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Ovarian Hormones Elicit Phosphorylation of Akt and Extracellular-Signal Regulated Kinase in Explants of the Cerebral Cortex

Meharvan Singh

Center for Reproductive Sciences and Department of Obstetrics and Gynecology, Columbia University, College of Physicians and Surgeons, New York, NY

Estradiol and progesterone both have been demonstrated to afford neuroprotection against various insults. In an attempt to identify potential mechanisms underlying these neuroprotective effects, two key elements within signal transduction pathways linked to neuroprotection were evaluated. In mouse cerebral cortical explants, both estradiol and progesterone elicited the phosphorylation of Akt, a downstream effector of the phosphoinositide-3 (PI-3) kinase pathway. Progesterone also elicited the phosphorylation of extracellular-signal regulated kinase (ERK), a component of the mitogen-activated protein kinase (MAPK) pathway. These effects were not inhibited by the progesterone receptor antagonist, RU486. However, inhibition of either MAPK/ERK kinase with PD98059 or PI-3 kinase with LY294002 successfully inhibited progesterone's actions on ERK and Akt, respectively. Collectively, the data offer novel mechanisms for both progesterone and estrogen action in the central nervous system, demonstrating the functional and mechanistic diversity of gonadal hormones and supporting their neuroprotective potential for such neurodegenerative disorders as Alzheimer disease.

Key Words: Progesterone; estrogen; Akt; extracellular-signal regulated kinase; neuroprotection; signal transduction.

Introduction

Gonadal hormones exert their actions not only in hypothalamic structures related to neuroendocrine function but in extrahypothalamic regions of the central nervous system (CNS) as well, supporting their roles in nonreproductive functions and behaviors. Estrogen, e.g., has been demonstrated to promote neurite outgrowth *(1,2)*; regulate cognitive performance *(3,4)*; and afford neuroprotection against various toxic insults, including hypoglycemia *(5)*, glutamate *(6)* or amyloid toxicity *(7–9)*, ischemia *(10,11)*, and oxidative stress *(9,12)*.

Progesterone has also been shown to have neuroprotective effects and can reduce neuronal loss resulting from neurotoxic and traumatic insults to the CNS. These effects include the ability of progesterone to reduce hippocampal neuronal vulnerability following such insults as glutamate, $FeSO_4$, and β-amyloid toxicity *(9)*. In addition, secondary neuronal loss following cortical contusion injury and resulting cognitive impairment were significantly reduced in rats that received progesterone treatment relative to untreated controls *(13,14)*. Progesterone has even been described to have antioxidant effects *(15)* that may also contribute to neuronal survival following injury.

The mechanisms for gonadal hormone action have also expanded beyond the classic genomic mechanism of steroid hormone action in which the activated receptor regulates gene expression through its interaction with the transcriptional machinery of cells (for a review see refs. *16* and *17*). For example, estrogen has been shown to recruit cyclic adenosine monophosphate (cAMP)/protein kinase A–dependent pathways *(18–20)*, regulate Ca^{2+} mobilization *(21–23)*, and elicit the activation of the mitogen-activated protein kinase (MAPK) pathway *(24–27)*. The ability of gonadal hormones to recruit or converge with these signal transduction cascades may not only provide a basis for their rapid and "transcriptionally independent" effects, but may also implicate these mechanisms in the survival-promoting effects of estrogen and progesterone.

In view of the neuroprotective effects of progesterone, the mechanisms underlying these actions were hypothesized to include activation of signaling elements within the MAPK and phosphoinositide-3 (PI-3) kinase pathways, two pathways linked to the promotion of neuronal survival. In addition, the mechanisms underlying estrogen's neuroprotective effects were hypothesized to include regulation of the PI-3 kinase pathway as well, extending previously documented mechanisms of estrogen action.

Activation of the MAPK pathway has not only been shown to promote survival of cells following such insults as growth factor or serum withdrawal; it has also been described to favor the catabolism of the amyloid precursor protein (APP) toward the generation of a soluble, nonamy-

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Author to whom all correspondence and reprint requests should be addressed: Meharvan Singh, PhD, Columbia University, Department of Obstetrics and Gynecology, 650 West 168th Street, Black Building 1615, New York, NY 10032. E-mail: ms435@columbia.edu

loidogenic peptide *(28,29)*, rather than β-amyloid (Aβ) *(29)*, a protein of considerable focus in the pathobiology of Alzheimer disease. On the other hand, activation of Akt, a downstream effector of the PI-3 kinase pathway, promotes cell survival through its participation in the inhibition of apoptosis (for a review see ref. *30*). Once activated, Akt can phosphorylate the proapoptotic molecule BAD *(31)*, rendering it unable to dimerize with the survival-promoting proteins, BCL-X_L and BCL-2 *(32)* and, as a consequence, affording protection against apoptotic cell death.

The PI-3 kinase pathway has been shown to be involved in insulin-like growth factor-1-dependent survival of cerebellar granule neurons *(33)* and the promotion of sensory neuron survival *(34)*. Furthermore, overexpression of Akt can overcome trophic factor withdrawal-induced cerebellar neuron apoptosis, while expression of a dominant negative form of Akt interferes with growth factor–induced survival of these neurons *(35)*.

