Resveratrol stimulates nitric oxide production by increasing estrogen receptor α-Src-caveolin-1 interaction and phosphorylation in human umbilical vein endothelial cells

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ABSTRACT Epidemiological studies correlate moderate red wine consumption to reduced incidence of cardiovascular disease. Resveratrol is a polyphenolic compound in red wine that has cardioprotective effects in rodents. Although endothelial cell (EC) studies indicate that micromolar resveratrol has diverse biological activities, these concentrations are not physiologically relevant because human oral ingestion provides only brief exposure to nanomolar plasma levels. Previously, we reported that nanomolar resveratrol activated ERK1/2 signaling in bovine aortic ECs (BAECs). The goal of this study was to determine the mechanisms by which nanomolar resveratrol rapidly activates endothelial nitric oxide synthase (eNOS) in human umbilical vein ECs (HUVECs). We report for the first time that resveratrol increased interaction between estrogen receptor α (ERα), caveolin-1 (Cav-1) and c-Src, and increased phosphorylation of Cav-1, c-Src, and eNOS. Pretreatment with the lipid raft disruptor beta-methyl cyclodextrin or Gαi inhibitor pertussis toxin blocked resveratrol- and E2-induced eNOS activation and NO production. Depletion of endogenous ERα, not ERβ, by siRNA attenuated resveratrol- and E2-induced ERK1/2, Src, and eNOS phosphorylation. Our data demonstrate that nanomolar resveratrol induces ERα-Cav-1-c-SRC interaction, resulting in NO production through a Gα-protein-coupled mechanism. This study provides important new insights into mechanisms for the beneficial effects of resveratrol in ECs.—Klinge, C. M., Wickramasinghe, N. S., Ivanova, M. M., Dougherty, S. M. Resveratrol stimulates nitric oxide production by increasing estrogen receptor α-Src-caveolin-1 interaction and phosphorylation in human umbilical vein endothelial cells. FASEB J. 22, 2185–2197 (2008)

Key Words: nongenomic estrogen action • membrane-associated estrogen receptor • red wine polyphenols

Epidemiological studies have demonstrated that the consumption of polyphenolic-rich foods, i.e., fruits and vegetables, and drinking purple grape juice or moderate amounts of red wine reduces the incidence of mortality and morbidity from coronary heart disease and improves endothelial function (1). Regular, moderate intake of wine (0.7–1.1 oz/day) or alcohol (1.1–1.8 oz/day) has been reported to decrease coronary artery disease (2). A recent crossover study of 35 healthy premenopausal women found that two 100-ml glasses of red wine per day (20 g ethanol/day) for 4 wk resulted in a greater reduction in inflammatory biomarkers, cellular adhesion molecules (i.e., VCAM-1 and E-selectin), and monocyte adhesion to endothelial cells (ECs) compared to white wine (3). A key polyphenol implicated in the cardioprotective effects of red wine is trans-resveratrol, i.e., trans-3,5,4′-trihydroxystilbene (4). A study in healthy men of the oral absorption of 25 mg trans-resveratrol/70 kg administered in white wine, grape juice, and vegetable juice showed peak serum resveratrol concentrations of 40 nM, ~30 min after consumption (5). Notably, these serum levels are inconsistent with most reported cell-based studies in which the biological activities of resveratrol were detected between 5 and 100 μM (5). However, circulating levels are consistent with our report that nanomolar resveratrol concentrations rapidly activated ERK1/2 (MAPK) in bovine aortic endothelial cells (BAECs) (6). Similar to estradiol (E2) (7), MAPK activation by resveratrol was dependent on the activities of estrogen receptor (ER), MEK-1, c-Src, matrix metalloproteinases (MMPs), and epidermal growth factor-receptor (EGF-R), and, in turn, activated endothelial nitric oxide synthase (eNOS) (6). The resveratrol concentration that activated nongenomic/membrane-initiated ER signaling was an order of magnitude less than that required for ER genomic activity as we (8, 9) and others (10, 11) previously reported. These data coincide with the recent report that low doses (0.2 mg/kg/day) of a

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doi: 10.1096/fj.07-103366
red wine polyphenolic extract (RWPC) were proangiogenic in posts ischemic rats, whereas high doses (20 mg/kg/day) were antiangiogenic (12).

E2 has proven cardioprotective activity in animal models (13). Estrogens have both genomic, i.e., transcriptional regulation, and "nongenomic," i.e., membrane-initiated intracellular signaling, activities mediated by direct binding to ERα and ERβ (14). Although nongenomic E2 activity is mediated in part by GPR30 (15, 16), E2 does not activate MAPK in ECs from ERα/ERβ double-knockout (DERKO) mice (17). Studies using carotid and femoral arteries isolated from DERKO mice indicate that both ERα and ERβ are responsible for E2-induced vasodilation (18).

Cumulative studies show that a subpopulation of intact ERs is associated with the endothelial plasma membrane and with caveolae (13). Recent electron microscopy studies revealed nuclear, cytoplasmic, and plasma membrane localization of both ERα and ERβ in human umbilical vein ECs (HUVECs) (19). Interestingly, there was no overlap of ERs and ERβ immunofluorescent signals, indicating that ERα and ERβ occupy discrete loci in HUVECs (19). Thus, we propose that ERα and ERβ may have different interaction partners and physiological effects in the vasculature, and the studies reported here address this hypothesis.

