Extracellular Nucleotides and Adenosine Independently Activate AMP-Activated Protein Kinase in Endothelial Cells
Involvement of P2 Receptors and Adenosine Transporters

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Abstract—AMP-activated protein kinase (AMPK) plays a key role in the regulation of energy homeostasis and is activated in response to cellular stress, including hypoxia/ischemia and hyperglycemia. The stress events are accompanied by rapid release of extracellular nucleotides from damaged tissues or activated endothelial cells (EC) and platelets. We demonstrate that extracellular nucleotides (ATP, ADP, and UTP, but not UDP) and adenosine independently induce phosphorylation and activation of AMPK in human umbilical vein EC (HUVEC) by the mechanism that is not linked to changes in AMP:ATP ratio. HUVEC express NTPDases, as well as 5’-nucleotidase; hence, nucleotides can be metabolized to adenosine. However, inhibition of 5’-nucleotidase had no effect on ATP/ADP/UTP-induced phosphorylation of AMPK, indicating that AMPK activation occurred as a direct response to nucleotides. Nucleotide-evoked phosphorylation of AMPK in HUVEC was mediated by P2Y1, P2Y2, and/or P2Y4 receptors, whereas P2Y6, P2Y11, and P2X receptors were not involved. The nucleotide-induced phosphorylation of AMPK was affected by changes in the concentration of intracellular Ca^{2+} and by Ca^{2+}/calmodulin-dependent kinase kinase (CaMKK), although most likely it was not dependent on LKB1 kinase. Adenosine-induced phosphorylation of AMPK was not mediated by P1 receptors but required adenosine uptake by equilibrative nucleoside transporters followed by its (intracellular) metabolism to AMP but not inosine. Moreover, adenosine effect was Ca^{2+} and CaMKK independent, although probably associated with upstream LKB1. We hypothesize that P2 receptors and adenosine transporters could be novel targets for the pharmacological regulation of AMPK activity and its downstream effects on EC function. (Circ Res. 2006;98:0:0.)

Key Words: endothelial cells • AMP-activated protein kinase • P2 receptors • adenosine • CaMKK

AMP-activated kinase (AMPK) is an evolutionarily conserved enzyme that acts as an ultrasensitive energy charge sensor and regulates cell energy metabolism.1 AMPK is a heterotrimeric Ser/Thr kinase consisting of a catalytic α subunit and regulatory β and γ subunits. AMPK is activated in response to ATP depletion associated with an increase in the intracellular AMP:ATP ratio. This ATP consumption can originate from pathological cellular stress, such as heat shock, hypoxia, or ischemia, and from physiological exercise-induced skeletal muscle contraction.2 AMPK can also be phosphorylated and activated by the mechanism independent of changes in AMP:ATP levels.3 AMPK is activated allosterically by AMP (up to 5-fold) and by phosphorylation of Thr172 on the α subunit (50- to 100-fold), which is catalyzed by upstream kinases, including LKB1 and CaMKK.4,5 Activated AMPK turns on catabolic pathways that generate ATP and turns off pathways that consume ATP by phosphorylation of multiple targets. These include glycogen synthase, acetylcoenzyme A carboxylase (ACC), and hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase. Because AMPK is central in controlling the metabolism of glucose and fatty acids, its role in obesity and type 2 diabetes is of major importance. AMPK is expressed in skeletal muscle, brain, liver, and pancreas. AMPK has been also localized in ECs; however, pathways of its activation, as well as the functions of this kinase in the endothelium, are still not well understood and remain to be determined.