The data presented here reveal that progesterone, like estradiol, is equally capable of eliciting phosphorylation of extracellular-signal regulated kinase (ERK) in slice cultures (explants) of the mouse cerebral cortex. Interestingly, progesterone also elicited the phosphorylation of Akt, an effect that has not been previously described in the CNS. Together with estrogen's ability also to elicit Akt phosphorylation, these novel mechanisms of action underscore the functional and mechanistic diversity of both gonadal hormones and provide a basis for their neuroprotective potential in such neurodegenerative disorders as Alzheimer disease.

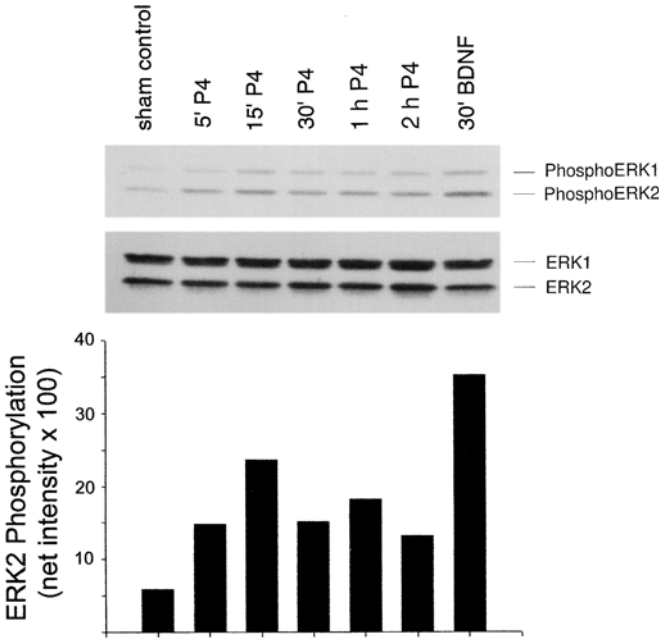

Fig. 1. Effect of progesterone on ERK phosphorylation. Cerebral cortical explants were treated with 100 n*M* progesterone for various lengths of time, resulting in a time-dependent phosphorylation of ERK1 and ERK2, as determined by Western blot analysis. The control lane shows the level of phosphoERK following a 15-min sham treatment (consisting of removal and replacement of the conditioned medium without the addition of hormone or growth factor). The lower panel represents reprobing of the phosphoERK blot for total ERK protein to verify equal loading of protein across lanes. Densitometric representation of the relative intensities of phosphorylated ERK2 is also provided. The numerical values in the bar graphs represent the net intensity for ERK2 phosphorylation normalized for ERK2 protein. The data shown are representative of three independent experiments.

Results

Effect of Progesterone on ERK Phosphorylation

Previous studies have documented that in cerebral cortical explants, 17β-estradiol elicits ERK phosphorylation in a time-dependent manner *(26,27)*. To evaluate whether progesterone elicits ERK phosphorylation as well, explants of the cerebral cortex were treated with 100 n*M* progesterone, resulting in a rapid and sustained pattern of ERK phosphorylation. The onset of phosphorylation was seen within 5 min, was maximal at 15 min, and remained above baseline for up to 2 h following treatment (Fig. 1).

Cellular Distribution and Intracellular Localization of Progesterone-Induced PhosphoERK

Using three-color confocal laser scanning microscopy for the simultaneous detection of nuclei (Sytox staining, green), microtubule-associated protein (MAP)-2B (a neuronal marker, presented as blue [Cy-5] in Fig. 2A or white in Fig. 2C,E), and the phosphoERK signal (red), the cell type (neuron vs glia) and intracellular localization of the progesterone-induced ERK phosphorylation was evaluated. Immunohistofluorescence revealed that the progesterone-induced phosphoERK signal was indeed present in neurons (Fig. 2B), as supported by both morphologic criteria (having a large nucleus, cellular processes, and ample cytoplasm) and the coexpression of the neuronal marker, MAP-2B. Progesterone elicited an increase in phosphoERK signal mainly in the cytoplasm, while nuclear accumulation of the signal was modest (Fig. 2B). By contrast, the neurotropin BDNF, serving as the positive control, resulted in a dramatic increase in phosphoERK signal in both the cytoplasm and the nucleus (Fig. 2D).

