Antiplatelet Actions of Statins and Fibrates Are Mediated by PPARs


Objectives—Statins and fibrates are hypolipidemic drugs which decrease cardiac events in individuals without raised levels of cholesterol. These drugs inhibit platelet function, but the mechanisms by which this pleiotropic effect is exerted are not known.

Methods and Results—We used a range of approaches to show statins inhibit human platelet activation in vitro while engaging PPARα and PPARγ. The effects of simvastatin were prevented by the PPARγ antagonist GW9662 or the PPARα antagonist GW6471. In a small-scale human study fluvastatin activated PPARα and PPARγ in platelets and reduced aggregation in response to arachidonic acid ex vivo. The effects of fenofibrate were prevented by PPARα antagonism with GW6471. Fenofibrate increased bleeding time in wild-type, but not in PPARα−/− mice. The inhibitory effect of fenofibrate, but not simvastatin, on aggregation was prevented by deletion of PPARα in murine platelets. PCKα, which influences platelet activation, associated and immune-precipitated with PPARγ in platelets stimulated with statins and with PPARα in platelets stimulated with fenofibrate.

Conclusions—This study is the first to provide a unifying explanation of how fibrates and statins reduce thrombotic and cardiovascular risk. Our findings that PPARs associate with PKCα in platelets also provide a mechanism by which these effects are mediated. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: platelets ■ statin ■ fibrate ■ PPARα, PPARγ
study we show that PPARs in platelets also interact and immune-precipitate with PKC\(\alpha\). Our findings that PPARs associate with PKC\(\alpha\) in platelets provide a mechanism through which statins and fibrates may produce antithrombotic effects.

Methods and Materials

Measurement of Aggregation in Whole Blood

Blood was collected into sodium citrate (3.2\% 1:9 v/v) and responses measured by changes in electric impedance using a Chrono-log 560 electric impedance aggregometer (Chrono-log Corporation, USA). Aliquots of 900 \(\mu\)L of blood (diluted 1:1 with saline) were placed in the aggregometer, at 37°C, with a stir bar revolution rate of 800 rpm. Aggregation was initiated after 5 minutes incubation with drugs by addition of ADP (10 \(\mu\)mol/L). Maximal responses after 15 minutes were recorded. All drugs were made up in DMSO (0.1\%), and vehicle controls were performed in all experiments.

Measurement of Platelet Aggregation in Platelet Rich Plasma

Platelet rich plasma (PRP) was prepared and aggregation measured as described previously. Briefly, 100 \(\mu\)L PRP was added to wells of 96-well plates with fluvastatin or vehicle control for 10 minutes at 37°C, followed by addition of either arachidonic acid or ADP.

Measurement of Platelet Adhesion

Platelet adhesion was measured by a modification of the methodology of Bellavite et al. PRP was diluted (1:4) with buffer containing 145 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L HEPES, 500 \(\mu\)mol/L NaHPO\(_4\), 5 mmol/L MgCl\(_2\), and 6 mmol/L glucose. 50 \(\mu\)L of diluted PRP was then incubated with drugs or vehicle for 1 hour at room temperature. Wells were washed with saline, followed by addition of substrate solution containing 1 mg/mL \(p\)-nitrophenyl phosphate dissolved in 0.1 mol/L sodium citrate, 0.1 mol/L citric acid, and 0.1% Triton X-100 (pH 5.4). Wells were incubated for 40 minutes on a shaker. The reaction was stopped by 2 mol/L NaOH and absorbance determined at a wavelength of 405 nm.

Measurement of Calcium in Platelets

Intracellular platelet calcium release was measured and calculated as previously described. Briefly, FLURA-2 loaded platelets were incubated with drugs for 5 minutes and stimulated with 10 \(\mu\)mol/L ADP.

PPAR Activation Assay

PPAR activation in platelets was measured as described previously. Briefly, platelets were isolated as for the calcium measurements, incubated with drugs for 5 minutes at 37°C, and lysed in buffer containing 10 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L MgCl\(_2\), 10 mmol/L KCl, 0.5 mmol/L DTT, 0.5\% Nonidet P-40, and 0.2 mmol/L PMSE. 20 \(\mu\)g of cell extract was used per well of a BD Transfector kit, and binding was determined as per manufacturer’s protocol.