Caveolin-1 (Cav-1) serves as a structural core for interaction of plasma-membrane-associated proteins, including the α-subunit of G-proteins, Ha-Ras, Src-kinases, eNOS, EGF receptors, some protein kinase-C isoenzymes (20), and ERα (20, 21). Binding of E2 to ERα in caveolae leads to Goi activation, MAPK and Akt signaling, and perturbation of the local Ca2+ environment, leading to eNOS phosphorylation and NO production (13). Endothelial plasma membrane-associated ERα is coupled via a Goi to MAPK and eNOS (22). Striatin couples ERα to Goi in EAhy926 immortalized ECs (23). The specific MEK inhibitor PD98059 blocked eNOS stimulation by E2 in HUVECs (24). Although no one has investigated endogenous ERβ-Cav-1 interaction in ECs, a recent study showed endogenous ERβ bound to overexpressed Cav-1 in human embryonic kidney-293 cells, and this interaction resulted in E2-induced activation of MAPK, AP-1, and vitamin D receptor expression (25). Lung tissue membranes isolated from βERKO mice showed reduced levels of Cav-1, indicating a role for ERβ in regulating Cav-1 expression (26). However, whether ERα and ERβ differentially regulate the rapid, nongenomic effects of estrogens, including phytoestrogens that bind ERβ with higher affinity than ERα (27), in ECs remains to be addressed.

The goal of the experiments reported in this paper was to elucidate the components of the signaling pathways involved in the ability of nanomolar concentrations of resveratrol to increase eNOS activity through a MAPK- and ER-dependent (6) mechanism in ECs. Specifically, we addressed the roles of ERα- and ERβ-caveolar interactions in the resveratrol-activated MAPK-eNOS pathway in ECs. In this study, we tested the hypotheses that 1) resveratrol increases ERα- and/or ERβ-Src-Cav-1 interaction, 2) resveratrol induces Cav-1 and eNOS phosphorylation, and 3) caveolae are required for rapid resveratrol-induced MAPK and eNOS activation. As described below, our results confirm the role of ERα in these interactions and reveal that ERβ does not appear to play a role in the rapid signaling and interactions stimulated by either resveratrol or E2 in HUVECs.

MATERIALS AND METHODS

Chemicals

E2, pertussis toxin (PTX), and methyl-β-cyclodextrin (beta-CD) were from Sigma (St. Louis, MO, USA). ICI 182,780 and PP2 were purchased from Tocris (Ellisville, MO, USA). Transresveratrol was generously provided by Royalmount Pharma (Montreal, QC, Canada). E2 and ICI 182,780 were dissolved in 100% ethanol (EtOH) and diluted in culture medium to a final 1:10,000 dilution (0.001% EtOH final).

Antibodies

Antibodies to Cav-1 and eNOS were obtained from BD Biosciences (San Jose, CA, USA); antibodies to Src, P-Tyr416-Src, P-Ser118-ERα, P-Tyr14-Cav-1, MAPK (ERK1/2), phospho-p44/42 MAPK(P-ERK1/2), P-Ser1177-eNOS, P-Thr495-eNOS, AKT, and P-Thr308-AKT were obtained from Cell Signaling Technology (Beverly, MA, USA). ERα monoclonal antibody AER320 was obtained from Neomarkers/Lab Vision (Fremont, CA, USA). ERβ polyclonal antibody H150 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ERβ polyclonal PA1–311 and monoclonal MA1–32317 antibodies were purchased from Affinity BioReagents (Golden, CO, USA). ERβ monoclonal antibody 6A12 (MS-ERB11-PX2) was from GeneTex (San Antonio, TX, USA).

Cell treatments

HUVECs were purchased from Cambrex BioScience (Walkersville, MD, USA), used between P3–8, and were maintained in EGM-2 (Cambrex). Cells were serum-starved for 24 h before each experiment and treated with vehicle (EtOH), 10 nM E2, 50 nM resveratrol, and other treatments, in EBM-2 medium without serum. Where indicated, cells were pretreated with 10 μM PP2 for 2 h, 100 nM ICI 182,780 for 30 min, 100 ng/ml PTX for 17 h, or 5 mM beta-CD for 30 min before the addition of EtOH, 50 nM resveratrol, and 10 nM E2. The concentrations of the chemical inhibitors were selected to minimize off-target effects, based on published reports: PP2, the selective Src family kinase inhibitor (28); PTX, which inhibits Gas and Goi, but not Goq (19); and methyl-beta-CD, which disrupts caveolae by binding and sequestering cholesterol (29, 30). HUVECs were treated with each inhibitor, e.g., PP2, ICI 182,780, beta-CD, and PTX, alone as well as in combination with EtOH, E2, or resveratrol, and cells were monitored by Brightfield microscopy. At the times and concentrations of each inhibitor used, there were no obvious changes in the morphology of the cells (Fig. 8).