Effect of Progesterone and Estradiol on Akt Phosphorylation

Treatment of cerebral cortical explants with 100 n*M* progesterone, a concentration previously reported to afford neuroprotection against $FeSO_4$, β-amyloid, and glutamate toxicity *(9)*, resulted in Akt phosphorylation within 5 min, was maximal between 15 and 30 min, and was sustained up to 2 h following treatment (Fig. 3A). Similarly, treatment with 10 n*M* 17β-estradiol induced a similar time-dependent phosphorylation of Akt that began within 5 min, reached

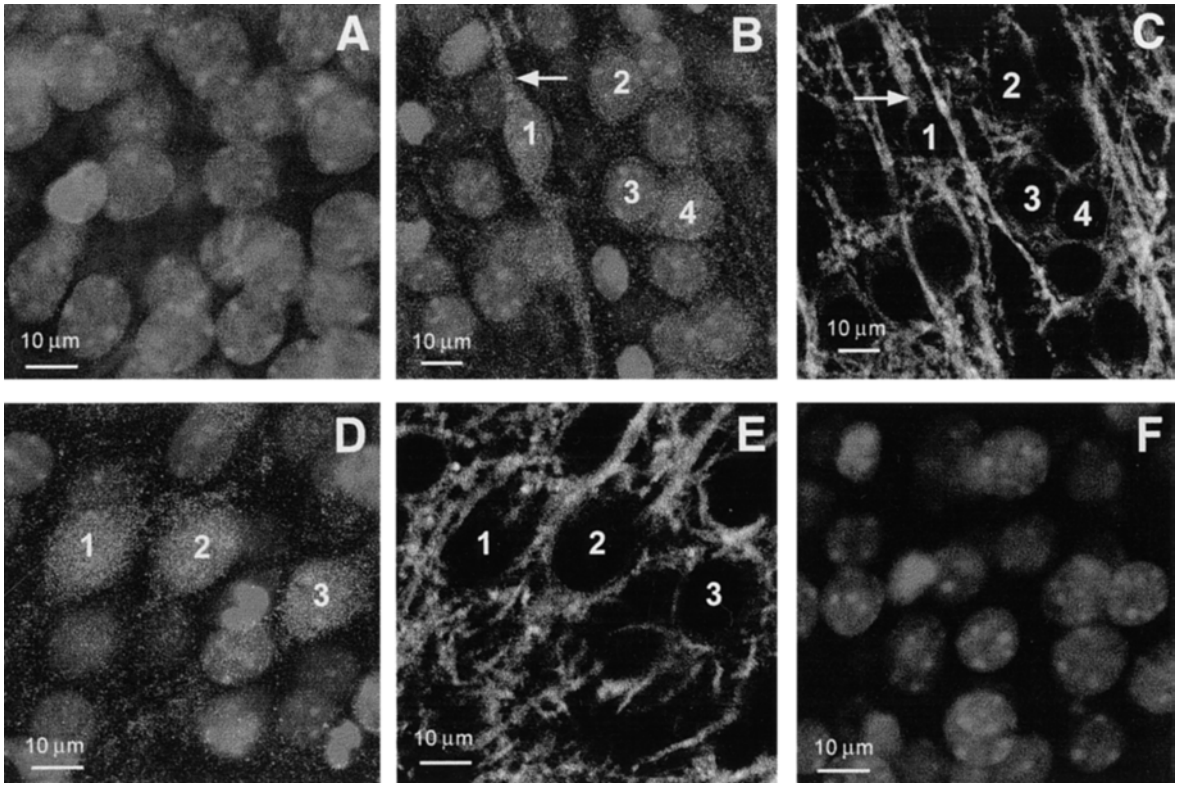

Fig. 2. Immunohistochemical evaluation of the cellular distribution and intracellular localization of the progesterone-induced phosphoERK signal. Three-color confocal laser scanning microscopy was used to detect cells that respond to progesterone treatment. (**A**) Untreated experimental control, showing only MAP-2B staining (in blue) and nuclei (green). Following 30 min of progesterone (100 n*M*) treatment (**B**), immunohistofluorescence revealed that the phosphoERK signal induced was present in cells that exhibited a prominent nucleus (Sytox staining, green), cellular processes, and ample cytoplasm. The phosphoERK signal, depicted by the red signal, was also found in cells that coexpressed the neuronal marker, MAP-2B (**C**). The numbers identify the same progesterone-responsive cells within the same field of the explant, labeled for either phosphoERK (B) or MAP-2B (C). The arrows in (B) and (C) identify the red cellular process in (B) as being positive for MAP-2B. Treatment of the explants with 100 ng/mL of BDNF served as the positive control (**D**), revealing not only a robust increase in cytoplasmic phosphoERK signal, but also a significant accumulation of the phosphoERK in the nucleus. The numbers identify the same BDNF-responsive cells within the same field of the explant, labeled for either phosphoERK (D) or MAP-2B (**E**). (**F**) Cerebral cortical explants that were processed without the addition of the primary antibody, serving as a methodologic control. Data are representative of two independent experiments.

maximal levels at 15 min, and remained above baseline for up to 2 h (Fig. 3B).

Pharmacologic Blockade of Progesterone's Effect on ERK and Akt

To establish the dependence of upstream elements within the MAPK and PI-3 kinase cascades, the effect of the MAPK/ERK kinase (MEK) inhibitor, PD98059 (100 μ*M*), and the PI-3 kinase inhibitor, LY294002 (15 μ*M*), were evaluated for their ability to inhibit progesterone-induced ERK and Akt phosphorylation, respectively. Inhibition of MEK, the signaling kinase immediately upstream of ERK, prevented progesterone-induced ERK phosphorylation (Fig. 4A). By contrast, the PI-3 kinase inhibitor, LY294002 (15 μ*M*), abolished the ability of progesterone to elicit Akt phosphorylation (Fig. 5), whereas the elicited ERK phosphorylation was left unaltered (Fig. 4B). The pharmacologic antagonist to the progesterone receptor (PR), RU486 (1 μ*M*), was also used to try to block the effect of progesterone on either ERK or Akt phosphorylation. Neither the progesterone-induced ERK phosphorylation (Fig. 4B) nor Akt phosphorylation (Fig. 5) was inhibited by RU486. Interestingly, RU486 actually stimulated Akt phosphorylation when administered in the absence of hormone (Fig. 5). By contrast, RU486 by itself had only a modest stimulatory effect on ERK phosphorylation (Fig. 4B).

