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Platelets and Blood Cells

Prolactin does not affect human platelet aggregation or secretion

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Summary

Platelets play an important role in the development of plaque formation and in the events after rupture of the atherosclerotic plaque, leading to atherothrombosis. Multiple hormones, either in excess or when deficient, are involved in the development of atherothrombotic disease, but, to which extent such hormones affect platelet function, is still controversial. It was the objective of this study to assess the ability of the pituitary hormone prolactin to affect platelet functions. Venous blood was collected from six healthy males. Platelet activation was studied by (i) flow cytometry in whole blood (exposure of P-selectin as a measure of platelet secretion, and binding of PAC-1 as a measure of ligand-binding conformation of $\alpha_{IIb}\beta_3$), and by (ii) optical aggrega-

tion and whole blood aggregation. All studies were performed without and with exposure to several concentrations of ADP (0.1, 0.5 and 1.0 μ M) and prolactin (50 and 1,000 μ g/l). The presence of the prolactin receptor was investigated by Western blot and flow cytometry. In response to either 50 or 1,000 μ g/l prolactin, no evidence of platelet activation or aggregation was found. In addition, ADP-induced platelet activation or aggregation was not enhanced by prolactin. Finally, prolactin receptors could not be detected on the surface of platelets. The present data indicate that prolactin does not directly modulate platelet function.

Keywords

Atherothrombosis, prolactin, platelet activation, platelet aggregation, prolactin receptor

Thromb Haemost 2009; 101: 1119–1127

Introduction

Elevated levels of the pituitary hormone prolactin have been reported in patients suffering from either stroke or an acute coronary syndrome. Also, hyperprolactinemic patients often have increased levels of risk factors known to contribute to the development of atherothrombosis, such as insulin resistance and impaired endothelial function (1–3). Moreover, several types of cells involved in the atherothrombotic process, e.g. endothelial cells, natural killer cells and lymphocytes, express functional prolactin receptor (4–7). In fact, it has been shown that prolactin triggers migration of macrophages and stimulates proliferation of smooth muscle cells (8, 9). Overall, these findings suggest that prolactin may play a role in atherothrombosis through effects at multiple levels of atherogenesis and thrombus formation.

Platelets are involved in the development of plaque formation and rupture, and are an important source of inflammatory mediators and matrix metalloproteinases (10, 11). To which extent pro-

lactin might activate human platelets, and whether such activation would contribute to the development of atherothrombosis, however, is controversial. Wallaschofski et al. have reported that prolactin by itself does not activate human platelets, but can enhance platelet activation in a synergistic fashion with ADP (12). Furthermore, these authors showed that a correlation exists between plasma levels of prolactin and ADP-induced platelet activation in subjects with high levels of prolactin, including pregnant women and patients with pituitary tumours (13). In contrast, Atmaca et al. found no association in pregnant women between levels of prolactin and platelet function. These investigators could not show any relationship between the levels of prolactin and β -thromboglobulin, an overall measure of the platelet activation status *in vivo* (14). These data, taken together, demonstrate that the role of prolactin in human platelet biology is unclear.

To investigate this further, we evaluated the effects of prolactin on human platelet activation and aggregation in an ex-vivo model.

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Methods

Blood collection and isolation of human platelets

The study was performed in accordance with ethical principles that have their origin in the Declaration of Helsinki and are consistent with ICH/ Good Clinical Practice. All participants provided informed consent. Healthy normoprolactinemic male subjects ($n=6$), who denied to have taken any medication during the previous 10 days were recruited by advertisement between January 2008 and March 2008. Their prolactin levels were 4.6 ± 1.2 $\mu\text{g/l}$ (mean \pm SD). Venous blood was collected from the antecubital vein with minimal venous occlusion into 1/10th volume of 10.9 mM tri-sodium citrate (BD Biosciences, San José, CA, USA) from fasting subjects, before 11.00 a.m. Platelet-rich plasma (PRP) was obtained by centrifugation for 15 minutes (min) at 180g (20°C).