siRNA knockdown

HUVECs were transfected with siRNA duplexes obtained from New England Biolabs (Ipswich, MA, USA; ERα ShortCut
HUVECs were pretreated for 30 min with 100 μM resveratrol for 10 and 20 min. For the inhibitor studies, were treated with 10 nM E2, 50 nM resveratrol, or EtOH for 24 h prior to loading with 3 μM DAF-2-DA for 30 min at 37°C. Cells were rinsed 3× with HEPES buffered physiological salt solution (PSS). Cells were then treated with EtOH, 10 nM E2 or 50 nM resveratrol for 10 and 20 min. For the inhibitor studies, HUVECs were pretreated for 30 min with 100 μM nitro-l-arginine methyl ester (L-NAME, Sigma), a NOS inhibitor; 5 mM beta-CD for 30 min, or 100 ng/ml PTX for 17 h before stimulation. After stimulation, the cells were fixed in 2% paraformaldehyde for 5 min at 4°C. The fixed cells were examined using a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Thornwood, NY, USA) with an ×10 objective lens, using an FL bulb and fluorescein isothiocyanate (FITC) filters with a peak excitation wavelength of 494 nm and a peak emission wavelength of 517 nm for DAF-2DA fluorescence. For Brightfield images, the light source was a halogen bulb and Brightfield filter in the same microscope. Images were captured using Axio Camera HRm and AxioVision Release 4.3 software (Carl Zeiss). All images were captured using identical microscope imaging settings with an exposure time of 256 μs.

Measurement of NO

NO production was assessed using the NO-specific fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2 DA; Sigma) (31). In brief, HUVECs were incubated in serum-free EB M-2 medium with 100 μM l-arginine (Sigma) for 24 h prior to loading with 3 μM DAF-2-DA for 30 min at 37°C. Cells were rinsed 3× with HEPES buffered physiological salt solution (PSS). Cells were then treated with EtOH, 10 nM E2 or 50 nM resveratrol for 10 and 20 min. For the inhibitor studies, HUVECs were pretreated for 30 min with 100 μM nitro-l-arginine methyl ester (L-NAME, Sigma), a NOS inhibitor; 5 mM beta-CD for 30 min, or 100 ng/ml PTX for 17 h before stimulation. After stimulation, the cells were fixed in 2% paraformaldehyde for 5 min at 4°C. The fixed cells were examined using a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Thornwood, NY, USA) with an ×10 objective lens, using an FL bulb and fluorescein isothiocyanate (FITC) filters with a peak excitation wavelength of 494 nm and a peak emission wavelength of 517 nm for DAF-2DA fluorescence. For Brightfield images, the light source was a halogen bulb and Brightfield filter in the same microscope. Images were captured using Axio Camera HRm and AxioVision Release 4.3 software (Carl Zeiss). All images were captured using identical microscope imaging settings with an exposure time of 256 μs.

Preparation of WCEs, communoprecipitation, and Western blot analysis

HUVECs were lysed in cold lysis buffer (10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 0.5 mM EGTA; 1 mM EDTA; 1% Triton X-100; 1% Na cholate; 0.5 mM PMSF) supplemented with protease and phosphatase inhibitors (Sigma). Insoluble material was removed by centrifugation, and 150 μg WCE was incubated with 1.5 μg of caveolin-1 (Cav-1) antibody (BD Biosciences), ERα, or ERβ antibodies overnight (17 h) with rotation at 4°C. Protein A-Sepharose 4B (Zymed, San Francisco, CA, USA) was added for 2 h with rotation at 4°C. The beads were sedimented at 10,000 g and washed with lysis buffer 4×, resuspended in 75 μl of Laemmli buffer, and boiled; 30 μl was separated on duplicate 10% SDS-PAGE gels and electrophoresed onto polyvinylidene difluoride (PVDF) membranes (Pall Corporation, Pencacola, FL, USA) as described (6). Membranes were probed first for phosphoproteins (P-ERα, P-Src, P-Cav-1, P-eNOS) and then stripped and reprobed for ERα, ERβ, Src, Cav-1, and eNOS. Mouse IgG TrueBlot (eBioscience, San Diego, CA, USA) was used as secondary antibody for some experiments. Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) was used to detect protein bands. Immunoblots were scanned into Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA) using a Microtek ScanMaker III scanner (Microtek, Carson, CA, USA) and Un-Scan-It 5.1 (Silk Scientific, Orem, UT, USA) was used to digitize the data (6). The treatment-specific P-protein pixels were divided by concordant nonphosphoprotein pixels and by Cav-1 in the same blot. In all experiments, ratios of P-protein/total protein were normalized to EtOH, which was set to 1, to obviate any effect of the vehicle in which E2 and resveratrol were dissolved.

Statistical analysis

Statistical analyses were performed using Student’s 2-tailed t test or 1-way ANOVA followed by Dunnett’s multiple comparison with GraphPad Prism (GraphPad, San Diego, CA, USA). A value of P < 0.05 was considered statistically significant.

