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Ligand-specific glucocorticoid receptor activation in human platelets

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Few studies have addressed the effects of classical anti-inflammatory glucocorticoids on platelet function. Here, we report for the first time that human platelets contain the glucocorticoid receptor (GR) as identified by a combination of biochemical and functional techniques. Ligand-binding studies revealed the presence of a high- and low-affinity binding site for [^{3}H]-dexamethasone in platelets. The 2 GR ligands prednisolone and dexamethasone competed for [^{3}H]-dexamethasone binding, as did the mineralocorticoid aldosterone. However, while prednisolone (1-10 μM) reduced adenosine diphosphate (ADP, 4 μM) and thromboxane A_2 receptor agonist U46619 induced platelet aggregation (up to 75%), dexamethasone had no effect. The inhibition produced by prednisolone was reversed by preincubation with the GR antagonist mifepristone (10 μM; RU486), suggesting the functional importance of the ligand-receptor complex. In addition, prednisolone caused a marked (~ 50%) reduction in thromboxane B_2 levels, whereas dexamethasone was without effect. The apparently anomalous binding data were clarified by the fact that washed platelets (1) contained mineralocorticoid receptor and that (2) it was associated with GR. Taken together, our data suggest that platelet GR forms a heterodimeric complex with the mineralocorticoid receptor that is susceptible to differential activation by specific receptor ligands. (Blood. 2005;106:4167-4175)

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Introduction

The glucocorticoid receptor (GR) is a member of the nuclear hormone receptor superfamily that encompasses, among others, estrogen receptor and peroxisome proliferators–activated receptor. Natural and synthetic glucocorticoids bind to GR, producing major conformational changes that result in nuclear translocation or transrepression of transcription factors, including nuclear factor κB.[1,2] In all cases, the majority of the ensuing glucocorticoid actions are mediated by GR and culminate in a modification of the synthesis and expression of pivotal mediators of innate and adaptive immunity.[3]

Despite their extensive use in cardiovascular and immune pathologies, glucocorticoids have been reported to be largely inactive on platelet function. Human studies in healthy volunteers have shown the lack of effect of dexamethasone (Dex) treatment in inhibiting renal excretion of the thromboxane A_2 (TxA_2) stable metabolite, thromboxane B_2 (TxB_2).[4] Similarly, conventional clinical doses of prednisone do not impair platelet function or enhance primary hemostasis in healthy subjects, as measured by the bleeding time.[5] Since changes in de novo protein synthesis are the basis for glucocorticoid effectiveness, the lack of a modulatory effect on platelet functions is not surprising given that these cells have been regarded as incapable of synthetic activity. More recently, though, platelets have been shown to produce and release several mediators by de novo synthesis from preformed mRNA.[6]

It has recently emerged that glucocorticoids can modulate cell functions via nongenomic processes.[7,8] Thus, glucocorticoid interaction with GR can lead to rapid effects such as the direct inhibitory action on nuclear factor κB activation.[9] The ability of the ligand/GR complex to directly affect several distinct signal pathways has been reported in several studies, such that their actions may be considered 2-fold. On one hand, it produces rapid changes in cell behavior, while, on the other, it modulates transcription factor activation with consequent alteration of protein synthesis without the necessity of nuclear localization.[10,11] Nongenomic glucocorticoid effects modulate the function of immune cells and are emerging as important contributors to the overall control on cellular function.[12]

Until recently, platelets were thought to be involved solely in the maintenance of vessel homeostasis and repair, possessing little other activity that could impact the inflammatory process. However, these anuclear cells are comparable with other immune cells as rich sources of inflammatory mediators, recently reviewed in Weyrich and Zimmerman.[13] Thus, platelets can rapidly release high levels of CXC ligand 4 (CXCL4), CXCL5, and CC chemokine ligand 5 (CCL5), and contribute to neutrophil and monocyte recruitment. Similarly, apart from the effect of antiplatelet therapy for thrombotic disorders, platelets—and drugs that affect them—may play a role in other types of vascular inflammation. As an example, recent studies have highlighted the role of platelets in leukocyte adhesion to activated postcapillary venule endothelium[14] as well as in other models of chronic inflammatory pathologies.[15-17]

In the present study, we report the presence of functional GR in human platelets. Using an integrated approach based on a series of functional and biochemical analyses, we provide evidence that platelet GR can interact with synthetic ligands in a fashion that is distinct from that reported for other blood cells.

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Materials and methods

Chemicals

Thromboxane B_2 (TxB_2), prostaglandin B_1 (PGB_1), acetylsalicylic acid (aspirin or ASA), citric acid, and the TxA_2 receptor agonist U46619 were purchased from Sigma-Aldrich (Poole, United Kingdom). 12-R-hydroxyeicosatetraenoic acid (12-R-HETE), arachidonic acid (AA), and 12-S-hydroxyheptadecatrienoic acid (12-S-HHTrE) were purchased from Cayman Chemical (Ann Arbor, MI). Acetonitrile (high-performance liquid chromatography [HPLC] grade), methanol (HPLC grade), pyridine, and acetic anhydride were from VWR (Lutterworth, United Kingdom). 9-anthryl diazomethane (ADAM) was obtained from Molecular Probes (Leiden, the Netherlands). Water was purified with a Millipore Milli-Q water purification system (Millipore, Watford, United Kingdom). The glucocorticoids, dexamethasone, prednisolone, hydrocortisone, and aldosterone were obtained from Sigma-Aldrich, while NCX-1015 (prednisolone 21-[4′-(nitrooxymethyl)benzoate) was a generous gift from Dr E. Ongini (NicOx SA, Milan, Italy).

Platelet preparations

Experiments with healthy volunteers were approved by the local research ethics committee (P/00/029, East London and City Health Authority [ELCHA]). Venous blood (40 mL) was taken from consenting healthy male volunteers and transferred immediately to citrated tubes. Samples were centrifuged for 10 minutes at 650*g* at 25°C. Using a Pasteur pipette, platelet-rich plasma (PRP) was carefully removed to separate polypropylene tubes and retained at room temperature until required (usually < 1 hour). Platelet-poor plasma (PPP) was prepared from PRP by centrifugation at 650*g* for 5 minutes at room temperature. For the washed platelet preparation, prostacyclin (300 ng/mL) was added to PRP prior to room temperature centrifugation (2400 rpm for 10 minutes) at room temperature to sediment the platelets. Two similar washes were conducted in Tyrode, and platelets were stored at 4°C prior to use.

