Estrogen Receptor Alpha Inhibits the Estrogen-Mediated Suppression of HIV Transcription in Astrocytes: Implications for Estrogen Neuroprotection in HIV Dementia

Paula M. Heron,¹ Jadwiga Turchan-Cholewo,² Annadora J. Bruce-Keller,^{2,3} and Melinda E. Wilson¹

Abstract

Many human immunodeficiency virus (HIV) proteins including Tat are produced by HIV-infected astrocytes and secreted into the brain resulting in extensive neuronal damage that contributes to the pathogenesis of HIV dementia. The neuroprotective hormone 17 β -estradiol (E2) is known to negatively regulate the HIV transcriptional promoter in human fetal astrocytes (SVGA cell line) in a Tat-dependent manner. In the present study we extended our investigation in HIV-infected SVGA cells and found a reduction in HIV p24 levels following E2 treatment in comparison to control. Although many E2-mediated events occur through estrogen receptor alpha (ER α), we found low levels of ER α mRNA and failed to detect ER α protein in SVGA cells. Paradoxically, when ER α was overexpressed the E2-mediated decrease in Tat transactivation of the promotor was prevented. To determine whether ER α expression is altered in the human brain following HIV infection, postmortum hippocampal tissue was obtained from cognitively normal HIV⁻ and HIV⁺ patients, patients diagnosed with either mild cognitive/motor disorder (MCMD) or HIV-associated dementia (HAD). Immunohistochemistry and quantitative real-time PCR (qRT-PCR) for ER α and glial fibrillary acidic protein (GFAP) showed that ER α mRNA levels were not significantly different between groups, while GFAP increased in the hippocampus in the HIV⁺ compared to the HIV⁻ group and was decreased in the MCMD and HAD subgroups compared to HIV⁺ controls. Notably the ratio of ER α -positive reactive astrocytes to total reactive astrocytes increased and significantly correlated with the severity of cognitive impairment following HIV infection. The data suggest that E2 would have the most dramatic effect in reducing HIV transcription early in the disease process when the subpopulation of astrocytes expressing ER α is low.

Introduction

HIV-ASSOCIATED DEMENTIA (HAD) develops in approximately 30% of all HIV-infected patients, while more than 60% of all HIV-infected patients develop neurological impairment.¹ Astrocytes are important participants in the neuropathogenesis of HAD and unlike microglia, harbor a nonproductive infection whereby viral proteins are expressed but replication of the viral genome does not occur.² One particular viral protein produced by astrocytes is Tat. Tat is essential for viral replication and activates the transcription of all viral proteins^{3–5} by binding the transactivation response region (TAR) within the 5' terminus of all retroviral mRNAs^{6,7} and stimulating the elongation efficiency of RNA polymerase II.^{8,9} In addition, Tat is secreted by infected astrocytes into the

brain and causes extensive neuronal damage¹⁰ directly or indirectly by stimulating the release of neurotoxic factors from infected astrocytes.^{11,12} Given the many ways in which Tat and astrocytes together participate in the pathogenesis of HAD, effective neuroprotective strategies are needed that either target inflammation and neuronal damage directly or indirectly by inhibiting the production of toxic viral proteins.

17 β -Estradiol (E2) is one factor that has been shown to be neuroprotective and antiinflammatory.^{13,14} E2 protects against Tat-induced neurotoxicity,^{15–17} oxidative stress-induced damage, microglial activation, and concomitant secretion of inflammatory mediators^{13,14,18} and alters neuronal morphology in regions of the brain involved in cognition.^{19,20} Furthermore, our laboratory previously reported that E2 attenuated Tat-induced HIV long terminal repeat (LTR)

¹Department of Physiology, University of Kentucky Medical Center, Lexington, Kentucky 40536-0298.

²Department of Anatomy and Neurobiology, University of Kentucky Medical Center, Lexington, Kentucky 40536-0298.

³Current address: Pennington Biomedical Research Center/Louisiana State University, Baton Rouge, Louisiana 70808.

promoter activation in SVGA cells.²¹ Because estrogen receptors are expressed by neurons and glia, estrogen may mediate neuroprotection through receptor-dependent processes. Indeed the protective effect of estrogen against ischemia-induced damage is dependent on the presence of estrogen receptor alpha (ER α).^{22,23} Furthermore, astrocytes have been implicated in the neuroprotective effects of estrogen in the brain.^{24–26} The fact that astrocyte expression of ER α is upregulated following injury to the rat,²⁴ primate,²⁷ and human brain²⁸ suggests a role for astrocyte-derived ER α in estrogen-mediated neuroprotection. The present study was designed to investigate whether the E2-mediated suppression of Tat transactivation in astrocytes was dependent on the activation of estrogen receptors and, furthermore, to test whether ER α is upregulated in astrocytes in the HIV brain.

Materials and Methods

Cell culture and ELISA

An immortalized astrocyte cell line, SVGA,²⁹ stably transfected with the HIV-LTR promoter driving chloramphenicol acetyltransferase (CAT) (SVGA-CAT) was a kind gift from A. Nath, Johns Hopkins University. HEK293 cells were a kind gift from J. Satin, University of Kentucky, HeLa cells were a kind gift from A. Nath and MCF-7 cells were a kind gift from M. Kilgore. Cells were grown in DMEM + 10% charcoal stripped fetal bovine serum (FBS) + 1% penicillin/streptomycin and maintained at 37°C in 5% CO₂. E2 solutions were prepared in ethanol and diluted so that the final ethanol concentration was 0.01%. Cells were seeded into 24-well plates and treated with E2 or vehicle. After 48 h cells were transfected with 0.8 μ g Tat1-72 or pcDNA (empty vector control) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24–48 h cells were harvested and assayed for CAT levels by ELISA (Roche Applied Science, Indianapolis, IN) as per the manufacturer's instructions. To determine the effect of estrogen on HIV transcription SVGA cells were pretreated with 1 nM doses of E2 or vehicle for 48 h and then infected for 2 h with 2000 pg/ml p24 collected from the supernatant of H9/HTLV-III_B (NIH 1983) cells.³⁰ SVGA infection was monitored by the measurement of p24 protein by ELISA (Perkin-Elmer, Waltham, MA) as per the manufacturer's instructions.

