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Estrogen Receptor β Signaling through Phosphatase and Tensin Homolog/Phosphoinositide 3-Kinase/Akt/Glycogen Synthase Kinase 3 Down-Regulates Blood-Brain Barrier Breast Cancer Resistance Protein

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ABSTRACT

Breast cancer resistance protein (BCRP) is an ATP-driven efflux pump at the blood-brain barrier that limits central nervous system pharmacotherapy. Our previous studies showed rapid loss of BCRP transport activity in rat brain capillaries exposed to low concentrations of 17-β-estradiol (E2); this occurred without acute change in BCRP protein expression. Here, we describe a pathway through which sustained, extended exposure to E2 signals down-regulation of BCRP at the blood-brain barrier. Six-hour exposure of isolated rat and mouse brain capillaries to E2 reduced BCRP transport activity and BCRP monomer and dimer expression. Experiments with brain capillaries from estrogen receptor (ER)α and ERβ knockout mice and with ER agonists and antagonists showed that E2 signaled through ERβ to down-regulate BCRP expression. In rat brain capillaries, E2 increased unphosphorylated, active phosphatase and tensin homolog (PTEN); decreased phosphorylated, active Akt; and increased phosphorylated, active glycogen synthase kinase (GSK)3. Consistent with this, inhibition of phosphoinositide 3-kinase (PI3K) or Akt decreased BCRP activity and protein expression, and inhibition of PTEN or GSK3 reversed the E2 effect on BCRP. Lactacystin, a proteasome inhibitor, abolished E2-mediated BCRP down-regulation, suggesting internalization followed by transporter degradation. Dosing mice with E2 reduced BCRP activity in brain capillaries within 1 h; this reduction persisted for 24 h. BCRP protein expression in brain capillaries was unchanged 1 h after E2 dosing but was substantially reduced 6 and 24 h after dosing. Thus, E2 signals through ERβ, PTEN/PI3K/Akt/GSK3 to stimulate proteasomal degradation of BCRP. These in vitro and in vivo findings imply that E2-mediated down-regulation of blood-brain barrier BCRP has the potential to increase brain uptake of chemotherapeutics that are BCRP substrates.

BCRP is an ATP-driven drug efflux pump at the blood-brain barrier (Cooray et al., 2002; Eisenblätter and Galla, 2002; Hartz et al., 2010). Recent studies with BCRP-null mice and with drugs that specifically inhibit this transporter show that it limits the ability of several chemotherapeutics, e.g., topotecan, imatinib, dasatinib, and lapatinib, to cross the brain capillary endothelium and enter the CNS (Breedveld et al., 2006; de Vries et al., 2007; Chen et al., 2009; Polli et al., 2009; Zhou et al., 2009). As a consequence, anticancer drugs that are BCRP substrates do not reach the tumor target tissue in the brain at all or only do so at subtherapeutic levels (Pal and Mitra, 2006). BCRP also underlies drug resistance in brain tumor cells, and an emerging concept suggests that one protective mechanism used by cancer stem cells is drug efflux mediated by ABC transporters, including BCRP (Dean, 2009). In addition, recent reports indicate that

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ABBREVIATIONS: BCRP, breast cancer resistance protein; CNS, central nervous system; ABC, ATP-binding cassette; E2, 17-β-estradiol; KO, knockout; ER, estrogen receptor; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase; GSK, glycogen synthase kinase; PPT, propylpyrazoletriol; DPN, diarylpropionitrile; MPP, methylpiperidinopyrazole; ICI, fulvestrant; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; XIII, 5-methyl-1*H*-pyrazol-3-yl)-(2-phenylquinazolin-4-yl)amine; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PCR, polymerase chain reaction; RT, reverse transcription; FTC, fumitremorgin C; ELISA, enzyme-linked immunosorbent assay; bp, base pair(s).

at the blood-brain barrier, BCRP works in concert with other ABC transporters such as P-glycoprotein (de Vries et al., 2007; Chen et al., 2009; Polli et al., 2009; Zhou et al., 2009; Kodaira et al., 2010). Thus, BCRP poses a serious problem for delivering certain chemotherapeutics across the blood-brain barrier into the CNS and into brain cancer tissue and brain cancer stem cells.

One potential strategy to overcome drug resistance due to BCRP is to target signals that regulate transporter activity and expression. However, little is known about the regulation of BCRP at the blood-brain and blood-tumor barriers. In several peripheral tissues, BCRP expression is under control of estrogen, but the underlying mechanism(s) is not well defined (Merino et al., 2005; Tanaka et al., 2005). We recently found that low nanomolar concentrations of 17-β-estradiol (E2) rapidly and reversibly reduced BCRP-mediated transport in isolated brain capillaries from rats and mice (Hartz et al., 2010). This down-regulation of transport activity occurred without reduction of BCRP protein expression. Experiments with receptor-specific agonists and antagonists and with ER knockout (KO) mice showed that these rapid effects of E2 on BCRP activity were signaled through both ERα and ERβ. In the present study, we extend these observations in three ways. First, we show that rapid loss of BCRP activity in brain capillaries is followed by a period of sustained down-regulation of activity and eventual reduction in transporter protein expression. Second, we identify ERβ, PTEN/PI3K/Akt/GSK3, and proteasomal degradation as key steps in the pathway that signals the loss of BCRP expression in brain capillaries. Third, we demonstrate that the time course of change in blood-brain barrier BCRP activity and expression observed in vitro is recapitulated in mice dosed with E2. We speculate that targeting the E2-dependent signaling pathway at the blood-brain barrier described here could provide an opportunity to improve CNS delivery of chemotherapeutics and thus improve chemotherapy of brain tumors.

Materials and Methods

Chemicals. BCRP antibody (BXP53) and fumitremorgin C were purchased from Alexis-Axxora (San Diego, CA). Estrogen receptor α, estrogen receptor β, Akt, phospho-Akt, phospho-GSK3 (α and β), and β-actin antibodies were from Abcam Inc. (Cambridge, MA). BODIPY FL prazosin was obtained from Invitrogen (Carlsbad, CA); PTEN and phospho-PTEN antibodies, the PTEN inhibitor bpV(HOpic), and the GSK3 inhibitor XIII were from EM Scientific (Gibbstown, NJ). Propylpyrazoletriol (PPT), diarylpropionitrile (DPN), methylpiperidinopyrazole (MPP), and ICI182,780 were purchased from Tocris Bioscience (Ellisville, MO). LY294002, triciribine, and lactacystin were obtained from Calbiochem-Novabiochem (San Diego, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Animals. Male Sprague-Dawley rats (retired breeders; 6 months old; average body weight: 500 g) were purchased from Taconic Farms (Germantown, NY) and from Charles River Breeding Laboratories (Portage, MI). Male BALB/c mice were obtained from Charles River Breeding Laboratories (10 weeks old; average body weight: 20 g). Ten-week-old male and female estrogen receptor α-deficient (ERα KO; B6.129-*Esr1*^{tm1Ksk} N10), ERβ KO (B6.129-*Esr2*^{tm1Unc} N9), and wild-type mice (C57BL/6 background) were a kind gift from Dr. Kenneth Korach (Laboratory of Reproductive and Developmental Biology, Receptor Biology Group, National Institute of Environmental Health Sciences, Research Triangle Park, NC) (Lubahn et al., 1993). The average body weight of female mice was 20 g; the average body weight of male mice was 25 g. Animal housing protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota and the National Institutes of Health National Institute of Environmental Health Sciences and were in accordance with National Institute of Environmental Health Sciences guidelines. All animal experiments were conducted in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care regulations and the Guides of Animal Use of the University of Minnesota and the National Institutes of Health *Guide for the Care and Use of Animals*.