Reagents

For detection of platelet activation by flow cytometry, thrombin receptor activating peptide (TRAP, SFLLRN) was obtained from Bachem AG (Bubendorf, Switzerland) and adenosine diphosphate (ADP) was purchased from Sigma (St Louis, MO, USA). Human recombinant prolactin was either purchased from Sigma (St Louis, MO, USA) or prepared by the Inserm lab (V. Goffin) as previously described (15). Results shown in this paper were obtained using the latter prolactin. Phycoerythrin (PE)-labelled CD62P, clone CLB-thromb/6, isotype IgG₁, was obtained from Immunotech, Beckman Coulter (Fullerton, CA, USA). Allophycocyanin (APC)-labelled CD61, clone Y2/51, isotype IgG₁, was from DakoCytomation (Glostrup, Denmark). Fluorescein isothiocyanate (FITC)-labelled anti-PAC1, clone PAC1, PE-labelled and FITC-labelled IgG₁, clone X40, isotype IgG_{2bκ}, clone MCP-11, and anti-mouse IgG_{2bκ}-FITC, clone R12-3, were obtained from BD Biosciences. The monoclonal antibody directed against the extracellular domain of the human prolactin receptor, clone 1A2B1, isotype IgG_{2bκ}, was obtained from Zymed, Invitrogen Corporation (Carlsbad, CA, USA).

Whole blood flowcytometry (platelet activation status)

Dilutions of prolactin were prepared in HEPES-buffer (NaCl 137 mM, KCl 2.7 mM, MgCl₂ 1 mM, HEPES 20 mM, NaH₂PO₄ 3.3 mM, bovine serum albumin 1 g/l, D-glucose 5.6 mM, pH 7.4). Citrate anticoagulated whole blood (300 μl) was diluted with 300 μl pre-warmed (37°C) saline 0.9 % (according to the manufacturers' instruction) and pre-incubated for 5 min with prolactin (50 and 1,000 $\mu\text{g/l}$ final concentration) at 37°C and stirred at 900 rpm in a whole-blood aggregometer.

From this diluted blood, 5 μl was added to 35 μl HEPES buffer, containing prolactin (final prolactin concentration 50 or 1,000 $\mu\text{g/l}$). Subsequently, CD61-APC was added, in combination with either CD62P-PE and anti-PAC1-FITC, or with CD63-PE only, without or with ADP (0.1, 0.5 or 1.0 μM). Maximum platelet activation was determined in the presence of TRAP (final concentration 15 mM) without prolactin. After incubation for 30 min, activation was terminated by addition of 2.5 ml 0.3% paraformaldehyde-containing HEPES-buffer. Flow cytometry was performed on a Calibur flowcytometer (Becton

Dickenson) by counting in a life gate for 5,000 CD61-APC positive events. Data were analysed by Cellquest Pro (version 4.02) (Becton Dickinson).

Optical aggregation

Optical platelet aggregation was performed on the aggregometer AGGRAM (Helena Laboratories Corp, Beaumont, TX, USA) at 800 rpm and 37°C according to the manufacturers' instruction. PRP was pre-incubated for 5 min with prolactin (50 or 1,000 $\mu\text{g/l}$ final concentration) under stirring conditions, before addition of ADP (0.1, 0.5 or 1.0 μM final concentration). Maximum platelet activation was determined in the presence of TRAP (15 μM) without prolactin. Recordings were stopped after 10 min.

Whole blood aggregation

Whole blood aggregation was performed on a whole blood platelet function analyser Multiplate from Dynabyte GmbH (Munich, Germany) by the impedance method. Citrate anticoagulated whole blood (300 μl) was diluted with 300 μl pre-warmed (37°C) saline 0.9 % (according to the manufacturers' instruction) and pre-incubated for 5 min with prolactin (50 or 1,000 $\mu\text{g/l}$ final concentration) at 37°C and stirred at 900 rpm. Induction of aggregation by addition of ADP and determination of maximum platelet aggregation with TRAP was performed as mentioned above (optical aggregation). Recordings were stopped after 5 min.

