Estradiol-Mediated Endothelial Nitric Oxide Synthase Association With Heat Shock Protein 90 Requires Adenosine Monophosphate–Dependent Protein Kinase

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Background—Estradiol activates endothelial nitric oxide synthase (eNOS) by mechanisms that involve estrogen receptor-α (ERα), protein kinase B/Akt, mitogen-activated protein kinases, and heat shock protein 90 (HSP90). Recently, AMP-activated protein kinase (AMPK), an enzyme that plays a crucial role in cellular adaptation to metabolic stress, has been implicated in physiological eNOS activation by the hormones adiponectin and insulin. We therefore investigated whether AMPK is activated by estradiol in endothelial cells and plays a role in estradiol-induced eNOS activation.

Methods and Results—Porcine aortic endothelial cells exhibited time- and concentration-dependent AMPK activation as determined by phosphorylation of AMPK and its downstream target acetyl coenzyme A carboxylase in response to estradiol (1 nmol/L to 10 μmol/L, 1 to 30 minutes). AMPK activation by estradiol was independent of both AMP levels and ERα but required estradiol conversion to its catechol metabolites. Estradiol treatment increased eNOS catalytic activity, an effect that was largely reversed when endothelial cells were infected with an AMPK dominant-negative adenovirus. However, inhibition of AMPK did not alter estradiol-induced eNOS phosphorylation at serine 1177 or threonine 495 but decreased eNOS interaction with HSP90. Consistent with this observation, blood vessels from α1-AMPK–null mice exhibited defective eNOS-mediated NO production in response to estradiol.

Conclusions—Taken together, these data indicate that AMPK activity is essential for estradiol-induced eNOS activation via the promotion of eNOS interaction with HSP90. These data point to a novel role for AMPK in modulating endothelial cell NO bioactivity and HSP90 function. (Circulation. 2005;111:3473-3480.)

Key Words: endothelium ■ nitric oxide synthase ■ signal transduction ■ adenylyl kinase ■ heat shock proteins

The acute administration of estradiol leads to a rapid increase in coronary blood flow that is now known to result from activation of the endothelial isoform of nitric oxide synthase (eNOS). Although endothelial cells express membrane-associated estrogen receptors (ERs) of both α- and β-isomers, only the α-isofrom mediates eNOS activation. Ligand engagement of ERα initiates its association with the p85α subunit of phosphoinositol 3-kinase and c-Src, leading to Akt activation. This event promotes eNOS phosphorylation at serine 1177, a key event that stimulates enzyme activation and enhances its sensitivity to calcium. The entire process of ERα-mediated eNOS activation also involves the mitogen-activated protein kinase (MAPK) pathway, as MAPK/extracellular signal-regulated kinase (ERK) inhibition effectively attenuates the effects of estradiol. Although the mechanism for this observation is not clear, MAPK has been implicated in ERα translocation among cellular compartments, and ERα movement into caveolae may be involved in eNOS activation. These data underscore the pleiotropic effects of ERα in modulating endothelial cell NO bioactivity.

Estradiol also promotes the association of eNOS with heat shock protein 90 (HSP90), although the precise mechanism(s) involved in this process is not yet clear. Nevertheless, HSP90 is clearly required for maximal eNOS activation, and this chaperone exhibits a predilection for proteins involved in signal transduction, including hormone receptors. Indeed, HSP90 has been found in association with the ER and is implicated in steroid receptor action. Thus, there is precedent for involvement of HSP90 in hormone receptor responses. As its name suggests, HSP90 is also part of the cellular response to environmental stress. For example, hypoxia, oxidative stress, osmotic shock, and arsenic are all known to result in upregulation of HSP90 protein and chaperone activity. Among other cellular stress responses, activation...
of AMP-dependent protein kinase (AMPK) has recently garnered considerable interest. This enzyme is activated in response to both metabolic and oxidative insults and is also a key regulator of cellular energy metabolism.

