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

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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⁺]), 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 phosphoinositol 3-kinase (PI3K)/Akt, AMP-activated protein kinase (AMPK), extracellular signal-regulated kinase 1/2 (ERK), p38 mitogen-activated protein kinase.
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N-methoxyflavone (PD98059), extracellular Ca2+

to date. Although P2X receptors facilitate the entry of

P2Y6, and P2Y11 through P2Y14) have been characterized

UDP-glucose is the ligand of P2Y14. Most cells express

P2Y2 and P2Y4 receptors; UDP is the ligand of P2Y6; and

P2Y2, P2Y11, and P2Y13 receptors; UTP is the ligand of

ligand of P2Y1 and P2Y12 receptors; ATP is the ligand for

respond to both purine (ATP, ADP) and pyrimidine (UTP,

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. Extracel-

lar nucleotides function as paracrine or autocrine mediators via activation of their respective purinergic P2

receptors. On the basis of molecular structure and func-

tion, P2 receptors are classified into 2 main groups: P2X,

ligand-gated ion channels, and P2Y, G protein–coupled

receptors. 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. P2X receptors are

exclusively activated by ATP, whereas P2Y receptors

respond to both purine (ATP, ADP) and pyrimidine (UTP,

UDP). Specifically, ADP is the preferential ligand of P2Y1 and

P2Y12 receptors; ATP is the ligand for P2Y2, P2Y11, and

P2Y13 receptors; UTP is the ligand of P2Y2 and P2Y4 recep-
tors; UDP is the ligand of P2Y6; and UDP-glucose is the ligand of P2Y14. Most cells express multiple P2 receptor subtypes. In particular, ECs express P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 receptors.

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. 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 PKCδ.

Methods

Reagents

ATP, ADP, UDP, 2′,3′-O-4(4-benzoylbenzoyl)-ATP (BzATP), 1-[N-

O-bis-(5-isoxazolinesulfonyl)-N-methyl-1-tosyl-4-phenylpiperazine

(KN62), 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochlo-

ride (LY294002), wortmannin, adenosine 5′-[3′,5′-O-[(ethyl-]

benzimidazol-2-yl)carbonyl]-3′-dThymidine (BIM), myristoylated

3-(4,4-difluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole

(STO-609) were purchased from Sigma-Aldrich Co (St

Louis, Mo), Compound C, bisindolylmaleimide I (BIM), myristoylated

PKA inhibitor (amide 14-22 cell permeable), phospho-eNOS (Ser-

1177) (Upstate Laboratories, San

Diego, Calif), and GAPDH (Calbiochem/

EMD Biosciences, Inc) were used. Membranes were incubated with secondary donkey anti-rabbit– or goat anti-mouse–horseradish peroxi-
dase–conjugated antibody (Pierce 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–horseradish peroxi-
dase–conjugated antibody (Pierce Biotechnology, Rockford, Ill) for 1 hour at room temperature. Protein bands were detected with enhanced chemiluminescence followed by 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 PKCδ 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 I–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), ac-
cording 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

Cell Culture

Human umbilical vein ECs (Cambrex, Walkersville, Md) were

cultured in an EGM-2 Bullet Kit medium containing human epider-

mal 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 exper-

iments, 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 000g 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, 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 Lae-
mlli. Antibodies were separated on 4% to 15% polyacrylamide
gel and transferred to polyvinylidene difluoride membrane by

semidry electrobobting. 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 Technol-

ogy, Beverly, Mass), human phospho-ERK1/2 (Yyr-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–horseradish peroxi-
dase–conjugated antibody (Pierce Biotechnology, Rockford, Ill) for 1 hour at room temperature. Protein bands were detected with enhanced chemiluminescence followed by exposure to autoradiography film. Immuneblots were scanned and analyzed with ImageJ 1.33u software (National Institutes of Health, Bethesda, Md).
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 μmol/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 μmol/L for 20 minutes) did not decrease nucleotide-induced phosphorylation of eNOS (Figure 2).

