British Journal of Pharmacology (2003) 139, 321–328
www.nature.com/bjp

Mechanisms involved in the stimulation of prostacyclin synthesis by human lymphocytes in human umbilical vein endothelial cells

[1,3]Faten Merhi-Soussi, [2,3]Zury Dominguez, [1]Olga Macovschi, [1]Madeleine Dubois, [1]Georges Nemoz, [1]Michel Lagarde & *,[1]Annie-France Prigent

[1]INSERM U352, Laboratoire de Biochimie et Pharmacologie, Bâtiment Louis Pasteur, Institut National des Sciences Appliquées de Lyon, 69621 Villeurbanne, France; and [2]Cátedra de Patología General y Fisiopatología, Instituto de Medicina Experimental, Facultad de Medicina, Universidad Central de Venezuela, Caracas, Venezuela

1 Endothelial cells play an important role in the modulation of vascular tone because of their ability to produce vasoactive substances such as prostacyclin (PGI_2). Cell–cell contact between human umbilical vein endothelial cells (HUVEC) and peripheral blood lymphocytes has been shown to stimulate endothelial PGI_2 synthesis by increasing free arachidonic acid availability through endothelial cytosolic phospholipase A2 ($cPLA_2$) activation. In this study, we sought to determine whether phospholipase C (PLC) and D (PLD) activation also contributes, besides $cPLA_2$, to the lymphocyte-induced PGI_2 synthesis in HUVEC, and to delineate further the potential mechanisms of $cPLA_2$ activation triggered by the interaction of HUVEC with lymphocytes.

2 Pretreatment of endothelial cells with the PI-PLC inhibitor U-73122 before the coincubation with lymphocytes markedly inhibited the PGI_2 output whereas the diacylglycerol (DAG) lipase inhibitor RHC 80267 and ethanol had no effect. These results suggest that PLC may be involved through inositol trisphosphate generation and calcium mobilization, and that neither DAG nor phosphatidic acid (PtdOH) was used as sources of arachidonic acid.

3 The stimulated PGI_2 synthesis was protein kinase C (PKC)-independent but strongly inhibited by the mitogen-activated protein kinase kinase (MEK) inhibitors PD98059 and U-0126 and by the Src kinase inhibitor PP1.

4 Immunoblot experiments showed an increased phosphorylation of the extracellular signal-regulated kinases 1/2 (ERK1/2) upon lymphocyte addition till 4 h coincubation. Phosphorylation was markedly inhibited by U-0126 and PP1 addition.

5 Collectively, these results suggest that the signaling cascade triggered by lymphocytes in endothelial cells involves an Src kinase/ERK1/2 pathway leading to endothelial $cPLA_2$ activation.
British Journal of Pharmacology (2003) **139,** 321–328. doi:10.1038/sj.bjp.0705253

Keywords: Prostacyclin; lymphocyte–endothelial cell interactions; ERK1/2 MAP kinase; $cPLA_2$; atherothrombogenesis

Abbreviations: DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; HUVEC, human umbilical vein endothelial cells; MAPK, mitogen-activated kinase; MEK, mitogen-activated protein kinase kinase (MAPK/ERK kinase); PBL, peripheral blood lymphocytes; PKC, protein kinase C; PtdOH, phosphatidic acid; $cPLA_2$, cytosolic phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PTK, protein tyrosine kinase.

Introduction

Endothelial cells play an important role in hemostasis and in the modulation of vascular tone because of their ability to produce a large variety of vasoactive substances (Vane & Botting, 1992). Among them, prostacyclin (PGI_2) is a potent vasorelaxant and an inhibitor of platelet aggregation. Prostacyclin is synthesized from free arachidonic acid, released from membrane phospholipids, which is converted into prostaglandin endoperoxide (PGH_2) by prostaglandin synthases (PGHS). PGH_2 is subsequently metabolized to prostacyclin by prostacyclin synthase (Wu *et al*., 1992). We have recently shown that cell–cell contact between human umbilical vein endothelial cells (HUVEC) and peripheral blood lymphocytes (PBL) markedly stimulates endothelial PGI_2 synthesis (Merhi-Soussi *et al*., 2000). This lymphocyte-induced PGI_2 synthesis presumably involved endothelial cytosolic phospholipase A_2 ($cPLA_2$) activation because it was almost totally suppressed by calcium chelators and by the PLA_2 inhibitor MAFP (Merhi-Soussi *et al*., 2000). Cytosolic PLA_2 activation is known to be mediated by several distinct agonist- and cell-specific intracellular signals that involve guanine nucleotide-binding proteins (G-proteins), increases in intracellular calcium level, and activation of kinases such as the extracellular signal-regulated kinases (ERK) and protein kinase C (PKC) (Leslie, 1997; Hirabayashi & Shimizu, 2000). Current data indicate that phosphorylation of specific sites on the enzyme is essential for $cPLA_2$ activation, in addition to its calcium-induced translocation to the membrane compartment. One of the best characterized phosphorylation sites is Ser^{505}, which is located in a consensus site for ERK phosphorylation (Hirabayashi & Shimizu, 2000). Phosphorylation of Ser^{505} by ERK1/2 increases $cPLA_2$ activity in acellular systems as well as in intact cells (Hirabayashi & Shimizu, 2000). Ser^{727} can also be

*Author for correspondence; E-mail: prigent@insa.insa-lyon.fr
[3]Contributed in equal part to this work.

phosphorylated by PKC or PKA *in vitro* but the physiological relevance of this phosphorylation remains to be elucidated (Leslie, 1997; Hirabayashi & Shimizu, 2000).

Agonist-induced phosphorylation and activation of $cPLA_2$ correlate with the activation of ERK1/2 in many cell models including endothelial cells (Clark *et al*., 1995; Sa *et al*., 1995; Kan *et al*., 1996; Wheeler-Jones *et al*., 1997). MAP kinases are known to be organized in modules, each containing at least three protein kinases that work in series (English *et al*., 1999). In the ERK1/2 module, Raf-1 is the first kinase, which phosphorylates kinases known as MAPK/ERK kinases (MEK) on serine or threonine residues. Activated MEK phosphorylate and activate in turn their specific targets ERK1 and 2 (Crews *et al*., 1992). At the beginning of the MAP kinase cascade, Raf-1 activity is submitted to complex regulation involving multiple phosphorylations by PKC, tyrosine kinases and p21-activated kinases in addition to Ras interaction (Morrison & Cutler, 1997). Furthermore, it has also been reported that phosphatidic acid (PtdOH), resulting from phospholipid hydrolysis by phospholipase D (PLD), is able to induce Raf-1 translocation to the plasma membrane with a subsequent activation of the MAP kinase cascade (Rizzo *et al*., 1999). This may well explain why PLA_2 and PLD are often jointly activated in some cells such as neutrophils (Fujita *et al*., 1996). Although PLA_2 activation is the most direct route for arachidonic acid release in response to various stimuli, alternative pathways exist in some cells. Arachidonic acid may be released from diacylglycerol (DAG), generated via phospholipase C (PLC) activation, by the enzyme DAG lipase (Balsinde *et al*., 1991). DAG may also result from PLD activation, which generates PtdOH, the latter being subsequently converted to DAG by phosphatidate phosphohydrolase (Wright & Malik, 1996).

