Estrogen increases retrograde labeling of motoneurons: evidence of a nongenomic mechanism

Alexander K. Murashov, Rustem R. Islamov, Roger J. McMurray, Elena S. Pak, and Douglas A. Weidner

Departments of Physiology and Microbiology and Immunology, The Brody School of Medicine, East Carolina University, Greenville, North Carolina 27858

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Murashov, Alexander K., Rustem R. Islamov, Roger J. McMurray, Elena S. Pak, and Douglas A. Weidner. Estrogen increases retrograde labeling of motoneurons: evidence of a nongenomic mechanism. Am J Physiol Cell Physiol 287: C320–C326, 2004. First published March 24, 2004; 10.1152/ajpcell.00542.2003.—Estrogen has a variety of neurotrophic effects mediated via different signaling cascades, including ERK and phosphatidylinositol 3-kinase (PI3K) pathways. In this study, we investigated effects of estrogen and inhibitors for ERK and PI3K applied directly onto the cut sciatic nerve on retrograde labeling of lumbar motoneurons. A mix of retrograde tracer (Fluorogold) and 17β-estradiol, in combination with an antagonist for estrogen receptors ICI 182,780, an inhibitor of ERK1/2 pathway (U0126), an inhibitor of PI3K (LY-294002), or a protein synthesis inhibitor (cycloheximide), was applied to the proximal stump of the transected sciatic nerve for 24 h. Coapplication of Fluorogold with 17β-estradiol produced a significant increase in the number of retrograde-labeled lumbar motoneurons, compared with Fluorogold alone. Estrogen potentiation of retrograde labeling was inhibited by application of ICI 182,780, U0126, LY-294002, and cycloheximide. Immunohistochemical analysis of the sciatic nerve, 24 h following crush injury, revealed accumulation of phospho-ERK in regenerating nerve fibers. The data suggest a role for estrogen, ERK, PI3K, and protein synthesis in the uptake and retrograde transport of Fluorogold. We propose that estrogen action in peripheral nerve fibers is mediated via the ERK and PI3K signaling pathways and is reliant on local protein synthesis.

Sciatic nerve; estrogen receptor; extracellular signal-regulated kinase

ESTROGEN HAS A VARIETY OF beneficial effects on the nervous system, including modulation of mental and neurodegenerative conditions, such as schizophrenia, Parkinson’s disease, and Alzheimer’s disease (8, 11), protection from trauma (6, 36), potentiation of neurite growth and synaptogenesis (3, 27, 46), and stimulation of peripheral nerve regeneration (17, 18, 44). Estrogen action in the nervous system has been implicated in greater resistance of females to neurological diseases and injury and in better recovery rates (7, 36). The diverse effects of estrogen on a variety of tissues are mediated via intracellular estrogen steroid receptors. Two estrogen receptors (ER), ER-α and ER-β, have been characterized to date, and there is evidence of another type of ER possibly existing (33, 45). Estrogen may trigger “genomic” and “nongenomic” course of events (31, 39). The classic genomic estrogenic action involves ER acting as a transcriptional enhancer, requiring direct interaction with DNA with subsequent changes in gene expression. The nongenomic mechanism includes interactions of the ER system with different intracellular signaling pathways (21, 26, 45, 46). Some of the factors and pathways implicated in estrogen signaling include neurotrophins (41), IGF-I (1), cAMP/ protein kinase A (3), N-methyl-D-aspartate receptors (26), a family of stress-activated protein kinase and MAPKs, including ERK (40, 45) and p38 kinase (23, 50), and phosphatidylinositol 3-kinase (PI3K)/Akt signaling cascade (19). Recent observations suggest that some of these signaling pathways are possibly involved in regulation of axonal transport (13, 24). In particular, PI3K has been implicated in actin phosphorylation and reorganization of microfilaments, which might be essential for regulation of axonal transport (32, 35, 48). In developing sympathetic neurons, PI3K was shown to be critical for retrograde neurotrophin signaling and survival (13, 22). The ERK/microtubule-associated protein 2 kinase has also been shown to have an effect on axonal transport by altering the state of phosphorylation of microtubule-associated protein 2 (37, 47). Moreover, signaling endosomes containing ERK1/2 were shown to be retrogradely transported in the isolated sciatic nerve in vitro (10). Therefore, both PI3K and ERK signaling pathways could be involved in regulation of axonal transport. Whereas the mechanism of retrograde signaling is not completely understood, substantial evidence supports a model in which the endosome-based transport of neurotrophins-Trk receptor tyrosine kinase complexes transmits a survival signal that involves both ERK and PI3K (13). The nonvesicular retrograde transport is likely to function in parallel, although its mechanism remains far from clear.