Isolation of Brain Capillaries. Brain capillaries from rats and mice were isolated as described previously (Hartz et al., 2008). For each isolation, 10 rats or 20 mice, respectively, were euthanized by CO_2 inhalation and then decapitated. Brains were dissected and homogenized in ice-cold PBS buffer supplemented with 5 mM D-glucose and 1 mM sodium pyruvate (2.7 mM KCl, 1.46 mM KH_2PO_4, 136.9 mM NaCl, and 8.1 mM Na_2HPO_4, pH 7.4). The homogenate was mixed with Ficoll (final concentration: 15%) and centrifuged at 5800*g* for 20 min at 4°C. The pellet was re:suspended in 1% BSA and the capillary suspension was passed over a glass bead column. Capillaries adhering to the glass beads were collected by gentle agitation in 1% BSA. Capillaries were washed with PBS and used for transport experiments, Western blotting, immunostaining, or RNA isolation.

Reverse Transcription-Polymerase Chain Reaction. Total RNA was isolated from rat brain capillaries, brain, choroid plexus, kidney, and liver using TRIzol reagent (Invitrogen). The samples were further purified using the RNeasy mini kit (QIAGEN, Valencia, CA), and reverse transcription was performed using the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA). PCR of RT products was performed with *Taq* DNA Polymerase from Promega (Madison, WI) using primers for rat ERα (forward, 5′-CTATGGCCAGTCGAGCATCACTTAC-3′; reverse, 5′-GATCATGTTTCCCTTCTCGCTGCTG-3′) and rat ERβ (forward, 5′-GAATGGTCAAGTGTGGATCCAGGAG-3′; reverse, 5′-CTCCATCCAGCAGCTTTCTAAGAGC-3′). All primers were custom-synthesized by QIAGEN Operon (Alameda, CA).

Western Blotting. Protein expression in brain capillary membranes and tissue samples was analyzed by Western blotting as described previously (Hartz et al., 2006, 2008; Bauer et al., 2007). In brief, isolated brain capillaries, liver, kidney, brain, and choroid plexus tissue samples were homogenized in lysis buffer (Sigma-Aldrich) containing Complete protease inhibitor (Roche Diagnostics, Mannheim, Germany). Samples were centrifuged at 10,000*g* for 15 min, and denucleated supernatants were used as brain, choroid plexus, and capillary lysates. Denucleated supernatants of liver, kidney, and brain capillaries were centrifuged at 100,000*g* for 90 min to obtain crude membrane fractions. Crude plasma membrane pellets were resuspended in buffer and protein concentrations were determined. The Invitrogen NuPage Bis-Tris electrophoresis and blotting system (Invitrogen) was used for Western blotting. After transfer of the protein, blotting membranes were blocked and incubated with primary antibody. Membranes were washed and incubated for 1 h with secondary antibody (horseradish peroxidase-conjugated ImmunoPure secondary IgG; 1:15,000; Thermo Fisher Scientific, Waltham, MA). SuperSignal West Pico Chemoluminescent Substrate (Thermo Fisher Scientific) was used for detection. Bands were visualized and recorded using a Gel Doc 2000 gel documentation system (Bio-Rad Laboratories, Hercules, CA). Digital analysis of the molecular weights for the two ERβ isoforms in total brain tissue and in brain capillary lysates was performed with QuantityOne 1-D, version 4.6.5 software (Bio-Rad Laboratories). Rainbow recombinant protein molecular weight marker RPN800 used for analyses was from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

BCRP Transport Assay. BCRP-mediated transport in isolated capillaries was performed as described previously (Shukla et al., 2009; Hartz et al., 2010). Isolated brain capillaries were transferred to glass coverslips and incubated for 1 h at room temperature with the fluorescent BCRP substrate BODIPY FL prazosin (2 μM). For each treatment, images of 10 capillaries were acquired by confocal

microscopy [C1 laser scanning confocal microscope unit (Nikon Instruments Inc., Melville, NY), TE2000 inverted microscope (Nikon Instruments Inc.), 40× oil immersion objective, 1.3 numerical aperture × 1.3, 488 nm line of an argon laser (model 163C; Spectra Physics, San Jose, CA), 515/30 nm band pass filter or 543 nm line of an HeNe laser (Nikon Instruments Inc., Melville, NY); or a 410 invert laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY), 40× oil immersion objective, numerical aperture × 1.2, 488 nm line of argon laser (Carl Zeiss Inc.)]. Images were analyzed by measuring luminal BODIPY FL prazosin fluorescence using Scion Image software (Scion Corporation, Frederick, MD), and specific, BCRP-mediated accumulation of fluorescent BODIPY FL prazosin in capillary lumens was determined by taking the difference between total luminal fluorescence and fluorescence in the presence of the BCRP inhibitor fumitremorgin C (FTC; 5 μM) as described previously (Shukla et al., 2009; Hartz et al., 2010).

Immunostaining. Freshly isolated brain capillaries were fixed for 20 min with 3% paraformaldehyde and 0.2% glutaraldehyde at room temperature. After washing with PBS, capillaries were permeabilized for 30 min with 0.1% (v/v) Triton X-100 in PBS and blocked for 30 min with 1% BSA in PBS. Brain capillaries were incubated for 1 h at 37°C with the primary antibody ERβ (1:50; 20 μg/ml). After washing with 1% BSA, capillaries were incubated for 1 h at 37°C with an Alexa Fluor 488-conjugated secondary IgG (1:1000, 2 μg/ml; Invitrogen); negative controls were incubated with secondary antibody only. Nuclei were counterstained with 5 μg/ml propidium iodide for 15 min. ERβ staining was visualized using a 510 meta laser scanning confocal microscope (510 NLO laser scanning confocal microscope, 40× water immersion objective, numerical aperture × 1.2, 488 nm line of argon laser; Carl Zeiss Inc.).

17-β-Estradiol Dosing. Male BALB/c mice were dosed with a single intraperitoneal injection of 0.1 mg/kg E2 in safflower oil; control animals received vehicle intraperitoneal injections. Mice were euthanized by CO_2 inhalation after 1, 6, and 24 h and then decapitated. Trunk blood, brains, and tissue samples were collected for further analysis.

E2 ELISA. E2 plasma levels were determined using an E2 ELISA kit (Calbiotech, Spring Valley, CA) according to the manufacturer's protocol. Plasma was obtained from blood samples by centrifugation at 5000*g* for 5 min at 4°C. Undiluted plasma samples were used for ELISA assays.

Statistical Analysis. Data are presented as mean ± S.E.M. Two-tailed unpaired Student's *t* test was used to evaluate differences between controls and treated groups; differences were considered to be statistically significant when $P < 0.05$.

