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Effect of the statin atorvastatin on intracellular signalling by the prostacyclin receptor *in vitro* and *in vivo*

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1 Prostacyclin plays a central role within the vasculature. We have previously established that the prostacyclin receptor (IP) undergoes isoprenylation, a lipid modification obligate for its function. The aim of the current study was to investigate the effect of the hydroxy methyl glutaryl co-enzyme A reductase inhibitor atorvastatin on signalling and function of the IP expressed in mammalian whole cells and in platelets isolated from patients undergoing therapeutic intervention with atorvastatin.

2 Initially, the effect of atorvastatin on signalling by the human (h) and mouse (m) IP overexpressed in human embryonic kidney 293 cells and the hIP endogenously expressed in human erythroleukaemic 92.1.7 cells was investigated. Atorvastatin significantly reduced IP-mediated cAMP generation (IC_{50} 6.6–11.1 μM) and $[Ca^{2+}]_i$ mobilization (IC_{50} 7.2–16.4 μM) in a concentration-dependent manner, but had no effect on signalling by the nonisoprenylated β_2 adrenergic receptor or the α or β isoforms of the human thromboxane A_2 receptor (TP).

3 Moreover, atorvastatin significantly reduced IP-mediated crossdesensitization of signalling by TPα (IC_{50} 10.4 μM), but not by TPβ.

4 In contrast to the whole-cell data, atorvastatin therapy did not interfere with IP-mediated cAMP generation or IP-induced inhibition of TP-mediated aggregation of platelets isolated from human volunteers undergoing therapeutic intervention with atorvastatin (10–80 mg per daily dose).

5 In conclusion, while data generated in whole cells indicated that atorvastatin significantly impairs signalling by both the hIP and mP, the *in vivo clinical* data indicated that, at the administered therapeutic dose, atorvastatin does not significantly compromise IP signalling and function in humans.

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Abbreviations: β_2AR, β_2 adrenergic receptor; $[Ca^{2+}]_i$, intracellular calcium; cAMP, adenosine 3′,5′-cyclic monophosphate; FBS, foetal bovine serum; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GPCR, G protein-coupled receptor; HA, haemagglutinin; HEK, human embryonic kidney; HEL, human erythroleukaemia; IP, prostacyclin receptor; PLC, phospolipase C; TP, prostanoid TXA_2 receptor; TXA_2, thromboxane A_2

Introduction

Prostaglandin (PG)I_2 or prostacyclin, mainly produced by vascular endothelial cells (Narumiya *et al*., 1993; 1999), acts primarily as an inhibitor of platelet aggregation, as a potent vasodilator, and is implicated in the control of circulatory homeostasis and in the prevention of cardiovascular disease (Vane & Botting, 1995). The prostacyclin receptor (IP), a G protein-coupled receptor (GPCR), primarily couples to adenylyl cyclase (AC) activation (Coleman *et al*., 1994; Wise & Jones, 1996; Narumiya *et al*., 1999) but may also couple to phospholipase (PL) Cβ activation, leading to phosphatidyl inositol turnover and mobilization of intracellular calcium ($[Ca^{2+}]_i$), perhaps as a secondary effector system (Namba *et al*., 1994; Wise & Jones, 1996).

Protein isoprenylation, a post-translational lipid modification, involves the covalent attachment of either a carbon-15 farnesyl or carbon-20 geranylgeranyl isoprenoid derived from the mevalonate/cholesterol biosynthetic pathway to the target protein (Zhang & Casey, 1996). These isoprenoids are typically attached to a conserved cysteine located within a distinct carboxyl-terminal '*CaaX motif*', where C represents the acceptor cysteine (Moores *et al*., 1991; Reiss *et al*., 1991). The recent identification of a putative CaaX motif, -*CSLC*, within the C-termini of the IP from a number of species led to the establishment that both the human (h) and mouse (m) IPs are isoprenylated *in vivo* (Hayes *et al*., 1999; Miggin *et al*., 2002). Site-directed mutagenesis established that, while C-15 farnesylation of either the hIP and mIP has no influence on ligand binding, it is *absolutely* required for efficient receptor–effector coupling and, hence, for IP function (Hayes *et al*., 1999; Miggin *et al*., 2002).

Epidemiological studies have confirmed that elevated cholesterol and, to a lesser extent, elevated triglycerides are among the most important risk factors of coronary heart disease (Smith, 2000; Kreisberg & Oberman, 2002). They

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accelerate the development of atherosclerosis and enhance the adverse effects associated with other risk factors such as smoking, obesity, diabetes and hypertension (Smith, 2000; Kreisberg & Oberman, 2002). Unquestionably, the greatest advance to current lipid-lowering regimes involved the discovery of 'statins' (Kreisberg & Oberman, 2002). Statins, which include compactin, lovastatin, fluvastatin, pravastatin, atorvastatin, cerivastatin, simvastatin and rosuvastatin, act by blocking the endogenous synthesis of cholesterol by 3-hydroxy-3-methylglutaryl Co-enzyme A (HMG-CoA) reductase, the rate-limiting enzyme of hepatic cholesterol biosynthesis (Farnier & Davignon, 1998).

Detailed analysis of large clinical trials suggests that the clinical outcome of statins is not solely associated with baseline cholesterol levels or the degree of cholesterol reduction (Liao, 2002). Thus, it is now believed that the clinical effects of the statins may also involve non-lipid-related mechanisms that modify endothelial function, inflammatory responses, foam cell formation, smooth muscle activation, plaque stability and thrombus formation independent of their cholesterol/lipid-lowering properties (Corsini *et al*., 1999; Bellosta *et al*., 2000; Koh, 2000; Laufs & Liao, 2000; Palinski, 2000; LaRosa, 2001; Lefer *et al*., 2001; Liao, 2002; Mason, 2003). For example, mevalonate, the product of the HMG-CoA reductase reaction, is not only a precursor of cholesterol but also of a number of nonsteroidal isoprenoid compounds that are essential for normal cellular activity, including the isoprene donors farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Thus, the finding that the prostacyclin receptor (IP) is isoprenylated, a step obligatory for IP function (Hayes *et al*., 1999; Miggin *et al*., 2002), coupled to the fact that statins can interfere with protein isoprenylation through depletion of FPP and GGPP (Maltese, 1990), indicates that the clinical use of statins could interfere with IP signalling and, therefore, could influence platelet and/or vascular smooth muscle (VSM) function.

Hence, given that atorvastatin is among the most widely prescribed cholesterol-lowering agents and that it can achieve relatively high systemic concentrations *in vivo* in humans (Cilla *et al*., 1996), the aim of the current study was to investigate the effects of atorvastatin on IP signalling and function in mammalian cells and in human subjects. Hence, we investigated the effect of atorvastatin on expression and signalling by both the mIP and hIP either overexpressed in human embryonic kidney (HEK) 293 cells or endogenously expressed in the megakaryocytic human erythroleukaemic 92.1.7 (HEL) cell line. In addition, the effect of atorvastatin on IP expression, intracellular signalling and platelet aggregation was investigated in platelets isolated from humans undergoing therapeutic intervention with atorvastatin (0–80 mg daily dose). While atorvastatin was found to significantly impair signalling by both the hIP and mP in cultured whole cells, the *in vivo clinical* data indicated that it did not significantly affect IP signalling and function in human subjects.

Methods

Materials

Iloprost, [^{3}H]iloprost (15.3 Ci mmol^{-1}) and [^{3}H]CGP-12177 (41.0 Ci mmol^{-1}) were purchased from Amersham Pharmacia Biotech, Buckinghampshire, U.K. [^{3}H]adenosine 3′,5′-cyclic monophosphate (cAMP) (15–30 Ci mmol^{-1}) was purchased from American Radiolabeled Chemicals Inc., St Louis, U.S.A. Isoproterenol was purchased from Sigma, St Louis, Missouri, U.S.A. Fura 2/AM St Louis, U.S.A. and U46619 were purchased from Calbiochem, Darmstadt, Germany. Atorvastatin was obtained from Pfizer Inc., U.S.A. Cicaprost was obtained from Schering AG (Berlin, Germany). All other reagents were ANALAR or molecular biology grade and were used without further purification.