Human tissue samples

A total of 13 autopsy cases (3 from HIV-negative controls and 10 from HIV-infected individuals) were used in the present study. Frozen blocks of postmortem human hippocampal tissue as well as 5- μ m-thick paraffin-embedded human hippocampal sections were obtained from the National NeuroAIDS Tissue Consortium (NNTC; Federal grant number N01MH32002). Fresh hippocampal tissue for RNA isolation and formalin-fixed paraffin-embedded hippocampal sections for immunohistochemical analysis were obtained from age-matched male and female subjects with minimal neuropathological diagnosis. Fresh and fixed tissue was obtained from three HIV-negative patients, three HIV-infected patients with normal cognitive function, three HIV-infected patients with mild cognitive or motor disorder (MCMD), and four HIV-infected patients with HAD. Prior approval for the use of human material in our studies was obtained from the University of Kentucky Medical Institutional Review Board.

Quantitative real time RT-PCR

Total RNA from SVGA, HEK293, or MCF-7 cells was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA). Total RNA from blocks of frozen human hippocampal tissue weighing between 11 mg and 311 mg was isolated according to the method published by Chomczynski and Sacchi.³¹ Briefly, tissue was homogenized in 250 μ l guanidinium thiocyanate buffer containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 0.7% 2-mercaptoethanol in DEPC water followed by phenol-chloroform extraction. First strand cDNA synthesis was carried out with SuperScript II and random hexamers (Invitrogen, Carlsbad, CA). Reverse transcription was performed at 42°C for 50 min followed by 70°C for 15 min. cDNA was quantified using the Quant-iT Oligreen ssDNA Assay Kit (Molecular Probes, Invitrogen, Carlsbad, CA). Primer sets used in the real time PCR reactions were as follows: ER α 5' GTGCCTGGCTAGAGATCCTG and 3' GAT GTGGGAGAGGATGAGGA, GFAP 5' CTGGAGGTTGAGA GGGACAA and 3' TCCTCCAGCGACTCAATCTT. Primers for reference gene expression (HPRT, RPL32, and H3.3) were obtained corresponding to published sequences.^{32,33} Amplification was performed in Mx3000P 96-well plates (Stratagene) in triplicate in a Mx3000P Sequence Detection System (Stratagene, La Jolla, CA) using 1 ng cDNA, 125 nM forward and reverse primer, 0.75 μ l Reference Dye (1:500), and 25 μ l 2 $\times$ SYBRGreen Brilliant Master Mix (Stratagene, La Jolla, CA) in a 50 μ l reaction. Thermalcycler conditions were 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s and 1 cycle of 95°C for 1 min and 55°C for 30 s. MCF-7 breast cancer cells were used as templates for ER α , H3.3, RPL32, and HPRT mRNA standard curves and HIV-negative human hippocampus was used for the generation of GFAP mRNA standard curves. Real time PCR data were normalized to the geometric mean of all reference genes according to the method previously described.³⁴

Immunocytochemistry

SVGA cells were plated into 12-well plates and transfected with 1 μ g ER α using Lipofectamine 2000. After 48 h the cells were fixed with 1:1 methanol:acetone, rinsed, blocked in 5% normal donkey serum (Jackson Immuno Research, Westgrove, PA), and incubated overnight at 4°C in rabbit anti-ER α (MC-20) (1:200; Santa Cruz Biotech, Santa Cruz, CA, catalog #sc-542). ER α was visualized with the use of antirabbit Alexa 594-conjugated secondary antibody (1:500) applied for 1 h at room temperature (RT). Cells were rinsed and left in phosphate-buffered saline (PBS) while images were captured. Then 5- μ m-thick paraffin-embedded human hippocampal sections were processed for ER α and GFAP immunohistochemistry with the use of the Antibody Amplifier (ProHisto, Columbia, SC). Sections were incubated at 60°C for 30 min prior to deparaffinization in xylene and ethanol. Sections were hydrated and double-label fluorescence immunohistochemistry was performed.³⁵ Briefly, sections were rinsed in 0.05 M Tris-buffered saline (TBS) pH 7.2 (3 $\times$ 10 min) and boiled in 0.05 M citric acid-buffered saline (CBS) pH 6.0 for 2 $\times$ 10 min in a conventional microwave oven. After rinsing, sections were incubated for 60 min at RT in 5% normal goat serum (Jackson ImmunoResearch, Westgrove, PA) and incubated at 4°C for 36 h in rabbit anti-ER α (1:400 MC-20, Santa Cruz Biotech, Inc., Santa Cruz, CA, catalog #sc-542). Visualization was achieved

with the use of antirabbit biotinylated (1:200 applied for 1 h at RT) followed by Cy3-conjugated streptavidin (1:500 applied for 45 min at RT) secondary antibodies. Sections were rinsed and incubated in 0.05 M glycine-HCl buffer saline pH 2.2 for 2 h at RT. After rinsing, sections were incubated in monoclonal anti-GFAP (1:800 Sigma, St. Louis, MO, catalog #G3893) for 60 min at 37°C followed by 4°C overnight. Visualization was achieved with the use of FITC-conjugated goat antimouse (1:200, Jackson ImmunoResearch, Westgrove, PA). After extensive washing, sections were coverslipped with Vectashield Hardset Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Peroxidase staining for ER α and GFAP was performed.^{36,37} Sections were deparaffinized, boiled in CBS as before, and incubated in 0.3% hydrogen peroxide in TBS containing 0.3% Triton X-100 for 30 min. After rinsing in TBS, sections were blocked in 5% normal goat serum for 1 h at RT followed by incubation at RT overnight in rabbit anti-ER α (1:75 MC-20, Santa Cruz Biotech. Inc., Santa Cruz, CA, catalog #sc-542) or monoclonal anti-GFAP (1:1000 Sigma, St. Louis, MO, catalog #G3893). Visualization was achieved with anti-rabbit and antimouse biotinylated secondary antibodies, respectively (1:200 applied for 1 h at RT). After several washes in TBS, sections were incubated in avidin-biotin peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) in TBS for 45 min at RT and developed using 3,3'-diaminobenzidine (DAB) tetrahydrochloride tablets (Sigma, St. Louis, MO).