Results

E2 Signals BCRP Down-Regulation at the Blood-Brain Barrier. We previously validated a confocal imaging-based assay to measure BCRP transport activity in isolated rat and mouse brain capillaries (Shukla et al., 2009; Hartz et al., 2010). This assay involves measurement of the accumulation of the fluorescent BCRP substrate BODIPY FL prazosin in capillary lumens. We showed that such accumulation was highly concentrative and sensitive to inhibition by micromolar concentrations of the BCRP-specific inhibitors FTC and Ko143, and the metabolic inhibitor NaCN. Figure 1A shows representative confocal images of rat brain capillaries preincubated with 2 μM BODIPY FL prazosin for 1 h to establish steady-state luminal accumulation of the substrate (control) and then exposed to 10 nM E2 for 1 to 6 h in the presence of 2 μM BODIPY FL prazosin. For comparison, an image of a capillary exposed to 2 μM BODIPY FL prazosin plus FTC for 1 h is also shown. In agreement with previous work (Hartz et al., 2010), 1-h exposure to E2 reduced luminal

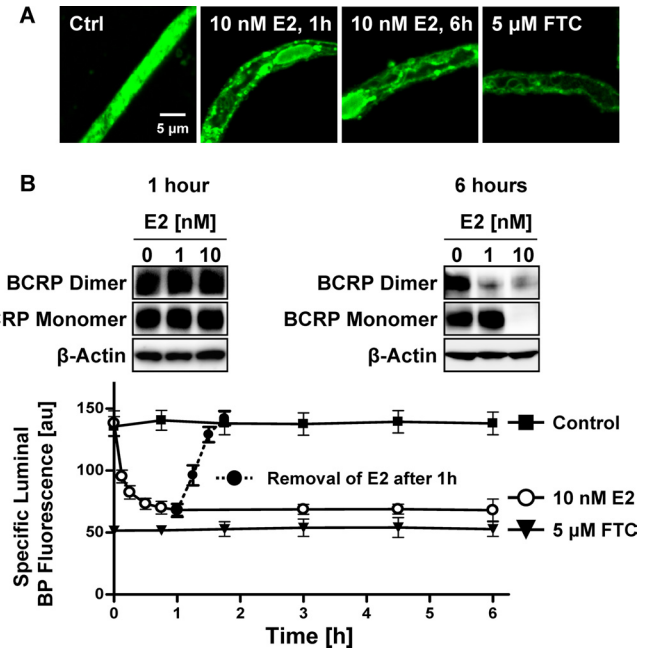

Fig. 1. E2-mediated down-regulation of BCRP. A, representative images of isolated rat brain capillaries that were incubated with 2 μM BODIPY FL prazosin for 1 h and then exposed to 10 nM E2 for 1 or 6 h, or to the BCRP inhibitor FTC. Note that E2 decreased luminal BODIPY FL prazosin fluorescence to levels comparable with those observed with BCRP inhibition by FTC. B, Western blots showing BCRP monomer and dimer protein expression in brain capillaries exposed to E2 for 1 and 6 h and time course of BODIPY FL prazosin luminal fluorescence in control-, FTC-, and E2-treated rat brain capillaries. For specific luminal BODIPY FL prazosin (BP) fluorescence, each data point represents the mean ± S.E.M. for 10 capillaries from a single preparation (pooled tissue from 10 rats). Units are arbitrary fluorescence units (scale, 0–255).

fluorescence roughly to the level seen in the FTC-treated capillary BCRP protein expression was not affected. Luminal fluorescence remained reduced after 6 h of exposure.

Figure 1B shows the full, extended time course of 10 nM E2 action on rat brain capillaries. For this experiment, capillaries were preincubated for 1 h with 2 μM BODIPY FL prazosin to establish steady-state before addition of E2 to the medium. As expected, luminal fluorescence in control capillaries did not vary over the 6-h experiment. Exposure of isolated rat brain capillaries to 10 nM E2 reduced BCRP transport activity within minutes, with maximal transporter inhibition occurring after 30 min; BCRP transport activity remained depressed for the full 6-h E2 exposure period (Fig. 1B). From 30 min on, transport activity in E2-exposed capillaries approached the reduced levels that were found with the BCRP inhibitor FTC. Consistent with our previous study, removing E2 from the incubation medium after 1 h completely restored BCRP transport activity to control levels within an additional hour of incubation in E2-free medium (Fig. 1B; Hartz et al., 2010).

BCRP is an ABC half-transporter that is only functional as a homodimer or multimer (Nakanishi et al., 2003; Xu et al., 2004). In Western blots, both monomer and dimer can be detected. As shown previously (Hartz et al., 2010), BCRP protein expression (monomer and dimer) in capillary membranes was not altered after 1 h of E2 exposure. In contrast, with 6 h of E2 exposure BCRP protein expression had decreased. BCRP dimer was reduced in a concentration-dependent manner by 1 and 10 nM E2; monomer was reduced by 10

nM E2 (Fig. 1B). Note that with 6 h of E2 exposure, BCRP transport activity correlates with the expression of the BCRP dimer, which is responsible for BCRP-mediated transport (Xu et al., 2004). For the remainder of the in vitro studies reported here, exposures were to 10 nM E2 for 6 h. In addition, transport results are reported as specific BODIPY FL prazosin luminal fluorescence, which is the FTC-inhibitable portion of total luminal fluorescence (Fig. 1B).

Expression of ERα and ERβ in Brain Capillaries. E2 signals through two classical nuclear receptors, ERα and ERβ. Here we show, for the first time, ERα and ERβ expression in isolated rat brain capillaries by RT-PCR, Western blots and immunofluorescence. Using RT-PCR, we detected a strong signal for ERα mRNA at 310 bp in brain capillaries, choroid plexus, kidney, and liver (Fig. 2A). No signal was found in brain tissue from adult rats used in our experiments, which is consistent with previous reports (Laflamme et al., 1998; Wilson et al., 2008). The Western blot in Fig. 2B shows ERα protein expression in choroid plexus and crude membranes from liver and kidney. At longer exposure times, ERα protein was also detected in brain capillaries and brain capillary membranes (Fig. 2C). Consistent with the RT-PCR data, we detected no ERα signal in total brain tissue. ERα immunostaining in brain capillaries was weak and diffuse consistent with low levels of ERα expression in the brain capillary endothelium (data not shown).

