Mechanism of Purinergic Activation of Endothelial Nitric Oxide Synthase in Endothelial Cells

Cleide Gonçalves da Silva, PhD; Anke Specht, MS; Barbara Wegiel, PhD; Christiane Ferran, MD, PhD; Elzbieta Kaczmarek, PhD

Background—Decreased endothelial nitric oxide (NO) synthase (eNOS) activity and NO production are critical contributors to the endothelial dysfunction and vascular complications observed in many diseases, including diabetes mellitus. Extracellular nucleotides activate eNOS and increase NO generation; however, the mechanism of this observation is not fully clarified.

Methods and Results—To elucidate the signaling pathway(s) leading to nucleotide-mediated eNOS phosphorylation at Ser-1177, human umbilical vein endothelial cells were treated with several nucleotides, including ATP, UTP, and ADP, in the presence or absence of selective inhibitors. These experiments identified P2Y1, P2Y2, and possibly P2Y4 as the purinergic receptors involved in eNOS phosphorylation and demonstrated that this process was adenosine independent. Nucleotide-induced eNOS phosphorylation and activity were inhibited by BAPTA-AM (an intracellular free calcium chelator), rottlerin (a protein kinase C inhibitor), and protein kinase Cδ siRNA. In contrast, blockade of AMP-activated protein kinase, calcium/calmodulin-dependent kinase II, calcium/calmodulin-dependent kinase kinase, serine/threonine protein kinase B, protein kinase A, extracellular signal-regulated kinase 1/2, and p38 mitogen-activated protein kinase did not affect nucleotide-mediated eNOS phosphorylation.

Conclusions—The present study indicates that extracellular nucleotide–mediated eNOS phosphorylation is calcium and protein kinase Cδ dependent. This newly identified signaling pathway opens new therapeutic avenues for the treatment of endothelial dysfunction. (Circulation. 2009;119:871-879.)

Key Words: endothelium • nitric oxide synthase type III • nucleotides • protein kinase C • receptors, purinergic P2

Nitric oxide (NO) is an important signaling molecule, regulating inflammation, angiogenesis, and thrombosis. However, in certain diseases, including atherosclerosis and diabetes mellitus, endothelial NO synthase (eNOS) expression and activity are strongly attenuated, impairing NO production. NO generated and released from the endothelium promotes vasorelaxation, protects endothelial cells (ECs) from apoptosis, inhibits vascular smooth muscle cell proliferation, blocks platelet activation, and enhances vascular growth. NO synthesis requires the activation of a family of enzymes called NOSs. Three NOS isoforms have been identified: 1 inducible form and 2 constitutive forms, neuronal NOS and eNOS. The expression and function of eNOS are regulated at multiple levels, namely transcriptional, posttranscriptional (mRNA stability), and post-translational (O-glycosylation, myristoylation, palmitoylation, and phosphorylation). Additional regulation occurs through eNOS dimerization and interaction with regulatory proteins such as calmodulin, caveolin-1, and heat-shock protein 90, as well as eNOS subcellular targeting to caveolae, other cellular membranes, or the cytoplasm. eNOS is activated by various stimuli, including thrombin, bradykinin, vascular endothelial growth factor (VEGF), tumor necrosis factor-α, histamine, insulin, acetylcholine, endothelin-1, and angiotensin II. Agonist stimulation of the specific receptors activates phospholipase Cγ, which is followed by an increase in diacylglycerol and an increase in intracellular free calcium concentration ([Ca2+]i), activating calmodulin. Ca2+/calmodulin binds to the calmodulin-binding domain in eNOS and allosterically activates eNOS. In addition, calmodulin can activate calcium/calmodulin-dependent kinase (CaMK) II, which further activates eNOS by phosphorylation of Ser-1177. Although the main signal transduction pathway of agonist-stimulated eNOS activation depends on Ca2+/calmodulin, eNOS activity can be additionally regulated via its phosphorylation by various other kinases, including phosphoinositide 3-kinase (PI3K)/Akt, AMP-activated protein kinase (AMPK), extracellular signal-regulated kinase 1/2 (ERK), p38 mitogen-activated protein kinase.
(MAPK), protein kinase C (PKC), and protein kinase A (PKA).6–12 Classically, eNOS phosphorylation at Ser-1177 is mandatory for its activation, whereas phosphorylation of Thr-495 by PKCζIII has been linked to loss of eNOS activity.13,14

