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Resveratrol and Estradiol Rapidly Activate MAPK Signaling through Estrogen Receptors α and β in Endothelial Cells*

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Vascular endothelial cells (EC) are an important target of estrogen action through both the classical genomic (*i.e.* nuclear-initiated) activities of estrogen receptors α and β (ERα and ERβ) and the rapid "nongenomic" (*i.e.* membrane-initiated) activation of ER that stimulates intracellular phosphorylation pathways. We tested the hypothesis that the red wine polyphenol *trans*-resveratrol activates MAPK signaling via rapid ER activation in bovine aortic EC, human umbilical vein EC, and human microvascular EC. We report that bovine aortic EC, human umbilical vein EC, and human microvascular EC express ERα and ERβ. We demonstrate that resveratrol and estradiol (E_2) rapidly activated MAPK in a MEK-1, Src, matrix metalloproteinase, and epidermal growth factor receptor-dependent manner. Importantly, resveratrol activated MAPK and endothelial nitric-oxide synthase (eNOS) at nM concentrations (*i.e.* an order of magnitude less than that required for ER genomic activity) and concentrations possibly achieved transiently in serum following oral red wine consumption. Co-treatment with ER antagonists ICI 182,780 or 4-hydroxytamoxifen blocked resveratrol- or E_2-induced MAPK and eNOS activation, indicating ER dependence. We demonstrate for the first time that ERα- and ERβ-selective agonists propylpyrazole triol and diarylpropionitrile, respectively, stimulate MAPK and eNOS activity. A red but not a white wine extract also activated MAPK, and activity was directly correlated with the resveratrol concentration. These data suggest that ER may play a role in the rapid effects of resveratrol in EC and that some of the atheroprotective effects of resveratrol may be mediated through rapid activation of ER signaling in EC.

Epidemiological studies have indicated that the consumption of red wine reduces the incidence of mortality from coronary heart disease (CHD)[1] (1, 2). The cardioprotective effect has been attributed to the polyphenol fraction of red wine (1). A key polyphenol in red wine is resveratrol, *trans*-3,5,4′-trihydroxystilbene, from grape skin. Red wine contains 1–75 mg of *trans*-resveratrol/liter (3). Studies in male rats demonstrated that an alcohol-free red wine extract and resveratrol protect the heart from ischemia reperfusion injury (4). Rodent studies showed that orally administered resveratrol is absorbed in the gut, has high affinity for heart and liver (5, 6), and is metabolized to glucuronides that have a $t_{1/2}$ of ~1.5 h (7). A recurrent question is whether resveratrol, at concentrations present in red wine, is effective *in vivo*. The oral absorption of 25 mg of *trans*-resvertrol/70 kg subject in white wine, grape juice, and vegetable juice was studied in healthy men (8). Peak serum resveratrol was 40 nM at ~30 min after consumption (8). High concentrations of resveratrol (*i.e.* 10–100 μM) induced endothelial nitric-oxide synthase (eNOS) gene expression in cultured endothelial cells (EC), suggesting that resveratrol may provide cardioprotection by increasing nitric oxide (NO) levels (9–11). Additionally, resveratrol acutely increased NO production in human umbilical vein endothelial cells (HUVEC) after a 2-min exposure (11). However, the mechanism for this acute activation is unknown.

Resveratrol binds and increases the transcriptional activity of estrogen receptors α and β (ERα and ERβ) at 50–100 μM (12, 13). Estrogens were long thought to have beneficial effects on cardiovascular parameters. However, the Women's Health Initiative prospective hormone replacement therapy trial was recently terminated because of increased CHD risk (relative risk = 1.29) in the hormone replacement therapy group (14) and pulmonary embolism in both the hormone replacement therapy (14) and estrogen replacement therapy groups; these findings raise questions regarding the role of estrogens in cardiovascular function and disease. It is noteworthy that estrogen replacement therapy did not alter CHD risk and that some clinicians believe hormone replacement therapy can be effective in the prevention of CHD (15). ERα gene polymorphisms are postulated to play a role in CHD and may be part of the reason for

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[1] The abbreviations used are: CHD, coronary heart disease; NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; ER, estrogen receptor; hER, human ER; rhER, recombinant hER; MMP, matrix metalloproteinase; siRNA, small interfering RNA; EGF, epidermal growth factor; EGF-R, EGF receptor; BAEC, bovine aortic endothelial cell; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; HMEC, human microvascular endothelial cell; WCE, whole cell extract; R,R-THC, *R,R*-5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol; PPT, 4,4′,4″-(4-propyl-c-pyrazole-1,3,5-triyl)tris-phenol (ERα agonist); DPN, 2,3-*bis*(4-hydroxyphenyl)-propionitrile (ERβ agonist); DN, dominant negative; ERK, extracellular signal-regulated kinase; IOD, integrated optical density; P, phospho; E_2, estradiol; HB, heparin-binding.

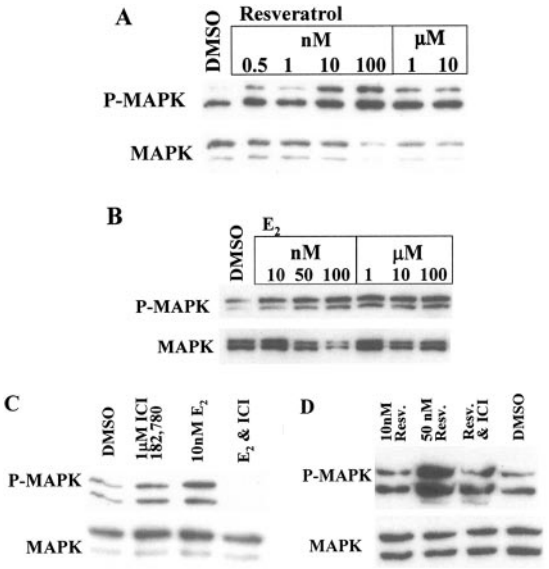

FIG. 1. **Nanomolar concentrations of resveratrol and E_2 rapidly increase MAPK phosphorylation in BAEC.** BAEC were treated with the indicated concentrations of the following for 20 min: *A*, *trans*-resveratrol or EtOH; *B*, E_2 or dimethyl sulfoxide (*DMSO*); *C*, 10 nM E_2; or *D*, 50 nM resveratrol (*Resv.*) ± 1 μM ICI 182,780 (*ICI*). Western blot analysis of P-MAPK and MAPK is described under "Experimental Procedures." These data are representative of at least three experiments.

