Estrogen is important for the primary prevention of vascular disease in young women, but the mechanisms of protection at the vascular cell are still largely unknown. Although traditionally thought of as a nuclear transcription factor, the estrogen receptor has also been identified in the cell plasma membrane to signal but serve largely undefined roles. Here we show that estradiol (E2) rapidly activates p38β mitogen-activated protein kinase in endothelial cells (EC), which activates the mitogen-activated protein kinase-activated protein kinase-2 and the phosphorylation of heat shock protein 27. The sex steroid preserves the EC stress fiber formation and actin and membrane integrity in the setting of metabolic insult. E2 also prevents hypoxia-induced apoptosis and induces both the migration of EC and the formation of primitive capillary tubes. These effects are reversed by the inhibition of p38β, by the expression of a dominant-negative mitogen-activated protein kinase-activated protein kinase-2 protein, or by the expression of a phosphorylation site mutant heat shock protein 27. E2 signaling from the membrane helps preserve the EC structure and function, defining potentially important vascular-protective effects of this sex steroid.

E2 has been reported to serve a significant function in the vasculature to prevent the primary development of cardiovascular disease in women (1). This occurs through multiple potential and described mechanisms leading to the modulation of vascular cell function and blood vessel tone and remodeling (2). The known mechanisms that underlie the preservation of vascular function include E2 inducing a favorable lipid profile, serving as an anti-oxidant, inhibiting the synthesis of pro-thrombotic proteins, and stimulating nitric oxide generation. However, the cell-based actions of E2, which lead to the preservation of the structure and function of important vascular cells, such as endothelial cells (EC), are not well understood. These actions are important because it has recently been appreciated that endothelial cell dysfunction underlies many acute and chronic vascular diseases (3).

Steroid hormones have traditionally been thought to act exclusively by binding to nuclear receptors, which then trans-activate target genes (4). Recent evidence has additionally supported rapid, non-genomic actions of several steroids (5, 6). Moreover, the estrogen receptor (ER) has also been identified in the cell plasma membrane (7), serving largely undefined roles. Some effects of E2 appear to originate from the cell membrane, probably through binding plasma membrane ER that activates downstream signaling (8–11). However, the range of signal transduction pathways activated by the membrane ER has not been defined, and the potential roles of these pathways to mediate important E2 actions in target cells are largely unknown. Here, we investigated potential roles of the ER in the function of the EC. We report the novel observation that E2 induces the activation of the p38β member of the mitogen-activated protein kinase family, leading to the activation of the serine/threonine kinase, MAPKAP-2 kinase, and the phosphorylation of heat shock protein 27. This signal transduction pathway in the EC substantially underlies the ability of E2 to preserve the actin cytoarchitecture during metabolic stress, rescue EC from hypoxia-induced apoptosis, and induce the migration of EC leading to tube formation. These findings implicate the membrane ER in the vascular cell protective actions of this steroid.

EXPERIMENTAL PROCEDURES

Materials—A p38β dominant-negative adenoviral vector was obtained from Jiahuai Han (Scripps), a dominant-negative MAPKAP-2 kinase expression plasmid was from Matthias Gaestel (Max Delbuck Center for Molecular Medicine, Berlin, Germany), and a triple serine mutant, non-phosphorylatable HSP27 expression vector was a gift from Lee Weber (University of Nevada).

Vascular Endothelial Cultures—Bovine aortic EC cultures were prepared as described previously (12) and plated as primary cultures in phenol red-free medium and in serum stripped with charcoal to remove steroids.

p38 and MAPKAP-2 Kinase Studies—EC were synchronized in the absence of serum for 24 h. For p38 or MAPKAP-2 kinase activity assays, the cells were incubated with E2 (10 nM) for 10 min before preliminary time course studies. The cells were lysed, and the lysate was immunoprecipitated with protein A-Sepharose conjugated to antiseraum for p38 or MAPKAP-2 kinase. Immunoprecipitated kinases were washed and then added to the proteins ATP-2 (for p38) or HSP27 (for MAPKAP-2) for in vitro kinase assays, similarly as described previously (12). This procedure was followed by SDS-polyacrylamide gel electrophoresis separation and autoradiography/laser densitometry.