The purpose of this study was to determine whether PLC and PLD activation also contributed, besides $cPLA_2$, to the lymphocyte-induced PGI_2 synthesis in HUVEC and to delineate further the potential mechanisms of $cPLA_2$ activation triggered by the interaction of HUVEC with lymphocytes.

Methods

Reagents and chemicals

RPMI 1640 medium with 25 mM HEPES and bicarbonate and M-199 medium (containing 25 mM HEPES buffer, Earle's salts, L-glutamine and L-amino acids), heat-inactivated newborn calf serum, L-glutamine, penicillin – streptomycin, gentamicin, endothelial cell growth factor, collagenase type IA, trypsin – EDTA solution, gelatin type B, phorbol 12-myristate 13-acetate (PMA), pertussis toxin (PTX), genistein, NaCl, dextran, Histopaque-1077, trypan blue, phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin and tris[hydroxymethyl]aminomethane were all purchased from Sigma-Aldrich (L'Isle d'Abeau, France). Glycerol was from SDS (Peypin, France). Fetal bovine serum was purchased from Biomedia (La Verpillère, France). PGI_2 EIA assay kits and compound U-73122 were from SPI-Bio (Massy, France). MP Hyperfilm, ECL, HRP-conjugated anti-mouse or anti-rabbit IgG antibodies were from Amersham-Pharmacia Biotech (Orsay, France). Compound PD98059 was from Calbiochem. Compounds RHC80267 and U-0126 were from TEBU (Le Perray-en-Yvelines, France). Anti p-ERK (sc-7383) and anti ERK1 (sc-93) antibodies from Santa Cruz Biotechnology were supplied by TEBU (Le Perray-en-Yvelines, France). Compounds PP1 and bisindolylmaleimide I hydrochloride (BIM) were from Alexis Biochemicals (Coger, Paris, France).

HUVEC isolation and culture

Human umbilical cords were collected soon after birth and processed within 24 h. Cells were isolated from umbilical cord veins by digestion with collagenase IA as described by Jaffe *et al*. (1973). Endothelial cell cultures were grown to subconfluence on T-25 Flasks (Falcon, VWR, Strasbourg, France) coated with 2% gelatin, in a humid atmosphere containing 5% CO_2, at 37°C. Culture medium consisted of M-199 medium containing 20% heat-inactivated newborn calf serum, 100 $\mu g\,ml^{-1}$ streptomycin, 100 $U\,ml^{-1}$ penicillin, 100 $\mu g\,ml^{-1}$ gentamicin and 1% endothelial cell growth factor. The identity of the endothelial cells was checked by their cobblestone appearance under phase contrast microscopy. After trypsin – EDTA treatment, endothelial cells were subcultured in 24-well gelatin-coated plates (Corning, VWR, Strasbourg, France), allowed to grow to confluence (10^5 cells per well) under the conditions described above, and used at this first passage.

Preparation of human PBL

Mononuclear cells were isolated from peripheral venous blood of healthy subjects who had not taken any medication for 2 weeks prior to blood donation (Etablissement français du sang, Lyon, France). Venous blood was drawn into citrate – phosphate – dextrose anticoagulant, and mononuclear cells were isolated by dextran sedimentation at 37°C for 30 min followed by Histopaque-1077 density gradient centrifugation for 20 min at $600 \times g$. Depletion of monocytes was performed by adhesion onto culture polystyrene flasks as follows: cells were adjusted to 1×10^6 cells ml^{-1} in RPMI 1640 supplemented with 10% fetal calf serum, antibiotics (100 $\mu g\,ml^{-1}$ streptomycin, 100 $U\,ml^{-1}$ penicillin) and 2 mM L-glutamine and then incubated for 2×1 h in a 75 cm^2 tissue culture flask (Falcon) in standard conditions, with flask change between the incubations. Nonadherent PBL were collected by gentle aspiration, recovered by centrifugation on Histopaque-1077 density gradient, washed three times with RPMI 1640, resuspended in serum-free medium (RPMI 1640 supplemented with 100 $U\,ml^{-1}$ penicillin, 100 $\mu g\,ml^{-1}$ streptomycin and 2 mM L-glutamine) and used for interaction with HUVEC. The purity of these lymphocyte-enriched preparations was assessed by flow cytometry analysis using CD14 mAb (Leu M3, Becton Dickinson). CD14-positive cells (monocytes) were less than 2.5%. Viability was greater than 95% according to the trypan blue exclusion test.

PGI_2 assay in supernatants from HUVEC coincubated with lymphocytes

Confluent HUVEC cultured in 24-well gelatin-coated plates were washed twice with RPMI 1640. Thereafter, monolayers were incubated at 37°C under standard conditions, with either serum-free medium (control) or lymphocytes (HUVEC to lymphocyte ratio 1 : 15), for the indicated times, in a final

volume of 0.5 ml. At the end of the incubation period, supernatants were harvested, centrifuged at $400 \times g$ in order to remove lymphocytes and stored at $-20°C$ until assayed for PGI_2 content. In some experiments, confluent HUVEC were pretreated with the following inhibitors: PLC inhibitor: U-73122, 5 μM for 15 min (Smallridge *et al*., 1992); DAG lipase inhibitor: RHC 80267, 10 μM for 30 min (Sutherland & Amin, 1982); PKC inhibitor: BIM 1 μM for 30 min (Martiny-Baron *et al*, 1993); MEK inhibitors: PD98059, 25 μM (Dudley *et al*., 1995) and U-0126, 10 μM (Favata *et al*., 1998) for 30 min; protein tyrosine kinase inhibitor: genistein, 100 μM for 30 min (Wheeler-Jones *et al*., 1996); Src kinase inhibitor: PP1, 50 μM for 30 min (Hanke *et al*., 1996), before coincubation with lymphocytes for 4 h at 37°C. Inhibitors were dissolved in dimethylsulfoxide (DMSO) and DMSO solutions were then appropriately diluted with culture medium. The final DMSO concentration was 0.5%. This amount of DMSO did not significantly alter the PGI_2 synthesis induced by lymphocyte contact. In the experimental conditions setup, no cell mortality could be observed. It was also verified that these inhibitors did not alter the conversion of exogenous arachidonic acid to PGI_2, and thus, did not affect endothelial PGH synthases. In experiments designed to evaluate the contribution of PLD, HUVEC were pretreated with 1% ethanol for 60 min before coincubation with lymphocytes in the presence of 1% ethanol. In all these experiments, PGI_2 released in the supernatant was quantified by enzyme immunoassay (EIA) as its stable breakdown product, 6-oxo-prostaglandin $F_{1\alpha}$(6-oxo-$PGF_{1\alpha}$). Cross reactivity with PGE_2 was lower than 1%.