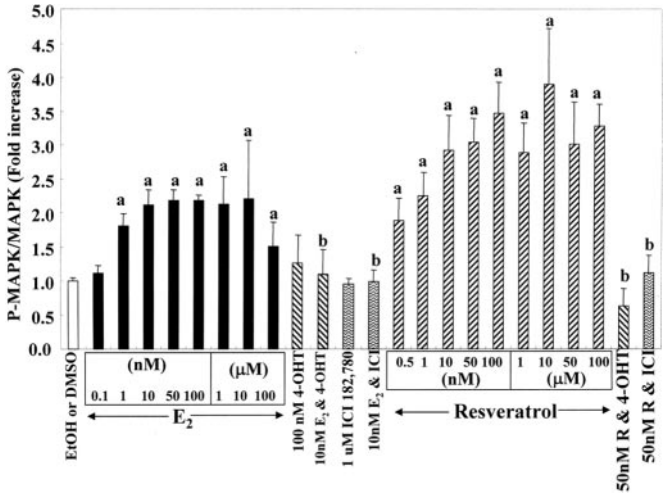

FIG. 2. **Resveratrol and E_2 rapidly activate MAPK via ER interaction.** Data were quantitated from Western blots of P-MAPK/MAPK in BAEC treated with the indicated concentrations of E_2, 4-hydroxytamoxifen (*4-OHT*), ICI 182,780, or resveratrol (*R*), alone or in combination. Each *bar* is the mean ± S.E. of 3–25 independent experiments. *a*, statistically different from the EtOH control, $p < 0.05$; *b*, statistically different from the value for the indicated ligand treatment alone, $p < 0.05$.

the Women's Health Initiative trial results (16). Further, numerous animal studies consistently demonstrate that E_2 inhibits the progression of atherosclerosis (reviewed in Ref. 17).

The vasculature is an important target of estrogen action through both the classical genomic pathways involving regulation of gene transcription by ERα and ERβ as well as rapid, non-genomic activation of intracellular signaling pathways (reviewed in Ref. 18). The rapid vasodilating effect of E_2 is related to its ability to increase NO by stimulating eNOS expression and activity (reviewed in Ref. 19). A subpopulation of ERα is associated with caveolae in the endothelial plasma membrane (reviewed in Ref. 20). Membrane ERα is coupled via a $G\alpha_i$ protein to MAPK and eNOS in EC (21) and to Src, MMP-9, MMP-2, EGF-R, and MAPK in breast cancer cells and BAEC (22). Membrane ERα activates ERK, c-Jun NH_2-terminal kinase, and p38 mitogen-activated protein kinases (23). Understanding how ERα, which has nuclear localization sequences (24) and is, whether liganded or not, predominantly nuclear (25), localizes to the cell membrane was recently elucidated. Transfection studies in Chinese hamster ovary cells demonstrated that serine 522 in the ligand binding domain of ERα interacts with Caveolin-1 (23). Caveolin-1 is a structural protein in caveolae that binds Src, Grb7, Raf, Ras, MEK, EGF-R, and ERα at the plasma membrane forming a "signalsome" for rapid activation of intracellular signaling (22). The goal of the present study was to determine whether resveratrol has rapid effects on MAPK and eNOS activities in EC and to determine whether common intermediates in the MAPK signaling pathway were involved in resveratrol and E_2 action.

EXPERIMENTAL PROCEDURES

Cell Treatments—BAEC were provided by Dr. Yang Wang at passage 2 (26) or purchased from Cambrex (Walkersville, MD). HUVEC were purchased from Cambrex. Human microvascular endothelial cells (HMEC) were provided by Dr. J. Steven Alexander of the Louisiana State University. BAEC were used between passage 3 and 8 and maintained in RPMI 1640 (Invitrogen) supplemented with 15% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin-streptomycin (Invitrogen). HMEC was maintained in MCDB 131 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 10 ng/ml EGF (Sigma), 1 mg/ml hydrocortisone (Sigma), and 1% penicillin-streptomycin (Invitrogen). HUVEC were used between passage 2 and 8 maintained in EGM-2 (Cambrex) supplemented with 2% fetal bovine serum. EC were serum-starved for 24 h prior to each experiment and treated in medium without serum plus the indicated chemical for 20 min. Whole cell extracts (WCE) were prepared in radioimmune precipitation assay buffer (27). Protein concentration was determined by a Bio-Rad protein assay.

Chemicals—trans-Resveratrol was generously provided by Pharma Science (Montreal, Canada). *R*,*R*-5,11-*cis*-Diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (R,R-THC) was generously provided by Dr. John A. Katzenellenbogen (28). GV (white) and LM (red) wine extracts were kindly provided by Dr. Alois Jungbauer (29). The following treatments were purchased: E_2 (Sigma); 4-hydroxytamoxifen (Research Biomedicals International); ICI 182,780, 4,4′,4″-(4-propyl-c-pyrazole-1,3,5-triyl)-tris-phenol (PPT), and 2,3-*bis*(4-hydroxyphenyl)-propionitrile (DPN) from Tocris (Ellisville, MO). TAPI-1 was from Peptides International (Louisville, KY). Tyrphostin AG1478 and PP2 were from Calbiochem.

Transient Transfection—HMEC were transiently transfected with pcDNA3 (Invitrogen), pUSE-Src-K297R, kinase inactive (Upstate Biotechnology, Lake Placid, NY), p3XFLAG-CMV7-ERK2 (wild-type rat ERK2), or p3XFLAG-CMV7-ERK2-K52R dominant negative (DN) generously provided by Dr. Melanie Cobb (30) using FuGENE 6 (Roche Applied Science). HUVEC were transfected with small interfering RNA (siRNA) to MEK1 (siGENOME SMARTpool™ reagent, catalogue number M-003571-00, Dharmacon, Lafayette, CO) using Nucleofection (Amaxa). HUVEC were treated with EtOH or resveratrol 48 h after transfection as indicated in the Fig. 5 legend.

Western Blot Analysis—WCE (40 μg of protein) were separated on 10% SDS polyacrylamide gels, electroblotted onto polyvinylidene difluoride membranes (31), and probed as indicated in the figure legends. Antibodies for MAPK(ERK1/2) and phospho-p44/42 MAPK(P-ERK1/2) were from Cell Signaling Technology (Beverly, MA). ERα AER320 and ERβ H150 antibodies were from Neomarkers (Fremont, CA) and Santa Cruz Biotechnologies (Santa Cruz, CA), respectively. Baculovirus-expressed rhERα was prepared from Sf21 cells as described (32). Baculovirus-expressed rhERβ (catalogue number P2718, *i.e.* hERβ1 (long form, 530 kDa)) was purchased from Panvera (Madison, WI). Phospho-ERα (Ser-118) antibody was from Cell Signaling. Glyceraldehyde-3-phosphate dehydrogenase and β-actin antibodies were from RDI (Flanders, NJ) and Sigma, respectively. Immunodetection employed Super Signal West Pico chemiluminescent substrate (Pierce) on Kodak BioMaxML film (Eastman Kodak Co.) (33). The resulting films were scanned into Adobe Photoshop version 7.0 using a Microtek ScanMaker III scanner. Un-Scan-It (Silk Scientific, Orem, UT) was used to quantitate the integrated optical densities (IOD) for each band. Immunoblots were first probed for P-MAPK and then stripped and reprobed for MAPK. IOD were added; ERK-1 plus ERK-2 equaled MAPK and P-