Imaging and quantification

Sections colabeled for ER α and GFAP were imaged on a Nikon Eclipse TE 2000-E inverted fluorescence microscope (Nikon Instruments, Inc., Melville, NY) with the use of a 10 $\times$ objective and filters equipped with excitation/emission spectra to detect fluorochromes FITC, Cy3, and DAPI. Images were captured and merged using Spot RT Software v3.5.1 through a Spot RT color camera (Diagnostic Instruments Inc., Sterling Heights, MI). Images were opened in Adobe Photoshop v7 and adjusted for brightness and contrast. Using the zoom tool, GFAP-immunoreactive (IR) cells containing ER α per unit area (0.9 mm $\times$ 0.65 mm) were marked and manually counted in each image. Three to five regions within the dentate gyrus were analyzed for each section. Sections processed for peroxidase staining of ER α or GFAP were examined by light microscopy and digitalized through a Spot RT camera.

Western blotting

Nuclear and cytoplasmic fractions were isolated from cell lysates using the NE-PER kit (Pierce, Rockford, IL). Total protein was determined by a microplate Lowry-based protein assay (Bio-Rad). Twenty five micrograms of protein from each sample was separated on a 10% Tris-HCl polyacrylamide precast gel (Bio-Rad). The separated proteins were then transferred to nitrocellulose membranes and blocked in Odyssey blocking reagent for 1 h at RT and the following primary antibodies applied: rabbit anti-ER α (1:200, Santa Cruz Biotech., Santa Cruz, CA, catalog #sc-542) and monoclonal antiactin (1:500; Sigma, St. Louis, MO, catalog #A4700). Fluorescently labeled secondary antibodies (Rockland IRDye 800) were diluted 1:5000 in a 1:1 solution of PBS and Odyssey blocking reagent containing 0.2% Tween and incubated at RT

for 1 h. The membranes were rinsed and visualized on an Odyssey Infrared Imaging System (Li-Cor Biosciences).

Statistical analysis

CAT enzyme levels in response to E2 treatment were analyzed by paired *t*-test compared to vehicle treatment. Quantitative RT-PCR for ER α mRNA in different cell types was analyzed by ANOVA followed by Fishers LSD post hoc test. $p < 0.05$ was considered statistically significant. ER α and GFAP mRNA expression as well as cell counts obtained for each patient group were analyzed by one-way ANOVA followed by Fishers LSD post hoc analysis where appropriate. Correlation analysis between data obtained for HIV-infected

FIG. 1. 17 β -Estradiol reduced Tat-induced activation of the HIV-LTR and viral transcription. (A) SVGA-LTR-CAT cells were treated with E2 (0.01–1 nM) 48 h before transfection with Tat plasmid DNA. Cells were collected, lysed, and analyzed for chloramphenicol acetyltransferase (CAT) protein by ELISA. CAT values are represented as mean $\pm$ SD relative to total protein and normalized to vehicle (0.01% ethanol). E2, at all doses, reduced CAT levels compared to vehicle ($n = 5$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ one-way ANOVA, Fishers LSD post hoc analysis). Transfection of the cells with the empty DNA vector that does not contain the Tat sequence was used as a control for Tat transactivation of the LTR. (B) SVGA cells were treated with E2 or vehicle prior to a 2-h infection with HIV. Cells were collected at the time points shown and assayed for p24 protein levels. E2-treated HIV-infected cells had reduced p24 levels at 3 days up to 1 week postinfection compared to controls ($n = 5$, * $p < 0.05$, two-way ANOVA, Fishers LSD post hoc analysis).

patient groups and the severity of dementia was determined by Spearman nonparametric correlation. $p < 0.05$ was considered statistically significant.

Results

E2 reduced Tat-induced activation of the HIV-LTR and viral transcription

Transfection of Tat plasmid DNA into SVGA-CAT cells results in the constitutive expression of Tat protein. Since these cells are stably transfected with the HIV-LTR promoter driving the CAT reporter enzyme, subsequent binding of Tat to the TAR region of the HIV-LTR results in transcriptional activation and the production of CAT enzyme. CAT levels were measured by ELISA and were shown to increase in cells expressing Tat protein compared to cells transfected with an empty vector control lacking the Tat coding sequence

(Fig. 1A). Similar to previously published data from our laboratory, we confirmed E2 (0.01–1 nM) treatment of SVGA-CAT cells attenuated the Tat-induced activation of the LTR. In addition, E2 treatment of HIV-infected SVGA cells resulted in a decrease in the expression of the HIV matrix protein, p24, at 3 days up to 1 week postviral infection compared to vehicle control (Fig. 1B).

SVGA cells express low levels of ER α mRNA and undetectable levels of ER α protein

To determine whether E2 suppressed Tat-induced LTR activation through an ER α -dependent mechanism, endogenous expression of ER α in SVGA cells was investigated. ER α mRNA expression in SVGA cells compared to ER α -positive HEK293 cells and MCF-7 breast cancer cells was determined by qRT-PCR. Results showed ER α mRNA was detected in

FIG. 2. SVGA cells contain low levels of ER α mRNA and undetectable levels of endogenous ER α protein. **(A)** Quantitative RT-PCR for ER α was performed on cDNA samples prepared from SVGA, HEK293, and MCF-7 cells. ER α expression was determined by a standard curve method and expressed relative to the geometric mean of expression levels obtained for reference genes, RPL32, HPRT, and H3.3. As shown, ER α mRNA was detected in all cell types. As expected, the ER α -negative HEK293 cells contained lower levels of ER α mRNA compared to SVGA ($n = 3$, $**p < 0.01$, Fishers LSD) while both cell types contained negligible levels of ER α compared to ER α -positive MCF7 cells. ER β mRNA was detected in SVGA and HEK293 cells; however, ER β mRNA levels were significantly lower than ER α mRNA levels ($n = 3$, $***p < 0.001$, Fishers LSD). All values represent mean $\pm$ SD. **(B)** Immunocytochemistry and Western blot for ER α protein were performed on SVGA, HeLa, and HEK293 cells. As shown, there was no clear evidence of ER α staining in any of the cell types analyzed (left panel), while ER α was clearly detected in cells overexpressing the protein (right panel). **(C)** Western blot analysis of nuclear (N) and cytoplasmic (C) fractions from SVGA, HEK293, and HeLa cells showed no detectable levels of ER α protein. HEK293 cells overexpressing ER α served as a positive control. The detection of actin (42 kDa) was used as a loading control.