Flow cytometry and confocal microscopy

Peripheral blood mononuclear cells (PBMCs) and washed platelets were seeded at 1×10^6 and 1×10^9 cells/well, respectively, in RPMI-1640 medium supplemented with 0.2% (wt/vol) bovine serum albumin (BSA) in 96-well tissue-culture plates. Cells were fixed with 2% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes at 4°C, and washed with PBS supplemented with 0.2% BSA. Cells were permeabilized with 0.02% (wt/vol) saponin (Sigma-Aldrich) in PBS/BSA for 5 minutes at 4°C. All subsequent steps were performed with 0.02% saponin added to all reagents. In order to detect cell-surface staining, some samples were not subjected to the permeabilization procedure and kept on ice. In all cases, nonspecific binding sites were blocked with human immunoglobulin G (IgG, 15 mg/mL) in PBS/BSA. For single-staining experiments, appropriately diluted polyclonal rabbit antibody to human GR (PA-512; Affinity Bioreagents, Golden, CO) was added to the cell suspension. After 1 hour at 4°C, cells were washed with PBS/BSA and incubated with $F(ab')_2$ fluorescein-isothiocyanate (FITC)–conjugated goat anti–rabbit IgG (Caltag, Burlingame, CA) for a further 45 minutes. For 2-color staining experiments, washed platelets at 1×10^9 cells/well were incubated with appropriately diluted polyclonal rabbit antibody to human GR (PA-512; Affinity Bioreagents) and either monoclonal antibody to P-selectin CD62P (Serotec, Oxford, United Kingdom) or monoclonal antibody to human mineralocorticoid receptor (MR, H10E4C9F; QBiogene-Alexis, Nottingham, United Kingdom). Thereafter, cells were washed with PBS/BSA, resuspended in PBS/BSA (without saponin), and fixed with an equal volume of 2% paraformaldehyde, prior to analysis. Controls included cells incubated in the presence of an IgG isotype-specific primary antibody of irrelevant specificity for monoclonal antibodies and rabbit IgG for polyclonal antibodies. After 1 hour at 4°C, cells were washed with PBS/BSA and incubated with respective secondary antibodies, $F(ab')_2$ phycoerythrin (PE)–conjugated or cyanin 5 (Cy5)–conjugated goat anti–rabbit IgG, and $F(ab')_2$ FITC-conjugated goat anti–mouse IgG (Caltag), for a further 45 minutes. Flow cytometry was performed on a FacsCalibur 4-color analyzer (Becton Dickinson, Oxford, United Kingdom). Platelets and mononuclear leukocytes were discriminated by forward and 90° light scatter. Fluorescence values of GR expression for platelets and PBMCs in the fluorescence channel 1 were reported in median fluorescence intensity (MFI) units. Elsewhere, 2-dimensional (2D) dot plots of fluorescence intensity were generated with appropriate compensation to determine specific single- and dual-color staining profiles of isolated, washed platelets.

For confocal analyses, whole blood was cytospun (at 200*g* for 4 minute) onto glass slides, air dried, then fixed in ice-cold acetone-methanol (50:50 vol/vol) at -20°C for 10 minutes. Cells were stained for GR using a specific rabbit polyclonal antibody (PA-512; Affinity Bioreagents) and for P-selectin CD62P with a mouse monoclonal antibody (Serotec) using a previously described protocol for intracellular fluorescence staining using propidium iodide as a nuclear counterstain.[18] Stained cells were observed by confocal microscopy using a Leica TCSSP confocal microscope equipped with a PL FLUROTAR 40× oil-immersion objective lens with a numerical aperture between 1.00 and 0.50 (all from Leica, Solms, Germany). Images were taken using a dual-band 488-nm argon and 514-nm krypton laser and were collected using TCSNT software (Leica).

Western blotting (WB) and immunoprecipitation (IP) studies

Washed platelets (1 mL containing $\sim 10 \times 10^6$ platelets), PBMCs (prepared as in Ito et al[18]), or the kidney cell line M-1 (European Collection of Cell Cultures [ECACC], United Kingdom) was incubated with or without glucocorticoids for either 0.5 or 2 hours at 37°C in 5% CO_2 atmosphere, and then lysed in 0.01 M Tris (tris(hydroxymethyl)aminomethane)–HCl (pH 8) supplemented with 0.15 M NaCl and a protease inhibitor cocktail (Hoffmann-La Roche, Basel, Switzerland). Lysates (100 μg protein) were diluted with 100 μL mild IP buffer (plus 0.5% nonidet P-40 [NP-40]) and incubated on ice for 30 minutes. Extracts were precleared with 20 μL protein A/G with agarose (50:50 mix; Santa Cruz Biotechnology, San Diego, CA) and 5 μL normal mouse IgG (500 μg/mL) for 1 hour at 4°C. After microcentrifugation, 20 μL protein A/G with agarose was conjugated with 5 μg anti–human GR (clone 8E9; Serotec) or the respective anti–heat shock protein (HSP) and incubated overnight (4°C) with constant rotation. Polyclonal antibodies, 3 μg of each per sample, were used for immunoprecipitation for human HSP70, HSP90 (Stressgen, Victoria, BC, Canada), FK506 binding protein (FKBP) 51 or FKBP52 (Merck Biosciences, Nottingham, United Kingdom). Immune complexes were resolved by gel electrophoresis on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels; after transfer onto nitrocellulose membranes, Western blot analysis was carried out using the anti–human MR (1:2000, clone H10E4C9F; Abcam, Cambridge, United Kingdom) or anti–human GR antibody (1:1000; Santa Cruz Biotechnology). In all cases, the signal was amplified with a horseradish peroxidase–linked goat antirabbit secondary antibody (1:2000; Dako, Cambridgeshire, United Kingdom) and visualized using BioMax MR-1 film (Kodak, Rochester, NY) after incubation with Luminol (enhanced chemiluminescence [ECL]; Amersham Biosciences, Oxford, United Kingdom).