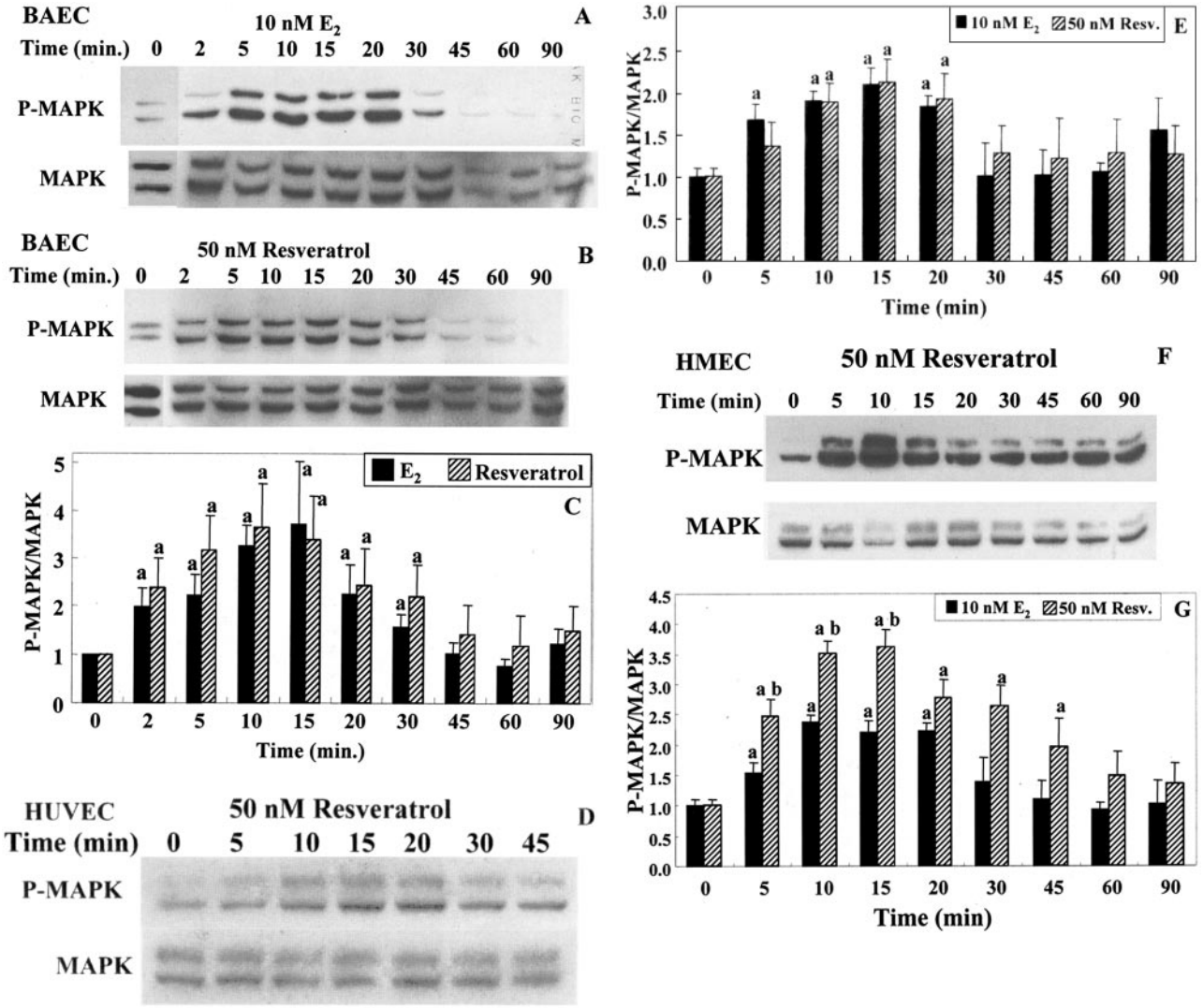

FIG. 3. **Resveratrol and E_2 rapidly activate MAPK in bovine and human EC.** BAEC (*A–C*), HUVEC (*D* and *E*), and HMEC (*F* and *G*) were treated with 10 nM E_2 (*A*) or 50 nM resveratrol (*B*, *D*, and *F*) for the indicated times. Western blot analysis of P-MAPK and MAPK and data quantitation (*C*, *E*, and *G*) are described under "Experimental Procedures." The data from 3–5 separate experiments were summarized as the mean ± S.E. *a*, statistically different from the EtOH control; *b*, statistically different from the E_2 value at that time point, $p < 0.05$.

ERK1 plus P-ERK2 equaled P-MAPK. The treatment-specific P-MAPK IOD were divided by concordant MAPK IOD in the same blot and normalized to EtOH or Me_2SO (vehicle control), which was set to 1. Anti-Src and anti-FLAG antibodies were from Upstate Biotechnology and Sigma, respectively. Anti-phospho-Src (Tyr-416) and anti-MEK1 antibodies were from Cell Signaling.

Immunoassay—HUVEC were preincubated with 10 μg/ml mouse IgG, ERα antibody Ab10 (Neomarkers), or ERβ antibody H150 for 2 h prior to 15 min treatment with EtOH, E_2 or resveratrol. P-ERK and total ERK were measured in WCE by TiterZyme enzyme immunoassay from Assay Designs, Inc. (Ann Arbor, MI) according to the manufacturer's protocol.

eNOS Assay—eNOS activity was measured by the conversion of [^{14}C]arginine monohydrochloride (50 μCi/ml, Amersham Biosciences) to [^{14}C]citrulline using the NOS assay kit from Cayman Chemical (Ann Arbor, MI) according to the manufacturer's instructions.

Statistical Analysis—Statistical analyses were performed using Student's two-tailed *t* test or one-way analysis of variance followed by Dunn's multiple comparison test with GraphPad prism.

RESULTS

Resveratrol and E_2 Stimulate MAPK Activation—E_2 rapidly activates one or more G proteins via membrane ERα, resulting in activation of Src, which in turn increases the activity of MMP-2 and MMP-9, resulting in stimulation of heparin-binding (HB)-EGF secretion and activation of EGF-R, MEK-1, and MAPK in MCF-7 and ZR-75–1 breast cancer cells and in BAEC (22). We hypothesized that resveratrol would act by this same pathway in EC. Short term resveratrol, like E_2, stimulated MEK-1 in BAEC in a concentration-dependent manner as evidenced by phosphorylation of ERK1/2 (Fig. 1). Repeated experiments and statistical evaluation are summarized in Fig. 2. It is noteworthy that activation of MAPK required only nM concentrations of resveratrol, which is 1,000–10,000-fold lower than the concentrations of resveratrol needed for other cellular responses, *e.g.* transcription activity of ERα or ERβ (13), antioxidant activity (34), and inhibition of cell proliferation (reviewed in Refs. 35 and 36). ER antagonists ICI 182,780 and 4-hydroxytamoxifen inhibited MAPK activation by resveratrol and E_2, indicating that resveratrol- and E_2-induced MAPK activation is mediated by binding ER (Figs. 1, *C* and *D*, and 2). Fig. 2 summarizes data showing the concentration-dependent activation of MAPK in BAEC by resveratrol and E_2 and inhibition of ligand-activated MAPK by ICI 182,780 and 4-hydroxytamoxifen.