Immunoblot analysis of ERK1/2

In time course experiments where ERK1/2 expression and phosphorylation were analyzed, 1.5×10^6 HUVEC were cultured in T-25 flasks with 1.35×10^7 lymphocytes (lymphocytes to HUVEC ratio = 9) for increasing periods of time up to 20 h. In experiments aiming at investigating the influence of U-0126 and PP1 on ERK1/2 phosphorylation, HUVEC were pretreated for 30 min with the inhibitors (10 μM U-0126 or 50 μM PP1) before coincubation with lymphocytes for 4 h at 37°C. Then, HUVEC layers were thoroughly washed, trypsinized and lysed in 50 mM Tris-HCl buffer, pH 7.4, containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitors (aprotinin, leupeptin, pepstatin, each at 1 μg ml^{-1}). Proteins from endothelial cell lysates (20 – 30 μg) were separated by SDS – PAGE and electrotransferred onto Immobilon-P membranes (Millipore). Phosphorylated ERK1/2 bands were detected using 1 : 2000 dilution of antiphospho-ERK monoclonal and HRP-conjugated anti-mouse IgG antibody, and visualized by ECL. Then, membranes were stripped and ERK1/2 bands were detected with an anti-ERK1/2 polyclonal antibody (dilution 1 : 2000) and HRP-conjugated anti-rabbit IgG antibody, and visualized as above. Blots were quantitated using a cooled digital CCD camera system (ImageMaster VDS-CL, Amersham Biosciences) and ImageQuant software.

Statistical analysis

Values are presented as means ± s.e. of n independent experiments. All data were compared by ANOVA (Statview II for Macintosh) followed by a protected t-test. P-values of 0.05 or less were considered to be statistically significant.

Results

Contribution of the PLC and PLD pathways to the lymphocyte-induced PGI_2 synthesis

When confluent HUVEC were coincubated with human lymphocytes for 4 h in a serum – free medium, PGI_2 synthesis was increased by about 10-fold over the basal level measured in HUVEC incubated alone (Figure 1). To examine a possible contribution of the PLC pathway to the endothelial synthesis of PGI_2 triggered by lymphocyte contact, HUVEC were pretreated with the PLC inhibitor U-73122 before coincubation experiments. As shown in Figure 1a, U-73122 (5 μM) strongly inhibited the lymphocyte-induced PGI_2 synthesis. Since PIP_2 hydrolysis by PLC results in DAG and IP_3 generation, we next examined the hypothesis that newly synthesized DAG may be a source of arachidonic acid via DAG lipase. Results shown in Figure 1b indicate that pretreatment of HUVEC with the DAG lipase inhibitor RHC 80267 (10 μM) did not significantly reduce endothelial PGI_2 synthesis. These results suggest that DAG was not used as a major source of arachidonate for this synthesis, but that PLC may be involved through IP_3 generation and calcium mobilization. Indeed, the lymphocyte-induced PGI_2 synthesis proved to be highly calcium-dependent as it was totally suppressed by calcium chelators (Merhi-Soussi *et al*., 2000). The product of PLD, PtdOH, can also be a source of arachidonic acid through PLA_2 hydrolysis. Its endogenous synthesis can be very specifically decreased by primary alcohols, which give rise to phosphatidylalcohol at the expense of PtdOH because of the transphosphatidylation properties of PLD (Yu *et al*., 1996; Morris *et al*., 1997). The pretreatment of HUVEC with 1% ethanol before their coincubation with lymphocytes in the presence of 1% ethanol only marginally reduced the lymphocyte-induced PGI_2 synthesis (Figure 1c). Furthermore, ethanol did not alter the basal PGI_2 synthesis of HUVEC incubated alone, which rules out possible PLD-independent effects of ethanol. To confirm the lack of PLD involvement, we have measured the PLD activity of [^{3}H] arachidonate-labeled HUVEC incubated in the presence of 1% butanol for 4 h either alone or with unlabelled lymphocytes. HUVEC incubated with lymphocytes produced the same amount of phosphatidylbutanol (2% of total labeled phospholipids) as control HUVEC (not shown), indicating that the contact with lymphocytes did not activate a PLD pathway in HUVEC.

PKC is not involved in the activation of endothelial $cPLA_2$ by lymphocytes

$cPLA_2$ activation has been reported to involve both PKC-dependent and PKC-independent mechanisms depending on the cell type considered (Kramer & Sharp, 1997). To assess the role of PKC in the regulation of the lymphocyte-induced PGI_2 synthesis, HUVEC were either pretreated with 1 μM of the PKC inhibitor BIM for 30 min, or with 100 nM PMA for 18 h to downregulate PKC, before their coincubation with lymphocytes. Neither the PKC inhibitor (Figure 2a) nor PKC downregulation (Figure 2b) affected the endothelial PGI_2 synthesis.

