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Biochemical Pharmacology 63 (2002) 1137–1142

Biochemical Pharmacology

Lack of involvement of extracellular signal-regulated kinase (ERK) in the agonist-induced endothelial nitric oxide synthesis

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Received 9 October 2001; accepted 12 December 2001

Abstract

In a recent paper, it was shown that stimulation of endothelial cells with bradykinin (BK) leads to phosphorylation of endothelial nitric oxide synthase (eNOS) mediated by extracellular signal-regulated kinase (ERK) (J. Biol. Chem. 275 (2000) 30707). Since *in vitro* phosphorylation by ERK reduced the catalytic activity of eNOS, it was suggested that this mechanism may be an important determinant of nitric oxide signalling in endothelial cells. To explore the physiological role of ERK as regulator of nitric oxide synthesis in intact cells, we measured the effects of the kinase inhibitor PD 98059 on BK- and ATP-induced nitric oxide formation in cultured endothelial cells and isolated vascular smooth muscle strips. PD 98059 completely inhibited ERK activation by BK and ATP in porcine aortic endothelial cells without affecting eNOS activation. Moreover, PD 98059 did not potentiate relaxation of isolated porcine pulmonary arteries to BK or ATP, indicating that ERK-catalysed eNOS phosphorylation does not contribute to the regulation of nitric oxide formation in intact cells or tissues.

Keywords: Nitric oxide; Endothelial nitric oxide synthase; Phosphorylation; Extracellular signal-regulated kinase; Endothelial cells; Smooth muscle relaxation

1. Introduction

Nitric oxide (NO) is a major signal and effector molecule that is involved in a variety of important physiological processes like vascular relaxation, platelet activation, neurotransmission, and unspecific immune response [1–4]. In the cardiovascular system, the formation of NO is catalysed by eNOS present in endothelial cells and cardiac myocytes. In addition to being controlled by Ca^{2+}/calmodulin (CaM) [5,6], eNOS activity is regulated by a direct interaction of the enzyme with cellular proteins, such as heat-shock protein 90 [7], caveolin-1 [8–11], and G protein-coupled receptors [12,13]. In the last few years much evidence has accumulated that endothelial NO formation is also regulated by eNOS phosphorylation catalysed by protein kinase B (Akt), AMP-activated protein kinase (AMPK), cAMP-dependent protein kinase (PKA), and protein kinase C (PKC). Activation of Akt is induced by a variety of stimuli such as shear-stress [14,15], vascular endothelial growth factor [16,17], estrogen [18,19], or BK [20] and leads to phosphorylation of eNOS at Ser-1177 (human sequence), resulting in an increase in the catalytic activity of eNOS and a decrease in the dependence of the enzyme for Ca^{2+}/CaM [15–17,21]. Phosphorylation of Ser-1177 is also mediated by PKA in response to cAMP-increasing drugs [22] and by AMPK in the course of metabolic stress [23]. The second phosphorylation site in eNOS that is involved in enzyme regulation is Thr-495. Phosphorylation of this residue is mediated by PKC and leads to decrease in eNOS activity [22]. Since phosphorylation of eNOS at Ser-1177 is accompanied by dephosphorylation at Thr-495, and *vice versa*, the phosphorylation and dephosphorylation reactions at the two sites are apparently coordinated [20,22,24]. In a recent paper, it was reported that activation of the MAP kinase cascade by stimulation of endothelial cells with BK also results in eNOS phosphorylation [25]. The effect was blocked by the MAP kinase kinase inhibitor PD 98059, indicating that BK-induced phosphorylation of eNOS was catalysed by ERK. Since *in vitro* phosphorylation of eNOS by recombinant ERK diminished enzyme activity, it was proposed that ERK-catalysed phosphorylation may serve as

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Abbreviations: NO, nitric oxide; eNOS, endothelial nitric oxide synthase; L-NNA, N^G-nitro-L-arginine; CaM, calmodulin; BK, bradykinin; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase.

