Thrombin and histamine stimulate endothelial nitric-oxide synthase phosphorylation at Ser1177 via an AMPK mediated pathway independent of PI3K-Akt

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Abstract Histamine and thrombin cause phosphorylation and activation of endothelial NO-synthase (eNOS) on Ser1177. We tested the role of various protein kinases in mediating this effect in human umbilical vein endothelial cells. Inhibition of the Ca^{2+}/calmodulin-dependent protein kinase II or phosphoinositide 3-kinase (PI3K) had no effect. H89, an inhibitor of both protein kinase A (PKA) and 5′-AMP-activated protein kinase (AMPK), strongly inhibited phosphorylation and activity of eNOS. Conversely, the PKA inhibitor Rp-adenosine 3′5′-cyclic monophosphate (cAMPS) had no effect and eNOS was not phosphorylated by treatments that affect cAMP levels. Thrombin and histamine caused phosphorylation of AMPK on Thr172 as well as on its downstream target acetyl-CoA carboxylase. Activation of AMPK using AICAR or CCCP also resulted in eNOS phosphorylation. We conclude that histamine and thrombin cause eNOS phosphorylation in an AMPK mediated manner, independent of P13K-Akt.

Keywords: eNOS; AMPK; Histamine; Thrombin; Endothelial cell

1. Introduction

Given the central position of the vascular endothelium in early atherogenesis and the vast number of blood borne chemical, cellular and rheological stimuli that continuously affect endothelial cells, the signal transduction mechanisms involved in linking exposure to endothelial response have in recent years attracted considerable attention. Among the numerous biologically important molecules produced by the vascular endothelium, nitric oxide (NO) seems to play a particularly important role in regulating cardiovascular homeostasis. It affects blood vessel dilation and hence vascular resistance and blood pressure, angiogenesis, apoptosis, adhesion of platelets and monocytes to the endothelium, endothelin-1 generation and vascular smooth muscle cell proliferation [1].

The major source of NO is endothelial NO-synthase (eNOS) which is regulated by a complex battery of regulatory mechanisms, including subcellular localization in caveoli where interaction of eNOS with caveolin and heat shock protein 90 exemplifies a regulatory protein-protein interaction [2]. Regulation with phosphorylation has also been reported and recently specific sites for phosphorylation of eNOS and specific protein kinases mediating the phosphorylation have been identified. These include protein kinase B (PKB)/Akt [3], protein kinase A (PKA) [4], protein kinase C (PKC) [5], 5′-AMP-activated protein kinase (AMPK) [6,7] and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [8], Michell and coworkers have published data suggesting that regulation of eNOS activity involves coordinated phosphorylation and dephosphorylation of Ser1179 and Thr497 residues on bovine eNOS by multiple protein kinases and phosphatases with stimulation, involving phosphorylation at Ser1179 and dephosphorylation at Thr497 [5]. Bradykinin has been shown to activate eNOS phosphorylation at Ser1179 either in an Akt [9] or CaMKII [8] dependent manner while stimulating dephosphorylation of Thr497 through a calcineurin mediated, Akt independent mechanism [9]. Adiponectin was also recently shown to cause eNOS phosphorylation at Ser1179. This phosphorylation was inhibited by wortmannin but was still Akt independent and mediated partially by AMPK [7]. Others have implicated Akt in eNOS phosphorylation caused by adiponectin, demonstrating a cross-talk between AMPK and Akt in endothelial cells [10].

AMPK is the central component of a protein kinase cascade that plays an important role in the regulation of energy metabolism, often referred to as the cell's metabolic master switch [11]. In response to a decrease in the energy state of a cell, AMPK is phosphorylated and activated by a still not fully characterized upstream pathway. Once activated, AMPK phosphorylates multiple targets, all aimed at restoring adenosine 5′-triphosphate (ATP) levels. These include acetyl CoA carboxylase (ACC), hydroxymethylglutaryl-CoA

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Abbreviations: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carbozamide-1-β-4 ribofuranoside; AMP, adenosine 5′-monophosphate; AMPK, 5′-AMP-activated protein kinase; ATP, adenosine 5′-triphosphate; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; cAMP, adenosine 3′5′-cyclic monophosphate; EGF, epidermal growth factor; eNOS, endothelial NO-synthase; HUVEC, human umbilical vein endothelial cells; NO, nitric oxide; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; ROCK, Rho-dependent protein kinase

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(HMG-CoA) reductase, glycogen synthase and eNOS [11]. The phosphorylation of eNOS at Ser1177 by AMPK is Ca^{2+}/CaM dependent [6].

