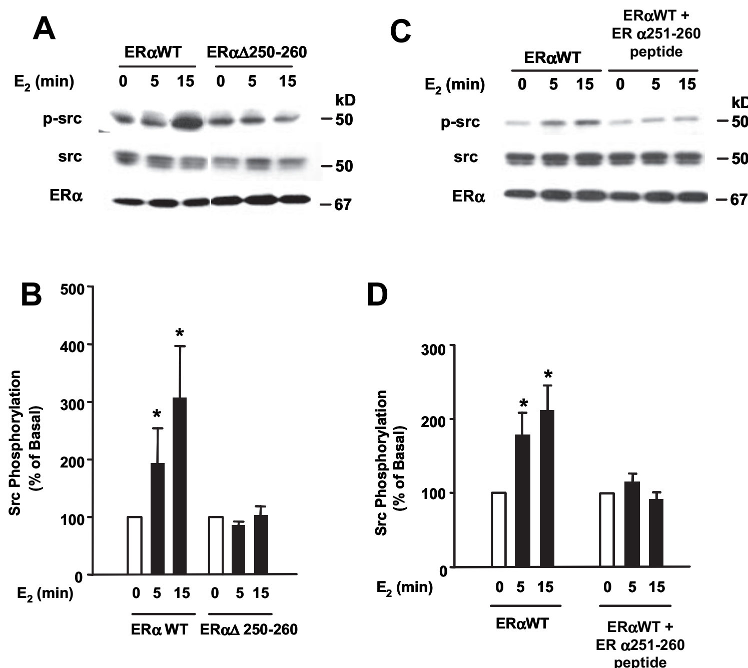

Fig. 4. Disruption of ERα Interaction with Gαi Prevents src Activation by E_2
A, COS-7 cells were transfected with plasmid encoding WT ERα or ERαΔ250–260 and incubated with 10^{-8} M E_2 for 0–15 min, and src activation was evaluated by immunoblot analyses of cell lysates using anti-phospho-src (p-src) polyclonal and anti-src monoclonal antibodies. B, Cumulative results for src phosphorylation expressed as percentage in nontreated cells (basal) for three independent studies (mean ± SEM; *, $P < 0.05$ *vs.* basal). C, COS-7 cells were transfected with plasmid encoding WT ERα and either empty vector or a plasmid encoding His-tagged peptide comprised of amino acids 251–260 of ERα, incubated with 10^{-8} M E_2 for 0–15 min, and evaluated for src activation. D, Cumulative results for src phosphorylation for three independent studies (mean ± SEM; *, $P < 0.05$ *vs.* basal).

are G protein dependent (16–19, 21, 22). With a focus on ERα, we have demonstrated for the first time direct interactions between an SHR and G proteins, and have determined that such interactions are critically involved in nongenomic steroid hormone signaling.

In pull-down experiments with purified recombinant proteins, we first showed that there is a direct protein-protein interaction between ERα and monomeric Gαi, which is enhanced specifically by E_2. We also demonstrated that the interaction is altered by modifications of Gαi that govern its interactions with classical GPCRs (10). Using PTX in studies of src phosphorylation, we further determined that Gαi interaction with ERα is an essential proximal process in membrane ERα signaling.

In experiments evaluating whether the interaction observed between ERα and Gαi is shared by other SHRs capable of nongenomic signaling involving G proteins (1, 2, 17–19), we found that ERβ and AR also display direct binding with Gαi that is enhanced by their respective steroid hormone ligands. These observations are consistent with the parallel capacity of membrane-associated ERα and ERβ to promote signaling to eNOS in cultured endothelial cells (21), and the ability of androgens to mediate PTX-sensitive signaling in cell types as diverse as neurons and skeletal muscle (17, 22). In contrast, we observed that GR and VDR do not bind to Gαi. Thus, direct interaction with Gαi is a shared feature of ERα and select SHRs that initiate signaling at the plasma membrane. Direct interactions with Gαs or Gαq may be operative in the nongenomic functions of other SHRs such as GR and VDR. Consistent with the latter possibility, it has been demonstrated that Gαq is required for VDR-induced nongenomic signaling during matrix biogenesis by chondrocytes (19).

