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p21-Activated kinase mediates rapid estradiol-negative feedback actions in the reproductive axis

Zhen Zhao, Cheryl Park, Melissa A. McDevitt, Christine Glidewell-Kenney, Pierre Chambon, Jeffrey Weiss, J. Larry Jameson, and Jon E. Levine

Nonclassical estrogen receptor \( \alpha \) (ER\( \alpha \)) signaling can mediate E\(_2\) negative feedback actions in the reproductive axis; however, downstream pathways conveying these effects remain unclear. These studies tested the hypothesis that p21-activated kinase 1 (PAK1), a serine/threonine kinase rapidly activated by E\(_2\) in nonneuronal cells, functions as a downstream node for E\(_2\) signaling pathways in cells of the preoptic area, and it may thereby mediate E\(_2\) negative feedback effects. Treatment of ovariectomized (OVX) rats with estradiol benzoate (EB) caused rapid and transient induction of phosphorylated PAK1 immunoreactivity in the medial preoptic nucleus (MPN) but not the arcuate nucleus. To determine whether rapid induction of PAK phosphorylation by E\(_2\) is mediated by nonclassical [estrogen response element (ERE)-independent] ER\( \alpha \) signaling, we used female ER\( \alpha \) null (ER\( \alpha \)\(^{-/-}\)) mice possessing an ER knock-in mutation (E207A/G208A; AA), in which the mutant ER\( \alpha \) is incapable of binding DNA and can signal only through membrane-initiated or ERE-independent genotropic pathways (ER\( \alpha \)^{AA} mice). After 1-h EB treatment, the number of pPAK1-immunoreactive cells in the MPN was increased in both wild-type (ER\( \alpha \)^{+/+}) and ER\( \alpha \)^{AA} mice but was unchanged in ER\( \alpha \)^{-/-} mice. Serum luteinizing hormone (LH) was likewise suppressed within 1 h after EB treatment in ER\( \alpha \)^{+/+} and ER\( \alpha \)^{AA} but not ER\( \alpha \)^{-/-} mice. In OVX rats, 5-min intracerebroventricular infusion of a PAK inhibitor peptide but not control peptide blocked rapid EB suppression of LH secretion. Taken together, our findings implicate PAK1 activation subsequent to nonclassical ER\( \alpha \) signaling as an important component of the negative feedback actions of E\(_2\) in the brain.

GnRH | LH | estrogen receptor \( \alpha \)

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This article contains supporting information online at www.pnas.org/cgi/content/full/0812597106/DCSupplemental.

Results

E\(_2\) Rapidly Induces PAK1 Phosphorylation In Vivo. The effects of EB on the expression of pPAK1 were assessed by peroxidase immunohistochemical analyses of the lateral and medial subdivisions of medial preoptic nucleus (MPN) and MPNm, respectively and the arcuate nucleus (AN). Treatment with EB but not oil vehicle produced a rapid and transient increase in the number of phosphorylated PAK1. To distinguish relative contributions of classical versus nonclassical ER\( \alpha \) signaling to E\(_2\) actions in vivo, Jakacka et al. (6) developed a gene knockin mouse that expresses a mutant (E207A/G208A; AA) form of ER\( \alpha \) with disrupted classical (ERE-dependent) but intact nonclassical (ERE-independent) ER\( \alpha \)-signaling capacities. By using animals in which the AA mutant allele was introduced onto the ER\( \alpha \)-null (ER\( \alpha \)^{-/-}) mutant background (ER\( \alpha \)^{-AA} mice), we determined that nonclassical ER\( \alpha \) signaling can rescue the majority of E\(_2\) negative feedback effects that are present in wild-type (ER\( \alpha \)^{+/+}) and completely absent in ER\( \alpha \)^{-/-} mice (7).