We have previously shown in primary cultures of human umbilical vein endothelial cells (HUVEC) that thrombin and histamine, although inhibiting Akt phosphorylation when applied in cotreatment with epidermal growth factor, stimulate eNOS phosphorylation at Ser1177 [12].

In this paper, we explore the role of various protein kinases in mediating the stimulatory effects of the G-protein activators thrombin and histamine on eNOS Ser1177 phosphorylation. Our results demonstrate that AMPK mediates the stimulatory effects of both agonists through a phosphoinositide 3-kinase (PI3K)-Akt independent pathway.

2. Materials and methods

2.1. Materials

Morgan's medium 199, fetal bovine serum and Penicillin–Streptomycin were purchased from Gibco-BRL, Life Technologies. Tissue culture plates (35 mm) were from Nunc, Cryotin X (collagenase) from cod was provided by The Science Institute of Iceland. Epidermal growth factor (EGF), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), histamine, thrombin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 1-isobutyl-3-methyl xanthine (IBMX), 8-bromo-adenosine 3′5′-cyclic monophosphate (cAMP), Rp-cAMPS, A23187, U73122, forskolin and wortmannin were purchased from Sigma. H89 was from Biomol. 5-aminoimidazole-4-carbozamide-l-β-4 ribofuranoside (AICAR), BAPTA/AM, KN-93 and Y 27632 were from Calbiochem. Myo[^{3}H]inositol, Hybond ECL Nitrocellulose membrane (6×8 cm), ECL + PLUS Western blotting detection system, Hyperfilm ECL High performance chemiluminescence film and L-(^{3}H) arginine (61.0 Ci/mmol) were from Amersham Pharmacia Bioteck. Antibodies against phospho-eNOS (Ser1177), AMPK, phospho-AMPK (Thrl72) and Anti-Rabbit IgG/HRP-linked came from Cell Signaling Technology. eNOS antibody came from Transduction Laboratories and pACC (Ser79) and ACC antibodies from Upstate, Poly-prep prefilled chromatography columns were from BioRad.

2.2. Endothelial cell culture

Endothelial cells were cultured from human umbilical veins by a modification of the method of Jaffe et al. as previously reported [13]. The cells were harvested by Cryotin X collagenase digestion and seeded on 35 mm culture dishes in medium 199 containing 20% fetal bovine serum and antibiotics (penicillin, 100 units/mL and streptomycin, 100 μg/mL). The culture dishes were incubated at 37 °C in humidified air with 5% CO_2. The medium was changed 24 h after seeding the cells and every 2–3 days thereafter until the cell culture reached confluence (after ~7 days).

When confluent, the cells were washed with Morgan's medium 199 and then placed in 1.0 mL Morgan's medium 199 with or without inhibitors at the indicated concentrations. 10–20 min later, agonist was added in a concentration calculated to reach the intended concentration for each experiment and left on for additional 2–20 min. The agonists were then removed along with the medium and cellular reactions terminated by adding 250 μL SDS sample buffer. The samples were boiled for 5 min and centrifuged for 10 min at 3000 rpm. After that, the samples were ready to be used or could be stored at −20 °C.

2.3. Electrophoresis and immunoblotting

Samples (8 μL) were resolved by SDS–PAGE (10%). The gels were blotted and the proteins thereby transferred to nitrocellulose. The membranes were hybridized with the indicated antibodies and later with a secondary antibody (Anti-Rabbit IgG/HRP-linked). The immuno-complexes were detected with ECL+PLUS Western blotting detection system and developed onto a film. Equal loading was ascertained by hybridizing membranes with antibodies against unphosphorylated protein.