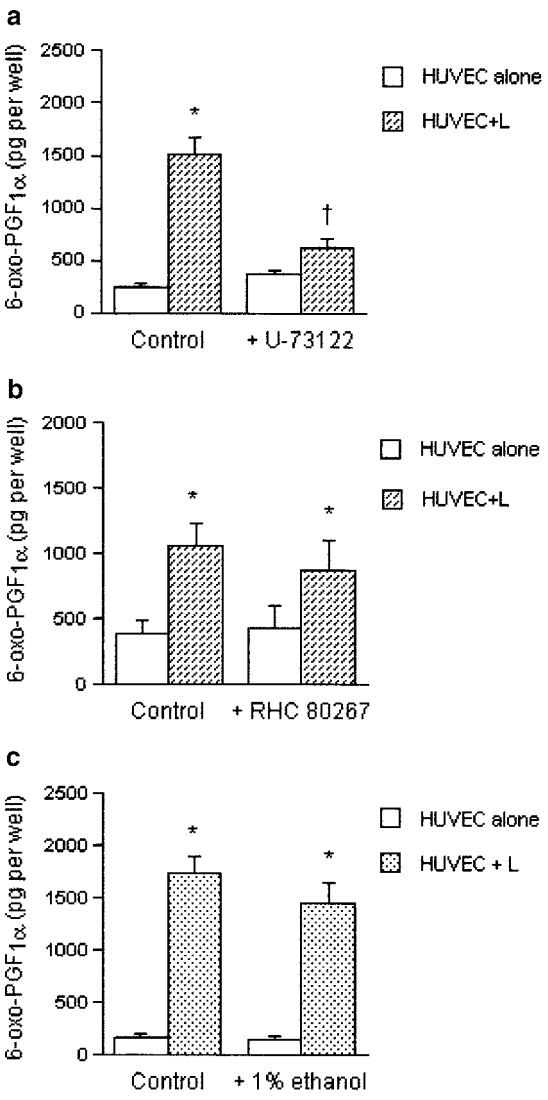

Figure 1 Contribution of the PLC and PLD pathways to the lymphocyte-induced PGI_2 synthesis. Confluent HUVEC (10^5 cells per well) either untreated (control) or pretreated with (a) 5 μM U-73122 (+U-73122) for 15 min, (b) 10 μM RHC 80267 (+RHC 80267) for 30 min or (c) 1% ethanol (+1% ethanol) for 1 h were incubated alone or in the presence of lymphocytes (lymphocyte to HUVEC ratio = 15) in a serum-free medium for 4 h at 37°C. In (c), ethanol when present was maintained during the 4 h coincubation. 6-oxo-$PGF_{1\alpha}$ was measured by EIA in supernatants. Results are expressed as picograms 6-oxo-$PGF_{1\alpha}$ per well and are means ± s.e. of four (b, c) to five (a) separate experiments performed in triplicate. Data were analyzed by ANOVA and the means compared by Scheffé's test. *Significantly different from control HUVEC incubated alone; †Significantly different from untreated HUVEC coincubated with lymphocytes.

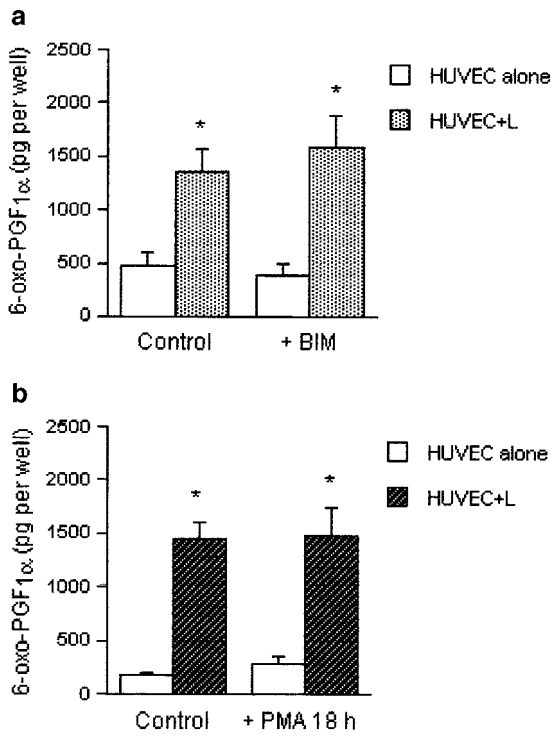

Figure 2 The lymphocyte-induced PGI_2 synthesis is independent of PKC. Confluent HUVEC (10^5 cells per well) either untreated (control) or pretreated with (a) 1 μM BIM (+ BIM) for 30 min or (b) 100 nM PMA for 18 h (+ PMA 18 h) were incubated alone or in the presence of lymphocytes (lymphocyte to HUVEC ratio = 15) in a serum-free medium for 4 h at 37°C. 6-oxo-$PGF_{1\alpha}$ was measured by EIA in supernatants. Results are expressed as picograms 6-oxo-$PGF_{1\alpha}$ per well and are means ± s.e. of five (a) or three (b) separate experiments performed in triplicate. Data were analyzed by ANOVA and the means compared by Scheffé's test. *Significantly different from control HUVEC incubated alone.

MAP kinases ERK1/2 have a pivotal role in the activation of endothelial $cPLA_2$ by lymphocytes

$cPLA_2$ activation has been shown to involve ERK1/2 activation in a large variety of cell types including endothelial cells (Sa *et al*., 1995; Leslie, 1997). To examine whether the MAP kinase cascade was involved in the $cPLA_2$ activation triggered by lymphocyte contact, HUVEC were pretreated with the MEK inhibitors PD98059 and U-0126 before their coincubation with lymphocytes. These compounds have been shown to prevent the activation of ERK1/2 and the subsequent phosphorylation of ERK1/2 substrates both in acellular systems and in intact cells (Dudley *et al*., 1995; Favata *et al*., 1998). As shown in Figure 3, pretreatment of HUVEC with the MEK inhibitors significantly reduced the synthesis of PGI_2 stimulated by lymphocyte contact. This synthesis was even totally suppressed if the inhibitor was maintained in the medium during the coincubation (not shown). These results suggest that lymphocytes were able to induce ERK1/2 phosphorylation and activation in endothelial cells. Indeed, immunoblot experiments using a monoclonal antibody that recognizes Tyr 204-phosphorylated ERK1/2 showed that ERK1/2 phosphorylation induced by lymphocytes was already slightly increased after 1 h of coincubation, maintained at a high level (two-fold increase) till 4 h, and then declined to the basal level after 8 h of coincubation (Figure 4).

Pathways of ERK1/2 activation stimulated by lymphocytes

Agonists of Gi-coupled receptors have been shown to stimulate MAP kinases in several cell types (English *et al*., 1999). Thus, in hepatocytes, ERK1/2 is activated in a PTX-sensitive way by some agonists such as vasopressin, angiotensin II or α1-adrenergic agonists (Melien *et al*., 1998).

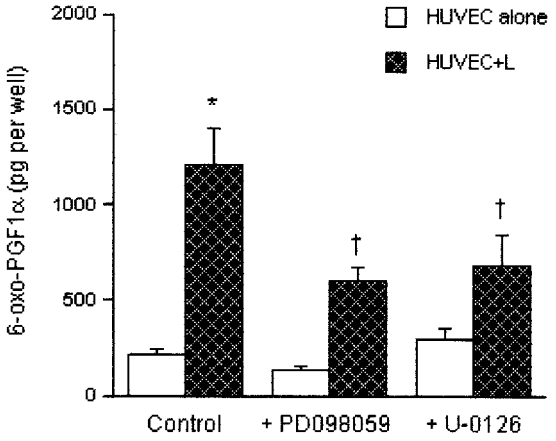

Figure 3 Effect of MEK inhibitors PD98054 and U-0126 on lymphocyte-induced PGI_2 synthesis. Confluent HUVEC (10^5 cells per well) either untreated or pretreated with 25 μM PD98059 or 10 μM U-0126 for 30 min were incubated alone (HUVEC) or with lymphocytes (HUVEC + L) (lymphocyte to HUVEC ratio = 15) for 4 h at 37°C. 6-oxo-$PGF_{1\alpha}$ was measured by EIA in supernatants. Results are expressed as picograms 6-oxo-$PGF_{1\alpha}$ per well and are means ± s.e. of five separate experiments performed in triplicate. Data were analyzed by ANOVA and the means compared by Scheffé's test. *Significantly different from control HUVEC incubated alone; †Significantly different from HUVEC coincubated with lymphocytes in the absence of inhibitor.