Stable cell lines

HEK.mIP, HEK.hIP, HEK.TPα and HEK.TPβ cells stably overexpressing haemagglutinin (HA) epitope-tagged forms of the wild-type mouse (m), human (h) prostacyclin receptor (IP) and the human thromboxane (TX) A_2 receptor (TP) α and β isoforms, respectively, have been described previously (Hayes *et al*., 1999; Lawler *et al*., 2001). HEK.β_2 adrenergic receptor (β_2AR) cells stably overexpressing the human β_2AR in HEK 293 cells were described previously (Lawler *et al*., 2001).

Clinical study design

In all, 32 randomly selected healthy volunteers, 20 men and 12 women (median age 54 ± 2.2 years; age range 27–75 years) participated in this study conducted in conjunction with Dr John Erwin, Consultant Cardiologist, St Vincent's University Hospital, Dublin. The study protocol was approved by the St Vincent's University Hospital Ethics Committee, and written informed consent and patient history were obtained from all participants. Potential donors were excluded if they were receiving any nonsteroidal anti-inflammatory drugs (NSAIDs) or had ingested such drugs during the previous 14 days.

Determination of cholesterol and lipid profiles

Quantitative determination of total cholesterol, HDL, LDL and triglycerides in whole blood was carried out using a Cholestech L·D·X® Lipid profile-II analyzer (Pfizer Inc.) according to the manufacturer's recommendations.

Platelet preparation and aggregations

Preparation of platelets was carried out as described previously (McNicol, 1996). Briefly, blood (40 ml) was drawn *via* venipuncture from human volunteers who had not taken any medication for at least 10 days into syringes containing indomethacin (10 μM) and 3.8% sodium citrate (9 : 1 v v^{-1}) (final concentration, 0.38% sodium citrate). The blood was centrifuged twice for 10 min at $160 \times g$ and the platelet-rich plasma (PRP) was removed and re-centrifuged for 2 min at $160 \times g$ to remove contaminating red blood cells. For aggregation studies, PRP was diluted to approximately 10^8 platelets ml^{-1} in platelet resuspension buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 5.5 mM glucose, pH 7.4); 0.5 ml aliquots were pre-incubated at 37°C for 2 min before addition of the aggregating agent (1 μM U46619, 1 μM cicaprost or 1 μM BW245C). Platelet aggregations were monitored using a Chronolog Platelet Aggregometer.

Measurement of cAMP generation

To investigate the effect of atorvastatin on agonist-mediated cAMP generation, cells were pre-incubated in the presence or absence of atorvastatin (20 μM) for 16 h prior to harvesting. For concentration–response studies, cells were pre-incubated for 16 h in the presence of atorvastatin (0–20 μM). Thereafter, nonviable cells were removed and cAMP assays were preformed as described previously (Hayes *et al*., 1999). For platelet studies, platelets were harvested from PRP by centrifugation at $900 \times g$ for 10–15 min and were washed in platelet resuspension buffer. Thereafter, approximately 3.7×10^8 platelets were resuspended in 200 μl of HEPES-buffered saline and cAMP assays were preformed as described previously (Hayes *et al*., 1999). HEK.hIP, HEK.mIP, HEL cells and platelets were stimulated with cicaprost (1 μM) or in the case of the HEK.β_2AR cells with isoproterenol (10 μM). In each case, basal cAMP levels (pmol cAMP mg^{-1} cell protein $\pm$ s.e.m.) were determined by exposing the cells to the vehicle HBS under identical incubation conditions. Levels of cAMP produced in ligand-stimulated cells relative to basal cAMP levels, in vehicle-treated cells, are expressed and presented as fold stimulation of basal (fold increase in cAMP $\pm$ s.e.m., $n=4$).

Measurement of intracellular [Ca^{2+}] mobilization

Measurements of $[Ca^{2+}]_i$ mobilization in Fura2/AM-preloaded cells was carried out essentially as described previously (Kinsella *et al*., 1997). Cells were pre-incubated for 16 h with either atorvastatin (10 μM) or with the vehicle, 0.001% methanol. For concentration–response studies, cells were pre-incubated with atorvastatin (0–20 μM) for 16 h. Thereafter, Fura2/AM-preloaded cells were stimulated with the IP agonist cicaprost (1 μM) or, as a control, with the TP agonist U46619 (1 μM). In separate experiments, to examine the concentration-dependent effect of atorvastatin on IP-mediated counter-regulation of TP-mediated signalling, HEK.TPα cells and HEK.TPβ cells, transiently transfected with pCMV:Gα_q, were pre-incubated in the presence and absence of atorvastatin (10 μM) for 16 h. For concentration–response studies, cells were pre-incubated with atorvastatin (0–20 μM) for 16 h. Thereafter, Fura2/AM preloaded cells were stimulated with the IP agonist cicaprost (1 μM), followed by stimulation with the TP agonist U46619 (1 μM). The results, representative of four independent experiments, are plotted as changes in $[Ca^{2+}]_i$ mobilization ($\Delta[Ca^{2+}]_i \pm$ s.e.m., nM, $n=4$) as a function of time (s).

Radioligand-binding studies

Radioligand-binding assays of the IP were carried out on crude cell membranes (P_{100}) using 4 nM [^{3}H]iloprost essentially as described previously (Hayes *et al*., 1999). Where specified, cells were pre-incubated with either atorvastatin (20 μM) or with the vehicle (0.001% methanol) for 16 h prior to harvesting. For platelet-binding studies, platelets (1×10^8 platelets assay^{-1}) were harvested from PRP by centrifugation at $900 \times g$ for 10–15 min and washed once in platelet resuspension buffer plus 0.1% indomethacin. For IP radioligand-binding studies, platelets were re-centrifuged and resuspended in 1 ml of hypotonic buffer (10 mM HEPES, 5 mM KCl, 5.5 mM glucose, pH 7.4) and incubated on ice for 30 min. Platelets were then homogenized and centrifuged at $10{,}000 \times g$ for 30 min at 4°C. The resulting pellet fraction (P_{100}) was resuspended in resuspension buffer for IP-binding studies. For TP-binding studies, whole-cell or platelet pellets were resuspended in HBSSHB buffer and [^{3}H]SQ29548-binding studies were carried out as described previously (Kinsella *et al*., 1997). β_2AR radioligand-binding assays were carried out on whole cells using 25 nM [^{3}H]CGP-12177 essentially as described previously (Gagnon *et al*., 1998).

Data analysis

Statistical analysis was carried out using the unpaired Student's *t*-test using GraphPad Prism V2.0. programme (GraphPad Software Inc., San Diego, CA, U.S.A.). *P*-values ≤ 0.05 were considered to indicate a statistically significant difference.

Results

Effect of atorvastatin on IP-mediated cAMP generation

The effect of atorvastatin on prostacyclin receptor (IP) signalling was initially investigated in HEK 293 cells stably overexpressing both the human (h) IP (HEK.hIP cells) and the mouse (m) IP (HEK.mIP cells), or in HEL cells which endogenously express the hIP at high levels. As controls, HEK 293 cells stably expressing the human β_2AR (HEK.β_2AR cells) and the human TPα and TPβ isoforms of the thromboxane A_2 (TXA_2) receptor (HEK.TPα and HEK.TPβ cells) were also examined. Initial cytotoxicity studies established that 63.8 $\pm$ 2.33% ($n=4$) of the parental HEK 293 cell line and 66.9 $\pm$ 3.69% ($n=4$) of HEL cells remained viable following 24 h exposure to atorvastatin (20 μM). Additionally, pre-incubation of cells with atorvastatin (20 μM) had no significant effect on [^{3}H]iloprost binding by the IP over-expressed in HEK.hIP ($P=0.4263$), HEK.mIP ($P=0.7619$) or endogenously expressed in HEL ($P=0.1503$) cells (data not shown). Similarly, atorvastatin (20 μM) had no significant effect on [^{3}H]CGP-12177 and [^{3}H]SQ29548 binding by the control HEK.β_2AR cells ($P=0.4536$) or by the HEK.TPα ($P=0.1732$) and HEK.TPβ ($P=0.2594$) cells, respectively (data not shown).

