Protein tyrosine kinases regulate agonist-stimulated prostacyclin release but not von Willebrand factor secretion from human umbilical vein endothelial cells

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The rapid synthesis and release of prostacyclin (PGI_2) and the exocytotic secretion of von Willebrand Factor (vWF) elicited by activation of G-protein-coupled receptors on endothelium occur via signalling mechanisms which are incompletely defined. Activation of protein tyrosine kinases (PTKs) and modulation of the tyrosine-phosphorylation state of endogenous proteins have been implicated in several cellular processes including arachidonate release and exocytosis. In the present study we have examined the regulatory role of PTKs in agonist-stimulated release of PGI_2 and vWF from human umbilical vein endothelial cells (HUVECs) using two chemically and mechanistically dissimilar PTK inhibitors (genistein and ST271). Genistein, but not the less active analogue daidzein, dose-dependently attenuated PGI_2 release in response to thrombin and histamine (IC_{50} approx. $20 \mu\text{M}$), and to the thrombin-receptor-activating peptide. A more potent inhibition of thrombin- and histamine-induced PGI_2 synthesis was observed in cells exposed to ST271. In contrast, neither genistein nor ST271 modulated agonist-drive

vWF secretion. At concentrations that abolished PGI_2 release, genistein blocked thrombin- or histamine-evoked tyrosine phosphorylation of a 42 kDa protein. Ca^{2+} ionophore-induced PGI_2 generation, but not vWF secretion, was also inhibited by both genistein and ST271, suggesting that these agents modulate PGI_2 synthesis by acting at, or distal to, agonist-induced changes in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$). In fura-2-loaded HUVECs genistein partially reduced the histamine-induced peak $[\text{Ca}^{2+}]_i$ but had no effect on the thrombin response. Ca^{2+} -induced PGI_2 release from electrically permeabilized HUVECs was abolished in the presence of ST271 or genistein, but not daidzein. The generation of PGI_2 in response to exogenous arachidonic acid was not modulated by genistein or ST271, suggesting that PTK inhibitors do not directly inhibit cyclo-oxygenase activity. Taken together, these results suggest that PTKs regulate PGI_2 synthesis and release in HUVECs by modulating, directly or indirectly, a Ca^{2+} -sensitive step upstream of cyclo-oxygenase.

INTRODUCTION

Vascular endothelial cells respond to external stimuli by altering the secretion or surface expression of biomolecules that regulate vascular tone and permeability, leucocyte traffic, platelet function, blood coagulation and fibrinolysis [1]. Recent studies in endothelial cells have focused on the signalling mechanisms controlling the rapid receptor-mediated release of prostacyclin (PGI_2), a potent vasodilator and inhibitor of platelet function, and the regulated exocytosis of von Willebrand factor (vWF), a major glycoprotein cofactor promoting adhesion of platelets to extracellular matrix after vascular injury [2,3]. Studies using both intact and electrically permeabilized endothelial cells have shown that agonist-driven PGI_2 synthesis and vWF secretion are dependent on changes in the level of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) [4,5]; PGI_2 release is primarily driven by the transient release of Ca^{2+} from intracellular stores [6,7] whereas agonist-induced vWF secretion shows an additional dependence on the sustained Ca^{2+} -influx component [8,9]. Further investigations using activators and selective inhibitors of the serine/threonine kinase, protein kinase C (PKC), indicate that PKC activation can modulate, but is not obligatory for, agonist-stimulated PGI_2 synthesis [10].

Similarly, histamine-induced vWF secretion is only partially dependent on PKC activation whereas thrombin-driven secretion apparently occurs via PKC-independent pathways which may in part depend on the activation of calmodulin-dependent kinases [3,11]. In addition, studies in permeabilized cells indicate that Ca^{2+} -driven PGI_2 synthesis is modulated by G-proteins, perhaps acting on phospholipase A_2 (PLA_2) [4]. Thus the signalling mechanisms controlling the release of vasoactive mediators from human endothelium are still incompletely understood.

In addition to a number of classes of serine/threonine kinases, human endothelial cells of diverse origin express several receptor protein tyrosine kinases (PTKs), some of which are endothelium-specific [12]. Furthermore, cytosolic PTKs including members of the *src* [13,14] and JAK kinase families (R. Soldi and F. Bussolino, personal communication) have also been identified in endothelial cells. Ligand binding to endothelial cell receptor PTKs, in common with a number of other tissues, results in the rapid tyrosine phosphorylation of various endogenous proteins [12,15]. Studies in several cell types [16,17], including endothelial cells [18], indicate that agonists that operate via binding to G-protein-linked receptors also enhance the tyrosine-phosphorylation state of key cellular proteins, including the

Abbreviations used: HUVECs, human umbilical vein endothelial cells; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; AEBSF, [4-(2-aminoethyl)]benzenesulphonyl fluoride; DMEM, Dulbecco's modified Eagle's medium; PGI_2 , prostacyclin; 6-keto- $\text{PGF}_{1\alpha}$, 6-ketoprostaglandin $\text{F}_{1\alpha}$; vWF, von Willebrand factor; PKC, protein kinase C; PTK, protein tyrosine kinase; IBMX, 3-isobutyl-1-methylxanthine; cNOS, constitutive nitric oxide synthase; MAP kinase, mitogen-activated protein kinase; p42^{mapk} , 42 kDa isoform of mitogen-activated protein kinase; PLA_2 , phospholipase A_2 ; ECL, enhanced chemiluminescence; TRAP, thrombin-receptor-activating peptide; IP_3 , inositol trisphosphate.

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42 kDa isoform of mitogen activated protein (MAP) kinase (p42^{mapk}). To date, the role of PTKs in the signalling pathways controlling endothelial cell function have been examined principally in the context of long-term cellular responses such as those elicited by stimulation of receptor PTKs or by exposure to cytokines. Thus PTK-dependent phosphorylation events may be essential for endothelial cell proliferation [15], for neutrophil adhesion to activated endothelium [19,20] and for cytokine-induced synthesis and secretion of plasminogen activator inhibitor [21]. Accumulating evidence, however, suggests that PTKs may also regulate rapid post-receptor events such as Ca²⁺ mobilization, exocytosis and arachidonic acid release. For example, PTK inhibitors, in addition to inhibiting tyrosine phosphorylation, attenuate several agonist-driven responses in human platelets including Ca²⁺ influx, aggregation and dense-granule secretion [22–24] and are known to influence rapid exocytosis in a number of secretory tissues [25,26]. PTK activation may therefore offer an alternative mechanism for controlling rapid responses mediated by activation of G-protein-coupled receptors. In the present study, we have employed two chemically and mechanistically dissimilar PTK inhibitors; genistein inhibits tyrosine kinases by competing directly with ATP whereas the inhibitory action of the tyrphostin-like compound ST271 can be attributed principally to its ability to bind to substrate protein(s) [27,28]. These inhibitors have been used to probe the involvement of PTKs in the regulation of PGI₂ synthesis/release and in the exocytotic secretion of vWF from human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Materials