Extracellular tri- and diphosphate nucleosides are released into tissue fluids and plasma from aggregated platelets and activated leukocytes and endothelial cells (EC), in response to various proinflammatory stimuli, tissue damage, and cell death.7 They generate a variety of responses within the vasculature, including platelet activation, changes in smooth muscle contractility, and vasodilation. Early cellular responses to extracellular nucleotides include elevation of the intracellular free calcium ion concentration ([Ca^{2+}]) caused by calcium influx or release from intracellular stores. Extracellular nucleotides exert their ef-
fects through purinergic P2 receptors, which are classified into 2 main groups: P2X, which are ligand-gated ion channels; and P2Y, G protein–coupled receptors. Analyses of P2 receptors at the RNA and protein levels, as well as evaluation of their pharmacological characteristics, indicate that P2X4, P2Y11, P2Y1, and P2Y2 are the most abundant in HUVEC. However, other receptors, including P2Y4, P2Y6, P2X7, and some other P2X receptors, have also been identified in EC. ADP is a specific agonist of P2Y1 receptors; UTP is known to stimulate P2Y2 and P2Y4 receptors, whereas UDP is an agonist of P2Y6 receptors. ATP can stimulate P2Y2 receptors, whereas ATP and its analog BzATP are ligands for P2Y11 and most of P2X receptors.

EC express CD39, a NTPDase that hydrolyzes tri- and diphosphate nucleosides to mononucleosides, as well as 5′-nucleotidase (CD73), which can further hydrolyze AMP to adenosine. This adenosine can exert cellular responses using 2 mechanisms, acting externally through P1 receptors or after being internalized through nucleoside transporters.

The aim of the present study was to determine whether extracellular nucleotides and their metabolites can be released in response to cellular stress are involved in activation of AMPK. We demonstrate that phosphorylation and activation of AMPK is induced by 2 independent pathways: the first being mediated by extracellular nucleotides associated with P2Y1, P2Y2, and/or P2Y4 receptors; and the second being initiated by adenosine, with the involvement of adenosine transporters and its metabolism by adenosine kinase. Our results suggest that extracellular nucleotides, P2 receptors, and adenosine transporters could be novel factors modulating EC energy metabolism through AMPK activation.

Materials and Methods

Reagents

Antibodies to human AMPK-α and phospho-AMPK-α(Thr172) (P-AMPK) were purchased from Cell Signaling Technology (Beverly, Mass). Rabbit anti-human phospho-ACC(Ser79) (P-ACC) was from Upstate Laboratories (San Diego, Calif). Streptavidin–horseradish peroxidase (HRP)–conjugated antibody was from Amersham Biosciences Co (Piscataway, NJ). Secondary donkey anti-rabbit–HRP-conjugated antibody was purchased from Pierce Biotechnology (Rockford, Ill). Bisindolylmaleimide I (BIM) was from Calbiochem Biosciences Co (Piscataway, NJ). Secondary donkey anti-rabbit–HRP–conjugated antibody was purchased from Pierce Biotechnology (Rockford, Ill). Bisindolylmaleimide I (BIM) was from Calbiochem Biosciences Co (Piscataway, NJ). Secondary donkey anti-rabbit–HRP–conjugated antibody was purchased from Pierce Biotechnology (Rockford, Ill). Bisindolylmaleimide I (BIM) was from Calbiochem Biosciences Co (Piscataway, NJ). Secondary donkey anti-rabbit–HRP–conjugated antibody was purchased from Pierce Biotechnology (Rockford, Ill). Bisindolylmaleimide I (BIM) was from Calbiochem Biosciences Co (Piscataway, NJ).

Western Blot

Cell lysates (30 μg protein/well) were analyzed under reducing conditions by SDS-PAGE performed according to Laemmli. Proteins were separated on 4% to 15% polyacrylamide gel and transferred to polyvinylidene difluoride membrane by semidry electoblotting. Membranes were blocked with 5% (wt/vol) nonfat dry milk and probed with specific antibodies overnight at 4°C. Membranes were incubated with HRP-conjugated goat anti-rabbit IgG (diluted 1:5000) for 1 hour at room temperature. Total ACC was visualized with streptavidin-HRP (diluted 1:1000 in TBS buffer 0.4% Tween 20), which detects the biotin part in the ACC molecule. Proteins were detected with ECL followed by exposure to the autoradiography film. Immoblot images were scanned and bands were densitometrically analyzed using Imagej 1.33u software (NIH). The P-AMPK/total AMPK ratio was determined and results were expressed as percentage of the control (untreated cells) AMPK relative phosphorylation.