In further studies of ERα, pull-down experiments performed with Gαi and mutant receptor proteins revealed that the region of ERα between amino acids 250 and 260 mediates the direct binding to Gαi. In addition, a peptide representing amino acids 251–260 of ERα disrupted the interaction between the wild-type receptor and Gαi. Furthermore, the ERα mutant lacking amino acids 250–260 was incapable of activating src in COS-7 cells, and in cells expressing wild-type ERα the coexpression of an HA-tagged peptide representing amino acids 251–260 blocked nongenomic signaling to src. Thus, we have identified amino acids 251–260 of ERα as a Gαi binding domain that is critically involved in nongenomic signaling by the receptor. Because there is negligible homology between these amino acids and the corresponding regions of ERβ and AR, which we show also bind directly to Gαi, detailed mutagenesis will now be required to identify

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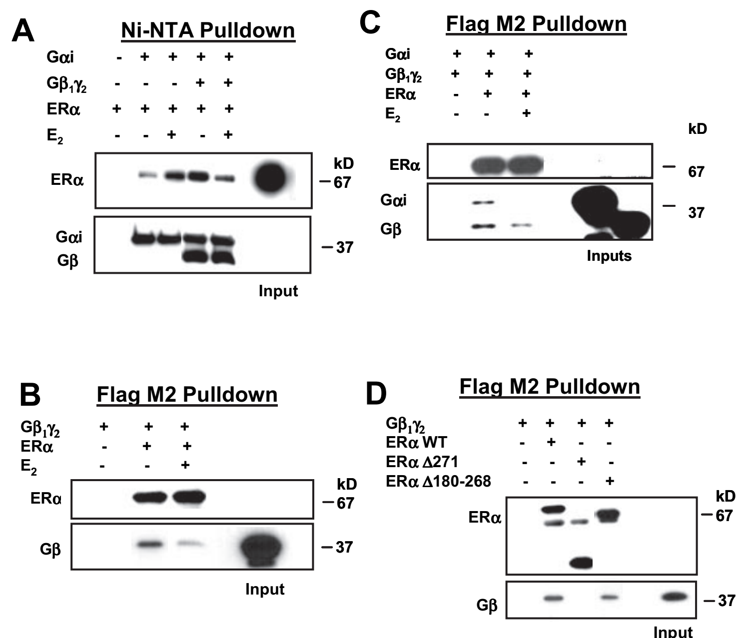

Fig. 5. ERα Interacts Directly with Gβγ

A, Recombinant ERα protein was pulled down with myristoylated His_6-Gαi-GDP using Ni-NTA in the absence or presence of recombinant $G\beta_1\gamma_2$ and 10^{-8} M E_2. Immunoblot analyses were performed for ERα, Gαi, and Gβ. B, Purified $G\beta_1\gamma_2$ was pulled down with recombinant Flag-tagged wild-type ERα in the absence or presence of E_2. C, Purified Gαi-GDP and $G\beta_1\gamma_2$ were pulled down using Flag-tagged wild-type ERα bait in the absence or presence of E_2. D, Comparisons were made in simultaneous $G\beta_1\gamma_2$ pull-downs with recombinant Flag-tagged wild-type ERα (WT) *vs.* ERαΔ271–595 *vs.* ERαΔ180–268, with a nonspecific band noted at 50 kDa in all samples in immunoblots for ERα.

the domains within ERβ and AR mediating this interaction. Interestingly, the Gαi binding domain of ERα resides within nuclear localization signal 3 (23), raising the intriguing possibility that there are competitive mechanisms dictating the relative function of the receptor at the plasma membrane and in the nucleus.