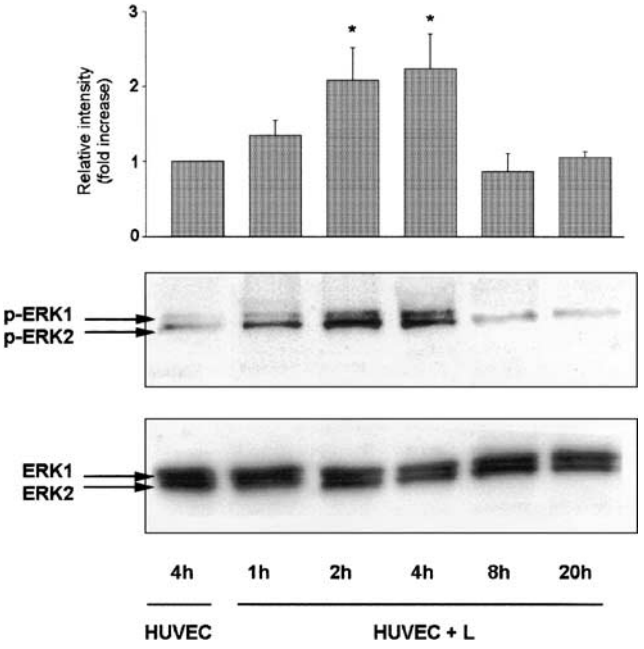

Figure 4 Immunoblot analysis of phospho-ERK1/2 and ERK1/2 from HUVEC coincubated with resting lymphocytes. Confluent HUVEC were incubated in the absence (HUVEC) or presence of resting lymphocytes (HUVEC + L) (lymphocyte to HUVEC ratio = 9) for the indicated periods of time. Monolayers were then thoroughly washed to remove lymphocytes; cells were lysed and proteins (30 μg protein per lane) were resolved by 10% SDS–polyacrylamide gel electrophoresis as described in Methods. The blots represent one of three separate experiments giving similar results. The upper panel shows the relative intensity of p-ERK1/2 bands normalized to their corresponding ERK1/2 controls. Values are means ± s.e. of three separate experiments. *Significantly different from control HUVEC incubated alone.

To examine the role of Gi in the signaling induced by HUVEC – lymphocyte contact, we studied how PTX pretreatment affected endothelial PGI_2 synthesis stimulated by lymphocytes. The pretreatment of HUVEC by 50 ng ml^{-1} PTX for 1 h before lymphocyte addition and a further 4 h coincubation with lymphocytes, either in the presence or absence of PTX, did not inhibit PGI_2 synthesis in three separate experiments (not shown), which indicates that the lymphocyte-triggered PGI_2 synthesis did not occur through a PTX sensitive pathway. Activation of protein tyrosine kinases (PTK) and modulation of the tyrosine phosphorylation state of endogenous proteins have been implicated in prostacyclin release from endothelial cells (Wheeler-Jones *et al*., 1996). To examine whether lymphocytes trigger a tyrosine kinase-dependent pathway to stimulate PGI_2 synthesis in HUVEC, we studied the influence of some tyrosine kinase inhibitors on the lymphocyte-induced PGI_2 synthesis. As shown in Figure 5, genistein, a tyrosine kinase inhibitor of broad specificity (Fleming *et al*., 1995), and PP1, an Src kinase-specific inhibitor (Viola *et al*., 1999) markedly inhibited (−88 and −90%, respectively) the endothelial production of PGI_2 stimulated by lymphocyte contact. Furthermore, PP1 also markedly decreased the phosphorylation of ERK1/2 induced by lymphocyte contact (Figure 6). This compound was even more efficient than the MEK inhibitor U-0126 at inhibiting ERK1/2 phosphorylation. Collectively, these results indicate that the activation of the MAP kinase/$cPLA_2$ cascade induced by lymphocytes requires the activation of upstream tyrosine kinases, presumably of the Src kinase family.

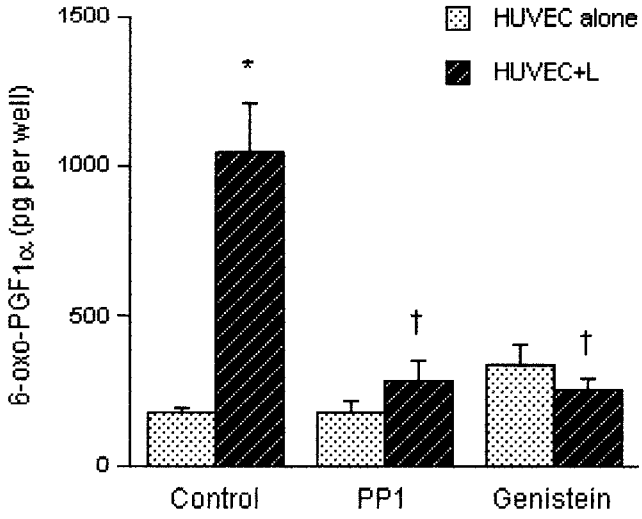

Figure 5 Effects of the tyrosine kinase inhibitor genistein and the Src kinase inhibitor PP1 on lymphocyte-induced PGI_2 synthesis. Confluent HUVEC (10^5 cells per well), either untreated or pretreated with 100 μM genistein or 50 μM PP1 for 30 min, were incubated alone (HUVEC) or with lymphocytes (HUVEC + L) (lymphocyte to HUVEC ratio = 15) for 4 h at 37°C. 6-oxo-$PGF_{1\alpha}$ was measured by EIA in supernatants. Results are expressed as picograms 6-oxo-$PGF_{1\alpha}$ per well and are means ± s.e. of three separate experiments performed in triplicate. Data were analyzed by ANOVA and the means compared by Scheffé's test. *Significantly different from control HUVEC incubated alone; †Significantly different from HUVEC coincubated with lymphocytes in the absence of inhibitor.

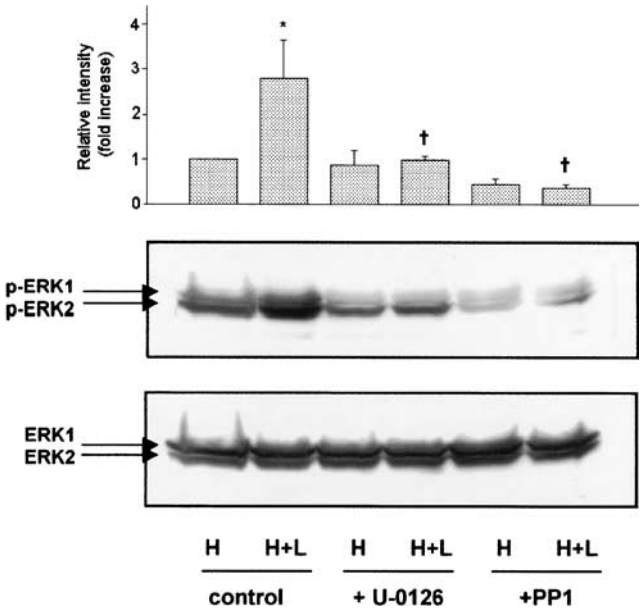

Figure 6 Inhibition of ERK phosphorylation by U-0126 and PP1. Confluent HUVEC (3×10^5 cells per well) either untreated or pretreated with 10 μM U-0126 or 50 μM PP1 for 30 min were incubated alone (H) or with lymphocytes (H + L) (lymphocyte to HUVEC ratio = 10) for 4 h at 37°C. Monolayers were then thoroughly washed to remove lymphocytes; cells were lysed and proteins (20 μg protein per lane) were resolved by 10% SDS–polyacrylamide gel electrophoresis as described in Methods. The blots represent one of four separate experiments giving similar results. The upper panel shows the relative intensity of p-ERK1/2 bands normalized to their corresponding ERK1/2 controls. Values are means ± s.e. of four separate experiments. *Significantly different from control HUVEC incubated alone; †Significantly different from control HUVEC incubated with lymphocytes in the absence of inhibitor.

