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Inhibition of MAP kinase kinase (MEK) blocks endothelial PGI_2 release but has no effect on von Willebrand Factor secretion or E-selectin expression

Caroline P.D. Wheeler-Jones*, Michael J. May, Rebecca A. Houliston, Jeremy D. Pearson

Vascular Biology Research Centre, King's College London, Campden Hill Road, Kensington, London W8 7AH, UK

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Abstract We have examined the potential role of MAP kinase in the regulation of endothelial cell PGI_2 synthesis, vWF secretion and E-selectin expression using the specific MEK inhibitor PD98059. PD98059 dose-dependently attenuated the tyrosine phosphorylation and activation of $p42^{mapk}$ in response to thrombin or inflammatory cytokines. Inhibition of thrombin-induced $p42^{mapk}$ activation was paralleled by an inhibitory effect of PD98059 on thrombin-driven PGI_2 generation but not on vWF secretion or IL-1α/TNFα-induced E-selectin expression. These results provide evidence for a key role for $p42^{mapk}$ in the acute regulation of PGI_2 synthesis in human endothelial cells and suggest that activation of the MAP kinase cascade is not obligatory for cytokine-stimulated E-selectin expression.

Key words: Human endothelial cell; MAP kinase; Thrombin; Cytokine; Prostacyclin; Adhesion molecule

1. Introduction

Endothelial cells play a major role in regulating vascular homeostasis by modulating the secretion or surface expression of local mediators in response to diverse physiological stimuli [1]. Our recent studies in human umbilical vein endothelial cells (HUVEC) have implicated tyrosine kinases as regulators of (i) the rapid synthesis and release of prostacyclin (PGI_2) in response to G protein-coupled agonists (GPCA) [2], and (ii) the expression of leucocyte adhesion molecules following exposure to inflammatory cytokines [3]. Thus, we have shown that thrombin, histamine or cytokines promote changes in the phosphotyrosine content of endogenous HUVEC proteins and that inhibitors of tyrosine kinases can attenuate distinct agonist-induced tyrosine phosphorylation events in parallel with PGI_2 release [2] or adhesion molecule expression [3]. One of the major tyrosine phosphorylated proteins observed in GPCA- [2] or cytokine-stimulated HUVEC [4] corresponds to the 42 kDa form of MAP kinase ($p42^{mapk}$) which is activated by phosphorylation on tyrosine and threonine residues catalysed by an upstream, dual-specificity MAP kinase kinase (MEK) [5]. In addition, there is a strong correlation between agonist-induced $p42^{mapk}$ activation and some of the functional responses of HUVEC and we have hypothesised that activation of the MAP kinase cascade may be necessary both for GPCA-driven PGI_2 synthesis [2], and for cytokine-induced adhesion molecule expression by human endothelium [4].

In the present study we have employed a highly specific, non-competitive inhibitor of MEK (PD98059 [6–8]) to address directly the role of $p42^{mapk}$ in thrombin-induced PGI_2 generation and in E-selectin expression evoked by IL-1α or TNFα.

2. Materials and methods

2.1. Materials

^{125}I-labelled 6-keto $PGF_{1\alpha}$ was purchased from Metachem Diagnostics Ltd., Piddington, Northampton, UK. Histamine dihydrochloride, human α-thrombin and bovine serum albumin (BSA; fraction V) were from Sigma (Poole, Dorset, UK). The monoclonal anti-$p44^{mapk}$ antibody (which cross-reacts with $p42^{mapk}$) and the polyclonal anti-phosphotyrosine antibody were from Affiniti Research Products Ltd., Nottingham, UK. Horseradish peroxidase conjugated goat anti-rabbit and rabbit anti-mouse immunoglobulins were purchased from Pierce and Warriner (Chester, Cheshire, UK.). Human recombinant IL-1α and TNFα, and monoclonal anti-E-selectin antibody were from R&D Systems Ltd. (Oxford, UK). Reagents for SDS-PAGE were from National Diagnostics (Hessle, Hull, UK). Enhanced chemiluminescence (ECL) Western Blot detection reagent and Hyperfilm-ECL film were obtained from Amersham Int., Bucks, UK. Culture media were from Sigma or Life Technologies (Paisley, UK). PD98059 was kindly provided by Dr. A. Saltiel, Parke-Davis, MI, USA. Mouse monoclonal anti-von Willebrand Factor (vWF) antibody (CLBRAg35) was a gift from Dr. J.A. Van Mourik (Central Blood Laboratory, Amsterdam, The Netherlands). All other reagents were obtained from Sigma or BDH (Poole, Dorset, UK) at the equivalent of AnalaR grade.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as previously described [2]. Experiments were performed on HUVEC passaged twice from primary cultures; cells were used 4–5 days after seeding at confluent density onto the appropropriate tissue culture plasticware precoated with 1% (w/v) gelatin.