Cell Culture

HUVEC (Cambrex, Walkersville, Md) were cultured in EGM-2 Bullet Kit medium containing human epidermal growth factor, hydrocortisone, human fibroblast growth factor, vascular endothelial growth factor, ascorbic acid, gentamicin, amphotericin-B, human insulin-like growth factor, heparin and 2% (vol/vol) FBS at 37°C in a 5% CO2 humidified air incubator. Confluent cells at passage 4 were used for all experiments. HeLa cells were grown in DMEM containing 10% FBS.

Measurement of Intracellular Nucleotides

Cells were harvested, and levels of intracellular nucleotides were measured by high-performance liquid chromatography (HPLC), using a C18 reverse-phase column, as described earlier.

Statistical Analysis

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

Results

Extracellular Nucleotides and Adenosine Induce Phosphorylation and Activation of AMPK in HUVEC

We examined the effect of adenosine and extracellular nucleotides on phosphorylation of AMPK in HUVEC. Cells were incubated for 0 to 10 minutes in presence of ATP, ADP, UTP (100 μmol/L), or adenosine (25 μmol/L) and cell lysates were analyzed by Western blotting with antibodies to human P-AMPK-α(Thr172) and total AMPK-α. We observed a transient, statistically significant increase of phosphorylation of AMPK induced by extracellular ATP, ADP, UTP, and adenosine (Figure 1). The increase of AMPK phosphorylation was very rapid, reaching maximum stimulation after 1 minute.
AMPK phosphorylates its various downstream targets, including ACC (Ser79) (P-ACC) or streptavidin-HRP antibody (total ACC). E, Results are expressed as percentage of the control (0 minutes) AMPK relative phosphorylation. Values are presented as means±SEM of 3 to 4 independent experiments. *P<0.05, **P<0.01 compared with the control.

For ATP (up to 3.5-fold), UTP (up to 3.0-fold), and ADP (up to 2.6-fold), and the P-AMPK levels were still significantly higher than control after 10 minutes incubation with all nucleotides. Adenosine-induced AMPK phosphorylation was slower than that observed with nucleotides and was significantly increased after 5 minutes (2.1-fold).

In addition, incubation of HUVEC for 5 minutes with varying concentrations of nucleotides (1 to 250 μmol/L) showed that ATP, ADP, and UTP at 50 μmol/L and higher induced phosphorylation of AMPK in a concentration-dependent manner (β=0.849, P<0.001; β=0.751, P<0.001; β=0.714, P<0.001, respectively) (Figure 2). Adenosine-dependent phosphorylation of AMPK was maximal at 25 μmol/L adenosine (Figure 2).

It is well established that phosphorylation of AMPK on Thr172 is associated with AMPK activation.4 Active AMPK phosphorylates its various downstream targets, including ACC.6 Western blot analysis of lysates from HUVEC treated with ATP, ADP, UTP, or adenosine demonstrated phosphorylation of ACC (Figure 3) and hence additionally confirmed activation of AMPK by nucleotides and adenosine.

HUVEC express various NTPDases, including NTPDase1 (described as CD39), that can hydrolyze ATP and ADP to AMP and UTP to UDP and UMP.21 However, extracellular AMP (97.5±15.5% of control; n=2, P=0.718) or UDP (106±12.08% of control; n=5 P=0.549) (100 μmol/L, 5 minutes incubation) had no effect on phosphorylation of AMPK.

HUVEC also express 5'-nucleotidase, which can hydrolyze AMP to adenosine.22 To exclude the possible adenosine effect in nucleotide-induced AMPK activation, we inhibited 5'-nucleotidase with its specific inhibitor, adenosine-5’-α,β-methylene diphosphate (AOPCP).23 Preincubation of HUVEC with AOPCP (100 μmol/L, 15 minutes) did not reduce the nucleotide-induced phosphorylation of AMPK and ACC (Figure 4), indicating that this effect is in fact nucleotide dependent.