Discussion

The ability of both estradiol and progesterone to elicit Akt phosphorylation in the cerebral cortex offers a novel mechanism by which these hormones may play a role in neuroprotection. In addition, progesterone was shown to elicit MEK-dependent ERK phosphorylation in a manner similar to that previously described for estradiol *(26,27)*. The pattern of phosphorylation was rapid in onset and sus-

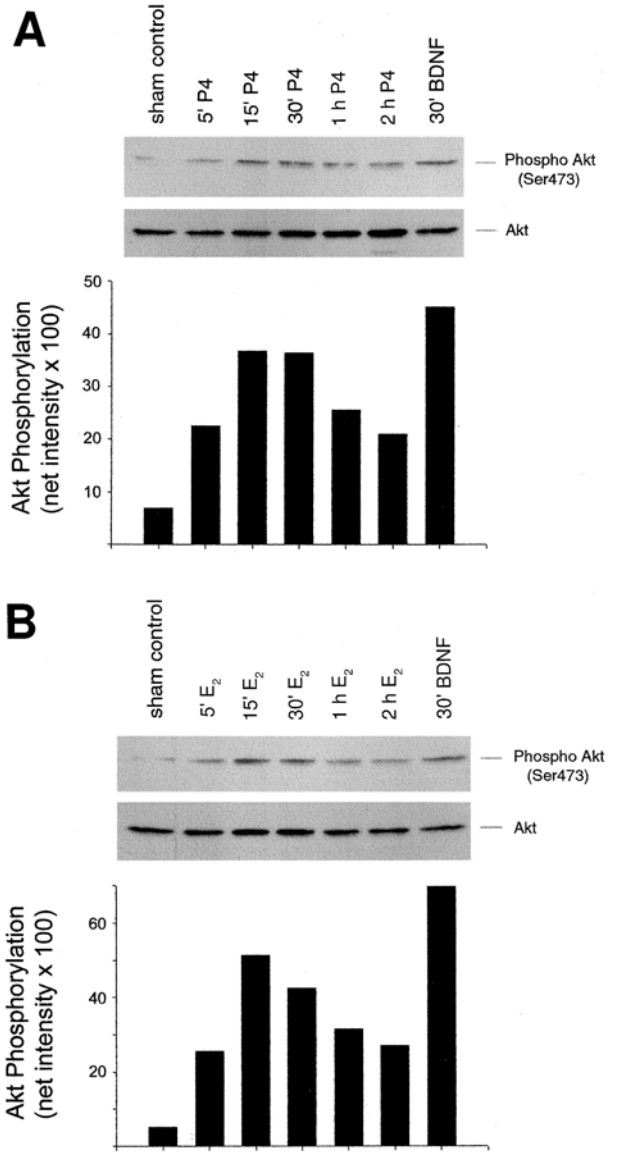

Fig. 3. Effect of progesterone and estradiol on Akt phosphorylation. Cerebral cortical explants were treated with 100 n*M* progesterone (**A**) or 10 n*M* 17β-estradiol (**B**) for various durations resulting in a time-dependent increase in Akt phosphorylation (Ser473), as determined by Western blot analysis. The onset of phosphorylation for both parameters was rapid and prolonged. The control lanes show the level of phosphoAkt following a 15- and 30-min sham treatment (consisting of removal and replacement of the conditioned medium without the addition of hormone or growth factor) for progesterone and estradiol, respectively. The lower panels in (A) and (B) represent reprobing of the phosphoAkt blot for total Akt protein to verify equal loading of protein across lanes. Densitometric analysis of the relative intensities of phosphorylated Akt, in graphic form, is also provided. The numerical values in the bar graphs represent the net intensity for Akt phosphorylation normalized for Akt protein (as seen in the second panel of the Western blot). Data shown are representative of two and three independent experiments for the effects of estrogen and progesterone, respectively.

tained, lasting for up to 2 hours following progesterone treatment. At least with respect to ERK activation, the prolonged phosphorylation observed following progesterone treatment is consistent with its potential neuroprotective/neurotrophic actions and distinguishes it from the more transient stimulation of ERK typically associated with an induction of proliferation (for a review see ref. *36*).

In view of the heterogeneous population of cells that comprise a cerebral cortical explant (consisting of both neurons and glia), immunohistochemical analysis was performed and revealed that progesterone treatment did in fact elicit an increase in the phosphoERK signal within neurons. Although there are well-characterized effects of progesterone and its 5α reduced metabolites on glial populations of the CNS (for a review see ref. *37* and *38*), the present result documents that neurons within the cerebral cortex are indeed responsive to progesterone. Thus, the neuroprotective potential afforded by progesterone's ability to elicit an increase in ERK phosphorylation may be a consequence of its direct action on neurons, in addition to potential indirect effects achieved via glia.