The kinetics of MAPK activation are important in cellular differentiation (37). As shown in Fig. 3, resveratrol and E_2 activated MAPK in BAEC, HUVEC, and HMEC in a time-dependent manner with a peak between 5 and 20 min. Activation

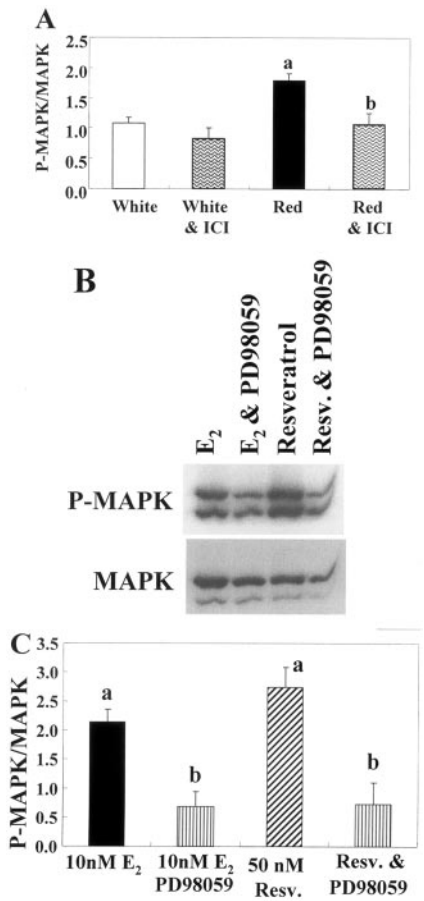

FIG. 4. **Red wine extract activates MAPK and resveratrol, and E_2 activation of MAPK is mediated by MEK1.** *A*, BAEC were treated for 20 min with a 1:1000 dilution of white or red wine extracts, prepared as described (29), alone or in combination with 1 μM ICI 182,780. The P-MAPK/MAPK ratio was quantitated from Western blot analyses. *B*, BAEC were pretreated with the MEK1 inhibitor 10 μM PD98059 for 2 h prior to the addition of 10 nM E_2 or 50 nM resveratrol for 15 min. *C*, quantitation of the P-MAPK/MAPK ratio. Each *bar* is the mean ± S.E. of at least four independent experiments. *a*, statistically different from the EtOH control, which was set to 1, $p < 0.05$; *b*, statistically different from the indicated ligand treatment alone, $p < 0.05$.

of MAPK returned to control levels by 30–45 min. Thus, rapid activation of MAPK by resveratrol and E_2 is similar in three different endothelial cell sources. Resveratrol gave a more robust activation of MAPK in HMEC than E_2 between 5 and 15 min and returned to control levels by 60 min.

Resveratrol and E_2 Activation of MAPK Is ER-, MEK-1-, MMP-, and EGF-R-dependent—The EC_{50} values for resveratrol and E_2 for P-MAPK/MAPK activation in BAEC were 1.33×10^{-8} and 1.56×10^{-10} M, respectively. These values are significantly less than the EC_{50} of 25 μM resveratrol for ERα and ERβ transcriptional activation of an estrogen response element reporter gene in transiently transfected MCF-7 cells (29). A red wine extract was shown previously to have higher ER transcriptional activity than a white wine extract, results correlating with the resveratrol content of 3.06 and 0.17 mg/liter in the red and white wine extracts, respectively (29). A 20-min treatment of BAEC with the red wine extract induced MAPK activation, whereas the white wine extract had no MAPK activity (Fig. 4*A*). The red wine extract-induced MAPK activity was inhibited by ICI 182,780, indicating that the effect was ER-mediated.

The MEK1 inhibitor PD98059 blocked resveratrol and E_2-induced MAPK phosphorylation (Fig. 4, *B* and *C*). Activation of Src is one of the initial steps in membrane-initiated ER-mediated cell signaling (38). PP2, a selective Src kinase inhibitor at nM concentrations, inhibited E_2- and resveratrol-induced MAPK activation in HUVEC (Fig. 5, *A* and *B*). To confirm the effects of the pharmacologic inhibitors, we performed transfection experiments in HMEC and HUVEC using DN or siRNA approaches. HMEC and HUVEC were selected for these experiments because these cells showed responses to resveratrol and E_2 that were similar to BAEC (Fig. 3), and they have a higher transfection efficiency (up to 40%, data not shown) than BAEC. Transient transfection of HMEC with DN point mutants of Src or ERK2 inhibited basal and resveratrol- and E_2-induced MAPK phosphorylation (Fig. 5*C*). Levels of Src and ERK overexpression were similar in all cases, indicating the effect was due to DN function of the protein (Fig. 5*D*). These data confirm the results of the chemical inhibitor studies. Transient overexpression of wild-type rat ERK2 increased basal MAPK activation, and neither resveratrol nor E_2 gave a further stimulation of MAPK in these cells. Similarly, transfection of HUVEC with siRNA directed against MEK1, decreasing MEK1 protein expression a minimum of 60%, inhibited basal and resveratrol-induced MAPK activation (Fig. 5*D*). These data indicate that the increase in P-MAPK/MAPK with resveratrol or E_2 treatment is mediated by activation of Src and MEK1.

Recent data demonstrated that E_2 activation of membrane ER in breast cancer cells and BAEC leads to activation of MMP-2 and MMP-9, release of HB-EBF, and activation of EGF-R, which in turn activates MAPK (22). We hypothesized that resveratrol would act by this same pathway. TAPI-1, an MMP inhibitor, and tyrphostin AG1478, an EGF-R tyrosine kinase inhibitor, decreased both resveratrol- and E_2-induced MAPK phosphorylation (Fig. 5, *E–G*). Taken together, these data indicate that resveratrol, like E_2 (22), activates a cascade of events, *i.e.* binding ER and activating MMP, EGF-R, Src, and MEK1, leading to MAPK activation.

Resveratrol and E_2 Increase ERα Ser-118 Phosphorylation in EC—One downstream target of activated MAPK is ERα (39). MAPK phosphorylates Ser-118 in the N-terminal activation function 1 domain of ERα (39). We observed that resveratrol and E_2 rapidly induced phosphorylation of Ser-118 in ERα in HMEC, with a peak in P-ERα detected between 10 and 30 min (Fig. 6, *A* and *B*). Similar results were detected in HUVEC and BAEC (data not shown). Pretreatment of HMEC with PD98059 (data not shown) or the Src kinase inhibitor PP2 (Fig. 6*C*) blocked E_2 or resveratrol stimulation of ERα phosphorylation. Others have reported that P-ERα has enhanced transcriptional activity relative to non-P-ERα (39).