FIG. 3. E2 attenuation of Tat-induced LTR activation does not occur in SVGA cells overexpressing ER α or in HEK293 cells in the presence or absence of ER α . **(A)** SVGA cells were treated with E2 or vehicle prior to cotransfection with ER α and Tat plasmid DNA. Forty-eight hours later cell lysates were analyzed for CAT protein levels by ELISA. As expected, E2 decreased Tat-induced LTR activation as shown by a decrease in CAT protein levels compared to vehicle ($n=5$, $**p < 0.01$, paired t -test). E2 treatment of SVGA cells overexpressing ER α alone, in the absence of Tat, had no significant effect on LTR activation compared to vehicle. In the presence of ER α , E2 treatment of SVGA cells failed to significantly reduce Tat-induced LTR activation compared to vehicle treatment. **(B)** In HEK293 cells E2 had no effect on Tat-induced LTR activation as shown by CAT protein levels compared to vehicle. E2 treatment of HEK293 cells overexpressing ER α alone, in the presence or absence of Tat, also had no significant effect on LTR activation compared to vehicle.

SVGA cells and HEK293 cells (Fig. 2A, left); however, in comparison to MCF-7 breast cancer cells, which express abundant levels of ER α mRNA and protein, the ER α mRNA levels detected in SVGA cells and HEK293 cells were negligible (Fig. 2A, middle). Furthermore, ER β mRNA levels were

detected in all three cell types but were even lower than ER α mRNA levels (Fig. 2A, right). Since ER α mRNA was detected in SVGA cells, albeit at low levels, endogenous expression of ER α protein in SVGA cells compared to ER α -negative HeLa and HEK293 cells was determined by immunocytochemistry (Fig. 2B) and Western blot (Fig. 2C). As shown, there was no clear evidence of ER α expression in SVGA, HeLa, or HEK293 cells compared to cells overexpressing ER α in which obvious nuclear localization of the protein was observed. Western blot analysis of ER α in nuclear and cytoplasmic fractions isolated from the various cell types as indicated (Fig. 2C) revealed a protein band ~ 66 kDa (the molecular weight of ER α) in HEK293 cells overexpressing ER α protein. However, no ER α protein was detected in SVGA cells.

ER α prevents E2-mediated suppression of Tat-induced LTR activation in SVGA cells

To investigate the role of ER α in E2 suppression of Tat-induced LTR activation, SVGA cells were transfected with plasmid DNAs encoding for Tat and ER α prior to CAT analysis. As expected, treatment of SVGA cells with 1 nM E2 reduced Tat-induced CAT expression by $\sim 40\%$ compared to vehicle. However, surprisingly, the presence of ER α negated this effect and no differences in Tat-induced CAT expression were found following E2 treatment compared to vehicle. E2 treatment of SVGA cells in which ER α was overexpressed, in the absence of Tat, appeared to result in an increase in CAT expression compared to vehicle, but this was not significant (Fig. 3A). ER α protein levels were monitored by Western blot analysis (data not shown). To investigate whether the E2-mediated suppression of Tat-induced LTR activation occurs only in SVGA cells, HEK293 cells were transfected in the same way as SVGA cells with the exception that all HEK293 cells were also transfected with the LTR-CAT construct since the HEK293 cell line lacked a stably transfected LTR reporter. CAT assays showed that E2 had no effect on Tat-induced activation of the LTR compared to vehicle (Fig. 3B). In addition, the presence of ER α in the presence or absence of E2 had no effect on LTR activation.

GFAP but not ER α mRNA expression changes following HIV infection

To understand the significance of our findings for the human HIV-infected brain, we next measured the levels of ER α mRNA and protein in postmortem human hippocampal tissue. Quantitative real-time PCR of cDNA generated from human hippocampal tissue revealed no change in ER α mRNA levels between any of the patient groups (Fig. 4A). However, GFAP mRNA levels were shown to increase in tissue obtained from HIV-infected patients compared to uninfected controls ($p < 0.001$). Interestingly, both MCMD and HAD groups showed a decline in GFAP mRNA compared to the HIV $^{+}$ control group ($p < 0.05$) with levels similar to those from uninfected patient samples (Fig. 4B). Analysis of the severity of cognitive decline following HIV infection was shown to negatively correlate with the level of GFAP mRNA in the hippocampus ($p = 0.005$, Spearman's nonparametric correlation). This suggests a significant relationship between GFAP mRNA levels and neurological progression from HIV infection to HAD.

FIG. 4. Analysis of ER α and GFAP expression in the human hippocampus. RNA was extracted from hippocampal tissue, reverse transcribed to cDNA, and the levels of ER α mRNA (**A**) or GFAP mRNA (**B**) were determined by quantitative real time PCR relative to the geometric mean of RPL32, HPRT, and H3.3 reference genes. (**A**) No differences in ER α expression were detected between patient groups with normal cognitive status ($n = 3$ HIV $^{-}$; $n = 2$ HIV $^{+}$) compared to those with minor (MCMD, $n = 3$) or severe cognitive decline (HAD, $n = 4$). In contrast, GFAP mRNA levels were significantly higher in the HIV $^{+}$ patient samples compared to HIV $^{-}$ patient samples (**B**) ($***p < 0.001$, Fishers LSD). The MCMD and HAD groups did not differ from the HIV $^{-}$ group but were significantly lower than the HIV $^{+}$ group ($\#p < 0.05$, Fishers LSD). Values represent mean $\pm$ SD. MCMD, mild cognitive and motor disorder; HAD, HIV-associated dementia. (**C**) Representative fluorescent images of the dentate gyrus from patients in each group showing the colocalization of ER α (red), GFAP (green), and DAPI (blue). (**D**) Magnified image of a GFAP $^{+}$ astrocyte containing ER α .

ER α is expressed in neurons and astrocytes in the human hippocampus

Human hippocampal sections were processed for immunocytochemical detection of ER α or GFAP protein. ER α was detected in pyramidal neurons of ammons horn as well as dentate granule cells and hilar neurons (data not shown). To determine whether hippocampal astrocytes express ER α , we performed localization analysis of immunofluorescent labeled sections. Images of GFAP (green), ER α (red), and DAPI (blue) labeling were captured. Images revealed the presence of numerous GFAP-IR cells positive for ER α in each patient group (Fig. 4C). A magnified image of a cell that was

identified as an ER α -IR astrocyte is shown in Fig. 4D, in which cytoplasmic labeling of ER α is shown.