Thereafter, the effect of atorvastatin on IP signalling was determined by examining agonist-mediated cAMP generation in HEK.hIP, HEK.mIP and in HEL cells and, as a control for a nonisoprenylated G_s/adenylyl cyclase coupled receptor, in HEK.β_2AR cells. Initial stimulation of both HEK.hIP and HEK.mIP cells with the selective IP agonist cicaprost produced 11.4 $\pm$ 0.53- and 12.7 $\pm$ 0.41-fold increases in cAMP, respectively (Figure 1, panel a). However, pre-incubation of cells with atorvastatin (10 μM) significantly impaired signalling by both the hIP (5.4 $\pm$ 1.16 cAMP fold increase; $P=0.0094$) and mIP (3.4 $\pm$ 0.73 cAMP fold increase; $P<0.0001$) when compared to nontreated cells (Figure 1, panel a). Stimulation of the hIP endogenously expressed in HEL cells with cicaprost yielded a 23.8 $\pm$ 0.84-fold increase in cAMP generation (Figure 1, panel a). Consistent with the data generated in the clonal HEK 293 cell lines, pre-incubation of HEL cells with atorvastatin (10 μM) for 16 h significantly reduced cAMP generation (6.0 $\pm$ 0.99 cAMP fold increase; $P=0.0002$) when compared to nontreated cells (Figure 1, panel a). In contrast, while stimulation of the nonisoprenylated control HEK.β_2AR cells with isoproterenol (10 μM) yielded substantial increases in

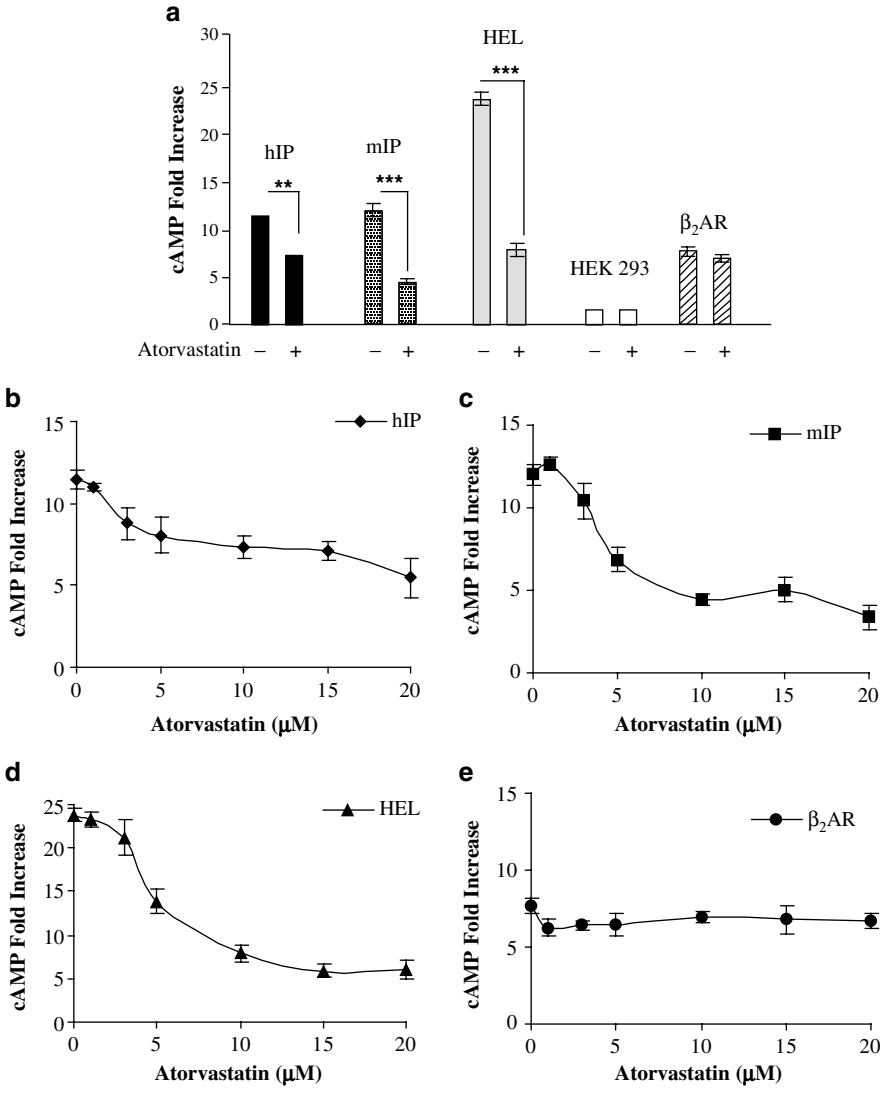

Figure 1 Effect of atorvastatin on IP-mediated cAMP generation. HEK.hIP cells (hIP), HEK.mIP cells (mIP), HEL cells (HEL), HEK 293 cells or, as controls, HEK.β_2AR cells (β_2AR) were pre-incubated with (+) or without (−) 10 μM atorvastatin for 16 h prior to harvesting (panel a). HEK.hIP cells (panel b), HEK.mIP cells (panel c), HEL cells (panel d), or HEK.β_2AR cells (panel e) were pre-incubated with 0–20 μM atorvastatin for 16 h prior to harvesting. Thereafter, cells were stimulated with 1 μM cicaprost or, in the case of the HEK.β_2AR cells, with 10 μM isoproterenol. In each case, basal cAMP levels were determined by exposing the cells to the vehicle HBS under identical incubation conditions. Levels of cAMP produced in ligand-stimulated cells relative to basal cAMP levels, in vehicle-treated cells, were expressed as fold stimulation of basal (fold increase in cAMP $\pm$ s.e.m., $n=4$). The asterisks (*) indicate that the level of cicaprost-mediated cAMP generation was significantly reduced in the presence of atorvastatin compared to vehicle-treated cells, where ** indicates $P \leqslant 0.001$, and *** indicates $P \leqslant 0.0001$. Basal levels of cAMP in HEK.hIP, HEK.mIP, HEL, HEK 293 and in HEK.β_2AR cells were 0.72 ± 0.21 nmol mg^{-1} cell protein, 0.86 ± 0.33 nmol mg^{-1} cell protein, 0.88 ± 0.04 nmol mg^{-1} cell protein, 0.42 ± 0.09 nmol mg^{-1} cell protein and 0.40 ± 0.09 nm mg^{-1} cell protein, respectively.

cAMP generation (7.7 ± 0.48 cAMP fold increase), pre-incubation of those cells with atorvastatin (10 μM) had no significant effect on signalling by the β_2AR (6.72 ± 0.53 cAMP fold increase; $P = 0.2538$) (Figure 1, panel a).