Measurement of cAMP in Platelets

PRP was preincubated with IBMX (0.5 mmol/L) for 2 minutes to inhibit phosphodiesterase activity. Drugs were added and incubated for 5 minutes before the reaction was stopped by addition of lysis reagent, followed by rapid freezing using liquid N\(_2\). cAMP levels were measured using a commercial ELISA according to manufacturer’s instructions.

Western Blotting

PRP was incubated with drugs for 0.5 to 30 minutes, and samples were lysed and run as described previously. Phosphor-(Ser) PKC substrate antibody in 5\%w/v BSA and 0.1\% Tween-20 was incubated overnight at 4°C followed by peroxidase-conjugated secondary antibody.

Immunoprecipitations

PRP was incubated with drugs for 10 minutes at 37°C. Control samples (labeled WB:PKC\(\alpha\)) were prepared as for Western blotting. Immunoprecipitation samples (labeled IP:PPAR) were incubated with Protein A/G beads and normal rabbit IgG for 1 hour on a rotator at 4°C. Primary antibody (1 \(\mu\)g) was added with Protein A/G beads and incubated overnight on a rotator at 4°C. Samples were centrifuged and resuspended in PBS. Western blots were performed for PKC\(\alpha\).

Measurement of Bleeding Time In Vivo

Male PPAR\(\alpha\)\(^{-/-}\) (on a SVEV129 background) or SVEV129 wild-type control mice (22 to 22g) were anesthetized with ketamine and xylazine (i.p.), and blood was collected by cardiac puncture into heparin. 25 \(\mu\)L PRP was placed in wells of a half area 96-well plate and aggregation measured in the same way as for human PRP.

Murine Platelet Aggregation

PRP from mice was isolated as reported previously. Briefly, male PPAR\(\alpha\)\(^{-/-}\) and SVEV129 wild-type control mice (18 to 22g) were anesthetized with urethane (i.p.), and blood was collected by cardiac puncture to heparin. 25 \(\mu\)L PRP was placed in wells of a half area 96-well plate and aggregation measured in the same way as for human PRP.

Clinical Study Design

Male white volunteers aged 18 to 40 years old and with a BMI value of 18 to 30 were given fluvastatin (LescolXL, Novartis) 80 mg O.D. for 7 days. Blood samples were taken at baseline and on day 7 of drug. Platelet aggregation and PPAR activation were measured as previously described above. The study was performed in accordance with St. Thomas’s Hospital Research Ethics Committee (07/Q0702/24).

Reagents

PPAR\(\alpha\)\(^{-/-}\) mice were purchased from the John Radcliff Hospital (Oxford, UK). ADP and collagen were from Chrono-log and arachidonic acid from Axxora. Statins (active sodium salt forms) were from Calbiochem, fenofibric acid was from ABCR, and fenofibrate was from LKT Laboratories. GW9662 was from Tocris. PPAR transfactor kit was from BD Biosciences. cAMP kit was from Amerham Biosciences. Protein A/G beads, normal rabbit IgG, PKC\(\alpha\) antibody, secondary antibody, and ECL reagent were from Santa Cruz. Rabbit anti-PPAR-\(\alpha\), \(\beta\), \(\gamma\) antibodies were from Abcam. Phospho-(Ser) PKC substrate antibody was from New England Biolabs. All other reagents were from Sigma.

Statistics

Statistical analysis was performed as described for each figure using a computerized software package (GraphPad Prism version 4.0). All values are expressed as mean±SEM. A value of \(P<0.05\) was considered significant by statistical test as appropriate.

Results

Effects of Statins and Fibronefrate on Platelet Activity

Pravastatin, fluvastatin, and simvastatin all inhibited aggregation of human whole blood induced by ADP (log IC\(_{50}\) -4.53±0.45 mol/L, -4.19±0.52 mol/L, and -4.13±0.93 mol/L respectively; Figure 1A) but had no effects in the absence of agonist. For further in vitro experiments simvastatin was used as a representative of the class. Similarly to the statins, fibronefrate inhibited platelet aggregation in whole
Role of PPAR Activation in the Inhibitory Effects of Simvastatin and Fenofibrate on Platelets In Vitro and In Vivo