Increased percentage of ER α -IR astrocytes correlates with the severity of dementia following HIV infection

Cell counts of dentate granule cells that expressed both ER α and GFAP as well as total GFAP-IR cells revealed no difference in the density of ER α -GFAP-IR cells between groups (Fig. 5A), while the density of GFAP-IR cells increased in HIV $^{+}$ cognitively normal patients compared to uninfected controls but was decreased in both MCMD and HAD compared to the HIV $^{+}$ cognitively normal group (Fig. 5B). These

FIG. 5. The percentage of ER α -immunoreactive astrocytes correlates with the severity of dementia following HIV infection. ER α -immunoreactive astrocytes (A) and GFAP-immunoreactive astrocytes (B) were counted in the dentate gyrus of each patient group. No significant difference in the density of ER α ⁺ astrocytes was found between patient groups. However, the density of GFAP-IR astrocytes increased following HIV infection compared to uninfected controls (* $p < 0.05$, Fishers LSD), while the MCMD and HAD groups showed a decrease in GFAP density compared to HIV⁺ (# $p < 0.05$) but were not significantly different from uninfected controls. (C) Analysis of the ratio of ER α ⁺ astrocytes to total astrocytes showed an attenuation following HIV infection compared to uninfected controls (* $p < 0.05$, Fishers LSD) while the HAD patient group showed an increase compared to HIV⁺ (# $p < 0.05$, Fishers LSD), which was restored to levels seen in the HIV⁻ group. Values represent mean $\pm$ SD.

findings are in agreement with the mRNA expression of ER α and GFAP we observed in the hippocampus. However, the density of ER α -GFAP-IR cells normalized to total GFAP-IR cells was decreased by 23% in the HIV⁺ control group compared to the HIV⁻ group (Fig. 5C, $p < 0.05$), but was increased in the HAD group compared to the HIV⁺ control group ($p < 0.05$). In addition to the quantification analysis of the data, a significant correlation between the severity of dementia following HIV infection and the density of ER α -IR astrocytes was observed ($p = 0.0049$, Spearman nonparametric correlation).

Discussion

The principal finding of the present study was that E2 suppressed Tat-induced HIV LTR activation through an ER α -independent mechanism. Paradoxically, we found that the overexpression of ER α negated the E2 effect in reducing Tat transactivation in a manner that was cell type specific. In addition, the data showed an increased percentage of ER α -IR astrocytes in the hippocampus of individuals with HAD in

comparison to cognitively normal HIV⁺ patients. Despite abundant data illustrating extensive astrogliosis in HIV brain and the increase in ER α expression within reactive astrocytes associated with neurodegenerative disease or injury, this is the first study to investigate changes in the expression and distribution of ER α in the hippocampus of HIV-infected patients and the relationship between ER α expression and cognitive function.

In general, estrogen action is mediated through the binding of either of the identified estrogen receptors, ER α and ER β . The ER α agonist (PPT) mimics E2 in suppressing Tat transactivation, which suggests that ER α may be involved in Tat-mediated transcription.²¹ In the present study we found ER α mRNA in SVGA and HEK 293 cells, however, these levels were negligible compared to MCF-7 cells, which express abundant levels of ER α mRNA and protein. HEK293 cells express ER α mRNA but lack ER α protein³⁸ whereas HeLa cells lack both ER α mRNA and protein. For this reason, HeLa and HEK293 cells were chosen as negative controls. Similarly, ER β mRNA levels were analyzed by qRT-PCR and found to be negligible compared to the levels of ER α mRNA in SVGA

FIG. 6. A Schematic diagram illustrating the potential protective effects of E2 on HIV protein expression following HIV infection. The diagram illustrates three brains, each representing a different disease state. In the HIV⁻ brain 22 reactive astrocytes are shown (circles) of which 13 express ER α (shaded) while the rest are ER α negative (open circles). In the absence of viral infection no HIV protein is found. As shown, the HIV⁺ brain contains 35 reactive astrocytes, 10 of which express ER α . The increase in the number of HIV-infected astrocytes leads to a substantial increase in HIV protein production. In contrast, the number of reactive astrocytes is decreased in the HAD brain and thus only moderate levels of HIV proteins are produced. As shown, the HAD brain contains 15 reactive astrocytes of which 9 express ER α . In the presence of E2, no change occurs in the normal brain. However, in the HIV⁺ brain 25 of 35 reactive astrocytes do not express ER α and therefore based on data previously shown, E2 would suppress Tat-induced LTR activation and reduce HIV protein production. In the HAD brain, however, only 6 of 15 cells do not express ER α , resulting in a slight reduction in HIV protein expression.

cells. For this reason we did not further investigate a role for ER β in mediating the effects of estrogen on attenuating HIV LTR activation in SVGA cells.

In agreement with reported observations no ER α protein was detected in HeLa or HEK293 cells by immunocytochemistry or Western blot. Interestingly, although SVGA cells appeared to have detectable levels of ER α mRNA, we failed to detect ER α protein in these cells. This result was surprising given that astrocytes and neurons express ER α and ER β .^{35,40} This may be explained by the properties of SVGA cells compared to primary astrocytes. SVGA cells were originally derived from human fetal brain tissue that was transfected with a replication-deficient mutant of SV40.²⁹ This resulted in the creation of an immortalized but not transformed cell line consisting of a homogeneous population of glial cells, identified by their immunoreactivity to GFAP.²⁹ SVGA cells were derived from fetal human brain tissue isolated at 8–12 weeks gestation, a time when ER α expression in the cortex has started to decline, while expression in the hippocampus has yet to occur.^{37,41} Together this suggests that the absence of detectable levels of ER α in SVGA cells may be because the cells are not fully differentiated into mature astrocytes. Indeed, SVGA cells have been described as a presumptive progenitor cell of the CNS that morphologically does not appear differentiated.⁴² Furthermore, despite the astrocytic phenotype the

cells adopt when cultured in 10% serum-containing media in the present studies, SVGA cells can alternatively be cultured as neuronal cells in the presence of N2-supplemented neurobasal media.^{42–44}

Since many astrocytes *in vivo* express ER α , we investigated the effect of ER α overexpression in SVGA cells on the effect of E2 in decreasing Tat-mediated HIV promoter activation. Results showed that in the presence of ER α no E2-mediated decrease in LTR activation by Tat was observed. E2 treatment of cells transfected with ER α but not Tat DNA appeared to increase LTR activation but did not reach significance. Interestingly E2 had no effect in the absence or presence of ER α in HEK293 cells, indicating that the effect of E2 is cell type specific. The E2-mediated suppression of Tat-induced HIV LTR activation in astrocytes in the human brain would have tremendous therapeutic potential because this would lead to a decrease in HIV protein production and concomitant neuronal damage. To address the relevance of our data illustrating the effect of ER α in negating the effect of E2 we investigated the expression of ER α protein in astrocytes in the HIV-infected human brain and how this relates to cognitive function.