Interestingly, extracellular nucleotides also have been implicated in activation of eNOS, leading to NO generation and vasodilation.15 Nucleotides are released into extracellular fluids by exocytosis from nucleotide-containing granules, by efflux through a membrane transport system in response to cell activation, or as a consequence of cell death. Extracellular nucleotides function as paracrine or autocrine mediators via activation of their respective purinergic P2 receptors.16 On the basis of molecular structure and function, P2 receptors are classified into 2 main groups: P2X, ligand-gated ion channels, and P2Y, G protein–coupled receptors.17 Seven P2X receptor subtypes (P2X1 through P2X7) and 8 P2Y receptor subtypes (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 through P2Y14) have been characterized to date. Although P2X receptors facilitate the entry of extracellular Ca2+, activation of P2Y receptors results in Ca2+ release from intracellular stores.18 P2X receptors are exclusively activated by ATP, whereas P2Y receptors respond to both purine (ATP, ADP) and pyrimidine (UTP, UDP) nucleotides. Specifically, ADP is the preferential ligand of P2Y1 and P2Y12 receptors; ATP is the ligand for P2Y2, P2Y11, and P2Y13 receptors; UDP is the ligand of P2Y2 and P2Y4 receptors; UDP is the ligand of P2Y6; and UDP-glucose is the ligand of P2Y14.17 Most cells express multiple P2 receptor subtypes. In particular, ECs express P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 receptors.19

Activation of the various P2 receptors by nucleotides initiates multiple signaling pathways, including the Akt and focal adhesion kinase/paxillin pathways, as well as the activation of several kinases such as AMPK, ERK, p38, and Jun N-terminal kinase.20–22 This leads to cytoskeletal changes and modulation of expression of several genes involved in the regulation of cell proliferation and apoptosis.

The aim of the present study was to elucidate the signaling pathway(s) involved in nucleotide-mediated phosphorylation of eNOS at Ser-1177. Our results unravel a novel nucleotide-triggered pathway that phosphorylates eNOS by increasing [Ca2+], and activating PKCζ.

**Methods**

**Reagents**

ATP, ADP, UTP, UDP, 2′,3′-O-(4-benzoylbenzoyl)-ATP (BzATP), 1-[N-O-bis(5-isouquinolinesulfonyl)l-N-methyl-l-tyrolyl]-4-phenylpiperazine (KN62), 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride and 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002), wortmannin, adenosine 5′-(3′,5′-dibutyryl) cAMP (BAPTA-AM), and 7-Oxo-7H-benzimidazo[2,1-a]benz[e]isouquinoline-3-carboxylic acid (STO-609) were purchased from Sigma-Aldrich Co (St Louis, Mo). Compound C, bisindolylmaleimide I (BIM), myristoylated PKA inhibitor (amide 14-22 cell permeable), staurosporine, 2′-amino-3′-methoxyflavone (PD98059), N-vinyl-4/0/(amine methyl ester (L-NAMe), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole (SB203580), rottlerin, G69683, and 1,2-bis[2-(aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetraethoxyxymethylester (BAPTA-AM) were purchased from Calbiochem/EMD Biosciences, Inc (San Diego, Calif). Recombinant human VEGF was from R&D Systems (Minneapolis, Minn), and the enhanced chemiluminescence kit was from Perkin Elmer Life Science (Boston, Mass).