¹²⁵I-labelled 6-ketoprostaglandin F_{1α} (6-keto-PGF_{1α}) was purchased from Metachem Diagnostics, Piddington, Northants., U.K. Fura-2 acetoxymethyl ester and fura-2 (pentapotassium salt) were from Molecular Probes (Eugene, OR, U.S.A.). Hepes (free acid), [4-(2-aminoethyl)]benzenesulphonyl fluoride (AEBSF), ionomycin, ionophore A23187, genistein and daidzein were from Calbiochem-Novabiochem (U.K.) Ltd., Beeston, Notts., U.K. ST271 and Ro 31-8220 were kindly provided by Dr. L. Garland, Wellcome Research Laboratories, Beckenham, Kent, U.K. and Dr. T. Hallam, Roche Products, Welwyn Garden City, Herts., U.K. respectively. Histamine dihydrochloride, human α-thrombin, sodium arachidonate and BSA (fraction V) were from Sigma (Poole, Dorset, U.K.). EGTA was obtained from Fluka (Gillingham, Dorset, U.K.) at puriss. grade. Polyclonal anti-phosphotyrosine and monoclonal anti-(MAP kinase) antibodies were from Affiniti Research Products Ltd., Nottingham, U.K. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin was purchased from Pierce and Warriner (Chester, Cheshire, U.K.). Reagents for SDS/PAGE were from Bio-Rad (Hemel Hempstead, Herts., U.K.) or National Diagnostics (Hessle, Hull, U.K.). ¹²⁵I-labelled cGMP (approx. 2000 Ci/mmol), enhanced chemiluminescence (ECL) Western-blot detection reagent and Hyperfilm-ECL film were obtained from Amersham International, Amersham, Bucks., U.K. Culture media were from Sigma or Life Technologies (Paisley, Scotland, U.K.). All other reagents were obtained from Sigma or BDH (Poole, Dorset, U.K.) at the equivalent of AnalaR grade.

Cell culture

HUVECs were isolated from umbilical veins by collagenase treatment essentially as described by Jaffe et al. [29] and cultured in medium 199 supplemented with 10% (v/v) fetal calf serum,

10% (v/v) newborn calf serum, L-glutamine (4 mM), penicillin (100 units/ml), streptomycin (100 units/ml), porcine heparin (90 µg/ml; Sigma) and endothelial cell growth supplement (20 µg/ml). Endothelial cell growth supplement was prepared from porcine brain as described by Macaig and Weinstein [30]. Tissue culture plasticware was coated with gelatin (1%) before plating. All experiments were performed on cells passaged twice from primary cultures and cells were used for experimentation 4–5 days after plating.

Measurement of vWF secretion and PGI₂ release

Intact cells

Confluent cultures of HUVECs in 24-well tissue culture trays were washed twice in serum-free Dulbecco's modified Eagle's medium (DMEM; pH 7.4) containing 20 mM Hepes. Cell monolayers were then exposed to medium containing various concentrations of agonist in the absence or presence of the appropriate inhibitor. In those experiments in which vWF secretion and PGI₂ release were assessed simultaneously, the supernatant fraction was sampled after a 60 min exposure to agonist. In some experiments only PGI₂ release was quantified, in which case supernatants were removed from above the monolayer after a 10 min challenge.

Release of vWF into the cell supernatants was measured using an ELISA [31,32] with a lower limit of detection of approx. 1.0 m-unit/ml. The mouse monoclonal anti-vWF CLBRAg35 (coating antibody; [33]) was a gift from Dr. J. A. Van Mourik (Central Blood Laboratory, Amsterdam, The Netherlands). The PGI₂ content of cell supernatants was quantified using a specific radioimmunoassay for 6-keto-PGF_{1α}, the stable hydrolysis product of PGI₂. The assay procedure was similar to that described previously [34] except that the immunoprecipitation was carried out using 15% (w/v) poly(ethylene glycol) 6000 in PBS (no Ca²⁺ or Mg²⁺) containing 1 mg/ml γ-globulin and 0.05% (v/v) Tween 20.

Permeabilized cells

Confluent cell monolayers in 24-well plates were washed twice with serum-free DMEM followed by a further wash with a permeabilization buffer comprised of 280 mM glycine, 20 mM glutamate (potassium salt), 20 mM Hepes (potassium salt), 2 mM magnesium acetate and 2.5 mM EGTA (potassium salt) (pH 7.4). Cells in each well were permeabilized as previously described [4]. At 5 min after electropore permeabilization, the buffer above the monolayer was replaced with a Ca²⁺-containing stimulation buffer {pH 7.4; 280 mM glycine, 20 mM glutamate (potassium salt), 4 mM magnesium acetate, 7.5 mM EGTA (potassium salt), 2.5 mM EDTA (potassium salt), 20 mM Hepes (potassium salt) and CaCl₂ to give free [Ca²⁺] of between 0.05 and 10 µM} supplemented with either ST271, genistein or daidzein. In some experiments, inhibitors were also present during permeabilization and/or for 15 min before permeabilization. Cell supernatants were sampled 60 min after addition of stimulation buffer and the vWF and PGI₂ contents assayed as described above for intact cells.

Determination of intracellular [Ca²⁺] ([Ca²⁺]_i)

Measurements of [Ca²⁺]_i were performed on HUVECs (passage 1/2) 2–4 days after seeding on to glass coverslips [35]. Cells were loaded with fura-2 by incubation for 30 min (room temperature) in Hepes (20 mM)-buffered DMEM containing 20% fetal calf serum and 1 µM fura-2 acetoxymethyl ester. After loading, the cells were maintained at room temperature in balanced salt

solution supplemented with 1 % (w/v) BSA before use (< 3 h after loading). The composition of the balanced salt solution was (mM): NaCl (145), KCl (5), MgSO_4 (1), CaCl_2 (1), Hepes (10), glucose (10), BSA (0.1 %, w/v), pH 7.4. $[\text{Ca}^{2+}]_i$ was measured in patches (approx. 10) of superfused endothelial cells at 37 °C using conventional epifluorescence microscopy with a data-acquisition rate of 2 Hz [35]. Cells were excited alternately at 340 and 380 nm and emission collected at > 470 nm. Genistein had no appreciable effect on fura-2 fluorescence in this system which circumvented the problems associated with the use of this compound in population measurements in a cuvette; daidzein, an analogue of genistein, could not be used in either system because of excessive interference with the fluorescence signal (results not shown). At the end of each run, the autofluorescence was estimated by the addition of 1 μM ionomycin and 2 mM Mn^{2+} which quenches fura-2 [36]. $[\text{Ca}^{2+}]_i$ was calculated as described by Grynkiewicz et al. [37]. Peak changes in $[\text{Ca}^{2+}]_i$ or the 340/380 nm ratio elicited by the addition of histamine or thrombin refer to the maximum change as defined by the peak height minus the value immediately before the addition. Because of recent doubts surrounding the validity of calibrating fura-2 signals, the data are shown in the Figures as changes in 340/380 nm ratio and have been converted into $[\text{Ca}^{2+}]_i$ in the text.