2.3. Immunoblotting

Confluent HUVEC in 60 mm dishes (approx 1×10^6 cells/dish) were serum-deprived for 12–16 h in M199 containing 5 mM glutamine (M199G). Monolayers were washed twice with Krebs-Ringer bicarbonate buffer (118 mM NaCl, 4.75 mM KCl, 1.2 mM $MgCl_2$, 1.0 mM $CaCl_2$, 25 mM $NaHCO_3$, pH 7.4, 37°C), and subsequently challenged with agonist. In some experiments a portion of the cell supernatant was retained for measurement of 6-keto $PGF_{1\alpha}$ content. For experiments employing cytokines, incubations were carried out in M199G. Incubations were terminated and whole cell lysates prepared as previously described [2]. Proteins were resolved by SDS-PAGE (10%) and transferred onto PVDF (Immobilon-P^T) membrane (Millipore, Bedford, MA). Membranes were blocked for 3 h in TBST (50 mM Tris, 150 mM NaCl, 0.02% (v/v) Tween 20, pH 7.4) containing 3% (w/v) BSA and probed with anti-phosphotyrosine or anti-$p44/p42^{mapk}$ antibody (diluted in TBST/0.2% (w/v) BSA). Blots were washed in TBST (6×10 min) and subsequently incubated (1 h) in TBST/0.2% BSA containing HRP-conjugated goat anti-rabbit or anti-mouse IgG as appropriate. Immunoreactive proteins were visualised using ECL ac-

*Corresponding author. Fax: (44) (171) 333-4008.

Abbreviations: HUVEC, human umbilical vein endothelial cell; MAP kinase, mitogen activated protein kinase; MEK, MAP kinase kinase; $p42^{mapk}$, 42 kDa MAP kinase; $p44^{mapk}$, 44 kDa MAP kinase; PGI_2, prostacyclin; vWF, von Willebrand Factor; GPCA, G protein-coupled agonist

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cording to the manufacturer's instructions. Where indicated blots were stripped by incubation (50°C, 30 min) in a buffer comprised of 62.5 mM Tris-HCl (pH 6.7), 2% (w/v) SDS and 0.7% (v/v) ß-mercaptoethanol and reprobed following extensive washing in TBST.

2.4. Measurement of PGI_2 release and vWF secretion

Confluent cultures of HUVEC in 24-well tissue culture trays were washed twice in serum-free Dulbecco's Modified Eagles Medium (DMEM; pH 7.4) containing 20 mM HEPES. Cell monolayers were then exposed to agonist in the absence or presence of PD98059 or vehicle alone. The supernatant fraction was sampled following a 60 min exposure to agonist and assayed for 6-keto $PGF_{1\alpha}$ (the stable breakdown product of PGI_2) and vWF using, respectively, radioimmunoassay and enzyme-linked immunosorbent assay procedures as previously described [2]. Supernatants from quiescent HUVEC monolayers used in the immunoblotting studies were also assayed routinely for 6-keto $PGF_{1\alpha}$ content; vWF secretion could not be accurately assessed in these cells due to the inhibitory effects of serum deprivation on cellular vWF content [9].

2.5. Measurement of E-selectin expression

Confluent monolayers of HUVEC in 96-well tissue culture trays were exposed to PD98059 for 30 min prior to incubation with cytokine for 4 h in the continued presence of inhibitor. Agonist-induced expression of E-selectin was then assessed by enzyme-linked immunosorbent assay [3].