Statistics

Data were analysed by two-tailed Student t-test (GraphPad Prism Software version 5.0; GraphPad Software, Inc, CA, USA). A probability value of <0.05 was considered to confer statistical significance.

Western blot

PRP from all volunteers were centrifuged at 800g for 20 min, at 20°C. Platelets were resuspended in phosphate-buffered saline (PBS) with ACD (trisodium citrate 85 mM, citric acid 71 mM, D-glucose 110 mM; 1:5 v/v). After centrifugation (20 min 800g), platelets were resuspended in PBS and diluted in two-fold concentrated Laemmli buffer containing β -mercaptoethanol, final average concentration was 0.1×10^6 cells/ μl , and we loaded 3.5×10^6 cells per lane. Lysates from both the human breast cancer cell line MCF7 (1.0×10^6 cells per lane) and transfected human embryonic kidney cells (HEK293) (0.50×10^6 cells per lane) stably expressing the long isoform of the human prolactin receptor (16, 17) were used as positive controls.

Proteins were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane (Biorad, Hercules, CA, USA). After blocking (1 hour at room temperature) in TBS-Tween 0.01% (NaCl 137 mM, Tris-HCl 20 mM; pH 7.6) containing 5% (w/v) non-fat milk, membranes were incubated with a mouse monoclonal antibody directed against the extracellular domain of the human prolactin receptor (Zymed Laboratories – Invitrogen Immunodetection, 35–9200, 1:1,000 v/v) overnight at 4°C. After washing with TBS-Tween, membranes were incubated with anti-mouse antibody conjugated to horseradish peroxidase (GE Healthcare, NXA931; 1:4,000 v/v), for 1 hour at room temperature. All antibodies were diluted in TBS-Tween

containing 3% bovine serum albumin (Sigma) (w/v). Immunoreactive proteins were detected using an Enhanced Chemiluminescence Detection System (ECL Western blotting detection reagents Amersham).

Flowcytometric detection of the prolactin receptor

To validate the detection of the prolactin receptor by flow cytometry, we used MCF7 cells, a human breast cancer cell line, as positive control, since MCF7 cells constitutively express and expose the prolactin receptor (16). To detect the prolactin receptor in lysates of MCF7 cells and platelets, an antibody was used that

is directed against the extracellular domain of the human prolactin receptor.

Confluent MCF7 cells were detached by adding EDTA 2 mM (pH 7.4). Cells were washed two times in 25 ml PBS-BSA 0.5% and centrifuged (10 min, 180g). After the second centrifugation, cells were resuspended in 2 ml PBS-BSA (0.5% w/v), and aliquots of 50 µl were incubated with either anti-human prolactin receptor antibody (Zymed) or the IgG_{2bK} isotype control (1:50 v/v final concentration) for 30 min (4°C). After labelling, aliquots were washed in 1 ml PBS-BSA 0.5% and centrifuged (10 min, 180g). The remaining volume of approximately 50 µl, containing the cell