Interestingly, progesterone did not elicit nuclear translocation of phosphorylated ERK to the same extent as that induced by the neurotrophin BDNF. It is possible that this difference reflects a distinct mechanism by which progesterone signals, where the phosphorylation and activation of additional signaling proteins, may be required in order to elicit changes in transcriptional activity. For example, activated ERK can translocate into the nucleus to phosphorylate transcription factors such as Elk-1 *(39–41)* and cAMP response element binding protein *(42,43)*, leading to the regulation of gene transcription *(44,45)*. Alternatively, activated ERK could also regulate cell function by phosphorylating additional downstream signaling kinases such as Rsk *(46,47)*, which, in turn, can translocate to the nucleus to regulate transcriptional activity. Based on the present data, this latter mechanism may be more relevant to progesterone's mechanisms of action.

It is important to acknowledge, however, that although upstream members of the MAPK cascade, such as Ras, have been demonstrated to be important for cell survival *(34)*, the significance of the activation of its downstream effector, ERK, is more equivocal and is likely dependent on the nature of the insult, the neuroprotective agent used, and possibly the cellular context (PC12 cells vs primary sensory neurons vs cerebral cortical neurons). For example, the ability of *N*-acetyl cysteine to protect PC12 cells against nerve growth factor (NGF) withdrawal has been shown to require the activation of ERK *(48)*. On the other hand, in NGF-deprived primary sensory neurons *(49)*, or in PC12 cells deprived of serum *(49,50)*, ERK activation, although induced by NGF, was not required for NGF-induced cell survival. Rather, it was the activation of the PI-3 kinase pathway that seemed to be necessary for PC12 cell survival *(50)*. These observations are in contrast to those observed in oxidative stress models, in which increased ERK activity (through the expression of dominant positive MEK constructs in NIH 3T3 cells) was shown to afford greater resis-

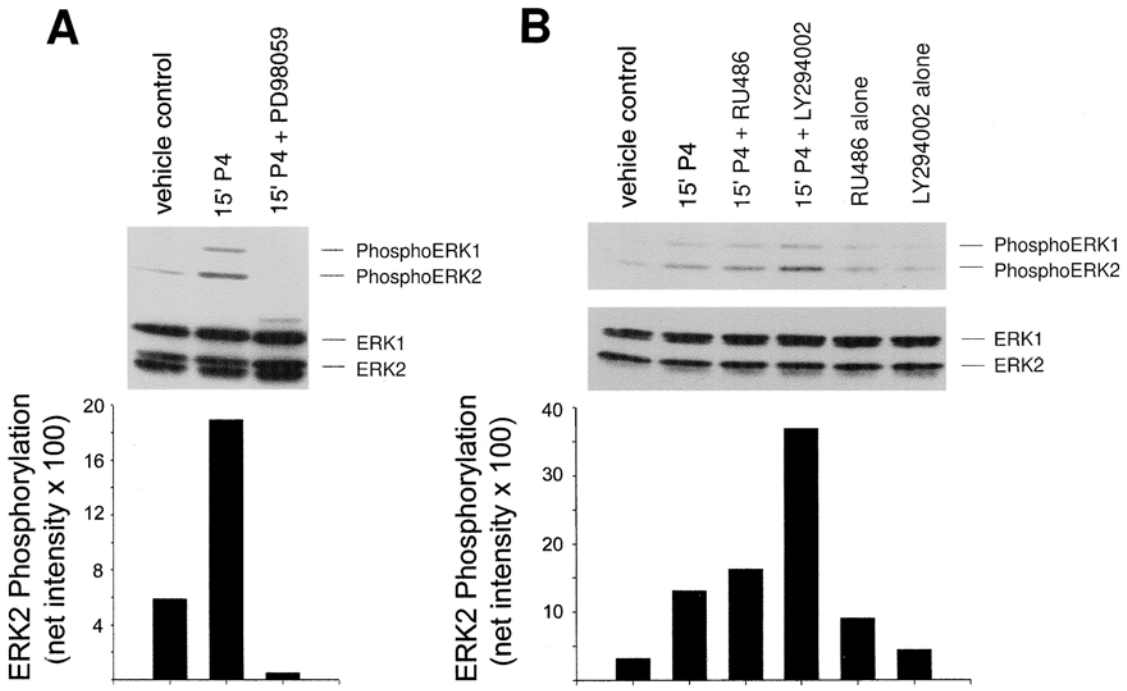

Fig. 4. Progesterone-induced ERK phosphorylation is inhibited by MEK blockade but not by RU486. Cerebral cortical explants were pretreated with the MEK inhibitor, PD98059 (100 μ*M*), for 30 min, prior to the administration of 100 n*M* progesterone for an additional 15 min. Western blot analysis revealed that PD98059 successfully inhibited the progesterone-induced response (**A**), whereas the PI-3 kinase inhibitor, LY294002 (15 μ*M*), was without effect (**B**). Interestingly, RU486 did not alter the ability of progesterone to elicit ERK phosphorylation. The vehicle control lane shows the level of phosphoERK following a 45-min treatment with 0.1% dimethylsulfoxide (DMSO) (simulating a mock 30-min pretreatment, and 15-min treatment). The lower panel represents reprobing of the phosphoERK blot for ERK protein to verify equal loading of protein across lanes. Densitometric representation of the relative intensities of phosphorylated ERK2 is also provided. The numerical values in the bar graphs represent the net intensity for ERK phosphorylation normalized for ERK protein (as seen in the second panel of the Western blot). Data shown are representative of two independent experiments.