Discussion

Results of the present study show that the ability of human lymphocytes to increase endothelial prostacyclin synthesis that we have recently described (Merhi-Soussi *et al*., 2000) mainly occurs through endothelial ERK1/2 MAP kinase and $cPLA_2$ activation, by a protein tyrosine kinase-dependent and PKC-independent mechanism, and that the other known pathways of arachidonic acid liberation are not involved. As we have shown in our previous work, the arachidonic acid used for the lymphocyte-stimulated PGI_2 synthesis is exclusively from endothelial origin even though a minor part of endothelial PGH_2 can be metabolized by the lymphocyte PGI_2 synthase through transcellular exchange (Merhi-Soussi *et al*., 2000). Thus, the main factor that regulates the PGI_2 output induced by lymphocyte contact is the availability of endothelial arachidonic acid to PGH synthase. Besides $cPLA_2$, PLC and PLD signaling pathways can also be involved in arachidonic acid release through DAG generation and hydrolysis by DAG lipase. For instance, arachidonic acid released from rat microvascular endothelial cells by interferon-γ originates from membrane phosphatidylcholine through the sequential activation of PLD, PtdOH phosphatase and DAG lipase (Mattila *et al*., 1993). Prostacyclin synthesis induced by endothelin-1 in rat aorta has also been shown to be mediated via PLD and not PLC or PLA_2 activation (Wright & Malik, 1996). However, the release of arachidonic acid triggered by human lymphocytes in HUVEC was clearly independent of PLD because no PLD activation could be observed in HUVEC upon lymphocyte addition. Furthermore, the lymphocyte-induced PGI_2 synthesis was not altered by the presence of ethanol, which is known to reduce PtdOH synthesis when PLD is activated.

Although the PIP_2-PLC pathway clearly contributed to the lymphocyte-induced PGI_2 synthesis, as shown by the marked inhibitory effect of the PIP_2-PLC inhibitor U-73122, the generated DAG does not seem to be an effective source of arachidonic acid because of the lack of effect of the DAG lipase inhibitor RHC 80267. It is thus very likely that PIP_2-PLC contributes to PGI_2 synthesis through its calcium mobilizing effect. Consistent with the idea that DAG is not directly involved in the lymphocyte-induced PGI_2 synthesis was the lack of conventional PKC involvement in this process. Indeed, neither the pretreatment of endothelial cells with the PKC inhibitor BIM, nor the PKC downregulation with PMA affected the lymphocyte-stimulated PGI_2 synthesis. Unlike bradykinin (Higaki *et al*., 1999) or lysophosphatidylcholine (Wong *et al*., 1998) that activate HUVEC $cPLA_2$ through PKC activation, the stimulatory effect of lymphocytes on endothelial $cPLA_2$ is clearly PKC-independent. Such a PKC-independent mechanism of PGI_2 synthesis has been described for acetylcholine in rabbit coronary endothelial cells where $cPLA_2$ translocation and activation were not blocked by PKC inhibitors (Kan *et al*., 1996).

Although lymphocytes did not modify the basal expression of ERK1/2 in HUVEC, they were able to induce a rapid and sustained activation of both kinases as shown by immunoblot analyses using an antiphospho-ERK1/2 monoclonal antibody. The involvement of ERK1/2 activation through phosphorylation in the lymphocyte-induced PGI_2 synthesis was further supported by the results obtained with the MEK1/2 inhibitors PD98059 and U-0126. Indeed, U-0126, which markedly reduced ERK1/2 phosphorylation induced by lymphocyte contact, significantly inhibited PGI_2 synthesis by 60%. Similar results on PGI_2 output were obtained with the other MEK inhibitor PD98059. Collectively, these results indicate that the activation of endothelial $cPLA_2$ induced by lymphocytes results from ERK1/2 activation. Several signaling pathways are known to activate Raf-1, the upstream kinase in the cascade leading to ERK1/2 activation (Cobb, 1999; English *et al*., 1999). Besides the classical Ras-dependent mechanism, Raf-1 may also be activated directly by PKC phosphorylation (Cai *et al*., 1997; Carroll & May, 1994) or by phosphatidic acid binding (Daniel *et al*., 1999; Rizzo *et al*., 1999). As discussed above, our results allow ruling out any PKC- or PLD-dependent mechanisms.

In some cell types such as hepatocytes, hormone-stimulated ERK1/2 activity in response to activation of heptahelical receptors has been shown to involve Gi proteins (Melien *et al*., 1998). Lymphocytes clearly did not activate a Gi-dependent signaling pathway because the PGI_2 synthesis that they induced was not PTX-sensitive. On the other hand, this stimulated PGI_2 synthesis was strongly inhibited by the tyrosine kinase inhibitor genistein and the more specific Src kinase inhibitor PP1. Src-family kinases phosphorylate membrane-associated scaffolding proteins to recruit Shc–Grb2–Sos complexes. Sos then stimulates the exchange of GDP for GTP in Ras, which in turn activates Ras and the MAP kinase cascade (English *et al*., 1999). Results of the present study

suggest that the lymphocyte to endothelial cell contact triggers a Src-dependent activation of ERK1/2 because PP1 strongly inhibited ERK1/2 phosphorylation and reduced PGI_2 synthesis by 90%. Src kinase activation is usually triggered by Gi proteins, via a mechanism involving their free $\beta\gamma$ subunits (English *et al*., 1999). This pathway can be excluded, because of the insensitivity of the lymphocyte-induced PGI_2 synthesis to PTX. However, we cannot rule out the involvement of other PTX insensitive G proteins acting through their $\beta\gamma$ subunits. Finally, increased calcium level may directly be coupled to Ras activation and the kinase cascade by a PKC-independent mechanism involving calcium-sensitive Ras guanine nucleotide exchange factors (Cobb, 1999). The lymphocyte-induced PGI_2 synthesis here described, which required ERK1/2 phosphorylation, was strictly calcium-dependent and PKC-independent. Further experiments are needed to determine whether the increase in calcium concentration induced by lymphocytes only favors $cPLA_2$ translocation or whether other calcium-stimulated pathways such as those involving calcium-sensitive Ras guanine nucleotide exchange factors or calcium-dependent tyrosine kinase (Lev *et al*., 1995) are also involved.