0006-2952/02/$ – see front matter
PII: S0006-2952(01)00936-4

a negative feedback mechanism to attenuate Ca^{2+}-induced eNOS activation. To test the hypothesis of a functional role of ERK in endothelial NO signalling, we studied the effects of PD 98059 on Ca^{2+}-induced NO formation in cultured endothelial cells and isolated vascular smooth muscle strips.

2. Materials and methods

2.1. Materials

Cell culture media, antibiotics and foetal calf serum were purchased from PAA Laboratories GmbH. L-[2,3,4,5-^{3}H]-Arginine hydrochloride (40–80 Ci/mmol) was purchased from American Radiolabeled Chemicals, anti-active MAP kinase antibody from Promega, PD 98059 from Calbiochem, and the ECL Western blotting detection system from Amersham. All other chemicals were obtained from Sigma. For determination of cGMP by radioimmunoassay, ^{125}I-labelled succinyl cGMP tyrosine methyl ester (prepared as described in [26]) and polyclonal anti-cGMP antibodies raised in rabbits in our laboratory were used.

2.2. Cell culture

Porcine aortic endothelial cells were isolated as described [27] and incubated at 37° and 5% CO_2 in Dulbecco's modified Eagle's medium, containing 10% heat-inactivated foetal calf serum, 100 unit/mL penicillin, 0.1 mg/mL streptomycin, and 1.25 μg/mL amphotericin B. After 3–5 days, primary cells were subcultured in petri dishes (determination of ERK phosphorylation), 6-well plates (determination of L-citrulline formation), or 24-well plates (determination of cGMP accumulation). Prior to experiments, cells were incubated for 24 hr in serum-free medium.

2.3. 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 and accumulation of intracellular cGMP. For measurement of L-citrulline formation, endothelial cells grown in 6-well plastic plates were washed and preincubated for 30 min at 37° in incubation buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 3 mM $CaCl_2$) in the absence and presence of 10 μM PD 98059. Reactions were started by addition of L-[^{3}H]-arginine (~10^6 dpm) and BK or ATP. After the indicated period of time, cells were washed with chilled Ca^{2+}-free incubation buffer followed by addition of 1 mL HCl (10 mM). Subsequent to lysis of the cells (~1 hr after addition of HCl), an aliquot was removed for determination of incorporated radioactivity. To the remaining sample, 200 mM sodium acetate buffer (pH 13) containing 10 mM L-citrulline was added (final pH ~5), and L-[^{3}H]-citrulline separated from L-[^{3}H]-arginine by cation exchange chromatography as described [28,29]. For measurement of intracellular cGMP accumulation, endothelial cells grown in 24-well plastic plates were washed and preincubated for 30 min at 37° in incubation buffer, containing 1 mM isobutylmethylxanthine in the absence and presence of 10 μM PD 98059. Reactions were started by addition of BK or ATP and terminated after 3 min by removal of the incubation medium and addition of 1 mL HCl (10 mM). Subsequent to lysis of the cells, cGMP was measured in the supernatant by radioimmunoassay.

2.4. Relaxation studies

Porcine pulmonary arteries were isolated daily from fresh lungs obtained from a local slaughterhouse. Preparation of artery strips and organ bath studies were performed as described [30]. Briefly, the main pulmonary artery was removed, cleared of extraneous connective tissue and adhering fat, and cut in a zigzag form. These strips were connected to a hook under a tension of 1 g in 5 mL organ baths containing oxygenated (95% O_2, 5% CO_2) Krebs solution (118 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 10 mM glucose) at 37° and connected to a transducer for isotonic registration. After equilibration (~1.5 hr), 10 μM indomethacin was added and vessels were contracted by addition of 1 μM norepinephrine. Relaxation was induced by cumulative addition of either BK (1 nM–1 μM) or ATP (10 μM–1 mM). To test for the effects of PD 98059, the vessels were washed with Krebs solution, equilibrated, recontracted with norepinephrine, incubated for 30 min with 10 μM PD 98059, followed by a second cumulative concentration–response curve to BK or ATP. At the end of experiments, 0.3 μM papaverine was added to obtain maximal relaxations. For control, successive concentration–response curves were also established in the absence of the inhibitor and revealed no difference between the first and second application of the agonist.