Thereafter, the concentration-dependent effects of atorvastatin (0–20 μM) on IP-mediated cAMP generation were examined (Figure 1). Atorvastatin caused concentration-dependent decreases in cAMP generation by both the hIP and mIP overexpressed in HEK.hIP cells and HEK.mIP cells, respectively, and by the hIP endogenously expressed in HEL cells (Figure 1, panels b–d). From the concentration–response curves, the IC_{50} (inhibitory concentration 50) values were found to be 6.6, 8.5 and 11.1 μM atorvastatin in HEK.hIP, HEK.mIP cells and HEL cells, respectively (Figure 1, panels b–d). Conversely, atorvastatin had no effect on isoproterenol-mediated cAMP generation by HEK.β_2AR even at the highest atorvastatin concentrations employed (Figure 1, panels a and d; $P \geqslant 0.05$).

Effect of atorvastatin on IP-mediated $[Ca^{2+}]_i$ mobilization

Thereafter, the effect of atorvastatin on the ability of the IP to couple to the G_q/PLCβ activation was examined by analysing cicaprost-mediated $[Ca^{2+}]_i$ mobilization by the hIP and mIP overexpressed in HEK.hIP. cells and HEK.mIP cells,

respectively, and in HEL cells. HEK 293 cells overexpressing the β isoform of the human TXA_2 receptor (TPβ) served as a control for a nonisoprenylated G_q/PLC-coupled receptor. Consistent with previous studies, HEK.hIP cells (Figure 2, panel a; $\Delta[Ca^{2+}]_i = 139 \pm 12$ nM), HEK.mIP cells (Figure 2, panel c; $\Delta[Ca^{2+}]_i = 152 \pm 16$ nM) and HEL cells (Figure 2, panel e; $\Delta[Ca^{2+}]_i = 83.7 \pm 4.0$ nM) showed efficient $[Ca^{2+}]_i$ mobilization in response to cicaprost (1 μM). In contrast, prior

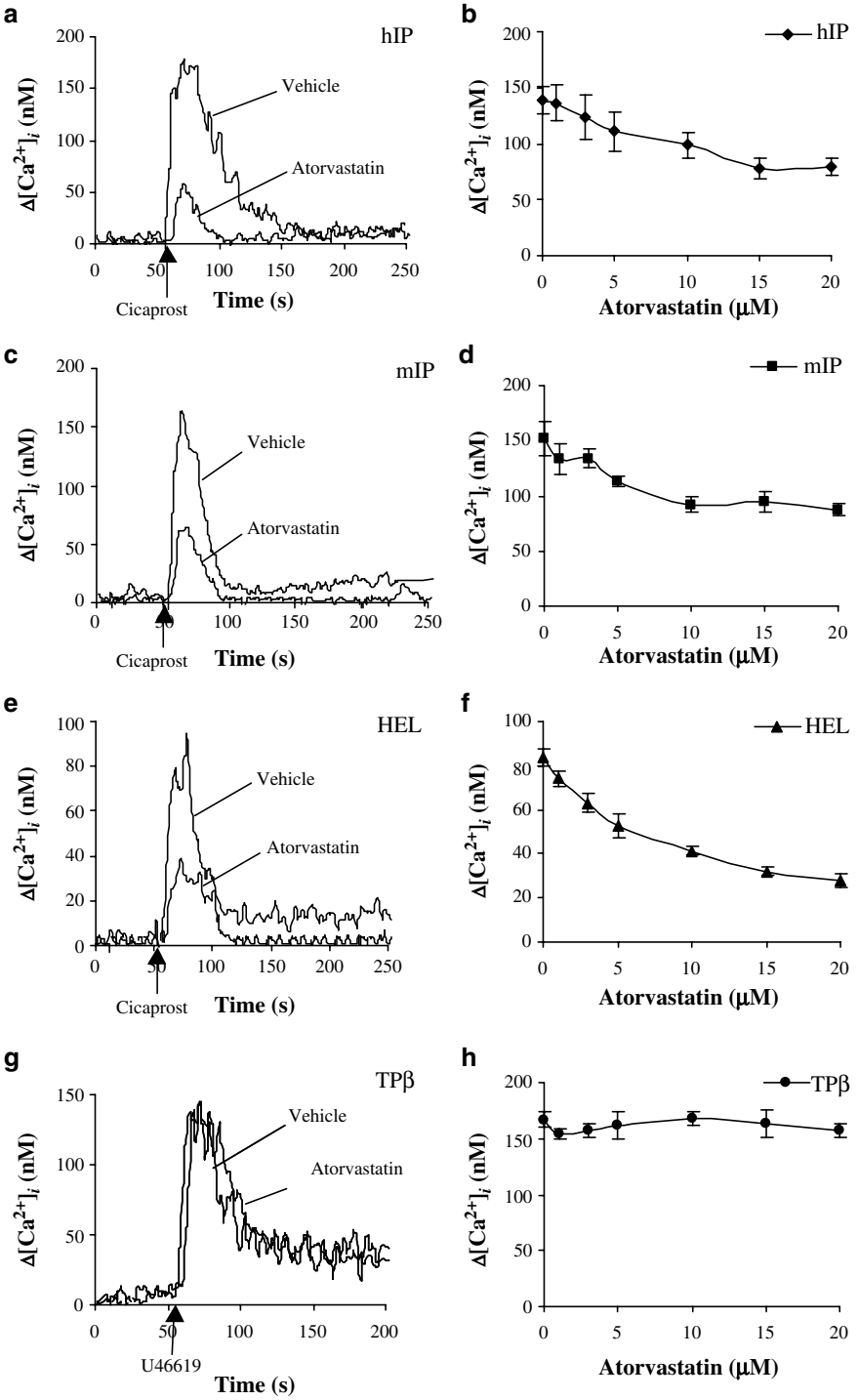

Figure 2 Effect of atorvastatin on IP-mediated $[Ca^{2+}]_i$ mobilization. HEK.hIP cells (panels a and b), HEK.mIP cells (panels c and d), HEL cells (panels e and f) and, as controls, HEK.TPβ cells (panels g and h) were pre-incubated with either 10 μM atorvastatin (Atorvastatin) or with 0.001% methanol (Vehicle) (panels a, c, e and g) or, alternatively, with 0–20 μM atorvastatin (panels b, d, f and h) for 16 h prior to harvesting. Cells, preloaded with Fura2/AM, were stimulated at approximately 50 s with 1 μM cicaprost (panels a, c and e) or with 1 μM U46619 at 50 s (panel g), as indicated by the arrows. Data presented in panels a, c, e and g are representative of at least four independent experiments and are plotted as changes in $[Ca^{2+}]_i$ mobilization ($\Delta[Ca^{2+}]_i \pm$s.e.m., nM, $n = 4$) as a function of time (s). In panels b, d, f and h, mean changes in $[Ca^{2+}]_i$ mobilization ($\Delta[Ca^{2+}]_i \pm$s.e.m., nM, $n = 4$) are plotted as a function of atorvastatin concentration (μM).

treatment of cells with atorvastatin (10 μM) for 16 h significantly reduced $[Ca^{2+}]_i$ mobilization in HEK.hIP cells (Figure 2, panel a; $\Delta[Ca^{2+}]_i = 79.5 \pm 7.5$ nM, $P = 0.0147$), in HEK.mIP cells (Figure 2, panel c; $\Delta[Ca^{2+}]_i = 87.4 \pm 4.9$ nM, $P = 0.0187$) and in HEL cells (Figure 2, panel e; $\Delta[Ca^{2+}]_i = 28.0 \pm 3.3$ nM, $P < 0.0001$). From concentration–response studies, IC_{50} values were found to be 16.4, 7.2 and 8.7 μM atorvastatin in HEK.hIP, HEK.mIP cells and HEL cells, respectively (Figure 2, panels b, d and f). In contrast, while stimulation of the nonisoprenylated control HEK.TPβ cells, transiently co-transfected with $G\alpha_q$, with the TXA_2 mimetic U46619 (1 μM) resulted in efficient rises in $[Ca^{2+}]_i$ mobilization (Figure 2, panels g and h; $\Delta[Ca^{2+}]_i = 134 \pm 9.12$ nM), pretreatment of cells with atorvastatin (10 μM) had no effect on the ability of TPβ to mobilize $[Ca^{2+}]_i$ (Figure 2, panels g and h; $\Delta[Ca^{2+}]_i = 117 \pm 3.4$ nM, $P = 0.1637$).

