

# Nongenomic Mechanisms of Endothelial Nitric Oxide Synthase Activation by the Selective Estrogen Receptor Modulator Raloxifene

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**Background**—Nontranscriptional signaling through estrogen receptors (ERs) is important in the cardiovascular system. In particular, estrogen stimulates endothelial NO synthase (eNOS) via the phosphatidylinositol 3-kinase (PI3K) pathway. The selective estrogen receptor modulator (SERM) raloxifene is effective for the treatment of postmenopausal osteoporosis, but its ability to activate eNOS via PI3K is unknown.

**Methods and Results**—Human umbilical vein endothelial cells were cultured in estrogen-deprived, phenol red-free medium. Raloxifene stimulated eNOS in a concentration- and time-dependent manner. Activation of eNOS by raloxifene was blocked by the PI3K inhibitor wortmannin and by the ER antagonist ICI 162,780 but not by transcriptional or translational inhibitors. Coimmunoprecipitation studies demonstrated that, in a ligand-dependent manner, raloxifene increased ER $\alpha$ -associated p85 $\alpha$ , p110 $\alpha$ , and PI3K activity. This correlated temporally with increases in the serine and threonine phosphorylation and activation of protein kinase Akt.

**Conclusions**—Our findings indicate that nongenomic ER signaling triggered by a SERM leads to a rapid activation of NO synthesis in human endothelial cells. The ability of raloxifene to facilitate ER $\alpha$ -PI3K interaction may provide additional insight into the structure-function relationship of specific SERMs, which promote the nontranscriptional effects of ER. (*Circulation*. 2002;105:1368-1373.)

**Key Words:** endothelium ■ receptors ■ nitric oxide ■ nitric oxide synthase ■ signal transduction

Cardiovascular disease is the major cause of mortality in Western countries.<sup>1</sup> Epidemiological studies show a strong correlation between menopause and cardiovascular diseases, and controversy exists as to the potential cardiovascular benefits of hormone replacement therapy (HRT) in postmenopausal women.<sup>2,3</sup> In the search for more selective agents, molecules exerting estrogenic effects in certain tissues while having neutral or antiestrogenic actions in others have been developed. Raloxifene is one of these selective estrogen receptor modulators (SERMs) and has been approved for the treatment of postmenopausal osteoporosis.<sup>4</sup> The therapeutic benefits of raloxifene presently are under evaluation in a large trial in postmenopausal women (Raloxifene Use in The Heart trial), with cardiovascular and breast cancer outcomes as primary end points. However, little is known about the cardiovascular effects of SERMs and their mechanism of action.

Raloxifene therapy after menopause is associated with a complex modification of cardiovascular risk markers, including decreased total and LDL cholesterol concentrations without changes in triglycerides and HDL cholesterol levels<sup>5</sup> as well as reduced lipoprotein(a).<sup>6</sup> Serum levels of fibrinogen<sup>6</sup>

and homocysteine<sup>7</sup> are also decreased by raloxifene. Furthermore, raloxifene is associated with vascular anti-inflammatory effects as well with the absence of proinflammatory changes shown by conventional HRTs, such as C-reactive protein increase.<sup>7</sup>

Recent studies suggest that most of the potential anti-atherogenic effects of estrogen may be related to its direct effects on the vascular wall.<sup>8,9</sup> Raloxifene has antiatherogenic effects in ovariectomized cholesterol-fed rabbits,<sup>10</sup> but no such effect has been found in primates,<sup>11</sup> thus posing a question concerning its possible effects in humans. In vitro studies suggest that raloxifene directly regulates vascular cells, rapidly stimulating endothelium-derived NO synthesis<sup>12</sup> and relaxing isolated rabbit coronary arteries.<sup>13</sup> Furthermore, recent data show that raloxifene therapy increases NO concentration and flow-mediated vasodilation in healthy postmenopausal women.<sup>14</sup>

Nongenomic signaling through the estrogen receptor (ER) accounts for relevant estrogen-dependent processes in the vessels, such as rapid activation of endothelial NO synthase (eNOS) in endothelial cells.<sup>15</sup> Activation of eNOS by estrogen occurs through the ERK-1/2 pathway<sup>16</sup> as well as via the

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phosphatidylinositol 3-kinase (PI3K)/protein kinase Akt pathway.<sup>15,17,18</sup> The recruitment of this latter cascade relies on the ligand-dependent association of ER $\alpha$  with PI3K.<sup>15</sup>

Nothing is presently known regarding the potential effects of SERMs on the nongenomic signaling via ER. Because NO plays an important role in cardiovascular disease, we tested the hypothesis that the SERM raloxifene can activate eNOS via a nontranscriptional signaling pathway involving PI3K/Akt.