**Cell Culture**

Human umbilical vein ECs (Cambrex, Walkersville, Md) were cultured in an EGM-2 Bullet Kit medium containing human epidermal growth factor, hydrocortisone, human fibroblast growth factor, VEGF, ascorbic acid, gentamicin, amphotericin-B, human insulin-like growth factor, heparin, and 5% (vol/vol) FBS at 37°C in a 5% CO2 humidified air incubator. Confluent cells at passage 4 or 5 were used in all experiments.

**Cell Treatment and Cell Lysate Preparation**

Cells were incubated with ATP, ADP, and UTP (1 to 250 μmol/L) for various periods of time (1 to 10 minutes). In some experiments, cells were preincubated with specific inhibitors for 20 minutes. After incubation, cells were placed on ice, washed with ice-cold Tris-buffered saline (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl), suspended in cell lysis buffer (20 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 1% [vol/vol] Nonidet P40, 1 mmol/L sodium orthovanadate, 100 mmol/L sodium fluoride, 2 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 2.5 mmol/L EDTA, and 1 mmol/L EGTA), scraped, incubated on ice for 20 minutes, and centrifuged at ∼14 000 g for 5 minutes at 4°C. Supernatants were kept at −80°C until used for Western blot analysis. Protein concentration was measured by the modified method of Lowry et al,23 using a detergent-compatible protein assay kit (Bio-Rad, Hercules, Calif).

**Western Blot**

Cell lysates (30 μg protein per well) were analyzed under reducing conditions by SDS-PAGE performed according to Laemmli.24 Proteins were separated on 4% to 15% polyacrylamide gel and transferred to polyvinylidene difluoride membrane by semidy electrodeblotting. Membranes were blocked with 5% (wt/vol) nonfat dry milk and probed overnight at 4°C with specific primary antibodies. Antibodies against human eNOS and phospho-eNOS (Ser-1177), human phospho-AMPKα (Thr-172) (Upstate Laboratories, San Diego, Calif), human phospho-Akt (Ser-473) (Cell Signaling Technology, Beverly, Mass), human phospho-ERK1/2 (Tyr-204) (Tyr-204; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), and GAPDH (Calbiochem/EMD Biosciences, Inc) were used. Membranes were incubated with secondary donkey anti-rabbit– or goat anti-mouse–horse radish peroxidase–conjugated antibody (Pierce Biotechnology, Rockford, Ill) for 1 hour at room temperature. Protein bands were detected with enhanced chemiluminescence following exposure to autoradiography film. Immunoblots were scanned and analyzed with ImageJ 1.33u software (National Institutes of Health, Bethesda, Md).

**EC Transfection With siRNA**

Cells were transfected with PKCζ, 7-HP–validated siRNA or an AllStars negative control siRNA (Qiagen, Valencia, Calif) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, Calif). Briefly, 50% to 60% confluent ECs grown in 6-well culture plates were transfected in 1 mL Opti MEM–reduced serum medium with 5 μL Lipofectamine 2000 and 100 pmol of the indicated siRNA per well. The cells were incubated at 37°C for 4 to 6 hours, and medium was changed to growth medium. After 48 hours, cells were stimulated with 100 μmol/L ATP or UTP for 1 minute, harvested, and analyzed by Western blotting.

**Measurement of Intracellular cGMP**

ECs were incubated with 100 μmol/L ATP or UTP for 20 minutes at 37°C, and intracellular cGMP was measured by a competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, Mich), according to the manufacturer’s instructions.