2.4. Determination of eNOS activity

eNOS activity in intact cells was determined by monitoring the conversion of incorporated L-(^{3}H) arginine into L-(^{3}H) citrulline as described by Schmidt and Mayer [14]. Briefly, the cells were washed and equilibrated for 20 min in incubation buffer (50 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 5 mM KC1, 1 mM $MgCl_2$, and 2 mM $CaCl_2$) with or without BAPTA or H89. Reactions were started by the addition of L-(^{3}H) arginine (61.0 Ci/mmol, 1 μCi, final concentration 16 nM) and the agonist. After 3 min the cells were washed with chilled incubation buffer containing 0.1 mM EGTA instead of $CaCl_2$, followed by the addition of 1 mL of 10 mM HCl. An hour later, an aliquot was removed for determining the incorporated radioactivity. To the remaining sample a solution of 200 mM sodium acetate, 20 mM NaOH containing 10 mM citrulline was added (final pH approx. 5.0) and L-(^{3}H) citrulline was separated from L-(^{3}H) arginine by cation exchange chromatography [14].

3. Results

3.1. Effects of EGF, histamine and thrombin on eNOS phosphorylation

Fig. 1 demonstrates the effects of EGF, histamine and thrombin on Serl177 phosphorylation of eNOS. As seen, the PI3K inhibitor wortmannin totally blocked the effect of EGF whereas phosphorylation by histamine and thrombin was unaffected, demonstrating different pathways mediating phosphorylation by these agonists.

3.2. Histamine and thrombin mediated eNOS phosphorylation is dependent on Ca^{2+} but independent of CaMKII

As shown in Fig. 2A, U73122, an inhibitor of phospholipase Cβ (PLCβ), inhibited the histamine mediated phosphorylation of eNOS. Conversely, the effects of the Ca^{+2} ionophore A23187 were not affected. The calcium chelator BAPTA blocked the phosphorylation by thrombin and histamine as well as that caused by A23187 (Fig. 2A).

One of the downstream effectors of Ca^{2+}/CaM is CaMKII, which has been implicated in eNOS phosphorylation after bradykinin treatment [8]. As seen in Fig. 2A, the CaMKII inhibitor KN-93 had no effect on eNOS phosphorylation caused by histamine or thrombin. The results suggest that

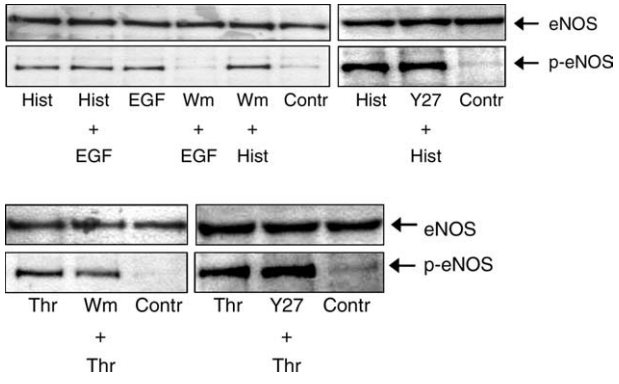

Fig. 1. The effect of EGF, histamine or thrombin on eNOS phosphorylation (Ser1177). Cells were treated with wortmannin (100 nM) for 10 min before treatment with EGF (10 ng/mL, 5 min), histamine (10 μM, 10 min) or thrombin (1 U, 5 min). The effect of Y 27632 (10 μM, 20 min) on histamine or thrombin mediated eNOS phosphorylation. Confluent endothelial cells were stimulated as indicated, lysates were electrophorized and blotted as described in methods and detected using antibody against eNOS and the Serl177 phosphorylated eNOS. Each Western blot is representative of three independent experiments.