In the present study, we examined whether estrogen may influence retrograde axonal transport via ERK and/or PI3K signaling cascades in motoneurons. The effect of 17β-estradiol was evaluated on the model of retrograde labeling of lumbar motoneurons in ovariec-tomized female mice after sciatic nerve axotomy. A mix of retrograde tracer (Fluorogold) and 17β-estradiol, in combination with an antagonist for ER ICI 182,780, an inhibitor of ERK1/2 pathway (U0126), an inhibitor of PI3K (LY-294002), or a protein synthesis inhibitor (cycloheximide), was applied to the proximal stump of the transected sciatic nerve for 24 h. Our results showed that 17β-estradiol applied directly onto the cut sciatic nerve produced a significant increase in the number of Fluorogold-labeled lumbar motoneurons. Estrogenic stimulation of retrograde labeling was inhibited by application of ICI 182,780, U0126, LY-294002, and cycloheximide. The present results indicate a role for estrogen, ERK, and PI3K signaling cascades in the uptake and retrograde transport of Fluorogold in motoneurons.

Address for reprint requests and other correspondence: A. K. Murashov, East Carolina Univ. School of Medicine, Brody Bldg., 600 Moye Blvd., Greenville, NC 27858 (E-mail: murashoval@mail.ecu.edu).

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MATERIALS AND METHODS

Animals and surgical procedure. Female, 8-wk old ICR mice were obtained from Charles River Laboratories (Wilmington, MA). Animals were kept, one per cage, under standard laboratory conditions with unlimited access to food and water and a 12:12-h light-dark schedule. Ketamine-xylazine anesthesia (1.5 mg/100 g ip) was used in all surgical procedures. To evaluate the role of estrogen on axonal transport, under controlled conditions, bilateral ovariectomy was performed first (17).

Two weeks after ovariectomy, the right sciatic nerve was cut in the midthigh, and a 7-mm Silastic tube with a 1.47-mm inner diameter was applied to the proximal nerve stump (28, 48). Afterward, the fluorescent dye Fluorogold or a mixture of Fluorogold with pharmacological agents was administered into the tube (7 μl total volume). The lower end of the tube was sealed with a petroleum jelly (Vaseline), the tube was glued to the surrounding skeletal muscles with tissue adhesive (3M Vetbond), and the incision was closed with wound clips. After application of Fluorogold for 24 h, animals were killed. In every experiment, we verified that the tube was still in place and that Fluorogold did not stain the adjacent tissue. Only the mice, which had the yellowish proximal nerve stump sited inside the tube (~90% of animals), were employed in the study.

For immunohistochemical study, 2 wk after ovariectomy, the right sciatic nerves were crushed in the midthigh for 15 s with a fine hemostat (n = 3). In sham operations, right sciatic nerves were shortly exposed, the wounds were closed, and the mice were allowed to recover (17). The duration of the crush injury was 24 h. Animal protocols were approved by the East Carolina University Animal Care and Use Committee.