PRP aggregation assay

PRP (500-μL aliquot) was added to Payton aggregation module (dual channel), and the instrument was calibrated as specified by the manufacturer, normalizing maximum transmittance on the PPP value recorded from a cuvette without stirring. Following a 3-minute stabilization period at 37°C, either vehicle or glucocorticoids were added in a 10-μL volume followed after 5 minutes by adenosine diphosphate (ADP, 4 μM). This concentration was chosen following an examination of the dose-response curve in preliminary experiments and gave approximately 80% of the maximal response (not shown). In selected experiments, 2 glucocorticoids (eg, RU486 and prednisolone) were added at 5-minute intervals. The platelet aggregation response was recorded to plateau or for a maximum of 5 minutes. The degree of platelet aggregation with the vehicle was then

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compared with that measured after treatment, and percentages of aggregation were calculated. In selected experiments, the TxA_2 receptor agonist U46619 (1 μM) or collagen (20 μg/mL) was used.[19]

HPLC determination of lipid release

Quantification of lipid release from resting and activated platelets was performed using a recently described chromatographic method.[20] Before HPLC analysis, lipid products were derivetized with ADAM reagent (1 μg/μL) before injection into an HPLC system consisting of a 4.6 × 100-mm 3-μm octadecyl silane Spherisorb column (phase Sep; Waters, Herts, United Kingdom), a 2150 pump (LKB, Bromma, Sweden), an FP-920 fluorescence detector (Jasco Tokyo, Japan), and a SCL-6B auto injector (Shimadzu, Kyoto, Japan). As mobile phase for the HPLC, a mixture of acetonitrile and water (85/15 vol/vol) was used. Integration was performed with Prime Software (HPLC Technology, Welwyn Garden, United Kingdom). Unknown samples were determined against standard curves prepared by spiking blank PPP with TxB_2 or AA, with a range from 6.25 to 125 ng/mL in both cases or a range from 5 to 40 ng/mL for 12-R-HETE and 12-S-HHTrE.

Ligand-binding assays

The GR-binding assay was conducted as recently described.[21] Briefly, platelets (1 mL containing ~ 10×10^6 platelets) were incubated with [^{3}H]-dexamethasone (50 nM; specific activity, 89 Ci [3.33×10^{12} Bq]/mmol) with or without cold glucocorticoid, for 1 hour at 37°C. After washing with 0.01 M ice-cold PBS, cell-bound [^{3}H]-dexamethasone (Amersham Biosciences) was quantified by liquid scintillation counting and the specific concentration calculated. Scatchard plot analysis was performed to determine dissociation constant (K_d) and binding maximum (B_{max}) values using a concentration range of 1.57 to 50 nM [^{3}H]-dexamethasone.

The binding to the MR was conducted in a similar fashion, using [^{3}H]-aldosterone (Amersham Biosciences) over a similar concentration range.

Data handling and statistical analysis

Experiments were conducted in triplicate and repeated at least 3 times with different cell preparations (GR-binding assay; immunoprecipitations; GR nuclear translocation). For IP experiments, Western blots were scanned using Epson *perfection* U1200 scanner (Singapore, Singapore), and densitometric analysis was completed using SCION image (National Institutes of Health [NIH], Bethesda, MD). Differences between the experimental groups were determined by one-way analysis of variance (ANOVA) followed by the Dunnett posthoc test, taking a P value less than .05 as significant.

Results

GR ligands and platelet activation

Addition of ADP to PRP provoked a marked aggregation of human platelets, as expected. Preincubation with 10 μM prednisolone, but not dexamethasone, significantly reduced the platelet response to ADP (Figure 1A). This inhibitory effect was restricted to prednisolone and its nitric oxide–releasing derivative NCX-1015, as hydrocortisone and dexamethasone were inactive (all GR ligands tested at 10 μM; Figure 1B). Prednisolone caused a concentration-dependent inhibition of ADP-induced platelet aggregation that was approximately 80% maximal at 3 μM; a 1-μM concentration was without effect (Figure 1C). The GR antagonist RU486 (10 μM) was inactive by itself on the ADP response; however, it reversed prednisolone-mediated inhibition of platelet aggregation, implying that this was a receptor-mediated event.