**Methods**

**Materials**

Cell culture reagents were obtained from Invitrogen, and compound C was a kind gift from Merck Research Laboratories (Piscataway, NJ). We obtained LY294002, 4-amino-5-(4-chlorophenyl)-7-((3-buty1)pyrazolo[3,4-b]pyrimidine (PP2), bisindolylmaleimide, PD98059, SB202190, and 17β-estradiol from Calbiochem. The estradiol metabolites 2-hydroxyestradiol and 2-methoxyestradiol were purchased from Steraloids. The ER antagonist ICI 182,780 was obtained from Tocris. Polyclonal antibodies against phospho-AMPK (Thr-172), α-AMPK, phosphoacetyl coenzyme A carboxylase (ACC) (Ser-79), and phospho-Akt (Ser-473) were from Cell Signaling Technology. Antibodies against ACC, α-AMPK, phospho-ACC, and phospho-eNOS (Ser-1177) were from Cell Signaling Technology, and the specific AMPK target peptide (SAMSGLHVLVKRR) (SAMS peptide) were from Upstate Biotechnology. The monoclonal eNOS antibody was from BD Biosciences, and the monoclonal ERα antibody was from NeoMarkers. We obtained [32P]ATP (250 µCi, 10 mCi/mL) and [3H]-l-citrulline (1 mCi/mL) from Perkin Elmer Life Sciences. 1-AMPK–null mice were kindly provided by Dr B. Viollet (Institut Cochin, Paris, France) and have been described.

**Adenovirus Transfection**

A dominant-negative α-AMPK mutant—expressing adenovirus that is known to inhibit basal and stimulated AMPK activity was obtained from Dr Morris J. Birnbaum, University of Pennsylvania. Cells were infected at a multiplicity of infection of 50 as described, and a β-galactosidase (LacZ)–expressing adenovirus (50 multiplicity of infection) was used as a control.

**Cell Culture**

Porcine aortic endothelial cells (PAECs) were harvested from juvenile male pigs and cultured on fibronectin-coated tissue-culture flasks in M199 supplemented with 20% fetal bovine serum, 50 µg/mL heparin sulfate, 2 mM/L L-glutamine, 100 µg/mL penicillin, and 100 µg/mL streptomycin. Before all experiments with eigrostogens, cells were cultured for at least 12 hours in phenol-free medium (Opti-MEM, Invitrogen) supplemented with 1% charcoal-stripped dextran (Sigma). Cyclic GMP levels were measured in vessel extracts with a Bioluminescent Somatic Cell Assay Kit (Sigma). Aliquots of the plasma were added to the agonist and incubated for 30 minutes. The product, adenyl-luciferin, causes light emission by its reaction with ambient O2. Luminescence was recorded every 10 seconds for 1 minute in a Turner Designs luminometer. Determination of AMPK catalytic activity in endothelial cells was performed by incorporation of radioactively labeled [3H]l-citrulline. Confluent PAECs in 6-well plates were washed twice and equilibrated for 30 minutes in PSS. The cells were then treated with estradiol or buffer immediately before the addition of 10 µmol/L L-arginine with 3.3 µCi of [3H]l-citrulline. After 15 minutes, cells were washed with PSS and lysed with 100 µL of 100% ethanol, and [3H]l-citrulline was determined by anion-exchange chromatography as previously described.

**Immunoblotting**

After various treatments, cells were washed twice with cold PSS and lysed in 100 µL of loading buffer containing 50 mM/L Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 200 mM/L dithiothreitol, 20% glycerol, and 0.1% bromophenol blue. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 7.5% to 12% gels, transferred to a nitrocellulose membrane, and incubated with the primary antibody overnight (1:1000). After washing and addition of the secondary antibody, protein bands were visualized with ECL reagent (Amersham Biosciences).

**Reverse Transcription–Polymerase Chain Reaction**

Human aortic endothelial cells in 100-mm dishes were washed in phosphate-buffered saline and subjected to RNA isolation. After RNA extraction, reverse transcription–polymerase chain reaction was performed with forward (sense) and reverse (antisense) primers. Nucleotide sequences for human AMPK isoforms were designed as follows: α1 sense, 5′-GAG TCT CCT GAG AGG and α1 antisense, 5′-AGG GGA AGG GTT CCA CAT AAT; α2 sense, 5′-GAG GGT GAA GTA TTA TTG and α2 antisense, 5′-CCA TTC ATC TCT TAT; β1 sense, 5′-CAA GGA GGT TGT TCC GAT GGA and β2 antisense, 5′-TAA AGG TGA TGA CTT ACT TCT; β1 sense, 5′-TCC GAT TTG GAG GAC TCC GTA and β2 antisense, 5′-AGG CAT CAC CAT ACT GTC; γ1 sense, 5′-GAG AAC GAT TCT CGC TGC and γ1 antisense, 5′-CCA ATC TGC AGC TCT GCC; γ1 sense, 5′-AGG CAT CAC CAT ACT GTC; γ1 sense, 5′-GAG AAC GAT TCT CGC TGC and γ1 antisense, 5′-CCA ATC TGC AGC TCT GCC; γ1 sense, 5′-AGG CAT CAC CAT ACT GTC; γ1 sense, 5′-GAG AAC GAT TCT CGC TGC and γ1 antisense, 5′-CCA ATC TGC AGC TCT GCC.
by phosphorylation of AMPK and its downstream target ACC (Figure 1A and 1B). Similar results were obtained when AMPK activity was measured by incorporation into the AMPK-specific SAMS peptide (Figure 1C). Thus, estradiol activates AMPK over a physiological concentration range.