Retrograde labeling. For retrograde labeling of motoneurons, 5% Fluorogold (Fluorochrom, Denver, CO) in vehicle was applied to the proximal stump of the nerve for 24 h. Retrograde labeling of motoneurons was performed to evaluate the efficiency of axonal transport in the presence of 10 nM 17β-estradiol (Sigma) (40); 1 μM ICI 182,780 (40), an ER antagonist (Tocris); 100 μM U0126 (9), an inhibitor of mitogen-activated protein kinase kinase (MEK)1/2 (Cell Signaling); 100 μM LY-294002 (38), an inhibitor of PI3K (Calbiochem); and 10 μg/ml cycloheximide (29), an inhibitor of protein synthesis (Sigma). 17β-Estradiol, ICI 182,780, and U0126 were initially dissolved in 100% DMSO and further diluted with saline; thus the final concentration of DMSO was the same in all mixtures and did not exceed 2%, which has no effect on uptake and retrograde transport of Fluorogold (48). In all experiments, inhibitors were applied to the nerve stump 15 min before application of a mixture containing Fluorogold with an inhibitor, or Fluorogold with an inhibitor and 17β-estradiol. To evaluate whether systemic estrogen treatment might affect retrograde axonal transport, some mice were injected subcutaneously with 17β-estradiol (0.1 ml; 500 μg/kg) (25).

Morphometry of retrograde-labeled motoneurons. Twenty-four hours postsurgery, mice were euthanized and perfused with cold PBS, followed by cold 4% paraformaldehyde in PBS (pH 7.4). Lumbar spinal cords were removed and postfixed in 4% paraformaldehyde in PBS (pH 7.4). Lumbar spinal cords were removed and postfixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, and embedded in Tris-buffered saline tissue-freezing medium (Triangle Biomedical Science, Durham, NC). Cryostat serial coronal 30-μm sections were analyzed with fluorescence microscopy by using a wideband ultraviolet filter. Fluorogold-labeled motoneurons in the ipsilateral side were recognized by their size, shape, and location (Fig. 1) and counted in every section without allowing for split nuclei, according to the protocol described elsewhere (28, 48). The images were analyzed for the mean cross-sectional area of motoneurons by using ImageJ (NIH Image). We observed no difference in the mean cross-sectional area of labeled cells between control and treated animals, which is why we did not correct the neuronal counts using Abercrombie’s formula. All values are represented as the means ± SE. Statistical analysis was performed with one-way ANOVA Newman-Keuls multiple-comparison test by using Prism (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA).

Tracing of radiolabeled estrogen in spinal cord. Radiolabeled estrogen ([2,4,6,7,16,17-3H]estradiol) was obtained from PerkinElmer Life Science (Boston, MA). The right sciatic nerve was cut, and a Silastic tube containing 7 μl of 10 nM 17β-[3H]estradiol (specific activity 110 Ci/mmol) was applied to the proximal nerve stump for 24 h. Animals (n = 2) were euthanized, and sciatic nerves (8-mm piece of the proximal nerve stump) and the whole spinal cords were removed. Dissected lumbar parts of spinal cords were divided into right (injured) and left (contralateral) sides; sciatic nerve proximal stumps were divided into two 4-mm portions, proximal and distal (immersed in 17β-[3H]estradiol). Tissue homogenized in PBS was transferred to liquid scintillation fluid (5 ml) and counted with TRI-CARB 2100TR analyzer (Packard). The probes from different animals were counted separately. Control tubes contained 7 μl of 10 nM 17β-[3H]estradiol or a vehicle. For all radioactive experiments, cages designated for radioactively contaminated animals were used.

Immunohistochemistry. All tissues for immunohistochemistry were collected 24 h after nerve crush. The staining was performed according to our laboratory protocol, as described previously (18). Mice were perfused with cold PBS, followed by cold 4% paraformaldehyde in PBS (pH 7.4). Sham sciatic nerves (at the midthigh level) and crushed nerves (a 5-mm piece with crush site in the middle) were removed and postfixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, and embedded in Tris-buffered saline tissue-freezing medium (Triangle Biomedical Science). Frozen 10-μm longitudinal sections of sciatic nerves were processed for immunostaining. Rabbit phospho-ERK antibodies (Cell Signaling) were applied at a 1:200 dilution. Sections were stained by using the Elite ABC kit (Vector Laboratories), and tissue antigens were visualized with a DAB substrate kit for peroxidase (Vector Laboratories). Densitometric analysis of the immunoprecipitate intensity was performed by using Kodak Digital Science 1D Image Analysis software (Eastman Kodak, Roch-
estrogen on retrograde labeling was established when a mixture of Fluorogold with 17β-estradiol was applied to the transected sciatic nerve \((n = 4)\). Both systemic and local delivery of estrogen enhanced retrograde labeling of Fluorogold (Fig. 3A). After 24 h, the total number of Fluorogold-labeled motoneurons was nonsignificantly elevated in systemically treated mice \((1,314 \pm 155\) vs. \(963 \pm 118; P < 0.1\)) and was significantly higher in mice with local application of estrogen \((1,449 \pm 159; P < 0.05)\) compared with control \((963 \pm 118)\), which had Fluorogold alone \((n = 7)\).