## Methods

### Cell Cultures

Human umbilical vein endothelial cells (HUVECs) from male or female newborns were harvested with type IA collagenase (1 mg/mL)<sup>12</sup> and maintained in phenol red-free DMEM (Gibco) containing HEPES (25 mmol/L), heparin (50 U/mL), ECGF (50  $\mu$ g/mL), L-glutamine (2 mmol/L), antibiotics, and 5% estrogen-deprived FBS. FBS was deprived of estrogen by charcoal stripping. Before every experiment, HUVECs were serum starved for 8 hours in phenol red-free DMEM containing no FBS. HUVECs were grown to confluence and used up to passage 2 for the experiments.

### eNOS Activity Assay

HUVECs were harvested in PBS with 1 mmol/L EDTA. eNOS activity was determined in cell lysates as conversion of [<sup>3</sup>H]-arginine to [<sup>3</sup>H]-citrulline.<sup>15</sup> Extracts incubated with the eNOS inhibitor L-NAME (1 mmol/L) served as blank. eNOS activity was obtained, subtracting the blank to the samples.

### Nitrite Assay

NO production in the culture medium was determined by a nitrite assay using 2,3 diaminonaphthalene.<sup>12</sup> Fluorescence of 1-(H)-naphotriazole was measured with excitation and emission wavelengths of 365 and 450 nm. Standard curves were constructed with sodium nitrite. Nonspecific fluorescence was determined in the presence of LNMA (3 mmol/L).

### PI3K Assay

HUVECs were harvested in lysis buffer (137 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4, 1 mmol/L CaCl<sub>2</sub>, 1 mmol/L MgCl<sub>2</sub>, 0.1 mmol/L Na<sub>2</sub>VO<sub>4</sub>, and 1% NP-40). Equal amounts of cell lysates were immunoprecipitated with TE111 mAb (NeoMarkers, Union City, Calif), recognizing a C-terminal (aa 302-595) fragment of ER $\alpha$ . PI3K activity was assayed in ER $\alpha$  immunoprecipitates using phosphatidylinositol-4,5-bis phosphate as substrate (Biomol, Plymouth Meeting, Pa).<sup>15</sup> Phospholipids were extracted with chloroform/methanol (1:1, vol/vol) and separated with borate thin-layer chromatography.<sup>15</sup>

### Immunoprecipitations

HUVECs were harvested in 100 mmol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 1 mmol/L Na<sub>2</sub>VO<sub>4</sub>, 1 mmol/L NaF, and 1 mmol/L PMSF. Equal amounts of cell lysates were incubated with 1  $\mu$ g of precipitating ER $\alpha$  (clone TE111), ER $\beta$  (clone L-20, Santa Cruz Biotechnology, Santa Cruz, Calif) or eNOS (Transduction Laboratories, Lexington, Ky) antibody for 1 hour at 4°C under gentle agitation. Next, 25  $\mu$ L of a 1:1 protein A agarose slurry was added, and the samples were rolled at 4°C for another hour. The samples were then pelleted, washed, and resuspended in 50  $\mu$ L of  $\times$ 2 Laemmli buffer for immunoblotting.

### Immunoblotting

Protein extracts (25  $\mu$ g) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Antibodies (0.4  $\mu$ g of antibody/mL) versus p110 $\alpha$  or p85 $\alpha$  (Upstate Biotechnology, Lake Placid, NY), eNOS (Transduction Laboratories), Akt, (Ser 473) phospho-Akt, or (Thr 308) phospho-Akt (New England BioLabs, Beverly, Mass) were incubated with the blots overnight at 4°C. The blots were washed and incubated with secondary antibodies (0.1  $\mu$ g of antibody/mL) coupled to horseradish peroxidase. Immunodetection was accomplished using the ECL kit (Amersham).