Effect of atorvastatin on IP-mediated desensitization of TP signalling

It has previously been established that signalling by the TPα, but not by the TPβ, isoform of the human TXA_2 receptor (TP) is subject to IP-mediated counter-regulation or heterologous desensitization that occurs through a mechanism involving direct cAMP-dependent protein kinase (PK)A phosphorylation of TPα at Ser^{329} within its carboxyl-terminal tail (Walsh *et al*., 2000). Thus, we next investigated the effect of atorvastatin on IP-mediated desensitization of TP receptor responses. Consistent with previous studies (Walsh *et al*., 2000), stimulation of both HEK.TPα and HEK.TPβ cells, each transiently co-transfected with $G\alpha_q$, showed efficient $[Ca^{2+}]_i$ mobilization in response to U46619 (1 μM; Figure 3, panels a and b; $\Delta[Ca^{2+}]_i = 139 \pm 2.2$ nM for TPα and $\Delta[Ca^{2+}]_i = 133.9 \pm 9.12$ nM for TPβ, respectively). While cicaprost (1 μM) did not yield significant rises in $[Ca^{2+}]_i$ mobilization in either cell type, it significantly reduced $[Ca^{2+}]_i$ mobilization by TPα in response to secondary stimulation of HEK.TPα cells with U46619 (Figure 3, panel a; $\Delta[Ca^{2+}]_i = 61.3 \pm 4.4$ nM, $P < 0.0001$). In contrast, U46619-mediated signalling by HEK.TPβ cells was unaffected by cicaprost (Figure 3, panel b; $\Delta[Ca^{2+}]_i = 136 \pm 14.6$ nM, $P = 0.9010$). Pre-incubation of HEK.TPα cells with atorvastatin (10 μM) had no significant effect on U46619-induced $[Ca^{2+}]_i$ mobilization *per se* (Figure 3; compare values for U46619-mediated $[Ca^{2+}]_i$ mobilization,

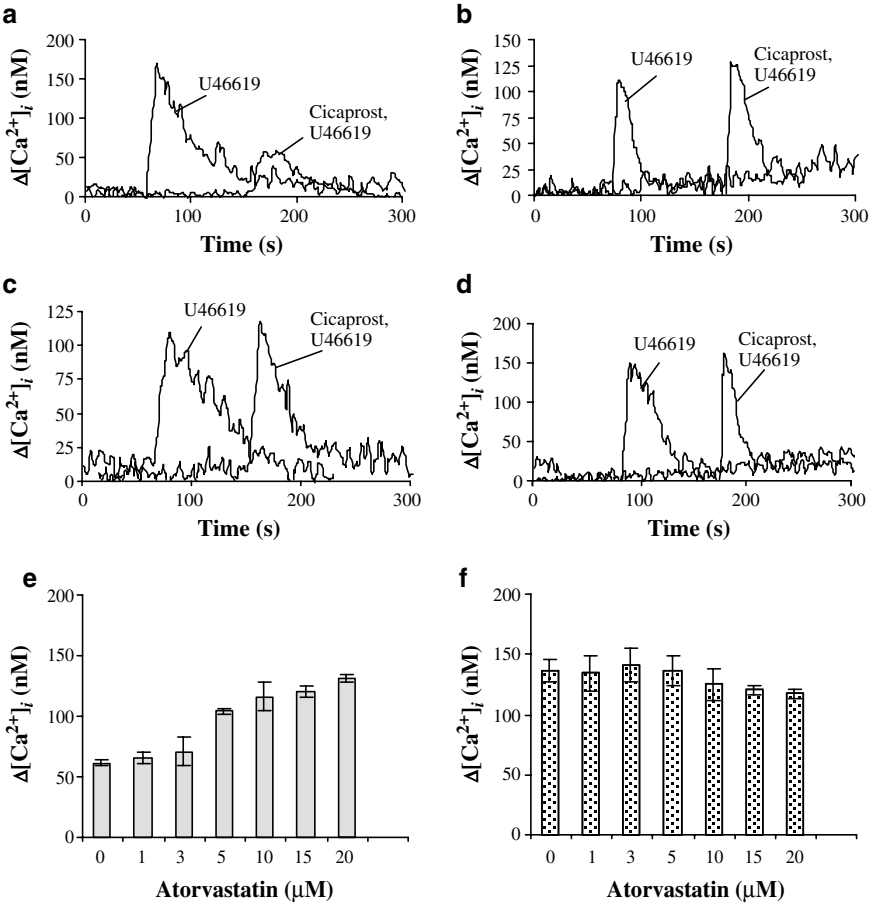

Figure 3 Effect of atorvastatin on IP-mediated desensitization of TP-mediated signalling. HEK.TPα cells (panels a, c and e) and HEK.TPβ cells (panels b, d and f), transiently transfected with pCMV:$G\alpha_q$, were pre-incubated with either the vehicle 0.001% methanol (panels a and b), with 10 μM atorvastatin (panels c and d) or with 0–20 μM atorvastatin (panels e and f) for 16 h prior to harvesting. Cells were preloaded with Fura2/AM and were stimulated with 1 μM U46619 at 50 s (U46619) or with 1 μM cicaprost at 50 s followed by 1 μM U46619 at 150–200 s, approximately (Cicaprost, U46619). Data presented in panels a–d are each representative of four independent experiments and are plotted as changes in $[Ca^{2+}]_i$ mobilization ($\Delta[Ca^{2+}]_i \pm$s.e.m., nM, $n = 4$) as a function of time (s). In panels e and f, data are plotted as mean changes in $[Ca^{2+}]_i$ mobilization ($\Delta[Ca^{2+}]_i \pm$s.e.m, nM, $n = 4$) as a function of atorvastatin concentration (μM).

$\Delta[Ca^{2+}]_i = 139 \pm 2.2$ nM (panel a) *versus* $\Delta[Ca^{2+}]_i = 129 \pm 3.6$ nM (panel c) in the absence and presence of atorvastatin, respectively, $P = 0.0768$). However, atorvastatin (10 μM) significantly impaired the level of cicaprost-induced desensitization of TPα signalling relative to vehicle-treated cells (Figure 3, panel c; $\Delta[Ca^{2+}]_i = 116 \pm 4.9$ nM, $P = 0.0011$), restoring U46619-mediated $[Ca^{2+}]_i$ mobilization to 84.2 ± 4.26% of that originally observed in the absence of cicaprost (Figure 3, panels a and c). From concentration–response studies, an IC_{50} value for atorvastatin inhibition of cicaprost desensitization of TPα signalling was found to be 10.4 μM (Figure 3, panel e). Consistent with previous reports, U46619-mediated signalling by HEK.TPβ cells was unaffected by cicaprost irrespective of pre-incubation of cells with or without atorvastatin (Figure 3, panels b, d and f; $P > 0.5$).

Donor volunteer characteristics

Thereafter, the *in vivo* effects of statins on IP function in platelets isolated from human volunteers undergoing therapeutic intervention with atorvastatin were investigated. In all, 32 randomly selected healthy volunteers participated in this study (Table 1). Recruitment was designed to include three groups of volunteers: Group A: volunteers currently on atorvastatin therapy (10–80 mg daily) to lower their blood cholesterol; Group B: as controls, age-matched sero-cholesterol volunteers who are not currently on statin therapy to lower their blood cholesterol; Group C: as a second control group, age-matched normo-cholesterol volunteers. A brief medical history was obtained from all subjects: four of the subjects were receiving ACE inhibitors and angiotensin II antagonists for hypertension and one was receiving a fibrate for coronary artery disease. All other subjects were not on any other continuous medication apart from those in Group A receiving atorvastatin. Initially, fasting lipid and lipoprotein levels were quantified using a Cholestech L·D·X® Lipid profile-II analyzer to monitor the specific cholesterol and triglyceride profiles of all volunteers (Table 1).