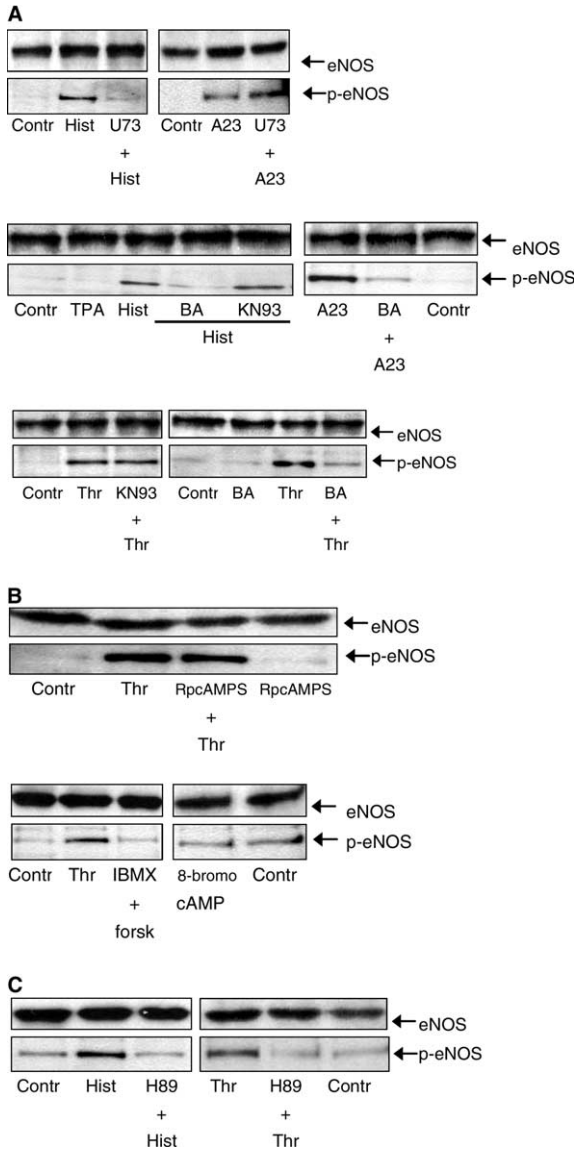

Fig. 2. The role of various protein kinases in eNOS phosphorylation (Serl177). (A) The cells were treated with U73122 (10 μM) for 20 min before treatment with histamine (10 μM, 3 min) or A23187 (200 nM, 3 min). The effect of a 20 min pre-treatment with BAPTA (30 μM) or KN-93 (30 μM) on histamine (10 μM, 5 min), thrombin (1 U, 5 min) or A23198 (200 nM, 3 min) mediated eNOS phosphorylation. The effect of TPA (100 ng/mL, 7 min) on eNOS phosphorylation. (B) The effect of Rp-cAMPS (10 μM, 20 min) on eNOS phosphorylation after thrombin (1 U, 2 min). The effect of BMX + forskolin (100 μM/10 μM, 20 min), thrombin (1 U, 10 min) and 8-bromo-cAMP (100 μM, 10 min) on eNOS phosphorylation. (C) Cells were treated with H89 (20 μM) for 10 min before treatment with histamine (10 μM, 3 min) or thrombin (1 U, 10 min). Samples were separated by SDS–PAGE and analyzed by Western blotting. Each Western blot is representative of three independent experiments.

histamine and thrombin mediated phosphorylation of eNOS is dependent on a Ca^{2+} signal without involving CaMKII.

3.3. PKC and PKA are not involved in histamine or thrombin-induced eNOS phosphorylation at Ser 1177

In addition to Ca^{2+} elevation, activation of PI specific PLC in HUVEC leads to activation of PKC. Therefore, we tested the effect of the PKC activator TPA on eNOS phosphorylation. As seen in Fig. 2A, TPA had no effect.

To test the possible effect of PKA on eNOS phosphorylation, the cells were treated either with substances increasing intracellular cAMP (IBMX and forskolin or 8-bromo-cAMP) or with the PKA inhibitors Rp-cAMPS or H89. None of the substances increasing cAMP levels had any effect on eNOS phosphorylation nor did Rp-cAMPS prevent the phosphorylation of eNOS after thrombin treatment (Fig. 2B). Conversely, H89 totally inhibited both histamine and thrombin stimulated eNOS phosphorylation (Fig. 2C). The inhibition was 96±4% based on scanning three independent gels.

3.4. The role of AMPK in eNOS phosphorylation caused by histamine or thrombin

Although marketed as "a selective and potent inhibitor of PKA", Cohen and coworkers [15] have shown that H89 inhibits several protein kinases to a similar degree, including AMPK. As AMPK has been shown to phosphorylate eNOS [6,7], we tested the effects of histamine and thrombin on AMPK activity.