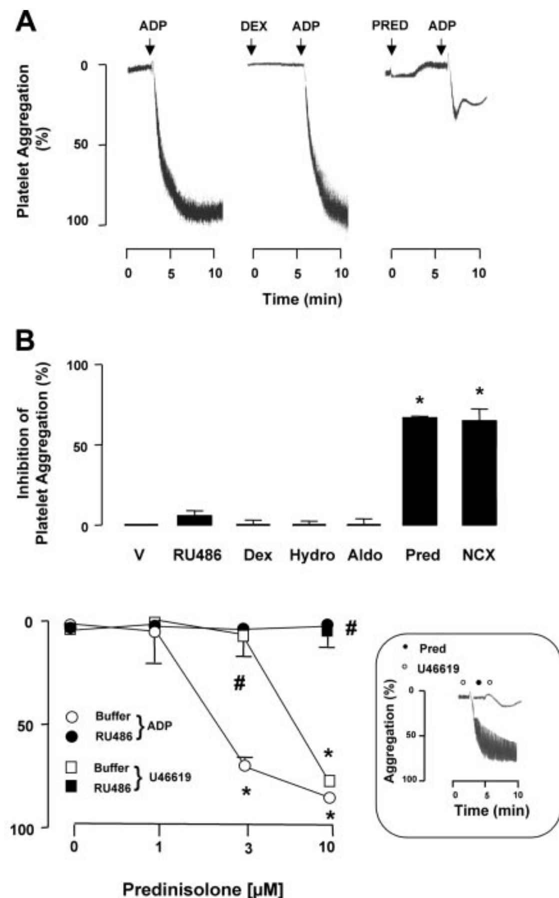

Figure 1. Prednisolone inhibits platelet aggregation. Human PRP was incubated with the reported glucocorticoid for 5 minutes before addition of 4 μm ADP, and platelet aggregation was monitored. (A) Prednisolone (PRED, 10 μM), but not dexamethasone (DEX, 10 μM), inhibited ADP-induced aggregation. Traces are representative of 6 distinct experiments. (B) Prednisolone and its nitro-derivative NCX-1015 are effective in inhibiting ADP-induced aggregation, whereas dexamethasone (Dex), hydrocortisone (Hydro), aldosterone (Aldo), and RU486 are inactive (all compounds tested at 10 μM). (C) Concentration response for prednisolone (added 5 minutes earlier) against 4 μM ADP- or 1 μM U46619-induced platelet aggregation. The effect of RU486 (10 μM; 5 minutes prior to prednisolone) is also shown, which blocks the inhibitory action of prednisolone on either stimulant. Inset: representative traces showing prednisolone's (10 μM) effect upon U46619-induced platelet aggregation. In all cases, data are mean ± SEM of 4 experiments with distinct platelet donors and performed in duplicate. *$P < .05$ versus control aggregation; #$P < .05$ versus respective prednisolone + buffer values.

The antiaggregatory effect of prednisolone was also observed on U46619-induced platelet aggregation (Figure 1C), although only at 10-μM concentration. Similar inhibition of collagen (20 μg/mL)–induced aggregation was seen, observing 20% ± 2% and 65% ± 4% inhibition for 3 μM and 10 μM prednisolone, respectively (data from 3 distinct donors).

The inhibitory effect of prednisolone on platelet function was not restricted to aggregation. Table 1 summarizes HPLC analyses for lipid metabolites in ADP-stimulated platelets. At concentrations congruent with inhibition of aggregation, prednisolone inhibited TxB_2 and 12-S-HHTrE, but not 12-R-HETE or AA, generation (Table 1). The nitro-derivative NCX-1015, tested only at 10 μM, mimicked the effect of prednisolone, whereas dexamethasone was markedly less active. When tested at 3 μM, the latter glucocorticoid failed to affect the release of any of the metabolites under analysis, with a modest effect on 12-S-HHTrE observed at 10 μM (Table 1). Aspirin was used as positive control in these experiments, and it inhibited TxB_2 and 12-S-HHTrE formation by 50% or more.

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Table 1. Modulation of lipid metabolism in ADP-stimulated human platelets

Treatment	TxB_2, ng/mL	12-S-HHTrE, ng/mL	12-R-HETE, ng/mL	Arachidonic acid, ng/mL
None	10 ± 1	14 ± 5	13 ± 2	72 ± 20
Aspirin	4 ± 1*	7 ± 2*	12 ± 3	105 ± 22*
Dexamethasone				
1 μM	9 ± 2	10 ± 3	7 ± 1*	69 ± 15
3 μM	9 ± 1	9 ± 2	8 ± 2	66 ± 10
10 μM	7 ± 2	7 ± 2*	9 ± 2	77 ± 16
Prednisolone				
1 μM	6 ± 2*	10 ± 2	7 ± 2*	78 ± 12
3 μM	5 ± 1*†	6 ± 3*	8 ± 2	84 ± 15
10 μM	5 ± 2*	5 ± 2*	10 ± 2	69 ± 5
NCX-1015	4 ± 2*†	6 ± 2*	10 ± 2	55 ± 15

Lipid release was measured in human PRP by HPLC ("Materials and methods"). Basal values in unstimulated platelets were (ng/mL): 4 ± 1 for thromboxane B_2 (TxB_2), 6 ± 2 for 12-S-hydroxyheptadecatrienoic acid (12-S-HHTrE), 6 ± 1 for 12-R-hydroxyeicosatetraenoic acid (12-R-HETE), and 1 ± 1 for arachidonic acid (n = 6). Dexamethasone, prednisolone, aspirin, or NCX-1015 was added to PRP for 5 minutes prior to ADP (4 μM) addition and extracts were prepared 10 minutes later. Aspirin and NCX-1015 (nitro-prednisolone) were used at 10 μM. Data are mean ± SEM of 6 experiments performed with PRP collected from distinct donors.

*$P < .05$ versus ADP alone.

†$P < .05$ versus corresponding Dex concentrations.

Detection of GR in human platelets

Immunochemical staining of washed platelets illustrated GR expression in permeabilized, but not nonpermeabilized, platelets (Figure 2). Incubation with an anti–P-selectin antibody produced positive staining of both preparations, whereas the anti-GR antibody was selective in its immunoreactivity only in permeabilized platelets. Dual-color imaging demonstrated the authenticity of the GR staining for platelets.

This crucial finding was confirmed by flow cytometric analysis, where human isolated platelets expressed GR at levels comparable with that of PBMCs; in freshly isolated blood from 4 volunteers tested, platelet GR immunoreactivity gave a value (median intensity fluorescence) of 163 ± 18 versus values of 237 ± 45 in the PBMC population. Figure 3A illustrates data and confirms the dual staining with the anti–P-selectin antibody, as determined on permeabilized washed platelets. To further characterize platelet GR, Western blotting analysis was performed. These data demonstrate that platelet GR has an apparent molecular weight (~ 97 kDa) similar to that found for PBMC GR (Figure 3B).

Biochemical characterization of GR in platelets

Scatchard plot analysis reported in Figure 4A clearly shows the negative hyperbole that was obtained, suggesting 2 binding sites for dexamethasone: a high affinity site with an estimated K_d of 1.96 nM and B_{max} of 1.07 pM and a low-affinity binding site with an approximate K_d of 86.2 nM and B_{max} of 23.9 nM. This result was unexpected and different from previous studies in PBMCs, U937 cells, and endothelial cells (to quote our own studies in which the identical protocol was applied).[21] As MR was also found to be present in platelets, but not PBMCs (Figure 3B), Scatchard analysis was then repeated with aldosterone as a tracer, and the results obtained indicate the existence of a single binding site for this mineralocorticoid with an apparent K_d of 4.88 nM and B_{max} of 4.062 pM (Figure 4B).