**Statistical Analysis**

Results are presented as mean±SE. Data were analyzed by 1-way ANOVA followed by the posthoc Duncan multiple range test when F was significant. Concentration-dependent effects were tested by...
regression analysis. Differences between groups were rated significant at values of \( P < 0.05 \).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Extracellular Nucleotides Induce eNOS Phosphorylation at Ser-1177**

ECs were incubated for 1 to 10 minutes with selected nucleotides, ATP, UTP and ADP at concentrations ranging from 1 to 250 \( \mu \text{mol/L} \). Results demonstrate that ATP, ADP, and UTP stimulated eNOS phosphorylation in time- and concentration-dependent manners, with maximal phosphorylation occurring within 1 to 2 minutes (Figure 1A and 1B). ADP effects were relatively weaker compared with ATP and UTP. In contrast to these nucleotides, similar concentrations of UDP did not induce eNOS phosphorylation (data not shown).

To evaluate a contribution of adenosine originating from extracellular nucleotides hydrolyzed by combined action of ectoenzymes, nucleoside triphosphate diphosphohydrolases, and 5' nucleotidase to eNOS phosphorylation, we used an inhibitor of 5'-nucleotidase, AOPCP. Preincubation of ECs with AOPCP (100 \( \mu \text{mol/L} \) for 20 minutes) did not decrease nucleotide-induced phosphorylation of eNOS (Figure 2).

**Increase in \([\text{Ca}^{2+}]_i\) Is Required for eNOS Phosphorylation After Treatment With Extracellular Nucleotides**

Because eNOS is a calcium/calmodulin-dependent enzyme, it is activated by various agonists that increase \([\text{Ca}^{2+}]_i\).\textsuperscript{25} Because one of the first signals of P2 receptor stimulation is an
increase in $[\text{Ca}^{2+}]_i$, we investigated the role of $\text{Ca}^{2+}$ in the nucleotide-induced eNOS phosphorylation. ECs were preincubated with a chelator of intracellular $\text{Ca}^{2+}$, BAPTA-AM (10 $\mu$mol/L), or an inhibitor of CaMK II, KN62 (10 $\mu$mol/L). Cell lysates were immunoblotted with antibodies to phospho-eNOS (P-eNOS; Ser-1177) and eNOS. Results were analyzed statistically and expressed as mean±SEM of 3 to 4 independent experiments. **$P<0.01$ vs control cells (C).

Figure 3. Calcium flux is required in extracellular nucleotide–induced eNOS phosphorylation in ECs. Representative Western blots of ECs stimulated with ATP or UTP (1 minute at 100 $\mu$mol/L) following 20-minute pretreatments with (A) an intracellular calcium chelator, BAPTA-AM (10 $\mu$mol/L), or (B) an inhibitor of CaMK II, KN62 (10 $\mu$mol/L). Cell lysates were immunoblotted with antibodies to phospho-eNOS (P-eNOS; Ser-1177) and eNOS. Results were analyzed statistically and expressed as mean±SEM of 3 to 4 independent experiments. **$P<0.01$ vs control cells (C).

Figure 4. Extracellular nucleotide–induced phosphorylation of eNOS is independent of AMPK. Representative Western blots of ECs pretreated for 20 minutes with either an inhibitor of CaMKK, STO-609 (1 $\mu$g/mL), or an inhibitor of AMPK, compound C (CC; 20 $\mu$mol/L). Cell lysates were immunoblotted with anti–phospho-eNOS (P-eNOS; Ser-1177), anti–phospho-AMPK (P-AMPK; Thr-172), and anti-GAPDH antibodies. Results were analyzed statistically and expressed as mean±SEM of 3 independent experiments. *$P<0.05$, **$P<0.01$ vs control (C).

AMPK, Akt, ERK, and p38 MAPK Are Not Implicated in P2 Receptor–Mediated Phosphorylation of eNOS
We have recently shown that extracellular nucleotides activate AMPK in ECs. AMPK has been reported as an upstream kinase in the eNOS pathway. Accordingly, we
evaluated a possible role of AMPK in purinergic activation of eNOS. Inhibition of the AMPK upstream activator CaMKK with STO-609 (1 µg/mL for 20 minutes) or AMPK with compound C (20 µmol/L for 20 minutes) did not affect nucleotide-induced phosphorylation of eNOS (Figure 4A and 4B). The inhibitory effects of STO-609 and compound C were confirmed by lack of phosphorylation of AMPK (Figure 4A and 4B).