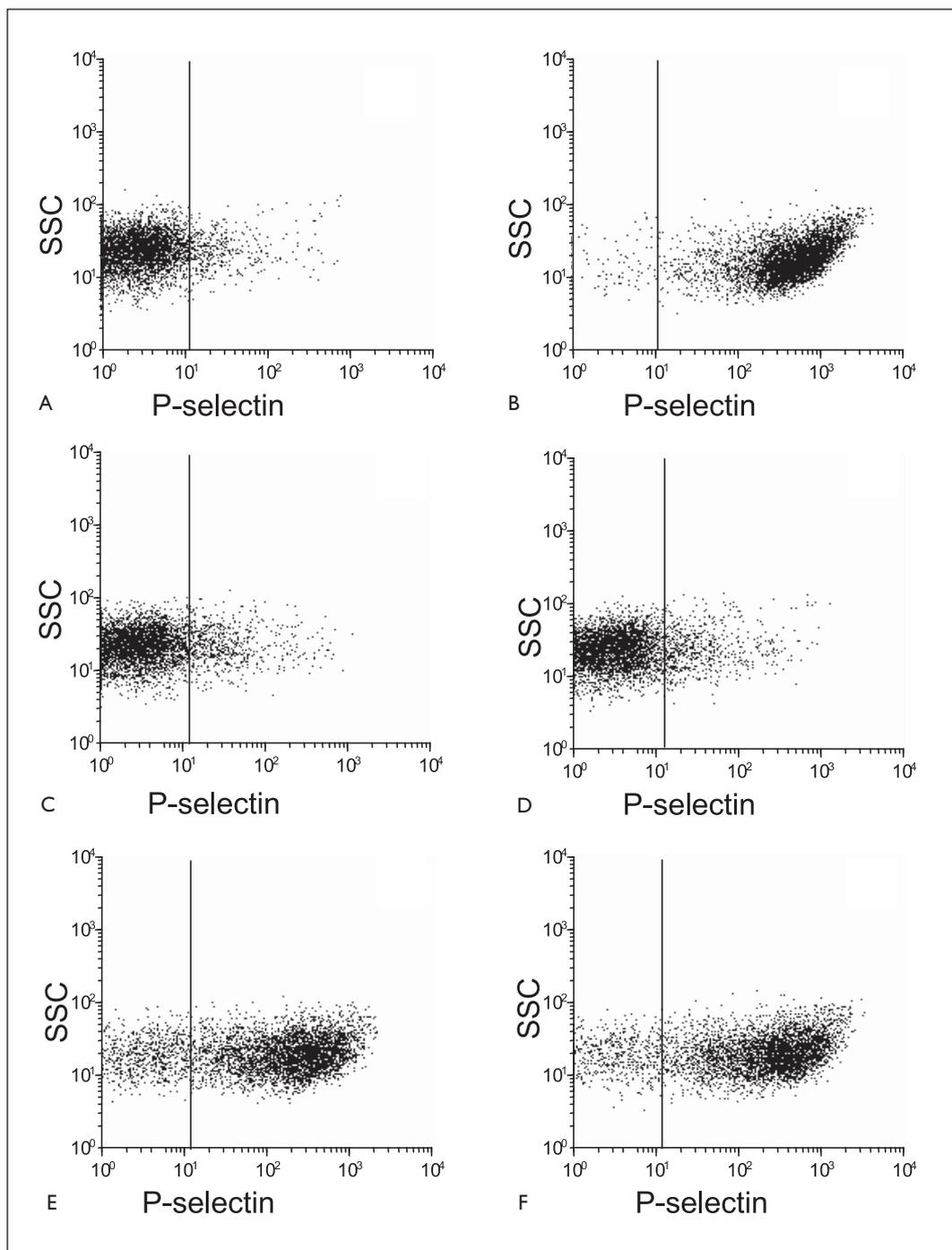


Figure 1: Effect of prolactin on platelet activation (exposure of P-selectin). Representative data of one donor. Flow cytometric dot plots showing exposure of P-selectin in the absence of prolactin and ADP (A), in the presence of 15 µM TRAP (B), 50 µg/l prolactin (C), 1,000 µg/l prolactin (D), or 0.5 µM ADP in the absence (E) or in presence of 1,000 µg/l prolactin (F).