RESULTS

Resveratrol and E2 induce ERα-Cav-1 interaction and phosphorylation

Although resveratrol is a low-affinity agonist for genomic ERα and ERβ activity (8–11, 32–34), no one has examined whether resveratrol affects ERα-interaction with Cav-1 in ECs. Serum-starved HUVECs were treated with 50 nM resveratrol or 10 nM E2 (positive control) for 2–30 min. Cellular proteins were immunoprecipitated with an antibody to Cav-1 and analyzed by immunoblotting with ERα and P-Ser-118-ERα. Resveratrol increased ERα-Cav-1 interaction with a peak at 5–20 min (Fig. 1A, B). Similar results were observed for E2 at the early time points, with a decrease in ERα-Cav1 interaction at 30 min followed by a return to basal ERα-Cav1 interaction by 45 min. Notably, the kinetics of direct ERα-Cav1 interaction in HUVECs was different in cells treated with E2 or resveratrol at the 30- and 45-min time points. The E2 data are in agreement with an E2-induced increase in ERα-Cav1 interaction in vascular smooth muscle cells (21).

We examined whether the increase in ERα-Cav1 interaction triggered by resveratrol was associated with increased ERα phosphorylation on Ser-118, a target of MAPK (35). Figure 1C–E shows that resveratrol and E2 rapidly increased the amount of P-ERα associated with Cav-1 and increased the P-ERα/ERα ratio. The amount of P-ERα associated with Cav-1 in resveratrol-treated HUVECs was increased between 5 and 30 min and then declined, with a second increase detected at 120 min. These data correlate with the ERα/Cav1 interaction data shown in Fig. 1A, B. Similar results were detected with E2. The ratio of P-ERα/ERα after Cav-1 immunoprecipitation (IP) showed a different pattern with a peak at 5 min, followed by a decrease, and then resveratrol treatment resulted in a second peak at 20 min. (Fig. 1E).
with Cav-1 by ~50% at the 5-min time point and that increased Src association with Cav-1 was sustained in resveratrol-treated HUVECs until after 20 min, whereas there was a decrease in Src-Cav-1 interaction at 10 min in E2-treated cells. Similarly, P-Tyr416-Src (active form of Src)-Cav-1 interaction peaked at 5 min in cells treated with either resveratrol or E2 and then decreased. Phosphorylation of c-Src kinase on Tyr416 results in disruption of the inhibitory/inactive conformation due to intramolecular and intermolecular interactions between the SH2 domain and P-Y527 and between its SH3 domain and the SH2-kinase linker domains and allows molecular recognition of c-Src substrates and binding partners (37). However, P-Src showed a second peak of interaction with Cav-1 at 20 min in resveratrol-treated cells. The ratio of P-Src/Src was increased after 2 min with either resveratrol or E2. The increase at the 20 min time point was not statistically significant. Pretreatment with the selective Src-kinase inhibitor PP2 blocked the increase in P-Src (Fig. 2D).

Both resveratrol and E2 increased the P-Cav-1/Cav-1 ratio at 10 and 120 min, indicating a biphasic effect on Cav-1 phosphorylation (Fig. 3A). The data in Fig. 3B, C compare the time course of phosphoprotein-Cav-1 interaction in HUVECs after resveratrol or E2 treatment. These data indicate different time kinetics of P-ERα/Cav-1 interaction with resveratrol showing an extended P-ERα/Cav-1 interaction compared to E2, which shows a peak at 5 min. Although the initial P-Src-Cav-1 peak at 5 min is similar for resveratrol and E2, resveratrol-treated HUVECs show a second P-Src-Cav-1 peak at 20 min not seen in E2-treated cells. Figure 3B indicates that ERα in the ERα-Cav-1 complex in resveratrol-treated cells is maintained in the phosphorylated active form for an extended period of time, which may indicate that resveratrol has a sustained effect on e-NOS phosphorylation and NO production.

**Resveratrol increases eNOS Ser-1177 phosphorylation**

Phosphorylation of eNOS at Ser-1177 indicates activation, whereas P-thr495 indicates inactive eNOS...
The effect of resveratrol on eNOS phosphorylation is unknown. Here, we observed that resveratrol increased P-Ser-1177-eNOS in 5 min of treatment with a peak at 10 min for both resveratrol and E2 (Fig. 4A, B). All data were normalized for the vehicle EtOH, which did not increase P-Ser-1177-eNOS (Fig. 4A, and data not shown for other time points up to 45 min). Wortmannin and PD98059 inhibited resveratrol- and E2-induced increase in P-Ser-1177-eNOS, indicating a role for both PI3K and MAPK pathways in eNOS activation. There was a concomitant decrease in P-thr495-eNOS after 10 min with resveratrol or cotreatment with E2 or resveratrol and wortmannin. In contrast, cotreatment with PD958059 increased P-thr495-eNOS (Fig. 4C). The increased P-Ser-1177-eNOS/eNOS at 5 and 10 min correspond to the time course of ERα/Cav-1 interaction and Cav-1 phosphorylation.

Figure 2. Resveratrol and E2 induce Src-Cav-1 interaction and phosphorylation. A) HUVECs were treated with EtOH (control), E2 (10 nM), or resveratrol (50 nM) for the indicated times. Where indicated, cells were pretreated with the Src kinase inhibitor PP2 (10 μM) for 1 h. Src-Cav-1, P-Src-Cav-1, and P-Src-Src interactions were examined by IP as in Fig. 1. Representative Western blots at 5-min time point are shown. B–D) Quantitation of the Src/Cav-1 (B), P-Src/Cav-1 (C), and P-Src/Src ratios (D). Values are means ± se from 3 separate experiments, except 60 min is from a single IP; *P < 0.05 vs. t0. †P < 0.05 vs. same treatment without PP2 pretreatment.