Additional pull-down experiments focused on the role of Gβγ in ERα-Gαi coupling. We found that Gβγ interacts directly with ERα via a receptor domain(s) residing within amino acids 271–595, which is distinct from the Gαi binding domain, that the interaction with Gβγ promotes receptor interaction with Gαi, and that the complex formed by ERα and heterotrimeric GDP-bound Gαβγ is disrupted upon E_2 activation of the receptor. We also observed that Gβγ is liberated from ERα by E_2 in the absence of Gαi. In studies of the kinetics of Gαi nucleotide binding, the activation of the M2 muscarinic receptor serving as a positive control promoted rapid GTPγS binding to Gαi, whereas E_2 stimulation of ERα did not. Therefore, Gβγ is released by ERα independently of conventional GTP binding to Gαi and the resulting conformational change in Gαi that disassociates the βγ dimer during signaling by classical GPCRs (10, 11). We postulate that the liberation of Gβγ is mediated alternatively by conformational changes that occur in ERα upon ligand binding. Similar changes in ERα conformation are known to modify the interaction of the receptor with nuclear cofactors (24, 25). Evidence that conformational changes may impact on ERα-G protein interactions lies in our findings in pull-downs with ICI 182,780, which modify ERα conformation in a manner that is unique compared with E_2 (24, 25). Under certain conditions, ICI 182,780 reversed E_2 effects on ERα-G protein interactions, under other conditions the ICI compound independently altered ERα-G protein interactions, and the modulation of ERα-G protein interactions by both E_2 and ICI 182,780 differed whether or not the G proteins were in heterotrimeric form. Furthermore, experiments in ERα-expressing COS-7 cells showed that cotransfection with βARK-ct attenuates E_2-induced srk and erk activation, indicating that the liberated Gβγ modulates downstream signaling. Thus, we have identified a novel means of G protein activation that provides greater diversity of function of an SHR.

The importance of direct ERα-G protein interactions to cell function was addressed in studies of ERα activation of eNOS in cultured endothelium. This process is critically involved in the vascular actions of E_2 that underlie the lower risk of cardiovascular disease in premenopausal women *vs.* men and the potential of estrogen replacement therapy to be cardioprotective (26). The disruption of ERα-Gαi interaction prevented E_2-induced eNOS activation, which was ER dependent, and it also negated the resulting attenuation of monocyte adhesion that is highly relevant to the initiation of atherosclerosis (26). As such, ERα-Gαi interaction plays an important

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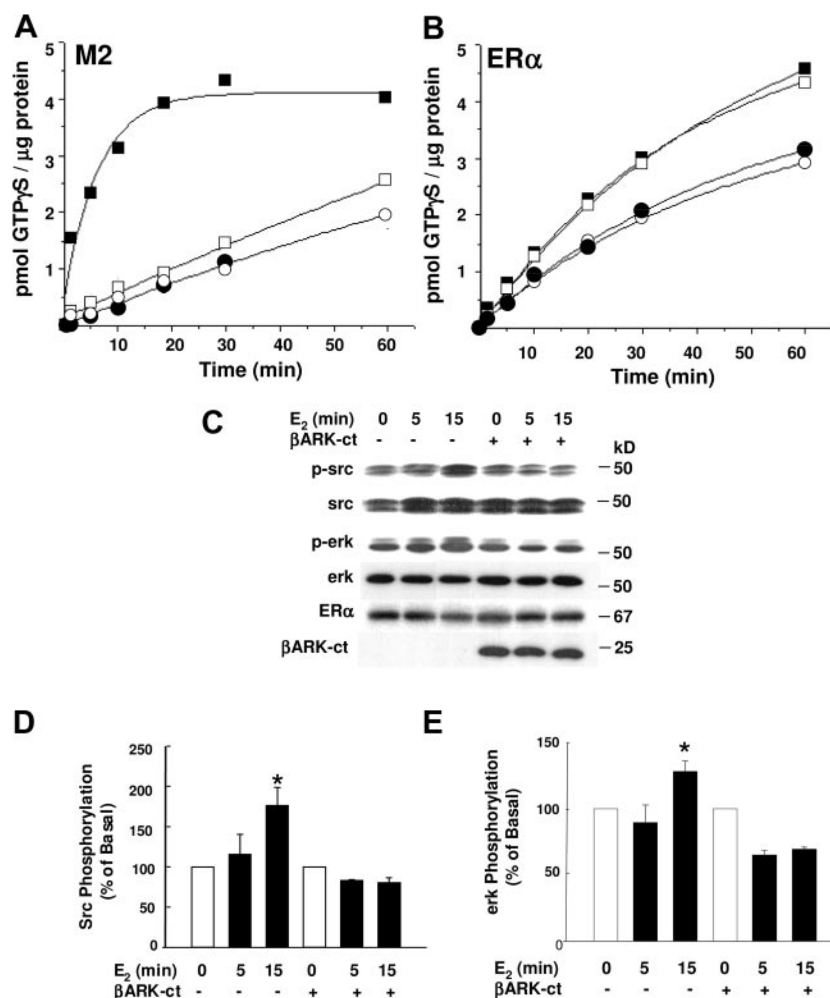