Transient Transfections—BAEC (passages 4–5) were grown to 40–50% confluence and then transiently transfected with 1.5–10 μg of fusion plasmids depending upon the plate size and the amount of cells. Plasmids for transfection included dominant-negative MAPKAP-2 kinase (MK2-R76K) (13), control plasmids pKS and pcDNA3, or a phosphorylation mutant HSP27 (pS2711–3G) (14). Cells were incubated with LipofectAMINE liposome-DNA complexes at 37 °C for 5 h followed by overnight recovery in Dulbecco’s modified Eagle’s medium contain-
ing 10% fetal bovine serum. The cells were synchronized for 24 h in serum-free Dulbecco's modified Eagle's medium and then experimentally treated. For the p38β dominant-negative, a recombinant adenovirus expressing this construct (p38TY) (15) was added to subconfluent EC in Dulbecco's modified Eagle's medium with 2% serum, and infection occurred at a multiplicity of 50–100 particles/cell for 12 h. The cells were then cultured in serum-free medium for 36 h before experimentation.

Cytoskeletal Actin—Non-transfected or transfected EC were grown to confluence on poly-D-lysine-coated glass coverslips and then exposed to

FIG. 1. A, 17-β-E2 or E2-BSA inhibits p38α but stimulates p38β activity in EC. EC were incubated with steroid for 10 min, and p38α (left) and p38β (right) were immunoprecipitated from the lysed cells and then used for in vitro assay of kinase activities directed against the ATF-2 substrate protein. Equal amounts of p38 protein are shown by the Western blot below the kinase activity. B, E2 or E2-BSA activates MAPKAP-2 kinase activity via p38β. SB203580 (SB) or infection of EC with a p38β dominant-negative (dn) adenoviral vector inhibited kinase activity. The bar graphs represent three experiments combined. *, p < 0.05 by analysis of variance plus Scheffe's test for control versus condition; †, p < 0.05 for 17-β-E2 versus steroid plus ICI 182780 (ICI); left and 0.05 for 17-β-E2 versus 17-β-E2 plus SB203580 or dominant-negative p38β (p38TY) (right). E2 or E2-BSA stimulates the phosphorylation of endogenous HSP27 via p38α (C) and MAPKAP-2 (D) kinase. EC were either labeled with [32P]orthophosphoric acid and then exposed to SB203580 or first transfected with a dominant-negative MAPKAP-2 kinase (MK2-R76K) followed by E2. *, p < 0.05 by analysis of variance plus Scheffe's test for control versus condition; †, p < 0.05 for 17-β-E2 versus 17-β-E2 plus SB203580 or dominant-negative MAPKAP-2 kinase.
20 μM CCCP, an uncoupler of oxidative phosphorylation in the presence of 40 ng/ml glucose for 2 h. Some cells were exposed to concomitant E2 (10 nM) or E2-BSA (100 nM). The cells were permeabilized, washed, and then stained with fluorescent-labeled phallolidin (Molecular Probes). Actin distribution was examined under a Nikon epifluorescent microscope.

**Endothelial Cell Migration Assay**—Non-transfected or transfected EC were grown to confluence on 6-well plates in Dulbecco’s modified Eagle’s medium with 10% serum. The cells were synchronized for 24 h in the absence of serum, and a wound was created by scraping the monolayer with a single-edge razor blade. Selected reagents were added to the wounded BAEC for 24 h at 37 °C. The cells were then fixed in 3.7% formaldehyde and assessed for migration. BAEC migration was measured using an image analyzer system composed of an inverted microscope and a 20–24-inch digitizing board (Jandel Scientific, Corte Madera, CA) attached to an IBM computer. The Sigma Scan program (Jandel) was used for analysis of measurements of the distance traveled by the cells within the calibrated area adjacent to the wound. Five measurements/well were taken, and the results from three separate experiments contributed to the bar graph (see Fig. 4). Apoptosis Studies—Non-transfected or transfected EC were placed into an anaerobic chamber for 24 h (Gas Pack System, Becton-Dickinson), which was purged with 95% N2, 5% CO2 and sealed with an oxygen-consuming palladium catalyst. This created hypoxic conditions of 35 mm Hg PO2. Some cells were exposed to 10 nM E2 or 100 nM E2-BSA. The control cells were subjected to normoxia (atmospheric air/5% CO2, 150 mm Hg PO2) in the absence of E2. Apoptosis was assessed by TUNEL staining, and the cells undergoing programmed cell death were counted in five separate fields/experiment. Data from three experiments constitute the bar graph (see Fig. 3).