Akt is another potential kinase involved in eNOS phosphorylation at Ser-1177 in ECs. However, in ECs, nucleotide-induced eNOS phosphorylation usually precedes that of Akt (1 to 2 minutes versus 5 to 10 minutes), which would argue against the implication of Akt in this system. The lack of involvement of Akt was directly validated by our data showing that preincubation of ECs with LY294002 (10 µmol/L for 20 minutes), a selective inhibitor of the Akt upstream kinase, PI3K, did not affect phosphorylation of eNOS by ATP or UTP (Figure 5A).

Other signaling molecules, including ERK and p38 MAPK that have been implicated in eNOS phosphorylation, also are activation targets of P2 receptor signaling. ECs were preincubated for 20 minutes with the ERK and p38 MAPK inhibitors PD98059 (50 µmol/L; inhibits upstream ERK kinase) and SB203580 (10 µmol/L), respectively. None of these inhibitors decreased eNOS phosphorylation in response to extracellular nucleotides (Figure 5B and data not shown).

The PKCδ Isoform Is Responsible for Nucleotide-Mediated eNOS Phosphorylation and Activation in EC

PKC has been shown to increase eNOS phosphorylation and activity in ECs. P2Y receptors activate phospholipase C, which hydrolyzes phosphatidylinositol bisphosphate into IP3 and diacylglycerol to induce PKC activation. We investigated the role of PKCs in nucleotide-mediated eNOS phosphorylation using pharmacological inhibitors of the various PKC isoforms.

Staurosporine (200 nmol/L; 20-minute preincubation), which originally was recognized as a PKC inhibitor (IC50, 5 nmol/L), completely inhibited nucleotide-induced eNOS phosphorylation (Figure 6A). However, staurosporine has a much broader specificity and may inhibit PKA (IC50, 15 nmol/L), protein kinase G (PKG; IC50, 18 nmol/L), and/or CaMK II (IC50, 20 nmol/L). We excluded any role for PKA and PKG in nucleotide-induced phosphorylation of eNOS by using their respective inhibitors, 14-22 amide (PKA inhibitor; 10 µmol/L) and PKG inhibitor (100 µmol/L), which did not attenuate eNOS phosphorylation (Figure 6B and data not shown). In contrast, we confirmed the participation of PKC in eNOS phosphorylation by using BIM, a non–subtype-selective PKC inhibitor. Preincubation with BIM (5 µmol/L for 20 minutes) significantly decreased eNOS phosphorylation in ECs (Figure 6C).

Having confirmed PKC involvement in nucleotide-induced phosphorylation of eNOS, we sought to identify the PKC isoform that was responsible. We focused on PKCα and PKCδ, which have previously been implicated in eNOS activation in ECs. ECs were preincubated with rottlerin (5 µmol/L for 20 minutes), a PKCδ-selective inhibitor, and G66976 (1 µmol/L for 20 minutes), a PKCα-specific inhibitor. Rottlerin significantly reduced nucleotide-mediated phosphorylation of eNOS, whereas G66976 had no effect (Figure 6D and 6E). In addition, we verified that extracellular nucleotides induced phosphorylation of PKCα at Thr-505, a residue localized in the activation loop of the catalytic domain of this enzyme (Figure 6E, inset).

Finally, to confirm participation of PKCδ in nucleotide-mediated eNOS phosphorylation, we used siRNA specific to PKCδ. Transfection of ECs with PKCδ siRNA significantly
reduced extracellular nucleotide–induced eNOS phosphorylation, whereas nontarget, negative control siRNA had no effect (Figure 6F).