Another question that remains to be addressed concerns the identity of the molecules present on the cell surface of endothelial cells and lymphocytes that trigger the PGI_2-stimulated synthesis. Since a direct physical contact between the two cell types is necessary to trigger the PGI_2 synthesis (Merhi-Soussi *et al*., 2000), one may speculate that some adhesion molecules are involved. As we have recently shown that none of the well-characterized adhesion molecules E-selectin, ICAM-1 and VCAM-1 contribute to the lymphocyte-induced PGI_2 synthesis (Dominguez *et al*., 2001), further studies are required to clarify this issue.

Figure 7 integrates the results of the present study and proposes an interpretation of the mechanisms involved. According to this partially hypothetical schema, lymphocyte contact with HUVEC, through putative and still unidentified receptor(s), recruits and activates an Src tyrosine kinase. This Src kinase, in turn, recruits scaffolding proteins and SOS complexes, which activate the Ras/MAP kinase cascade, and ultimately lead to cPLA2 activation and arachidonic acid release. This pathway is strongly inhibited by PP1 at the level of Src kinase and by MEK1/2 inhibitors. PLCγ also contributes to PGI_2 synthesis, but very likely through IP_3 generation and increased intracellular calcium concentrations required for $cPLA_2$ activation rather than through DAG synthesis and PKC activation.

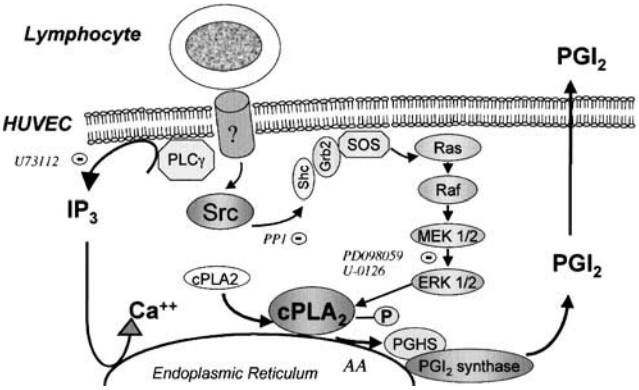

Figure 7 Proposed model for endothelial PGI_2 synthesis induced by lymphocyte contact. See the discussion for further explanation. ?, unidentified receptor or adhesion molecule; AA, arachidonic acid.

We thank the Tonkin Hospital, Villeurbanne, France for kind donations of fresh human umbilical cords. This work was supported by INSERM and by 'the Région Rhône-Alpes'. Z.D, Assistant Professor on leave from Venezuela to France, was supported by the Consejo de Desarrollo Científico y Humanístico, CDCH-Universidad Central de Venezuela.

References

BALSINDE, J., DIEZ, E. & MOLLINEDO, F. (1991). Arachidonic acid release from diacylglycerol in human neutrophils. Translocation of diacylglycerol-deacylating enzyme activities from an intracellular pool to plasma membrane upon cell activation. *J. Biol. Chem.,* **266,** 15638 – 15643.

CAI, H., SMOLA, U., WIXLER, V., EISENMANN-TAPPE, I., DIAZ-MECO, M.T., MOSCAT, J., RAPP, U. & COOPER, G.M. (1997). Role of diacylglycerol-regulated protein kinase C isotypes in growth factor activation of the Raf-1 protein kinase. *Mol. Cell. Biol.,* **17,** 732 – 741.

CARROLL, M.P. & MAY, W.S. (1994). Protein kinase C-mediated serine phosphorylation directly activates Raf-1 in murine hematopoietic cells. *J. Biol. Chem.,* **269,** 1249 – 1256.

CLARK, J.D., SCHIEVELLA, A.R., NALEFSKI, E.A. & LIN, L.L. (1995). Cytosolic phospholipase A2. *J. Lipid Mediat. Cell. Signal.,* **12,** 83 – 117.

COBB, M.H. (1999). MAP kinase pathways. *Progr. Biophys. Mol. Biol.,* **71,** 479 – 500.

CREWS, C.M., ALESSANDRINI, A. & ERIKSON, R.L. (1992). The primary structure of MEK, protein kinase that phosphorylates the ERK gene product. *Science,* **258,** 478 – 480.

DANIEL, L.W., SCIORRA, V.A. & GHOSH, S. (1999). Phospholipase D, tumor promoters, proliferation and prostaglandins. *Biochim. Biophys. Acta.,* **1439,** 265 – 276.

DOMINGUEZ, Z., MERHI-SOUSSI, F., MACOVSCHI, O., NÉMOZ, G., LAGARDE, M. & PRIGENT, A.F. (2001). Endothelial cell prostacyclin synthesis induced by lymphocytes is independent of the membrane fatty acid composition of both cell types and of E-selectin, VCAM-1 or ICAM-1 mediated adhesion. *Br. J. Haematol.,* **113,** 521 – 532.

DUDLEY, D.T., PANG, L., DECKER, S.J., BRIDGES, A.J. & SALTIEL, A.R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 7686 – 7689.

ENGLISH, J., PEARSON, G., WILSBACHER, J., SWANTEK, J., KARANDIKAR, M., XU, S. & COBB, M.H. (1999). New insights into the control of MAP kinase pathways. *Exp. Cell Res.,* **253,** 255 – 270.

FAVATA, M.F., HORIUCHI, K.Y., MANOS, E.J., DAULERIO, A.J., STRADLEY, D.A., FEESER, W.S., VAN DYK, D.E., PITTS, W.J., EARL, R.A., HOBBS, F., COPELZND, R.A., MAGOLDA, R.L., SCHERLE, P.A. & TRZASKOS, J.M. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.,* **273,** 18623 – 18632.

FLEMING, I., FISSLTHALER, B. & BUSSE, R. (1995). Calcium signaling in endothelial cells involves activation of tyrosine kinases and leads to activation of mitogen-activated protein kinases. *Circ. Res.,* **76,** 522 – 529.

FUJITA, K., MURAKAMI, M., YAMASHITA, F., AMEMIYA, K. & KUDO, I. (1996). Phospholipase D is involved in cytosolic phospholipase A2-dependent selective release of arachidonic acid by fMLP-stimulated rat neutrophils. *FEBS Lett.,* **395,** 293 – 298.

HANKE, J.H., GARDNER, J.P., DOW, R.L., CHANGELIAN, P.S., BRISSETTE, W.H., WERINGER, E.J., POLLOK, B.A. & CONNELLY, P.A. (1996). Discovery of a novel, potent, and Src

family selective tyrosine kinase inhibitor. *J. Biol. Chem.,* **271,** 695 – 701.