We detected ERβ mRNA at 374 bp in brain capillaries, brain, and choroid plexus but not in liver or kidney (Fig. 2D). By Western blotting, we found two strong bands for ERβ protein in total brain tissue and in brain capillary lysates. The molecular weights of the two bands were determined to be 55 and 60 kDa by digital molecular weight analysis (Fig. 2E), a finding consistent with previous reports demonstrating expression of multiple ERβ isoforms in most tissues (Hirata et al., 2001; Leung et al., 2006). Weak ERβ signals were found in liver and brain capillary membranes. In crude kidney membranes, one band at 55 kDa was present; no signal was detected in choroid plexus. By immunostaining,

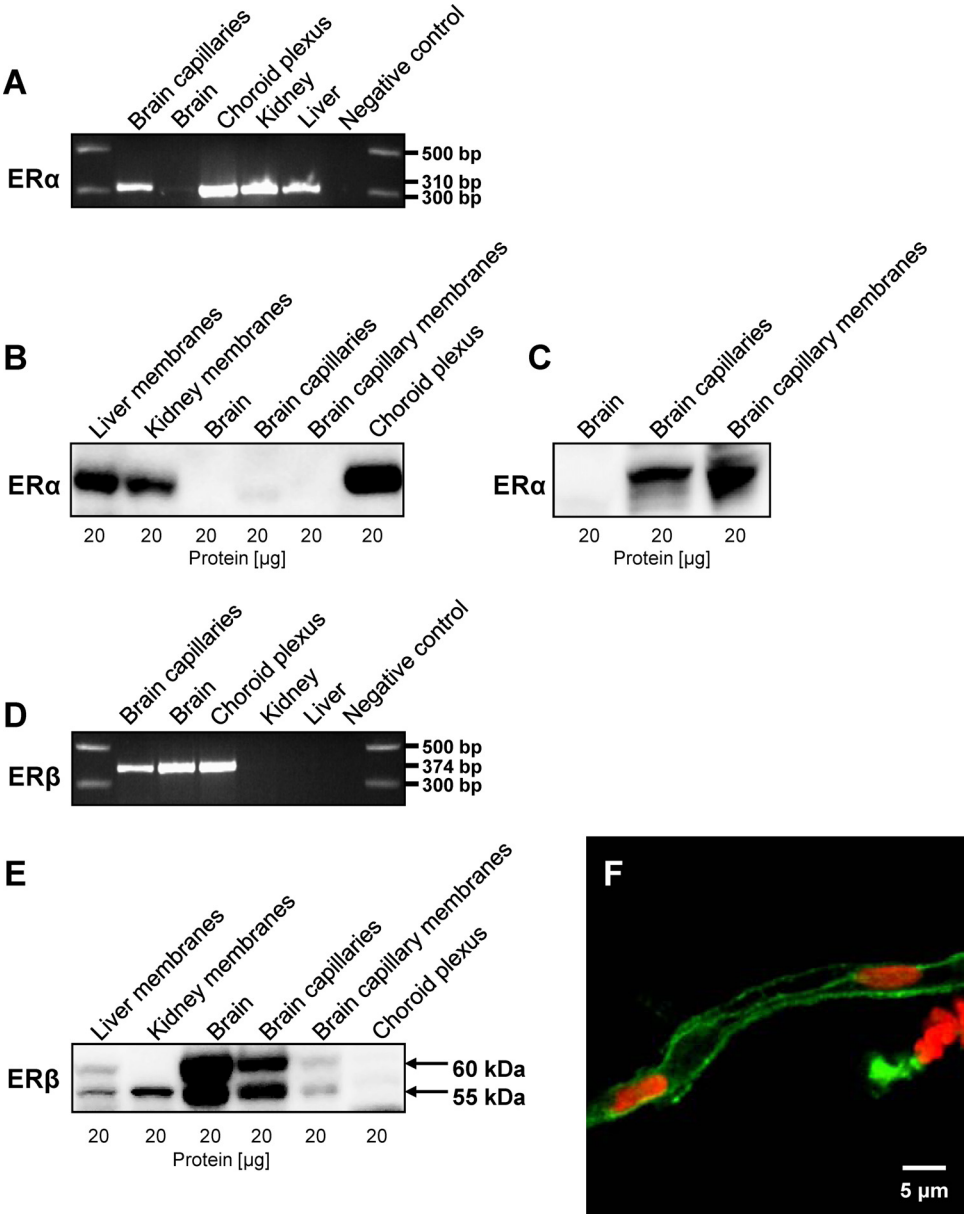

Fig. 2. Expression of ERα and ERβ in isolated rat brain capillaries. A, RT-PCR for ERα (310-bp amplicon). B, Western blot showing ERα in liver, kidney, and choroid plexus. C, Western blot (longer exposure of blotting membrane) showing ERα in brain capillaries and brain capillary membranes, but not in total brain. D, RT-PCR for ERβ (374-bp amplicon) shows expression in brain capillaries, brain, and choroid plexus. E, ERβ protein expression in kidney, brain, and brain capillaries. Two bands were detected at 55 and 60 kDa (determined by digital molecular weight analysis). F, representative ERβ immunostaining of a brain capillary (green); nuclei were counterstained with propidium iodide (red).

we found strong and distinct ERβ staining in isolated rat brain capillaries (Fig. 2F). Thus, although both ERs are expressed in brain capillaries, our data suggest that ERβ is expressed at higher levels.

E2 Signals through ERβ to Down-Regulate BCRP. To determine through which ER E2 signaled to BCRP in brain capillaries over 6 h, we first used agonists and antagonists for ERα and ERβ. Exposing isolated brain capillaries for 6 h to 1 nM PPT, an ERα agonist, did not alter BCRP expression or transport activity (Fig. 3A). Consistent with this, 100 nM MPP, an ERα antagonist, did not block E2-mediated BCRP down-regulation (Fig. 3B). In contrast, the ERβ agonist DPN (10 nM) decreased BCRP transport activity in isolated brain capillaries and expression of BCRP monomer and dimer in capillary membranes (Fig. 3C). Blocking ERβ with 1 μM ICI182,780 abolished E2-mediated down-regulation of BCRP protein expression and restored BCRP transport activity (Fig. 3D). Taken together, these data strongly suggest that E2 signaled BCRP down-regulation through ERβ but not ERα.

Experiments with brain capillaries isolated from male and female ERα and ERβ KO mice confirmed this conclusion.