To investigate the role of ERs in potentiation of retrograde labeling in estrogen-treated mice, ER antagonist ICI 182,780 was applied to the proximal nerve stump in the mice with systemic \((n = 4)\) and local delivery of estrogen \((n = 3)\). In the experiment with local administration of estrogen, ICI 182,780 was applied to nerve stump 15 min before administration of Fluorogold. The results indicated that ICI 182,780 caused a significant reduction of labeled motoneurons in the mice with systemic estrogen treatment \((550 \pm 184\) vs. \(1,314 \pm 155; P < 0.01\)) (Fig. 3A). However, in the experiment with a local application of estrogen, ICI 182,780 produced a nonsignificant decrease in the number of retrogradely labeled motoneurons \((1,065 \pm 62\) vs. \(1,449 \pm 159; P < 0.1\)). Thus local application of estrogen negated the inhibiting effect of ICI 182,780 on ER function. These results suggest that estrogen action on retrograde labeling may be mediated via activation of ERs.

Local application of PI3K inhibitor LY-294002 \((n = 3)\) to the proximal stump of the sciatic nerve had no direct effect on retrograde labeling. Indeed, the number of labeled motoneurons did not differ from control values \((949 \pm 130\) vs. \(963 \pm 118\)) (Fig. 3B). At the same time, 17β-estradiol mixed with LY-294002 \((n = 3)\) did not increase the number of Fluorogold-labeled cells \((891 \pm 253)\) compared with control \((963 \pm 118; P > 0.05)\) or LY-294002-treated mice \((949 \pm 130; P > 0.05)\). Thus these results indicate that PI3K inhibitor LY-294002 blocks the estrogen-induced increase in retrograde labeling.

In contrast, MEK1/2 inhibitor U0126 \((n = 4)\) markedly affected retrograde labeling. Application of U0126 alone caused a significant decrease in the number of labeled motoneurons \((405 \pm 57\) vs. \(963 \pm 118, P < 0.05)\) (Fig. 3B). Estrogen coapplied to the nerve stump with U0126, 15 min after preincubation with the inhibitor \((n = 4)\), failed to increase the retrograde labeling \((353 \pm 115\) vs. \(405 \pm 57; P > 0.05)\).

The two selected inhibitors have shown a noticeably different impact on retrograde labeling. Obviously, the PI3K inhibitor did not have a direct effect on labeling compared with control, whereas the MEK1/2 inhibitor alone significantly reduced the number of Fluorogold-labeled cells. Coapplication of estrogen, in combination with any of the inhibitors, did not increase retrograde labeling. These data indicate a role of both MEK/ERK and PI3K signaling in regulation of peripheral nerve function.

Within the axon, the local translation of mRNA is an important source of proteins during axon growth \((12)\). In this study, we investigated Fluorogold backlabeling, under conditions of inhibited protein synthesis. Application of the protein synthesis inhibitor cycloheximide alone to the proximal stump of the nerve \((n = 4)\) showed a significant decrease in the number of labeled motoneurons, compared with control \((490 \pm 93\) vs. \(963 \pm 118; P < 0.05)\) (Fig. 3C). Estrogen applied to the proximal nerve stump after 15-min preincubation
with cycloheximide \((n = 4)\) partially removed the blocking effect of the protein synthesis inhibitor on retrograde labeling. The number of labeled motoneurons was not statistically different from that of control mice and constituted \(594 \pm 179\).

The data indicate that the local inhibition of protein synthesis reduced retrograde transport of Fluorogold, whereas estrogen coapplied with cycloheximide partially negated the action of the cycloheximide.