Effect of atorvastatin on radioligand binding to the IP in human platelets

To monitor the effects of atorvastatin therapy on IP endogenously expressed in platelets, radioligand-binding assays using the specific [^{3}H]iloprost were carried out on crude platelet membrane (P_{100}) fractions. Consistent with previous whole cell data, atorvastatin had no significant effect on [^{3}H]iloprost binding by IP endogenously expressed in platelets isolated from Group A ($P = 0.3887$) compared to those isolated from control Groups B ($P = 0.3793$) and C ($P = 0.2577$) (Table 2). Similarly, [^{3}H]SQ29548 binding by the nonisoprenylated control TP receptor in Group A was unaffected by atorvastatin therapy compared to that observed in platelets isolated from control Groups B ($P = 0.1324$) and C ($P = 0.2581$) (Table 2).

Table 1 Lipid and cholesterol profiles

	Group A	*Group B*	*Group C*
No. of donors	10	10	12
Sex (M/F)	6/4	7/3	7/5
Age (years)	54.2 ± 2.9	55.3 ± 4.6	48.3 ± 7.6
Total (mmol l^{-1})	5.3 ± 0.39	6.7 ± 0.59	4.9 ± 0.12
HDL (mmol l^{-1})	1.4 ± 0.12	1.3 ± 0.07	1.8 ± 0.11
LDL (mmol l^{-1})	2.7 ± 0.33	4.3 ± 0.54	1.5 ± 0.76
Triglycerides (mmol l^{-1})	3.4 ± 0.45	2.4 ± 0.32	1.9 ± 0.06

Recruitment of volunteers into the study was designed to include three groups: Group A, volunteers currently on atorvastatin (10–80 mg daily); Group B, sero-cholesterol volunteers and Group C, normo-cholesterol volunteers. Fasting lipid and lipoprotein levels of all volunteers were determined using a Cholestech L·D·X® Lipid profile-II Analyzer. Data presented are the absolute numbers or mean ± s.e.m. HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Effect of atorvastatin on IP- and DP-mediated coupling to adenylyl cyclase in human platelets

Consistent with previous data, stimulation of IP endogenously expressed in platelets from control Groups B and C produced 9.1 ± 1.3- and 8.3 ± 1.0-fold increases in cAMP, respectively (Figure 4, panel a). In contrast to our previous data in HEK 293 and HEL cells (Figure 1), cicaprost-induced cAMP generation was not significantly affected in platelets isolated from Group A (8.6 ± 0.38 cAMP fold increase) compared to those isolated from Groups B ($P = 0.6968$) and C ($P = 0.7635$) (Figure 4, panel a). Similar to that of the IP, the primary intracellular pathway used by the nonisoprenylated prostaglandin D_2 receptor (DP) is stimulation of adenylyl cyclase with concomitant rises in cAMP. While stimulation of platelets from the control Groups B and C with the DP agonist BW245C (1 μM) yielded substantial increases in cAMP generation (6.7 ± 0.94- and 7.4 ± 1.27-fold increases in cAMP, respectively), atorvastatin therapy had no significant effect on cAMP generation by the nonisoprenylated DP in platelets from Group A (7.7 ± 0.44 cAMP fold increase; $P = 0.3339$ comparing Groups A and B and $P = 0.8486$ comparing Groups A and C, respectively; Figure 4, panel b).

Effect of atorvastatin on IP and DP desensitization of TP-mediated aggregation in platelets

We extended our studies to investigate the ability of atorvastatin therapy to interfere with IP-mediated inhibition

Table 2 Prostacyclin receptor (IP) and thromboxane A_2 receptor (TP) expression in platelets

Group type	*[^{3}H]iloprost bound*[a] (fmol mg^{-1} protein)	*[^{3}H] SQ29 548 bound*[b] (fmol mg^{-1} protein)
Group A	356 ± 33.8	386 ± 35.5
Group B	458 ± 108	510 ± 70.2
Group C	398 ± 12.2	476 ± 68.4

[a]To monitor IP expression levels, radioligand-binding assays were carried out on P_{100} crude platelet membrane fractions from statin donor platelets (Group A), sero-cholesterol donor platelets (Group B) and normo-cholesterol donor platelets (Group C) using the specific IP radioligand [^{3}H]iloprost (4 nM) for 1 h at 30°C.
[b]TP radioligand assays were out on whole platelets from statin donor platelets (Group A), sero-cholesterol donor platelets (Group B) and normo-cholesterol donor platelets (Group C) in the presence of the specific TP agonist [^{3}H]SQ29 548 (20 nM) for 30 min at 30°C. Data are presented as the mean ± s.e.m. ($n = 10$ for Groups A and B and $n = 12$ for Group C).

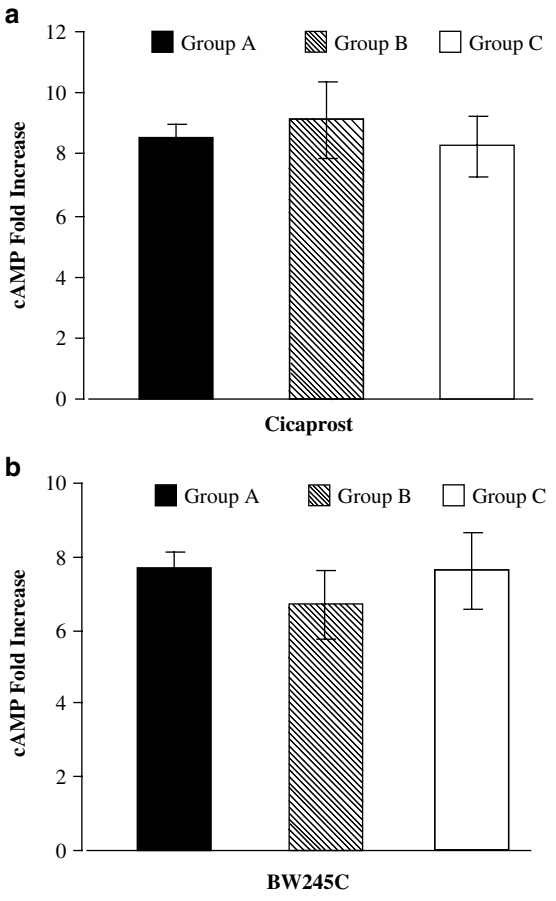

Figure 4 The effect of statin therapy on IP- and DP-mediated cAMP generation and inhibition of TP-mediated aggregation in human platelets. Statin donor platelets (Group A), sero-cholesterol donor platelets (Group B) and normo-cholesterol donor platelets (Group C) were stimulated at 37°C for 10 min with 1 μM cicaprost (panel a) or 1 μM BW245C (panel b). In each case, basal cAMP levels were determined by exposing the cells to the vehicle HBS under identical incubation conditions. Levels of cAMP produced in ligand-stimulated cells relative to basal cAMP levels, in vehicle-treated cells, were expressed as fold stimulation of basal (fold increase in cAMP $\pm$ s.e.m., $n = 10$ for Groups A and B and $n = 12$ for Group C). Basal levels of cAMP in platelets were 1.23 ± 0.56 nmol mg^{-1} cell protein.

of TP-induced platelet aggregation from all the three volunteer groups recruited in the clinical study. Platelets exhibited efficient aggregation in response to 1 μM U46619 (Figure 5, panels a, d, g, j and k; 76 ± 8.6, 80.5 ± 4.7 and 77.7 ± 3.0% for Groups A, B and C, respectively). While cicaprost and BW245C at 1 μM did not induce platelet aggregation *per se*, they completely blocked aggregation in response to secondary stimulation of cells with U46619 (Figure 5, panels b, c, e, f, h, i, j and k). Thus, in accordance with the platelet cAMP data, atorvastatin therapy had no significant effect on IP-mediated inhibition of platelet aggregation compared to aggregations from platelets isolated from Groups B ($P = 0.6519$) and C ($P = 0.8552$) (Figure 5, panels b, e, h and j). In all cases, DP-mediated inhibition of platelet aggregation from platelets isolated from Group A was not significantly different from control donor platelets (Group B, $P = 0.5563$; Group C, $P = 0.3104$) (Figure 5, panels c, f, i and k).