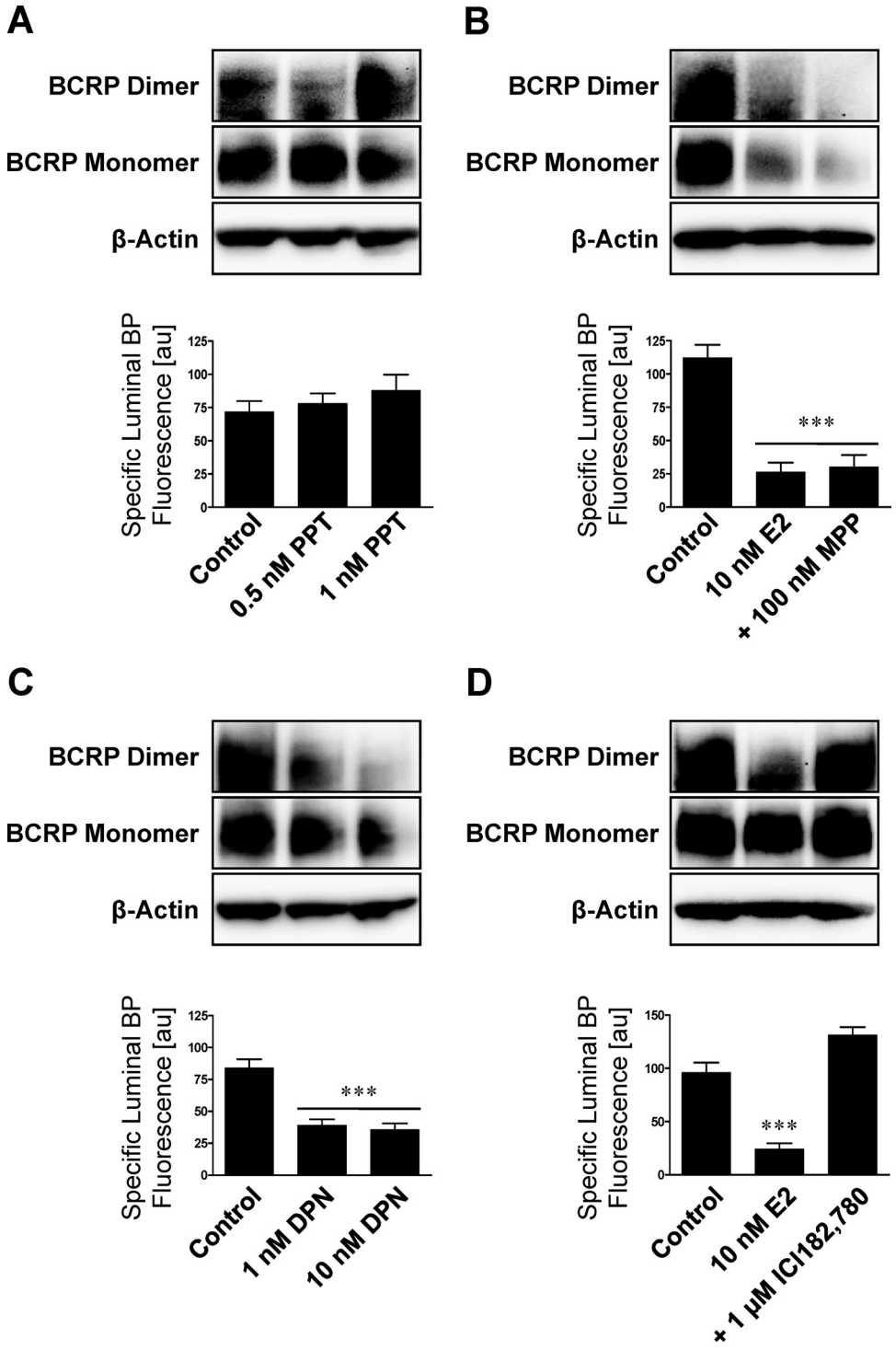

Fig. 3. E2 signals through ERβ to down-regulate BCRP protein expression and specific transport function. A, ERα activation with 1 nM PPT had no effect on BCRP expression and function within 6 h. B, 100 nM MPP, an ERα antagonist, did not prevent E2-mediated BCRP down-regulation. C, ERβ agonist DPN (10 nM) reduced BCRP expression and transport activity within 6 h. D, blocking ERβ with 1 μM ICI182,780 abolished E2-mediated down-regulation of BCRP. For specific luminal BODIPY FL prazosin (BP) fluorescence, each data point represents the mean ± S.E.M. for 10 capillaries from a single preparation (pooled tissue from 10 rats). Units are arbitrary fluorescence units (scale, 0–255). ***, $P < 0.001$, significantly lower than controls.

Note that previously we found no difference in BCRP protein expression and transport activity in brain capillary membranes from male and female rats (Hartz et al., 2010); transport assays and Western blots appear to confirm this finding for wild-type, ERα KO, and ERβ KO mice (Fig. 4). In addition, we found no male-female differences in responses to 6-h exposure to 10 nM E2 in capillaries isolated from wild-type, ERα KO, and ERβ KO mice. That is, E2 exposure reduced BCRP transport activity and protein expression in capillaries from male and female wild-type mice and male and female ERα KO mice (Fig. 4, A and C). Importantly, E2 exposure did not reduce BCRP transport activity and protein expression in capillaries from male and female ERβ KO mice (Fig. 4, B and D). Thus, in capillaries from male and female mice, signaling through ERβ, but not ERα, is essential for E2-mediated down-regulation of BCRP activity and expression.

E2 Signaling through PTEN/PI3K/Akt/GSK3 Activates Degradation of BCRP. In several tissues, estrogen signaling is linked to the PTEN/PI3K/Akt/GSK3 pathway (Hisamoto et al., 2001; Guzeloglu-Kayisli et al., 2003; Choi et al., 2004; Varea et al., 2009). This also appears to be the case in rat brain capillaries. We first conducted experiments with the potent PI3K inhibitor LY294002 and the Akt inhibitor triciribine that by themselves reduced BCRP transport activity and protein expression (Fig. 5A). Further experiments demonstrated that the PTEN inhibitor bpV(HOpic) and the GSK3 inhibitor XIII reversed the E2 effect and restored BCRP protein expression and transport activity (Fig. 5, B and C). To confirm involvement of this pathway, we assayed phosphorylation of PTEN, a negative, intracellular regulator of Akt and found that 10 nM E2 exposure shifted band intensity from inactive, phosphorylated PTEN to active PTEN (Fig. 5D). Consistent with E2-mediated activation of PTEN, E2 decreased the level of active, phosphorylated Akt and increased the level of inactive Akt (Fig. 5E), and it slightly increased the level of active, phosphorylated GSK3-α