**Accumulation of phospho-ERK1/2 in the sciatic nerve 24 h after crush injury.** Our previous study \((18)\) has demonstrated a 1.8-fold increase in the level of activated ERK1/2 after crush injury of the sciatic nerve. The data obtained in this study showed that the MEK1/2 inhibitor significantly reduced retrograde labeling of motoneurons. To evaluate the possible involvement of ERK in axonal transport, we analyzed localization of phospho-ERK in the regenerating sciatic nerve 24 h after crush injury.

Staining of sham sciatic nerves revealed slightly immunopositive nerve fibers (Fig. 4A). In the injured nerve, the proximal part (above the crush site) had a weak staining of the nerve fibers as well. Interestingly, in the distal portion (below the crush site) of the nerve, we observed strongly ERK-positive regenerating axons (Fig. 4B).

Densitometric analysis of the immunoprecipitate revealed a significant increase in the level of intensity in the regenerating portion of the nerve, compared with the portion of the nerve above the crush site and intact nerves (Fig. 5). Thus the histological and densitometric analyses clearly showed prominent accumulation of phospho-ERK1/2 in the distal portion of the proximal stump of the sciatic nerve.

**DISCUSSION**

Spinal motoneurons extend their axons over long distances from perikarya to skeletal muscle fibers, and efficiency of axonal transport in both anterograde and retrograde directions
is a crucial parameter in motoneuronal function. The axonal transport machinery is composed of cytoskeletal and motor proteins, driven by a variety of protein kinases and phosphatases modifying phosphorylation states of membrane and cytoplasmic proteins.

Retrograde axonal transport of target-derived neurotrophic factors is pivotal for survival, axon growth, and navigation during development and regeneration of motoneurons. Neurotrophins, such as nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3, are imported into the cell by receptor-mediated internalization and can affect cell function locally or be delivered to the perikarya by retrograde axonal transport (10, 13, 22, 24, 49).

Our laboratory’s recent studies suggest a positive role of estrogen on regeneration of peripheral nerves (17, 18). Systemic delivery of the estrogen significantly enhanced regeneration of the sciatic nerve in ovariectomized female mice. Macroarray analysis in the lumbar spinal cord revealed upregulation of a wide array of genes promoting axonal growth, including genes encoding motor and cytoskeletal proteins, as well as kinesin motor protein C2, neuronal kinesin heavy chain, Rac1, and cortactin (18). Furthermore, we observed increased expression of genes for ER-α and ER-β in axotomized lumbar motoneurons and accumulation of ERs in the regenerating axons.

Both genomic and nongenomic mechanisms of estrogen action could be implicated in the regulation of retrograde axonal transport. Our experiments with ER antagonist ICI 182,780 applied to the cut sciatic nerve demonstrated that the local inhibition of ERs blocked the effect of systemically delivered estrogen on retrograde delivery of Fluorogold. Therefore, estrogen potentiation of retrograde labeling was mediated via a local signaling mechanism. Indeed, the tracing of [3H]estradiol applied to proximal stump of the severed sciatic nerve showed no retrograde transport of [3H]estradiol to the soma of the lumbar motoneurons.

Interestingly, estrogen coapplied to the nerve stump with ICI 182,780 abolished the blocking effects of the ER antagonist on axonal transport. This may be a result of competitive interaction between estrogen and ICI 182,780, which confirms that estrogen action on retrograde labeling of motoneurons is mediated via local ERs.

Estrogen has been shown to activate PI3K (19) and ERK signaling cascades in neuronal cells (40). The PI3K activity plays an important role in neuronal polarity (38), retrograde axonal transport (48), and neuroprotection (14, 16). In sympathetic neuron cultures, the PI3K pathway was shown to be critical for neurotrophin retrograde signaling and neuronal survival (22). The ERK signaling cascade was shown to be involved in neuronal differentiation (2), local protein synthesis (29), dendrite formation (47), and cellular viability (4, 15). In addition, it has been demonstrated that PI3K and ERK pathways might cooperatively affect neuronal function (5) and regulate retrograde neurotrophin signaling via an endosome-based mechanism (10, 13, 49).