pellet, was incubated with anti-mouse IgG_{2bκ}-FITC labelled antibody for 30 min at 4°C. Subsequently, aliquots were centrifuged (3 min, 390g) and 300 µl PBS-BSA 0.5% was added to end the incubation. PRP was prepared as previously described. 200 µl PRP was incubated for 30 min at room temperature with either anti-human prolactin receptor antibody or the IgG_{2bκ} isotype control (1:50 v/v final concentration), in the absence or presence of ADP (10 µM final concentration), TRAP (15 µM final concentration). After incubation, 1 ml HEPES buffer (pH 7.4) and 0.2 ml ACD was added and the mixture was centrifuged for 20 min (800g, 20°C). Supernatant was discarded. Platelets were resuspended in a total volume of 200 µl HEPES buffer. Aliquots of 50 µl were added to the following combinations of antibodies (5 µl per antibody): CD61-APC plus CD62P-PE or CD63-PE or IgG₁-PE, in addition 5 µl of anti-mouse IgG_{2bκ}-FITC-labelled antibody was added to all tubes. After 30 min of incubation at room temperature, 1 ml of HEPES buffer was added to all tubes. Flow cytometry was performed on a Calibur flowcytometer by counting in a life gate for 5,000 CD61-APC positive events. Data were analysed by Cellquest Pro (version 4.02).

Results

Effect of prolactin on platelet activation

To study the effects of prolactin on platelet activation, in a first set of experiments, platelets were incubated with prolactin in the

absence or presence of various concentrations of ADP. Arbitrarily, a fluorescent threshold was set to distinguish platelets exposing P-selectin (activated platelets) from platelets not or hardly exposing P-selectin (non-activated platelets). Figure 1A shows flowcytometric dot plots of platelet P-selectin in the absence of prolactin, ADP or TRAP. Figure 1B shows the exposure of P-selectin on TRAP (15 µM)-activated platelets (positive control). When platelets were incubated with a high physiological concentration of prolactin (50 µg/l and 1,000 µg/l, respectively), no increase in exposure of P-selectin was observed (Fig. 1C and D), indicating that prolactin by itself does not activate platelets. Since prolactin alone did not trigger exposure of P-selectin, we tested its ability to enhance platelet activation triggered by various concentrations of ADP. Figure 1E shows that ADP alone (0.5 µM) triggered a modest exposure of P-selectin, which was not enhanced by prolactin (1,000 µg/l) (Fig. 1F).

Overall data with regard to exposure of P-selectin and binding of PAC-1 are summarised in Figures 2A and 2B, respectively. Platelet activation with TRAP was arbitrarily set at 100% per each volunteer. Addition of either 50 or 1,000 µg/l prolactin alone, i.e. in the absence of ADP, induced no platelet activation as measured by the percentage exposure of P-selectin (mean ± SEM of column) (Fig. 2A) or binding of PAC-1 (Fig. 2B). Whereas platelet activation increased upon incubation with increasing concentrations of ADP, neither the exposure of P-selectin (Fig.