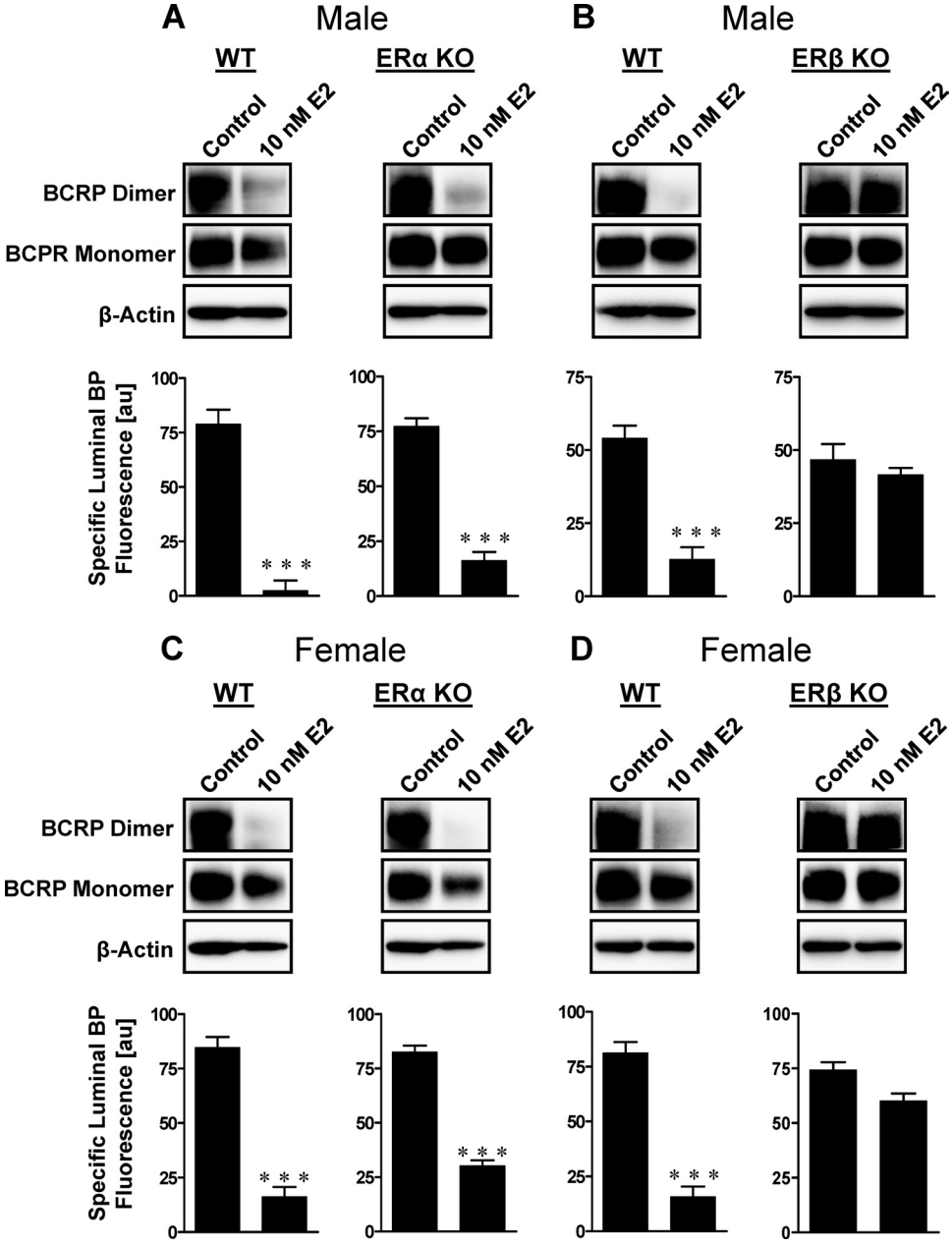

Fig. 4. E2 down-regulates BCRP in isolated brain capillaries from ERα knockout mice but not ERβ knockout mice. A, E2 decreased BCRP expression and specific transport activity in capillaries isolated from male wild-type and ERα knockout mice. B, E2 had no effect in male ERβ knockout mice. C, in isolated capillaries from female wild-type and ERα knockout mice E2 also down-regulated BCRP. D, as in isolated brain capillaries from male mice, E2 did not down-regulate BCRP protein expression or transport activity in capillaries from female ERβ knockout mice. For specific luminal BODIPY FL prazosin (BP) fluorescence, each data point represents the mean ± S.E.M. for 15 capillaries from a single preparation (pooled tissue from 20 mice per group). Units are arbitrary fluorescence units (scale, 0–255). Statistical comparison: ***, $P < 0.001$, significantly lower than controls.

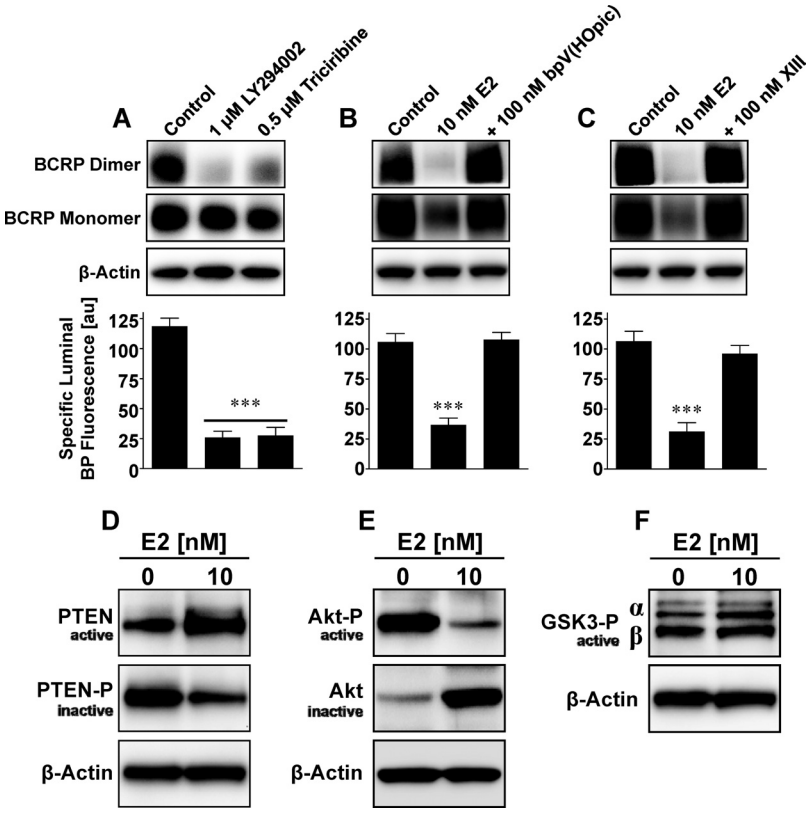

Fig. 5. E2 signals BCRP down-regulation through PTEN/PI3K/Akt/GSK3. A, PI3K inhibitor LY294002 and the Akt inhibitor triciribine reduced BCRP specific transport activity and BCRP dimer expression in isolated rat brain capillaries. B and C, PTEN inhibitor bpV(HOpic) and the GSK3 inhibitor XIII abolish the E2 effect on BCRP and restore monomer and dimer expression as well as transporter function. D, 6-h exposure of brain capillaries to 10 nM E2 caused a shift from inactive, phosphorylated PTEN to active PTEN. E, E2-mediated activation of PTEN increased the level of inactive Akt and decreased the level of active, phosphorylated Akt. F, E2 also increased the level of active, phosphorylated GSK3-α and GSK3-β. For specific luminal BODIPY FL prazosin (BP) fluorescence, each data point represents the mean ± S.E.M. for 10 capillaries from a single preparation (pooled tissue from 10 rats). Units are arbitrary fluorescence units (scale, 0–255). ***, $P < 0.001$, significantly lower than controls.

and GSK3-β (Fig. 5F). Finally, exposing capillaries to the proteasome inhibitor, lactacystin, abolished E2-mediated down-regulation of BCRP transport activity and dimer expression (Fig. 6). This latter result suggests that BCRP was internalized from the membrane and directed to the proteasome for degradation.

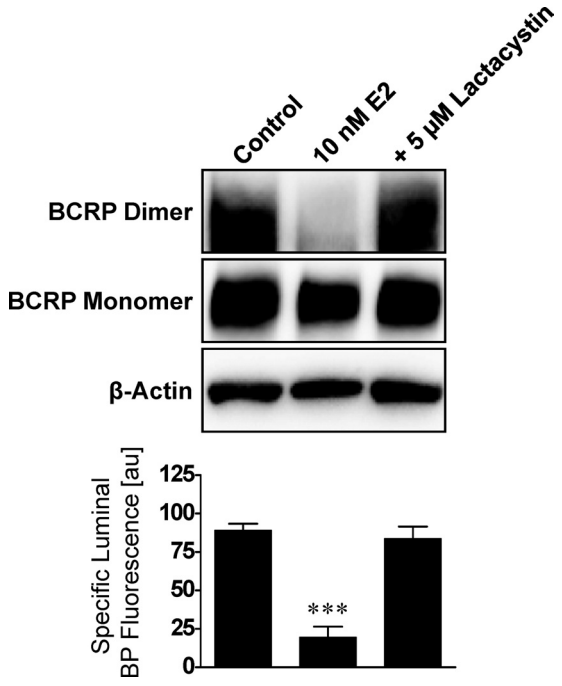

Fig. 6. E2-mediated BCRP down-regulation involves proteasomal degradation. The proteasome inhibitor lactacystin abolished E2-mediated down-regulation of BCRP-specific transport activity and dimer expression. For specific luminal BODIPY FL prazosin (BP) fluorescence, each data point represents the mean ± S.E.M. for 10 capillaries from a single preparation (pooled tissue from 10 rats). Units are arbitrary fluorescence units (scale, 0–255). Statistical comparison: ***, $P < 0.001$, significantly lower than controls.