Discussion

Statins, competitive inhibitors of hydroxymethylglutaryl coenzyme A (HMG CoA) reductase, are widely used in the treatment of hypercholesterolaemia (Farnier & Davignon, 1998). However, concerns have been raised about their potential to elicit adverse effects outside their cholesterol-lowering roles (Newman & Hulley, 1996; Guallar & Goodman, 2001; Lawler *et al*., 2001). In 1998, for example, over 175,000 prescriptions for statin medications were issued under the Irish General Medical Services (GMS) scheme (Heerey *et al*., 2000). These drugs, accounting for over 96% of all lipid-lowering agents prescribed, were generally well tolerated, but adverse effects were noted in approximately 3% of these patients (Heerey *et al*., 2000). Furthermore, in a recent study, the statins cerivastatin and lovastatin were shown to impair mIP and hIP coupling to G_s- and G_q-mediated effector signalling in whole-cell studies, most likely by inhibiting the endogenous production of mevalonate and thus potentially interfering with downstream isoprenylation (Lawler *et al*., 2001).

Atorvastatin is the most widely indicated statin in the therapeutic intervention of hyperlipidaemia. Clinically, atorvastatin is prescribed at 10–80 mg daily (Bulletin, 2001). While not as potent as cerivastatin for example, the half-lives of atorvastatin are 14 and 24 h for its active metabolites, ortho- and parahydroxylated derivatives, which account for approximately 70% of its circulating HMG-CoA reductase activity (Cilla *et al*., 1996; Christians *et al*., 1998; Stern *et al*., 2000; Williams & Feely, 2002). Given its long elimination half-life and the presence of detectable plasma levels of the active metabolites, atorvastatin can accumulate in the plasma, achieving a steady-state drug concentration after multiple doses (Cilla *et al*., 1996; Christians *et al*., 1998; Stern *et al*., 2000; Williams & Feely, 2002). Indeed, maximum plasma concentrations of 0.2 μM (252 μg l^{-1}) and the 24 h area under the curve (AUC_{0-24}) of 1.1 μM (1293 μg l^{-1}) were achieved *in vivo* in humans for atorvastatin following a daily dosing of 80 mg for 14 days (Cilla *et al*., 1996).

Given that atorvastatin is among the most widely prescribed lipid-lowering agents and that it can achieve a relatively high systemic concentration *in vivo* in humans, herein we chose to examine the effects of atorvastatin on IP signalling. Hence, the effect of atorvastatin on mIP and hIP coupling to G_s- and G_q-mediated effector signalling was initially examined in HEK.hIP cells, HEK.mIP cells and in HEL cells as an essential prelude to clinical studies carried out on human platelets. Pre-incubation of HEK.hIP, HEK.mIP and HEL cells with atorvastatin yielded concentration-dependent reductions in cAMP ($IC_{50} = 6.6$, 8.5 and 11.1 μM atorvastatin, respectively) and $[Ca^{2+}]_i$ mobilization ($IC_{50} = 16.4$, 7.2 and 8.7 μM atorvastatin, respectively). On the other hand, atorvastatin had no effect on signalling by the nonisoprenylated β_2AR or by the TPβ isoform of the TXA_2 receptor, confirming that the effect of atorvastatin is selectively inhibiting IP-mediated signalling and is not targeted to the inhibition of some other nonspecific component of the signalling system. Moreover, pretreatment of cells had no significant effect on radioligand binding by the IP or by the nonisoprenylated β_2AR or by the TPα or TPβ isoforms of the TXA_2 receptor. These data exclude the possibility that the effect of atorvastatin on IP signalling may be due to an overall reduction in IP expression levels.