ERα and ERβ Activate MAPK—The laboratory of John Katzenellenbogen has developed ERα- and ERβ-selective agonists and antagonists (40–43). PPT and DPN selectively activate ERα and ERβ at nM concentrations, respectively, whereas μM concentrations are needed to activate the opposite subtype (44). The ERα- or ERβ-selective agonists PPT (40) or DPN (42) rapidly induced MAPK phosphorylation in BAEC with the EC_{50} = 3.16 and 9.16 nM, respectively (Fig. 7, *A* and *B*). These concentrations are within the range selective for each ER subtype (44). The ER antagonist ICI 182,780 and MEK inhibitor PD98059 inhibited PPT- or DPN-induced MAPK phosphorylation, indicating that the effects of PPN and DPN were mediated by ER binding and MEK activation. The ERβ-selective antagonist/ERα agonist R,R-THC (28) inhibited DPN-induced MAPK phosphorylation, implying that a DPN-ERβ interaction was responsible for the increase in P-MAPK. Fig. 7*C* summarizes these data. Notably, R,R-THC also inhibited PPT-induced MAPK phosphorylation. Because R,R-THC and PPT are both ERα agonists, this result was unexpected. A possible explana-

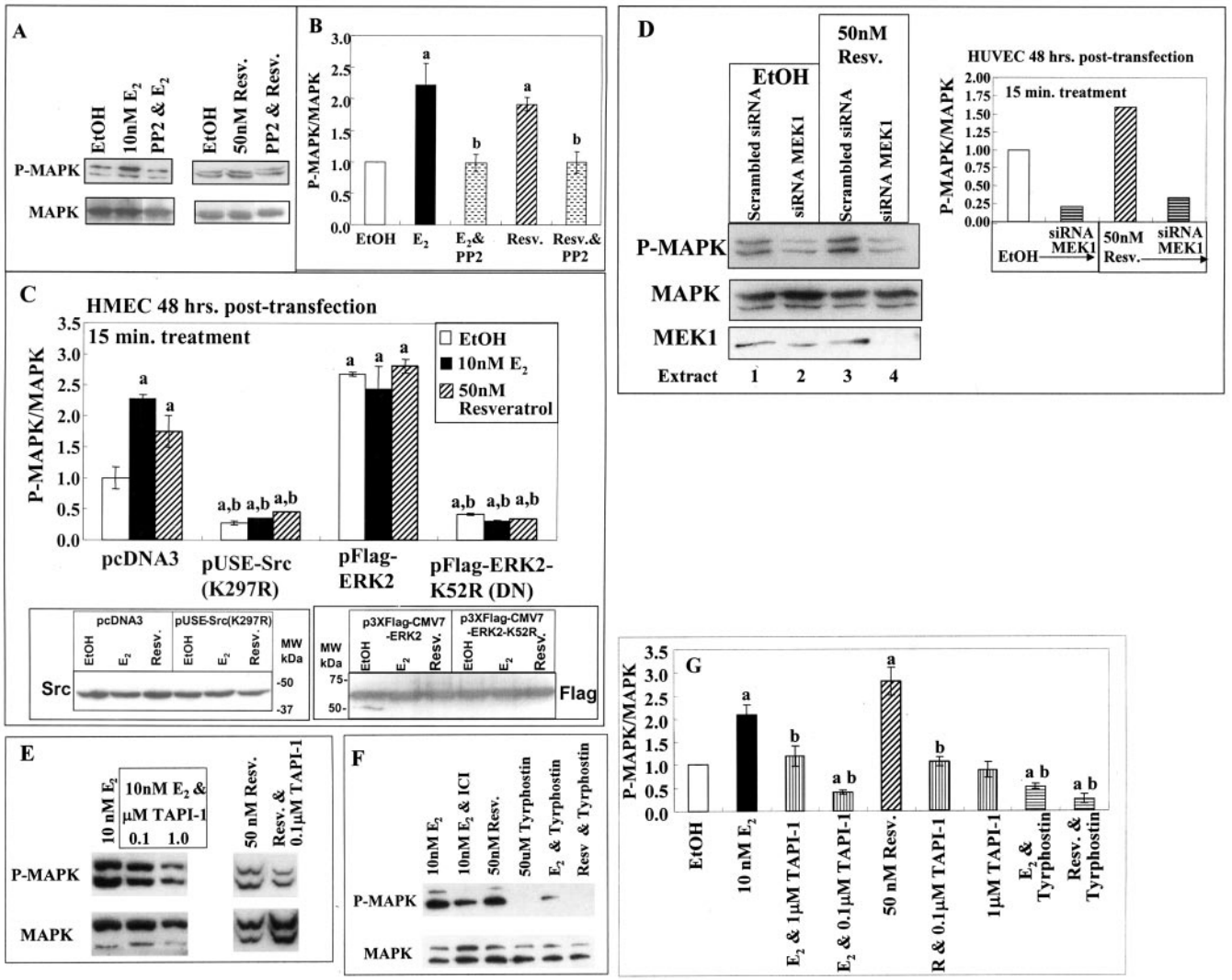

FIG. 5. **Inhibition of Src, ERK2, MEK1, MMP, and EGF-R kinase blocks resveratrol and E_2 activation of MAPK.** *A*, HUVEC were treated with EtOH, 10 nM E_2, or 50 nM resveratrol (*Resv.*) for 15 min or were preincubated with the Src kinase inhibitor 10 nM PP2 for 1 h prior to treatment, as indicated. Blots were probed for P-MAPK, stripped, and reprobed for MAPK. *B*, quantitation of the P-MAPK/MAPK ratio as the mean ± S.E. of four independent experiments. *C*, HMEC were transfected with pcDNA3, DN-Src (pUSE-Src-K297R), wild-type FLAG-tagged ERK2 (pFLAG-ERK2), or DN-ERK2 (pERK2-K52R) for 48 h and treated for 15 min with EtOH, E_2, or resveratrol (as indicated). P-MAPK/MAPK was assayed by immunoassay. Immunoblot analysis showed no alteration in total Src and that cells transfected with FLAG-ERK2 expressed the expected size of FLAG fusion protein (Western blot *panels* below the P-MAPK/MAPK *bar graphs*). *D*, HUVEC were transfected with siRNA to MEK1 for 48 h and treated for 15 min with EtOH or 50 nM resveratrol. Western blot analysis for P-MAPK, total MAPK, and MEK1 proteins and quantitation of the P-MAPK/MAPK ratio are shown. MEK1 expression was decreased 64% in *extract 2* and 87% in *extract 4*. *E*, BAEC were treated with E_2 or resveratrol ± the indicated MMP inhibitor TAPI-1 for 15 min. *F*, BAEC were treated with E_2, resveratrol, and ICI 182,780 in the presence or absence of 50 μM tyrphostin AG1478 for 15 min. *G*, quantitation of the P-MAPK/MAPK ratio. *a*, statistically different from the EtOH control; *b*, statistically different from the concordant E_2 or resveratrol value without PP2 treatment, $p < 0.05$.

tion for this observation is that both ERα and ERβ, perhaps as heterodimers, are involved in non-genomic activation of MAPK and that inhibiting one ER subtype impedes the activity of the other. Alternatively, the selectivity of these agents may be different for extranuclear (non-genomic) *versus* nuclear (genomic) ER activity. R,R-THC inhibited resveratrol-induced MAPK phosphorylation, implicating resveratrol-ERβ interaction in MAPK activation. Preincubation of HUVEC with antibodies to ERα or ERβ blocked E_2- and resveratrol-induced MAPK phosphorylation, implicating both ERα and ERβ in the activation of ERK by resveratrol and E_2 (Fig. 7*D*).