### Akt Kinase Assay

HUVECs were harvested in 50 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.5 mol/L Na<sub>2</sub>VO<sub>4</sub>, 0.1% (vol/vol)  $\beta$ -ME, 1% Triton X-100, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 10 mmol/L sodium glycerophosphate, 0.1 mmol/L PMSF, 1  $\mu$ g/mL of aprotinin, pepstatin, leupeptin, and 1  $\mu$ mol/L microcystin. Akt activity was determined in cell lysates using the PKB/Akt kinase assay kit (Upstate Biotechnology). The peptide RPRAAATF corresponding to the Akt phosphorylation site of glycogen synthase kinase-3 was used as substrate. The cpm of the control samples was taken as 1, and the other values were normalized and expressed as fold versus control.

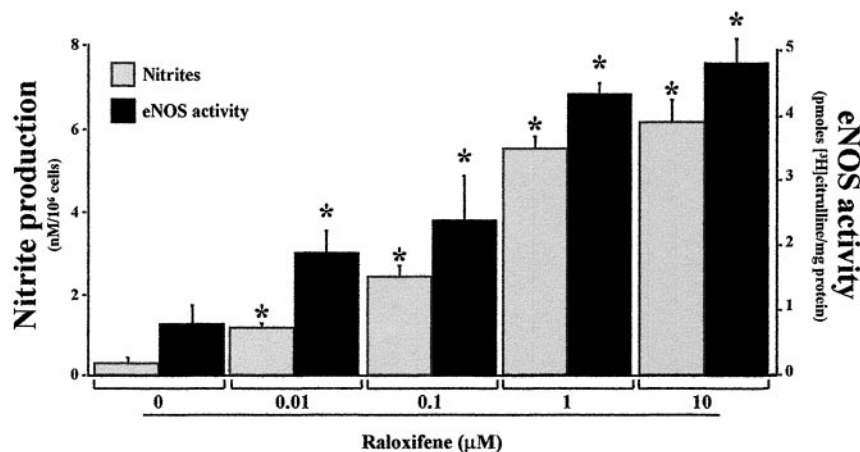
### Statistical Analysis

All values are expressed as mean $\pm$ SD. Statistical differences between mean values were determined by ANOVA, followed by the Fisher's protected least-significance difference test for comparison of mean values.

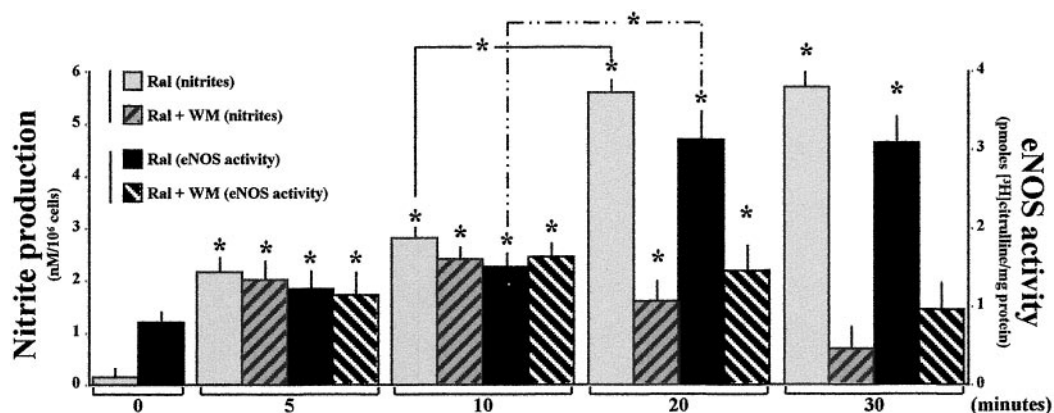
## Results

### Raloxifene Activates eNOS and NO Synthesis in HUVECs

As we recently showed in another study,<sup>12</sup> a 30-minute raloxifene treatment of estrogen-deprived, serum-starved HUVECs produced a concentration-dependent increase in NO synthesis in the culture medium (Figure 1) attributable to