Simvastatin activated PPARα and PPARγ (Figure 2A) but not PPARβ (data not shown) in human platelets. Similarly pravastatin and fluvastatin activated PPARα and PPARγ in platelets (PPARα: 0.097±0.012, 0.138±0.011, and 0.138±0.005 arbitrary densitometry units for control, pravastatin, and fluvastatin, respectively; PPARγ: 0.082±0.01, 0.15±0.011, and 0.167±0.004 arbitrary densitometry units for control, pravastatin, and fluvastatin, respectively). Fenofibrate only activated PPARα, and not PPARγ (Figure 2A) or PPARβ (data not shown). Selective antagonists of PPARγ, GW9662 (10 μmol/L), or PPARα, GW6471 (10 μmol/L), which alone had no effect on platelet activation (platelet aggregation/adhesion; data not shown or platelet cAMP release; Figure 2D), reversed the inhibitory effects of simvastatin on platelet adhesion (Figure 2B), whereas GW6471 reversed the inhibitory effects of fenofibrate (Figure 2C). The increase in cAMP induced by simvastatin was inhibited by GW9662, but not GW6471 (control 26.8±2 pmol/mL versus 17±1.1 pmol/mL and 21.9±1.7 pmol/mL for GW6692 and GW6471, respectively; Figure 2D), whereas the increase in cAMP induced by fenofibrate was only inhibited by GW6471 (control 23.8±1.2 pmol/mL versus GW6471 13.3±1.5 pmol/mL). When simvastatin and fenofibrate were added together, there was a greater increase in cAMP production than when they were added separately, and this release was inhibited by both GW9662 and GW6471 (control 32.7±5 pmol/mL versus 15±2.3 pmol/mL and 19.1±2.4 pmol/mL for GW6692 and GW6471, respectively). In experiments to study the effective concentration ranges of GW9662 and GW6471, at concentrations of more than 30 μmol/L both drugs had direct inhibitory effects on platelet function. Furthermore, when platelets were cotreated with GW9662 and GW6471, platelet function was greatly reduced, precluding the use of these drugs at higher concentrations or in combination (data not shown).

PPARα knock-out mice, but unfortunately not PPARγ knock-out mice, have been generated, and we used these to further characterize platelet responses to fenofibrate. These studies showed a distinct phenotype effect on “basal” bleeding time which was elevated in PPARα−/− compared to wild-type control mice (Figure 3A and 3B). Fenofibrate (10 mg/kg) increased bleeding time in wild-type, but not in PPARα−/− mice. By way of a control, we found that bleeding time was increased in both types of mice by the prostacyclin-mimetic treprostinil sodium (40 μg/kg; Figure 3A and 3B), which inhibits platelet activation independently of PPARα.17 Incubation of fenofibrate or simvastatin at 30 μmol/L with platelets from wild-type mice resulted in an inhibition of collagen-induced aggregation by 53±7% and 66±9%,
respectively (Figure 3C). In platelets from PPARα−/− mice, the amount of inhibition of aggregation was significantly reduced to 23% for fenofibrate, but there was no difference on simvastatin-induced inhibition of aggregation.

In humans, fluvastatin therapy for 1 week resulted in a moderate but detectable and statistically significant activation of PPARs in platelets (Figure 4A). Furthermore, oral fluvastatin therapy resulted in a reduced platelet response to arachidonic acid (1 mmol/L; Figure 4B). As expected, fluvastatin inhibited arachidonic acid (1.6 mmol/L) induced activation of platelets in vitro (Figure 4C). Oral therapy with fluvastatin did not, however, influence platelet responses to ADP (Figure 4D).

**Interactions Between PKCα and PPARs**

As previously reported, human platelets express abundant amounts of PKCα, PPARα, and PPARγ. Other studies have shown that inhibition of PKCα in platelets inhibits activation.19,20 PKCα immunoprecipitated with PPARγ and PPARα in a manner that increased concentration-dependently when platelets were treated in vitro for 10 minutes with simvastatin (PPARγ) or fenofibrate (PPARα; Figure 5A). Similar results were seen with fluvastatin and immunoprecipitation for PPARγ. To measure PKC activation, Western blots were performed with a phosphor-(Ser) PKC substrate antibody which only recognizes classical PKC substrates containing phosphorylated serine residues, hence PKC activity (Figure 5B). Optimal PKC inhibition was observed after 10 minutes incubation of platelets with either simvastatin or fenofibrate. Total PKCα was also measured as a control.