Both histamine and thrombin caused phosphorylation of AMPK (Thrl72) and the AMPK substrate ACC (Ser79) (Fig. 3A). The phosphorylation of AMPK and ACC was unaffected by wortmannin, but inhibited by the Ca^{2+} chelator BAPTA (Fig. 3A). Furthermore, H89 inhibited ACC phosphorylation caused by histamine or thrombin (97 ± 3% inhibition, quantified by scanning three independent gels), whereas AMPK phosphorylation was not affected (Fig. 3A). AMPK, eNOS and ACC phosphorylation was also caused by CCCP, which activates AMPK by lowering the ATP/AMP ratio [16] (Fig. 3B). AICAR, which mimicks the activating effect of AMP on AMPK without affecting the AMP/ATP ratio [17] also caused phosphorylation of AMPK, eNOS and ACC (Fig. 3C). All the effects of thrombin, histamine and CCCP on eNOS phosphorylation were independent of PI3K (Figs. 1 and 3B), whereas H89 inhibited phosphorylation of eNOS after all these treatments (Figs. 2C and 3B). Finally, the Ca^{2+} ionophore A23187 mimicked the effects of thrombin and histamine on the phosphorylation of AMPK, eNOS and ACC (Fig. 4). These effects of A23187 on eNOS phosphorylation were inhibited by H89 and BAPTA but unaffected by wortmannin (Figs. 2A and 4A). BAPTA also prevented the phosphorylation of AMPK by A23187 (Fig. 4B). As with eNOS, the PLC inhibitor U73122 prevented the histamine mediated AMPK phosphorylation but had no effect on the phosphorylation mediated by A23187 (Fig. 4B).

As H89 has been shown to inhibit Rho-dependent protein kinase II (ROCK-II) [15] as well as AMPK, and ROCK-II can regulate eNOS expression and phosphorylation [18], we tested the effects of the ROCK-II inhibitor Y 27632. As seen in Fig. 1, inhibiting ROCK-II had no effect on eNOS phosphorylation.

3.5. eNOS activation in response to histamine or thrombin

In order to determine whether histamine or thrombin treatment led to the activation of eNOS, we studied their effect on the conversion of L-(^{3}H) arginine to L-(^{3}H) citrulline. Both agonists caused threefold increase in citrulline formation (Fig. 5) and this activation was largely prevented by pretreatment with H89 or BAPTA. BAPTA also caused a decrease in the basal rate of conversion to L-(^{3}H) citrulline. As