Competition experiments with unlabeled glucocorticoid revealed unexpected results. Whereas prednisolone was able to compete for dexamethasone binding to platelets (Figure 4C), this glucocorticoid was inactive on aldosterone binding (Figure 4D). $[H^3]$-dexamethasone was only partially displaced by prednisolone,

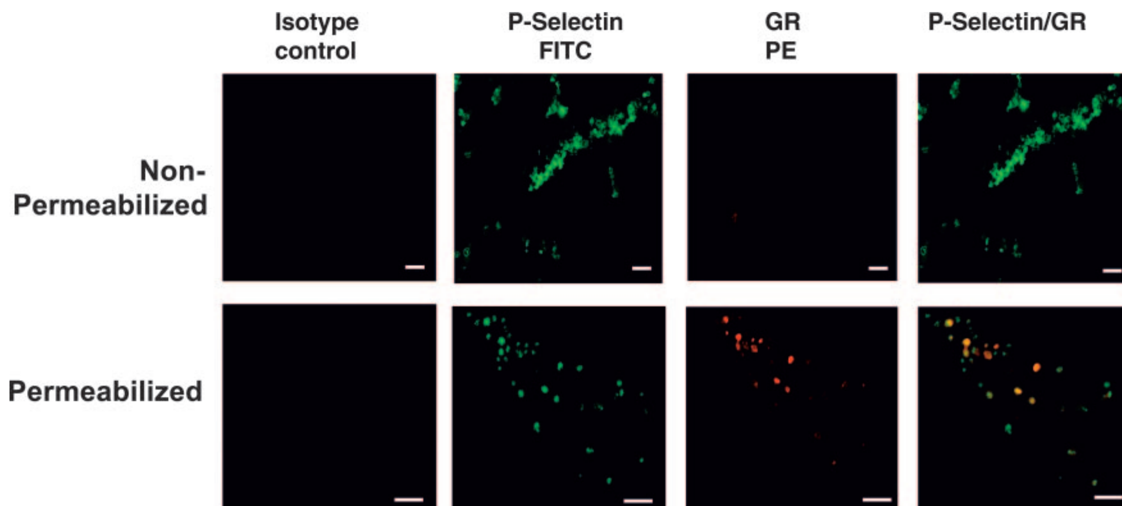

Figure 2. Detection of GR in human platelets by confocal analysis. Washed platelets were permeabilized with saponin or left intact ("Materials and methods") and stained with FITC-conjugated anti–P-selectin antibody or phycoerythrin (PE)–conjugated anti-GR antibody. Single-color images are shown, as well as the combined image (far-right panels). Note the large presence of yellow staining (dual labeling) in these 2 panels. Bars, 10 μm.

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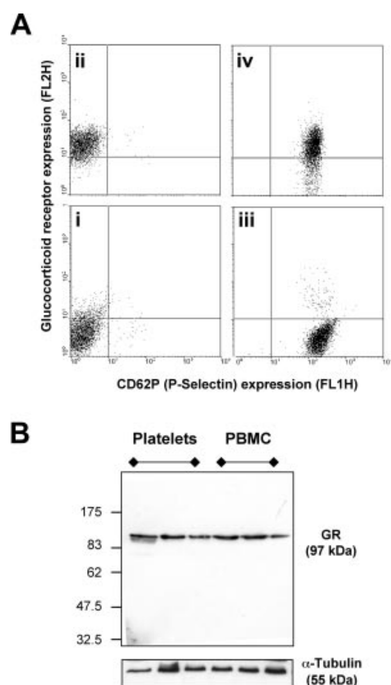

Figure 3. Detection of GR in human platelets by flow cytometry and Western blotting analyses. (A) Washed platelets were permeabilized with saponin ("Materials and methods") and stained with FITC-conjugated anti–P-selectin antibody or PE-conjugated anti-GR antibody. (Ai) Negative primary antibody control; (Aii) positive staining for GR; (Aiii) positive staining for P-selectin; and (Aiv) 2-color staining demonstrating dual positivity of platelets for P-selectin and GR (representative of data from 3 donors). (B) Human washed platelets and correspondent PBMC samples express GR; platelet GR has identical MW to PBMC GR. Blot is with samples from 3 distinct donors, and it is representative of 3 separate experiments.

especially if compared with the efficacy of cold dexamethasone or aldosterone (Figure 4C). In contrast, the latter 2 steroids were able to compete against [H^3]-aldosterone almost to an equal extent, though the homologous competitor appeared to be more effective at 1-μM concentration (Figure 4D).

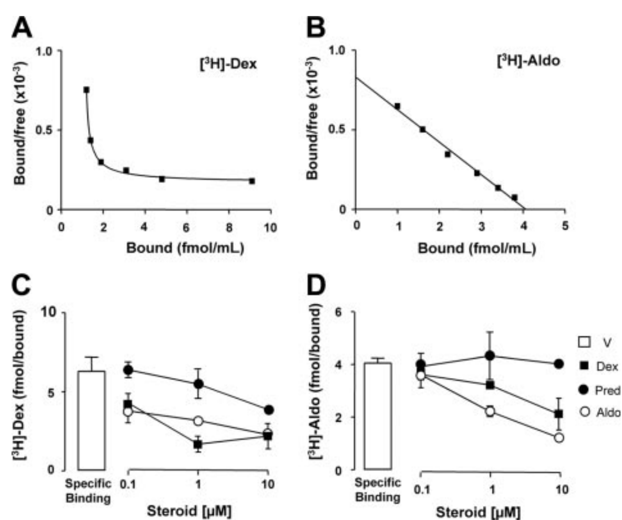

Figure 4. GR- and MR-binding assay. Human PRP was incubated with [H^3]-dexamethasone or [^{3}H]-aldosterone, and binding assays were performed as detailed in "Materials and methods." (A) Scatchard analysis of [^{3}H]-dexamethasone binding. (B) Scatchard analysis for [^{3}H]-aldosterone binding. (C) The effect of cold prednisolone (Pred), dexamethasone (Dex), and aldosterone (Aldo) on [^{3}H]-dexamethasone binding to platelets. (D) As in panel C, using [^{3}H]-aldosterone as tracer. V indicates vehicle. Data are mean ± SEM of 2 experiments with different PRP preparation for the Scatchard analyses, and of 4 experiments performed in duplicate for the competition analyses.