In response to injury mediated by trauma, ischemia, or neurodegenerative disease ER α expression in the brain is increased.^{22,35,45,46} ER α expression increases in reactive astrocytes in the rodent, primate, and human brain,^{24,27} and

astrogliosis is a common pathological finding following injury to the CNS.⁴⁷ With the use of GFAP immunolabeling, we identified reactive astrocytes in hippocampal samples obtained from each patient. GFAP-IR cells were detected mainly in the dentate gyrus, a pattern that has been reported for HIV-infected brains.⁴⁸ As expected, GFAP expression was higher in the HIV⁺ samples compared to uninfected controls, indicative of astrogliosis, while in HIV⁺ samples derived from cognitively impaired patients, GFAP expression was decreased in the dentate gyrus compared to patients classified as cognitively normal. Interestingly, the decline in reactive astrocytes following the initial viral-induced increase in astrogliosis is correlated with neurological progression to HAD as shown by the directly proportional relationship between cognitive status in HIV and reduced GFAP expression. These findings are in agreement with a report describing a correlation between neurological progression and astrocyte apoptosis in HAD.⁴⁹

Immunohistochemical analysis of the distribution of ER α and GFAP colabeled cells in the hippocampus showed that both the density of ER α -IR cells and ER α mRNA expression in the dentate gyrus did not differ between cognitively impaired and cognitively normal HIV-infected patients. This finding was unexpected and deserves further investigation since ER α expression is increased in the rat following ischemia⁵⁰ and the human brain in Alzheimer's disease (AD).⁵¹ However, the percentage of ER α -GFAP-IR cells decreased in HIV-infected patients compared to uninfected controls and was increased in HIV⁺ patients with HAD compared to cognitively normal patients. The increase in the percentage of the ratio of ER α -GFAP-IR cells was progressive and was shown to correlate with the severity of cognitive impairment following HIV infection.

It has previously been reported that in the hippocampus of AD patients the density of ER α -IR astrocytes is increased while the percentage of ER α -IR astrocytes remains unaltered compared to nondemented control subjects.³⁵ It is possible that the role of ER α expression in the hippocampus in neurological progression to HAD following HIV infection may differ from the development of dementia in AD. Of importance are the data supporting a role for estrogen neuroprotection in delaying the onset and progression of AD,⁵² which for HIV is not entirely clear at present. The increase in the ratio of ER α -IR astrocytes in the hippocampus of HAD patients may provide a desirable target for estrogen action. In support of this, an increase in ER α -IR astrocytes has been reported in the rat following a stab wound and excitotoxic injury²⁴ and estrogen has been shown to provide neuroprotection in both of these injuries.^{53–55} Therefore, the fact that we did not observe the same trend in our HIV samples implies that ER α upregulation in astrocytes is not a general response to injury but appears to be injury specific.

Despite supporting evidence for E2 as a neuroprotective agent in the brain, gender differences in the development and severity of cognitive decline in HIV-infected women are not entirely clear; however, it has been reported that women on hormone replacement therapy were less likely to develop HAD than those who were not.⁵⁶ Overall, too few studies have been conducted to determine a neuroprotective role for estrogen in HIV dementia; however, it is possible that E2 could play a protective role in the neuropathogenesis of HAD by attenuating the Tat-induced activation of the HIV promoter

resulting in reduced HIV protein synthesis and concomitant neuronal damage. However, based on the data presented in this study, the time at which E2 would be most beneficial in the reduction of HIV transcription would be at early stages of disease progression when patients have yet to develop cognitive impairment (illustrated in Fig. 6). At this stage, HIV entry into the brain has occurred, the numbers of reactive astrocytes that do not express ER α far exceed those that do, and E2 treatment would significantly lower the levels of Tat-induced viral transcription. In contrast, a patient suffering HAD is unlikely to benefit from E2 treatment because even though astrocyte cell death leads to reduced levels of HIV proteins, the damage caused by the initial increase in reactive astrocytes has already occurred and a slight decrease in HIV protein expression at this stage is unlikely to provide adequate neuroprotection. Although we showed that E2 suppressed Tat-induced LTR activation in an astrocyte cell line, it remains to be determined whether this occurs in astrocytes in the human brain and furthermore whether endogenous ER α expression in astrocytes interferes with this effect.

Since our data suggest that ER α is not responsible for mediating the effects of E2 LTR activation, the mechanism behind the E2-mediated suppression of HIV transcription remains a mystery. The G-protein-coupled receptor, GPR30, is a recently identified receptor shown to bind E2 with high affinity leading to the activation of MAP kinase⁵⁷ and PI3 kinase pathways in MCF-7 cells.⁵⁸ PI3K activation has been shown to inhibit Tat-induced LTR activation⁵⁹ and GPR30 is expressed and is activated by E2 in SVGA cells (unpublished observations). Therefore a role for GPR30 in mediating the effects of estrogen in the attenuation of HIV transcription cannot be ruled out. Our data suggest that estrogen can act as a protective agent in the brain of HIV-infected individuals; however, more extensive clinical studies are needed to address gender differences in HIV dementia.

Acknowledgments

The authors would like to thank the National NeuroAids Tissue Consortium for supplying the postmortem human tissue. We would also like to thank Dr. S. Estus for supplying the GFAP primers used for qRT-PCR and help with RT-PCR data analysis, Dr. T. McClintock for help with RT-PCR experimental design and data analysis, Ms. C. Long for technical assistance with tissue sectioning, and Dr. R. Kryscio for help with statistical analysis. This work was supported by Grant P20 RR 15592 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH).

Disclosure Statement

No competing financial interests exist.

References

1. Fischer-Smith T and Rappaport J: Evolving paradigms in the pathogenesis of HIV-1-associated dementia. *Expert Rev Mol Med* 2005;7(27):1–26.
2. Saito Y, Sharer LR, Epstein LG, *et al.*: Overexpression of nef as a marker for restricted HIV-1 infection of astrocytes in postmortem pediatric central nervous tissues. *Neurology* 1994;44(3Pt. 1):474–481.