To examine whether eNOS phosphorylation in response to nucleotides corresponds to eNOS activation and NO generation, we measured NO-induced cGMP production by ELISA 20 minutes after stimulation with nucleotides. ATP/UTP treatment increased intracellular cGMP, reaching levels comparable to those obtained after addition of VEGF (50 ng/mL), a well-known eNOS activator in ECs (Figure 6G). cGMP accumulation in control and nucleotide-stimulated ECs was abolished by preincubation with PKCδ-specific inhibitor rottlerin (ROT; 5 μmol/L) and incubated with or without nucleotides (100 μmol/L for 1 minute). Activation of PKCδ by extracellular nucleotides was confirmed by its phosphorylation at Thr-505 (E, inset). Cell lysates were immunoblotted with anti–P-eNOS (Ser-1177), anti-eNOS, anti–phospho-PKCδ (P-PKCδ; Thr-505), and anti-PKCδ antibodies. Results were statistically analyzed and expressed as mean±SEM of 3 to 7 independent experiments. *P<0.05 vs ROT. F, Representative Western blots of nontransfected (NT) ECs and ECs transfected for 48 hours with PKCδ-specific or negative control (−) siRNA and incubated with or without nucleotides (100 μmol/L for 1 minute). Cell lysates were immunoblotted with antibodies to P-eNOS (Ser-1177), eNOS, PKCδ, and GAPDH (loading control). Results were statistically analyzed and expressed as mean±SEM of 4 independent experiments. G, eNOS activity, expressed as cGMP levels, is PKCδ-dependent. ECs were incubated for 20 minutes with 100 μmol/L ATP or UTP, with and without L-NAME (100 μmol/L) or rottlerin (ROT; 5 μmol/L), and cGMP production was measured by the enzyme immunoassay. VEGF (50 ng/mL) was used as a positive control. Results were analyzed statistically and expressed as mean±SEM of 2 to 4 independent experiments. *P<0.05, **P<0.01 vs control.
the eNOS inhibitor L-NAME (100 µmol/L for 20 minutes), indicating that cGMP is a proper marker of NO production. PKCδ inhibition by rottlerin significantly attenuated nucleotide-induced cGMP production (Figure 3A).

**Discussion**

The participation of extracellular nucleotides in vessel relaxation has been described and accounted for by NO generation in response to a conformational activation of eNOS by Ca\(^{2+}\)/calmodulin.\(^{26,27}\) Besides conformation-based activation, eNOS activity is modulated by its phosphorylation at Ser-1177. Phosphorylation of this residue is recognized as a positive regulator of eNOS activity.\(^{6}\) The impact of extracellular nucleotides on eNOS phosphorylation has not been clearly defined. In this study, we investigated the mechanism(s) of P2 receptor signaling leading to eNOS phosphorylation and activation in ECs.

We demonstrate that P2 receptor–mediated eNOS phosphorylation at Ser-1177 in ECs is calcium and PKCδ dependent. We also report that AMPK, Akt, PKG, CaMK II, CaMKK, p38, and ERK, which are activated by extracellular nucleotides and potential upstream kinases for eNOS, do not participate in this process.

Our results demonstrate that the extracellular nucleotides ATP, UTP, and ADP induce eNOS phosphorylation in time- and concentration-dependent manners (Figure 1). Furthermore, we established that the prompt nucleotide-mediated eNOS phosphorylation is strictly nucleotide and not adenosine dependent (Figure 2).