2.5. Determination of ERK phosphorylation

Endothelial cells grown in petri dishes were washed, preincubated for 30 min at 37° in 50 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 3 mM $CaCl_2$ in the absence and presence of 10 μM PD 98059 and stimulated with 1 μM BK or 1 mM ATP. After 3 min, cells were washed with chilled phosphate-buffered saline, harvested, centrifuged for 5 min at 1000 *g*, and the pellet was resuspended in 0.1 mL of a 50 mM triethanolamine buffer, pH 7.4, containing 0.5 mM EDTA and 12 mM 2-mercaptoethanol. After disruption of the cells by sonication, an aliquot of 20 μL was removed for protein determination [31], and the remaining sample was mixed

with 16 μL of a 5-fold concentrated Lämmli buffer and boiled for 5 min at 95°, followed by electrophoresis on 10% SDS-polyacrylamide gels as described [32]. The separated proteins were blotted onto nitrocellulose membranes in 25 mM Tris buffer, pH 8.3, containing 0.2 M glycine and 20% (v/v) methanol at 250 mA for 90 min. Unspecific binding sites were saturated by over-night incubation of the membranes at 4° in Tris-buffered saline, containing 1% (w/v) bovine serum albumin. Subsequently, the membranes were washed twice for 5 min, followed by incubation for 2 hr with the anti-active MAP kinase antibody diluted 1:5000 in TBST (Tris-buffered saline containing 0.2% (v/v) Tween 20), containing 0.1% (w/v) bovine serum albumin. The membranes were then washed twice for 15 min with TBST and incubated for 1 hr with horseradish peroxidase labelled anti-rabbit-IgG antibody which had been diluted 1:8000 in TBST, containing 0.1% (w/v) bovine serum albumin. Finally, the membranes were washed three times for 20 min with TBST and processed with the ECL Western blotting detection system according to the recommendations of Amersham.

3. Results and discussion

To probe the cultured porcine aortic endothelial cells used in this study for a proper function of the MAP kinase cascade, cells were preincubated for 30 min in the absence and presence of 10 μM PD 98059, stimulated for 3 min with 1 μM BK, followed by measuring EKR activation by immunoblotting with an antibody specific for active (phosphorylated) ERK. As shown in Fig. 1, stimulation of the cells with BK led to pronounced activation of ERK, a response that was completely blocked by the kinase inhibitor PD 98059 (10 μM). In accordance with other reports demonstrating that agonist-induced ERK phosphorylation is triggered by an increase in the intracellular Ca^{2+} concentration [33,34], a marked activation of ERK was also obtained when ATP was used instead of BK as a Ca^{2+}-mobilizing agonist. Based on these data, we used both BK and ATP to investigate whether an activation of the MAP kinase cascade is involved in the regulation of agonist-induced NO formation in endothelial cells.

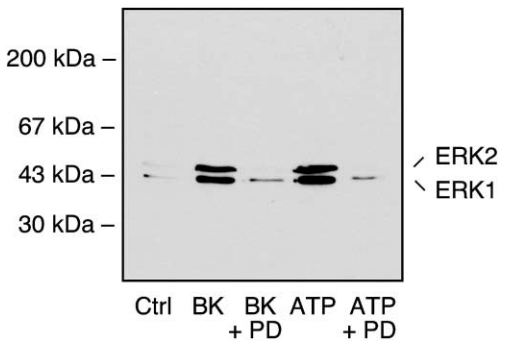

Fig. 1. Activation of ERK by BK and ATP in cultured endothelial cells. Endothelial cells were preincubated for 30 min in the absence or presence of 10 μM PD 98059 (PD) and then incubated further for 3 min in the absence (Ctrl) or presence of 1 μM BK or 1 mM ATP. Cell lysates (50 μg) were resolved by SDS-PAGE, and immunoblots probed using an anti-active MAP kinase antibody. The blot shown is representative of three independent experiments.

