PPARβ/δ Agonists Modulate Platelet Function via a Mechanism Involving PPAR Receptors and Specific Association/Repression of PKCα

Ferhana Y. Ali, Matthew G. Hall, Béatrice Desvergne, Timothy D. Warner, Jane A. Mitchell

Objectives— Peroxisome proliferator-activated receptor β/δ (PPARβ/δ) is a nuclear receptor found in platelets. PPARβ/δ agonists acutely inhibit platelet function within a few minutes of addition. As platelets are anucleated, the effects of PPARβ/δ agonists on platelets must be nongenomic. Currently, the particular role of PPARβ/δ receptors and their intracellular signaling pathways in platelets are not known.

Methods and Results— We have used mice lacking PPARβ/δ (PPARβ/δ−/−) to show the effects of the PPARβ/δ agonist GW501516 on platelet adhesion and cAMP levels are mediated specifically by PPARβ/δ, however GW501516 had no PPARβ/δ-specific effect on platelet aggregation. Studies in human platelets showed that PKCα, which can mediate platelet activation, was bound and repressed by PPARβ/δ after platelets were treated with GW501516.

Conclusions— These data provide evidence of a novel mechanism by which PPAR receptors influence platelet activity and thereby thrombotic risk. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: platelets ■ PPARβ/δ ■ PKCα ■ knockout mice

Peroxisome proliferator-activated receptor β/δ (PPARβ/δ) is a nuclear receptor present in platelets. We have previously shown PPARβ/δ ligands inhibit platelet function via a nongenomic pathway which is independent of nuclear events.1 In addition to synthetic ligands, PPARβ/δ can be also be activated by the antithrombotic hormone prostaglandin (PG) I2.1,2 The specific role of PPARβ/δ receptors in the antiplatelet effects of synthetic agonists is not known. Here, we have used platelets from mice lacking PPARβ/δ (PPARβ/δ−/−) to show this receptor mediates in part the antiplatelet effects of GW501516. We have gone on to show in human or mouse platelets that GW501516 activates PPARβ/δ receptors to bind and repress PKCα, thereby providing a novel yet plausible mechanism for how PPAR receptors produce nongenomic responses in platelets.

Methods

All experiments were performed according to ethical guidelines, and a full experimental methods section is available as supplemental material (available online at http://atvb.ahajournals.org).

Immunoprecipitations were performed in human platelet rich plasma (PRP) or human/mouse washed platelets incubated with GW501516. Samples were incubated with antibody against PPARβ/δ and Western blots performed with anti-PKCα antibody. To measure PKC activity a phosphor-(Ser) PKC substrate antibody was used, which measures phosphorylation of PKC target proteins.

Mouse (20 to 25g) PRP was pooled and incubated with GW501516. ADP was added and aggregation measured over 16 minutes using a standard light transmission plate reader at 595 nm. Adhesion was measured using a buffer containing p-nitrophenyl phosphate. cAMP release was measured by ELISA.

Results

To investigate the effect of PPARβ/δ activation on PKCα, human PRP was incubated with GW501516, proteins were immunoprecipitated with PPARβ/δ antibodies, and complexes analyzed by Western blot with primary antibodies to PKCα (Figure 1A). GW501516 significantly increased the association between PPARβ/δ and PKCα (Figure 1B). In addition, this protocol was repeated in human and murine washed platelets and provided similar results (see supplemental materials). In line with these observations, PKCα activity was reduced by GW501516, with maximal inhibition seen after 10 minutes incubation with human PRP (Figure 1C) and murine PRP (see supplemental materials).