Here, we attempt to identify a downstream mediator of nonclassical ER\( \alpha \) signaling mechanisms conveying E\(_2\) negative feedback in the brain. E\(_2\) can modulate dendrite morphogenesis and induce synaptogenesis in hypothalamic (8–11) and extrahypothalamic neuronal populations (12, 13), and such structural plasticity may mediate E\(_2\) feedback effects (14). That the ER\( \alpha \) isoform appears to mediate many of these effects rapidly (15) is consistent with the idea that membrane-initiated, nonclassical ER\( \alpha \) signaling can rapidly induce the actin-cytoskeletal reorganization required for synaptic remodeling (16). In nonneuronal cells, ER\( \alpha \) signaling produces rapid alterations in cell shape, polarity, and motility by activating p21-activated kinase 1 (PAK1) (17), the best-characterized member of a family of conserved mammalian serine/threonine kinases that function as downstream effectors of activated Rho GTPases, Rac1 and Cdc42, as well as phosphatidylinositol 3-kinase (PI3K) (18). Because nonclassical ER\( \alpha \) signaling and activated PAK1 share common effects on neuronal morphology (19), and because E\(_2\) can activate PAK1 through nonclassical mechanisms (20), we tested the hypothesis that the nonclassical negative feedback actions of E\(_2\) are conveyed in part via ER\( \alpha \)-mediated activation of PAK1.


The authors declare no conflict of interest.

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Representative tissue sections containing the AN from an were without effect on the number of pPAK1-Ir cells in the AN. Controls. In the same OVX animals, the oil and EB treatments significantly different from corresponding values in oil-treated tissues at each time point (Fig. 2). The number of pPAK1-Ir neurons in the AN was not significantly different after EB treatment compared with corresponding oil treatment (Table B). Data are represented as the mean ± SEM (n = 6–9).

ERα signaling mediates pPAK1 activation in preoptic cells. To assess the role of nonclassical ERα signaling, we compared the ability of 1 μg of EB to induce pPAK1-Ir in the preoptic areas of ERα+/+, ERα−/−, and ERα−/− mice within 1 h of treatment. Consistent with our findings in OVX rats, acute E2 treatment induced a significant increase in the number of pPAK1-Ir cells within 1 h in the MPNl and MPNm in OVX wild-type ERα+/+ mice (P < 0.01; Fig. 3 B and C). This effect was completely absent in the ERα−/− mice (Fig. 3 B and C), confirming the obligatory involvement of ERα in E2-mediated action. In the OVX ERα−/− mice, the ability of E2 to induce pPAK1 was restored to levels observed in the ERα+/+ mice (P < 0.05; Fig. 3 B and C), indicating that nonclassical ERα signaling is sufficient to mediate E2 effects on pPAK1 in the MPN. Representative photomicrographs of pPAK1-Ir in the MPN of the 3 genotypes are provided in Fig. 3D. The summary values for pPAK1-Ir in the 3 genotypes are depicted in Fig. 3B and C. In contrast to the effects of E2 in the preoptic area, E2 was without any effect on pPAK1-Ir in the AN in any of the groups (Fig. 4).

Nonclassical ERα Signaling Mediates Rapid Negative Feedback Actions of E2. If E2 can rapidly activate pPAK1 through nonclassical ERα signaling, and this mechanism is integral to E2 negative feedback, then it should be true that E2 can engage this mechanism to effect a rapid suppression of LH secretion. We therefore tested the ability of acute EB injections to rapidly suppress LH by nonclassical ERα signaling. To examine the rapid feedback actions of E2 on LH release, ERα+/+, ERα−/−, and ERα−/− female mice were ovariectomized, and 7 days later (1,000–1,200 h) they received s.c. injections of 1 μg of EB or oil vehicle. Animals were killed 1 h after the injection. A total of 3 blood samples were collected from each mouse—one at the time of OVX, one just before the EB injection, and one at sacrifice 1 h later. LH RIA of the plasma samples revealed that LH levels before OVX were low in ERα+/+, slightly elevated in ERα−/−, and greatly increased in ERα−/− mice, as reported previously (7). The serum LH levels were significantly elevated in both ERα+/+ (P < 0.001; Fig. 5, a) and ERα−/− (P < 0.05; Fig. 5, b) mice at 7 days after OVX compared with pre-OVX levels. In contrast, LH levels in ERα−/− mice were elevated before OVX and remained at these levels at 7 days after OVX. Treatment of OVX ERα+/+ mice with EB resulted in a suppression of LH