It has been demonstrated that estrogen cross talks with both cascades (19, 40). The estrogen-induced activation of the ERK-signaling pathway in neurons is well established, although the exact role of estrogen in ERK activation is far from clear. Recent evidence of ERK-dependent translation of mRNA within axons (29), and the fact that ERK can be transported in the anterograde (34) and retrograde (10) directions, suggest that ERK may regulate axonal transport in two aspects. It may activate the local synthesis of specific proteins and phosphorylate cytoskeletal and motor proteins.

**Fig. 6.** Hypothetical mechanism of E2 signaling in peripheral nerves. MAP-2, microtubule-associate protein-2.
Thus both ERK and PI3K are likely to contribute to the internalization of Trk-containing endosomes and the maturation and retrograde transport of signaling vesicles critical for neuronal survival (13).

To investigate the possible mechanism of estrogen action on retrograde labeling, PI3K inhibitor (LY-294002) or MEK1/2 inhibitor (U0126) was applied to the proximal stump of the severed sciatic nerve alone or in the presence of estrogen. Interestingly, that PI3K inhibitor (LY-294002) did not impair the retrograde labeling in our experiments, whereas it attenuated retrograde signaling in sympathetic neuron culture (22). The discrepancy could be explained by using different experimental models. It is likely that the effect of LY-294002 in sympathetic neuron culture was somewhat stronger than on axotomized sciatic nerve in live animal. Another possible explanation is that the effect of LY-294002 on retrograde signaling could be more pronounced in sympathetic neurons, which depend on nerve growth factor for their survival, than in motoneurons. The failure of estrogen to increase retrograde labeling in the presence of U0126 and LY-294002 indicates the possible cross talk among estrogen, ERK, and PI3K signaling pathways in peripheral nerve fibers. Therefore, our observations suggest that estrogen may trigger synergistic effects of PI3K and MEK-ERK cascades on retrograde transport.

The recent data on local translation of mRNA within axons indicate that local protein synthesis is an essential event during nerve regeneration (12, 42, 43, 51). Based on this and the data that the estrogen-activated MEK-ERK cascade may affect local protein synthesis (29), we investigated the effects of the protein synthesis inhibitor cycloheximide on axonal transport. Cycloheximide alone significantly reduced the number of labeled motoneurons. Co-application of estrogen with cycloheximide partially removed the blocking effect of the inhibitor on retrograde labeling of motoneurons. The data suggest that impaired local protein synthesis severely affects retrograde transport and that estrogen may support protein synthesis and/or stimulate axonal transport via activation of ERK. Thus the ER antagonist (ICI 182,780), inhibitors for MEK1/2 (U0126) and PI3K (LY-294002), and a protein synthesis inhibitor (cycloheximide) blocked the retrograde labeling at different levels (Fig. 6).

In our laboratory’s previous studies, we observed the anterograde transport and accumulation of heat shock protein 25, p38 MAPK, and ERs in regenerating sciatic nerve fibers by immunohistochemistry and Western blot analysis (18, 30). Immunoblot analysis of activated ERK1/2 revealed a significant increase in the level of the protein in the regenerating portion of the sciatic nerve after crush injury (18). In the present study, using immunohistochemistry, we demonstrated accumulation of phospho-ERK in the distal portion of the proximal stump. Although ERK may be transported in both anterograde (34) and retrograde (10) directions, our unpublished observations with transection of the sciatic nerve showed that ERK is transported in the anterograde direction to the site of the injury. Taken together, these data indicate that the estrogen-activated ERK pathway might play an important role during axon regeneration. While the present study was being prepared for publication, Jezierski and Sohrabji (20) published complementary observations of similar, but not identical, potentiation of retrograde axonal transport by estrogen in the rodent forebrain. These data further demonstrated that estrogen stimulates retrograde axonal transport within the mammalian nervous system, which may be related to neurotrophic properties of this hormone.

Our observations demonstrated a role for estrogen, ERK, PI3K, and local protein synthesis in the retrograde labeling of axotomized lumbar motoneurons. Therefore, we propose that estrogen-induced potentiation of retrograde labeling of lumbar motoneurons is mediated via ERK and PI3K signaling pathways and is reliant on local protein synthesis.

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