EC Express ERα and ERβ—HUVEC, HMEC, BAEC, and MCF-7 (used as a positive control because this cell line expresses both ERα and ERβ proteins (45)) express the expected masses of ERα, ERβ1, and ERβ1s proteins, *i.e.* 67, 60, and 53 kDa, respectively (Fig. 8). Based on quantitation of Western blots with rhERα and rhERβ as standards and normalization of the data to β-actin as a loading control, we estimated that HMEC, HUVEC, BAEC, and MCF-7 express an average of 3.2, 4.3, 3.4, and 2.8 pmol of ERα (monomer)/μg of WCE and 2.5, 2.7, 2.4, and 2.3 pmol of ERβ1 (monomer)/μg of WCE, respectively. Neither the values of ERα nor those of ERβ expression were significantly different between the cell lines with an $n = 4-8$. The ERα values agree with the levels of ERα expression in rat aorta (46). To our knowledge, this is the first estimation of the levels of ERβ expression in EC. To demonstrate the specificity of the antibodies used in Western blotting, baculovirus-expressed recombinant human ERα and ERβ1 were Western blotted (Fig. 8*C*). AER320 recognized only ERα but not ERβ, and H150 recognized only ERβ but not ERα. Thus, the detection of both ERα and ERβ in EC is not an artifact of antibody cross-reactivity.

Resveratrol and E_2 Activate eNOS—Previous studies reported that E_2-activated eNOS in EC was fully inhibited by concomitant treatment with tamoxifen or ICI 182,780, indicat-

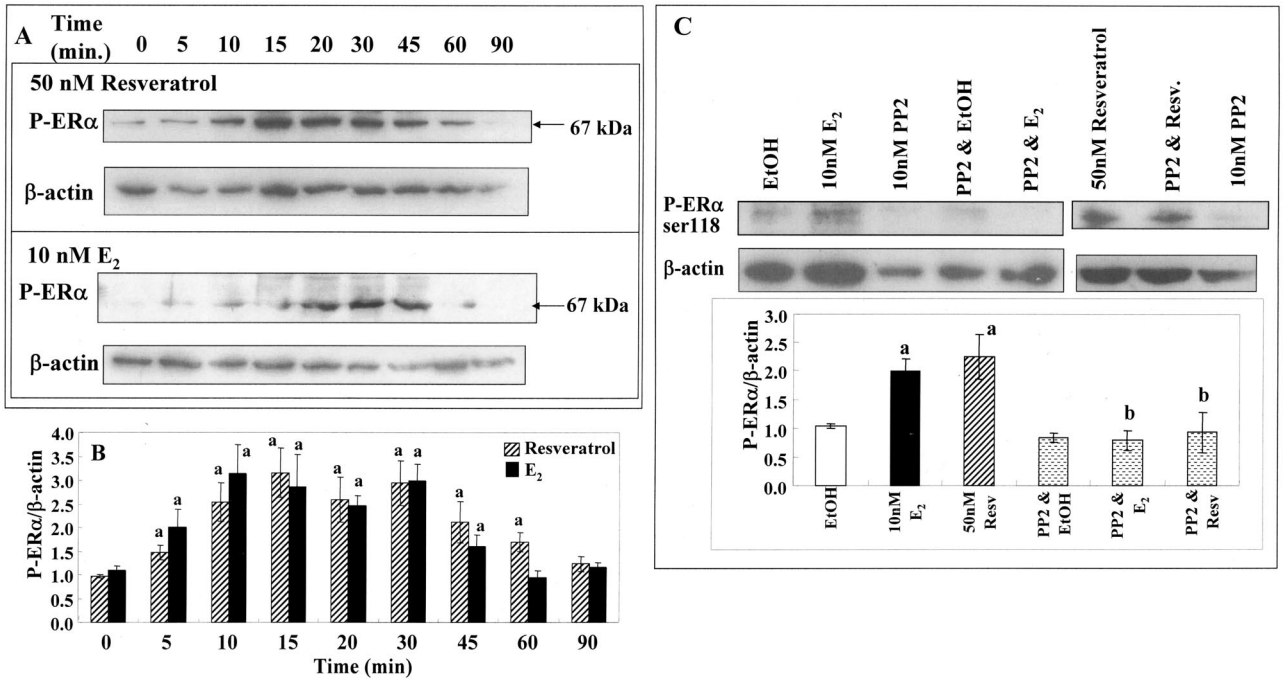

FIG. 6. **Resveratrol and E_2 rapidly increase ERα phosphorylation.** *A*, HMEC were treated with resveratrol or E_2 as indicated. The blot was probed with phospho-Ser-118 ERα antibody, stripped, and reprobed for β-actin. *B*, quantitation of the data in *A*. Each *bar* is the mean ± S.E. of at least four independent experiments. *a*, statistically different from the EtOH control, which was set to 1, $p < 0.05$; *b*, statistically different from the indicated ligand treatment alone, $p < 0.05$. *C*, a 60-min pretreatment with Src kinase inhibitor PP2 (10 nM) inhibited 10 nM E_2 and 50 nM resveratrol (*Resv*)-induced phosphorylation of ERα. This blot is representative of two similar experiments. Quantitation of the mean ± S.E. of three independent experiments is shown. *a*, statistically different from the EtOH control; *b*, statistically different from the concordant E_2 or resveratrol value without PP2 treatment, $p < 0.05$.