These data suggest the existence of both GR and MR in platelets, and that dexamethasone, but not prednisolone, could act at both receptors. Western blotting analyses of washed platelets revealed the clear presence of MR at the expected MW (~ 110 kDa; Figure 5A), a finding potentially related to the unusual binding profiles. Kidney M-1 cells, used as positive control,[22] were also positive for MR (Figure 5A) and GR (not shown). Flow cytometry confirmed the presence of both MR and GR in human permeabilized and washed platelets (Figure 5B).

In some cellular systems, GR and MR can form heterodimers (for a review, see Trapp and Holsboer[23]), thus we tested whether this combination could also be present in platelets. When compared with PBMCs prepared from the same donors, platelet GR also coprecipitates with MR. This is shown in a representative experiment in Figure 6A (positive GR/MR association observed in 5 distinct preparations). The same results were not obtained with PBMCs or when isotype IgG2a control was used (Figure 6A). The expression of MR in platelets raises the obvious question about its function(s). Addition of 10 μM aldosterone to PRP did not activate platelet aggregation by itself (not shown), nor did it affect the response to a submaximal concentration of ADP (Figure 6B). However, platelet incubation with aldosterone unmasked a modest yet significant inhibitory effect of dexamethasone (Figure 6B-C).

Platelet GR and accessory proteins

In the final part of the study, we monitored the effect of prednisolone and dexamethasone on GR association with known chaperone proteins. Figure 7 shows that in resting platelets GR immunoprecipitated with the chaperone HSP70, HSP90, and the immunophilins FKBP51 and FKBP52. As internal controls, we

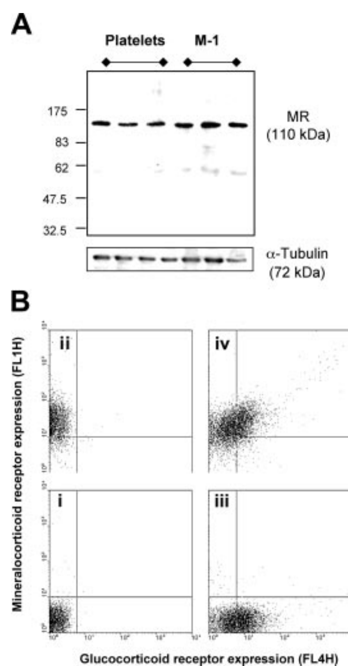

Figure 5. MR expression in human platelets. (A) Protein extracts from washed platelets or HEK cells were prepared as detailed in "Materials and methods" and analyzed by WB for MR expression; a band corresponding to the expected MW was observed. Blot is with samples from 3 distinct cell preparations, and it is representative of 3 separate experiments. (B) Washed platelets were permeabilized with saponin ("Materials and methods") and stained with FITC-conjugated antibody to MR or Cy5-conjugated anti-GR antibody. (Bi) Negative primary antibody control; (Bii) positive staining for MR; (Biii) positive staining for GR; and (Biv) 2-color staining demonstrating platelet dual positivity for MR and GR (representative of data from 3 donors).

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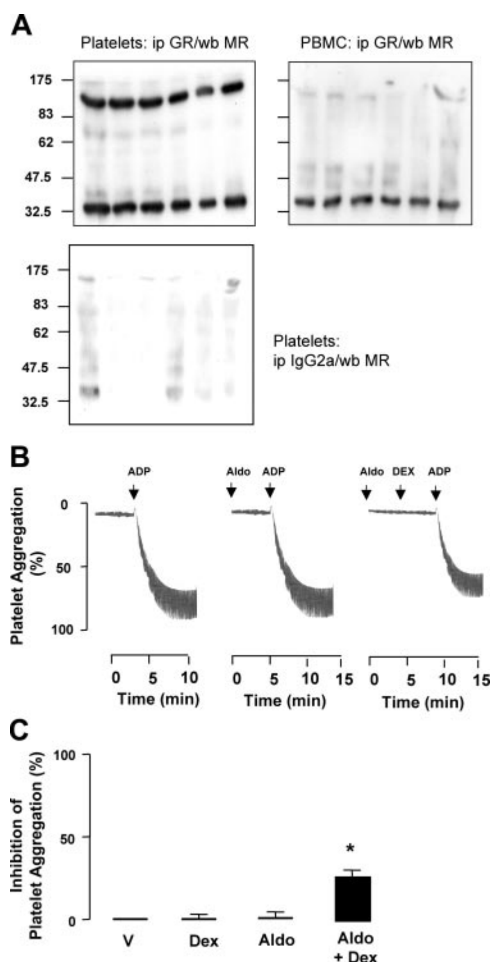

Figure 6. MR/GR association and aldosterone effect in human platelets. (A) Washed platelet extracts were immunoprecipitated with anti-GR antibodies that coprecipitated MR; the same did not occur for PBMC extracts (prepared from the same donors) or when an IgG2 isotype was used. Blots are representative of 3 distinct determinations. (B) Human PRP was incubated with 10 μM aldosterone (Aldo) before addition of 4 μm ADP; alternatively (right-hand panel), dexamethasone (10 μM) was added after Aldo. (C) Cumulative data from the experiments depicted in panel B. Data are mean $\pm$ SEM of 3 experiments with distinct platelet donors and performed in duplicate. $^*P < .05$ versus control ADP-induced platelet aggregation.

also ran comparative PBMC samples, in which GR is known to be associated with all these accessory proteins.[24]

The kinetics of this association was then analyzed. Addition of dexamethasone or prednisolone to PRP provoked a time-dependent

Table 2. Time-dependent glucocorticoid-mediated dissociation of GR chaperon proteins

Time and treatment	Densitometric analysis, arbitrary units: HSP70	HSP90	FKBP51	FKBP52
0.5 h				
Control	119 ± 12	104 ± 9	139 ± 16	94 ± 35
Dex	124 ± 9	127 ± 9*	144 ± 20	130 ± 32
Pred	156 ± 9*	136 ± 8*	112 ± 15	131 ± 34
2 h				
Control	103 ± 9	76 ± 7	142 ± 15	160 ± 11
Dex	108 ± 29	144 ± 4*	114 ± 18*	140 ± 32*
Pred	76 ± 10*	130 ± 16*	102 ± 9*	161 ± 20*

PRP was incubated with vehicle (control), 10 μM dexamethasone (Dex), or 10 μM prednisolone (Pred) for the time indicated. Then, immunoprecipitation with specific antibodies to HSP70, HSP90, FKBP51, or FKBP52 was conducted as described in "Materials and methods," and WB analysis for GR was applied to reveal direct protein association. See Figure 7 for an example of basal detection of GR association with each single chaperone. Data, reported as arbitrary units, are mean $\pm$ SEM of at least 3 distinct experiments.