Measurement of cGMP accumulation

HUVECs in 24-well trays were washed with serum-free DMEM (pH 7.4), preincubated for 15 min (37 °C) in DMEM containing 3-isobutyl-1-methylxanthine (IBMX; 500 μM), IBMX plus genistein or IBMX plus daidzein. Cells were subsequently exposed to thrombin or histamine in the continued presence of genistein, daidzein or vehicle alone (DMSO; final concentration 0.1 %) for 10 min (37 °C). At the end of the incubation period, cell supernatants were retained for the determination of 6-keto-PGF_{1 α} content, and the cGMP was extracted from resting and stimulated cells by the addition of 0.1 M HCl (1 ml/well) directly to the monolayer. Samples were stored at -20 °C until analysis. After acetylation [38] cGMP was measured in duplicate in appropriately diluted aliquots of cell extract by radioimmunoassay. Acetylated standards/samples (100 μl), ^{125}I -labelled cGMP (5000 d.p.m./100 μl) and antiserum (100 μl ; 1:10000 dilution) were incubated (4 °C) overnight and the immunoprecipitation was carried out as described for measurement of 6-keto-PGF_{1 α} .

Immunoblotting

Confluent HUVECs in 60 mm dishes (approx. 1×10^6 cells/dish) were serum-deprived for 12–16 h in medium 199 containing 5 mM glutamine. 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 thrombin or histamine. In some experiments a portion of the cell supernatant was retained for measurement of 6-keto-PGF_{1 α} content. Incubations were terminated by rapid aspiration of the cell supernatant followed by washing with ice-cold PBS 'A' containing 20 μM sodium orthovanadate. Cells were lysed in buffer containing 63.5 mM Tris/HCl, pH 6.8, 10 % glycerol, 2 % SDS, 5 % 2-mercaptoethanol, 1 mM sodium orthovanadate, 1 mM AEBSF and 50 $\mu\text{g}/\text{ml}$ leupeptin. Proteins in aliquots of cell lysates were separated by SDS/PAGE (10 %) and transferred to 0.2 μm nitrocellulose membrane (Anderman and Co., Kingston-upon-Thames, Surrey, U.K.). Membranes were blocked for 2 h in TBST [50 mM Tris/HCl, 150 mM NaCl, 0.02 % (v/v) Tween 20,

pH 7.4] containing 3 % (w/v) BSA and subsequently probed overnight with anti-(MAP kinase) or anti-phosphotyrosine antibody [1:2500 dilution in TBST/0.2 % (w/v) BSA]. Blots were washed in TBST (6 $\times$ 10 min) and incubated in TBST/0.2 % BSA containing horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10000) for 1 h. After further washing (8 $\times$ 10 min) in TBST, immunoreactive bands were visualized using ECL according to the manufacturer's instructions.

Assay of PKC activity

Confluent monolayers of HUVECs in 60 mm dishes were detached by brief exposure to 0.1 % trypsin/0.02 % EDTA, washed twice by centrifugation (200 g; 5 min) in serum-containing medium 199 and twice in PBS 'A'. The cell pellet was resuspended in 200 μl of buffer A [20 mM Tris/HCl, pH 7.4, 2 mM EDTA, 0.5 mM EGTA, 50 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM PMSF and 1 % (v/v) 2-mercaptoethanol]. HUVECs were disrupted by ultrasonication on ice (4 $\times$ 10 s), and sonicates from two dishes were combined and applied to a DEAE-cellulose column (0.4 ml) pre-equilibrated with buffer A. Columns were washed and the enzyme was eluted with 150 μl of buffer A containing 120 mM NaCl. The enzyme activity in 10 μl aliquots of column effluent was assessed in the absence or presence of various kinase inhibitors by measuring the incorporation of ^{32}P from [γ - ^{32}P]ATP into histone H1 substrate [39]. PKC activity was calculated from the difference in ^{32}P incorporation measured in the presence and absence of phosphatidylserine and dioleoin. Results are expressed as pmol of ^{32}P incorporated/min per μg of HUVEC protein.

Measurement of cell viability

HUVEC viability in the presence of PTK inhibitors was assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma) as described by Mossman [40]. Absorbance was measured at 540 nm using a Titertek Multiscan PLUS MkII microtiter reader (INN Flow, Irvine, Scotland, U.K.).

Statistical analysis

Student's *t* test was used to compare means of groups of data. *P* < 0.05 was considered statistically significant.

RESULTS

Effects of genistein on agonist-driven PGI₂ release and vWF secretion

The ability of thrombin and histamine to promote transient synthesis and release of PGI₂ [41,42] and to induce vWF secretion from human endothelium is well documented [2,3,8,11]. Maximal levels of PGI₂ release are generally observed at lower thrombin concentrations (0.5–1.0 unit/ml) than maximal vWF secretion (approx. 2.5 units/ml) whereas both vWF and PGI₂ release are maximally stimulated after exposure to 10 μM histamine. Figure 1(A) depicts the effects of various concentrations of genistein on PGI₂ (6-keto-PGF_{1 α}) release and vWF secretion from HUVEC monolayers stimulated with a maximally effective dose of human α -thrombin (2.5 units/ml). Although basal release was unaffected (not shown), genistein dose-dependently inhibited thrombin-driven PGI₂ release (Figure 1A) with near-complete inhibition achieved at 100 μM genistein; the IC₅₀ value derived from three experiments (2.5 units/ml thrombin) was $47 \pm 4 \mu\text{M}$ (mean $\pm$ S.E.M.). Daidzein, a structurally similar but less active analogue of genistein [27], had no significant effect on PGI₂ release, although a slight potentiation of release was observed with low concentrations (Figure 1B). At concentrations of

Figure 1 Effects of genistein and daidzein on thrombin-induced PGI₂ release and vWF secretion