Fig. 1. Time dependence of E2-induced pPAK1-Ir in the MPN of female rats. OVX females rats treated with oil or EB were killed at 0.5, 1, 2, and 4 h after injection. (A) Representative photomicrographs of pPAK1-Ir cells in the MPN and adjacent regions of OVX rats 0.5 h after oil or EB injection. (Scale bar: 100 μm.) The number of pPAK1-Ir cells in the MPN (B) and MPNm (C) was significantly greater after 0.5 and 1 h of EB treatment compared with corresponding oil treatment (n = 6–9). Data are represented as the mean ± SEM (***, P < 0.001; **, P < 0.01; *, P < 0.05).

Nonclassical ERα Signaling Mediates Rapid E2 Induction of pPAK1-Ir. ERα can mediate negative feedback actions of E2 by nonclassical signaling mechanisms, and rapid, nonclassical ERα-mediated activation of pPAK1 has been shown to occur in breast cancer cells (17). We therefore sought to determine whether nonclassical

Fig. 2. E2-independent pPAK1-Ir in the AN of female rats. Animals were treated as described in Fig. 1. (A) Representative photomicrographs of pPAK1-Ir in the AN and adjacent regions of OVX rats 1 h after oil or EB injection. (Scale bar: 100 μm.) The number of pPAK1-Ir neurons in the AN was not significantly different after EB treatment compared with corresponding oil treatment (B).
Suppression seen in the OVX ERα−/− mice 1 h after oil or EB injection. (Scale bar: 100 μm.) The number of pPAK1-Ir cells in the MPN of female ERα−/− mice constituted ∼70% of the suppression seen in the OVX ERα+/+ mice (P < 0.001; Fig. 5, c).

Inhibition of PAK Phosphorylation Blocks Acute EB Suppression of LH Secretions. Within the PAK kinases, a conserved, proline-rich sequence of 18 aa called PAK18 binds tightly to the SH3 domain of PAK-interacting exchange factor (PIX). The PIX–PAK interaction was shown to be essential for PAK activation (21). The PAK18 peptide has been used to interfere selectively with the activation of PAKs 1–3 in cell cultures (22–26) and in vivo (27). The PAK18 peptide but not the inactive peptide R192A has been shown to reduce pPAK levels in the hippocampus by 80% after intracerebroventricular (icv) treatments, an effect that was accompanied by drebrin loss, coflin pathology, and memory deficits (22). This suggests that PAK18 and R192A are valid tools for inhibiting PAKs. We first verified the ability of PAK18 to block PAK phosphorylation by using hypothalamic GT1-7 cells in vitro. Western blot analysis of PAK demonstrated that inhibition of PAK phosphorylation is rapid and significant when these cells are incubated with the peptide PAK18 (10 μM) for 1 h compared with R192A (P < 0.05; Fig. 6A). Subsequently, we infused peptide PAK18 (6 μg/μL, 1 μL/min in 5 min) or R192A in the lateral ventricle of OVX rats. The cerebrospinal fluid (CSF) volume was 250 μL per rat, with a physiological flow rate of 2.9 μL/min (30); therefore, the concentration of PAK18 and R192A in the CSF was ∼10 μM 1 h after the infusion, a dose found effective in vitro. Immediately after the peptide infusion, OVX animals were given an s.c. injection of EB (30 μg per rat). At 1 h after EB injection, animals were killed, and brains were removed rapidly to assess the ability of PAK18 to reduce pPAK1-Ir by immuno-
target genes (7). In the present study, we have further deter-
mination that (a) E2 treatments to suppress LH secretion in as little as 20 min (31). Although rapid steroid hormone effects on gene transcription are known, the rapid (<60 min) modulation of LH by E2 occurs within a temporal window that is generally held to be too short to additionally include RNA processing, translation, posttrans-
alional enzymatic processing, intracellular transport, and neu-
rosecretion. A variety of membrane-integrated or membrane-
associated receptors and cell signaling mechanisms have instead been suggested to mediate at least some of the E2 effects on GnRH and LH. Classic work by Kelly et al. (62) revealed rapid effects of E2 on neuronal firing in preoptic neurons that were best explained by activation of membrane receptors for E2. Further work demonstrated that GnRH neurons themselves are rapidly hyperpolarized by E2, even during tetrodotoxin-induced blockade of synaptic inputs (32), suggesting direct electrophysi-
ological suppression through membrane-associated receptors in GnRH neurons. The absence of ERα in GnRH neurons has led some to suggest that rapid, direct effects of E2 on GnRH neurons may be mediated by ERβ (33) and/or an unidentified G protein-
meditated suppression of GnRH neuronal function. We also cannot exclude the possibility that ERα-dependent mechanisms may up-regulate ERα-independent E2 signaling through other receptors, which may in turn mediate E2 feedback effects.