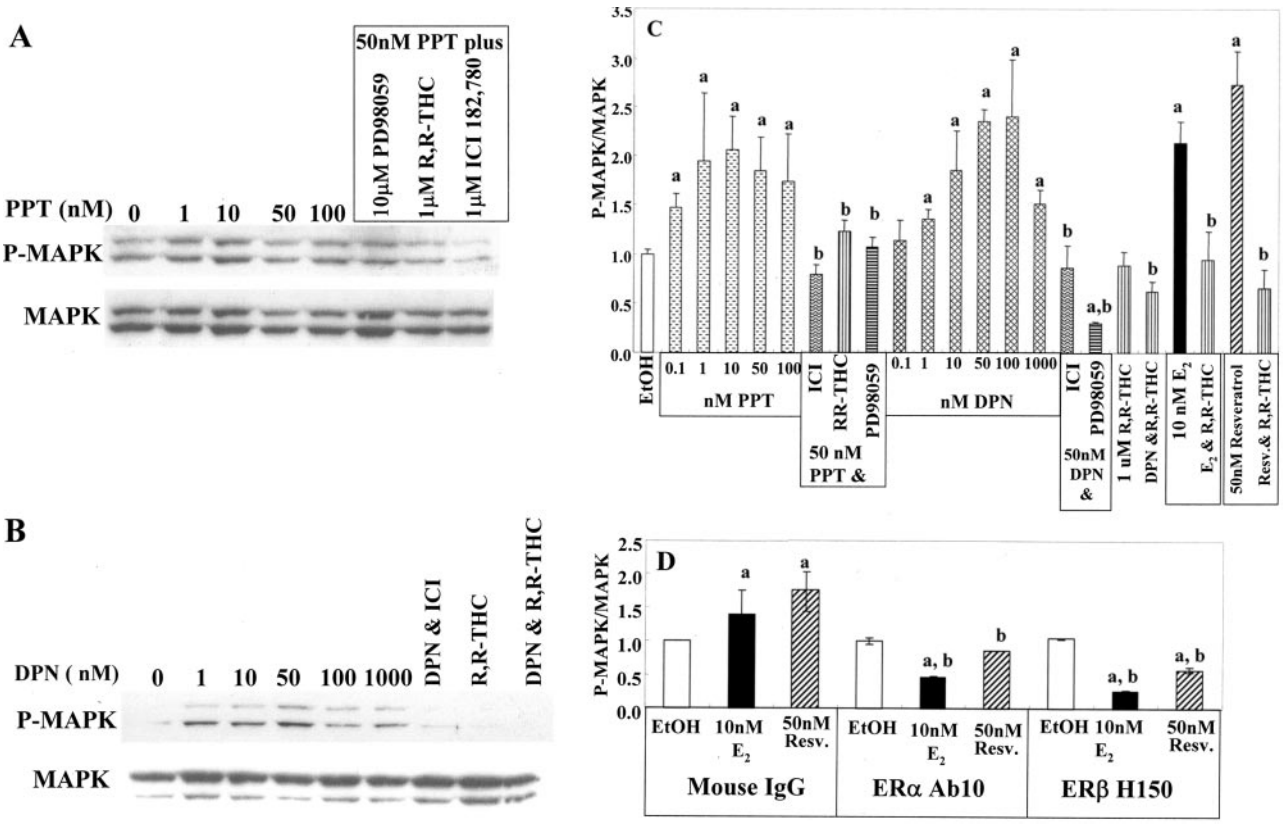

FIG. 7. **ERα and ERβ selective agonists rapidly activate MAPK in BAEC.** BAEC were treated with ERα agonist PPT (*A*) or ERβ agonist DPN (*B*) for 20 min. Where indicated, BAEC were pretreated with 1 μM PD98059 for 1 h or co-treated with 1 μM R,R-THC or 1 μM ICI 182,780. Western blot analysis of P-MAPK and MAPK and data quantitation (*C*) are described in the Fig. 1 legend and under "Experimental Procedures." Each *bar* is the mean P-MAPK/MAPK ± S.E. of 3–11 independent experiments. *D*, HUVEC were preincubated with mouse IgG, ERα antibody Ab10, or ERβ antibody H150 for 2 h prior to a 15-min treatment with EtOH, 10 nM E_2, or 50 nM resveratrol. P-MAPK/MAPK was determined from three samples by an immunoassay described under "Experimental Procedures." *a*, statistically different from the EtOH control; *b*, statistically different from the indicated ligand treatment alone, $p < 0.05$.

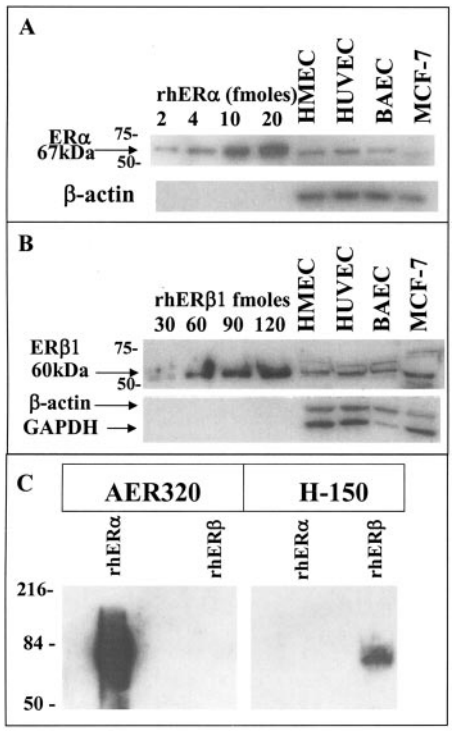

FIG. 8. **ERα and ERβ are expressed in EC.** Western blot of ERα (*A*) and ERβ (*B*) expression in WCE prepared from untreated HUVEC, HMEC, BAEC, and MCF-7 cells. The membranes were stripped and reprobed with antibodies to β-actin and/or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as indicated. *C*, the specificity of the ERα and ERβ antibodies AER320 and H150, respectively, using baculovirus-expressed rhERα (9 pmol) or rhERβ (6 pmol) from Panvera.

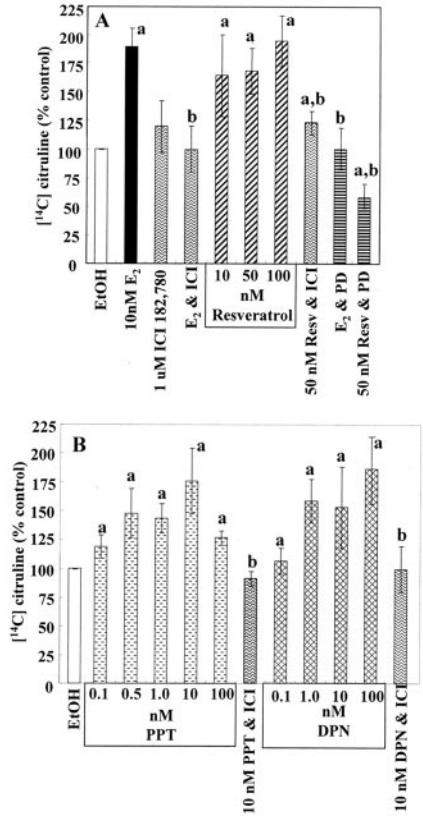

FIG. 9. **Resveratrol, E_2, PPT, and DPN rapidly activate eNOS in BAEC.** The conversion of L-[^{14}C]arginine to L-[^{14}C]L-citrulline was measured as an assay for eNOS activity after treating BAEC with the indicated concentrations of E_2, resveratrol (*Resv.*), or ICI 182,780 (*ICI*) (*A*) or with PPT or DPN, alone or in combination with ICI (*B*), for 15 min as described under "Experimental Procedures." The indicated cells (*A*) were pretreated with 20 μM PD98059 (*PD*) for 1 h prior to hormone treatment. Values are the percent of EtOH control and are the mean ± S.E. of 3–17 separate experiments. *a*, statistically different from the EtOH control; *b*, statistically different from the indicated ligand treatment alone, $p < 0.05$.

ing that direct E_2-ER interaction activates eNOS (47). Treatment of BAEC for 10 min with 10 nM E_2 or 50 nM resveratrol induced eNOS activity as measured by the conversion of [^{14}C]arginine to [^{14}C]citrulline (Fig. 9*A*). Concomitant treatment with ICI 182,780 inhibited E_2- and resveratrol-stimulated eNOS, indicating that the process is ER-dependent. Preincubation of BAEC with the MEK1 inhibitor PD98059 inhibited E_2- or resveratrol-induced eNOS activity, indicating that the MAPK pathway is involved in eNOS activation.

Treatment of BAEC with the ERα- or ERβ-selective agonists PPT (40) or DPN (42) rapidly induced eNOS activity (Fig. 9*B*). ICI 182,780 inhibited PPT- and DPN-stimulated eNOS, indicating ER dependence. These data indicate that agonist-occupied ERα and/or ERβ activate eNOS in BAEC.