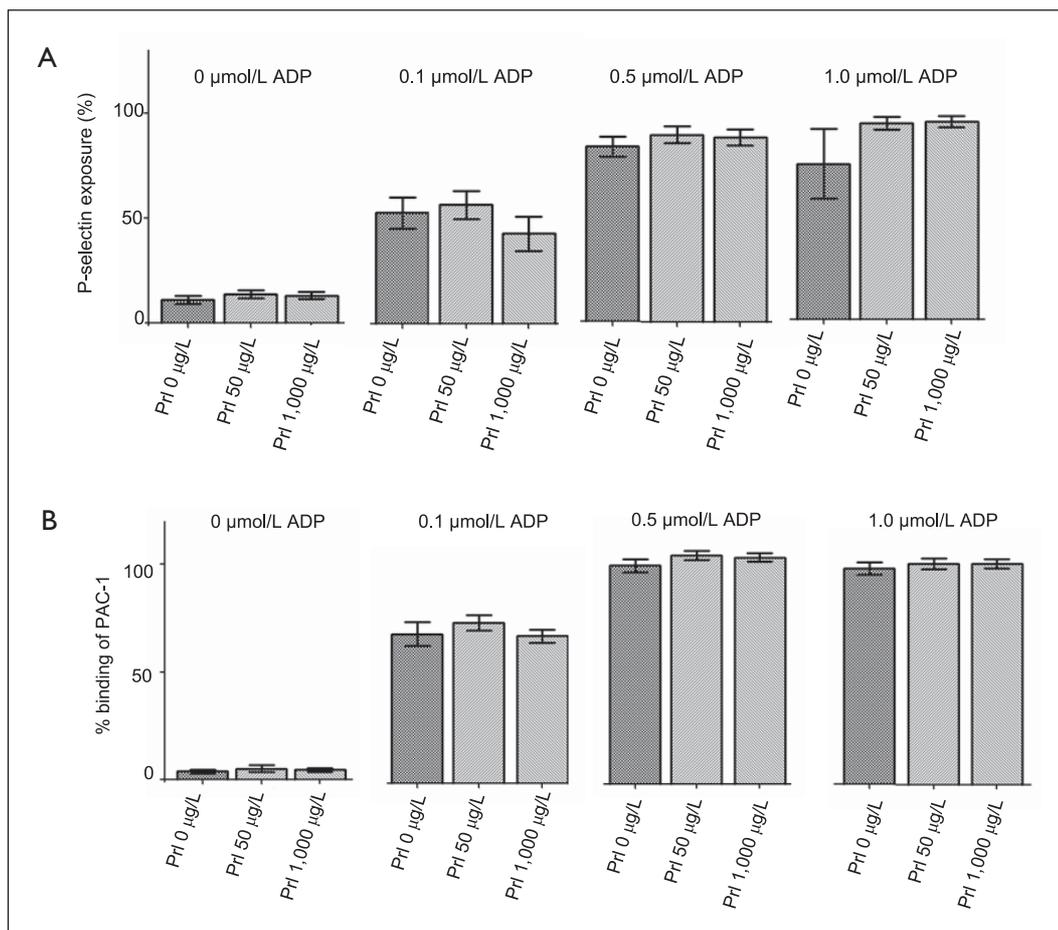


Figure 2: Effect of prolactin on platelet activation (exposure of P-selectin or binding of PAC-1, respectively). Data from all donors analysed. Flow cytometric data from all experiments, showing exposure of P-selectin (A) or binding of PAC-1 (B). Addition of either 50 or 1,000 µg/l prolactin alone, i.e. in the absence of ADP, induced no platelet activation. Upon incubation with increasing concentrations of ADP, exposure of P-selectin and binding of PAC-1 was increased, but this exposure was not further enhanced upon co-incubation with prolactin. Per ADP concentration, all p-values were > 0.05 for the detected difference between bars.

2A) nor the binding of PAC-1 (Fig. 2B) was further enhanced upon co-incubation with prolactin. From Figures 1 and 2 it is concluded that prolactin by itself or in the presence of ADP does not affect (additional) platelet activation.

Effect of prolactin on platelet aggregation

To examine whether prolactin affects platelet aggregation, PRP was incubated with prolactin in the absence or presence of various concentrations of ADP. Figure 3A shows platelet aggregation without any addition, i.e. without addition of either prolactin, ADP or TRAP. Figure 3B shows aggregation upon stimu-

lation with TRAP (15 μ M; positive control). When platelets were incubated with prolactin alone (50 μ g/l or 1,000 μ g/l), no optical platelet aggregation occurred (Fig. 3C and D, respectively). Thus, prolactin by itself does not induce platelet aggregation. In line with previous studies, we assessed the possibility that prolactin enhances ADP-induced platelet aggregation. Since it is known that there is a large variation between individuals in response to ADP-induced platelet activation and aggregation, we determined dose-response curves for each individual donor with increasing concentrations of ADP (0.1, 0.5 and 1.0 μ M). A representative example is shown in Figures 3E and 3F. Figure 3E