The mechanism of AMPK activation can be linked to the increase in the intracellular AMP:ATP ratio. We measured levels of intracellular AMP and ATP by HPLC in EC stimulated with ADP (100 μmol/L) and adenosine (25 μmol/L) for 5 and 10 minutes. No statistically significant changes in the AMP:ATP ratio (∼1.3-fold increase) were observed (Figure 5), even though some tendency to increase in AMP level (50%) was visible for ADP (after 5 minutes) or adenosine (after 10 minutes).

Identification of P2 Receptors Involved in Nucleotide-Induced Phosphorylation of AMPK

AMPK Activation by Nucleotides and Adenosine

Figure 1. Time-course studies of phosphorylation of AMPK induced by nucleotides and adenosine in HUVEC. Representative Western blots of HUVEC stimulated by 100 μmol/L ATP (A), 100 μmol/L UTP (B), 100 μmol/L ADP (C), or 25 μmol/L adenosine (ADO) (D) from 0 to 10 minutes. Cell lysates were immunoblotted with anti–phospho-AMPK (Thr172) (P-AMPK), anti–AMPK-α and anti–P-ACC antibodies. E, Results are expressed as percentage of the control (0 minutes) AMPK relative phosphorylation. Values are presented as means±SEM of 3 to 5 independent experiments. *P<0.05, **P<0.01 compared with control.

Figure 2. Concentration dependence of nucleotide- and adenosine-induced phosphorylation of AMPK in HUVEC. Representative Western blots of HUVEC stimulated for 5 minutes with the indicated concentrations of ATP (A), UTP (B), ADP (C), or adenosine (ADO) (D), ranging from 0 to 250 μmol/L. Cell lysates were immunoblotted with anti–P-AMPK (Thr172), anti–AMPK-α, and anti–P-ACC antibodies. E, Results are expressed as percentage of the control (0 μmol/L) AMPK relative phosphorylation. Values are means±SEM of 3 to 4 independent experiments. *P<0.05, **P<0.01 compared with control.

Figure 3. Extracellular nucleotides and adenosine induce phosphorylation of ACC. Western blot analysis of HUVEC stimulated by ADP (100 μmol/L), ATP (100 μmol/L), UTP (100 μmol/L), or adenosine (ADO) (25 μmol/L) for 5 minutes or ADO* for 30 minutes. Cell lysates were immunoblotted with anti–phospho-ACC (Ser79) (P-ACC) or streptavidin-HRP antibody (total ACC). Results are representative of 4 to 5 independent experiments. C indicates control.
express many subtypes of these receptors. To identify the receptors responsible for nucleotide-induced activation of AMPK, pharmacological analysis was done. As shown in Figures 1 through 3, ADP and UTP induced phosphorylation of AMPK and its downstream target, ACC. These data indicate that P2Y1, P2Y2, and/or P2Y4 receptors are involved in the activation of the AMPK signaling pathway. UDP had no effect on AMPK phosphorylation hence the involvement of the P2Y6 receptors can be excluded.

ATP significantly induced AMPK phosphorylation (Figures 1 and 2). However, ATP can activate a broad spectrum of P2 receptors expressed on HUVEC, including P2Y2, P2Y11, and all P2X receptors. Among P2X receptors, P2X7 is rather unique because it responds to high concentrations of ATP (millimolar range). Therefore, to study the involvement of P2X7 receptors in AMPK activation, we used 1 mmol/L ATP and additionally treated EC with KN62, the antagonist of P2X7 receptor.26 Even though high concentrations of ATP induced phosphorylation of AMPK, preincubation with KN62 (1 μmol/L, 20 minutes) did not affect the increase of P-AMPK and P-ACC levels (Figure 6), suggesting that P2X7 receptors are not involved in AMPK phosphorylation. Furthermore, we treated cells with BzATP, a potent agonist of P2X7 receptors, and a ligand for some other P2X and P2Y11 receptors.27–29 The treatment with BzATP did not trigger phosphorylation of AMPK and ACC (Figure 6), confirming that these subtypes of P2 receptors do not participate in ATP-induced activation of AMPK in HUVEC.

