Transcriptional Regulation of Endothelial Nitric-oxide Synthase by an Interaction between Casein Kinase 2 and Protein Phoshpatase 2A

(Received for publication, January 29, 1999, and in revised form, August 2, 1999)

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We previously demonstrated that lysophosphatidylcholine up-regulated endothelial nitric-oxide synthase promoter activity by increasing Sp1 binding via the action of protein serine/threonine phosphatase 2A (Cieslik, K., Zembowicz, A., Tang, J.-L., and Wu, K.K. (1998) J. Biol. Chem. 273, 14885–14890). To characterize the regulation of basal endothelial nitric-oxide synthase promoter activity and the signaling pathway through which lysophosphatidylcholine augments endothelial nitric-oxide synthase transcription, we used a casein kinase 2 inhibitor coupled with immunoprecipitation to demonstrate that basal Sp1 binding and endothelial nitric-oxide synthase promoter activity were controlled by casein kinase 2 complexed with protein serine/threonine phosphatase 2A. Casein kinase 2 catalyzed protein serine/threonine phosphorylation 2A phosphorylation thereby inhibiting its activity. Lysophosphatidylcholine selectively activated p42/p44 mitogen-activated protein kinase. Purified extracellular regulated kinase 2 blocked casein kinase 2 activity and increased protein serine/threonine phosphatase 2A activity, resulting in an increased Sp1 binding and endothelial nitric-oxide synthase promoter activity. These results indicate that Sp1 binding to its cognate site on the endothelial nitric-oxide synthase promoter and its transactivation of endothelial nitric-oxide synthase is regulated by post-translational Sp1 phosphorylation and dephosphorylation through a dynamic interaction between casein kinase 2 and protein serine/threonine phosphatase 2A.

Endothelial nitric-oxide synthase (eNOS) is a member of the NOS family, which catalyzes the oxidation of L-arginine to nitric oxide (NO) and L-citrulline (1–4). Nitric oxide induces smooth muscle cell relaxation, inhibits platelet aggregation, and protein serine/threonine phosphatase 2A. Casein kinase 2 catalyzed protein serine/threonine phosphorylation 2A phosphorylation thereby inhibiting its activity. Lysophosphatidylcholine selectively activated p42/p44 mitogen-activated protein kinase. Purified extracellular regulated kinase 2 blocked casein kinase 2 activity and increased protein serine/threonine phosphatase 2A activity, resulting in an increased Sp1 binding and endothelial nitric-oxide synthase promoter activity. These results indicate that Sp1 binding to its cognate site on the endothelial nitric-oxide synthase promoter and its transactivation of endothelial nitric-oxide synthase is regulated by post-translational Sp1 phosphorylation and dephosphorylation through a dynamic interaction between casein kinase 2 and protein serine/threonine phosphatase 2A.

EXPERIMENTAL PROCEDURES

Construction of eNOS 5′-Promoter in Luciferase Expression Vectors—A 5′-flanking fragment at nucleotide position from −1322 to +22 was obtained by polymerase chain reaction, using genomic DNA as a template and synthetic oligomers as primers: EN13F (5′-AAA-

GATCTTCCATCTCCCTCCTCCTG-3′) and EN3H (5′-GGGAAGCTT-
GTACTGTGCGTCCACTCTG-3′). The polymerase chain reaction product purified from agarose gel was digested with BglII/HindIII and cloned into the promoterless luciferase reporter vector pGL3. Cell Culture and Transient Expression—ECV-304 (spontaneously transformed human umbilical vein endothelial cell line) was cultured in complete Medium 199 (Life Technologies, Inc.) containing 10% fetal bovine serum. Transient transfection by Lipofectin (Life Technologies, Inc.) was performed as described (13). ECV-304 was incubated in serum-free medium containing a mixture of 10 μl of Lipofectin and 2 μg of promoter construct at 37 °C for 5 h. Medium was removed; cells were washed and incubated with fresh complete medium for 24 h. Cells were

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* This work was supported by United States Public Health Service Grants P50 NS23327 and RO1–50675 from National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. ¶ To whom correspondence should be addressed: Vascular Biology Research Center and Div. of Hematology, University of Texas-Houston Medical School, 6431 Fannin, MSB 5.016, Houston, TX 77030. Tel.: 713-500-6801; Fax: 713-500-6812; E-mail: kkwu@heart.med.uth.tmc.edu.