**Figure 1.** Estrogen-deprived, serum-starved HUVECs were treated for 30 minutes with increasing concentrations of raloxifene. Gray bars indicate nitrite concentrations in culture media; black bars show eNOS activity in cell lysates. \* $P$ <0.05 vs control. The experiments were repeated 3 times in quadruplicates (nitrite measurements) or triplicates (eNOS activity), with comparable results.



**Figure 2.** Estrogen-deprived, serum-starved HUVECs were treated with raloxifene (Ral) (1  $\mu\text{mol/L}$ ) for different times in the presence or absence of wortmannin (WM) (30 nmol/L, added 30 minutes before raloxifene). Nitrite concentrations in culture media and eNOS activity in cell lysates were measured. \* $P < 0.05$  vs control. The experiments were repeated twice in quadruplicates (nitrite measurements) or triplicates (eNOS activity), with equal results.

a rapid activation of eNOS (Figure 1).

### PI3K Mediates Nontranscriptional eNOS Activation by Raloxifene

Raloxifene (1  $\mu\text{mol/L}$ ) induced a time-dependent increase in NO release in the culture medium and in eNOS activity in cell lysates (Figure 2). The addition of the specific PI3K inhibitor wortmannin (30 nmol/L) 30 minutes before raloxifene treatment blocked NO synthesis increase and eNOS activation. However, wortmannin was only able to inhibit raloxifene-induced eNOS activity at 20 and 30 minutes.

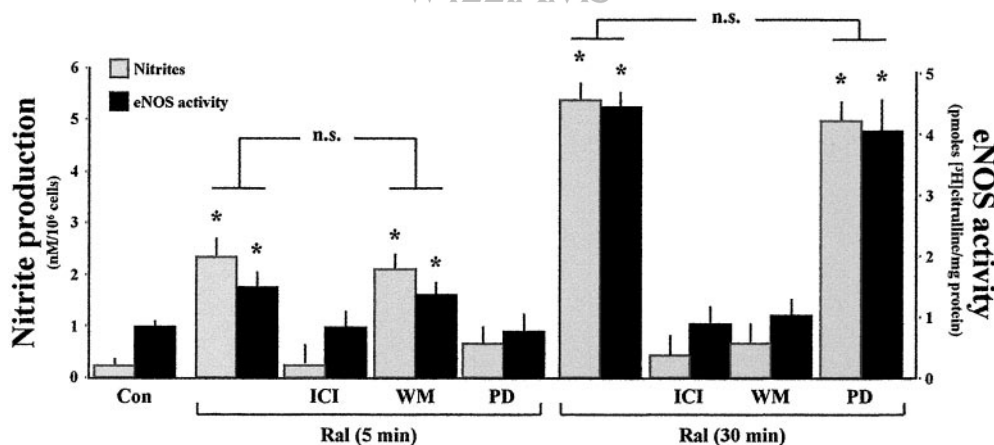
Pretreatment of HUVECs with a pure ER antagonist, ICI 182,780 (10  $\mu\text{mol/L}$ ), completely prevented raloxifene-induced NO release and eNOS activation (Figure 3). Additionally, the MEK-1/2 inhibitor PD 98059 (5  $\mu\text{mol/L}$ ) prevented the early (but not the later) rise in NO synthesis and eNOS activation, whereas opposite effects were seen with wortmannin (30 nmol/L) (Figure 3).