Platelet activation assays were performed in platelets from wild-type (WT) and PPARβ/δ−/− mice. cAMP release was stimulated in WT platelets after incubation with GW501516 (1 μmol/L), however this was significantly reduced in PPARβ/δ−/− platelets. The ability of GW501516 (10 μmol/L) to inhibit ADP-induced platelet adhesion was significantly reduced in tissue from PPARβ/δ−/− mice compared to WT animals. By contrast, no difference was seen in the ability of ADP to induce platelet aggregation in tissue from WT versus PPARβ/δ−/− mice (Figure 2).
Discussion

The mechanism by which PPARβ/δ inhibits platelet activation is unknown. However, as platelets are anucleated, inhibitory effects of PPARβ/δ agonists must clearly be independent of the nucleus. In the search for alternative signaling pathways we identified PKCα as a potential target.3,4 In nucleated cells, PPARγ blocks PKCα downstream signaling.5 We show a concentration-dependent binding of PPARβ/δ to PKCα and a time-dependent inhibition of platelet PKCα activity. This suggests that binding and repression of PKCα by PPARβ/δ could account for the nongenomic inhibitor effects of agonists such as GW501516 on platelets.

To establish whether GW501516 effects were mediated specifically by PPARβ/δ, murine PPARβ/δ−/− and WT platelets were incubated with GW501516. GW501516 increased cAMP levels in WT platelets but had little effect in PPARβ/δ−/− platelets. Adenylyl cyclase, which converts ATP to cAMP, is a downstream target of PKCα in nucleated cells.6,7 We found selective inhibitors of PKCα (Gö6976 and Gö6983) caused basal levels of cAMP in mouse platelets to increase significantly (basal cAMP, 106±6 fmol/mL; +Gö6976 (30 nmol/L), 241±9 fmol/mL; +Gö6983 (30 nmol/L), 236±18 fmol/mL). This suggests PKCα activity negatively regulates adenylyl cyclase, which is consistent with our hypothesis. GW501516 also inhibited platelet adhesion partially mediated by PPARβ/δ, as responses were significantly less in platelets from PPARβ/δ−/− compared to WT. PKCα has been shown to positively regulate platelet adhesion.8 This again is consistent with our hypothesis that PPARβ/δ binds PKCα and, via this mechanism, represses its function. In contrast, the inhibitory effects of GW501516 on aggregation were independent of PPARβ/δ receptors. This observation suggests that GW501516 can inhibit platelet functions independently of the target receptor by pathways yet to be established, but clearly independently of gene induction.

It should be noted that interpretation of our data are limited because platelet responses vary greatly with in vitro conditions, and ADP may only activate PKCα weakly. Nevertheless, in summary, we have shown for the first time that PPARβ/δ is a functionally active protein in platelets and that
direct binding and repression of PKCα is a plausible mechanism by which PPARβ/δ regulates platelet reactivity.

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**Disclosures**
None.

**References**
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Supplemental Material

Methods

Measurement of PKCα activity by Western blotting

Human blood was collected by venepuncture into citrated tubes (1:9 v/v) from volunteers who had not taken any drugs known to interfere with platelet activation. Platelet rich plasma (PRP) was prepared by centrifugation of blood at 175 g for 15 minutes. Murine blood was collected by cardiac puncture into heparin. Murine PRP was prepared by centrifugation of blood at 180 g for 6 minutes. The plasma, along with the top third of the red blood cell layer was centrifuged at 100 g for 5 minutes and PRP collected. PRP was then incubated with drugs for 0.5 – 30 minutes at 37°C after which time samples were lysed and then separated by gel electrophoresis as described previously\(^1\). PKCα activity was determined by probing the nitrocellulose membranes with an antibody which specifically recognises phosphorylated PKC substrates. Rabbit phosphor-(Ser) PKC substrate antibody in 5% w/v BSA and 0.1% Tween-20 was incubated with the membranes overnight at 4°C followed by peroxidase-conjugated goat anti-rabbit secondary antibody.