Confluent HUVECs in 24-well tissue culture trays were washed twice with serum-free DMEM (pH 7.4) and exposed to medium containing the indicated concentrations of either genistein (**A**) or daidzein (**B**) for 15 min. Monolayers were then incubated with human α -thrombin (2.5 units/ml) in the continued absence or presence of inhibitor, and the vWF (●) and 6-keto-PGF_{1α} (○) released into the supernatant fraction were measured after 60 min as described in the Materials and methods section. The open and closed bars represent the basal release of 6-keto-PGF_{1α} and vWF respectively. Vehicle alone (0.1% DMSO) affected neither basal nor thrombin-stimulated release/secretion, and basal values were not altered in the presence of genistein (not shown). Data are means $\pm$ S.E.M. ($n = 4$) from a typical experiment representative of three showing similar results. (**C**) HUVECs were exposed (15 min) to vehicle (■) or 50 μ M genistein (▲) and subsequently challenged with thrombin at the indicated concentrations in the continued presence of vehicle or genistein. The 6-keto-PGF_{1α} content of the medium above the monolayer was assayed after 10 min. Results are given as means $\pm$ S.E.M. and are from three experiments performed in triplicate. * $P < 0.001$ compared with vehicle alone.

daidzein exceeding 100 μ M a significant inhibitory effect on stimulated PGI₂ release was also observed (results not shown). Thus, in subsequent experiments genistein was routinely used at 50 μ M. Cell viability was assessed using a colorimetric cytotoxicity assay [40]; in cells exposed for 4 h to 100 μ M genistein or 100 μ M daidzein the A_{540} values were respectively 98 ± 7 and $91 \pm 8\%$ of that observed in vehicle-treated HUVECs. Figure 1(C) shows the results from experiments in which cells were challenged with increasing doses of thrombin in the presence or absence of a fixed dose of genistein (50 μ M). Genistein completely inhibited agonist-driven PGI₂ release at thrombin concentrations up to 1.0 unit/ml [$IC_{50} = 18 \pm 7$ μ M ($n = 3$); not shown] and this inhibitory effect, as previously noted (Figure 1A), was partially overcome in the presence of thrombin levels exceeding 1.0 unit/ml (Figure 1C).

In contrast with the marked attenuation of PGI₂ release, genistein did not modulate vWF secretion from thrombin-stimulated HUVECs (Figure 1). Similarly, when monolayers were exposed to thrombin-receptor-activating peptide (TRAP), a 14-amino acid peptide ligand of the cloned thrombin receptor [43] which has previously been shown to elevate $[Ca^{2+}]_i$ and promote PGI₂ release in HUVECs [44,45], incubation with genistein (50 μ M) caused a substantial attenuation of TRAP-stimulated PGI₂ release but had no significant effect on vWF

secretion measured at submaximal and maximal TRAP concentrations (Table 1).

To ascertain whether the inhibitory effects of genistein were restricted to thrombin-mediated signalling events, we also examined the effects of genistein (and daidzein) on PGI₂ release and vWF secretion from histamine-stimulated HUVECs (Figure 2). Genistein potently inhibited PGI₂ release with maximal effects observed at an inhibitor concentration of 50 μ M and an estimated IC_{50} of about 20 μ M (Figure 2A); daidzein (50 μ M) did not significantly attenuate release. Similarly, exposure of HUVECs to a fixed concentration of genistein completely abolished PGI₂ release induced by increasing doses of histamine (Figure 2B). Histamine-stimulated vWF secretion, in contrast, was not inhibited by genistein; in fact, at concentrations of genistein that maximally reduced PGI₂ release, a slight enhancement of secretion was observed. This effect is unlikely to reflect an inhibition of PTK activity since vWF secretion was also marginally increased in the presence of daidzein (Figure 2A).

When HUVEC phospholipid-dependent kinase activity was assessed in a partially purified preparation of PKC *in vitro*, the non-selective kinase inhibitor staurosporine and the highly selective PKC inhibitor Ro 31-8220 completely abolished PKC activity at concentrations of 100 nM and 10 μ M respectively (not shown). In contrast, both genistein and ST271 at supramaximal

Table 1 Effects of genistein on vWF secretion and PGI₂ release in TRAP-stimulated HUVECs

HUVEC monolayers were washed twice in serum-free Hepes-buffered DMEM (pH 7.4), preincubated (15 min) with vehicle (0.1% DMSO), genistein (50 μ M) or daidzein (50 μ M) and then exposed to thrombin or TRAP at the indicated concentrations. Cell supernatants were sampled after a 60 min incubation period (37 °C) and assayed for vWF and 6-keto-PGF_{1 α} as described in the Materials and methods section. Data are means $\pm$ S.E.M. of two experiments performed in triplicate. nd, Not determined. * P < 0.01, ** P < 0.0001 compared with agonist plus vehicle.

Treatment	vWF secretion (m-units/ml)		6-Keto-PGF _{1α} (pg/well)	
	Vehicle	Genistein	Vehicle	Genistein
Control	6.9 $\pm$ 0.7	6.0 $\pm$ 0.8	4.3 $\pm$ 0.4	3.9 $\pm$ 0.6
Thrombin (2.5 units/ml)	13.8 $\pm$ 1.1	nd	80.7 $\pm$ 7.8	29.2 $\pm$ 2.9*
TRAP (1 μ M)	9.4 $\pm$ 0.2	9.0 $\pm$ 0.4	7.0 $\pm$ 0.7	2.4 $\pm$ 1.0**
TRAP (10 μ M)	16.4 $\pm$ 2.6	13.4 $\pm$ 3.1	35.0 $\pm$ 2.6	2.6 $\pm$ 0.9**
TRAP (100 μ M)	16.6 $\pm$ 0.2	13.2 $\pm$ 2.7	64.9 $\pm$ 8.0	2.9 $\pm$ 1.0**

Figure 2 Effects of genistein on histamine-induced release of PGI₂

(A) HUVEC monolayers in 24-well tissue culture trays were preincubated with genistein (hatched bars) or daidzein (cross-hatched bars) at the indicated concentrations (μ M) and then exposed to histamine (10 μ M) in the continued presence of inhibitor. The vWF and 6-keto-PGF_{1 α} released into the supernatant fraction were assayed after a 60 min incubation period. (B) Cells were incubated with vehicle alone (■), genistein (▲) or daidzein (○; 50 μ M; 15 min) and then exposed to various concentrations of histamine. 6-Keto-PGF_{1 α} was measured after a 10 min incubation period. Data are means $\pm$ S.E.M. from two (A) or three (B) experiments performed in triplicate. * P < 0.05, ** P < 0.0001 compared with histamine alone.

concentrations only inhibited PKC activity *in vitro* by approx. 30%; thus genistein ($\geq$ 100 μ M) reduced phospholipid-dependent activity ($10^{-3} \times$ c.p.m.) from 21.4 ± 3.1 to 14.7 ± 2.0 and in

Figure 3 Effects of ST271 on agonist-induced PGI₂ release and vWF secretion

Confluent HUVECs were exposed to the indicated concentrations of ST271 for 15 min. Cells were then incubated with either 1.0 unit/ml thrombin (A) or 10 μ M histamine (B) in the continued presence of inhibitor. The vWF (●) and 6-keto-PGF_{1 α} (○) released into the supernatant fraction were measured after 60 min. The open and closed bars represent the basal release of 6-keto-PGF_{1 α} and vWF respectively. Data points are means $\pm$ S.E.M. of four observations from typical experiments which are each representative of two others showing similar results. * P < 0.05 compared with vehicle alone.

the presence of ST271 (100 μ M) activity was reduced to 15.4 ± 2.0 (mean $\pm$ S.E.M., n = 4).