**Tube Formation**—Human dermal microvascular EC were plated on growth factor-reduced Matrigel in the presence or absence of E2 and maintained for 6 h at 37 °C. The cells were fixed at 6 h (maximum tube formation), stained with Diff-Quik, and photographed and assessed at ×10 magnification using phase microscopy. Five random fields/condition were examined, the number of cords/tubes was counted in each, and the mean values were determined. The experiment was repeated twice additionally.

**RESULTS**

**E2 Induces Rapid Activation of p38β, MAPKAP-2, and Phospho-HSP27**—Endothelial cells have been previously demonstrated to contain ER (16). We first determined that in the primary cultures of bovine aortic endothelial cells, E2 stimulated the activity of the mitogen-activated protein kinase family member, p38β (Fig. 1A, right) while inhibiting p38α activity (Fig. 1A, left) maximally at 10-min incubation. This finding indicates a unique differential regulation of the isoforms of this kinase. E2 effects were significant at 1 nM and were substantially reversed by the specific ER antagonist, ICI 182,780. No effects were seen with 10 nM of the relatively inactive stereoisomer 17α-E2 or with testosterone, but a cell-impermeable membrane ER ligand, E2-BSA, affected the activity of the two isoforms of p38 comparable to E2. E2-BSA has been shown in several studies to neither enter the cell nor bind and/or activate the nuclear ER (10, 17, 18). These data implicate a membrane ER in the modulation of signaling, mainly based upon the rapid and specific effects of E2 but also based upon the lack of precedence for any nuclear receptor to rapidly signal, as well as the comparable effects of E2-BSA and E2.

Little is known in general about the cell biologic importance of p38β except that it might participate in cardiac hypertrophy (19). However, a known immediate downstream target of p38 is the serine/threonine kinase MAPKAP-2 (20). We found that E2 or E2-BSA was each capable of stimulating the activity of this kinase in EC directed against a substrate protein, exogenous heat shock protein 27 (Fig. 1B). The stimulation of MAPKAP-2 kinase activity was reversed by the soluble and specific inhibitor of p38 activity, SB203580 (21), or by infecting the EC with an adenoviral vector expressing a dominant-negative mutant p38β protein (15). The results indicate that the β isoform of p38 mediates the actions of E2 to phosphorylate exogenous HSP27.

We then sought to link this pathway to endogenous HSP27 phosphorylation, a known substrate for MAPKAP-2 kinase. E2 or E2-BSA stimulated the phosphorylation of endogenous HSP27, and this was reversed by SB203580 (Fig. 1C). Transiently transfecting and expressing a dominant-negative MAPKAP-2 protein MK2-R76K (13) also abrogated the phosphorylation of HSP27 in response to the steroid (Fig. 1D). This process identified a novel and rapid signal pathway for estrogen, potentially contributing to the steroid cellular actions.

**E2 Signals to the Preservation of the Actin Cytoskeleton**—HSP27 is phosphorylated by MAPKAP-2 at three serine residues at positions 15, 78, and 82 (22). HSP27 acts as a capping protein for the barbed ends of actin in the unphosphorylated state (23) but is believed to play a role as a phosphoprotein in stabilizing F-actin and allowing the polymerization of this myofilament (24). In quiescent EC, a predominantly cortical pattern of F-actin localization was observed (Fig. 2A, left). Upon exposure to E2, the actin filaments were localized to stress fibers and focal adhesions (Fig. 2B, left). We exposed the EC to an uncoupler of oxidative phosphorylation, CCCP (20 μM), in the pres-
ence of low glucose; this metabolic stress simulates an ischemic insult (25). The stress led to the distortion of the actin cytoskeleton, the severing of actin, and a severely disrupted stress fiber and focal adhesion formation (Fig. 2C, left). Incubation of the cells with E2 strongly preserved the cell membrane integrity and stress fiber/focal adhesion localization of the actin (Fig. 2D, left). However, treatment of the cells with SB203580 or expression of the dominant-negative MAPKAP-2 protein substantially prevented the effects of E2 (Fig. 2A, E, and F, left). Similarly, the expression of a triple serine mutant HSP27 that is unable to be phosphorylated reversed the ability of E2 to preserve cytoskeletal integrity in the stressed EC (Fig. 2G, left). Similar results were seen when the EC were exposed to E2-BSA (Fig. 2H, right). The stable and localized expression of actin to stress fibers is necessary to preserve cell morphology and function and prevent EC dysfunction, which is strongly implicated in the pathogenesis of vascular disease (3). This expression identifies a potentially important cell biologic effect of E2.