$^*P < .05$ versus respective control.

association of HSP90 (Table 2) that was different from PBMCs where this chaperone protein quickly dissociated from GR (Paul-Clark et al[21] and data not shown). However, the 2 glucocorticoids displayed different effects on HSP70 (Figure 7A shows examples of basal association with GR). In 3 distinct experiments, prednisolone was able to cause first hyperassociation (30 minutes) and then dissociation (2 hours) of GR from HSP70, whereas dexamethasone was inactive (Table 2). No major differences between prednisolone and dexamethasone were obtained with respect to FKBP51 and FKBP52; both drugs provoked significant dissociation after 2-hour incubation.

Discussion

In the present study, we report the existence of a functional GR in human platelets and describe some of its behavioral and activation characteristics that differ from those reported in other cell types. This is potentially related to the existence of GR/MR heterodimers in platelets and may explain the different effects produced by distinct GR ligands. It is proposed that the ability of prednisolone to down-regulate platelet activation here reported may have clinically important implications.

Platelets have long been recognized to have a pivotal role at the interface between coagulation and inflammation,[25] predominantly

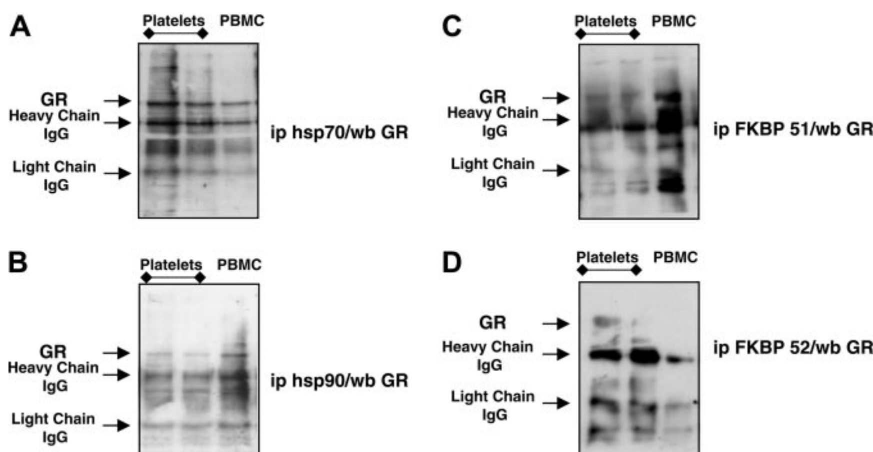

Figure 7. Detection of GR-associated proteins in human platelets. PRP extracts were immunoprecipitated with specific antibodies to (A) HSP70, (B) HSP90, (C) FKBP51, or (D) FKBP52 as described in "Materials and methods," and WB analysis for GR was applied to reveal direct protein association. PBMC extracts, prepared from the same donors, were run in parallel. Blots are representative of 4 distinct experiments.

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because they could serve as stores for proinflammatory mediators that are rapidly released at sites of endothelial activation/injury.[13,26] Classically, platelets have been considered unable to synthesize proteins and peptide mediators. For these reasons, we believe, the effects of glucocorticoids on platelets have been poorly investigated, since glucocorticoid-mediated GR activation has generally been linked to alterations of protein synthesis.[8] In addition, the few studies in which the effect of dexamethasone (often used as the "gold standard" glucocorticoid) was tested have found this glucocorticoid unable to alter platelet functions.[4] We provide here a novel biochemical mechanism that could be responsible for the paucity of data and contrasting findings reported in this field.

In contrast to the lack of effect of dexamethasone, addition of prednisolone to human PRP produced a concentration-dependent inhibition of second-phase ADP-induced aggregation. This result was surprising, since glucocorticoids have been thought to be inactive on platelet aggregation, unless used at high concentrations,[27] an effect possibly linked to membrane stabilization (often claimed for the neuroprotective actions of this group of drugs).[28] In our experimental conditions, though, several other glucocorticoids (tested up to 10 μM) were unable to affect platelet aggregation. This suggested some selectivity in the actions of prednisolone, a finding corroborated by 2 other sets of data: the blockade of the effect by the GR antagonist RU486[28] and the ability of a nitro-derivative of prednisolone[21] to inhibit platelet aggregation.

Pharmacologic analysis of the actions of prednisolone revealed a distinct concentration-dependent response against the aggregatory actions of ADP and U46619. This was supported by HPLC determination of different lipids generated from ADP-activated PRP, and the contrasting behavior displayed by prednisolone and dexamethasone on platelet aggregation was confirmed on other aspects of platelet biology. For instance, dexamethasone produced only minor alterations in lipid metabolism by activated platelets.[20] Comparative analyses of the data in Table 2 show that 1 to 10 μM prednisolone inhibited TxB_2 generation, whereas dexamethasone in the same concentration range was inactive. However, both glucocorticoids inhibited 12-S-HHrTe and 12-R-HETE formation, with almost equal efficacy. Thus, the ability to suppress TxB_2 generation is one of the discriminatory roots of prednisolone efficacy on platelet aggregation. This was achieved at clinically relevant concentrations, which are much lower than the millimolar concentrations used in the past to observe effect on platelet aggregation (eg, see Jorgensen and Stoffersen[27]). However, the efficacy of prednisolone on U46610-induced aggregation suggests interference with more than a single aspect of platelet biology that warrants further investigations.