Effects of ST271 on agonist-driven responses

In further studies we assessed the effects of ST271, a tyrphostin-like PTK inhibitor which is structurally distinct from genistein and which differs in its mode of inhibitory action [46], on the secretory responsiveness of HUVECs. Exposure to ST271 also caused a dose-dependent inhibition of both thrombin (1.0 unit/ml)- and histamine (10 μ M)-induced PGI₂ release without significantly affecting vWF secretion from the same monolayers (Figure 3). Estimated IC₅₀ values from two individual experiments were 5 and 7 μ M for thrombin-stimulated PGI₂ release, and 10 and 12 μ M for histamine-induced PGI₂ generation.

Effects of PTK inhibitors on PGI₂ release in response to Ca²⁺ ionophores

Since PTK inhibitors potently reduced PGI₂ synthesis/release in HUVECs stimulated with different Ca²⁺-mobilizing agonists, we examined the possibility that these agents may affect the release induced by elevated [Ca²⁺]_i in the absence of receptor stimulation.

Table 2 Effects of PTK inhibitors on ionomycin-induced PGI₂ release

HUVECs in 24-well tissue culture trays were incubated (15 min) with the indicated concentrations of genistein, daidzein or ST271 and subsequently challenged with ionomycin (1 μ M) in the continued presence of inhibitor. The 6-keto-PGF_{1 α} in the cell supernatant was measured after 60 min. Vehicle alone (0.1% DMSO or 0.1% ethanol) did not affect ionomycin-induced 6-keto-PGF_{1 α} formation (not shown). Data are means $\pm$ S.E.M. from two experiments with each inhibitor, each performed in quadruplicate. * P < 0.05, ** P < 0.02, *** P < 0.001 compared with ionomycin alone.

Treatment	6-Keto-PGF _{1α} (pg/well)
None	75 $\pm$ 15
Genistein (50 μ M)	65 $\pm$ 10
Ionomycin	1100 $\pm$ 80
Ionomycin + genistein (10 μ M)	660 $\pm$ 110**
Ionomycin + genistein (50 μ M)	150 $\pm$ 30***
Ionomycin + daidzein (10 μ M)	960 $\pm$ 80
Ionomycin + daidzein (50 μ M)	880 $\pm$ 30*
None	40 $\pm$ 10
ST271 25 μ M	45 $\pm$ 20
Ionomycin	595 $\pm$ 110
Ionomycin + ST271 (5 μ M)	505 $\pm$ 65
Ionomycin + ST271 (15 μ M)	210 $\pm$ 55***
Ionomycin + ST271 (25 μ M)	140 $\pm$ 45***

Table 2 shows the effects of genistein, daidzein and ST271 on PGI₂ formation elicited by the Ca²⁺ ionophore, ionomycin (1 μ M). At 50 μ M, genistein did not affect basal PGI₂ release but potentially inhibited ionomycin-induced release; release was also partially inhibited (approx. 10%) in the presence of an equimolar concentration of daidzein. A comparable inhibitory effect on ionomycin-induced release was also observed in the presence of ST271 (Table 2). Similarly, in cells treated with ST271 (25 μ M), A23187 (1 μ M)-induced PGI₂ release was reduced from 350 $\pm$ 41 to 93 $\pm$ 11 pg/well (basal 15 $\pm$ 3 pg/well, n = 4), whereas vWF secretion (m-units/well) from the same cells was unaffected (basal, 0.51 $\pm$ 0.08; A23187, 2.40 $\pm$ 0.04; A23187 plus ST271, 2.35 $\pm$ 0.16).

Ca²⁺-induced PGI₂ release in electrically permeabilized HUVECs is inhibited by genistein and ST271

PTK inhibitors clearly attenuate PGI₂ release elicited by receptor-independent elevations in [Ca²⁺]_i (Table 2), suggesting that these

agents may modulate PGI₂ synthesis/release by acting at, or distal to, agonist-induced changes in [Ca²⁺]_i. To probe further the potential involvement of PTKs in Ca²⁺-driven PGI₂ release we studied the effects of genistein, and of ST271, on Ca²⁺-induced release in electrically permeabilized HUVEC monolayers (Table 3). Addition of Ca²⁺ (1 μ M free Ca²⁺) to permeabilized cells promoted an approximately 2–4-fold increase in the accumulation of 6-keto-PGF_{1 α} in the cell supernatant; co-incubation with genistein or ST271, but not daidzein, dose-dependently attenuated Ca²⁺-induced PGI₂ release. Genistein also significantly inhibited the release measured in the absence of added Ca²⁺ (approx. 10 nM free Ca²⁺) (Table 3). The apparent potentiation of Ca²⁺-stimulated PGI₂ release seen at 10 μ M genistein was mimicked by an equimolar concentration of daidzein. When intact cells were pretreated (15 min) with genistein or ST271, followed by permeabilization and subsequent exposure to Ca²⁺ in the absence of inhibitor, no effect on Ca²⁺-induced PGI₂ release was observed (results not shown). Ca²⁺-driven vWF secretion, in common with agonist-stimulated secretion (Figure 1), was not modulated in the presence of PTK inhibitors (not shown).

Effects of genistein on agonist-induced Ca²⁺ mobilization

Several studies have emphasized the potential role of tyrosine kinases in the regulation of [Ca²⁺]_i influx [18,47–49]. Since, under most circumstances, an elevation in [Ca²⁺]_i is necessary for triggering PGI₂ release from endothelial cells [6,7], we examined whether PTK inhibitors modulated agonist-induced [Ca²⁺]_i changes in fura 2-loaded HUVECs. Figure 4 illustrates the effects of genistein (50 μ M) on the ratio of the fluorescence values at 340 and 380 nm evoked by histamine (10 μ M) and thrombin (1.0 unit/ml). Genistein had no appreciable effect on the peak response to thrombin but did significantly reduce the histamine-induced change in the 340/380 nm ratio (Figure 4a). When the ratio data shown in Figure 4 were expressed in terms of absolute [Ca²⁺]_i, genistein inhibited the histamine-induced Ca²⁺ transient from 2.58 $\pm$ 0.29 to 1.47 $\pm$ 0.5 μ M but had no effect on the peak [Ca²⁺]_i level (2.82 $\pm$ 0.39 μ M) in cells exposed to thrombin. The plateau phase of the response to both agonists (histamine, 0.24 $\pm$ 0.01; thrombin, 0.22 $\pm$ 0.02 μ M [Ca²⁺]_i) was inhibited by approx. 25–30% in genistein-treated cells (Figure 4b) and this effect was reversed after removal of the inhibitor from the superfusing solution (Figures 4c and 4d).