When HUVECs were stimulated with raloxifene for 30 minutes in the presence of transcriptional inhibitors, actinomycin D or 5,6-dichlorobenzimidazole riboside (DRB), or the

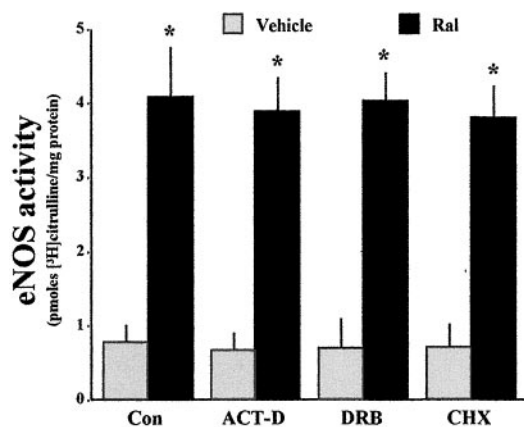
translational inhibitor cycloheximide (all added 30 minutes before raloxifene), neither actinomycin D (5  $\mu\text{mol/L}$ ), DRB (50  $\mu\text{mol/L}$ ), nor cycloheximide (10  $\mu\text{mol/L}$ ) affected raloxifene-stimulated eNOS activity (Figure 4), confirming that raloxifene activates eNOS via nontranscriptional mechanisms.

### Raloxifene Increases ER $\alpha$ -PI3K Association

The ER isoform, ER $\alpha$ , binds to the PI3K regulatory subunit p85 $\alpha$  in a ligand-dependent manner.<sup>15</sup> To determine whether raloxifene can also promote this interaction, we performed coimmunoprecipitation studies using a mAb versus a C-terminal fragment of ER $\alpha$ . Raloxifene increased the amount of p85 $\alpha$  associating with ER $\alpha$  (Figure 5A). Similar results were obtained for the PI3K catalytic subunit p110 $\alpha$  (Figure 5A). Both raloxifene-dependent p85 $\alpha$  and p110 $\alpha$  associations with ER $\alpha$  were reversed by ICI 182,780 (Figure 5A). The corresponding polyacrylamide gel was silver stained (Figure 5A), and comparable amounts of precipitating antibody and immunoprecipitated proteins were found in every condition. We additionally checked whether the PI3K



**Figure 3.** Estrogen-deprived, serum-starved HUVECs were treated with raloxifene (1  $\mu\text{mol/L}$ ) for 5 or 30 minutes in the presence or absence of ICI 182,780 (10  $\mu\text{mol/L}$ ), wortmannin (WM) (30 nmol/L), or PD 98059 (5  $\mu\text{mol/L}$ ) (all added 30 minutes before raloxifene). \* $P < 0.05$  vs control. n.s. indicates not significant. The experiment was repeated twice in quadruplicates (nitrite measurements, gray bars) or triplicates (eNOS activity, black bars), with equal results.



**Figure 4.** HUVECs were treated with raloxifene (Ral) (1  $\mu\text{mol/L}$ ) for 30 minutes in the presence or absence of actinomycin D (ACT-D) (5  $\mu\text{mol/L}$ ), 5,6-dichlorobenzimidazole riboside (DRB) (50  $\mu\text{mol/L}$ ), or cycloheximide (CHX) (10  $\mu\text{mol/L}$ ), and eNOS activity was assayed in cell extracts. Con indicates control. \* $P < 0.05$  vs control. The experiment was repeated 3 times in triplicates, with equal results.

effector Akt or eNOS can form a signaling complex with ER $\alpha$  and PI3K. No ER $\alpha$ -associated Akt or eNOS could be found either basally or on treatment (Figure 5A).

Additionally, because raloxifene has different affinities to ER $\alpha$  or ER $\beta$ <sup>19</sup> and induces different conformations from the ones induced by estradiol,<sup>20,21</sup> we investigated whether raloxifene has similar effects on ER $\beta$ -PI3K association. However, no association of p85 $\alpha$  with ER $\beta$  was found after stimulation with raloxifene (Figure 5B).

### Raloxifene Increases ER $\alpha$ -Associated PI3K Activity

PI3K is activated by the interaction of p85 $\alpha$  with regulatory molecules, such as IRS-1/2. We performed immunoprecipitations on HUVEC lysates using an anti-ER $\alpha$  antibody, and we then subjected the IPs to an in vitro PI3K activity assay. Raloxifene increased PI3K activity in ER $\alpha$  immunoprecipitates in a concentration-dependent manner (Figure 6A). Pretreatment of HUVECs with ICI 182,780 (10  $\mu\text{mol/L}$ ) or wortmannin (30 nmol/L) but not with PD 98059 (5  $\mu\text{mol/L}$ ) prevented raloxifene-induced PI3K activation (Figure 6A). Moreover, PI3K activity in ER $\alpha$  immunoprecipitates was increased by raloxifene in a temporal pattern consistent with the activation of eNOS (Figure 6B).