Fig. 6. Signal Initiation by E_2 and ERα Complexed with Gαβγ Is Mediated by Gβγ Independent of Guanine Nucleotide Exchange
A and B, The kinetics of GPCR and ERα stimulation of membrane-bound G protein GTP binding differ. A, Membranes from *Sf*9 cells infected with baculoviruses that express His_6-tagged-Gαi, Gβ, and Gγ, and either no additional virus or the M2 muscarinic receptor virus were incubated with [^{35}S]GTPγS at 30 C in the absence or presence of 10^{-6} M carbachol. Aliquots of reaction mixtures were taken at the indicated time points, quenched, and Ni-NTA resin was used to pull down the detergent-extracted Gαi. The amount of His-tagged G protein-bound [^{35}S]GTPγS was determined by liquid scintillation counting. B, Parallel studies of ERα were performed in the absence or presence of 10^{-8} M E_2. In A and B, results for Gαβγ alone are indicated by *circles* and those for Gαβγ plus receptor by *squares*, and *open* and *closed symbols* represent findings in the absence and presence of ligand, respectively. Values shown in A and B are means for n = 2, and results were confirmed in three separate experiments. C, COS-7 cells transfected with plasmid encoding wild-type ERα and either empty vector or the cDNA for the βARK-ct were incubated with 10^{-8} M E_2 for 0–15 min, and src and erk activation was evaluated by immunoblot analyses of cell lysates using anti-phospho-src (p-src), anti-phospho-erk (p-erk), or anti-src or anti-erk antibodies. Cumulative results are shown for src phosphorylation (D) and erk phosphorylation (E) expressed as percentage in non-E_2-treated cells (basal) for three independent studies (mean ± SEM; *, $P <$ 0.05 *vs.* basal).

role in dictating the phenotype of a cell type with well-recognized responses to E_2.

The mechanisms that we have elucidated in which E_2 initiates downstream nongenomic responses by liberating Gβγ from Gαi and ERα upon ligand activation independent of guanine nucleotide exchange add considerably to the processes described to date for nongenomic estrogen signaling. Another example is the potential role of direct estrogen binding to the GPCR GPR30. This event occurs in the endoplasmic reticulum, and it is thought to promote intracellular calcium mobilization and the generation of PIP3 in the nucleus (27). Additional mechanisms include the involvement of adaptor proteins such as the modulator of nongenomic actions of the ER (MNAR) and striatin, which bind to ER to promote the maintenance of a signaling module (28, 29). In the emerging field of nongenomic endocrinology, our discovery of direct ERα:G protein interactions provides important new understanding of the proximal mechanisms by which proteins classically known to serve as transcription factors exhibit a second fundamental capacity to ini-