E2 Prevents EC Cell Death Resulting from Hypoxia—We then explored other cell biologic effects of E2 in this context. EC become dysfunctional or undergo cell death after acute/chronic ischemia or hypoxia. We subjected the EC to 24 h of hypoxia in a chamber containing 1% O_2. This resulted in substantial apoptotic cell death as shown by TUNEL staining (Fig. 3, b versus control a). E2 was capable of rescuing the cells, preventing 64% of the hypoxia-induced apoptosis (Fig. 3c), but this was substantially reversed by inhibiting p38 or MAPKAP-2 kinase activation (Fig. 3, d and e) or by expressing the non-phosphorylatable HSP27 (Fig. 3f). Similar protection was afforded by the E2-BSA ligand (data not shown). Thus, this pathway serves an important function for cell survival.

E2 Stimulates EC Migration and Tube Formation—The migration of vascular cells is critical to the process of blood vessel remodeling. For instance, the migration of a subpopulation of endothelial cells is an important step in the process of angiogenesis. We determined whether E2 could promote EC migration via a signaling-related mechanism. E2 clearly promoted the migration of EC across a wound barrier (Fig. 4, A versus B and bar graph) and was not capable of stimulating EC proliferation in 24 h, which supported migration as the mechanism. The effect of E2 was prevented by 85% from the incubation of cells with SB203580 (Fig. 4C) and was substantially reversed by the expression of MAPKAP-2 dominant-negative protein.
Membrane Estrogen Receptors

FIG. 4. E2 induces the migration of EC that is prevented by the inhibition of the activity of p38 and MAPKAP-2 kinase and that is expressing a non-phosphorylatable HSP27 protein. Top, non-transfected or transfected BAEC were cultured on agar and were wounded by a razor blade. Migration across the wound was determined transfected or transfected BAEC were cultured on agar and were expressing a non-phosphorylatable HSP27 protein.

Steroids traditionally are thought to exclusively act in the cell as nuclear transcription factors, modulating target genes through complex interactions of steroid receptors, co-activators or co-repressor proteins, histone acetylase, and proteins comprising the basal transcriptional machinery (27). However, a functional plasma membrane ER was identified more than 20 years ago (7), and additional evidence has supported its existence and suggested a potential role in the biology of estrogen action (8–10). As a plasma cell membrane receptor, ER might be expected to modulate signal transduction, and several pathways have recently been identified as being activated by E2 (9–11, 28). Here, we report novel findings that E2 can activate a signal pathway that leads to the preservation of function and form of vascular endothelial cells (Fig. 6). These results define several mechanisms by which E2 can protect against EC dysfunction and resulting vascular disease (3).

We first found that E2 or E2-BSA activated the p38β isoform but inhibited p38α at 10 min. A specific ER antagonist reversed these effects. We, therefore, propose that a membrane ER rapidly regulates these kinases. Furthermore, mitogen-activated protein kinases localize to the plasma membrane where they are activated by upstream signal transduction pathways, which originate at this location (29). Activation of the p38β member of the mitogen-activated protein kinase family has not been reported for any steroid, and the differential modulation of p38α and p38β isoforms by any substance has not been previously reported. The p38 family consists of four isoforms (α, β, δ, γ), and the precise cellular roles of each are incompletely understood. p38α has been shown to participate in the induction of apoptosis (30, 31), whereas p38β is known to participate in cardiac hypertrophy (19) and mediates the transcriptional regulation of Bcl-2 and anti-apoptosis (32). Interestingly, we found that estrogen inhibits p38α in the EC. Taken together, modulation of these two isoforms of p38 may contribute to anti-apoptosis in the EC (see below).