tance to H_2O_2-induced cell death *(51)*. Furthermore, estrogen mediated neuroprotection of primary cortical neurons against glutamate toxicity *(6)*, and estrogen's ability to protect hippocampal slice cultures from NMDA- or kainate-mediated neurotoxicity *(52)* was found to correlate with ERK activation and was sensitive to MEK inhibition. Regarding potential protective mechanisms in Alzheimer disease, ERK activation seems to consistently favor the regulation of APP catabolism toward the generation of the less toxic, soluble form of APP *(28,29)* and has even been shown to correlate with a reduced secretion of the amyloidogenic peptide, Aβ *(29)*. Thus, progesterone's ability to elicit ERK phosphorylation in a manner similar to that elicited by estrogen may represent a relevant mechanism by which this hormone may also afford neuroprotection against various insults and could implicate progesterone treatment in the development of novel therapeutic strategies for Alzheimer disease.

The PI-3 kinase pathway, which includes the downstream effector, Akt (or PKB), is well characterized as being able to mediate inhibition of apoptosis and supports its role in mechanisms underlying neuronal survival *(31,35,53)*. Of the various cellular targets of Akt, BAD a member of the Bcl-2 family of genes, has been demonstrated to be an important target because it relates to the prevention of apoptosis (for a review see ref. *30*). BAD, when overexpressed, increases its association with antiapoptotic proteins within the Bcl-2 family, such as Bcl-X_L, blocking Bcl-X_L-dependent cell survival *(32,54)*. Phosphorylation of BAD, on the other hand, causes BAD to dissociate from Bcl-X_L and Bcl-2 and fosters its association instead with cytoplasmic 14-3-3 proteins *(32)*, favoring cell survival. Because Akt phosphorylation and activation leads to BAD phosphorylation, the ability of both estradiol and progesterone to phosphorylate Akt may represent an early event in the neuroprotective pathway induced by these ovarian hormones. In support of Akt's role in the neuroprotective actions of estrogen, a recent study demonstrates that the ability of estrogen to protect against glutamate toxicity was abrogated by an inhibitor of PI-3K activity *(55)*.

Recently, it has been proposed that an abnormality in the regulation of PI-3 kinase may contribute to the pathology in Alzheimer disease. Postmortem analysis of brains of individuals with Alzheimer disease revealed that the soluble form of PI-3 kinase was significantly reduced in the frontal cortex relative to controls *(56)*. As such, the requirement of PI-3 kinase activity for progesterone's ability to elicit Akt phosphorylation only further strengthens its therapeutic potential in Alzheimer disease.

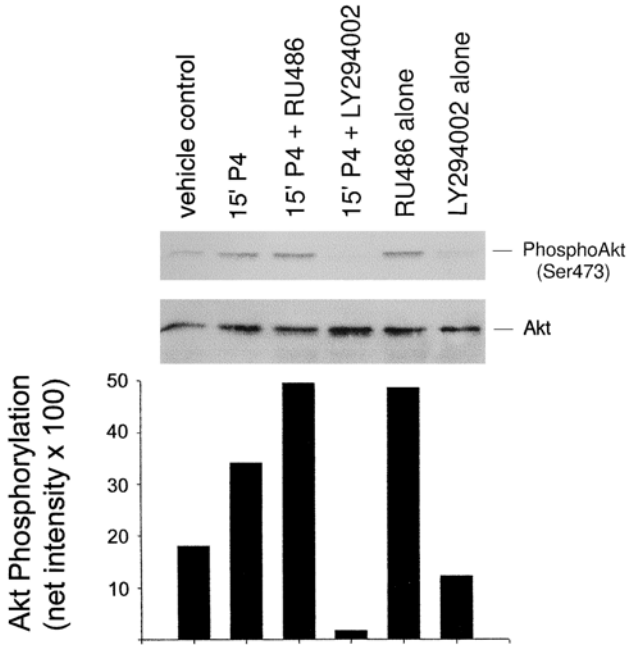

Fig. 5. Progesterone-induced Akt phosphorylation is dependent on PI-3 kinase activity but is insensitive to RU486. Cerebral cortical explants were pretreated with the PI-3 kinase inhibitor, LY294002 (15 μ*M*), for 30 min, prior to the administration of 100 n*M* progesterone. Western blot analysis revealed that LY294002 successfully inhibited the progesterone-induced response. Not only was the progesterone receptor antagonist, RU486, incapable of blocking the effect of progesterone on Akt, but it actually stimulated Akt phosphorylation in the absence of hormone. The vehicle control lane shows the level of phosphoAkt following 45 min of treatment with 0.1% DMSO (simulating a mock 30-min pretreatment, and 15-min treatment). The lower panel represents reprobing of the phosphoAkt blot for Akt protein to verify equal loading of protein across lanes. Densitometric representation of the relative intensities of phosphorylated Akt is also provided. The numerical values in the bar graphs represent the net intensity for Akt phosphorylation normalized for Akt protein (as seen in the second panel of each Western blot). Data shown are representative of two independent experiments.