† The abbreviations used are: eNOS, endothelial nitric-oxide synthase; lysoPC or LPC, lysophosphatidylcholine; CK2, casein kinase 2; PP2A, protein serine/threonine phosphatase 2A; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular regulated kinase 1 (p44) and 2 (p42); NE, nuclear extracts; ECL, enhanced chemiluminescence; IP, immunoprecipitation; BSA, bovine serum albumin; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; JNK, c-Jun N-terminal kinase; PAGE, polyacrylamide gel electrophoresis.
then washed and incubated in Medium 199 containing 0.5% fetal bovine serum for 16 h. The medium was replaced with fresh Medium 199 containing 5% fetal bovine serum in the presence or absence of PD 98059 (Calbiochem) or 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB, Calbiochem). After 1 h of incubation, 100 μM lysophosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) was added to the medium to a final concentration of 100 mM each and lysoPC incubated for an additional 6 h at 37 °C. The cells were harvested and luciferase activity was determined by luciferase assay in a luminometer (Analytical Luminescence Laboratories, Monolight model 2010) as described (17).

**Nuclear Extract (NE) Preparation**—NE was prepared by a procedure previously described (15). After completion of the experimental protocol, cells were harvested and suspended in 1 ml of NE lysis buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 300 mM sucrose, 0.5% Nonidet P-40, 1 μg/ml leupeptin, 30 μM aprotinin, 0.5 mM PMSF), and placed on ice for 10 min. Cells were centrifuged at 6500 rpm for 20 min, washed with 3/4 packed cell volume with buffer A, and spun down. Extraction buffer B (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 400 mM NaCl, 1 mM DTT, 0.2 mM EDTA, pH 8.0, 25% glycerol, 1 μg/ml leupeptin, 30 μM aprotinin, 0.5 mM PMSF) was added to the nuclear extract at 2/3 packed cell volume. Nuclei were passed 10 times through a 23-gauge needle and stirred on ice for 30 min. The nuclear debris was pelleted for 5 min at 12,000 rpm. The supernatant was diluted isovolumetrically in buffer D (20 mM HEPES, pH 8.0, 50 mM KCl, 1% glycerol, 50 μg/ml BSA, 6.0 mM MgCl₂, 0.5 mM DTT, 0.5 mM PMSF, 1 mM ZnCl₂, 1.5 μg of poly(dI-dC), 0.05% Nonidet P-40) and incubated on ice for 15 min. [γ-32P]ATP-labeled Sp1 oligonucleotide probe was added and incubated at room temperature for 15 min. The mixture was electrophoresed at 12.5 V/cm (5% polyacrylamide gel, 0.75% TBE, 0.075% Nonidet P-40, 0.1 mg/ml BSA) with buffer containing 0.5× TBE and 0.5× Nonidet P-40. The gel was vacuum-dried and autoradiographed.

**Western Blot Analysis**—Protein Phosphatase 2A Assay—PP2A assay kits were obtained from Promega. The assay is based on determining the amount of free phosphate generated in the reaction by measuring the absorbance of a molybdate malachite green-phosphate complex. 10 μg of NE was incubated on a 96-well plate together with a peptide substrate RRA(pT)VA and buffer (50 mM imidazole, pH 7.2, 0.2 mM EDTA, 0.02% β-mercaptoethanol, 0.1 mg/ml BSA) for 30 min at 30 °C. After incubation, the molybdate complex dye was added and incubated for an additional 30 min at room temperature for color development. The level of molybdate malachite green-phosphate complex formed was monitored at 630 nm.

**Immunoprecipitation**—5 μg of NE with 4 μl of anti-human CK2α-subunit rabbit polyclonal IgG1 (Upstate Biotechnology, Inc.) or 16 μl of anti-PP2A catalytic subunit rabbit polyclonal IgG (Promega) was incubated overnight at 4 °C in 1 ml of TBS buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5 mM leupeptin, 30 μM aprotinin, 0.5 mM PMSF). 20 μl of Protein G Plus-Agarose (Santa Cruz Biotechnology) was added to each sample and incubated for an additional 2 h. Immunoprecipitates (IP) were spun down at 2500 rpm for 5 min, washed four times with TBS, and resuspended in 100 μl of TBS or CK2 assay buffer.

**Luciferase Assay**—22) luciferase reporter activity in 10-fold more samples was measured using the dual luciferase reporter system (Promega 104 and 90 of Data not shown). The density of these two bands was enhanced by lysoPC treatment (Fig. 1A, lane 2 versus lane 3). To determine whether CK2 is involved in regulating the Sp1 binding activity, we pretreated ECV-304 cells with DRB, a selective CK2 inhibitor. DRB at 6 μM markedly increased the basal Sp1 binding activity (Fig. 1A, lane 4) and caused a lesser increase in lysoPC-induced Sp1 binding. DRB treatment also increased the basal promoter activity (Fig. 1B). These results suggest that the basal Sp1 binding to eNOS promoter region and consequently basal eNOS promoter activity was controlled by CK2. They are consistent with a previous report, which showed that CK2 was involved in Sp1 phosphorylation and lowered the Sp1 binding activity in liver differentiation (16).