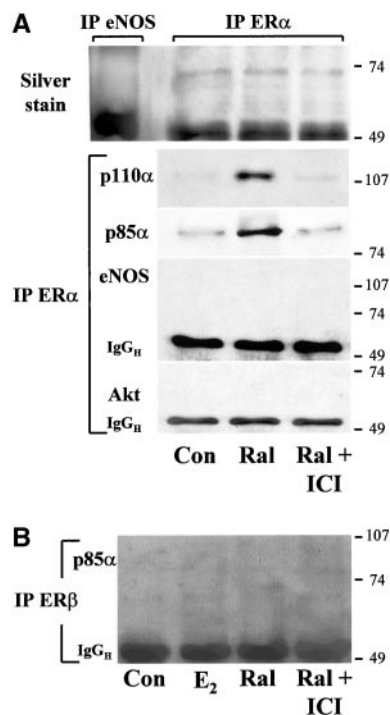
### Activation of Akt by Raloxifene

The main effector of PI3K is Akt, which phosphorylates and rapidly activates eNOS.<sup>22,23</sup> Akt is activated by 2 independent phosphorylations on serine-473 and threonine-308. We found that raloxifene (1  $\mu\text{mol/L}$ ) increased Akt serine and threonine phosphorylation, consistent with PI3K and eNOS activation (Figure 7A). Akt phosphorylations were inhibited by either ICI 182,780 or wortmannin. To determine whether Akt phosphorylation correlated with increased Akt activity, we performed Akt kinase activity assays on HUVECs. Raloxifene (1  $\mu\text{mol/L}$ , 20 minutes) increased Akt activity by 2.8-fold, which was completely abolished by ICI 182,780 (10  $\mu\text{mol/L}$ ) or wortmannin (30 nmol/L) (Figure 7B). In contrast,

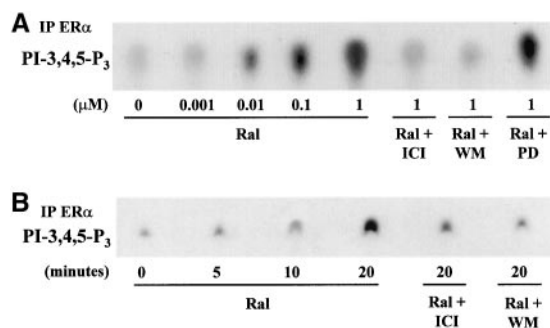
pretreatment with PD 98059 (5  $\mu\text{mol/L}$ ) had no effect on raloxifene-stimulated Akt kinase activity (Figure 7B).

### Discussion

In recent years, new models of signal transduction via the estrogen receptors have been identified that do not involve transcriptional gene regulation. These nontranscriptional or nongenomic mechanisms mediate relevant processes in the blood vessels, such as regulation of NO synthesis by estrogen, but the molecular basis has been clarified only in part.<sup>15</sup> Our findings suggest that SERMs trigger ER-mediated nontranscriptional signaling, similarly to estrogen. Raloxifene increases the association of ER $\alpha$  with the lipid kinase PI3K, resulting in PI3K recruitment and activation. PI3K, in turn, leads to the recruitment of phosphatidylinositol-dependent kinases and protein kinase Akt, which eventually phosphorylates and activates eNOS.<sup>22,23</sup> Although this pathway has been described for estrogen,<sup>15</sup> it is intriguing that raloxifene, which has a different chemical structure compared with estrogen, also activates this pathway. Indeed, compared with estrogen, SERMs induce different ER conformations, and, particularly, the positioning of helix 12 in the ligand binding domain is different with 17 $\beta$ -estradiol or raloxifene.<sup>20,21</sup> Nonetheless, raloxifene-induced ER $\alpha$  conformation is able to interact with PI3K. On the other hand, because estrogen