Interestingly, the PR antagonist, RU486, failed to inhibit progesterone's ability to elicit ERK or Akt phosphorylation. RU486 has been characterized as a PR antagonist principally on the basis of its ability to disrupt progesterone-induced gene transcription, by altering the receptor conformation and consequently rendering it unable to interact with the transcriptional machinery *(57)*. Because the effect of progesterone on ERK or Akt phosphorylation is upstream of any DNA interaction, it is possible that the *genomic* mechanism of RU486 action renders it incompatible with such *nongenomic* effects of the steroid. In addition, while RU486 is effective in inhibiting PR-mediated transcription, this compound has actually been shown to paradoxically promote the dissociation of heat-shock proteins from the PR and induce receptor dimerization *(57)*, cellular events typically associated with PR activation. Moreover, RU486 has even been shown to elicit the phosphorylation of the PR to a similar extent as that induced by the synthetic progestin R5020 *(58)*. These events that mimic the "cytosolic activation" of the PR may provide a basis for which RU486 elicited Akt phosphorylation in the cerebral cortical explants and suggests that RU486 could also be neuroprotective. Consistent with this hypothesis is the demonstration by Behl et al. *(59)* that RU486 does in fact exhibit protective effects against oxidative stress in hippocampal neurons. While the protection against oxidative stress has been principally related to the antiglucocorticoid properties of RU486, the ability of RU486 to elicit Akt phosphorylation may provide another important mechanism for its protective influence.

There is growing evidence supporting the use of postmenopausal hormone replacement therapy for the prevention of Alzheimer disease and suggests that the loss of ovarian hormones may render a woman more susceptible to the deficits associated with Alzheimer disease. For example, the increased risk of Alzheimer disease associated with menopause is greatly reduced in women who have had hormone replacement *(60)*. Furthermore, in animal studies, deficits in neurotrophin expression *(61,62)*, cholinergic activity *(4)*, and cognition *(3,4)* resulting from ovariectomy are improved following estrogen replacement. However, ovariectomy, like menopause, results in a precipitous decline of not only estrogen but also progesterone, suggesting that loss of progesterone may contribute to the deficits observed and could therefore be equally important in attenuating or delaying the onset of specific neuropathologic changes associated with Alzheimer disease. The data described here provide two novel mechanisms by which progesterone can elicit its effects in the CNS—through the activation of the MAPK and PI-3 kinase pathways—and supports its role in promoting cell survival. In addition, the observation that estradiol is equally capable of eliciting the phosphorylation of Akt offers another important means by which estrogen can afford neuroprotection and, thus, furthers our understanding of the molecular basis by which steroid hormones facilitate protection against various cytotoxic insults, underscoring their potential therapeutic relevance to such neurodegenerative diseases as Alzheimer disease.

Materials and Methods

Tissue Culture

Organotypic explants were derived from ~360 μm-thick hemicoronal slices of postnatal day (P) 2 (day of birth = P1) frontal and cingulate cerebral cortex, obtained from both male and female pups born of timed-pregnant C57Bl/6J mice (Jackson Laboratories). Explant slices were maintained as roller tube cultures *(63)* on rat tail collagen-coated/poly-D-lysine precoated glass cover slips and grown in steroid-deficient and phenol red–free maintenance medium (25% gelding serum, 22.5% Hank's balanced salt solution, 50% basal medium Eagles, 7.5 mg/mL of glucose, 2 m*M*

L-glutamine, 50 µg/mL of ascorbic acid) supplemented with 2 n*M* E_2. The cultures were maintained in vitro for 6 d, followed by a 24 h "washout" period to establish a baseline for the biochemical/enzymatic parameter being measured. Cultures were treated according to previously established methods *(26)*. Briefly, after the washout period, the conditioned medium was removed from the roller tube, spiked with the appropriate treatment, and returned to the tube for a specified length of time. Control cultures were sham treated (removal and replacement of conditioned medium, without the addition of hormone) to account for any consequences of procedural manipulation of the explants. The duration of sham treatment (30 min for estradiol, 15 min for progesterone) corresponded to the time during which either estradiol or progesterone elicited maximal ERK or Akt phosphorylation.