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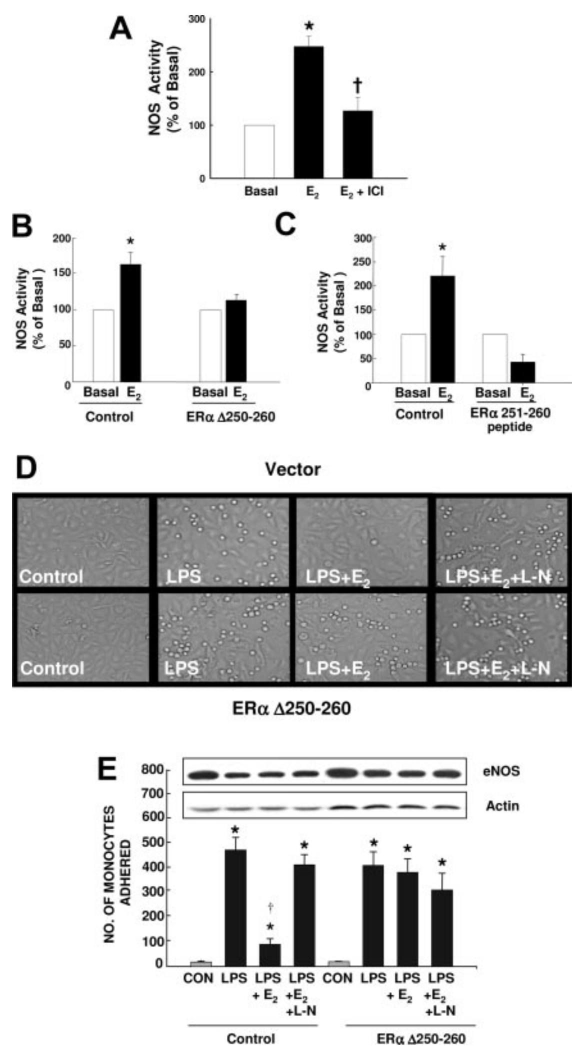

Fig. 7. Disruption of ERα-Gαi Interaction in Endothelial Cells Attenuates ER-Dependent E_2 Activation of eNOS and Resulting Antagonism of Monocyte Adhesion

A, eNOS activation in BAEC was assessed in the presence of buffer alone (basal), 10^{-8} M E_2, or E_2 plus 10^{-5} M ICI 182,780 for 15 min. B and C, BAEC were transfected with empty vector (control) *vs.* plasmid encoding ERαΔ250–260 (B), or with empty vector *vs.* a plasmid that expressed a peptide consisting of amino acids 251–260 of the receptor (ERα251–260) (C), and E_2-stimulated eNOS activity was assessed. In A–C, values are mean ± SEM; n = 6. *, $P < 0.05$ *vs.* basal; †, $P < 0.05$ *vs.* no ICI 182,780. D, Monocyte adhesion was assessed in BAEC transfected with an empty vector (*upper panels*) or a plasmid that expressed ERαΔ250–260 (*lower panels*) and treated with medium alone (control), LPS (100 ng/ml), LPS plus E_2 (10^{-8} M), or LPS plus E_2 plus nitro-L-arginine methyl ester (L-N) (2 mM). Images are representative optical fields. E, Cumulative findings for monocytes adhered per ×20 magnification field; mean ± SEM; n = 4–5. *, $P < 0.05$ *vs.* control; †, $P < 0.05$ *vs.* LPS alone. *Inset* shows immunoblot analyses for eNOS and actin in the various treatment groups.

tiate hormone signaling at the plasma membrane. This work also reveals a new role for G protein signaling outside of conventional GPCR activation. It is anticipated that future efforts in this realm will enable us to continue to reveal the intricacies of SHR biology dictating ultimate cellular responses.