**Figure 5.** Estrogen-deprived, serum-starved HUVECs were treated with raloxifene (Ral) (1  $\mu\text{mol/L}$ ) for 30 minutes in the presence or absence of ICI 182,780 (10  $\mu\text{mol/L}$ , 30 minutes before raloxifene). A, Cell extracts were immunoprecipitated with mAb vs ER $\alpha$  (IP ER $\alpha$ ) or vs eNOS (IP eNOS) and immunoblotted for p110 $\alpha$ , p85 $\alpha$ , eNOS, or Akt. Alternatively, immunoprecipitates were separated with SDS-PAGE and the gel was silver stained. B, HUVEC lysates were immunoprecipitated with anti-body vs ER $\beta$  and immunoblotted with anti-p85 $\alpha$ . E<sub>2</sub> indicates 17 $\beta$ -estradiol (10 nmol/L, 30 minutes). All blots are representative of at least 3 different experiments, with comparable results.



**Figure 6.** A, After treatment with raloxifene (Ral) (30 minutes) at increasing concentrations in the presence or absence of ICI 182,780 (10  $\mu\text{mol/L}$ ), wortmannin (WM) (30 nmol/L), or PD 98059 (5  $\mu\text{mol/L}$ ) (all added 30 minutes before raloxifene), HUVEC lysates were immunoprecipitated with antibody vs ER $\alpha$ . B, HUVEC lysates treated with raloxifene (1  $\mu\text{mol/L}$ ) for different durations in the presence or absence of ICI 182,780 (10  $\mu\text{mol/L}$ ) or of wortmannin (30 nmol/L), both added 30 minutes ahead of raloxifene, were immunoprecipitated with an anti-ER $\alpha$  antibody. The immunoprecipitates were assayed for PI3 kinase activity. Shown are the D-3 phosphorylated phosphoinositides. The experiments are representative of 4 different assays, showing similar results.

agonists and antagonists systematically induce different ER conformations,<sup>24</sup> it is possible that not all natural or synthetic estrogens activate this pathway with biological and clinical implications.

Raloxifene concentrations of 100 to 200 nmol/L have been shown to induce a 50% shift in estrogen receptor conformation.<sup>24</sup> On MCF-7 cells, even lower doses of raloxifene exert biologic effects, whereas concentrations between 100 nmol/L and 1  $\mu\text{mol/L}$  are needed to achieve ER occupancies between 80% and 100%.<sup>25</sup> In HUVECs, near-maximal stimulation of eNOS and NO synthesis were achieved with slightly higher concentrations (1  $\mu\text{mol/L}$ ) of raloxifene, possibly because of the lower cellular amount of ERs in endothelial compared with MCF-7 cells. The concentrations of raloxifene used in this study are consistent with the doses inducing endothelium-dependent relaxation in coronary arteries.<sup>13</sup> Because most of the experiments were performed using these concentrations, which are slightly higher than what is achieved in humans during standard therapies, potential nonspecific effects of raloxifene cannot be completely excluded.