Treatment of Cultures

For the evaluation of progesterone's ability to elicit ERK and Akt phosphorylation, explants were pulsed with 100 n*M* progesterone for 5, 15, and 30 min, 1 and 2 h and were compared with the sham-treated control. The neurotropin BDNF (100 ng/mL), administered for a duration of 30 min served as the positive control. The concentration of progesterone used represents a dose shown to elicit neuroprotective effects in hippocampal cultures *(9)*. To evaluate the contribution of MEK and PI-3 kinase, upstream elements of ERK and Akt, respectively, cultures were preincubated with the MEK inhibitor, PD98059 (100 µ*M*); the PI-3 kinase inhibitor, LY294002 (15 µ*M*); or the vehicle control (0.1% DMSO) for 30 min prior to the administration of progesterone. Similarly, the PR antagonist, RU486 (1 µ*M*) (gift from N. J. MacLusky, Columbia University, New York), or vehicle control (0.1% DMSO) was also administered for 30 min prior to the addition of progesterone. The control cultures were sham treated, consisting of treatment with 0.1% DMSO for 30 min (which paralleled the pretreatment), followed by an additional 15-min incubation in the continued presence of 0.1% DMSO (which paralleled the duration of progesterone treatment) to account for any effects of vehicle alone.

Western Blot Analysis

Explants were excised from the collagen substrate and placed in lysis buffer (50 m*M* Tris, pH 7.4; 150 m*M* NaCl; 10% glycerol; 1 m*M* EGTA; 1 m*M* Na_3VO_4; 5 µ*M* $ZnCl_2$; 100 m*M* NaF; 1% Triton X-100; 10 µg/mL of aprotinin; 1 µg/mL of leupeptin; and 1 m*M* phenylmethylsulfonyl fluoride) in which they were homogenized by trituration through a 22-gage needle and centrifuged at 100,000*g* for 15 min at 4°C. The resulting supernatants (lysates) were normalized for protein content (Lowry Assay, Bio-Rad Protein Assay Kit). Samples (25 µg each) were separated on a 10% polyacrylamide gel (sodium dodecyl sulfate polyacrylamide gel electrophoresis) followed by transfer onto polyvinyl difluoride membranes (0.22-µ*M* pore size; Bio-Rad, Hercules, CA). The membranes were then blocked overnight with 3% bovine serum albumin (Fraction V; Sigma, St. Louis, MO) in Tris-buffered saline containing 0.2% Tween-20 (TBS-T) and probed with the following:

1. For ERK phosphorylation: rabbit anti-phosphoMAPK (dual phosphospecific [Thr202/Tyr204] (1:1000) (New England Biolabs, Beverly, MA).
2. For ERK protein: rabbit anti-ERK1 (1:500), rabbit anti-ERK2 (1:500) (Santa Cruz Biotechnologies, Santa Cruz, CA).
3. For Akt phosphorylation: rabbit anti-phosphoAkt (recognizing Ser473 phosphorylation of Akt; 1:1000).
4. For Akt protein: rabbit anti-Akt (New England Biolabs).

Antibody binding to the membrane was detected using a secondary antibody (either goat antirabbit or donkey antigoat) conjugated to horseradish peroxidase (1:40,000) (Pierce, Rockford, IL) and visualized on autoradiographic film, using enzyme-linked chemiluminescence (Amersham, Arlington Heights, IL). All blots were reprobed with the appropriate antibody to verify equal loading of protein across lanes.

Immunohistofluorescence

The cultures were processed while still on the collagen-coated cover slips. After steroid treatment, the cultures were rinsed briefly with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 1 h at 4°C. After rinsing with PBS and blocking overnight with 10% donkey serum/5% nonfat dry milk in Tris-buffered saline containing 0.5% Triton X-100 (TBS-T), the cultures were incubated with the two primary antibodies; mouse anti-MAP-2B (Roche, Indianapolis, IN) coupled with rabbit anti-dual phosphospecific ERK (New England Biolabs) overnight. The cultures were washed with TBS-T, reblocked, and incubated with the secondary antibody (bridging antibody: goat antirabbit), followed again by thorough washing and subsequent reblocking. The fluorophore-conjugated antibodies, Cyanine-3-coupled donkey antigoat antibody (Jackson Immunoresearch, West Grove, PA), and Cyanine-5-coupled donkey antimouse (Jackson Immunoresearch) antibody, which recognize the secondary and MAP-2B antibodies, respectively, were added. After several washes with TBS-T, the nuclear dye (Sytox; Molecular Probes) was added for 30 min. After subsequent washing with TBS, the cultures were mounted onto glass slides using Vectashield mounting medium and viewed under a Zeiss Confocal Laser Scanning Microscope (confocal microscopy core facility, Columbia University). The nucleus appears green, the MAP-2B (neuronal) staining is represented by an intense blue, and the phosphoERK signal appears red.

Densitometric Analysis of ERK and Akt Phosphorylation

Autoradiograms were scanned using an HP Scanjet 6200C (Hewlett Packard, Greeley, CO) and analyzed using Kodak

1D Image Analysis software (Eastman Kodak, Rochester, NY). Net intensity values were calculated by subtracting the background within the area measured for each band from the total intensity within this same measured area in order to account for any variation in background intensity across the film. The numerical values in the bar graphs in Figs. 1, 3, 4, and 5 represent the net intensity for ERK or Akt phosphorylation normalized for ERK or Akt protein (as seen in the second panel of each Western blot).

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