Flow cytometry and confocal microscopic analyses confirmed that human platelets express GR; WB indicated that platelet GR was indistinguishable from PBMC GR in terms of WB; however, further investigations on the biochemical characteristics of platelet GR revealed other abnormalities compared with conventional GR biology.

GR functionality is tightly regulated by several receptor-associated proteins, of which several are heat shock proteins, such as HSP90 and HSP70, and the others are the immunophilins FKBP51, FKBP52, and Cyp40.[8,24] Although the number of receptor-associated proteins putatively associated with GR is ever increasing,[29] reconstitution experiments have shown that HSP90 and HSP70 are the major chaperones essential for unveiling the high-affinity receptor-binding cleft with GR, with other associated proteins determining GR stability and nuclear translocation.[30-32] Thus, in PBMCs and other nucleated cells, GR binding to its ligands (synthetic or natural glucocorticoids) causes HSP90 dissociation and exposure of the nuclear localization sequence[33]; the GR-ligand complex then dimerizes and translocates into the nucleus, where it modulates the transcription of the genes containing the glucocorticoid response element (GRE) within their promoter region. However, it is now clear that the GR-ligand complex can also affect gene expression indirectly by interfering with the function of cognate transcription factors.[8]

Experiments investigating GR binding in human platelets revealed several anomalies. For instance, Scatchard plot analysis indicated the existence of at least 2 binding sites for dexamethasone, whereas only 1 was evident for aldosterone. The profile for the steroids differs from that observed in PBMCs using the same whole-cell assay system, as well as in U937 cells,[21] endothelial cells,[34] and an osteoblast cell line (M.J.P.-C., unpublished data, October 2002). In line with this finding, prednisolone competed for [^{3}H]-dexamethasone for only approximately 50%, as did aldosterone. Congruently, dexamethasone, but not prednisolone, competed for aldosterone binding to MR. These unconventional binding data could be explained by the existence of a GR/MR heterodimer in platelets, a finding reported for other cell types.[23] In line with this hypothesis, GR and MR could be coimmunoprecipitated from human platelet, but not PBMC, extracts. It is of interest that dexamethasone binding to human MR has also been reported in transfected systems.[35,36] In addition, dexamethasone activates human MR to a significantly lower extent than GR, but, interestingly, with an affinity and potency very similar to aldosterone.[35] Thus, we wish to propose the hypothesis that addition of dexamethasone to platelets, by activating both GR and MR, produces a net null impact on activation. In contrast, the more selective GR ligand prednisolone inhibited the platelet response. In keeping with this hypothesis, platelet preincubation with aldosterone unmasked an antiaggregatory effect of dexamethasone. Although the latter effect was relatively modest compared with the antiaggregatory action of prednisolone, these results confirm the genuine inhibitory properties of platelet GR. More support for this conclusion may also be derived from experimental data obtained from a model of heart failure, in which an MR antagonist, eplerenone, prevented platelet activation.[37] A proinflammatory profile for chronic MR activation is now emerging that indicates a potential impact for this receptor on cardiovascular pathologies with an inflammatory etiology.[38,39]

Finally, analysis of HSP association with GR again revealed a discrepancy from the classic mode of GR activation. Human platelets are known to contain HSP90 and HSP70.[40,41] In the present study, we could also detect FKBP51 and FKBP52 in a physical association with GR. However, the dynamic of HSP interaction with GR was different from that reported for PBMCs and other cell types. In platelets, activation of the GR-ligand–binding domain did not cause HSP90 dissociation,[30-32] but rather the association increased in a time-dependent fashion, with a calculated increment of more than 30% from 30 minutes onward, perhaps suggesting that GR in the platelet is in an inactive or unfolded form and requires ligand to become functionally active and to reassociate with HSP. Of interest, the ability to increase HSP90 association was shared by both dexamethasone and prednisolone, whereas the dynamics of HSP70 interaction with GR were markedly affected by prednisolone but not dexamethasone. In particular, prednisolone addition to platelets caused a transient association with GR ($>$ 50% increase at 30 minutes) followed by a

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rebound dissociation; this is opposite to that described in PBMCs and U937 cells.[21] Thus, given the dichotomy of behavior with respect to TxB_2 formation, it is tempting to propose that prednisolone's ability to alter HSP70 association with GR could be at the basis of its inhibition of ADP-induced platelet activation. In resting platelets, HSP70 is bound in a large complex together with HSP90 and protein phosphatase 1.[42] This present study suggests that GR is also part of this complex. Upon platelet activation, dephosphorylation of HSP70 is one of the first events to occur, causing the complex to disintegrate.[42] It is noteworthy that GR contains numerous potential phosphorylation sites.[43] Evidence obtained during the past 10 years suggests that alteration in the GR phosphorylation status can affect GR-ligand binding, HSP90 interactions, receptor subcellular localization, nuclear-cytoplasmic shuttling, and transactivation potential.[44] The fact that we could not detect HSP90 dissociation is consistent with the hypothesis that platelet GR does not require nuclear translocation. Conversely, the ability of prednisolone to provoke HSP70 dissociation is in line with its profile of GR activation. Furthermore, it is highly likely that specific phosphorylation events regulate the spatial and temporal association between GR and its accessory proteins in platelets. Confirmation of this would be the objective of future studies.

To conclude, we report the detection of GR in human platelets, thus increasing the list of nuclear receptors expressed in these cell fragments.[41,45] As platelets have recently been shown to exhibit de novo protein synthesis,[13] it is possible that activation of these receptors might affect this specific response and consequent generation of proinflammatory cytokines and chemokines. In any event, platelet GR activation leads to a series of nongenomic responses, which require GR binding but are independent from activation of transcription/translation.[7,12] The data we present not only uniquely demonstrate that glucocorticoids can rapidly (< 1 hour) alter platelet function, but also indicate that these responses are ligand specific. Thus, at variance from the classical pharmacologic classification of glucocorticoids, mechanistic differences among specific compounds are now emerging (eg, see Croxtall et al[46] from our group), and this appears to be particularly evident in the case of platelet GR.

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