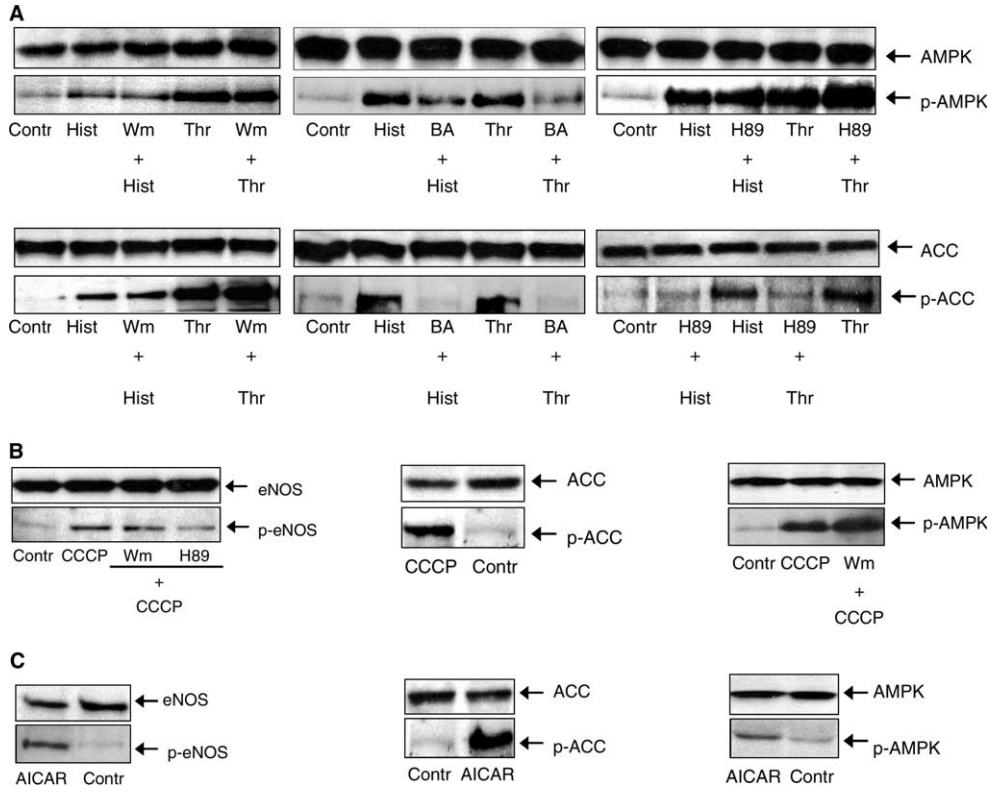

Fig. 3. The role of AMPK in eNOS phosphorylation (Ser1177). (A) The effect of wortmannin (100 nM, 10 min), BAPTA (30 μM, 10 min) or H89 (20 μM, 20 min) on histamine (10 μM, 5 min) or thrombin (1 U, 5 min) mediated phosphorylation ofAMPK (Thrl72) or ACC (Ser79). (B) The effect of a 20 min pre-treatment with wortmannin (100 nM) or H89 (20 μM) on eNOS or AMPK phosphorylation caused by CCCP (10 μM, 3 min). The effect of CCCP (10 μM, 10 min) on ACC phosphorylation. (C) Cells were treated with AICAR (2 mM) for 4 h (eNOS, ACC) or 6 h (AMPK). The AICAR treatments were performed in medium containing 20% serum. Samples were separated by SDS–PAGE and analyzed by Western blotting. Each Western blot is representative of three independent experiments.

described by Wagner et al. [19], using bradykinin or ATP as agonists, the activation was shortlived, being almost over in 3 min (not shown).

4. Discussion

In this paper, we show that both histamine and thrombin cause eNOS phosphorylation at Ser1177 in HUVEC in an AMPK mediated manner, independent of PI3K-Akt. Several protein kinases have been implicated in eNOS phosphorylation at Ser1177. We demonstrate that the phosphorylation caused by histamine and thrombin is Ca^{2+} dependent, without the involvement of PKC or CaMKII. Also, results from various manipulations affecting cAMP levels weighed against the possibility that PKA mediated the eNOS phosphorylation caused by these G-protein agonists. It has been shown that AMPK can phosphorylate eNOS on Ser1177 [6,20] and eNOS has been reported to be in a complex with AMPK [6,21]. We show that histamine and thrombin cause phosphorylation of AMPK on Thrl72 and that wortmannin had no effect on histamine or thrombin-mediated phosphorylation of either eNOS or AMPK. Other substances, such as the mitochondrial uncoupler CCCP and AICAR, which cause AMPK phosphorylation [16,17], also caused eNOS phosphorylation in our cells further supporting our conclusion that histamine and thrombin stimulate phosphorylation of eNOS in an AMPK-dependent manner. Using an L-(^{3}H) citrulline conversion assay, we demonstrate that treatment with histamine or thrombin actually leads to eNOS activation. The inhibition of AMPK phosphorylation as well as eNOS phosphorylation by U73122 suggests that histamine and thrombin mediate their activation by G_q. Histamine and thrombin are known to activate PLC in HUVEC [22] and G_q coupled receptors cause activation of AMPK [23].