MATERIALS AND METHODS

Production of Recombinant $G\alpha_i$, $G\beta\gamma$, ERα, and mutant ERα

His-tagged $G\alpha_{i1}$ was purified from *Escherichia coli* that had been transformed with a plasmid encoding rat $G\alpha_{i1}$ alone or together with a plasmid encoding yeast *N*-myristoyltransferase to produce myristoylated Gαi (30). $G\beta_1\gamma_2$ dimers were synthesized and purified from *Sf*9 cells as previously described (31). Baculoviruses encoding Flag-tagged wild-type human ERα and mutant ERα truncated proteins were produced and amplified using the Bac-to-Bac *Sf*9 cell transfection system (Invitrogen, Carlsbad, CA). To create the constructs for Flag-tagged wild-type ERα and the truncation mutants ERαΔ271–595, ERαΔ185–251, ERαΔ261–271, and ERαΔ250–260, the Flag-tag was first inserted N-terminally into the wild-type and the mutant plasmids in pCDNA3.1 using oligonucleotides encoding the heptapeptide tag MDYKDDDK and the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Using *Eco*R1 restriction sites, the wild-type and mutant receptor forms with Flag tags were transferred into pFASTBAC1 (Invitrogen) for expression in *Sf*9 cells. Constructs for Flag-tagged ERαΔ1–175 and ERαΔ185–268 were kindly provided by Dr. W. Lee Kraus (Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY). The sequence of all constructs was verified. To prepare the recombinant proteins, *Sf*9 cells growing in IPL41 medium were infected with baculovirus for 48 h, pelleted, and homogenized in lysis buffer [20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 1 mM dithiothreitol, and protease inhibitor cocktail (Calbiochem, San Diego, CA)], and lysates were subjected to centrifugation at 12,000 × *g* at 4 C. Lysates were incubated with Anti-Flag M2 affinity gel (Sigma, St. Louis, MO) at 4 C to isolate the Flag-tagged proteins. After four washes, the proteins were eluted by competition with flag peptide. Purity assessed by SDS-PAGE, and Coomassie blue staining was consistently greater than 95%.

Protein Interaction Analyses Using Pull-Downs

Purified myristoylated His-tagged $G\alpha_{i1}$ (300 nM) was incubated in 500 μl of 20 mM HEPES buffer (pH 8.0) containing 150 mM NaCl, 5 mM $MgCl_2$, 4% glycerol, 0.05% C12E10, and protease inhibitor cocktail (Calbiochem), with 30 μM GDP or GTPγS added for 1 h at 30 C. Purified Flag-tagged ERα proteins were added, plus or minus E_2 at 10^{-5}–10^{-8} molar concentrations, and reactions were incubated at 4 C for 1 h with gentle agitation. Further incubation was performed for 1 h with Ni-nitrilotriacetic acid (NTA) resin (Qiagen, Valencia, CA) to allow binding of His-tagged $G\alpha_{i1}$. Samples were washed with the 20 mM HEPES buffer, and the resin was pelleted and suspended in SDS-PAGE sample buffer. After resolution by 10% SDS-PAGE, immunoblot analyses were performed with the $G\alpha_{i1/2}$-specific antiserum B087 (32), and mouse monoclonal antibodies Ab-15 (Labvision, Fremont, CA) or AER320 (Labvision) directed against ERα. In selected experiments, the impact of ICI 182,780 (10^{-5} M) was determined. In other studies, a peptide representing amino acids 251–260 of ERα (MKGGIRKDRR) or scrambled peptide (GRGKRIRDKM) was added to the pull-downs (10× relative to wild-type ERα). Additional pull-downs were performed with myristoylated $G\alpha_{i1}$-GDP and recombinant ERβ (Invitrogen) or recombinant AR in truncated form (amino acids 606–

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902) (Invitrogen). Flag pull-down experiments were performed similarly using the Flag-tagged wild-type ERα and mutant ERα proteins, $G\alpha_i$ and/or $G\beta_1\gamma_2$ and Anti-Flag M2 affinity gel (Sigma). In additional experiments, protein interactions were evaluated using COS-7 cell plasma membranes. COS-7 cells were transfected with cDNAs for ERβ, AR (kindly provided by Dr. Michael McPhaul, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX), GR, or VDR, and 48 h later plasma membranes were isolated as previously described (21). The plasma membranes were then used in pull-down experiments with myristoylated $G\alpha_{i1}$-GDP done in the absence or presence of 10^{-5} M E_2, dihydrotestosterone, dexamethasone, or 1,25-dihydroxy vitamin D_3, respectively. The Ni-NTA eluted samples were resolved by 10% SDS-PAGE, and immunoblot analyses were performed with receptor-specific antibodies for ERβ and VDR (Affinity BioReagents, Golden, CO) and AR and GR (Santa Cruz Biotechnology, Santa Cruz, CA), or with the $G\alpha_{i1/2}$ protein-specific antiserum B087.