**Inhibition of PP2A Activity by CK2**—We then determined whether PP2A activity was influenced by DRB treatment. Pre-treatment of cells with DRB increased the basal PP2A activity (Fig. 2). LysoPC-induced PP2A activity was also elevated by DRB. These results are in agreement with those of Sp1 binding and eNOS promoter activity. CK2 and PP2A protein levels in NE were not affected by lysoPC treatment; nor were they increased by DRB treatment (Fig. 3).

**Complex of CK2 with PP2A in Nuclear Extracts**—It has been shown by several signaling systems that kinase forms a complex with its phosphatase partner in achieving a dynamic and specific on and off signaling (18–21). We suspected that CK2 might complex with PP2A in regulating Sp1-activated eNOS transcription. NE obtained from basal or lysoPC-treated cells were immunoprecipitated with anti-PP2A and the CK2 activity in IP was determined. The results from one of five experi-
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FIG. 1. Increased basal Sp1 binding and eNOS promoter activities by DRB, a CK2 inhibitor. A, Sp1 in nuclear extracts (NE) formed two complexes with a 22-mer Sp1 consensus sequence as indicated. Our previous study showed that both bands were competed out by a 100-fold molar excess of unlabeled probes or an eNOS promoter region containing canonical Sp1 sites (15). DRB at 6 μM increased the basal Sp1 binding. C denotes basal and L denotes lysoPC-treated cells. B, ECV-304 cells were transfected by lipofection with an eNOS promoter fragment from –1322/+22 constructed into a promoterless luciferase expression vector pGL3. Luciferase activity was expressed as relative light units (RLU/μg of NE protein). The concentrations of lysoPC (LPC) and DRB are 100 and 6 μM, respectively. Each bar is mean ± S.D. of three to five experiments.

FIG. 2. The effect of DRB on PP2A activity in cells treated with and without 100 μM of lysoPC. The concentrations of DRB are micromolar. Each bar represents mean ± S.D. of five separate experiments.

FIG. 3. PP2A and CK2 protein levels in NE determined by Western blot analysis. Lane 1 in panel A and lane 7 in panel B are PP2Ac and CK2α standards, respectively.

FIG. 4. Association of PP2A with CK2. A, Autoradiographs of a representative experiment. NE (C denotes basal and L denotes lysoPC-treated) or purified CK2 (0.4 μg, 10 units/μg) and PP2A (0.4 μg, 1 unit/μg) were immunoprecipitated with anti-PP2Ac, anti-CK2α, or pre-immune IgG (pre-IgG, only the basal results shown), and after washing, the CK2 activity of the immunocomplex was measured using casein as a phosphorylation substrate. Lane 1 shows addition of purified CK2 in the incubation buffer without subjecting to immunoprecipitation to serve as an internal positive control. B, densitometric analysis of casein phosphorylation in five experiments. Numbers shown on the horizontal axis correspond to those shown in A. Purified CK2 activity (lane 1) was arbitrarily set as 100%. The data denote mean ± standard deviation. Differences between lanes 4 and 5 (p < 0.01) and between lanes 8 and 9 (p < 0.01) are statistically significant.

lysoPC-treated ECV-304 (lane 5 versus lane 4). Basal NE immunoprecipitated with pre-immune IgG did not exhibit CK2 activity (lane 2) nor did purified PP2A immunoprecipitated with PP2Ac antibody (lane 3). As a positive control, purified CK2 immunoprecipitated with anti-CK2α, exhibited casein phosphorylation activity (lane 7). Basal NE, immunoprecipitated with anti-CK2α, showed a high CK2 activity which was
lowered by lysoPC treatment (lane 8 versus lane 9). Densitometric analysis of casein phosphorylation bands from five experiments shows an equivalent CK2 activity in complexes from unstimulated endothelial NE immunoprecipitated with anti-PP2Ac (Fig. 4, lane 4) or with anti-CK2a (Fig. 4B, lane 8). The CK2 activity in the complexes following lysoPC treatment was reduced to about half of the control (lane 5 versus lane 4 and lane 9 versus lane 8).