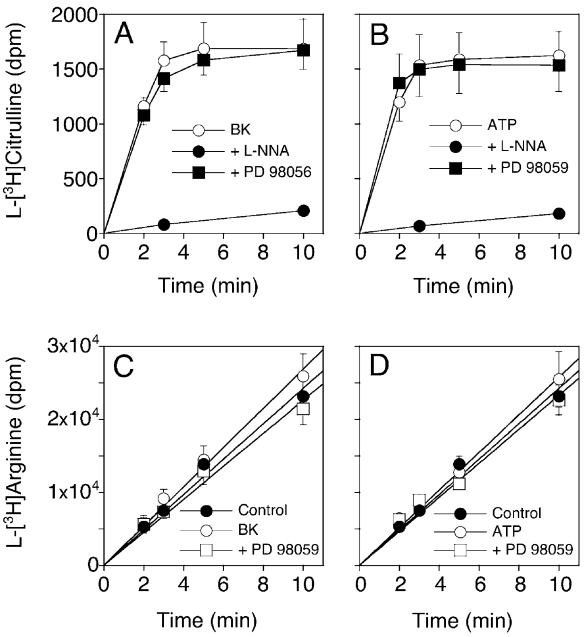

Fig. 2. Effects of PD 98059 on uptake of L-[^{3}H]-arginine and formation of L-[^{3}H]-citrulline. Endothelial cells were preincubated for 30 min in the absence or presence of 10 μM PD 98059 or 0.3 mM L-NNA and then stimulated with 1 μM BK (A and C) or 1 mM ATP (B and D) in the presence of L-[^{3}H]-arginine. At the time points indicated, cells were lysed and incorporation of L-[^{3}H]-arginine (C and D) or accumulation of L-[^{3}H]-citrulline (A and B) were determined as described in Section 2. Data are mean values ± SE of four independent experiments.

Since the time course of BK-induced phosphorylation of eNOS is rather slow (half-maximal phosphorylation after ~3 min, maximal phosphorylation after ~5 min) [35], it was suggested that this process may present a means for the de-activation of eNOS after agonist stimulation [25]. Therefore, we first investigated whether a pretreatment of endothelial cells with PD 98059 leads to a change in the time course of eNOS activation in response to maximally active concentrations of BK (1 μM) or ATP (1 mM). As shown in Fig. 2A and B, addition of the agonists evoked a pronounced, L-NNA inhibitable accumulation of L-[^{3}H]-citrulline that was linear for 2–3 min and then levelled off, giving a plateau after ~5 min. In contrast to L-[^{3}H]-citrulline accumulation, L-[^{3}H]-arginine uptake was linear for 10 min (Fig. 2C and D), indicating that the apparent decrease in L-[^{3}H]-citrulline formation observed after ~3 min was not due to a diminished availability of radioactive substrate. Since the time course of L-[^{3}H]-citrulline formation correlated with the transient intracellular Ca^{2+} signal,[1] our data suggest that receptor agonists activate

[1] Wagner, *et al.*, unpublished data.

