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;29:706-711.)

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 formation.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.3–5 Fibrates reduce triglycerides and increase high-density lipoprotein cholesterol. Importantly, statins and fibrates are preventative against heart attack and stroke, even in individuals with normal levels of circulating cholesterol.1,2,6 However, the mechanisms by which statins or fibrates cause these noncholesterol related, or pleiotropic, protective effects are not completely understood.

Interestingly both statins and fibrates inhibit platelet function,3,7 which is, of course, a widely recognized property of drugs such as aspirin and clopidogrel that are used to reduce the incidence of heart attacks and strokes.6,8 However, 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.1,3,9 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 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: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 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 μL 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**

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**PPAR Activation Assay**

PPAR activation in platelets was measured as described previously. Briefly, platelets were isolated as for the calcium measurements. PRP was incubated with drugs for 0.5 to 30 minutes, and samples were lysed and run as described previously. Phosphor-(Ser) PKC antibody, secondary antibody, and ECL reagent were from Amersham Biosciences. Protein A/G beads, normal rabbit IgG, PKCo antibody, secondary antibody, and ECL reagent were from Santa Cruz. Rabbit anti-PPAR-(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 for simvastatin) 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 μM/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 μM/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 μM/L), or PPARα, GW6471 (10 μM/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±2.2 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±2.5 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 μM/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−/− mice 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 i.v.).

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 by Dunnett 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.

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 activation19,20 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
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 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.²¹,²⁵,²⁶

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 nongenomic 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.15–18; notably, in platelets PKCα is an abundant isoform. Here we show that treatment of platelets with fluvastatin or simvastatin causes PPARγ to bind to PKCα. Similarly, in accordance with this, we found that in platelets treated with fenofibrate PPARα 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 (Ferhana Y. Ali, unpublished observations, 2008). Others have confirmed that inhibition of PKCα or removal of PKCα from platelets inhibits function19,20—we would 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.27,28 A recent study showed simvastatin inhibited collagen-induced platelet aggregation via activation of nitric oxide synthase and guanylyl cyclase.24 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 signalling 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 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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None.

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