One of the first cellular responses to extracellular nucleotides is an increase in [Ca\(^{2+}\)], which can originate either from the cell milieu (by P2X receptor signaling) or from the intracellular stores (by P2Y receptor signaling). In previous work, we demonstrated that in ECs Ca\(^{2+}\) is released from intracellular stores (endoplasmic reticulum) after stimulation with extracellular nucleotides.\(^{20}\) This led us to conclude that in ECs extracellular nucleotides signal mostly via P2Y receptors. Accordingly, extracellular nucleotides are likely to phosphorylate eNOS by engaging P2Y receptors. This conclusion is additionally supported by data showing that nucleotide-induced and NO-dependent vasodilation in human vessels is associated with P2Y and not P2X receptors.\(^{27}\) Here, we examined the effects of ATP (ligand for P2Y2 and P2Y11 receptors), ADP (ligand for P2Y1 and P2Y12 receptors, the latter not expressed in ECs), UTP (ligand for P2Y2 and P2Y4 receptors), and UDP (ligand for P2Y6 receptors) on eNOS phosphorylation. Our results indicate that P2Y1, P2Y2, and possibly P2Y4 receptors are the main receptors involved in purinergic activation of eNOS in ECs. We excluded P2Y6 and P2Y11 receptors because their respective ligands, UDP and BzATP, did not induce eNOS phosphorylation (data not shown).

Our results also demonstrate a crucial role for Ca\(^{2+}\) in nucleotide-induced eNOS phosphorylation. Indeed, eNOS phosphorylation was totally inhibited by chelating intracellular Ca\(^{2+}\) (Figure 3A). These results suggest that increased [Ca\(^{2+}\)], is necessary for the activation of an upstream kinase involved in eNOS phosphorylation and/or that eNOS cannot be phosphorylated unless calcium-related conformational changes have taken place. Other studies showing that mobilization of [Ca\(^{2+}\)] is a crucial step of eNOS activation support our conclusion.\(^{25}\)

Increased [Ca\(^{2+}\)] is usually associated with the activation of CaMK II and CaMKK, which, as stated earlier, could act as upstream kinases for eNOS.\(^{5}\) However, in our experimental system, these enzymes did not participate in the phosphorylation of eNOS (Figures 3B and 4A), even though we confirmed in previous work that extracellular nucleotides do activate CaMKK in ECs.\(^{21}\)

There are contradictory results with regard to the role of AMPK in eNOS phosphorylation in ECs. AMPK has been reported as a direct upstream activator of eNOS phosphorylation in ECs stimulated with thrombin and histamine.\(^{7}\) However, AMPK-independent activation of eNOS also was described in ECs treated with thrombin.\(^{11,28}\) Recently, we have shown that extracellular nucleotides activate AMPK in ECs.\(^{21}\) Therefore, we hypothesized that AMPK could act as an upstream activator of eNOS in our system. Our results disproved this hypothesis. Inhibition of AMPK activity with its pharmacological inhibitor, compound C, attenuated phosphorylation of AMPK but had no effect on nucleotide-induced eNOS phosphorylation (Figure 4B). Likewise, inhibition of CaMKK, an upstream AMPK activator, with STO-609 did not affect eNOS phosphorylation (Figure 4A). Moreover, dominant-negative AMPKα2 did not decrease eNOS phosphorylation by extracellular nucleotides (data not shown).

Although we have documented in a previous study that extracellular nucleotides do activate AMPK in ECs,\(^{21}\) in our experimental system, AMPK does not participate in the phosphorylation of eNOS.

Similarly, the participation of Akt in phosphorylation of human eNOS at Ser-1177 was shown in ECs treated with various agonists, including VEGF, thrombin, and histamine.\(^{6}\) However, here again, nucleotide-induced eNOS phosphorylation was not decreased by pretreatment of ECs with the PI3K inhibitors LY294002 (Figure 5A) and wortmannin (data not shown), excluding any participation of the PI3K and Akt kinases in this pathway. We also excluded ERK, p38, PKA, and PKG from any involvement in nucleotide-induced eNOS phosphorylation (Figure 5B and data not shown).