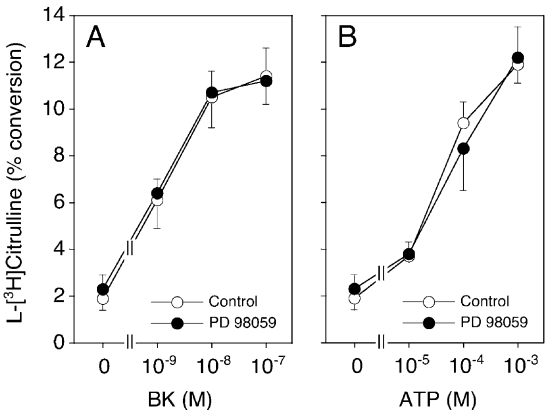

Fig. 3. Effect of PD 98059 on concentration-dependent activation of eNOS by BK and ATP. Endothelial cells were preincubated for 30 min in the absence or presence of 10 μM PD 98059 and then stimulated for 3 min with increasing concentrations of BK (A) or ATP (B) in the presence of L-[^{3}H]-arginine. Subsequent to lysis of the cells, the conversion of incorporated L-[^{3}H]-arginine into L-[^{3}H]-citrulline was determined as described in Section 2. Data are mean values ± SE of four independent experiments.

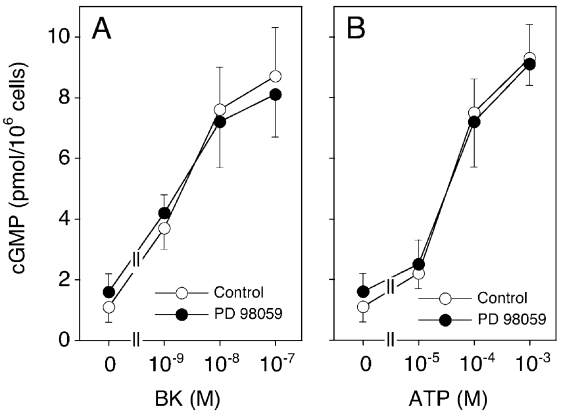

Fig. 4. Effect of PD 98059 on agonist-induced accumulation of endothelial cGMP. Endothelial cells were preincubated for 30 min in the absence or presence of 10 μM PD 98059 and then stimulated for 3 min with increasing concentrations of BK (A) or ATP (B). Subsequent to lysis of the cells, cGMP was determined by radioimmunoassay. Data are mean values ± SE of three independent experiments.

eNOS only during the initial 2–3 min, and enzyme activity then declines to basal values due to the decrease in intracellular Ca^{2+}. Although the decline in eNOS activity observed after ~3 min temporally correlated with the reported increase in enzyme phosphorylation [35], L-[^{3}H]-citrulline formation was not prolonged or enhanced in cells preincubated with PD 98059 (Fig. 2A and B), suggesting that ERK-catalysed eNOS phosphorylation does not substantially contribute to eNOS de-activation. However, these data do not exclude the possibility that phosphorylation by ERK inhibits eNOS activity by reducing the sensitivity of the enzyme to Ca^{2+}/CaM, an effect that may not be detectable in a fully activated system. We, therefore, also studied the effects of PD 98059 on the concentration-dependent activation of eNOS by BK and ATP. As shown in Fig. 3A, stimulation of endothelial cells for 3 min with increasing concentrations of BK enhanced the conversion of L-[^{3}H]-arginine into L-[^{3}H]-citrulline from 1.9 ± 0.5 to 11.4 ± 1.2% ($n = 4$ each). A virtually identical concentration-response curve was obtained with cells which had been pretreated for 30 min with 10 μM PD 98059. Similar to BK, ATP also elicited a pronounced formation of L-citrulline (11.9 ± 0.8% ($n = 4$) at 1 mM ATP) that was insensitive to PD 98059 (Fig. 3B). Since the time course of eNOS activation is faster than that of eNOS phosphorylation, cells were also stimulated for a prolonged period of time (6 min) with increasing concentrations of the agonists, but again, PD 98059 had no effect (data not shown). To corroborate these findings, we measured the accumulation of intracellular cGMP as a marker of NO formation (Fig. 4). In accordance with the results obtained with the L-arginine to L-citrulline conversion assay, stimulation of the cells with BK or ATP led to a marked increase in the accumulation of intracellular cGMP (from 1.1 ± 0.5 to 8.7 ± 1.6 and 9.3 ± 0.9 pmol/10^6 cells, respectively), which was not affected by preincubation of the cells with 10 μM PD 98059.

