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. PKCα, 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

Statins are widely prescribed cholesterol-lowering drugs that are first-line treatments for the prevention of coronary artery disease and atherosclerosis, reducing the incidence of thrombotic events such as heart attack and stroke.1 Statins inhibit the activity of a key enzyme in cholesterol synthesis within the body, 3-hydroxymethyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and so reduce cholesterol levels.2 Statins are classified as natural (eg, pravastatin), synthetic (eg, fluvastatin), or semisynthetic (eg, simvastatin). Like the statins, the fibrates are widely used lipid-lowering drugs that reduce the incidence of heart attack and stroke, even in individuals with normal levels of circulating cholesterol.1,2,6 However, the mechanisms by which statins or fibrates inhibit platelet function, but the mechanisms by which statins and fibrates inhibit platelets are unclear. It is not known whether they are a consequence of lowering cholesterol or mediated via some other mechanism.1,3 Here we show that statins and fibrates have rapid and direct inhibitory effects on platelet function in vitro and in vivo that cannot be attributed to effects on cholesterol levels.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that are involved in many biological processes, including lipid and energy metabolism,10,11 inflammation responses, and atherosclerotic plaque formation.12,13 Three PPARs are known: α, β (sometimes called δ), and γ. The better studied are PPARα and PPARγ. In cells which have a nucleus, statins are effective ligands for PPARα and PPARγ, whereas fibrates are ligands for PPARα.2,14,15 Recent literature indicates that PPARs are present in human platelets and that their activation inhibits platelet functions through nongenomic mechanisms.16,17 Here we show that inhibitory effects of simvastatin and fenofibrate on platelet function are mediated by PPARs. In nucleated cells PPARγ ligands cause PPARγ to interact and inhibit PKCα.15,18 In platelets, PKCα activation facilitates platelet aggregation.19,20 In the current

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Correspondence to Timothy D. Warner, The William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse Square, London, EC1M 6BQ, UK. E-mail t.d.warner@qmul.ac.uk

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study we show that PPARs in platelets also interact and immune-precipitate with PKCα. Our findings that PPARs associate with PKCα 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 µL 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 µmol/L). Maximal responses after 15 minutes were recorded. All drugs were made up in DMSO (0.1%), and buffer containing 10 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L NaHPO4, 5 mmol/L MgCl2, and 6 mmol/L glucose. 50 µL of diluted PRP was then incubated with drugs 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.22 PRP was diluted (1:4) with buffer containing 145 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L HEPES, 500 µmol/L NaHPO4, 5 mmol/L MgCl2, and 6 mmol/L glucose. 50 µL 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/L 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.17 Briefly, FLURA-2-loaded platelets were incubated with drugs for 5 minutes and stimulated with 10 µmol/L ADP.

PPAR Activation Assay
PPAR activation in platelets was measured as described previously.17 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 MgCl2, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.3% Nonidet P-40, and 0.2 mmol/L PMSF. 20 µg of cell extract was used per well of a BD Transfaktor 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 N2. 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.17 Phosphor-(Ser) PKC substrate antibody in 5% wt/vol 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:PKCα) 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 µg) 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 PKCα.

Measurement of Bleeding Time In Vivo
Male PPARα−/− (on a SVEV129 background) or SVEV129 wild-type control mice (22 to 25g) were injected i.v. with either fenofibrate (10 mg/kg), treprostinil sodium (40 µg/kg), or dimethylsulfoxide (DMSO, vehicle, 0.1 µL/g). After 10 minutes, mice were anesthetized with ketamine and xylazine (i.p.) and placed on a raised platform with their tails protruding over the end. Tails were positioned 5 mm above filter paper, and then a 2-mm cut from the tail tip was made. The bleeding time was recorded from the time bleeding started until it had completely stopped for more than 30 seconds.

Murine Platelet Aggregation
PRP from mice was isolated as previously reported.21 Briefly, female PPARα−/− and SVEV129 wild-type control mice (18 to 22g) were anesthetized with urethane (i.p.), and blood was collected by cardiac puncture in to heparin. 25 µL PRP was placed in to 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
PPARα−/− mice were purchased from the John Radcliffe Hospital (Oxford, UK). ADP and collagen were from Chrono-log and arachidonic acid from Axonora. 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 Amersham Biosciences. Protein A/G beads, normal rabbit IgG, PKCα antibody, secondary antibody, and ECL reagent were from Santa Cruz. Rabbit anti-PPARα-α, -β, -γ 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 Fenofibrate on Platelet Activity
Pravastatin, fluvastatin, and simvastatin all inhibited aggregation of human whole blood induced by ADP (log IC50 = −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, fenofibrate inhibited platelet aggregation in whole
blood (Figure 1A). Simvastatin elevated cAMP production to a similar amount as fenofibrate (Figure 1B) and reduced the increases in intraplatelet calcium that accompanied stimulation with ADP (10 μmol/L; Figure 1C). As patients are often cotreated with fibrates and statins we investigated the potential for interaction on platelet function. In the presence of a threshold concentration of fenofibrate (3 μmol/L) the inhibitory effects of simvastatin on platelet aggregation were significantly increased (Figure 1D).

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 GW9662 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 GW9662 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, PPARγ, and PPARα. 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. Similar results were seen with fluvastatin, as well as the active form of fenofibrate, fenofibric acid (data not shown), indicating that even though fenofibrate is a prodrug it is still capable of producing the same results as the active metabolite.

**Discussion**

Statins and fibrates act by separate mechanisms as lipid lowering drugs and so are used therapeutically both independently and in combination.2–4,7 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.23 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
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 PPARγ 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 and PPARγ 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 for why aggregation to ADP was not affected may come from a consideration of the roles of thromboxane A2. Simvastatin inhibits the formation of thromboxane A2,24 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
Fluvastatin or simvastatin causes PPAR isoform. Here we show that treatment of platelets with inhibitors of PKC in vitro and in vivo leading to inhibition of activation in a period of time; i.e., we would have noted a more pronounced effect on aggregation stimulated by arachidonic acid than on that potentially meaningful clinically because a similar profile would be attributed to gene induction. The mechanisms by which statins and fibrates increase cAMP are critical inhibitory secondary messengers 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 PKCa. 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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