The data suggest that P2Y1, P2Y2, and/or P2Y4 receptors are involved in phosphorylation of AMPK in HUVEC, whereas P2Y6, P2Y11, and P2X receptors probably do not play a role in this activation.

Mechanism of Nucleotide-Induced Phosphorylation of AMPK
One of the first indicators of P2 receptor stimulation is an increase in [Ca\(^{2+}\)]. Therefore, we investigated the role of Ca\(^{2+}\) in nucleotide-induced AMPK phosphorylation. HUVEC were preincubated with a chelator of intracellular Ca\(^{2+}\), BAPTA-AM (20 minutes, 10 μmol/L), followed by stimulation with 100 μmol/L of ATP or UTP for 1 minute. BAPTA-AM significantly attenuated nucleotide-induced phosphorylation of AMPK and ACC (Figure 7), suggesting that Ca\(^{2+}\) plays an important role in this activation.

Figure 4. Nucleotide-induced phosphorylation of AMPK is not adenosine dependent. Representative Western blot of HUVEC pretreated for 15 minutes with inhibitor of 5’-nucleotidase, AOPCP (100 μmol/L), and incubated with or without nucleotides (100 μmol/L, 5 minutes). Cell lysates were immunoblotted with anti-P-AMPK (Thr172), anti–AMPK-α, and anti–P-ACC antibodies. After quantification, values were statistically analyzed and are expressed as means±SEM of 2 to 4 independent experiments. *P<0.05, **P<0.01 compared with control; +P<0.05 compared with non-preincubated cells, treated with respective nucleotide. C indicates control.

Figure 5. Changes in AMP:ATP ratio do not affect AMPK activation by extracellular nucleotides and adenosine. HUVEC were stimulated with 100 μmol/L ADP (A) or 25 μmol/L adenosine (B) for 5 and 10 minutes, and concentrations of intracellular AMP and ATP were measured by HPLC. Data are expressed as means±SEM of 3 independent experiments. P>0.05 compared with control.

Figure 6. Role of P2X and P2Y11 receptors in AMPK phosphorylation. HUVEC were stimulated with ATP (1 mmol/L, 5 minutes) with and without preincubation with KN62 (1 μmol/L, 20 minutes), the antagonist of P2X7 receptor. Cells were also treated with BzATP (100 and 300 μmol/L, 5 minutes), an agonist for P2X and P2Y11 receptors. Cell lysates were analyzed by Western blotting using antibodies to P-AMPK (Thr172), AMPK-α, and P-ACC. Results obtained from 3 independent experiments are presented as means±SEM. **P<0.01 compared with control. C indicates control.
AMPK Activation by Nucleotides and Adenosine

d da Silva et al

Figure 7. Phosphorylation of AMPK induced by nucleotides depends on Ca$^{2+}$ originating from intracellular stores. Representative Western blots showing the effect of BAPTA-AM on nucleotide-induced AMPK and ACC phosphorylation. HUVEC were pretreated with BAPTA-AM (20 minutes, 10 μmol/L) and stimulated with ATP and UTP (1 minute, 100 μmol/L). Cell lysates were immunoblotted with antibodies to P-AMPK, AMPK-α, and P-ACC. After quantification, values were statistically analyzed and are expressed as means ± SEM of 3 to 6 independent experiments. *P<0.05 and **P<0.01 compared with untreated cells. C indicates control.

Figure 8. Nucleotide-induced phosphorylation of AMPK is mediated by CAMKK in HUVEC. Representative Western blot of HUVEC pretreated for 30 minutes with STO-609 (1 μg/μL), inhibitor of CAMKK, and incubated with or without nucleotides (100 μmol/L, 1 minute). Cell lysates were immunoblotted with anti-P-AMPK (Thr172), anti-AMPK-α, and anti-P-ACC antibodies. After quantification, values were statistically analyzed and are expressed as means ± SEM of 3 independent experiments. *P<0.05, **P<0.01 compared with control. C indicates control.