AMPK Activation by Estradiol Is Independent of AMP

To determine whether estradiol-induced AMPK activation depends on AMP, we used the adenylate kinase inhibitor 5-iodotubercidin to block intracellular AMP formation from ADP. We found that 5-iodotubercidin had no effect on PAEC AMPK activation (Figure 2A and 2B). As one might predict, estradiol also had no effect on cellular ATP levels that would ultimately result in AMP formation (Figure 2C). Thus, estradiol activates AMPK independent of AMP formation.

AMPK Activation by Estradiol Is Independent of ERs and Does Not Involve ERK, p38 MAPK, Phosphoinositol 3-Kinase, Src, or Protein Kinase C

Because functional ERs are required for nongenomic effects of estradiol, we sought to determine whether ERs mediate estradiol-induced AMPK activation. Despite robust expression of ERα (data not shown), estradiol-induced AMPK activation was not inhibited by the ER antagonist ICI182,780 (Figure 3A and 3B). In contrast, the AMPK inhibitor, compound C, potently suppressed basal and estradiol-
stimulated AMPK activity in endothelial cells (Figure 3C and 3D). These data indicate that estradiol activates AMPK independent of ERs.

To examine possible upstream signals that mediate AMPK activation by estradiol, we used inhibitors of several signaling pathways known to be modulated by estrogens. As shown in Figure 3E, inhibition of Src-family kinases (PP2), phosphoinositol 3-kinase (LY294002), ERK (PD98059), or protein kinase C (bisindolylmaleimide and Gö6893) had no material impact on estradiol-induced AMPK activation. These data indicate that estradiol must activate AMPK by mechanisms distinct from signals associated with ER activation.

**Estradiol-Induced AMPK Activation Is Mediated by Catechol Estradiol Metabolites**

Previous studies suggest that estradiol conversion to 2-hydroxyestradiol and 2-methoxyestradiol is involved in its antimitogenic activity in smooth muscle cells. These estradiol metabolites act largely in a receptor-independent manner, as they have only limited affinity for ERs. Estradiol hydroxylation at C-2 and C-4 via the cytochrome p450 class of enzymes yields the catechol estradiols 2- and 4-hydroxyestradiol that, in turn, may be converted into methoxyestradiols via catechol-O-methyltransferase (for review, see Dubey et al). We found that inhibition of hydroxyestradiol formation with the cytochrome p450 inhibitor 1-aminobenzotriazole attenuated estradiol-mediated AMPK activation (Figure 4A). In contrast, inhibition of
hydroxyestradiol conversion to their methoxy counterparts with the catechol-O-methyltransferase inhibitor Ro 41-0960 enhanced estradiol-induced AMPK activation (Figure 4A), suggesting that hydroxyestradiols mediate AMPK activation by estradiol. Consistent with this notion, 2-hydroxyestradiol significantly increased AMPK activity, whereas 2-methoxyestradiol was less effective (Figure 4B). Taken together, these results suggest that estradiol-induced AMPK activation is mediated by its conversion to hydroxyestradiols.

Estradiol-Induced eNOS Activation Requires AMPK
We next investigated the role of AMPK in estradiol-induced eNOS activation. Estradiol induced dose-dependent eNOS activation, as manifested by enhanced [3H]-L-citrulline production, eNOS Ser-1177 phosphorylation, and eNOS association with HSP90 (Figure 5A and 5B). We also found that AMPK exists in a complex with eNOS under basal conditions, and this did not change as a function of estradiol stimulation (Figure 5B). Inhibition of AMPK activity with either a dominant-negative AMPK mutant (Figure 5C) or compound C abrogated estradiol-mediated eNOS activation (Figure 5D). Thus, estradiol-mediated eNOS activation requires the catalytic activity of AMPK.