Interaction of Purified CK2 with PP2A—To confirm CK2 and PP2A complex formation, purified CK2 holoenzyme was incubated with PP2A holoenzyme or catalytic subunit PP2Ac and immunoprecipitated with anti-PP2Ac. CK2 activity was detected in IP from CK2 + PP2Ac (Fig. 5A, lane 3) as well as CK2 + PP2A (Fig. 5A, lane 5). Preimmune IgG did not bring down a complex with CK2 activity (Fig. 5A, lanes 2 and 4) while purified CK2 treated with anti-PP2Ac did not yield any precipitate with CK2 activity (Fig. 5A, lane 7). Thus, CK2 activity detected in PP2A immunoprecipitate was specific for CK2-PP2A complex. The stoichiometry of CK2 and PP2A activities in the complex appeared to be 1 to 1 (Fig. 5, B–D). As inhibition of CK2 by DBB increased PP2A activity (Fig. 2), we wondered whether CK2 catalyzes the phosphorylation of PP2A. Purified PP2Ac was incubated in CK2 assay buffer with purified CK2 and [γ-32P]ATP. PP2A phosphorylation was analyzed by SDS-PAGE and autoradiography. Fig. 5E shows phosphorylation of the 36-kDa catalytic subunit of PP2A.

Activation of ERK1/2 by Lyso-PC—To discern the signaling pathway through which lysoPC alters CK2-PP2A activities, we evaluated the effect of lysoPC on the MAPK pathways. LysoPC selectively induced ERK1 and ERK2 phosphorylation in a time- and concentration-dependent manner without altering their protein levels (Fig. 6, A and B). In accordance with ERK1/2 phosphorylation, ERK activity as evaluated by using Elk-1 as a substrate was increased by lysoPC in a concentration-related manner (Fig. 6C). LysoPC did not induce phosphorylation of JNK/SAPK or p38 MAPK (data not shown). Both ERK1/2 phosphorylation and ERK catalytic activity induced by lysoPC were blocked by PD 98059 (Fig. 6, B and C), a selective inhibitor of MEK-1, which is the upstream kinase that catalyzes ERK phosphorylation (18).

Effect of an MEK-1 Inhibitor on Sp1 Binding, eNOS Promoter Activity, and PP2A Activity—The results shown above are consistent with a selective activation of MEK-1 and consequently ERK1/2 by lysoPC. To ascertain that this pathway is involved in regulating Sp1 binding, we pretreated cells with PD 98059, stimulated them with and without lysoPC, and determined Sp1 binding activity in the nuclear extracts of these treated cells. PD 98059 at 10 μM reduced the lysoPC-induced Sp1 binding activity to approximately the basal level without affecting the basal binding activity, while at 50 μM, it suppressed both basal and lysoPC-induced Sp1 binding activities (Fig. 7A). PD 98059 at 10 μM similarly suppressed lysoPC-induced eNOS promoter activity without a significant effect on the basal promoter activity (Fig. 7B). These results indicate that lysoPC increases Sp1 binding and eNOS promoter activities through the MEK-1 signaling pathway. This signaling pathway was involved in modulating PP2A activity. PD 98059 at 10 μM suppressed PP2A activity to the basal level (Fig. 7C), without a significant effect on PP2A protein level (Fig. 3A). The CK2 protein levels were also unaffected by PD 98059 (Fig. 3B).

Effects of Purified Activated ERK2 on CK2 and PP2A Activities—Activated ERK1/2 are translocated to the nucleus where they target transcription factors including Elk-1 (18). However,
our results implied that ERK1/2 might target CK2 and/or PP2A. To discern this, we incubated purified activated ERK2 (p42 MAPK, the major isoform detected as shown in Fig. 6, A and B) with CK2 in the presence and absence of PP2A. The CK2 activity was reduced when CK2 alone or mixed with PP2A was coincubated with ERK2 (Fig. 8, A and B, lanes 2 and 3). By contrast, the PP2A activity was not suppressed when purified PP2A was incubated with ERK2 (Fig. 8C, lane 1 versus lane 3). PP2A activity was suppressed by CK2 (Fig. 8C, lane 1 versus lane 2), and this suppression was totally abrogated by omitting ATP from the assay mixture. In the absence of ATP, the PP2A activity of PP2A + CK2 mixture was not statistically different from that of PP2A alone (n = 3, p > 0.05). These results are consistent with the notion that CK2-catalyzed PP2A phosphorylation (Fig. 5E) is a key mechanism for PP2A inhibition by CK2. Addition of ERK2 to CK2/PP2A mixture increased the PP2A activity (Fig. 8C, lane 2 versus lane 4, p < 0.01). These results indicate that ERK2 targets primarily CK2. It reduces CK2 activity through which it increases PP2A activity.