3. Garber ME and Jones KA: HIV-1 Tat: Coping with negative elongation factors. *Curr Opin Immunol* 1999;11(4):460–465.
4. Karn J: Tackling Tat. *J Mol Biol* 1999;293(2):235–254.
5. Brigati C, Giacca M, Noonan DM, and Albini A: HIV Tat, its targets and the control of viral gene expression. *FEMS Microbiol Lett* 2003;220(1):57–65.
6. Dingwall C, Ernberg I, Gait MJ, *et al.*: Human immunodeficiency virus 1 tat protein binds trans-activation-responsive region (TAR) RNA in vitro. *Proc Natl Acad Sci USA* 1989;86(18):6925–6929.
7. Rosen CA and Pavlakis GN: Tat and Rev: Positive regulators of HIV gene expression. *AIDS* 1990;4(8):A51.
8. Garcia-Martinez LF, Mavankal G, Peters P, Wu-Baer F, and Gaynor RB: Tat functions to stimulate the elongation properties of transcription complexes paused by the duplicated TAR RNA element of human immunodeficiency virus 2. *J Mol Biol* 1995;254(3):350–363.
9. Wu-Baer F, Sigman D, and Gaynor RB: Specific binding of RNA polymerase II to the human immunodeficiency virus trans-activating region RNA is regulated by cellular cofactors and Tat. *Proc Natl Acad Sci USA* 1995;92(16):7153–7157.
10. Magnuson DS, Knudsen BE, Geiger JD, Brownstone RM, and Nath A: Human immunodeficiency virus type 1 tat activates non-N-methyl-D-aspartate excitatory amino acid receptors and causes neurotoxicity. *Ann Neurol* 1995;37(3):373–380.
11. Brack-Werner R: Astrocytes: HIV cellular reservoirs and important participants in neuropathogenesis. *AIDS* 1999;13(1):1–22.
12. Wang T, Rumbaugh JA, and Nath A: Viruses and the brain: From inflammation to dementia. *Clin Sci (Lond)* 2006;110(4):393–407.
13. Bruce-Keller AJ, Keeling JL, Keller JN, Huang FF, Camondola S, and Mattson MP: Antiinflammatory effects of estrogen on microglial activation. *Endocrinology* 2000;141(10):3646–3656.
14. Pozzi S, Benedusi V, Maggi A, and Vegeto E: Estrogen action in neuroprotection and brain inflammation. *Ann NY Acad Sci* 2006;1089:302–323.
15. Kendall SL, Anderson CF, Nath A, *et al.*: Gonadal steroids differentially modulate neurotoxicity of HIV and cocaine: Testosterone and ICI 182,780 sensitive mechanism. *BMC Neurosci* 2005;6(1):40.
16. Turchan J, Anderson C, Hauser KF, *et al.*: Estrogen protects against the synergistic toxicity by HIV proteins, methamphetamine and cocaine. *BMC Neurosci* 2001;2:3.
17. Wallace DR, Dodson S, Nath A, and Booze RM: Estrogen attenuates gp120- and tat1-72-induced oxidative stress and prevents loss of dopamine transporter function. *Synapse* 2006;59(1):51–60.
18. Morale MC, Serra PA, L'Episcopo F, *et al.*: Estrogen, neuroinflammation and neuroprotection in Parkinson's disease: Glia dictates resistance versus vulnerability to neurodegeneration. *Neuroscience* 2006;138(3):869–878.
19. de Lacalle S: Estrogen effects on neuronal morphology. *Endocrine* 2006;29(2):185–190.
20. Saenz C, Dominguez R, and de Lacalle S: Estrogen contributes to structural recovery after a lesion. *Neurosci Lett* 2006;392(3):198–201.
21. Wilson ME, Allred KF, Bisotti AJ, Bruce-Keller A, Chuahan A, and Nath A: Estradiol negatively regulates HIV-LTR promoter activity in glial cells. *AIDS Res Hum Retroviruses* 2006;22(4):350–356.
22. Dubal DB, Zhu H, Yu J, *et al.*: Estrogen receptor alpha, not beta, is a critical link in estradiol-mediated protection against brain injury. *Proc Natl Acad Sci USA* 2001;98(4):1952–1957.
23. Wilson ME, Dubal DB, and Wise PM: Estradiol protects against injury-induced cell death in cortical explant cultures: A role for estrogen receptors. *Brain Res* 2000;873(2):235–242.
24. Garcia-Ovejero D, Veiga S, Garcia-Segura LM, and DonCarlos LL: Glial expression of estrogen and androgen receptors after rat brain injury. *J Comp Neurol* 2002;450(3):256–271.
25. Dhandapani KM and Brann DW: Estrogen-astrocyte interactions: Implications for neuroprotection. *BMC Neurosci* 2002;3:6.
26. Azcoitia I, Garcia-Ovejero D, Chowen JA, and Garcia-Segura LM: Astroglia play a key role in the neuroprotective actions of estrogen. *Prog Brain Res* 2001;132:469–478.
27. Blurton-Jones M and Tuszynski MH: Reactive astrocytes express estrogen receptors in the injured primate brain. *J Comp Neurol* 2001;433(1):115–123.
28. Kim EJ, Oh CS, Kim J, Kim WH, Chung YH, and Shin DH: Reactive astrocytes expressing intense estrogen receptor-alpha immunoreactivities have much elongated cytoplasmic processes: An autopsy case of human cerebellar tissue with multiple genitourinary and gastrointestinal anomalies. *J Korean Med Sci* 2007;22(5):936–941.
29. Major EO, Miller AE, Mourrain P, Traub RG, de Widt E, and Sever J: Establishment of a line of human fetal glial cells that supports JC virus multiplication. *Proc Natl Acad Sci USA* 1985;82(4):1257–1261.
30. Nitkiewicz J, Chao W, Bentsman G, *et al.*: Productive infection of primary murine astrocytes, lymphocytes, and macrophages by human immunodeficiency virus type 1 in culture. *J Neurovirol* 2004;10(6):400–408.
31. Chomczynski P and Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162(1):156–159.
32. Kelley MR, Jurgens JK, Tentler J, Emanuele NV, Blutt SE, and Emanuele MA: Coupled reverse transcription-polymerase chain reaction (RT-PCR) technique is comparative, quantitative, and rapid: Uses in alcohol research involving low abundance mRNA species such as hypothalamic LHRH and GRF. *Alcohol* 1993;10(3):185–189.
33. Zhang X, Ding L, and Sandford AJ: Selection of reference genes for gene expression studies in human neutrophils by real-time PCR. *BMC Mol Biol* 2005;6(1):4.
34. Vandesompele J, De Preter K, Pattyn F, *et al.*: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3(7):RESEARCH0034.
35. Lu YP, Zeng M, Hu XY, *et al.*: Estrogen receptor alpha-immunoreactive astrocytes are increased in the hippocampus in Alzheimer's disease. *Exp Neurol* 2003;183(2):482–488.
36. Hu XY, Qin S, Lu YP, Ravid R, Swaab DF, and Zhou JN: Decreased estrogen receptor-alpha expression in hippocampal neurons in relation to hyperphosphorylated tau in Alzheimer patients. *Acta Neuropathol* 2003;106(3):213–220.
37. Gonzalez M, Cabrera-Socorro A, Perez-Garcia CG, *et al.*: Distribution patterns of estrogen receptor alpha and beta in the human cortex and hippocampus during development and adulthood. *J Comp Neurol* 2007;503(6):790–802.
38. Katagiri D, Hayashi H, Victoriano AF, Okamoto T, and Onozaki K: Estrogen stimulates transcription of human immunodeficiency virus type 1 (HIV-1). *Int Immunopharmacol* 2006;6(2):170–181.