Previously, it has been reported that the G-protein activator bradykinin stimulates phosphorylation of eNOS on Ser1177 via CaMKII [8]. However, while we found that histamine and thrombin mediated phosphorylation of eNOS was Ca^{2+} dependent, inhibited by the Ca^{2+} chelator BAPTA and mimicked by the Ca^{2+} ionophore A23187, it was unaffected by the CaMKII inhibitor KN-93. Treatment with TPA had no effects on eNOS Ser1177 phosphorylation, thereby ruling out PKC mediated effects of histamine or thrombin. The inhibition by H89, however, suggested a possible involvement of PKA as had been demonstrated for eNOS phosphorylation caused by shear stress and bradykinin [4,24]. However, H89 is not a specific PKA inhibitor and has been reported to inhibit several other protein kinases including AMPK and ROCK-II [15]. Also, Rp-cAMPS, a structurally unrelated PKA inhibitor, was without effect on eNOS phosphorylation. Finally, manipulation of cAMP levels had no effect on eNOS phosphorylation in our cells, indicating that the involvement of PKA in this pathway is unlikely. Therefore, we conclude that the H89 in-

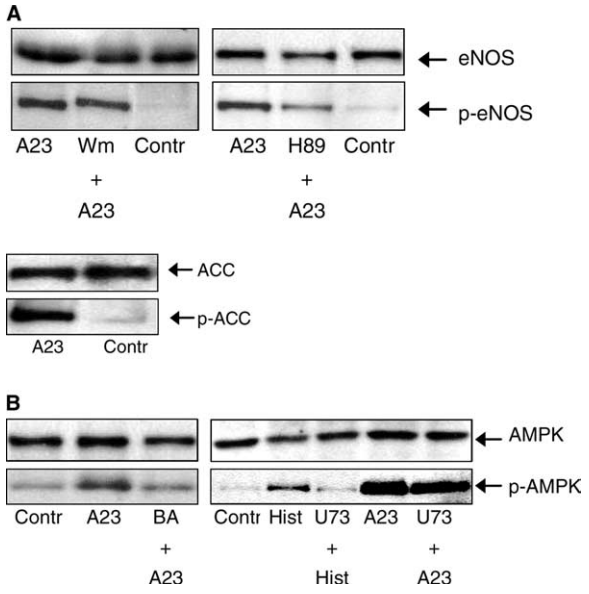

Fig. 4. The involvement of Ca^{2+} in eNOS (Ser1177) or AMPK (Thrl72) phosphorylation. (A) The effect of A23187 (200 nM, 3 min). The cells were treated with wortmannin (100 nM) or H89 (20 μM) for 20 min before treatment with A23187. (B) The cells were treated with BAPTA (30 μM) for 20 min before treatment with A23187 (200 nM, 3 min). The effect of U73122 (10 μM, 20 min) on histamine (10 μM, 3 min) or A23187 (200 nM, 3 min) mediated AMPK phosphorylation. Samples were separated by SDS–PAGE and analyzed by Western blotting. Each Western blot is representative of three independent experiments.

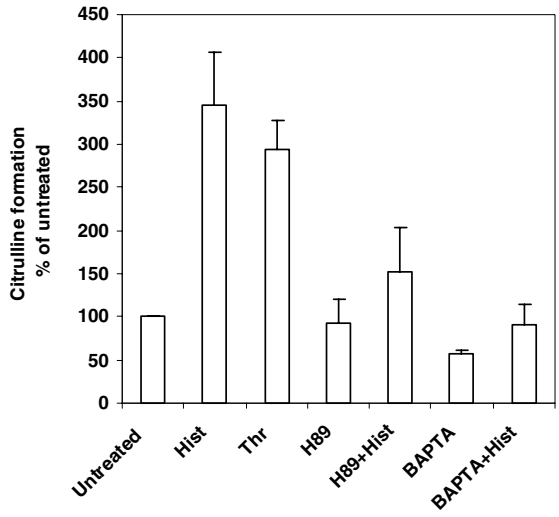

Fig. 5. eNOS activation in response to histamine or thrombin. Effect of histamine (10 μM, 3 min) or thrombin (1 U, 3 min) on eNOS activation and the effect of 20 min pre-treatment with H89 (20 μM) or BAPTA (30 μM) on eNOS activation caused by histamine. The results show the average ± S.D. of three experiments each done in duplicate.

hibition of eNOS phosphorylation is due to AMPK inhibition. This is further supported by our finding that H89 inhibits the phosphorylation of the AMPK substrate ACC but not the phosphorylation of AMPK itself.