**DISCUSSION**

Our findings shed light on how basal and lysoPC-induced eNOS transcriptions are controlled and regulated. Previous studies have shown that basal eNOS promoter activity depends almost entirely on binding of Sp1 to a cognate site at the proximal region of eNOS promoter (13, 14). Results from this study indicate that basal Sp1 binding activity is controlled by CK2, and lysoPC treatment of endothelial cells leads to suppression of the basal CK2 activity (Fig. 4) with an increase in PP2A activity (Fig. 2). These results implied inhibition of PP2A by CK2 at the basal state. This notion was supported by two
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LysoPC selectively activates the ERK1/2 pathway (Fig. 6, A–C) through which PP2A activity is enhanced in cells (Fig. 2). From these experimental results, a model for basal and lysoPC-induced eNOS transcription regulation is proposed as depicted in Fig. 9. According to this model, Sp1 binding to its DNA motif which has been suggested to be governed by a balance between Sp1 phosphorylation and dephosphorylation (16, 22) is controlled by CK2 interacting with PP2A. At the basal state, CK2 dominates over PP2A thereby maintaining a high level of Sp1 phosphorylation with a controlled basal Sp1 binding activity and eNOS promoter activity. Stimulation with lysoPC leads to a reduction of CK2 activity and increased PP2A activity via ERK activation, tilting the balance to favor Sp1 dephosphorylation and consequently unleashing the control accompanied by increased Sp1 binding and gene transcription.

PP2A occupies a pivotal position in eNOS transcription. Its activity is regulated by phosphorylation through its complex formation with CK2. This mode of PP2A regulation is in keeping with the PP2A properties. PP2A is a heterotrimer composed of a 36-kDa catalytic C subunit and a 65-kDa regulatory A subunit that form the core enzyme and a regulatory B subunit that binds to the core enzyme to form the holoenzyme (for a review, see Ref. 23). Phosphorylation of the catalytic or regulatory subunit has been reported to influence its catalytic activity (24–27). Our results are consistent with a previous report that CK2 catalyzes the phosphorylation of the PP2A catalytic subunit (21). Regulation of PP2A activity is facilitated by its binding to diverse proteins including several kinases (20, 21, 28, 29). A recent report indicates that it binds to CK2α but not CK2 holoenzyme and its activity is enhanced by complex with CK2α (21). This is contrary to our results, which show that it complexes with either CK2 holoenzyme or CK2α and its activity is suppressed by either form of CK2 in the complex. When ATP was omitted from the reaction mixture, the suppressing effect of CK2 on PP2A was abrogated. In our experimental system, CK2-catalyzed phosphorylation of PP2A is responsible for its inhibition of PP2A activity.

Our experimental data support the notion that Sp1 is a substrate for CK2. Recent reports showed that Sp1 is phosphorylated by CK2 and phosphorylated Sp1 has reduced DNA binding activity during terminal differentiation of the liver (16, 30). It was subsequently shown that Thr-579 located within the second zinc finger at the C-terminal region of Sp1 is actively phosphorylated by CK2 and mutation of this residue eliminated Sp1 phosphorylation and CK2-mediated inhibition of

\[ \text{FIG. 9. A model depicting regulation of Sp1 binding by PP2A interacting with CK2 at basal and MAPK-signaled activation of eNOS gene transcription.} \]
Sp1 binding activity (16). Other serine residues in this region are also phosphorylated, but their identities and their functional roles remain unclear. Two classes of protein serine/threonine phosphatases have been implicated to catalyze the dephosphorylation of CK2-mediated Sp1 phosphorylation. In the liver differentiation study, protein phosphatase 1 (PP1) was implicated based on okadaic acid inhibition of Sp1 binding activity (16). However, okadaic acid is a nonselective inhibitor for PP1 and PP2A (31). Our results provide direct evidence for the involvement of PP2A in regulating Sp1 binding activity.

LysoPC, a lipid mediator, is generated from phosphatidylcholine via the action of phospholipase A2 (32). It induces transcription of a series of endothelial genes (33–36). The signaling pathway through which lysoPC activates gene transcription has not been clearly defined. Several studies have shown that it activates protein kinase C (37, 38). Others have shown that it activates MAP kinases (39, 40). Our results demonstrate for the first time that ERK2 exerts a direct inhibitory effect on CK2. LysoPC induces MEK-1 and ERK1/2 activation probably by at least two upstream pathways: 1) through PKC activation, which may lead to Ras or Raf activation with subsequent MEK-1 and ERK1/2 activation, and 2) through Ras activation that results in Raf activation followed by MEK-1 and ERK activation.

Acknowledgment—We thank Susan Mitterling for editorial assistance.

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J. Biol. Chem. 1999, 274:34669-34675.
doi: 10.1074/jbc.274.49.34669

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