In Vivo Effect of E2 on Blood-Brain Barrier BCRP. To determine whether E2 exposure in vivo also reduced BCRP expression, we gave mice a single intraperitoneal dose of 0.1 mg/kg E2 and measured E2 plasma levels, BCRP protein expression, and transport activity in isolated brain capillaries after 1, 6, and 24 h. One hour after dosing, E2 plasma levels were significantly increased (125 ± 37 pg/ml; Fig. 7). At 6 and 24 h after E2 dosing, plasma levels were similar to those observed in vehicle-treated control mice (6 h, 13 ± 6 pg/ml; 24 h, 1.7 ± 0.3 pg/ml; vehicle-treated control mice, 1.7–4.8 pg/ml). In brain capillaries isolated from E2-dosed animals, we found decreased BCRP transport activity at all

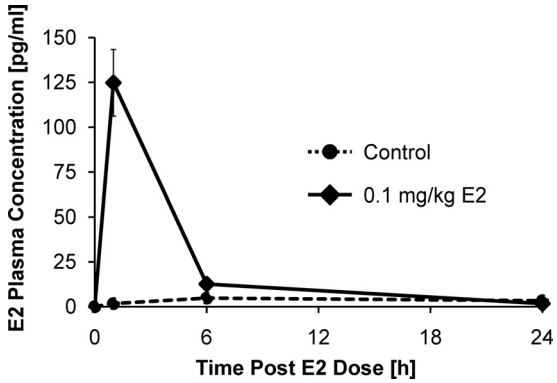

Fig. 7. Time course of changes in plasma E2 in mice after a single dose of 0.1 mg/kg E2 given by intraperitoneal injection. Data are given as mean ± S.E.M. ($n = 20$ mice per group; plasma from each mouse assayed at each time).

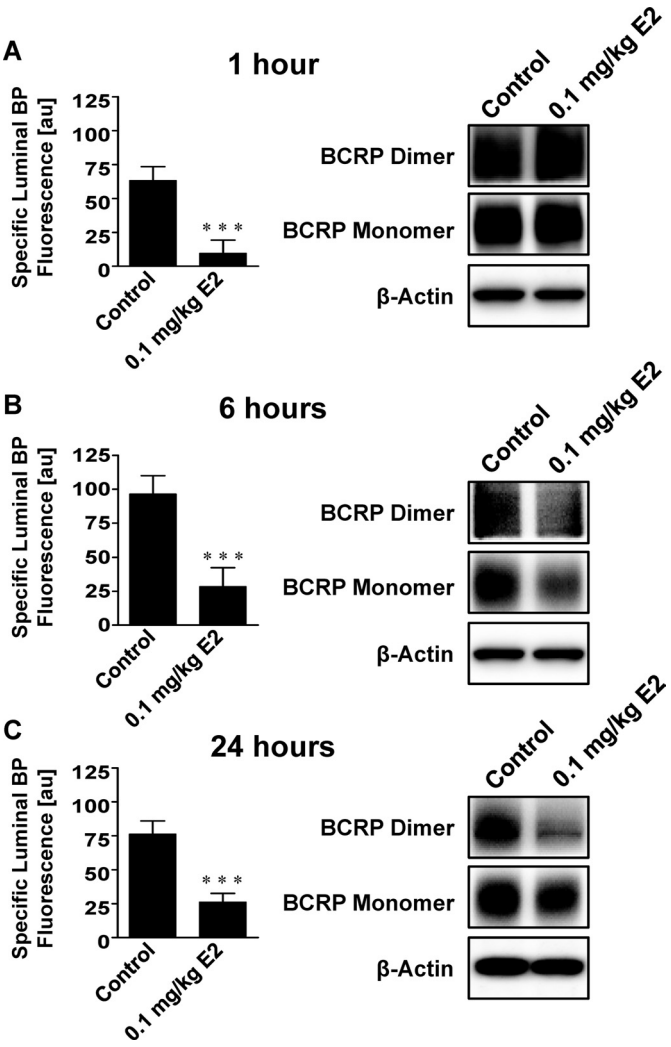

Fig. 8. In vivo time course of BCRP protein expression and transport activity in brain capillaries. A to C, dosing mice with 0.1 mg/kg E2 reduced BCRP transport activity in brain capillaries after 1, 6, and 24 h. BCRP protein expression was reduced 6 and 24 h after E2 dosing. For specific luminal BODIPY FL prazosin (BP) fluorescence, each data point represents the mean ± S.E.M. for 10 capillaries from a single preparation (pooled tissue from 10 rats). Units are arbitrary fluorescence units (scale, 0–255). ***, $P < 0.001$, significantly lower than controls.

time points and reduced BCRP dimer expression 6 and 24 h after E2 dosing (Fig. 8). It is important to note that these in vivo findings mirror the essential elements of the in vitro time course shown in Fig. 1.

Discussion

We recently reported that low nanomolar concentrations of E2 acting through ERα and ERβ rapidly (minutes) reduce BCRP transport activity in isolated brain capillaries and that BCRP protein expression is not altered by E2 exposures up to 1 h (Hartz et al., 2010; Fig. 9A). The present combined in vitro/in vivo study confirms and extends those findings. We show that E2-induced loss of BCRP transport activity was sustained for at least 6 h in vitro and for 24 h in vivo. At those longer exposure times, BCRP protein expression (both monomer and dimer) was also reduced. Experiments with selective pharmacological tools and ERα KO and ERβ KO mice showed that sustained loss of BCRP transport activity and reduction