We determined that E2 or E2-BSA comparably activated p38β and the downstream signaling and endothelial cell biology. Several laboratories (17, 33), in addition to our own (9, 10), have provided evidence that E2-BSA does not enter the cell to bind the nuclear ER or dissociate into E2 and BSA components as implicated in a single publication (34). Also, considering 1) the rapidity of E2 (or E2-BSA) effects, 2) that a specific ER antagonist can prevent the signaling by the steroid compounds actions, and 3) the fact that there is no precedent for nuclear receptors to rapidly signal, we therefore believe that these non-genomic effects of E2 are mediated at the membrane ER.

The activation of p38 by E2 then led to the activation of the MAPKAP-2 kinase, a member of a serine/threonine family of related kinases that is stimulated by a variety of cell stressors...
and growth factors. MAPKAP-2 kinase mediates the direct phosphorylation of transcription factors (cAMP-response element-binding protein, serum response factor) (35, 36) or acts indirectly through downstream kinases, such as MSK-1 (37). In our studies, E2 induced the phosphorylation of endogenous HSP27, and this required MAPKAP-2 kinase because the expression of a dominant-negative MAPKAP-2 significantly prevented HSP27 phosphorylation. Expression of this dominant-negative MAPKAP-2 construct has been previously shown to prevent the activation of this kinase (13), and we confirmed this in response to E2 or E2-BSA (data not shown).

What is the importance of the ability of estrogen to trigger this signaling pathway? The integrity of F-actin is necessary for the physiological functions of EC, such as creating a selective vascular permeability barrier to proteins (38, 39). Disruption of the actin cytoskeleton occurs after ischemia or other stresses and results in tissue edema formation or vascular thrombosis (40, 41). This disruption is because of ATP depletion during ischemic stress, a metabolic condition that we have simulated here with low glucose and an inhibitor of oxidative phosphorylation (25). Upon subjecting the EC to this stress, the F-actin integrity and localization were markedly disrupted. E2 or E2-BSA was capable of preventing this disruption through signaling to HSP27 phosphorylation. In this respect, EC survival is also related to our demonstrated effects of E2. HSP27 is a known survival factor for a variety of cells (42, 43) perhaps through helping to maintain cytoskeletal integrity, thus preventing apoptosis in response to several stresses in various cell types (44, 45).

ATP depletion results in cellular calcium overload, which can activate gelsolin, an actin-severing protein (46). It is unknown but possible that phosphorylated HSP27 can prevent gelsolin or related protein activation in this setting. Phosphorylated HSP27 also promotes EC migration that is (47) in part related to the ability of the phosphoprotein to allow F-actin polymeri-
zation (23), which is critical for the extension of cell processes, such as lamellodia (48). Cell migration is dependent on a complex series of events that include active signaling from components of the focal adhesion complex, such as Src family kinases and focal adhesion kinase (49). It has recently been shown that E2 can activate Src in MCF-7 cells (10, 50). Proximal signaling to the activation of the focal adhesion complex, possible activation of the small GTPases (Rac and Rho), and the activation of the p38-mediated signaling pathway described here may be linked or may occur in parallel in response to E2.

Cell migration is one important step in the process of angiogenesis. An excellent example of hormone-entrained neovascularization/regression of blood vessels occurs in the uterus during the menstrual cycle. Angiogenesis may result in part from the ability of E2 to stimulate local vascular endothelial growth factor production (51) and subsequent neovascularization. Here, we show a direct effect of E2 in stimulating primitive capillary tube formation, at least in part, through signaling to HSP27 posttranslational modification.

The actions of E2 to signal to cell biology through the membrane ER do not preclude important effects of the nuclear ER to transcribe genes that are critical to maintain EC function. For instance, E2 can transcriptionally activate the HSP27 gene through an Sp1-related mechanism (52). This indicates an important synergism where the nuclear ER promotes HSP27 protein synthesis, and the membrane ER probably triggers the rapid, function-regulating phosphorylation of the protein. In this way, acute and chronic modulation of HSP27 can be achieved by the potentially coordinated actions of distinct and compartmentalized pools of ER.

In total, these effects of E2 remarkably preserve EC form and function in the setting of several relevant in vitro stresses. This interaction may unfortunately extend to breast cancer where HSP27 expression and function correlate with the survival and invasiveness of tumors (53, 54), both of which are promoted by E2. This may be related to the dependence of the tumor vasculature on the survival and preservation of EC function that is important synergism where the nuclear ER promotes HSP27 posttranslational modification.

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