Figure 3. Resveratrol and E2 increase phosphorylation of Cav-1, Src, and ERα. HUVECs were treated with EtOH (control), E2 (10 nM), or resveratrol (50 nM) for the indicated times. P-Cav-1/Cav-1, P-ERα/Cav-1, P-Src/Cav-1 interactions were examined by IP with an antibody to Cav-1 as in Fig. 1. A) A representative Western blot at the 10-min time point for P-Cav-1/Cav-1. B, C) Quantitation of the P-ERα/Cav-1, P-Src/Cav-1, and P-Cav1/Cav-1 ratios in resveratrol-treated (B) and E2-treated cells (C). Values are means ± se from 3 separate experiments, except 60 min is from a single IP; *P < 0.05 vs. t0.
PTX blocks resveratrol-induced MAPK, Src, and eNOS phosphorylation

E2 stimulated the direct interaction of ERα with Gαi (22) via ERα-striatin interaction (23). To test the hypothesis that resveratrol’s activation of Src, MAPK, and eNOS is mediated by Gαi, HUVECs were pretreated with PTX (Fig. 4D, E). Pretreatment with PTX completely blocked resveratrol- and E2-stimulated Src, MAPK, and eNOS activation, indicating that signaling involves PTX-sensitive Gαi or Gαs, but not Goq (39) in HUVECs. Data are normalized to EtOH. Resveratrol did not act additively with E2 in activating Src, MAPK, or eNOS, as measured by the phosphoprotein/total protein ratio (Fig. 4E). Because we used individual concentrations of E2 and resveratrol at which MAPK activation is maximum (6), we suggest that the lack of additivity indicates that the response is mediated by a common pathway which is saturated.

Pretreatment with ICI 182,780 inhibits recruitment of ERα to Cav-1 and Cav-1 phosphorylation

To test the role of direct resveratrol-ERα binding in the rapid changes observed in Figs. 1–3, HUVECs were pretreated with 100 nM ICI 182,780 for 30 min before the addition of either 50 nM resveratrol or 10 nM E2. ICI 182,780 is a well-established antagonist of genomic ER that prevents coactivator recruitment (40), inhibits ERα dimerization (41), and enhances ERα proteosomal degradation after 6 h (40). In addition, ICI 182,780 inhibits the rapid E2 activation of membrane-associated ERα responses (13, 42) but not GPR30-mediated activity (16). Pretreatment of HUVECs with ICI 182,780 inhibited the resveratrol- and E2-induced increases in the P-Src/Src, P-Cav-1/Cav-1, P-ERα/Cav-1 and ERα/Cav-1 ratios (Fig. 5A, B). These data indicate that the increased phosphoprotein/protein ratios and interactions induced by both resveratrol and E2 are at least in part ERα-mediated.

Caveolae are required for resveratrol-induced ERα-Cav-1 interaction and eNOS activation

To determine whether caveolae are required for resveratrol-induced ERα-Cav-1 interaction and phosphorylation of Src, Cav-1, and ERα, serum-starved HUVECs were pretreated with EtOH (vehicle control) or 5 mM beta-CD for 30 min to disrupt caveolae by binding and sequestering cholesterol (29). Figure 5C, D shows that beta-CD inhibited resveratrol- and E2-induced ERα-Cav-1 interaction, and ERα, Src, and eNOS-Ser-1177 phosphorylation. Together, the results demonstrate that caveolae are required for Cav-1-ERα-Src interaction and resveratrol signaling, and disruption of caveolae with beta-CD inhibits ERα-Src-Cav-1 interaction and resveratrol- and E2-induced ERα phosphorylation and eNOS activation.

Resveratrol and E2 do not stimulate ERβ-Cav-1 interaction

No one has examined the effect of E2 or resveratrol on endogenous ERβ-Cav-1 interaction and downstream sig-
ERα protein of ERα binding sites revealed that HUVECs express 0.9 and 0.2 fmol/mg protein, followed by either a 5 min (for the P-Src/Src data, because that is the time of maximal P-Src/Src) or 10 min treatment with EtOH (control), E2 (10 nM), or resveratrol (50 nM) (for all other analyses indicated). The indicated interactions were examined by IP with an antibody to Cav-1 followed by immunoblotting with P-Src, P-Cav-1, or P-ERα as described in Fig. 1. The data for HUVECs not preincubated with the inhibitors are from Figs. 1–3 and are included for ease of visual comparison. Three representative Western blots are shown. Graphs show quantitation of the P-Src/ Src, P-Cav-1/Cav-1, P-ERα/ERα, P-ERα/Cav-1, and ERα/Cav-1 ratios (B, D). Values are means ± SE from 3 separate experiments; *P < 0.05 vs. EtOH; †P < 0.05 vs. same treatment without ICI, PTX, or βCD pretreatment.