2.6. Statistics

Statistical analysis was performed using the Peritz F multiple means comparison test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. PD98059 inhibits phosphorylation and activation of $p42^{mapk}$ in response to thrombin

Our previous studies in HUVEC have shown that thrombin and histamine promote the rapid tyrosine phosphorylation of a 42 kDa protein and have demonstrated using 'mobility-shift' and specific in vitro kinase assays that this protein corresponds to the activated form of $p42^{mapk}$ [2,10–12]. The effects of a 30 min preincubation with PD98059 (5–50 μM) on the tyrosine phosphorylation profile and on the electrophoretic mobility shift of $p42^{mapk}$ observed following thrombin stimulation of quiescent HUVEC are shown in Fig. 1. The upper, slower migrating form of $p42^{mapk}$ in Fig. 1B corresponds to the 42 kDa tyrosine phosphorylated protein in Fig. 1A. After a 1 min incubation, thrombin marginally enhanced the phosphorylation state of $p42^{mapk}$ and strongly increased phosphorylation of a protein migrating at approx. 95 kDa (p95), in addition to affecting the phosphorylation of a number of other proteins (≈60–70 kDa, ≈100–130 kDa; Fig. 1A, lane 2). Following 10 min exposure to thrombin (Fig. 1A, lane 8) there was a greater phosphorylation of $p42^{mapk}$, a delayed increase in phosphotyrosine content of proteins approximating 90 and 65–75 kDa, and no detectable phosphorylation of p95. A protein of approx. 44 kDa was also tyrosine phosphorylated in the presence of thrombin (Fig. 1A, lane 8).

The level of $p42^{mapk}$ phosphorylation observed in the absence of agonist varied between experiments (compare Fig. 1, lane 1 with Fig. 2, lane 1) and this basal activation could be reduced by removal of extracellular calcium or by exposure to the protein tyrosine kinase inhibitor genistein ([2] and data not shown). PD98059 (50 μM) abolished basal $p42^{mapk}$ phosphorylation and at 5 μM reduced agonist-stimulated $p42^{mapk}$ activation to a level equal to or below that observed in control cells (Fig. 1A,B). PD98059 also inhibited $p42^{mapk}$ activation following challenge with either histamine (1 μM) or the tyrosine phosphatase inhibitor pervanadate (1–50 μM; data not shown).

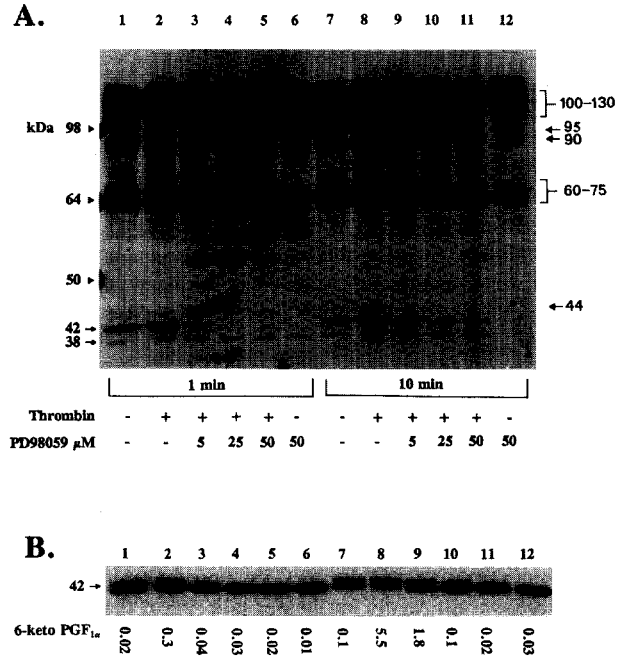

Fig. 1. PD98059 inhibits basal and thrombin-induced tyrosine phosphorylation and activation of $p42^{mapk}$. Confluent HUVEC in P60 tissue-culture dishes were serum-starved for 16 h in M199G. Washed monolayers were exposed to various concentrations of PD98059 (or to 0.001% DMSO) for 30 min and then challenged with thrombin (1.0 U/ml) in the continued presence of inhibitor. Incubations were terminated after 1 or 10 min exposure to agonist, lysed and subjected to SDS-PAGE followed by immunoblotting. (A) Blot was probed with anti-phosphotyrosine antibody and immunoreative proteins visualised using ECL. (B) The blot in A was stripped and reprobed with anti-MAP kinase antibody followed by ECL development. Data are from a representative experiment from a series of 3. Values for release of PGI_2 (6-keto $PGF_{1\alpha}$; ng/well) from HUVEC in each well are also shown.