Table 3 Effects of PTK inhibitors on Ca²⁺-induced PGI₂ release in electrically permeabilized HUVECs

Confluent HUVEC monolayers in 24-well trays were electrically permeabilized as described in the Materials and methods section and exposed to permeabilization buffer containing vehicle (0.1% DMSO), genistein, daidzein or ST271 at the indicated concentrations, in the absence of added Ca²⁺ (low Ca²⁺, approx. 10 nM), or in the presence of 1 μ M free Ca²⁺. The 6-keto-PGF_{1 α} in the cell supernatant was measured 60 min after the addition of Ca²⁺. Data are means $\pm$ S.E.M. of four observations from single experiments representative of three with each inhibitor. * P < 0.001 compared with vehicle alone (1 μ M Ca²⁺); † P < 0.01 compared with vehicle alone (low Ca²⁺).

Treatment	6-Keto-PGF _{1α} (pg/well)					
	Genistein		Daidzein		ST271	
Low Ca ²⁺	None	495 $\pm$ 26	None	562 $\pm$ 43	None	80 $\pm$ 15
	50 μ M	333 $\pm$ 21†	50 μ M	553 $\pm$ 30	25 μ M	78 $\pm$ 25
1 μ M Ca ²⁺	None	805 $\pm$ 31	None	940 $\pm$ 53	None	278 $\pm$ 21
	10 μ M	995 $\pm$ 84	10 μ M	1360 $\pm$ 84	5 μ M	367 $\pm$ 52
	20 μ M	556 $\pm$ 57*	20 μ M	945 $\pm$ 81	15 μ M	186 $\pm$ 21*
	50 μ M	438 $\pm$ 57*	50 μ M	914 $\pm$ 59	25 μ M	94 $\pm$ 5*

Figure 4 Effects of genistein on agonist-induced $[Ca^{2+}]_i$ mobilization

Fura-2-loaded HUVECs were pre-exposed to vehicle (open bars) or 50 μ M genistein (cross-hatched bars) for 5 min and then challenged with either histamine (10 μ M) or thrombin (1.0 unit/ml) in the continued presence of inhibitor. The effects of genistein on the peak (a) and plateau (b) fluorescence measurements expressed as the ratio of the values obtained at 340 and 380 nm are shown. Data are means $\pm$ S.E.M. of four to five observations from one cell preparation with similar results observed in three other experiments. (c) and (d) show fluorescence traces from thrombin- or histamine-stimulated HUVECs respectively taken from representative experiments. * P < 0.05, ** P < 0.01, *** P < 0.02 compared with agonist alone.

Table 4 Effect of PTK inhibitors on PGI₂ release in response to exogenous arachidonic acid

HUVECs were washed twice with serum-free DMEM (pH 7.4) and exposed (15 min) to genistein or ST271 at the indicated concentrations. Cells were then challenged with arachidonic acid (30 μ M) in the continued presence of vehicle or inhibitor. The supernatant fraction was assayed for 6-keto-PGF_{1 α} after a 10 min incubation. Data are means $\pm$ S.E.M. for the number of determinations given in parentheses from one experiment representative of three performed. * P < 0.05 compared with arachidonate alone.

Treatment	6-Keto-PGF _{1α} (pg/well)
None	20 $\pm$ 1 (8)
Arachidonate (30 μ M)	110 $\pm$ 10 (8)
+ 10 μ M genistein	120 $\pm$ 12 (4)
+ 20 μ M genistein	130 $\pm$ 20 (3)
+ 50 μ M genistein	151 $\pm$ 4 (4)*
+ 50 μ M daidzein	93 $\pm$ 3 (3)
+ 5 μ M ST271	111 $\pm$ 14 (4)
+ 10 μ M ST271	97 $\pm$ 8 (3)
+ 15 μ M ST271	103 $\pm$ 6 (4)
+ 25 μ M ST271	91 $\pm$ 8 (3)

Effects of PTK inhibitors on PGI₂ release induced by exogenous arachidonic acid

To examine the possibility that PTK inhibitors may modulate PGI₂ release via a direct effect on the activity of cyclo-oxygenase, the ability of these agents to affect 6-keto-PGF_{1 α} accumulation in the presence of exogenous arachidonic acid was determined. Table 4 shows the effects of a range of concentrations of genistein and ST271 on arachidonate-induced formation of 6-keto-PGF_{1 α} . Neither inhibitor attenuated PGI₂ synthesis under these condi-

Table 5 Effect of genistein on L-arginine-dependent cGMP accumulation

Confluent HUVECs in 24-well tissue culture trays were washed twice with serum-free DMEM (pH 7.4). Cells were then pre-exposed (15 min) to vehicle, nitro-L-arginine methyl ester (L-NAME; 100 μ M), genistein or daidzein at the indicated concentrations and subsequently challenged with either histamine or thrombin in the continued presence of inhibitor. The supernatant fraction was removed after a 10 min incubation and assayed for 6-keto-PGF_{1 α} ; cGMP was immediately extracted from the monolayer as described in the Materials and methods section. Basal levels of cGMP and 6-keto-PGF_{1 α} were not affected by genistein (not shown). Data are means $\pm$ S.E.M. from two experiments with quadruplicate determinations per treatment. * P < 0.02, ** P < 0.001, *** P < 0.05 compared with agonist alone; nd, not determined.

Treatment	GMP (fmol/well)	6-Keto-PGF _{1α} (pg/well)
None	101 $\pm$ 12	8 $\pm$ 4
Histamine (10 μ M)	351 $\pm$ 22	61 $\pm$ 6
Histamine + genistein (10 μ M)	342 $\pm$ 33	42 $\pm$ 7
Histamine + genistein (50 μ M)	420 $\pm$ 35	2 $\pm$ 1**
Histamine + daidzein (50 μ M)	273 $\pm$ 32***	42 $\pm$ 15
Histamine + L-NAME	143 $\pm$ 17**	nd
None	96 $\pm$ 13	4 $\pm$ 1
Thrombin (1.0 unit/ml)	384 $\pm$ 18	71 $\pm$ 6
Thrombin + genistein (10 μ M)	377 $\pm$ 42	42 $\pm$ 3*
Thrombin + genistein (50 μ M)	288 $\pm$ 27***	3 $\pm$ 2**
Thrombin + daidzein (50 μ M)	284 $\pm$ 29***	60 $\pm$ 5
Thrombin + L-NAME	110 $\pm$ 7**	nd

tions; in fact, genistein, but not daidzein, slightly potentiated release at the highest concentration employed (Table 4). Both inhibitors were also without effect on PGI₂ release induced by lower concentrations of arachidonate (5 and 15 μ M; results not shown).