Figure 9. Role of LKB1 in nucleotide and adenosine-mediated activation of AMPK. HeLa cells were incubated with extracellular nucleotides, ATP, UTP, and ADP (100 μmol/L, 2 minutes) or adenosine (25 μmol/L, 5 minutes). Representative Western blots show cell lysates immunoblotted with antibodies to P-AMPK (Thr172) and AMPK-α. Representative Western blots of 3 independent experiments. C indicates control.

Phosphatidylinositol 3-kinase (PI3K) has been demonstrated as an upstream activator of AMPK. Moreover, we have shown earlier that PI3K pathway was involved in extracellular nucleotide-induced EC migration. Thus, to establish the role of PI3K in nucleotide-induced activation of AMPK, 2 inhibitors of PI3K, LY294002 and wortmannin, were applied. Cell preincubation with LY294002 (LY) (10 μmol/L, 20 minutes) or wortmannin (W) (100 μmol/L, 20 minutes) before stimulation with ATP, UTP, or ADP had no significant effects on P-AMPK levels (ATP, 329 ± 21; UTP, 270 ± 21; ADP, 205 ± 22; W, 221 ± 38; results expressed as % of control ± SEM; n=3 to 6; P=0.179).

Stimulation of G protein–coupled P2Y receptors is linked to protein kinase C (PKC) activation. Furthermore, it has been shown that ischemic preconditioning induced AMPK activation in the PKC-dependent manner. To evaluate a role of PKC in the nucleotide-induced activation of AMPK, we used an inhibitor of PKC, bisindolylmaleimide I (BIM). Preincubation of EC with BIM (20 minutes, 100 μmol/L) did not change nucleotide-induced phosphorylation of AMPK (data not shown), indicating that PKC is not involved in the purinergic activation of AMPK.

Thus, we conclude that nucleotide-induced activation of AMPK in HUVEC is PI3K and PKC independent; however, increase in [Ca$^{2+}$], is crucial for this pathway. Ca$^{2+}$ interacts with calmodulin and activates Ca$^{2+}$/calmodulin-dependent kinase kinase (CaMKK) and Ca$^{2+}$/calmodulin-dependent kinase II (CaMK II), potential upstream regulators of AMPK, both expressed in HUVEC. KN62 (10 μmol/L), an inhibitor of CaMK II, had no effect on nucleotide-induced phosphorylation of AMPK (data not shown), whereas STO-609, a specific inhibitor of CAMKK (10 μmol/L), attenuated this response (and ACC phosphorylation) (Figure 8), indicating that CaMKK is the upstream kinase involved in extracellular nucleotides-mediated activation of AMPK.

Finally, to investigate a role of LKB1 in nucleotide-induced phosphorylation of AMPK, we used HeLa cells that are deficient in LKB1. All studied nucleotides (ATP, UTP, and ADP) were still able to stimulate the AMPK phosphorylation, even in the absence of LKB1. The nucleotide effects on AMPK were also inhibited by preincubation of HeLa cells with STO-609 (Figure 9). These results probably exclude LKB1 as an upstream kinase and confirm the involvement of CaMKK in nucleotide-induced AMPK phosphorylation.