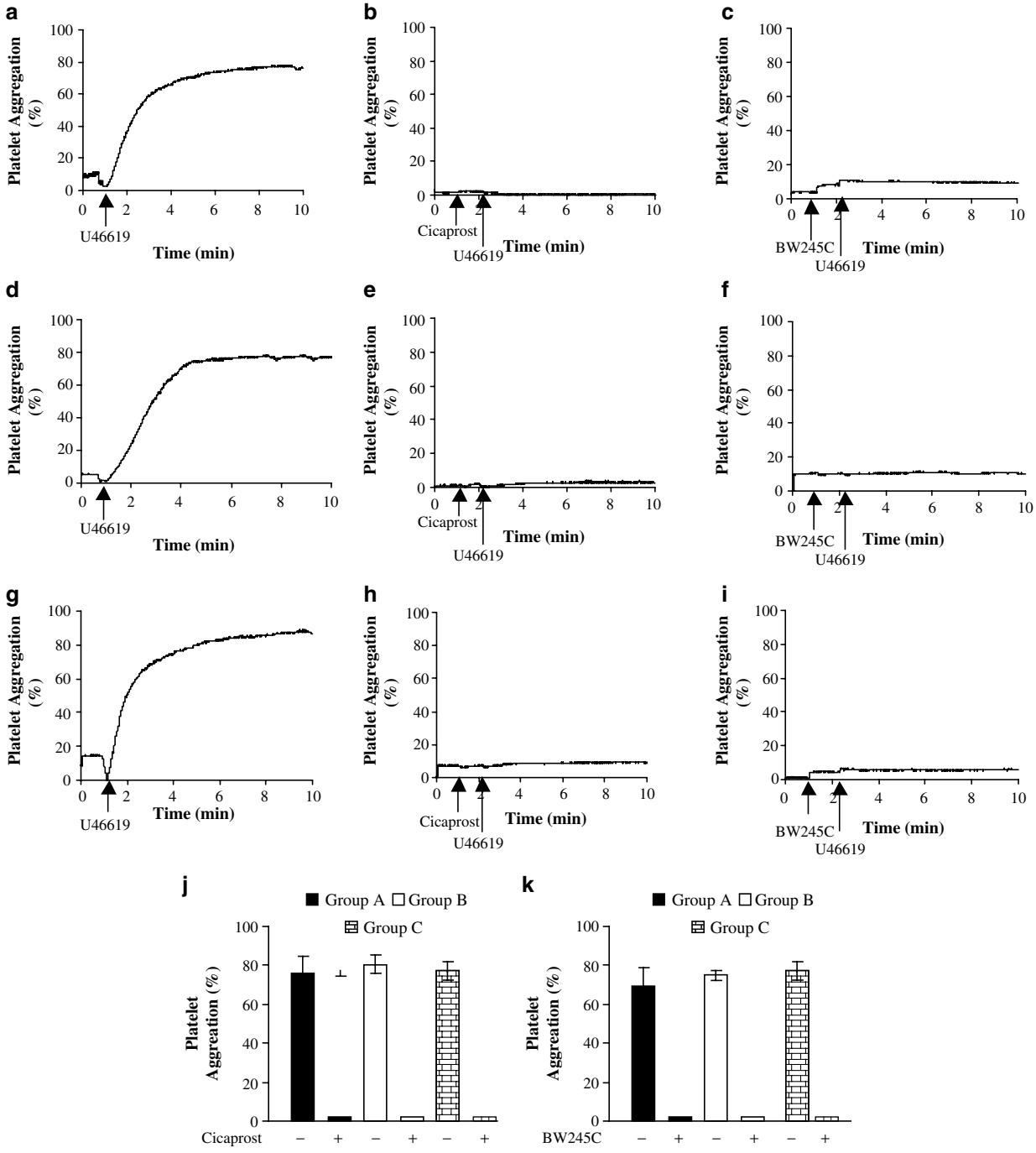

Figure 5 Effect of cicaprost and BW245C on U46619-mediated aggregation in human platelets. Statin donor platelets (Group A; panels a–c), sero-cholesterol donor platelets (Group B; panels d–f) and normo-cholesterol donor platelets (Group C; panels g–i) were stimulated with 1 μM U46619 (panels a, d, g), 1 μM cicaprost followed by 1 μM U46619 (panels b, e, h) or 1 μM BW245C followed 1 μM U46619 (panels c, f, i). Ligands were added at the times indicated by the *arrows* and platelet aggregations were monitored using a Biodata Pap 4 aggregometer. Data presented in panels a–i are plotted as percentage aggregation (%) as a function of time ($n=10$ for Groups A and B and $n=12$ for Group C). In panels j and k, platelets were stimulated with 1 μM U46619 alone (−, panels j and k), 1 μM cicaprost followed by 1 μM U46619 (+, panel j) or 1 μM BW245C followed by 1 μM U46619 (+, panel k) and data are plotted as mean percentage changes in platelet aggregation (% Platelet aggregation ± s.e.m.; $n=10$ for Groups A and B and $n=12$ for Group C).

Intramolecular crosstalk is widely known to occur between various signalling systems, and provides a mechanism to crossdesensitize or counter-regulate one signalling system by another, sometimes opposing, system. For example, such crossdesensitization has been widely documented to occur between the antiaggregatory adenylyl cyclase/cAMP-dependent PKA system modulated by prostacyclin and the proaggregatory PLCβ/PKC system modulated by TXA_2 in

platelets and vascular smooth muscle (Coleman *et al*., 1994; Cheng *et al*., 2002). Given the central role of the counter-regulation between IP : TP signalling within the vasculature, coupled to the finding that atorvastatin inhibits IP function, impairing IP-mediated activation of $G\alpha_s$/adenylyl cyclase and $G\alpha_q$/PLCβ effector systems, we sought to examine the effect of atorvastatin on IP-mediated desensitization of TP receptor responses. Both TPα and TPβ exhibited efficient U46619-induced $[Ca^{2+}]_i$ mobilization and, consistent with previous studies (Walsh *et al*., 2000), cicaprost reduced $[Ca^{2+}]_i$ mobilization by the TPα, but not by the TPβ, isoform of the TXA_2 receptor (TP). Pretreatment of HEK.TPα cells with atorvastatin significantly reduced the level of cicaprost-induced crossdesensitization of TPα signalling compared to cells not treated with atorvastatin, but had no effect on U46619-mediated signalling in HEK.TPβ cells. Hence, consistent with previous reports (Hayes *et al*., 1999; Lawler *et al*., 2001; Miggin *et al*., 2002), these data further confirm the absolute requirement for isoprenylation by the IP and indicate that impairment of isoprenylation by statin inhibition may have consequences on other signalling systems that are either directly or indirectly linked to IP signalling.

The central role of prostacyclin within the vasculature, coupled to the functional requirement for isoprenylation for IP signalling and the fact that atorvastatin significantly impairs IP signalling in whole cells, suggests that the clinical use of atorvastatin has the potential to interfere with IP function *in vivo*. Thus, to extend the studies carried out in whole cells, we investigated the *in vivo* effects of atorvastatin on IP function in human subjects undergoing therapeutic intervention with atorvastatin. In all, 32 volunteers were recruited; 10 volunteers currently on atorvastatin; 10 age-matched sero-cholesterol volunteers not on statin therapy and, finally, 12 age-matched normo-cholesterol volunteers. Five of the subjects were using other continuous medication, but only three were co-prescribed with atorvastatin. Of these three drugs, two ACE inhibitors and one angiotensin II antagonist, none had any previous reports of adverse events occurring with concomitant use of statins.

Initially, to establish whether atorvastatin therapy had any effect on IP expression levels, radioligand-binding assays were carried out on crude membrane fractions from platelets isolated from all three groups. Consistent with data obtained in whole cells, atorvastatin therapy had no significant effect on radioligand binding by the IP or by the nonisoprenylated TP, confirming that any potential effects of atorvastatin on IP signalling were not due to decreases in the overall levels of IP (data not shown). Thereafter, the effect of atorvastatin therapy on IP- and DP-mediated cAMP generation was investigated in platelets isolated from the three groups (groups A, B and C) recruited for the clinical study. In contrast to the data obtained in HEK 293 clonal cell lines and in HEL cells, IP-mediated cAMP generation in platelets isolated from Group A (those on atorvastatin) was not significantly different from that generated from platelets isolated from Groups B (sero-cholesterol volunteers) and C (normo-cholesterol volunteers).

Under normal situations, prostacyclin and PGD_2 act by inhibiting platelet aggregation in response to platelet agonists such as TXA_2, thrombin and collagen (Armstrong, 1996). This counter-regulation of responses was demonstrated in control platelets isolated from Groups B (sero-cholesterol volunteers) and C (normo-cholesterol volunteers) recruited in the clinical study, whereby IP- and DP-mediated inhibition of TP-mediated platelet aggregation was measured as a function of time. To investigate the potential effect of atorvastatin therapy on IP-mediated inhibition of platelet aggregation, the ability of cicaprost to inhibit U46619-mediated aggregation was examined in platelets isolated from Group A subjects. Platelets isolated from Group A (those on atorvastatin) exhibited efficient aggregation in response to U46619 and, like that of the control Groups B and C, this aggregation was completely abolished upon prior stimulation with cicaprost. Thus, in accordance with the *in vivo* clinical signalling data observed, atorvastatin therapy had no significant effect on IP-mediated inhibition of platelet aggregation. In all cases, DP-mediated inhibition of platelet aggregation from Group A was not significantly different from control donor platelets Groups B and C.

Although the statins have been proven relatively safe, the recent withdrawal of cerivastatin owing to 52 cases of fatal rhabdomyolysis, 12 of which involved concomitant use of gemfibrozil, has cast doubt on the world's most widely prescribed lipid-lowering agents (Furberg & Pitt, 2001; Lucas *et al*., 2002). Thus, there has been an explosion of studies describing the pleiotropic effects of the statins, and these studies are the subject of intense and justified interest. In conclusion to this study, while data generated in whole cells indicated that atorvastatin significantly impairs signalling (cAMP generation and $[Ca^{2+}]_i$ mobilization) by both the human and mouse IP, the *in vivo* clinical data indicated that, at the administered therapeutic dose, atorvastatin does not significantly interfere with IP-mediated cAMP generation, IP-regulated platelet aggregation responses or crossdesensitization of signalling between the IP and TP receptors in platelets isolated from human volunteers undergoing therapeutic intervention with the drug. It is noteworthy that the IC_{50} values for IP-mediated cAMP generation (IC_{50} 6.6–11.1 μM) and $[Ca^{2+}]_i$ mobilization (IC_{50} 7.2–16.4 μM) generated in whole cells are substantially lower than the maximum plasma concentrations of 0.2 μM atorvastatin (252 $\mu g\,l^{-1}$) and the 24 h area under the curve (AUC_{0-24}) of 1.1 μM atorvastatin (1293 $\mu g\,l^{-1}$) documented in the literature (Cilla *et al*., 1996), and may provide an explanation to the discrepancy seen between data generated in whole cells and to that generated with the *in vivo* clinical data. Whether these data transcend to other statin family members currently on the market, or to that of cerivastatin, requires further basic and clinical research.

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