DISCUSSION

The primary goal of this study was to determine whether resveratrol rapidly activated MAPK and eNOS via ER activation in EC. We report a number of novel observations. We demonstrated that nM concentrations of resveratrol, like E_2, rapidly activate MAPK in an ER-, MEK-, MMP-, Src-, and EGF-R-dependent manner in BAEC. These data suggest that resveratrol and E_2 activate the same non-genomic ER pathway that was elucidated for the rapid E_2 activation of MAPK in MCF-7 and ZR-75–1 breast cancer cells and BAEC (22). This activation pathway is diagrammed in Fig. 10 and shows the effect of antagonists and inhibitors used in this study. We are aware that resveratrol may also activate MAPK in an ER-independent manner because ICI 182,780 blocked only ~70% of the resveratrol-induced MAPK activity. Previously, 10–100 μM resveratrol was reported to activate eNOS (11), concentrations that are unlikely to be achieved by reasonable red wine consumption (8). Ours is the first report demonstrating that nM concentrations of resveratrol elicit biochemical activity in EC, concentrations achieved in human serum ~30 min post-resveratrol ingestion (8). Hence, we speculate that oral ingestion of beverages containing resveratrol, such as red wine or grape juice, could result in transient serum levels, leading to activation of membrane-initiated ER signaling in EC that would activate MAPK and eNOS. We suggest that differences in resveratrol purity (all-*trans versus* mixed *cis* and *trans*), time course of treatment, HUVEC source, or passage (11) may account for the difference in the concentrations of resveratrol required to achieve the biological activity obtained in our experiments and previous work (9, 48–51). Micromolar resveratrol activated MAPK in human DU145 prostate (48) and thyroid (49) cancer cell lines with a peak of P-MAPK/MAPK detected 4–8 h post-treatment. Conversely, resveratrol inhibited MAPK activity in human SH-SY5Ycells (50) and in porcine coronary arteries with an IC_{50} = 37 μM (51). Because resveratrol binds ERα and ERβ with a K_d in the μM range (13, 52, 53) and the EC_{50} for resveratrol activation of estrogen response element-driven reporter gene activity was 10 μM (13), our data suggest that resveratrol may be more selective for membrane-initiated *versus* nuclear ER. The mechanism accounting for this difference is unknown. Alternatively, it is possible that the low solubility of resveratrol (13) results in an underestimation of nuclear levels and an overestimation of the resveratrol concentration required for genomic ER activity.

A second novel observation presented here is that both resveratrol and E_2 rapidly activate eNOS in an ER- and MEK1-

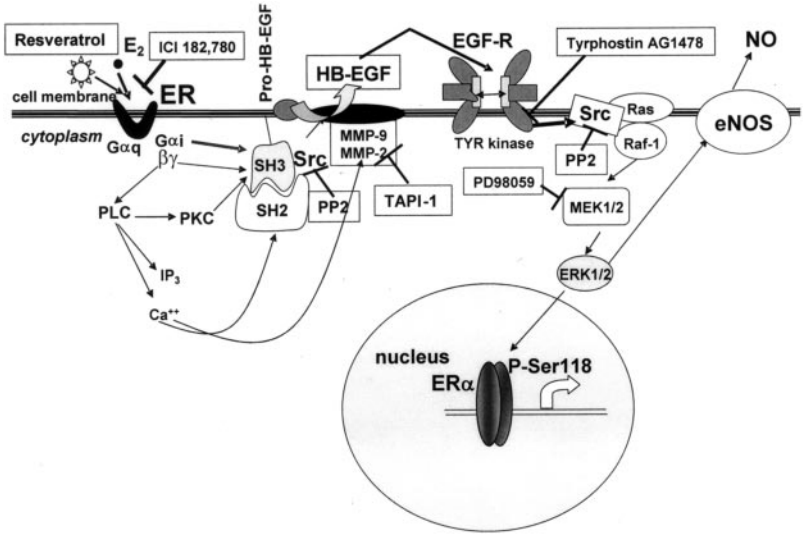

FIG. 10. **Model for resveratrol and E_2 activation of MAPK in EC.** ER is localized in a "signalsome complex" that includes Caveolin-1, which binds Src, Grb7, Raf, Ras, MEK, EGF-R, and ERα at the plasma membrane and may be coupled to G proteins (20) that activate MEK1/2 and that cause release of Ca^{2+} from intracellular pools. This model is based primarily on studies in breast cancer cells and BAEC (22), wherein E_2 binding to ER activates Src, which activates MMPs that cleave pro-HB-EGF to bioactivate HB-EGF, a ligand for EGF-R. Activation of EGF-R kinase in turn activates the MAPK pathway. MAPK activates eNOS, releasing NO (20). Chemicals that inhibit the activity of steps in the pathway used in experiments are indicated. The data presented here suggest that resveratrol also activates this pathway. *IP_3*, inositol 1,4,5-trisphosphate; *PLC*, phospholipase C; *PKC*, protein kinase C.

dependent manner because the ER antagonist ICI 182,780 and an inhibitor of MEK1, *i.e.* PD98059, inhibited the resveratrol- and E_2-induced eNOS activity. Furthermore, to our knowledge, we present the first demonstration that the ERβ- and ERα-selective agonists DPN and PPT activate eNOS in BAEC. Thus, we speculate that resveratrol and E_2 activate eNOS via their interaction with both ER subtypes in BAEC.

Reports on the endogenous expression of ERα or ERβ in EC are mixed. For example, although HUVEC reportedly expressed both ERα and ERβ at the mRNA level (54), no ERα or ERβ proteins were detected (55). Another study showed ERα protein expression in HUVEC and BAEC (56). Recently, ERβ but not ERα was detected in EC in endometrial tissues of humans and primates (57). Here we report that BAEC, HUVEC, and HMEC express full-length ERα and ERβ proteins. Among the possible explanations for these differences are the EC tissue source and the manner in which the cells are maintained, because passage number and cell density are inversely correlated with membrane-associated ERα expression (58).

Studies in male rats have demonstrated that an alcohol-free red wine extract and resveratrol can protect the heart from the detrimental effects of ischemia reperfusion injury as seen in reduced myocardial infarction and improved post-ischemic ventricular function (4). The cardioprotective effects of red wine and resveratrol have been associated with increased high density lipoprotein cholesterol (59); however, studies have shown that high density lipoprotein cholesterol levels explain only 50% of the protective effect of alcoholic beverages (2). The other 50% has been attributed to decreased platelet activity (2, 60) and to reduced vascular tension (61). The rapid vasodilating effect of E_2 is at least partially related to activation of eNOS and increased NO production (62–64). Because NO reduces vascular tension by causing vasodilation (20), the results reported here showing that nM concentrations of resveratrol rapidly activated eNOS are congruent with a role for resveratrol mediating cardioprotective activity via endothelial relaxation.

In conclusion, the data presented here demonstrate that nM concentrations of resveratrol and E_2 rapidly activate ER in EC, resulting in MAPK and eNOS activation. The physiological implications of the present findings are that the nM concentrations of resveratrol, which are found transiently in human circulation after modest wine consumption (8), offer a possible mechanism for observed vascular protective effects of resveratrol *in vivo*. The ability of selective inhibitors, DN expression constructs, and siRNA against proteins in the pathway elucidated for the rapid stimulation of intracellular signaling by E_2 via a plasma membrane ER in breast cancer cells (22) (*i.e.* MMP, EGF-R, Src, and MEK) to also inhibit resveratrol-mediated MAPK activation indicates that resveratrol and E_2 act via similar pathways in EC. Future studies directed toward elucidating the regulation of ERα and ERβ expression, their intracellular localization, and activity in the endothelium will provide new understanding of the physiological significance of the impact of resveratrol on vascular function.

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