Rapid inhibition of LH secretion can also occur via direct suppression of gonadotrope responsiveness to GnRH (35). It does not appear to be the case, however, that the nonclassical ERα signaling mechanisms described in the present studies are mediated by any such direct actions on the gonadotrope; pitu-
tary responsiveness to GnRH is not enhanced in ERα-null mutants (3), nor is it reduced by E2 in ERα-expressing afferents to GnRH neurons. The direct ERα-independent effects of E2 on GnRH neurons may provide additional components of negative feedback control of GnRH and LH release, or they may function to regulate other aspects of GnRH neuronal function. Our studies clearly implicate the ERα isoform, which is not expressed in GnRH neurons, in the rapid feedback actions of E2 in vivo, and thus alternatively suggest an indirect mechanism conveyed via ERα-expressing afferents to GnRH neurons. The direct ERα-independent effects of E2 on GnRH neurons may provide additional components of negative feedback control of GnRH and LH release, or they may function to regulate other aspects of GnRH neuronal function. Our findings are therefore consistent with the hypothesis that PAK activation in preoptic neurons mediates the negative feedback actions of E2 on GnRH release. Although these results do not reveal the cellular signaling pathways that mediate E2 activation of PAK1, the rapidity of this inhibition of PAK phosphorylation in preoptic-hypothalamic areas blocks the acute negative feedback actions of E2.

The involvement of a nongenotropic mechanism in E2 nega-
tive feedback has long been suspected, given the ability of acute E2 treatments to suppress LH secretion in as little as 20 min (31). Although rapid steroid hormone effects on gene transcription are known, the rapid (<60 min) modulation of LH by E2 occurs within a temporal window that is generally held to be too short to additionally include RNA processing, translation, posttrans-
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ological suppression through membrane-associated receptors in GnRH neurons. The absence of ERα in GnRH neurons has led some to suggest that rapid, direct effects of E2 on GnRH neurons may be mediated by ERβ (33) and/or an unidentified G protein-

Discussion

Our laboratories recently demonstrated that the majority of E2 negative feedback actions in the mouse can be exerted by a nonclassical ERα signaling mechanism that proceeds in the absence of direct binding of ERα to ERαEs in the promoters of target genes (7). In the present study, we have further determined that these inhibitory effects can occur within 1 h of E2 administration, and thus are likely exerted via nongenotropic signaling mechanisms. Our findings specifically implicate the PAKs as components of the nongenotropic ERα signaling pathways leading to the suppression of GnRH and LH, because they show that (i) E2 rapidly induces PAK1 phosphorylation in the MPN, (ii) nonclassical ERα signaling is sufficient to rescue rapid phosphorylation of PAK1 in preoptic cells, as well as suppression of LH secretion in ERα-null mutants, and (iii) acute

Fig. 6. Inhibition of PAK phosphorylation. Inhibition of PAK phosphorylation by treatment with PAK18 inhibitory peptide to GT1-7 cells (A) and in the lateral ventricle of OVX rats (B) for 1 h (n = 4–6). (Scale bar: 100 μm.) Data are represented as the mean ± SEM (**, P < 0.01; *, P < 0.05).