Mechanism of Adenosine-Induced Phosphorylation of AMPK

As we demonstrated in Figures 1 and 2, adenosine induced phosphorylation of AMPK. EC are known to have a very active adenosine metabolism, characterized by a large capacity for uptake and release of the nucleoside. Extracellular adenosine can be metabolized to inosine and hypoxanthine. To evaluate the possible effect of adenosine metabolites, HUVEC were incubated for 5 minutes with 25 μmol/L inosine or hypoxanthine. Neither of them had an effect on AMPK phosphorylation (inosine, 118.3 ± 16.84% [n=3, P=0.344]; hypoxanthine, 100.3 ± 14.4% of control [n=3, P=0.705]). These data suggest that the observed adenosine-induced AMPK phosphorylation is indeed adenosine specific.
Extracellular adenosine can exert its effect on cells either by P1 receptors or after entering the cells, which is mediated by adenosine transporters. To identify the mechanism of adenosine-induced AMPK phosphorylation, HUVEC were pretreated for 20 minutes with either an antagonist of P1 receptors, 8-SPT (30 μM), or inhibitor of adenosine transporter NBTI (10 μM), and incubated with adenosine (ADO) (25 μM, 5 minutes). Cell lysates were immunoblotted with antibodies to P-AMPK (Thr172), AMPK-α, and P-ACC. After quantification, values were statistically analyzed and are expressed as means ± SEM of 5 to 7 independent experiments. **P<0.01 compared with the control. C indicates control.

Intracellular adenosine can be metabolized by either adenosine deaminase that converts adenosine to inosine or adenosine kinase that converts adenosine to AMP. Inhibition of adenosine kinase with AMDA (10 μM, 30 minutes) completely blocked AMPK activation and ACC phosphorylation, whereas inhibition of adenosine deaminase with EHNA (10 μM, 30 minutes) did not affect adenosine-induced AMPK activation (Figure 11). Therefore, in adenosine-initiated pathway of AMPK phosphorylation, the metabolism of intracellular adenosine to AMP seems to play an important role.

In addition, we examined a role of possible upstream activators of AMPK, in adenosine-induced AMPK phosphorylation. Wortmannin and LY294002 (inhibitors of PI3K), KN62 (10 μM; CaMK II inhibitor), or STO-609 (1 μg/mL; CaMKK inhibitor) did not change the effect of adenosine (25 μM, 5 minutes) on AMPK phosphorylation in HUVEC (Figure 12). Therefore we conclude that PI3K, CaMK II, and CaMKK were not mediators of adenosine-initiated pathway of AMPK activation.
AMPK Activation by Nucleotides and Adenosine

Discussion

In the present study, we show for the first time that in HUVEC, extracellular nucleotides, and adenosine independently induce phosphorylation of AMPK via P2Y receptors and nucleoside transporters, respectively.

AMPK can be activated by the allosteric action of AMP and by phosphorylation of the Thr172 on the catalytic α subunit by upstream kinases. Extracellular nucleotides and adenosine induced phosphorylation of AMPK on Thr172 in a time- and concentration-dependent manner (Figures 1 and 2) and thus activated it. This functional AMPK activation has been additionally validated by experiments showing phosphorylation of the AMPK downstream target, ACC (Figures 1 through 10). Although nucleotides significantly phosphorylated AMPK at concentrations of 50 μmol/L and higher, the effect of adenosine was maximal at 25 μmol/L and decreased at 50 and 100 μmol/L. Adenosine that is transported into cells can be metabolized by different enzymes, including adenosine kinase and adenosine deaminase. At lower concentrations, adenosine is converted by adenosine kinase to AMP. However, adenosine kinase is substrate inhibited; thus, adenosine at concentrations exceeding several micromoles per liter is metabolized via adenosine deaminase to inosine, which has no effect on AMPK activation. Hence, our data are consistent with the kinetics of adenosine metabolism.

Extracellular nucleotides are sequentially hydrolyzed by NTPDases and 5′-nucleotidase to adenosine, which can also activate AMPK. However, phosphorylation of AMPK induced by ATP, UTP, and ADP was not diminished the inhibitor of 5′-nucleotidase, AOPCP (Figure 4); therefore, we concluded that extracellular nucleotides and not adenosine, originating from nucleotide hydrolysis, activate AMPK.