Effects of genistein on agonist-induced formation of GMP

Several agonists, including histamine and thrombin, concomitantly induce the synthesis and release of PGI₂ and NO via the constitutive Ca²⁺-sensitive NO synthase (cNOS). To determine the potential involvement of tyrosine kinases in agonist-induced NO release, we examined the effects of genistein and daidzein on cGMP accumulation (and PGI₂ release) in cells exposed to either thrombin or histamine (Table 5). These agonists each promoted an approximately fourfold increase in cGMP which was potently inhibited by treatment with nitro-L-arginine methyl ester (100 μ M), an inhibitor of endothelial cNOS. In contrast, elevated cGMP levels were not consistently affected by the presence of PTK inhibitors. For example, a maximal concentration of genistein partially inhibited thrombin-stimulated cGMP formation but this inhibition was mimicked by an equimolar concentration of daidzein. Similarly, daidzein, but not genistein, partly inhibited cGMP accumulation in the presence of histamine. Agonist-driven PGI₂ synthesis/release from the same monolayers was, in contrast, dose-dependently inhibited by genistein (Table 5).

Modulation of agonist-induced changes in tyrosine phosphorylation by genistein

Protein tyrosine phosphorylation was assessed by Western blotting with a specific anti-phosphotyrosine antibody. Figure 5(A) depicts the pattern of tyrosine phosphorylation in whole-cell lysates prepared from quiescent HUVECs exposed for 10 min to vehicle, thrombin or histamine; the effects of pre-exposure to genistein (50 μ M) or daidzein (50 μ M) on agonist-induced phos-

Figure 5 Effect of genistein on tyrosine phosphorylation of a 42 kDa protein in agonist-stimulated HUVECs

Confluent HUVECs in P60 dishes were serum-deprived for 16 h and subsequently treated with vehicle (0.1% DMSO), genistein (50 μ M) or daidzein (50 μ M) for 15 min before exposure (10 min) to either thrombin (1.0 unit/ml) or histamine (10 μ M) in the continued presence of inhibitor. Total lysate proteins were subjected to Western-blot analysis using a polyclonal anti-phosphotyrosine antibody (**A**) or a monoclonal anti-(MAP kinase) antibody (**B**) as outlined in the Materials and methods section. Lanes are as follows: 1, control; 2, thrombin; 3, thrombin plus genistein; 4, thrombin plus daidzein; 5, histamine; 6, histamine plus genistein; 7, histamine plus daidzein. Arrows denote approximate molecular mass in kDa.

phorylation are also shown. Both histamine and thrombin enhanced the tyrosine phosphorylation state of a number of HUVEC proteins including those with approximate molecular masses of 65–70 kDa (Figure 5A), 110–130 kDa (not shown), and a prominent band migrating at about 42 kDa (P42; Figure 5A). Figure 5(B) shows an anti-(MAP kinase) immunoblot obtained from the same samples as those depicted in Figure 5(A); the phosphorylated activated form of the 42 kDa MAP kinase (p42^{mapk}), which displays a lowered electrophoretic mobility, corresponds to the 42 kDa tyrosine-phosphorylated protein in Figure 5(A) and co-migrates precisely with P42 when immunoblots probed with anti-phosphotyrosine are stripped and reprobbed with the MAP kinase antibody [50]. In genistein-treated cells, neither thrombin nor histamine promoted the tyrosine phosphorylation of p42^{mapk}; daidzein at equimolar concentration slightly inhibited thrombin- but not histamine-induced phosphorylation of p42^{mapk} (Figure 5). PGI₂ synthesis by the same monolayers was abolished in the presence of genistein (results not shown).

DISCUSSION

Human vascular endothelial cells express several protein kinases with specificities for serine/threonine residues, and there is evidence that regulated serine/threonine kinases (e.g. PKC) can influence, but are not obligatory for, agonist-induced endothelial secretory events [3,6]. As yet, however, little is known about the involvement of PTKs in regulating the secretion of vasoactive mediators, the molecular mechanisms controlling PTK activation

or the identity of the cellular substrates phosphorylated in endothelial cells. In the present study, we have shown that thrombin and histamine, agonists that bind to receptors belonging to the G-protein-coupled receptor superfamily, promote the rapid tyrosine phosphorylation of several endothelial cell proteins (including p42^{mapk}) and that both agonist-induced changes in tyrosine phosphorylation and PGI₂ release are attenuated by PTK inhibitors. These results extend the recent finding by Fleming and co-workers [18] that activation of G-protein-linked receptors promotes changes in the level of phosphotyrosine in endothelium and also demonstrate that PTKs are important and selective regulators of PGI₂ synthesis.

The present studies employed two PTK inhibitors with distinct mechanisms of inhibitory action [27,28,46], both of which had qualitatively similar effects on PGI₂ release in response to receptor-dependent and -independent stimuli. At agonist concentrations that maximally induce PGI₂ release (10 μ M and 1.0 unit/ml for histamine and thrombin respectively), the estimated IC₅₀ values for genistein-mediated inhibition of thrombin- and histamine-induced PGI₂ release were similar (approx. 20 μ M). However, at higher doses of thrombin (> 1.0 unit/ml) the inhibitory effect of genistein was incomplete such that 40–50% of the response to 2.5 units/ml thrombin remained insensitive to genistein. These results demonstrate that the signalling pathways evoked by thrombin and histamine involve the activation of PTKs with similar sensitivities to genistein but suggest that high concentrations of thrombin can, in addition, activate a parallel PTK-insensitive pathway leading to PGI₂ synthesis. PGI₂ release in response to TRAP was also reduced by genistein, confirming that thrombin-driven PGI₂ release involves proteolytic activation of the receptor [44,45]. ST271 was a more potent inhibitor of PGI₂ release than genistein and, in addition, differentially affected the responses to thrombin and histamine, with IC₅₀ values compared with histamine being approximately twofold those seen in thrombin-stimulated HUVECs. This may reflect the ability of histamine and thrombin to activate different PTKs or to activate the same PTKs to differing extents, and suggests that there are distinctions between the signalling pathways triggered by thrombin and histamine in human endothelium. These findings reinforce recent observations indicating qualitative and quantitative differences between a number of other intracellular events, including phosphorylation of the PKC substrate MARCKS [51], generation of inositol trisphosphate (IP₃) [52] and the signalling pathways leading to vWF secretion [3] in thrombin- and histamine-stimulated HUVECs. The fact that genistein affected thrombin- and histamine-evoked responses equally, whereas ST271 discriminated between the two agonists, may be related to the distinct mechanisms of inhibitory action of the two agents; by competing with intracellular substrate(s), ST271 might be expected to exert more selective effects than genistein.