To scrutinize other potential kinases that are involved in nucleotide-induced eNOS phosphorylation, we investigated participation of the PKC isoforms in purinergic signaling. PKC is a family of serine-threonine–specific kinases, which is divided into 3 groups: the conventional PKCs (α, βI, βII, and γ), the novel PKCs (δ, ε, η, and θ) and the atypical PKCs (ζ and λ).\(^{29}\) The conventional PKCs depend on calcium and are activated by diacylglycerol; the novel PKCs are calcium independent (but some physiological concentration of Ca\(^{2+}\) is needed) and activated by diacylglycerol; the atypical PKCs are calcium independent and not activated by diacylglycerol.\(^{30}\) Different PKC isoforms have been shown to exert opposite effects on eNOS activity. Although PKCβII decreases eNOS activity by phosphorylating Thr-495,\(^{13,31}\) PKCα and PKCδ increase eNOS phosphorylation and activity in ECs in response to agonists such as thrombin.\(^{10,11}\)
In this study, we used an array of various pharmacological inhibitors of PKCs: staurosporine, a selective blocker of PKC; BIM, a PKC inhibitor; rottlerin, a PKCα inhibitor; and Gö6976, a PKCα inhibitor. Although staurosporine, BIM, and rottlerin significantly inhibited nucleotide-mediated phosphorylation of eNOS, with rottlerin also diminishing cGMP levels, Gö6976 did not affect eNOS phosphorylation (Figure 6). In addition, siRNA to PKCβ significantly attenuated eNOS phosphorylation induced by nucleotides. These combined data indicate that PKCβ is the main kinase activating eNOS in ECs exposed to extracellular nucleotides.

Accordingly, we propose that on coupling of P2Y1 and P2Y2 receptors to phospholipase C via Goα proteins, diacylglycerol is generated and the level of [Ca2+]i is increased, enabling calmodulin- and PKCβ activation and subsequent eNOS phosphorylation, as depicted in Figure 7. Our demonstration of this pathway clarifies the mechanisms involved in nucleotide-induced eNOS activation through combined Ca2+-dependent conformational modification, as well as PKCβ-dependent phosphorylation of eNOS.

**Conclusions**

Our observations strongly suggest that the phosphorylation of eNOS that follows the treatment of ECs with ATP, UTP, and ADP occurs via an increase in the intracellular calcium levels and the activation of PKCβ. We suggest that P2 receptors, by activating eNOS and increasing NO generation, represent novel therapeutic targets for the prevention and/or treatment of endothelial cell dysfunction associated with vascular diseases.

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**Disclosures**

None.

**References**

CLINICAL PERSPECTIVE

Decreased endothelial nitric oxide (NO) synthase activity and NO production are critical contributors to the endothelial dysfunction and vascular complications observed in many diseases, including atherosclerosis, diabetes mellitus, and hypertension. Here, we report a new pathway initiated by extracellular nucleotides and P2 purinergic receptors leading to endothelial NO synthase activation and NO generation. This signaling pathway does not involve activation of PI3K/Akt, extracellular signal-regulated kinase 1/2, or AMP-activated protein kinase, to name only a few kinases, the roles of which in endothelial NO synthase activation are well established. Instead, extracellular nucleotide–mediated endothelial NO synthase phosphorylation is calcium and protein kinase Cβ dependent. Extracellular nucleotides play a significant biological role in many tissues and cell types as signaling molecules that regulate cellular functions under both normal and pathophysiological conditions. Extracellular nucleotides exert their biological action via specific purinergic P2 receptors that are classified into 2 main groups: P2X, ligand-gated ion channels, and P2Y, G protein–coupled receptors. Most cells, including endothelial cells, express multiple P2 receptor subtypes. Many laboratories are developing chemical agonists and antagonists of P2 receptors to be used as potential pharmacological agents. Clopidogrel, a blocker of platelet P2Y12 receptor, has been used successfully as an antithrombotic drug. Similarly, denufosol tetrasodium, a nucleotide analog designed to treat cystic fibrosis, is in clinical trials. This indicates that P2 receptors, despite their ubiquitous distribution, constitute useful pharmacological targets for the management of various ailments. On the basis of our data, we propose that P2 receptors also can be new pharmacological targets for the treatment of endothelial dysfunction.
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