**Discussion**

Statins and fibrates act by separate mechanisms as lipid lowering drugs and so are used therapeutically both independently and in combination.2–4 These drugs markedly reduce the risk of heart attack and stroke, effects which are not fully explained by their influences on cholesterol levels—the so called pleiotropic effects. This study shows for the first time that statins and fibrates at therapeutic concentrations can activate PPARs in platelets to inhibit platelet function; activities which would be associated with a reduction in thrombotic events.

In the current study we found that a range of statins and fibrates, at therapeutically relevant concentrations, inhibited platelet activation in vitro measured by a number of functional indicators. Platelet activity is tightly regulated by intracellular cAMP levels.73 We found that statins and fibrates increase cAMP in platelets, which is likely to be an intermediate signaling event in the responses we report. The
It is important to note that basal bleeding time responses did differ between the drugs, PPARs, and platelet responses. It is noteworthy that bleeding time increased to similar levels (A). By contrast in mice lacking a functional PPARα, increased bleeding time (B). Data are represented as mean±SEM for n=4 to 5 mice for each genotype. In A, *, refers to differences between drugs and vehicle-treated animals. In B, * refers to differences between fenofibrate and treprostinil sodium. In C, * refers to differences between wild-type and PPARα−/−, as determined by t-test.

Figure 3. Effect of fenofibrate on bleeding time and platelet aggregation in wild-type and PPARα−/− mice. In wild-type mice fenofibrate (10 mg/kg; i.v.) or treprostinil sodium (40 μg/kg; i.v.) increased bleeding time to similar levels (A). By contrast in PPARα−/− mice only treprostinil sodium, but not fenofibrate, increased bleeding time (B). Data are represented as mean±SEM for n=4 to 5 mice for each genotype. C, Fenofibrate or simvastatin (both 30 μmol/L) incubated with PRP for 10 minutes and aggregation measured after stimulation with collagen (10 μg/ml). Data are represented as area under the curve for n=4 taken from 20 pooled mice for each genotype. Statistical difference was assumed where P<0.05 and was denoted by *. Data in A and B were compared by 1-way ANOVA followed by Bonferroni test. In A, * refers to differences between drugs and vehicle-treated animals. In B, * refers to differences between fenofibrate and treprostinil sodium. In C, * refers to differences between wild-type and PPARα−/−, as determined by t-test.

Figure 4. Effects of standard dosing with fluvastatin on human platelet reactivity ex vivo. Platelet aggregation in PRP from blood taken at baseline (pre-) and after 7 days of fluvastatin (80 mg/d) treatment (post-). PPAR activation was increased after fluvastatin (Figure 5A), and aggregation induced by AA (1.6 mmol/L) was inhibited after oral dosing in vivo (Figure 5B) and in separate experiments in vitro (Figure 5C). In contrast, aggregation induced by ADP after oral dosing with fluvastatin was not inhibited (Figure 5D). Data are presented as mean±SEM for (A) n=8 donors, (B) n=8 separate donors, (C) n=5 separate donors, (D) n=8 separate donors. Significant differences were calculated using paired t test (A), 2-way ANOVA (B and D), or 1-way ANOVA by Dunnett test (C) and significance assumed where P<0.05 and denoted by *.

Concentrations of the drugs required to inhibit activity was within the range expected after oral clinical dosing. In parallel with inhibition of platelet activation we found that PPARγ or PPARα receptors were activated in the platelet by statins and fibrates. Importantly the inhibitory effects of statins and fibrates on platelet activation in vitro were prevented by selective PPAR antagonists. To further validate our hypothesis we performed experiments with genetically modified mice lacking a functional PPARα receptor. There are no PPARα knock-out mice so we were not able to study those responses. From our human platelet in vitro experiments we concluded that the effects of fibrates were mediated by PPARα, but not PPARγ. This was confirmed by experiments in PPARα−/− mice, which demonstrated an absence of fenofibrate effects on bleeding time and platelet aggregation compared to wild type animals. These observations together with those using highly selective antagonists in human platelets clearly establish a "cause and effect relationship" between the drugs, PPARs, and platelet responses. It is important to note that basal bleeding time responses did differ in wild-type and PPARα knock-out mice, although understanding the nature of phenotypic changes that result in these baseline differences is beyond the scope of this study. Nevertheless we are confident that our conclusions are supported by our observations, as the prostacyclin-mimetic treprostinil sodium, which inhibits platelet activation independently of PPARα, increased bleeding time in both knock-out and wild-type animals.