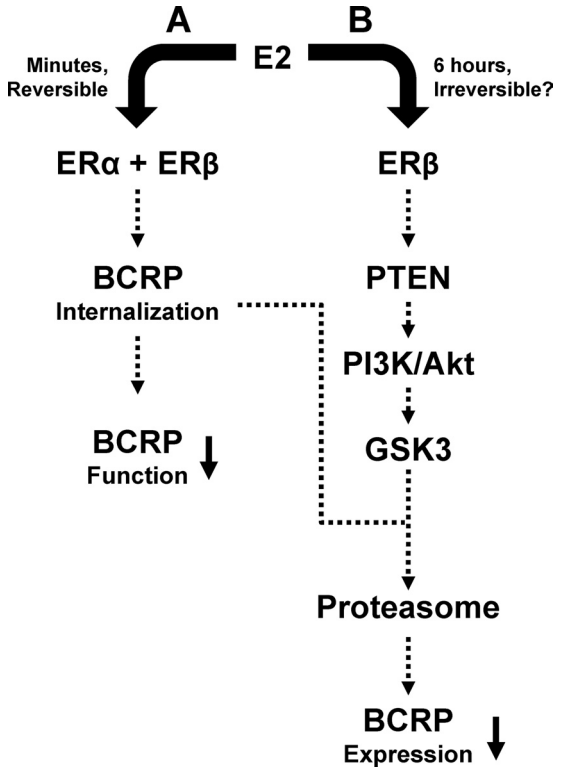

Fig. 9. Proposed signaling pathways through which E2 reduces BCRP transport activity and protein expression in brain capillaries. Pathway A was characterized previously (Hartz et al., 2010). Pathway B is described in the present study. We speculate that the two pathways are linked in that transporter internalization must precede proteasomal degradation. It remains to be determined whether PTEN/PI3K/Akt/GSK3 signaling directs internalized BCRP to the proteasome or whether it stimulates ubiquitination and processing of the protein.

in BCRP protein expression were signaled through ERβ, PTEN activation, PI3K/Akt inactivation, and GSK3-α and GSK3-β activation (Fig. 9B). Decreased BCRP expression probably reflected increased proteasomal degradation of the transporter protein. Thus, E2 acting though either ER can signal the initial loss of BCRP activity (Hartz et al., 2010), but only signaling through ERβ leads to reduced BCRP protein expression.

Our previous study implied that both ERα and ERβ are expressed in rat brain capillaries (Hartz et al., 2010). Consistent with this, we show here that rat brain capillaries contain mRNA and protein for both estrogen receptors. However, ERβ expression appeared to be substantially higher than that of ERα. We also found ERβ protein expression in total brain tissue but could not detect ERα protein. These observations agree with previous studies demonstrating that ERβ is the prominent estrogen receptor in the CNS, whereas ERα protein expression in the brain is scattered, region-dependent, and only found in discrete subcellular compartments (Mitra et al., 2003).

Initially, it was thought that estrogen receptors reside only within the cytosol and nucleus (Truss and Beato, 1993). However, it is now clear that estrogen receptors can also be associated with the plasma membrane, where they can initiate rapid estrogen-induced signaling that does not involve transcription (Simoncini and Genazzani, 2003). We previously demonstrated such rapid signaling to BCRP in brain capillaries (Hartz et al., 2010). It is likely that the sustained

E2/ERβ signaling documented in the present study also does not involve transcription, because BCRP degradation is the result. Note that in the present study we found two intense bands for ERβ protein in brain capillary lysate but only a marginal signal in brain capillary membranes. This observation and our immunostaining of ERβ in capillaries suggest a primarily submembranous localization of the receptor.

The present experiments with ER agonists and antagonists and ERα KO and ERβ KO mice clearly show that E2 signaling through ERβ caused the reduction in BCRP protein expression. Previous studies imply that the effects of E2 on BCRP expression are tissue-specific. In several human breast cancer cell lines, E2 exposure decreases BCRP protein expression and function, but it does this by acting through ERα not ERβ (Imai et al., 2005). However, E2 has also been reported to increase BCRP protein expression in a human breast cancer cell line by signaling through ERα (Zhang et al., 2006). In a human placenta cell line, E2 signaled through ERβ to up-regulate BCRP (Wang et al., 2008); and in mouse, BCRP and ERβ mRNA levels are positively correlated in placenta, whereas BCRP and ERα mRNA levels are positively correlated in liver (Wang et al., 2006). Thus, both ERα and ERβ can be involved in E2 regulation of BCRP, but the signals involved and the effect on BCRP (up- or down-regulation) seem to be tissue-specific.

Figure 9 shows the proposed signaling pathway through which E2 down-regulates BCRP in brain capillaries. Key to the pathway is ERβ activation of PTEN, which in turn inactivates PI3K/Akt leading to activation of GSK3-α and GSK3-β. PTEN is a tumor suppressor that blocks PI3K-mediated phosphorylation of Akt, inhibiting activation of Akt (Cantley and Neel, 1999). A recent study by Bleau et al. (2009) demonstrated that PTEN/PI3K/Akt signaling regulates BCRP activity in mouse and human gliomas. The authors found that signaling impaired BCRP function in glioma endothelial cells, corresponding to a disruption of blood-brain barrier integrity in the tumor. In contrast, in nonendothelial tumor cells that formed neurospheres and were highly tumorigenic, loss of PTEN elevated Akt activity and increased BCRP trafficking to the membrane as well as BCRP-mediated transport (Bleau et al., 2009). This latter result is particularly relevant to the present study, because we found that E2 signaling through ERβ activated PTEN, inactivated Akt, and activated GSK3-α/β, causing loss of BCRP transport activity and protein expression. In this regard, phosphorylation of proteins by the active form of GSK3 that we detected in E2-treated brain capillaries is an early event in the sequence of events that sends proteins to the proteasome for degradation (Oh et al., 2009).

Indeed, the present data suggest that sustained E2 signaling reduces BCRP activity expression through degradation of the transporter at the proteasome. This process, however, requires internalization of BCRP and transporter trafficking away from the plasma membrane before the transport protein is degraded at the proteasome. There is evidence in the literature for both elements of this proposed mechanism. First, signal-dependent internalization of ABC transporters has been demonstrated previously for ABC drug efflux transporters such as P-glycoprotein, multidrug resistance-associated protein 2, and bile salt export pump (Kipp and Arias, 2000). In this regard, we have previously suggested P-glycoprotein internalization and reduction of transporter functional activity in rat brain capillaries in response to tumor necrosis factor-α- and endothelin-1, and this has recently also been suggested for vascular endothelial growth factor- and protein kinase C-induced down-regulation of P-glycoprotein activity in rat brain capillaries (Hartz et al., 2004, 2006; Hawkins et al., 2010, Rigor et al., 2010). Second, Nakanishi et al. (2006) found that the proteasome is involved in the posttranslational regulation of BCRP, and our data support this conclusion. Clearly, additional experiments are required to elucidate the mechanism of BCRP internalization and its trafficking to the proteasome.

Finally, we show here that one-time dosing of mice with E2 transiently and substantially increased plasma E2 levels. This was accompanied first by a loss of BCRP transport activity in brain capillaries (within 1 h) and then by a loss of both BCRP transport activity and transporter expression 6 to 24 h after dosing. It is currently not known how long this E2 effect on BCRP lasts, but BCRP monomer protein expression seemed to have recovered 24 h after E2 dosing. Note that these events after E2 dosing closely recapitulated the time course of E2 action in isolated brain capillaries, that is, loss of transporter activity after 1 h and loss of activity and expression after 6 h. Our results suggest a therapeutic strategy in which ER-based signaling would be used to reduce BCRP transport activity and increase brain accumulation of chemotherapeutics that are BCRP substrates. Certainly, at this stage, we need to know more about E2 action on blood-brain barrier BCRP in vivo, including, the detailed time course of BCRP loss and recovery and E2 dose response. In addition, it remains to be shown whether E2 treatment could also be used to down-regulate BCRP in brain tumor cells and brain cancer stem cells.

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