Fig. 7. Inhibition of PAK phosphorylation blocks acute E2 suppression of LH secretions. The LH level was significantly decreased at 1 h after R192A icv infusion and EB sc. injection. In the contrast, the LH level was not significantly altered by treatment with PAK18 and EB (n = 4–6). Data are represented as the mean ± SEM (***, P < 0.001; *, P < 0.05).
process effectively limits the possibilities to those that are initiated at the plasma membrane or within the cytoplasm and that are independent of transcriptional modulation. The actions of E2 in MPN cells parallel those observed previously in breast cancer cells, where E2 was found to activate PAK1 through a rapid, nontranscriptional mechanism.

A number of kinases have been reported to phosphorylate PAK1 and regulate its activity (37), including the cyclin B-bound Cdc2, which phosphorylates PAK1 at Thr-212, a site also targeted by the p35-bound form of Cdk5, a neuron-specific protein kinase (38, 39). Moreover, extracellular signal-regulated kinase 2 (ERK2) mediates phosphorylation of PAK1 at Thr-212 (40). PAK1 is also known to function as an effector protein of PI3K (18, 41). In the present study, ERα signaling was found to induce rapid phosphorylation of PAK1 at Thr-212 in MPN cells. Because E2 can rapidly influence MAPK/ERK and PI3K/Akt signaling pathways in a variety of cell types (42, 43) and in a variety of brain regions, including the MPN (44), it is possible that ERα signaling induces phosphorylation of PAK1 by ERα-mediated activation of either or both intermediate signaling kinases.

Consistent with the hypothesis that PAK1 mediates E2 feedback effects, recent studies have revealed that many of the cellular actions of E2 in the CNS are shared by those of activated PAK1. PAK1 activity in MPN cells parallel those observed previously in breast cancer cells, where E2 was found to activate PAK1 through a rapid, nontranscriptional mechanism.

Materials and Methods

Animals. The ERα−/− and ERα−/−A mutant mice were generated as described (6, 58, 59). Further details appear in SI Materials and Methods.

Effects of EB Treatment on PAK1 Phosphorylation. Rats and mice were anesthetized and bilaterally OVX. On the morning of day 7 after OVX (0800–1000 hours), animals were given an s.c. injection of sesame oil vehicle or EB (10 μg per rat; 1 μg per mouse). Animals were anesthetized with 75 mg/kg i.p. ketamine (Fort Dodge Laboratories) and 5 mg/kg i.p. xylazine (BURNS Veterinary Supply Inc.) and transcardially perfused with 4% paraformaldehyde (Sigma), pH 7.4, at the following time points after injection: 0.5, 1, 2, or 4 h for rats; 1 h for mice (60). Further details appear in SI Materials and Methods.

Effects of OXV and Acute EB Treatment on LH Release. Female mice 2–4 months of age were anesthetized (0800–1000 hours), and blood samples were collected immediately before OXV. At 7 days after OXV, blood samples were obtained, and either 1 μg of EB or sesame oil vehicle injections was administered s.c. At 1 h after injections, blood samples were obtained by exanguination following cardiac puncture. Further details appear in SI Materials and Methods.

Effects of PAK18 Inhibitory Peptide on EB Suppression of LH Release. Details of stereotoxic surgery, PAK18 inhibitory peptide infusion, EB treatment, and blood sample collection can be found in SI Materials and Methods.

Cell Culture and Western Blot Analysis. The effectiveness of the PAK18 peptide in suppressing PAPK phosphorylation was tested in GT1-7 cells, an immortalized GnRH-producing cell line (61). Further details appear in SI Materials and Methods.

Immunohistochemistry and Analysis. Brain sections were processed for immunohistochemistry following standard procedures. Details can be found in SI Materials and Methods.

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