In addition to our observations on human platelets in vitro we performed a small-scale clinical study to investigate the effects of a standard dose of fluvastatin on PPAR activation in platelets in vivo and on responses of platelets to proaggregatory agents ex vivo. As was found in in vitro protocols, in vivo administration of fluvastatin to healthy human volunteers increased PPARα activation independently of PPARγ, as determined by 1-way ANOVA. PPARα receptor binding in platelets. Although 7 days of fluvastatin therapy did not influence platelet activation induced by ADP, it did significantly prevent activation induced by arachidonic acid. These observations serve to substantiate the notion that statins activate PPARs in platelets in vivo in man and reveal an interesting difference in sensitivity of platelet activation pathways studied either in vitro or in vivo. One possible explanation why aggregation to ADP was not affected may come from a consideration of the roles of thromboxane A2. Simvastatin inhibits the formation of thromboxane A2, which is the predominant mechanism of arachidonic acid-induced aggregation. ADP, however, stimulates platelets primarily through stimulation of P2Y receptors and promotion of granule secretion. We can be confident that our findings regarding the
effects of fluvastatin on responses to arachidonic acid are potentially meaningful clinically because a similar profile would be seen if our volunteers had taken low-dose aspirin for the same period of time; ie, we would have noted a more pronounced effect on aggregation stimulated by arachidonic acid than on that stimulated by ADP.

Clearly then, statins and fibrates activate PPARs in platelets in vitro and in vivo leading to inhibition of activation in response to some agonists. In nucleated cells statins and fibrates activate PPAR receptors, which regulate cell function by actions on gene transcription. However, the effects we report here are rapid and in cells with no nucleus, so cannot be attributed to gene induction. The mechanisms by which PPARs regulate platelet function are not known, and it is beyond the scope of this report to identify fully the pathway. However, we have revealed two important novel aspects of PPAR signaling in platelets that will certainly contribute to the mechanism by which statins and fibrates affect platelet function. PKC pathways are very active in platelets, where they facilitate platelet activation. In nucleated cells PPARs regulate platelet function are not known, and it is beyond the scope of this report to identify fully the pathway. However, we have revealed two important novel aspects of PPAR signaling in platelets that will certainly contribute to the mechanism by which statins and fibrates affect platelet function. PKC pathways are very active in platelets, where they facilitate platelet activation. In nucleated cells PPARγ ligands cause PPARγ to interact with and inhibit PKCα. Notably, in platelets PKCα is an abundant isoform. Here we show that treatment of platelets with fluvastatin or simvastatin causes PPARα to bind to PKCα. Similarly, in accordance with this, we found that in platelets treated with fenofibrate PPARα was bound to PKCα. For comparison we also examined the effects of the active form of fenofibrate, fenofibric acid, and found it to produce identical results to fenofibrate. Thus, even though fenofibrate is a prodrug it is still active in our model of platelet activation. We observed that 10 minutes of incubation was sufficient to cause optimal activation of PKCα in platelets for all the drugs tested. In preliminary observations we also found that platelets incubated with PPARβ selective ligands had PPARβ bound to PKCα (unpublished observations). Others have confirmed that inhibition of PKCα or removal of PKCα from platelets inhibits function—would we therefore hypothesize that statins and fibrates activate PPARs in platelets to inhibit platelet activation via a novel mechanism that involves binding and repression of PKCα.

In addition to repression of platelet PKCα we found that statins and fibrates increased the levels of cAMP. cAMP is a critical inhibitory secondary messenger in platelets, the elevation of which inhibits platelet activity. cAMP can synergize with cGMP to inhibit platelet function. A recent study showed simvastatin inhibited collagen-induced platelet aggregation via activation of nitric oxide synthase and guanylyl cyclase. Nitric oxide inhibitors did not completely reverse the inhibitory effects of simvastatin, and the authors suggest this pathway is not the only pathway involved in simvastatin signaling in platelets. Thus statins may be functioning by increasing both cAMP as well as cGMP pathways to inhibit platelet activation.

In conclusion, we show that the direct inhibitory effects of statins and fibrates on platelet activation are mediated by PPARs and involve an interaction with platelet PKCα. These data are the first to provide a unifying mechanism for the pleiotropic actions of these drugs and could be useful in the development of new and improved therapies.

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Disclosures
None.

References


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