Immunoprecipitations

Human PRP was incubated with GW501516 (1, 3, 10μM) for 10 minutes at 37°C. At this time some samples were removed for control measurements; the amount of protein in each sample was determined and samples were boiled with gel loading buffer. Remaining samples were incubated with Protein A/G beads and normal rabbit IgG for 1
hour on a rotator at 4°C before being centrifuged and resuspended in buffer. PPARβ/δ antibody (1μg) was then added with Protein A/G beads and samples incubated overnight on a rotator at 4°C. Samples were then centrifuged and resuspended in PBS. Western blots were performed with rabbit anti-PKCα antibody followed by peroxidase-conjugated goat anti-rabbit secondary antibody.

Washed human and murine platelets were prepared from PRP as described previously\(^1\)

### Murine platelet aggregation

Mice (20-25g) were anaesthetised with halothane and blood was removed by cardiac puncture into heparinised syringes. PPARβ/δ\(^{-/-}\) mice (from Professor Béatrice Desvergne, University of Lausanne, Switzerland) were backcrossed on a C57BLK6 background. PRP was prepared by centrifugation, as above, with all experiments being performed within 3 hours of blood collection. 25μl of PRP was added to individual wells of half-area 96-well plates which contained either drugs or vehicle controls and incubated at 37°C for 10 minutes according to protocols we have published previously\(^2\). Aggregation was stimulated by the addition of agonist (ADP, 10μM) and plates were immediately placed in a light transmission plate reader at 37°C. Measurements were taken at a wavelength of 595nm every 4 minutes with shaking in between the readings for 16 minutes. Aggregation was determined as the changes in light transmission over the 16 minute period.

### Platelet adhesion


The amount of platelet adhesion was measured in the wells from the platelet aggregation samples using an adapted method from Eriksson et al.\textsuperscript{3} published by our group recently\textsuperscript{2}. After the aggregation was complete, the wells were washed twice with saline and incubated with buffer containing the substrate p-nitrophenyl phosphate for 15 minutes at room temperature. Reaction was stopped with sodium hydroxide and plates were read at 405nm.

\textit{cAMP measurement}

PRP was prepared and 100µl added to the individual wells of 96-well plates. PRP was pre-incubated with IBMX (0.5mM) for 2 minutes to inhibit phosphodiesterase activity. GW501516 was then added and incubated for 5 minutes before the reaction was stopped by addition of lysis reagent, followed by rapid freezing using liquid N\textsubscript{2}. cAMP levels were measured in the lysates using a commercial ELISA according to the manufacturer’s instructions.

\textit{Reagents}

ADP was from Chrono-log (Manchester, UK) and GW501516 was from Axxora (Nottingham, UK). cAMP EIA kit was from Amersham Biosciences (Buckinghamshire, UK). Rabbit anti-PPAR\textgreek{β/δ} antibody was from Abcam (ab8937; Cambridge, UK). Phospho-(Ser) PKC substrate antibody was purchased from Cell Signalling Technology (MA, USA). All other Western blotting reagents were from Santa Cruz (Wiltshire, UK). p-nitrophenyl phosphate and all other reagents were from Sigma (Dorset, UK).
Statistics

Statistical analysis was performed as described for each figure using a computerised software package (GraphPad Prism version 4.0). All values are expressed as mean ± S.E.M, unless otherwise stated. A value of p<0.05 was considered significant by statistical test as described in figure legends.

Results

Figure S1. Immunoprecipitation in human washed platelets. Platelets were incubated with GW501516 and incubated with PPARβ/δ antibody followed by Western blotting for PKCα (homogenate IP). Control samples were not incubated with PPARβ/δ antibody (total homogenate). V, vehicle control; RM, molecular weight marker.

Figure S2. Immunoprecipitation in murine washed platelets. Platelets were incubated with GW501516 and incubated with PPARβ/δ antibody followed by Western blotting for
PKCα (homogenate IP). Control samples were not incubated with PPARβ/δ antibody (total homogenate). V, vehicle control; RM, molecular weight marker.

Figure S3. Measurement of PKC activity in murine PRP. PRP was incubated with GW501516 for 0-30 minutes and samples lysed and probed. Vehicle control (V) was taken at 30 minutes.

References