**Figure 5.** Pretreatment of HUVECs with ICI 182,780 or methyl-β-cyclodextrin (βCD) inhibits resveratrol- and E2-induced protein-protein interactions and phosphorylation. HUVECs were pretreated with 100 nM ICI 182,780 (A, B), or 5 mM βCD (C, D) for 30 min, followed by either a 5 min (for the P-Src/Src data, because that is the time of maximal P-Src/Src) or 10 min treatment with EtOH (control), E2 (10 nM), or resveratrol (50 nM) (for all other analyses indicated). The indicated interactions were examined by IP with an antibody to Cav-1 followed by immunoblotting with P-Src, P-Cav-1, or P-ERα antibodies and reprobing the same blots for Src, Cav-1, or ERα as described in Fig. 1. The data for HUVECs not preincubated with the inhibitors are from Figs. 1–3 and are included for ease of visual comparison. Three representative Western blots are shown. Graphs show quantitation of the P-Src/Src, P-Cav-1/Cav-1, P-ERα/ERα, P-ERα/Cav-1, and ERα/Cav-1 ratios (B, D). Values are means ± SE from 3 separate experiments; *P < 0.05 vs. EtOH; †P < 0.05 vs. same treatment without ICI, PTX, or βCD pretreatment.

**Knockdown of ERα and not ERβ inhibits rapid activation of MAPK, Src, and eNOS by E2 and resveratrol.**

To further examine the ER subtype specificity for the observed rapid stimulation of MAPK, Src, and eNOS phosphorylation in response to resveratrol and E2 in HUVECs, cells were transfected with control/nonspecific siRNA or siRNA targeting ERα or ERβ. Transfection of HUVECs with siRNA for ERα reduced ERα protein by 80% but had no significant effect on ERβ expression (Fig. 7A). Transfection of HUVECs with siRNA for ERβ reduced ERβ protein by 75%, but had no significant effect on ERα expression (Fig. 7B). Knockdown of ERα reduced the E2- or resveratrol-induced activation of MAPK, Src, and eNOS, as well as basal eNOS phosphorylation (Fig. 7C). In contrast, knockdown of ERβ did not significantly inhibit the E2- or resveratrol-induced activation of MAPK, Src, and eNOS. Together, these data indicate that ERα, not ERβ, plays the major role in the E2- or resveratrol-induced activation of MAPK, Src, and eNOS in HUVECs.

**Resveratrol stimulates NO production.**

We examined the acute effects of resveratrol and E2 on NO production using DAF-2 fluorescent dye. Resveratrol- and E2-induced DAF-2 fluorescence, indicative of NO production, was maximal after 10 min and was sustained after 20 min (Fig. 8). To verify that the increase in green fluorescence reflected NO production, HUVECs were pretreated with L-NAME, a NOS inhibitor. As expected, L-NAME blocked the increase in green fluorescence. Pretreatment of HUVECs with beta-CD and PTX also completely blocked resveratrol- or E2-induced NO production (Fig. 8). These data agree with resveratrol-induced Ser-1177 eNOS phosphorylation (Fig. 4A).
Laemmlı buffer (25 μl) was loaded in lane 4; Cav-1 antibody (0.5 μg) was diluted in Laemmlı buffer, boiled, and loaded into lane 5. Lanes 6 and 7 include 15 μg WCEs from EtOH- or E2-treated HUVECs, respectively. Baculovirus-expressed rhERβ (14 fmol) was included as a control (lane 8). B) Reverse IP: ERβ-Cav-1 interaction was examined by IP (as in A) with an antibody to ERβ antibody (MA-23217, lanes 1–3) and Western blot analysis for Cav-1. Baculovirus-expressed rhERα (558 and 1395 fmol, lanes 4 and 5) or rhERβ (355 and 710 fmol, lanes 6 and 7) were separated in parallel. Molecular weight (MW) marker size is indicated in kilodaltons. Antibody specificity is demonstrated: ERβ antibody H150 did not recognize ERα and ERβ antibody AER320 likewise did not bind ERβ. C) Quantitation of the ERβ/Cav-1 and Cav-1/ERβ ratios from 3 separate experiments for each IP (IP-Cav-1, probe ERβ; IP-ERβ, probe Cav-1). Values are means ± se[SCAP]; *P < 0.05 vs. EtOH (Student’s t test, 2-tailed). D) Western blot for ERα in WCEs of HUVECs: 40 μg WCE from MCF-7 (untreated) or HUVECs, untreated, or treated for 10 min with EtOH, 10 nM E2, or 50 nM resveratrol, was separated on a 10% SDS-PAGE gel. The indicated amount (fmol) of rhERα was added as a loading control. MW marker size is indicated in kilodaltons. The membrane was first probed with AER320 ERα antibody and then stripped and reprobed for α-tubulin. There is no evidence of the ~54 kDa band detected by AER320 in the IP for ERβ in B. We suggest that this is mouse IgG.