PD98059 had no effect on the rapid thrombin-induced phosphorylation of p95 (Fig. 1A, lanes 2–5) but reduced the thrombin-stimulated tyrosine phosphorylation of p90 and p65–75 with maximal inhibition achieved at 25 μM (Fig. 1A, lanes 8–11). The thrombin-induced phosphorylation of p44 (Fig. 1A, lane 8), which was not observed in control cells, was also inhibited in cells pretreated with PD98059 (Fig. 1A, lane 9).

3.2. PD98059 inhibits PGI_2 release but not vWF secretion

To determine whether abolition of $p42^{mapk}$ activity was associated with loss of secretory responsiveness, we examined the effects of PD98059 on thrombin- and histamine-induced PGI_2 release and vWF secretion (Table 1). PD98059 dose-dependently attenuated both basal and agonist-stimulated PGI_2 generation with maximal inhibition achieved at 20 μM. Comparable effects of PD98059 on PGI_2 synthesis were also observed in quiescent HUVEC (see Fig. 1) although the extent of both basal and agonist-driven release was greater than in cells maintained in serum-containing medium (data not shown). In contrast, neither basal nor agonist-driven vWF secretion was modified by a 30 min pre-exposure to PD98059 (Table 1). Similarly, PD98059 inhibited pervana-

date-induced PGI_2 release but not vWF secretion (data not shown).

3.3. PD98059 inhibits cytokine-mediated activation of $p42^{mapk}$ but not E-selectin expression

Preincubation with PD98059 (5–50 μM) completely blocked the phosphorylation of $p42^{mapk}$ induced by maximally effective concentrations of IL-1α (100 U/ml; Fig. 2) or TNFα (data not shown). Under the same conditions, however, E-selectin expression induced by either cytokine was unaffected by pre-exposure to PD98059 at concentrations up to 50 μM (Table 2).

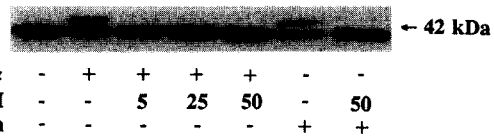

Fig. 2. Effects of PD98059 on IL-1α-induced phosphorylation of $p42^{mapk}$. HUVEC in P60 dishes were serum-starved for 16 h in M199G, washed, and incubated in M199G containing PD98059 (PD; 5–50 μM) or vehicle (0.001% DMSO) for 30 min. Cells were then exposed to IL-1α (100 U/ml, 30 min) or thrombin (Thr; 1.0 U/ml, 10 min) in the presence (or absence) of PD and cell lysates prepared as described in Section 2. The figure shows an anti-MAP kinase immunoblot developed using ECL. Results are representative of two similar experiments.