It has repeatedly been shown that regulation of eNOS is a complex, multifactorial process [2]. Harris et al. [9] concluded that bradykinin stimulates eNOS phosphorylation via PI3K/Akt. After bradykinin treatment they demonstrated Akt phosphorylation and eNOS phosphorylation which were inhibited by wortmannin [9]. However, others have demonstrated wortmannin sensitive phosphorylation of eNOS which is Akt independent. Thus, shear stress mediated eNOS phosphorylation is mediated by PKA [4] and adiponectin mediated eNOS phosphorylation is mediated by AMPK [7]. Further complications were recently reported by Ouchi et al. [10] who demonstrated that Akt functions downstream of AMPK in adiponectin stimulated endothelial cells, suggesting the following signaling pathway: adiponectin-AMPK-PI3K-Akt-eNOS. However, in all those cases, eNOS phosphorylation mediated by AMPK was P13K dependent. In contrast, in the experiments reported in this paper, wortmannin did not inhibit eNOS phosphorylation caused by histamine or thrombin, clearly demonstrating a P13K-independent pathway distinct from those activated by the other G-protein agonists, adiponectin and bradykinin. Our finding that BAPTA inhibits the phosphorylation of AMPK, eNOS and ACC together with the observation that all these phosphorylations are mimicked by treatment with the Ca^{2+} ionophore A23187, suggests that a Ca^{2+} signal is upstream of AMPK phosphorylation in thrombin or histamine stimulated endothelial cells.

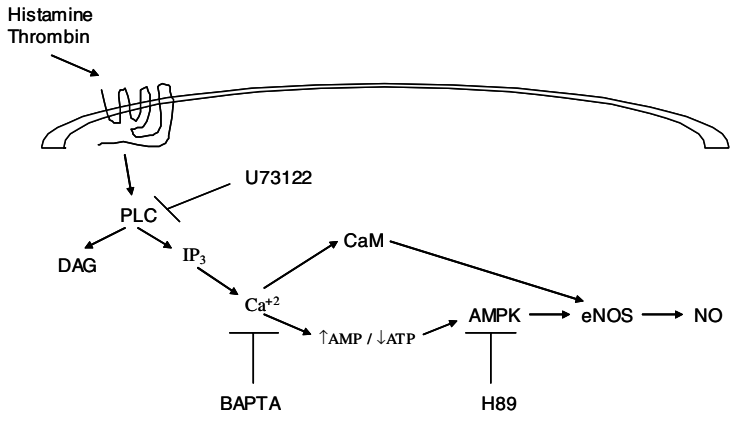

Fig. 6. A proposed pathway for thrombin and histamine-mediated eNOS activation. Thrombin and histamine activate PLC, causing formation of IPs and release of Ca^{2+} initiating energy requiring processes and activation of AMPK. AMPK phosphorylates eNOS and this together with increased Ca^{2+} levels activates eNOS to produce NO.

Finally, the inhibition by H89 and BAPTA in the citrulline assay suggests the importance of Ser1177 phosphorylation in the activation of eNOS following histamine treatment.

Thrombin, histamine and A23187 have previously been shown to cause a transient phosphorylation of elongation factor 2 (eEF2) [25] which is downstream of AMPK [26], and thus to temporarily inhibit protein synthesis. This effect is mimicked by treatments that lower ATP levels (oligomycin and CCCP) [16]. We speculate (Fig. 6) that the activation of AMPK that we describe in HUVEC is mediated by lowered ATP levels. Both thrombin and histamine cause activation of phospholipase C leading to a Ca^{2+} signal resulting in synthesis of prostacyclin and platelet activating factor, release of von Willenbrands factor and induction of shape change, all processes which require ATP. Inhibition of PLC by U73122 prevented the phosphorylation of both AMPK and eNOS after histamine stimulation but not after treatment with the Ca^{2+} ionophore A23187 (PLC independent).

In conclusion, in HUVEC we have demonstrated a role for AMPK in a pathway mediating stimulatory signals from G-protein coupled receptors causing eNOS phosphorylation in a PI3K-Akt independent manner.

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