Figure 2. Nucleotide-induced phosphorylation of eNOS is not adenosine dependent. Representative Western blots of ECs pretreated for 20 minutes with an inhibitor of 5'-nucleotidase, AOPCP (100 μmol/L), and incubated with or without nucleotides (100 μmol/L for 1 minute). Cell lysates were immunoblotted with anti–phospho-eNOS (P-NOS; Ser-1177) and anti-eNOS antibodies. Results were analyzed statistically and expressed as mean±SEM of 3 to 4 independent experiments. *$P<0.05$, **$P<0.01$ vs control cells.

Increase in [Ca²⁺] 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 [Ca²⁺]. Because one of the first signals of P2 receptor stimulation is an
increase in \([\text{Ca}^{2+}]\), 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 \(\mu\text{mol/L}\)) for 20 minutes, followed by stimulation with 100 \(\mu\text{mol/L}\) ATP or UTP for 1 minute. BAPTA-AM significantly attenuated nucleotide-induced phosphorylation of eNOS (Figure 3A), suggesting that an increase in \([\text{Ca}^{2+}]\), released from intracellular stores plays an important role in this activation.

Agonist-induced increase in \([\text{Ca}^{2+}]\), activates CaMK, CaMK II, and CaMK kinase (CaMKK), which could mediate or contribute to eNOS phosphorylation. However, a 20-minute preincubation with KN62 (10 \(\mu\text{mol/L}\)), an inhibitor of CaMK II, or STO-609 (1 \(\mu\text{g/mL}\)), a specific inhibitor of CaMKK, had no effect on nucleotide-induced phosphorylation of eNOS, demonstrating that these kinases are not involved in purinergic activation of eNOS in ECs (Figures 3B and 4A, respectively).

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.\(^{21}\) AMPK has been reported as an upstream kinase in the eNOS pathway.\(^{7}\) 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 PKCa 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 Gö6976 (1 μmol/L for 20 minutes), a PKCδ-specific inhibitor. Rottlerin significantly reduced nucleotide-mediated phosphorylation of eNOS, whereas Gö6976 had no effect (Figure 6D and 6E). In addition, we verified that extracellular nucleotides induced phosphorylation of PKCδ 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...
the eNOS inhibitor L-NAME (100 μmol/L for 20 minutes), indicating that gMP is a proper marker of NO production. PKCδ inhibition by rottlerin significantly attenuated nucleotide-induced cGMP production (Figure 6G).

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\textsuperscript{2+}/calmodulin.\textsuperscript{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.\textsuperscript{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, PKA, 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 (Figure 2).

One of the first cellular responses to extracellular nucleotides is an increase in [Ca\textsuperscript{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\textsuperscript{2+} is released from intracellular stores (endoplasmic reticulum) after stimulation with extracellular nucleotides.\textsuperscript{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.\textsuperscript{25} 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 extracellular nucleotides and potential upstream kinases for eNOS, do not participate in this process.

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Increased [Ca\textsuperscript{2+}], is usually associated with the activation of CaMK II and CaMKK, which, as stated earlier, could act as upstream kinases for eNOS.\textsuperscript{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.\textsuperscript{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.\textsuperscript{7} However, AMPK-independent activation of eNOS also was described in ECs treated with thrombin.\textsuperscript{11,28} Recently, we have shown that extracellular nucleotides activate AMPK in ECs.\textsuperscript{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,\textsuperscript{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.\textsuperscript{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, β1, βII, and γ), the novel PKCs (δ, ε, η, and θ) and the atypical PKCs (ζ and λ).\textsuperscript{29} The conventional PKCs depend on calcium and are activated by diacylglycerol; the novel PKCs are calcium independent (but some physiological concentration of Ca\textsuperscript{2+} is needed) and activated by diacylglycerol; the atypical PKCs are calcium independent and not activated by diacylglycerol.\textsuperscript{30} Different PKC isoforms have been shown to exert opposite effects on eNOS activity. Although PKCβII decreases eNOS activity by phosphorylating Thr-495,\textsuperscript{13,31} PKCα and PKCδ increase eNOS phosphorylation and activity in ECs in response to agonists such as thrombin.\textsuperscript{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 PKCδ 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 PKCδ significantly attenuated eNOS phosphorylation induced by nucleotides. These combined data indicate that PKCδ 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 Goq proteins, diacylglycerol is generated and the level of [Ca²⁺]i is increased, enabling calmodulin and PKCδ 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 PKCδ-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 PKCδ. 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.

Sources of Funding

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Disclosures

None.

References


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**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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