These results clearly demonstrate that inhibition of ERK with PD 98059 affects neither the potency of Ca^{2+}-mobilizing agonists on eNOS activation nor the kinetics of NO formation. In contrast to our results obtained with intact endothelial cells, Michel and co-workers demonstrated that eNOS immunoprecipitated from PD 98059-treated endothelial cells exhibited a slightly higher catalytic activity in enzyme assay than eNOS precipitated from untreated cells [25]. Intriguingly, this stimulatory effect of PD 98059 was observed even in resting endothelial cells (in which activated ERK was barely detectable) and less pronounced in cells stimulated with BK, raising the question whether the observed effect was indeed caused by inhibition of ERK-catalysed eNOS phosphorylation.

Since it was recently reported that inhibition of ERK does not affect the BK-induced relaxation of coronary artery rings [24], we also investigated the effect of PD 98059 on the relaxation of isolated porcine pulmonary arteries in response to BK and ATP. As shown in Fig. 5A, cumulative addition of BK elicited a concentration-dependent relaxation of the arteries with a maximal effect of 59 ± 4.6% and an EC_{50} of 3.5 ± 0.8 nM ($n = 8$). Preincubation of the arteries for 30 min with 10 μM PD 98059 did not significantly affect the maximal response to BK (54 ± 3.4%; $n = 14$) but slightly increased the EC_{50} to 7.6 ± 1.5 nM ($P = 0.02$, Student's t-test). In contrast to the results obtained with BK, the relaxation induced by ATP was not affected by PD 98059 (Fig. 5B). The mechanism underlying the minor inhibitory effect of PD 98059 on BK-induced relaxation is unclear, but is certainly not related to ERK-mediated eNOS phosphorylation as (i) a similar effect was not observed with ATP, and (ii) based on the previous report by Michel and co-workers [25], inhibition of ERK would be expected to enhance and not to diminish NO-meditated effects.

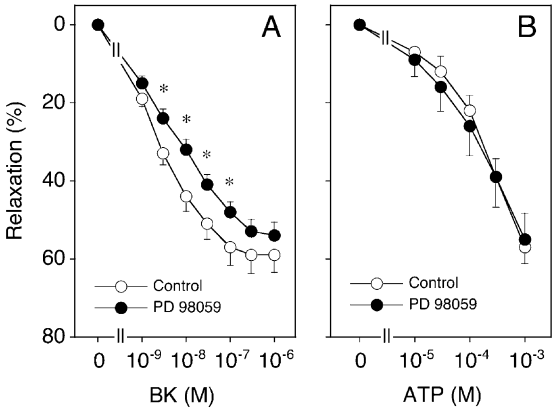

Fig. 5. Effect of PD 98059 on endothelium-dependent relaxation of porcine pulmonary arteries. Artery strips precontracted with 1 μM norepinephrine were incubated for 30 min in the absence or presence of 10 μM PD 98059, and then relaxation was induced by cumulative addition of BK (A) or ATP (B). Data (mean values ± SE; $n = 8$–14) were statistically evaluated by analysis of variance; significant differences ($P < 0.05$) are indicated with an asterisk.

In summary our data demonstrate that inhibition of ERK by PD 98059 neither prolongs nor potentiates the effects of Ca^{2+}-mobilizing agonists on endothelial NO formation in cultured cells or isolated blood vessels, indicating that ERK-catalysed eNOS phosphorylation does not contribute to the regulation of NO signalling *in vivo*.

Acknowledgments

H.D. Gibraeil is a recipient of a PhD fellowship from the Austrian Academic Exchange Service (ÖAD). We thank Margit Rehn for excellent technical assistance. This work was supported by the Fonds zur Förderung der Wissenschaftlichen Forschung in Österreich (Grants P14777-GEN (K.S.) and P13586-MED (B.M.)).

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