39. Watanabe A, Kinoshita Y, Hosokawa K, Mori T, Yamaguchi T, and Honjo H: Function of estrogen-related receptor alpha in human endometrial cancer. *J Clin Endocrinol Metab* 2006;91(4):1573–1577.
40. Savaskan E, Olivieri G, Meier F, Ravid R, and Muller-Spahn F: Hippocampal estrogen beta-receptor immunoreactivity is increased in Alzheimer's disease. *Brain Res* 2001;908(2):113–119.
41. Brandenberger AW, Tee MK, Lee JY, Chao V, and Jaffe RB: Tissue distribution of estrogen receptors alpha (ER-alpha) and beta (ER-beta) mRNA in the midgestational human fetus. *J Clin Endocrinol Metab* 1997;82(10):3509–3512.
42. Dowling-Warriner CV and Trosko JE: Induction of gap junctional intercellular communication, connexin43 expression, and subsequent differentiation in human fetal neuronal cells by stimulation of the cyclic AMP pathway. *Neuroscience* 2000;95(3):859–868.
43. Lawrence DM, Durham LC, Schwartz L, Seth P, Maric D, and Major EO: Human immunodeficiency virus type 1 infection of human brain-derived progenitor cells. *J Virol* 2004;78(14):7319–7328.
44. Lawrence DM, Seth P, Durham L, *et al.*: Astrocyte differentiation selectively upregulates CCL2/monocyte chemoattractant protein-1 in cultured human brain-derived progenitor cells. *Glia* 2006;53(1):81–91.
45. Kokiko ON, Murashov AK, and Hoane MR: Administration of raloxifene reduces sensorimotor and working memory deficits following traumatic brain injury. *Behav Brain Res* 2006;170(2):233–240.
46. Hoffman GE, Merchenthaler I, and Zup SL: Neuroprotection by ovarian hormones in animal models of neurological disease. *Endocrine* 2006;29(2):217–231.
47. Eng LF and Ghimikar RS: GFAP and astrogliosis. *Brain Pathol* 1994;4(3):229–237.
48. Vanzani MC, Iacono RF, Caccuri RL, Troncoso AR, and Berria MI: Regional differences in astrocyte activation in HIV-associated dementia. *Medicina (B Aires)* 2006;66(2):108–112.
49. Thompson KA, McArthur JC, and Wesselingh SL: Correlation between neurological progression and astrocyte apoptosis in HIV-associated dementia. *Ann Neurol* 2001;49(6):745–752.
50. Dubal DB, Rau SW, Shughrue PJ, *et al.*: Differential modulation of estrogen receptors (ERs) in ischemic brain injury: A role for ERalpha in estradiol-mediated protection against delayed cell death. *Endocrinology* 2006;147(6):3076–3084.
51. Ishunina TA and Swaab DF: Increased expression of estrogen receptor alpha and beta in the nucleus basalis of Meynert in Alzheimer's disease. *Neurobiol Aging* 2001;22(3):417–426.
52. Brann DW, Dhandapani K, Wakade C, Mahesh VB, and Khan MM: Neurotrophic and neuroprotective actions of estrogen: Basic mechanisms and clinical implications. *Steroids* 2007;72(5):381–405.
53. Green PS and Simpkins JW: Neuroprotective effects of estrogens: Potential mechanisms of action. *Int J Dev Neurosci* 2000;18(4–5):347–358.
54. Xia S, Cai ZY, Thio LL, *et al.*: The estrogen receptor is not essential for all estrogen neuroprotection: New evidence from a new analog. *Neurobiol Dis* 2002;9(3):282–293.
55. Weaver CE Jr, Park-Chung M, Gibbs TT, and Farb DH: 17beta-Estradiol protects against NMDA-induced excitotoxicity by direct inhibition of NMDA receptors. *Brain Res* 1997;761(2):338–341.
56. Clark RA and Bessinger R: Clinical manifestations and predictors of survival in older women infected with HIV. *J Acquir Immune Defic Syndr Hum Retrovirol* 1997;15(5):341–345.
57. Filardo EJ, Quinn JA, Frackelton AR Jr, and Bland KI: Estrogen action via the G protein-coupled receptor, GPR30: Stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol Endocrinol* 2002;16(1):70–84.
58. Prossnitz ER, Oprea TI, Sklar LA, and Arterburn JB: The ins and outs of GPR30: A transmembrane estrogen receptor. *J Steroid Biochem Mol Biol* 2008;109(3–5):350–353.
59. Cook JA, August A, and Henderson AJ: Recruitment of phosphatidylinositol 3-kinase to CD28 inhibits HIV transcription by a Tat-dependent mechanism. *J Immunol* 2002;169(1):254–260.

Address correspondence to:

Melinda E. Wilson

Department of Physiology

University of Kentucky Medical Center

Lexington, Kentucky 40536-0298

E-mail: melinda.wilson@uky.edu