DISCUSSION

Red wine and its key constituent resveratrol have a variety of activities associated with cardioprotective effects (2). Nonetheless, the exact mechanisms for red wine’s ability to prevent coronary artery disease and stroke is not fully elucidated. One major area of concern in studying resveratrol’s effects is the discrepancy between the high concentrations apparently needed to achieve biological activities in vitro or in animal models with the epidemiological effects seen at moderate red wine consumption that would achieve only nanomolar concentrations in vivo for a short time (5). The primary goal of this study was to determine the molecular mechanisms by which nanomolar concentrations of resveratrol, concentrations compatible with oral consumption (5), rapidly activate NO production in ECs by examining endogenous rather than overexpressed proteins. Here, we demonstrated that nanomolar concentrations of resveratrol, like E2 (43), increase ERα-Cav-1-Src interaction in lipid rafts/caveolae in HUVECs in a time-dependent manner leading to Cav-1, Src, ERK1/2, and eNOS phosphorylation, and NO production. Furthermore, we show that resveratrol’s activation of eNOS appears to be mediated by activation of Gα1 and/or Gαs proteins and that ERβ does not appear to be critical for these signaling events, since siRNA knockdown of ERβ did not prevent resveratrol or E2 activation of Src, MAPK, or eNOS in HUVECs.

Previously, we reported that nanomolar concentrations of resveratrol activated NO production in BAECs in an ERα, Src, MEKI/ERK1/2, MMP, and EGFR-dependent manner (6). However, the exact mechanisms by which resveratrol-ER interaction activated eNOS remained undefined. ERα localizes in caveolae of ECs (13), and the signal transduction mechanisms by which E2 rapidly (i.e., nongenomically) activates eNOS have been explored (reviewed in ref. 44). ERα interacts directly with c-Src, Cav-1, hsp90, and other proteins in caveolae of ECs and breast cancer cells (13, 20, 45–47). In response to E2, Src is rapidly activated, inducing formation of a complex that contains ERα, c-Src, and the p85 subunit of PI3K. These interactions are required for activation of eNOS (13, 42, 43). Our data are consistent with these reports, as we demonstrated that resveratrol, like E2, rapidly induces ERα-Cav-1 association, whereas neither resveratrol nor E2 stimulates ERβ interaction with Cav-1 interaction. Resveratrol, like E2 (43, 48), increased ERα and ERβ interaction with c-Src. Further, our data showing that ERα did not coimmunoprecipitate with ERβ, and vice versa, is also consistent with recent electron microscopy studies indicating that
ERα and ERβ occupy discrete loci in HUVECs (19). Thus, we conclude that ERα and ERβ have different interaction partners in HUVECs and speculate that this may apply to physiological interactions in the vasculature as well. Support for this idea at the genomic level is born out by recent microarray profiling of genes regulated by E2 in aortas from ovariectomized wild-type vs. ERα or ERβ knockout mice (49). ERα was responsible for the up-regulation of most of the identified E2 target genes in the aorta. In contrast, E2-ERβ actively repressed the transcription of nuclear-encoded mitochondrial respiratory chain genes. Because the animals were treated for 1 wk with E2, these data reflect not only primary target genes, but secondary and tertiary genes as well as integration of nongenomic and genomic actions of E2 in the aorta. That ERα and ERβ have selective actions in a cell-type-dependent manner is likewise indicated by the report that E2 and genistein, a phytoestrogen that is a selective ERβ agonist, but not propyl-pyrazole-triol, a selective ERα agonist, increased eNOS and decreased nNOS at the mRNA and protein levels in the rat hypothalamic paraventricular nucleus (50). Moreover, the ERβ-selective agonist diarylpropionitrile rapidly increased NO production, and E2-stimulated NO was inhibited by the selective ERβ antagonist R68 tetrahydrochrysene (51). In addition to E2 and synthetic ER ligands, a variety of phytochemicals and endocrine disruptors initiate second messenger-triggered signal cascades emanating from the plasma membrane that are mediated by ERα, ERβ, and/or GPR30 (52–57).

One novel finding in the present study is that nanomolar levels of resveratrol, like E2, caused a rapid association of Cav-1 with Src (Fig. 2B). However, resveratrol elicited a more sustained interaction (5–20 min), whereas E2 caused only transient interactions peaking at 5 and 20 min. Indeed, the chronological order of protein-protein interactions and phosphorylation stimulated by resveratrol and E2, while largely overlapping, is somewhat different (Fig. 3B, C). For example, E2 more rapidly increased ERα-Cav-1 interaction compared to resveratrol (Fig. 1B). Further, the E2-induced ERα-Cav-1 interaction was reduced below basal at 30 min, whereas the resveratrol-activated interaction was reduced at 45 min. The mechanism for the differences in chronology and the more sustained Cav-1-Src triggered by resveratrol interaction is unknown but may involve differences in the identity or kinetics of proteins in addition to those tested here and that are involved in the signalosome complex, such as MNAR/PELP and the p85 regulatory subunit of PI3K (58). Further studies are required to identify the proteins involved in differences between resveratrol and E2. Importantly, Src kinase activity appears to be involved in resveratrol- and E2-induced eNOS activation, since pretreatment with the selective Src kinase inhibitor PP2 completely blocked resveratrol- or E2-induced association of Src/Cav-1 (Fig. 2D). These data are consistent with previous reports on E2 activation of the Src/Cav-1-eNOS signaling pathway in rat lung ECs and MCF-7 human breast cancer cells (21, 59).