HIGAKI, T., SAWADA, S., KONO, Y., IMAMURA, H., TADA, Y., YAMASAKI, S., TORATANI, A., SATO, T., KOMATSU, S., AKAMATSU, N., TAMAGAKI, T., TSUDA, Y., TSUJI, H. & NAKAGAW, M. (1999). A role of protein kinase C in the regulation of cytosolic phospholipase A_2 in bradykinin-induced PGI_2 synthesis by human vascular endothelial cells. *Microvasc. Res.,* **58,** 144 – 155.

HIRABAYASHI, T. & SHIMIZU, T. (2000). Localization and regulation of cytosolic phospholipase A_2. *Biochim. Biophys. Acta.,* **1488,** 124 – 138.

JAFFE, E.A., NACHMAN, R.L. & MINICK, C.R. (1973). Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.,* **52,** 2745 – 2756.

KAN, Y., RUAN, Y. & MALIK, K.U. (1996). Involvement of mitogen-activated protein kinase and translocation of cytosolic phospholipase A_2 to the nuclear envelope in acetylcholine-induced prostacyclin synthesis in rabbit coronary endothelial cells. *Mol. Pharmacol.,* **50,** 1139 – 1147.

KRAMER, R.M. & SHARP, J.D. (1997). Structure, function and regulation of the Ca^{2+}-sensitive cytosolic phospholipase A_2 ($cPLA_2$). *FEBS Lett.,* **410,** 49 – 53.

LESLIE, C.C. (1997). Properties and regulation of cytosolic phospholipase A_2. *J. Biol. Chem.,* **272,** 16709 – 16712.

LEV, S., MORENO, H., MARTINEZ, R., CANOLL, P., PELES, E., MUSACCHIO, J.M., PLOWMAN, G.D., RUDY, B. & SCHLESSINGER, J. (1995). Protein tyrosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions. *Nature,* **376,** 737 – 745.

MARTINY-BARON, G., KAZANIETZ, M.G., MISCHAK, H., BLUMBERG, P.M., KOCHS, G., HUG, H., MARME, D. & SCHÄCHTELE, C. (1993) Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. *J. Biol. Chem.,* **268,** 9194 – 9197.

MATTILA, P., USTINOV, J. & RENKONEN, R. (1993). Interferon-γ induces a phospholipase D-dependent release of arachidonic acid from endothelial cell membranes: a mechanism for protein kinase C activation. *Scand. J. Immunol.,* **38,** 197 – 200.

MELIEN, Ø., THORESEN, G.H., SANDNES, D., ØSTBY, E. & CHRISTOFFERSEN, T. (1998). Activation of p42/p44 mitogen-activated protein kinase by angiotensin II, vasopressin, norepinephrine, and prostaglandin $F_{2\alpha}$ in hepatocytes is sustained, and like the effect of epidermal growth factor, mediated through pertussis toxin-sensitive mechanisms. *J. Cell. Physiol.,* **175,** 348 – 358.

MERHI-SOUSSI, F., DOMINGUEZ, Z., MACOVSCHI, O., DUBOIS, M., SAVANY, A., LAGARDE, M. & PRIGENT, A.F. (2000). Human lymphocytes stimulate prostacyclin synthesis in human umbilical vein endothelial cells. Involvement of endothelial $cPLA_2$. *J. Leukoc. Biol.,* **68,** 881 – 889.

MORRIS, A.J., FROHMAN, M.A. & ENGEBRECHT, J.A. (1997). Measurement of phospholipase D activity. *Anal. Biochem.,* **252,** 1 – 9.

MORRISON, D.K. & CUTLER, R.E. (1997). The complexity of Raf-1 regulation. *Curr. Opin. Cell. Biol.,* **9,** 174 – 179.

RIZZO, M.A., SHOME, K., VASUDEVAN, C., STOLZ, D.B., SUNG, T.C., FROHMAN, M.A., WATKINS, S.C. & ROMERO, G. (1999). Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent Raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. *J. Biol. Chem.,* **274,** 1131 – 1139.

SA, G., MURUGESAN, G., JAYE, M., IVASHCHENKO, Y. & FOX, P.L. (1995). Activation of cytosolic phospholipase A_2 by basic fibroblast growth factor via p42 mitogen-activated protein kinase-dependent phosphorylation pathway in endothelial cells. *J. Biol. Chem.,* **270,** 2360 – 2366.

SMALLRIDGE, R.C., KIANG, J.G., GIST, I.D., FEIN, H.G. & GALLOWAY, R.J. (1992). U-73122, an aminosteroid phospholipase C antagonist, noncompetitively inhibits thyrotropin-releasing hormone effects in GH3 rat pituitary cells. *Endocrinology,* **13,** 1883 – 1888.

SUTHERLAND, C.A. & AMIN, D. (1982). Relative activities of rat and dog platelet phospholipase A_2 and diglyceride lipase. Selective inhibition of diglyceride lipase by RHC 80267. *J. Biol. Chem.,* **257,** 14006 – 14010.

VANE, J.R. & BOTTING, R.M. (1992). Secretory functions of the vascular endothelium. *J. Physiol. Pharmacol.,* **43,** 195 – 207.

VIOLA, A., SCHROEDER, S., SAKAKIBARA, Y. & LANZAVECCHIA, A. (1999). T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science,* **283,** 680 – 682.

WHEELER-JONES, C., ABU-GHAZALEH, R., COSPEDAL, R., HOULISTON, R., MARTIN, J. & ZACHARY, Y. (1997). Vascular endothelial growth factor stimulates prostacyclin production and activation of cytosolic phospholipase A_2 in endothelial cells via p42-/p44 mitogen-activated protein kinase. *FEBS. Lett.,* **420,** 28 – 32.

WHEELER-JONES, C., MAY, M.J., MORGAN, A.J. & PEARSON, J.D. (1996). Protein tyrosine kinases regulate agonist-stimulated prostacyclin release but not von Willebrand factor secretion from umbilical vein endothelial cells. *Biochem. J.,* **315,** 407 – 416.

WONG, J.T., TRAN, K., PIERCE, G.N., CHAN, A.C. O, K. & CHOY, P.C. (1998). Lysophosphatidylcholine stimulates the release of arachidonic acid in human endothelial cells. *J. Biol. Chem.,* **273,** 6830 – 6836.

WRIGHT, H.M. & MALIK, K.U. (1996). Prostacyclin formation elicited by endothelin-1 in rat aorta is mediated via phospholipase D activation and not phospholipase C or A_2. *Circ. Res.,* **79,** 271 – 276.

WU, K.K., KULMACZ, R.J., WANG, L.-H., LOOSE-MITCHELL, D.S. & TSAI, A.-L. (1992). In: *Molecular Biology of Prostacyclin Biosynthesis*. ed. Rubanyi, G. & Vane, J.R. pp. 11 – 23. Amsterdam: Elsevier.

YU, C.H., LIU, S.Y. & PANAGIA, V. (1996). The transphosphatidylation activity of phospholipase D. *Mol. Cell. Biochem.,* **157,** 101 – 105.

(Received November 27, 2002
Revised February 14, 2003
Accepted February 21, 2003)