Cell Culture and Transfection

COS-7 cells (American Type Culture Collection, Manassas, VA) grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum were transfected with cDNA encoding wild-type human ERα or ERαΔ250–260 in pCDNA3.1 using LipofectAMINE Plus (Invitrogen). In selected studies, cells were cotransfected with either empty vector or pLP-CMV-HA(ERα251–260), an HA-tagged peptide comprised of amino acids 251–260 of ERα, or with empty vector *vs.* cDNA for βARK-ct kindly provided by Dr. Robert Lefkowitz (Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC) (33). Primary BAEC were cultured and maintained as previously described and used within seven passages (34). BAEC were transfected with either empty vector *vs.* ERαΔ250–260, or with empty vector *vs.* pLP-CMV-HA(ERα251–260). The sequences of all constructs were verified, and expression was confirmed by immunoblot analyses.

Src and erk Activation by Immunoblot Analyses

To assess src activation, COS-7 cells were treated with 10^{-8} M E_2 for 0–15 min and lysed, and immunoblot analyses were performed using anti-phospho-tyrosine-416 Src polyclonal antibody (Cell Signaling Technology, Danvers, MA) and anti-Src monoclonal antibody (Santa Cruz Biotechnology). To assess erk activation, immunoblotting was performed with anti-phospho-erk polyclonal antibody (Promega, Madison, WI) and anti-erk2 monoclonal antibody (Upstate Biotechnology, Charlottesville, VA).

GTPγS Binding Studies

Sf9 cells were grown in IPL41 medium and infected with baculoviruses that expressed His_6-Gαi, $G\beta_1$, and $G\gamma_2$, and either no additional virus or M2 muscarinic receptor or ERα baculoviruses. Forty-eight hours later, cell membranes were harvested, homogenized into buffer containing 20 mM HEPES, 150 mM NaCl, 2 mM $MgSO_4$, and 1 mM EDTA (pH 8.0) and used for GTPγS binding time course studies. [^{35}S]GTPγS was added to the membranes at 30 C in the presence or absence of ligand (10^{-6} M carbachol or 10^{-6} M E_2 or 10^{-5} M ICI 182,780) to initiate the reactions and aliquots were removed at specific time points. Each reaction aliquot was quenched in stop buffer (300 mM $MgCl_2$, 3.0 mM GDP, 3.0 mM GTP) and extracted with 1% sodium cholate for 1 h at 4 C. After centrifugation at 100,000 × *g* for 20 min, the extracts were adsorbed onto Ni-NTA in a buffer containing 20 mM HEPES (pH 8.0), 100 mM NaCl, 1 mM $MgCl_4$, 10 μM GTP, 0.5% C12E10 (Sigma) to pull down His-tagged Gαi-GTPγS. The amount of Gαi-GTPγS (picomoles per microgram of membrane protein) was determined by liquid scintillation counting.

NOS Activation

NOS activation was assessed in intact BAEC by measuring L-[^{14}C]arginine conversion to L-[^{14}C]citrulline using previously reported methods (35). Cells were treated with 10^{-8} M E_2 in the absence or presence of 10^{-5} M ICI 182,780. Stimulated activity is expressed as a percentage of basal activity, and results were confirmed in three independent experiments.

Monocyte Adhesion Assays

The adhesion of monocytes to BAEC was evaluated as previously described (35). Near-confluent BAEC were treated with medium alone or medium plus LPS (100 ng/ml) for 18 h in the absence or presence of 10^{-8} M E_2 with or without 2 mM nitro-L-arginine methyl ester. U937 cells (1 × 10^6 per 35-mm plate) were added to each monolayer under rotating conditions, nonadhering cells were removed by gentle washing with PBS, cells were fixed with 1% paraformaldehyde, and the number of adherent cells was counted per ×20 magnified field. eNOS and actin abundance was evaluated by immunoblot analyses in additional plates of BAEC treated in an identical manner.

Statistical Analysis

Comparisons were made between multiple groups by ANOVA with Neuman-Keuls *post hoc* testing. Significance was defined as $P < 0.05$.

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Address all correspondence and requests for reprints to: Philip W. Shaul, Department of Pediatrics, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390-9063. E-mail: philip.shaul@utsouthwestern.edu.

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