Interpretation of data obtained using kinase inhibitors requires caution since those that act by competing with ATP, including genistein, can potentially exert non-selective effects on a range of kinases. However, several lines of evidence indicate that genistein is acting specifically on tyrosine kinases under our experimental conditions: (i) ST271 exerts qualitatively similar effects on PGI₂ release; (ii) Ca²⁺-driven PGI₂ release in permeabilized cells is abolished in the presence of genistein or ST271 but not by a pseudosubstrate inhibitor of PKC (C. P. D. Wheeler-Jones and J. D. Pearson, unpublished work); (iii) inhibitors of PKC generally enhance agonist-induced changes in [Ca²⁺]_i [48], in contrast with the inhibitory action of genistein; (iv) genistein does not affect phorbol ester-induced vWF secretion whereas Ro 31-8220 treatment results in complete inhibition [3].

PTK inhibitors potently attenuated PGI₂ release induced by agonist or ionophore in intact HUVECs, or promoted by Ca²⁺ in permeabilized cells, but had no effect on vWF secretion from the same cells. Thus PTK activation is apparently not essential for the regulated exocytotic release of vWF from human endothelial cells. Since these measurements were routinely made after a 60 min incubation period, we cannot rule out the possibility that PTK inhibitors may, for example, influence the rate of vWF secretion at very early time points after agonist stimulation. In addition, it is possible that the secretory process may involve PTK(s) which are insensitive to the inhibitors employed in the present study.

PGI₂ synthesis in endothelial cells is highly dependent on agonist-induced increases in [Ca²⁺]_i and is primarily driven by release of Ca²⁺ from intracellular stores [6,7,10]. In the present study both PTK inhibitors dose-dependently attenuated ionophore-induced PGI₂ release with similar potencies to their reduction of agonist-stimulated release. These findings are consistent with a site of inhibition distal to elevation of [Ca²⁺]_i. However, PTK inhibitors have been reported to reduce agonist-induced [Ca²⁺]_i elevations in some cell types (e.g. thrombin-stimulated platelets [48,49]) but not in others (e.g. thrombin-stimulated fibroblasts [53]). In view of the recent demonstration that bradykinin-induced changes in [Ca²⁺]_i in human endothelial cells are significantly inhibited by genistein, apparently by a selective effect on Ca²⁺ influx [18], we examined the effects of genistein on [Ca²⁺]_i in HUVECs. At a concentration that abolished PGI₂ release in response to either thrombin or histamine, genistein had minimal and differential effects on the [Ca²⁺]_i changes evoked by these agonists. Thus genistein had no effect on the peak [Ca²⁺]_i rise in response to thrombin but did reduce the histamine-induced [Ca²⁺]_i elevation. A number of possible mechanisms could account for this inhibition, including a reduced generation of IP₃ and/or enhanced IP₃ metabolism. However, even in the presence of genistein, [Ca²⁺]_i in cells exposed to histamine remained well above the threshold level (about 800 nM) required for initiation of PGI₂ synthesis [7]. We conclude that the action of PTK inhibitors on agonist-induced PGI₂ release cannot be accounted for by modulation of [Ca²⁺]_i. Moreover, the observation that ST271 and genistein (but not daidzein) completely inhibited Ca²⁺-driven PGI₂ synthesis in electrically permeabilized HUVECs demonstrates conclusively that tyrosine kinases regulate PGI₂ synthesis downstream of raised [Ca²⁺]_i.

In agreement with other studies [18,47–49], the plateau phases of the Ca²⁺ response to both thrombin and histamine were partially and reversibly inhibited by pretreatment with genistein. These changes, however, were not sufficient to cause an inhibition of cNOS activity since L-arginine-dependent cGMP formation was not modified in genistein-treated cells. Similar findings have recently been reported in vascular smooth-muscle cells in which PTK inhibitors, including genistein, failed to affect cGMP accumulation elicited by endothelium-derived NO released basally or after stimulation with bradykinin [54]. The ability of PTK inhibitors to attenuate, albeit poorly, the plateau phase of the Ca²⁺ response in HUVECs suggests the existence of a Ca²⁺-influx pathway in these cells which may be controlled, at least in part, by changes in tyrosine kinase activity.

In contrast with their effects on agonist- or ionophore-induced PGI₂ synthesis, neither genistein nor ST271 affected PGI₂ production from exogenous arachidonic acid, indicating that these inhibitors are unlikely to be affecting the activity of the constitutively expressed cyclo-oxygenase. Thus, PTKs directly or indirectly modulate a Ca²⁺-regulated event in PGI₂ synthesis upstream of cyclo-oxygenase. The most likely candidate is the

cytosolic (85 kDa) Ca²⁺-dependent PLA₂ (cPLA₂) which is believed to be the principal PLA₂ responsible for arachidonate mobilization and hence PGI₂ production in human endothelial cells [55]. cPLA₂ is phosphorylated and its activity enhanced by p42^{mapk} [56]. Since p42^{mapk} is rapidly tyrosine-phosphorylated in histamine- and thrombin-stimulated HUVECs and this phosphorylation event is also inhibited by genistein, it is reasonable to postulate that PTK-dependent activation of p42^{mapk} may be a necessary component of the signalling pathway(s) regulating PGI₂ synthesis in human endothelial cells. Indeed, a recent study has proposed that a similar mechanism is involved in the regulation of basic fibroblast growth factor-induced arachidonate generation in bovine aortic endothelial cells [57]. Further evidence for the involvement of reversible tyrosine-phosphorylation events in the control of endothelial cell PGI₂ synthesis and release is provided by the ability of peroxovanadate, a potent inhibitor of tyrosine phosphatases, to enhance tyrosine phosphorylation and promote PGI₂ synthesis in intact and electrically permeabilized HUVECs (C. P. D. Wheeler-Jones, R. A. Houlston and J. D. Pearson, unpublished work).

In summary, we have shown that PTKs regulate PGI₂ release in HUVECs by modulating, either directly or indirectly, a Ca²⁺-sensitive step upstream of cyclo-oxygenase, possibly cPLA₂. In contrast, agonist-induced vWF secretion is entirely unaffected by inhibition of tyrosine kinase activity. The identity of the PTKs activated by stimulation of G-protein-coupled receptors in human endothelium remains unknown. Recent studies in other systems, however, indicate that thrombin can couple to several members of the *src* family of cytosolic tyrosine kinases [58,59]. We are currently attempting to identify the PTKs involved in the modulation of endothelial PGI₂ synthesis and to determine the importance and mechanism of cPLA₂ phosphorylation in this process.

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