Another novel observation is that the rapid effects of resveratrol are mediated by Cav-1-associated full-length ERα (66 kDa). Although the 46-kDa isoform of ERα lacking the N terminus (A and B domains) was reported to be preferentially associated with the plasma membrane in EA.hy926 cells (immortalized human ECs) (37, 60), our data show that full-length ERα and
ER/H9251 (61) were immunoprecipitated with Cav-1 in HUVECs. It is possible that the ER isoforms present in the plasma membrane differ in different ECs. Notably, pretreatment with ICI 182,780 before the addition of resveratrol or E2 and siRNA against ER/H9251, not ER/H9252, blocked the P-Src/Cav-1, P-Cav-1/Cav-1, P-ER/H9251/ER/H9251, P-ER/H9251/Cav-1, and ER/H9251/Cav-1 interactions (Fig. 5B) and phosphorylation of Src, Cav-1, eNOS, and ERα (Fig. 7).

Cumulatively, our data suggest that resveratrol and E2 activate a similar nongenomic ER pathway (Fig. 9) that accounts for rapid E2 activation of eNOS in ECs (22, 43, 45, 46). In contrast to our previous study in BAECs in which subtype-specific ligands indicated roles for both ERα and ERβ in eNOS activity (6), siRNA knockdown studies in HUVECs indicate that ERα plays a predominant role in the resveratrol and E2-activation of MAPK, Src, and eNOS. Coincidentally, while resveratrol and E2 increased ERα-Cav-1 interaction within 5 min, ERβ-Cav-1 interaction was decreased by ligand treatment (Fig. 6). Thus, it is tempting to speculate, on the basis of numerous studies in a variety of tissues/cell types establishing that ERβ is a down-regulator of responses up-regulated by ERα (62–71), that the decrease in ERβ-Cav-1 interaction may be part of the stimulatory mechanism.

Cav-1 is a substrate for Src kinase that phosphorylates Cav-1 on Tyr-14 (72). Increased P-Cav-1 has been suggested as an indicator of a reduced interaction between Cav-1 and other signaling proteins (20). Our data agree with these findings, as indicated by the inverse association of Cav-1 phosphorylation, i.e., at 10 min, with reduced P-Src-Cav-1 and P-ERα-Cav-1 association. However, when P-Cav-1/Cav-1 peaked again at 60 min and remained high up to 120 min in resveratrol-treated cells, P-ERα/Cav-1 was also high (Fig. 3B). The distinct difference between E2 and resveratrol induced effects in HUVECs is the extended P-ERα-Cav-1 association in resveratrol-, but not E2-treated cells (Fig. 3C).

Notably, beta-CD, which disrupts caveolae-like structures by binding to and sequestering cholesterol from the plasma membrane of intact cells (73), abolished resveratrol- and E2-induced protein-Cav-1 associations, eNOS phosphorylation, and NO production. These observations indicate that intact caveolar structure is required for resveratrol's rapid effects on ERα-Cav-1 interaction, eNOS activation, and NO production in HUVECs, similar to our observations reported here using E2 as a positive control established in ECs by other investigators (13, 20).

Because direct interactions between plasma membrane-associated ERα and Goi have been implicated in...
eNOS activation and NO production in COS-7 cells transfected with ERα and specific Gαi proteins (22), we investigated whether Gαi is involved in resveratrol-induced e-NOS activation in HUVECs. Our data demonstrate that pretreatment with PTX, a Gα inhibitor, blocked completely resveratrol’s activation of Src, MAPK, and eNOS, indicating that Gαi plays an important role in rapid resveratrol signaling in HUVECs. These findings are similar to E2 as shown here and reported by others (13, 20, 44, 74).

Previous studies showed that high concentrations (micromoles or even millimoles) of resveratrol have a variety of beneficial activities in cardiovascular tissues (75). For example, 10 μM resveratrol produced a powerful relaxation response in endothelium-containing rings of rat aorta (76). Likewise, 10 μM resveratrol caused rapid coronary vasodilation and increased coronary flow in Langendorff-perfused rat hearts (77). Recently, a 5-min treatment with 30 or 100 μM resveratrol suppressed ischemia-reperfusion-induced ventricular arrhythmias in perfused rat hearts (78). These studies are limited by nonphysiologically relevant resveratrol concentrations used (5). In addition to differences in resveratrol effects due to high concentrations used experimentally, we hypothesize that because of its rapid metabolism (79–81), resveratrol’s acute effects in the vasculature may contribute to its vascular/cardio-protective effects through different mechanisms than chronic/high dose effects. The resveratrol concentration used here is an order of magnitude less than that required for ER genomic activity as reported by us (8, 9) and others (10, 11).

In conclusion, the data presented here demonstrate that nutritionally relevant (nanomolar) concentrations of resveratrol rapidly activate plasma membrane-associated ERα in caveolae of HUVECs, leading to eNOS activation and NO production via activation of Gαi, Cav-1, Src, and MAPK in a manner similar to that elicited by E2. These studies imply that dietary intake of resveratrol may offer possible vascular protective effects in vivo.

We thank Royalmount Pharma (Montreal, QC, Canada) for providing trans-resveratrol for our study. We thank Dr. Wasana Sumanasekera for her assistance in the initial IP experiments. This research was supported by American Heart Association grant 0555270B to C.M.K. We thank Dr. Barbara J. Clark for her insightful suggestions on this manuscript.

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Received for publication December 4, 2007. Accepted for publication January 24, 2008.