Estradiol-Induced HSP90 Association With eNOS Requires AMPK
To determine the mechanism involved in AMPK-mediated eNOS activation, we examined eNOS phosphorylation status and HSP90 binding in response to estradiol. As expected, estradiol stimulated Akt, resulting in eNOS Ser-1177 phosphorylation (Figure 6). This process was not altered by either pharmacological or molecular inhibition of AMPK (Figure 6). In contrast, AMPK inhibition significantly attenuated eNOS association with HSP90 (Figure 6). These data implicate AMPK in the dynamic modulation of eNOS activity by HSP90.

Estradiol-Induced eNOS Activation In Vivo Requires α1-AMPK
We found in preliminary experiments with reverse transcription–polymerase chain reaction and immunoblotting that endothelial cells predominantly express the α1, β1, and γ1 isoforms of AMPK (data not shown). Accordingly, to determine the physiological significance of our in vitro results, we examined estradiol-mediated eNOS activation in intact aortic segments from wild-type and α1-AMPK–null mice. Wild-type mice exhibited estradiol-mediated eNOS activation manifested as an increase in aortic cGMP content (P<0.05, n=4) that was inhibited by 500 μmol/L nitro-L-arginine methyl nitro-L-arginine methyl

**Figure 5.** Estradiol-mediated eNOS activation involves AMPK. A, PAECs were equilibrated in PSS for 30 minutes. Directly after addition of [3H]-L-arginine, cells were treated with estradiol as indicated for 15 minutes, and [3H]-L-citrulline formation was determined as described in Methods. CTL indicates control. B, PAECs were treated with 100 nmol/L estradiol (E2) for indicated times and lysed, and eNOS was immunoprecipitated (IP). Precipitates were probed for eNOS, phospho-eNOS, α-AMPK, and HSP90 as indicated by immunoblotting (IB). C, PAECs were transfected (50 multiplicity of infection) with adenoviral vectors expressing either dominant-negative(DN) AMPK mutant or β-galactosidase (LacZ) in regular medium. After 48 hours, cells were equilibrated in PSS for 30 minutes, treated with 100 nmol/L estradiol (E2) for indicated time, and lysed, and AMPK activation was determined by immunoblotting as in Figure 1. D, PAECs treated as in C or incubated with 20 μmol/L compound C (20 minutes) were treated with 100 nmol/L estradiol (E2) or its vehicle (VEH) for 2 minutes, and eNOS activity was determined as in A. Data are mean±SEM of 4 experiments. *P<0.05 vs corresponding vehicle by 1-way ANOVA with a Dunnett comparison.
ester (Figure 7C). In contrast, α1-AMPK–null mice demonstrated no significant estradiol-mediated eNOS activation \((P=0.17, n=4)\) despite DEA-NONOate–induced cGMP accumulation that was comparable to that of wild-type mice (Figure 7C). These data indicate that estradiol-mediated eNOS activation in vivo requires α1-AMPK.

**Discussion**

The principal finding of our study is that AMPK is activated by estradiol and appears required for estradiol-mediated eNOS activation. This stimulation of AMPK did not depend on ERs, because it was insensitive to the ER antagonist ICI182,780. Instead, AMPK activation was related to the metabolism of estradiol to hydroxyestradiol. We also found that activation of AMPK was required for HSP90 association with eNOS, a well-known requirement for optimal eNOS-mediated NO production.13,28 In contrast, estradiol-mediated Akt activation was not dependent on AMPK catalytic activity, suggesting that estrogen promotes eNOS activation through 2 distinct, yet complementary, pathways (Figure 8). Common downstream targets of AMPK include key enzymes of glucose and lipid metabolism,29,30 and AMPK activation tends to suspend energy-consuming pathways and stimulate energy production.17 These properties of AMPK are consistent with its known role as an adaptation to metabolic stress, and others have speculated that this enzyme may be part of a generalized cellular stress response.17 It is also known that AMPK may be activated in response to hormones such as adiponectin31 and leptin,32 which are known to modulate fatty acid oxidation. We now add estradiol to the known hormonal stimuli that lead to AMPK activation. At first glance, this link between estradiol and AMPK may seem incongruous, as estrogen does not have an established role in the control of cellular energy status. However, recent studies with the aromatase-knockout mouse indicate that estrogen may have important implications for fatty acid oxidation. Animals lacking aromatase are unable to synthesize endogenous estradiol, and they exhibit hepatic steatosis33 and increased adiposity34 owing to impaired fatty acid oxidation.