4. Discussion

$p42^{mapk}$ is thought to represent a point of convergence for mitogenic signals originating from several classes of cell surface receptor including the receptor tyrosine kinases and those that couple to heterotrimeric G proteins [13]. Growing evidence from a number of cell types, including terminally differentiated cells, additionally implicates $p42^{mapk}$ in the control of acute responses to receptor stimulation such as exocytosis and arachidonic acid release [14,15]. In endothelial cells $p42^{mapk}$ can be activated by growth factors [16], GPCA [17,18], shear stress [19,20] or inflammatory cytokines (this study) but the functional relevance of stimulus-induced changes in $p42^{mapk}$ remains to be determined. In the present study we used a novel inhibitor of MEK to explore the potential involvement of $p42^{mapk}$ in thrombin-driven PGI_2 synthesis and in cytokine-induced expression of E-selectin. At a concentration (5 μM) previously reported to abolish insulin- or NGF-stimulated MAP kinase activation in adipocytes [7] or PC12 cells [21], respectively, PD98059 blocked activation of $p42^{mapk}$ following treatment with thrombin, histamine, inflammatory cytokines or pervanadate. In agreement with previously published reports [6] the degree of inhibition depended upon the strength of $p42^{mapk}$ activation; thus, 5 μM PD98059 abolished $p42^{mapk}$ activation induced by maximally effective doses of thrombin, histamine, or IL-1α whereas 10-fold higher concentrations were required to suppress activation induced by pervanadate (not shown) which promotes a near complete shift of $p42^{mapk}$ to its phosphorylated, active form. These results provide evidence for the effectiveness of PD98059 as an inhibitor of MEK, and hence $p42^{mapk}$, in human endothelial cells exposed to activators of the 'classical' MAP kinase cascade. We also observed marginal tyrosine phosphorylation of a ≈44 kDa protein in thrombin-stimulated cells which was also inhibited by PD98059. This protein may well correspond to $p44^{mapk}$ which is known to be activated in response to thrombin in other cell types, including smooth muscle [22]; inhibition by PD98059 would be consistent with previous observations that PD98059 totally blocks all measurable in vitro MAP kinase activity in adipocytes [8]. However, unlike the phosphorylation of the 42 kDa protein ($p42^{mapk}$), tyrosine phosphorylation of the 44 kDa substrate was not consistently observed in our experiments and, if present, (Fig. 1) was clearly only marginally phosphorylated compared to p42. The MAP kinase antibody used in the present studies is raised against human $p44^{mapk}$ but cross-reacts with $p42^{mapk}$. Our previous studies in HUVEC [2], and those of others in a HUVEC-derived cell line [17], indicate that this antibody does not detect a 44 kDa MAP kinase in human endothelial cells, but does so readily in bovine endothelial cells (BEC) [17] and human smooth muscle cells (R.A. Houliston et al., unpublished). Moreover, in BECs $p44^{mapk}$ is not activated under conditions which maximally activate $p42^{mapk}$ [17] whereas both isoforms are activated in human smooth muscle. These findings suggest that $p42^{mapk}$ is the major regulated form of MAP kinase in human endothelial cells.

In the present study PD98059 had differential effects on thrombin-stimulated tyrosine phosphorylation depending upon the time of exposure to agonist. For example, after a 10 min thrombin challenge, the inhibition of $p42^{mapk}$ phosphorylation was accompanied by reduced tyrosine phosphorylation of several higher molecular weight proteins. Given the exquisite specificity of PD98059 [6] these effects are unlikely to result from direct modulation of other kinases and presumably reflect phosphorylation events occuring downstream of

Table 1
Effects of PD98059 on PGI_2 release and vWF secretion from resting and agonist-stimulated HUVEC

Treatment	6-Keto $PGF_{1\alpha}$ (pg/well)	vWF (mU/ml)
None	39 ± 12	4.0 ± 0.3
5 μM PD	15 ± 6[a]	2.8 ± 0.3
20 μM PD	< 3	3.1 ± 0.2
50 μM PD	< 3	3.6 ± 0.4
Histamine	1845 ± 65	10.4 ± 1.7
His + 5 μM PD	204 ± 61[c]	10.2 ± 0.3
His + 20 μM PD	19 ± 1[c]	10.4 ± 1.4
His + 50 μM PD	< 3	11.0 ± 0.2
None	16 ± 3	0.5 ± 0.1
5 μM PD	7 ± 2[a]	0.7 ± 0.2
20 μM PD	5 ± 1[b]	0.6 ± 0.1
50 μM PD	5 ± 2[c]	0.6 ± 0.2
Thrombin	81 ± 6	6.4 ± 0.2
Thr + 5 μM PD	35 ± 2[c]	5.1 ± 0.6
Thr + 20 μM PD	6 ± 1[c]	3.9 ± 1.4
Thr + 50 μM PD	4 ± 1[c]	6.4 ± 1.8

Confluent HUVEC monolayers were exposed to the indicated concentrations of PD98059 (PD) or vehicle (0.001% DMSO) for 30 min prior to incubation with thrombin (Thr; 1.0 U/ml) or histamine (His; 1 μM) in the continued presence (or absence) of inhibitor. Cell supernatants were sampled after 60 min and assayed for 6-keto $PGF_{1\alpha}$ and vWF. Data shown are from two representative experiments (one with each agonist) from a series of three with each agonist and are given as mean ± S.E.M. with quadruplicate determinations per observation.
< 3 = below assay detection limit.
[a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ versus control.