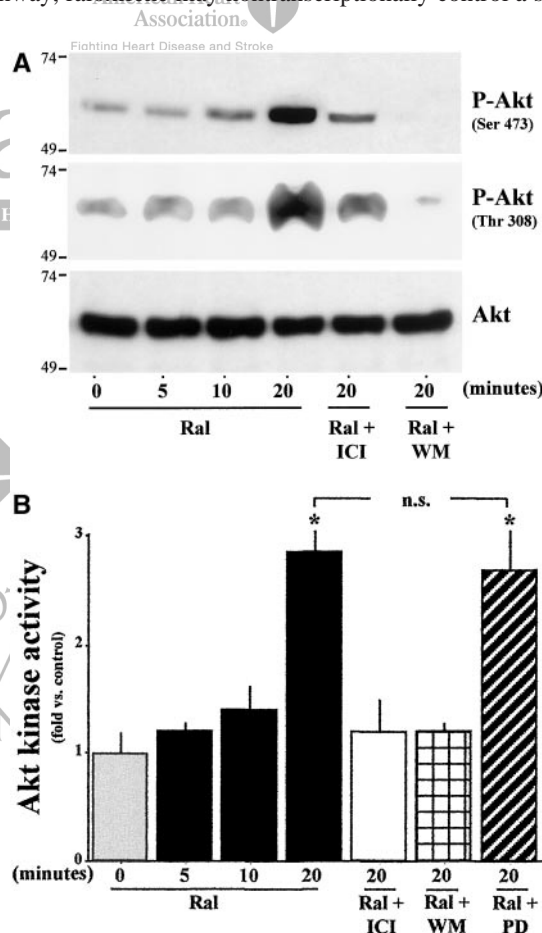
The understanding of the basis of ER $\alpha$ /PI3K interaction awaits the identification of the docking domains on the two proteins. The SH<sub>2</sub> and SH<sub>3</sub> domains on p85 $\alpha$  are not responsible for binding to ER $\alpha$ ,<sup>15</sup> but human p85 $\alpha$  contains two Leu-X-X-Leu-Leu sequences (PubMed P27986), the roles of which have not been investigated. ER $\beta$  is unable to interact with PI3K, suggesting that the site of interaction on ER $\alpha$  could be different from the DNA-binding domain, which shares the highest homology with ER $\beta$ .<sup>26</sup>

Our results also show that the initial rapid activation of eNOS by raloxifene can be blocked by a MEK-1/2 inhibitor, indicating that raloxifene may activate the mitogen-activated protein (MAP) kinase cascade through ERs in endothelial cells. The sequential inhibition of raloxifene-induced eNOS activation by PD 98059 and wortmannin thus stands for an activation of MAP kinases, followed by activation of PI3K on

raloxifene treatment of endothelial cells. However, MAP kinase activation is not required for the activation of PI3K.<sup>15</sup>

By controlling NO synthesis, raloxifene may exert vascular protective actions, because NO possesses anti-inflammatory and antiatherogenic effects.<sup>27</sup> Indeed, the loss of endothelium-derived NO leads to enhanced platelet aggregation, increased vascular smooth muscle cell proliferation, and endothelial-leukocyte interactions.<sup>27</sup> Consistent with this hypothesis is the inhibitory action of raloxifene on endothelial-leukocyte interaction<sup>28</sup> that could be attributable to NO production.<sup>29</sup> These antiatherogenic actions may be clinically important, because large studies show that HRTs are not cardioprotective when atherosclerotic lesions are established.<sup>30,31</sup> Thus, the potential cardiovascular benefits of estrogens may rely on the prevention of atherosclerosis rather than on actions on advanced atherosclerotic plaques.

Furthermore, through the activation of the ER $\alpha$ /PI3K/Akt pathway, raloxifene may nontranscriptionally control a series



**Figure 7.** HUVECs were treated with raloxifene (Ral) (1  $\mu\text{mol/L}$ ) for different times in the presence or absence of ICI 182,780 (10  $\mu\text{mol/L}$ ), wortmannin (WM) (30 nmol/L), or PD 98059 (in 7B) (all added 30 minutes before raloxifene). A, Cell extracts were immunoblotted with antibodies recognizing nonphosphorylated Akt (lower box), Thr-308-phosphorylated Akt (middle box), and Ser-473-phosphorylated Akt (upper box). The experiments are representatives of 3 replicates that provided the same results. B, Cell extracts were immunoprecipitated with an antibody vs Akt, and Akt kinase assays were performed. \* $P < 0.05$  vs control. n.s. indicates not significant. The experiment was repeated 2 times in triplicates, with equal results.

of cellular functions, such as cell survival, cellular uptake of glucose, glycogen synthesis, the lipolytic process, and gene transcription.<sup>32</sup>

In summary, we provide a mechanism by which SERMs elicit nontranscriptional signaling via ER. By promoting the interaction of ER $\alpha$  with PI3K, raloxifene rapidly activates eNOS and stimulates NO production in endothelial cells. This nontranscriptional signaling mechanism of ER may account for some of the cardiovascular protective effects of raloxifene as well as other SERMs.

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