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Because fatty acid oxidation is regulated in part through the action of AMPK,17 it is tempting to speculate that impaired fatty acid oxidation in aromatase deficiency is related to our observations that estradiol activates AMPK at physiologically relevant concentrations. The precise contribution(s) of estradiol-mediated AMPK activation, however, will need to be investigated in future studies.
The principal downstream target of estradiol-induced AMPK activation investigated in this study was eNOS. The link between AMPK activation and eNOS catalytic activity has been established before. In cardiac myocytes, AMPK is found as part of a complex with eNOS and is involved in its phosphorylation at either Ser-1177 or Thr-495, depending on the presence or absence of calmodulin, respectively. In the setting of hypoxia, AMPK activation is linked to angiogenesis, a process that is dependent in part on eNOS activation. Consistent with this notion, endothelial cell ischemia/reperfusion injury is associated with activation of AMPK and eNOS phosphorylation at Ser-1177. The data presented here add considerably to this body of literature, in that we have identified estradiol as an upstream stimulus involved in AMPK activation that has implications for eNOS catalytic activity. Thus, our study establishes AMPK as a physiological modulator of eNOS.

We found that estradiol-mediated AMPK activation was independent of ER ligand engagement and involved catechol metabolism of estradiol. A number of cytochrome P450 enzymes convert estradiol to its hydroxylated derivatives, with CYP1A1 and CYP1B1 notable for their specificity in producing catechol estradiols via C-2 or C-4 hydroxylation. These 2- and 4-hydroxyestradiol derivatives undergo subsequent O-methylation to their corresponding methoxy derivatives via the ubiquitous enzyme catechol-O-methyltransferase. Although this process has yet to be linked with AMPK activation, there is precedent for its role in modulating vascular phenotype. For example, catechol estradiols stimulate endothelial cell prostacyclin production. In insulin-resistant rats, chronic treatment with 2-hydroxyestradiol enhances endothelium-dependent arterial relaxation to acetylcholine. If one considers that AMPK activation is also thought to attenuate insulin resistance, it is consistent with this notion, endothelial cell ischemia/reperfusion injury is associated with activation of AMPK and eNOS phosphorylation at Ser-1177. The data presented here add considerably to this body of literature, in that we have identified estradiol as an upstream stimulus involved in AMPK activation that has implications for eNOS catalytic activity. Thus, our study establishes AMPK as a physiological modulator of eNOS.

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We found that estradiol-mediated AMPK activation was required for HSP90 association with eNOS. The generally accepted paradigm of estradiol-mediated eNOS activation is depicted in Figure 8. This model involves rapid activation of ERα via ligand engagement and the subsequent activation of Src and phosphoinositide 3-kinase, subsequently leading to Akt activation. Our data add to this knowledge by separating Akt activation from HSP90 association with the enzyme. This effect does not appear related to Akt-mediated AMPK activation, as IC1182,780 effectively inhibits estradiol-mediated Akt activation but is ineffective with regard to AMPK stimulation. Thus, our data provide a new mechanism for modulation of eNOS catalytic activity.

A broader implication for our work relates to the possibility of a role for AMPK in HSP90 chaperone function. In addition to eNOS, HSP90 is known to associate with a number of signaling proteins, including Src, ErbB2, Raf, and Akt, among others (reviewed in Pratt and Toft). The dissociation of HSP90 from its client proteins is associated with client protein degradation and initiation of a stress response. The chaperone function of HSP90 is an active process linked to cycles of ATP binding and hydrolysis. Moreover, HSP90 function is sensitive to the cellular energy charge, as HSP90 readily binds ADP, and this event promotes client protein degradation. Because AMPK activity is a key sensor of cellular energy state, its is plausible that the HSP90 chaperone function may be subject to regulation by AMPK. This hypothesis warrants further investigation.

In summary, the data presented here indicate that estradiol-mediated eNOS activation involves the synergistic action of ER-dependent and -independent mechanisms. Our scheme includes eNOS phosphorylation by a mechanism involving Eρα, phosphoinositide 3-kinase, and Akt, whereas HSP90 association with the enzyme involves an ER-independent mechanism that requires AMPK. These data provide a novel mechanism for the control of eNOS activity through modulation of HSP90 chaperone function and provide a new target for AMPK activity.

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