Table 2
PD98059 does not affect cytokine-induced expression of E-selectin

Treatment	E-selectin expression (% cytokine stimulated)
None	5 ± 2
IL-1	99 ± 5
IL-1 + 5 μM PD	99 ± 3
IL-1 + 25 μM PD	101 ± 4
IL-1 + 50 μM PD	96 ± 4
None	4 ± 2
TNFα	100 ± 4
TNFα + 5 μM PD	98 ± 3
TNFα + 25 μM PD	99 ± 3
TNFα + 50 μM PD	104 ± 3

HUVEC monolayers in 96-well trays were pre-incubated for 30 min in M199 containing the indicated concentrations of PD98059 (PD) or DMSO vehicle (0.001%). Cells were then challenged with either IL-1α (100 U/ml) or TNFα (100 U/ml) for a further 4 h in the continued presence or absence of PD and E-selectin expression measured by ELISA. Data are given as a percentage of the expression evoked by cytokine alone (100%) and are mean ± S.E.M. from 4 separate experiments with triplicate determinations per observation.

$p42^{mapk}$. In contrast, the rapid and prominent thrombin-induced phosphorylation of a ≈97 kDa protein was unaffected by exposure to PD98059 suggesting that this results from kinase activity situated upstream from $p42^{mapk}$ or in a parallel MAP kinase-independent pathway.

We have previously found that serum-deprived HUVEC exhibit enhanced basal and agonist-induced PGI_2 synthesis compared to cells maintained in serum-containing medium (C.P.D. Wheeler-Jones et al., unpublished). This is correlated with a more prominent basal and agonist-stimulated phosphorylation of $p42^{mapk}$, providing circumstantial evidence for a link between PGI_2 release and $p42^{mapk}$ activation. In the present study, inhibition of basal and agonist-induced $p42^{mapk}$ activation by PD98059 was accompanied by a parallel inhibition of PGI_2 release in both 'resting' and stimulated HUVEC. Our ability to detect both $p42^{mapk}$ activation and PGI_2 release in the absence of agonist is not unexpected; replacement of medium above endothelial cell monolayers subjects the cells to shear stress which is known to promote PGI_2 release [23] and to enhance phosphorylation and activation of $p42^{mapk}$ [19,20]. Taken together these results demonstrate a central role for $p42^{mapk}$ in the control of PGI_2 synthesis/release in HUVEC. Although we cannot exclude the potential involvement of a $p44^{mapk}$ in this response, our inability to detect basal phosphorylation of p44 together with the minor, and delayed thrombin-induced p44 phosphorylation, suggests that PGI_2 synthesis in human endothelium is regulated mainly by $p42^{mapk}$. In contrast, under conditions where thrombin-driven PGI_2 release and $p42^{mapk}$ activation were abolished, PD98059 had no effect on vWF secretion suggesting that activation of the MAP kinase cascade is not essential for regulated exocytosis in endothelial cells.

Inflammatory cytokines are known to activate a number of parallel kinase cascades in a variety of cell types [24]. In vitro studies have shown that one of the downstream targets of $p42^{mapk}$ is the transcription factor ATF-2 [25], the activation of which is required for cytokine-induced E-selectin expression [26]. However, at concentrations which effectively blocked IL-1α- and TNFα-induced $p42^{mapk}$ activation, PD98059 had no detectable effect on cytokine-stimulated E-selectin expression. The significance of the MAP kinase cascade for cytokine-mediated effects in endothelium remains unclear but the present study suggests that activation of $p42^{mapk}$ is not obligatory for expression of E-selectin.

In summary, we have shown that inhibition of the MAP kinase pathway selectively blocks PGI_2 release in response to G protein-coupled agonists, results which are consistent with a key role for $p42^{mapk}$ in the acute regulation of PGI_2 synthesis in human endothelial cells. Further studies are required to determine whether this results from $p42^{mapk}$-mediated phosphorylation of cytosolic phospholipase A_2 [16,27].

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