Extracellular nucleotides do not cross the cell membrane but exert their biological action as extracellular signaling molecules through ubiquitously expressed purinergic P2X and P2Y receptors. Our data showed a role for P2Y1 and P2Y2 and/or P2Y4 receptors in the activation of AMPK because ADP and UTP, the primary agonists of these receptors, induced phosphorylation of AMPK. Lack of response to UDP suggested that P2Y6 receptors were not involved in AMPK activation. Experiments with BzATP and KN62, an agonist and a noncompetitive antagonist of P2X7 receptors, respectively, excluded the role of P2X7 receptor in phosphorylation of AMPK (Figure 6). BzATP has been recognized as a selective agonist of P2X7 receptors; however, it can also activate P2Y11 receptors, as well as other P2X receptors. Lack of AMPK phosphorylation by BzATP excluded P2X and P2Y11 receptors from the list of receptors potentially involved in the AMPK activation.

We have previously shown that in HUVEC, extracellular nucleotide-induced increases in [Ca2+]i were linked to P2Y receptors. Here, we observed significant attenuation of ATP and UTP-induced phosphorylation of AMPK by BAPTA-AM (Figure 7), which indicates that Ca2+ released from the endoplasmic reticulum is essential for the activation of AMPK. These results confirm that P2Y (and not P2X) receptors are indeed responsible for nucleotide-induced activation of AMPK in HUVEC.
concentration of newly generated AMP might increase in some cellular compartments where AMPK activation takes place, and this could be a mechanism of adenosine-mediated AMPK activation. We hypothesize that the mechanism of adenosine-induced activation of AMPK is linked to LKB1, with possible effect of an increase in the intracellular AMP level.

There was also some (statistically insignificant) increase in the AMP level and in the AMP:ATP ratio in EC activated with ADP (Figure 5). We assume that the changes in intracellular nucleotide levels observed after incubation with ADP might have been, in fact, related to adenosine, originating from hydrolysis of ADP. This hypothesis is supported by published data,\textsuperscript{45,46} showing that inhibitor of adenosine transporter, dipyridamole, entirely blocks changes in the intracellular nucleotide levels in response to extracellular nucleotides.

AMPK can play an important role in the endothelium associated with regulation of EC metabolism and angiogenesis and can protect the endothelium from various stresses, including high levels of glucose and fatty acids. It regulates fatty acid metabolism in EC and therefore is involved in the cellular energy homeostasis.\textsuperscript{50} AMPK also induces NO generation, enhances the response to insulin, and protects EC from hyperglycemia-induced apoptosis.\textsuperscript{51,52}

AMPK has been shown to be activated by hypoxia, and AMPK signaling was identified as a new regulator of angiogenesis that is specifically required for EC migration and differentiation under conditions of hypoxia.\textsuperscript{53} Another study indicates that AMPK activated under hypoxia can be involved in coregulation of hypoxia induced factor-1 (HIF-1), a transcription factor that is critical for hypoxic induction of physiologically important genes, as well as secretion of vascular endothelial growth factor (VEGF) and glucose uptake.\textsuperscript{54} Further studies on the AMPK role in physiology and pathophysiology of the endothelium seem to be of great importance.

In summary, our findings demonstrate 2 distinct, but converging, pathways of AMPK activation in HUVEC: the first is induced by extracellular nucleotides, linked to P2Y1, P2Y2, and/or P2Y4 receptors, dependent on Ca\textsuperscript{2+} and CaMKK; and the second pathway is induced by adenosine uptake with the involvement of adenosine transporters, followed by the generation of (intracellular) AMP, and activation of AMPK, with LKB1 as a putative upstream kinase. In an in vivo situation, nucleotides are finally hydrolyzed to adenosine. Therefore, the 2 mechanisms of AMPK activation, nucleotide and adenosine dependent, can operate at the same time. Extracellular nucleotides and adenosine, whose local concentrations under pathophysiological conditions can be substantially increased, could play an important role in restoring the energy balance by AMPK activation that modulates glucose uptake and fatty acid metabolism. We propose that P2Y receptors and nucleoside transporters could be novel targets for a pharmacological intervention to regulate AMPK and hence EC metabolism and function.

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