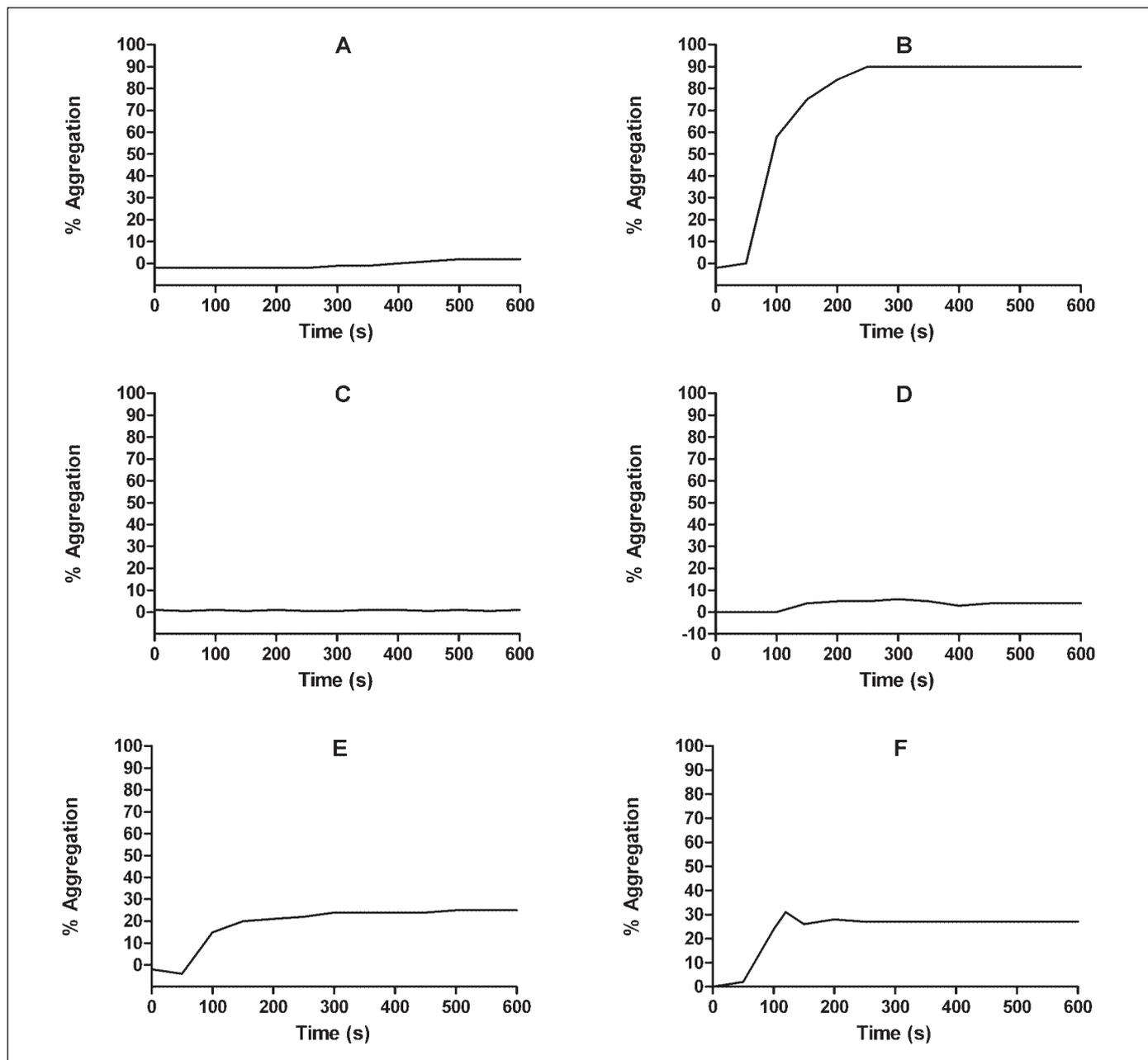


Figure 3: Effect of prolactin on platelet aggregation (optical aggregation). Representative data of one donor. Optical aggregation data from one representative experiment, showing optical aggregation in the absence of prolactin and ADP (A), in the presence of 15 μ M TRAP (B), 50 μ g/l prolactin (C), 1,000 μ g/l prolactin (D), or 0.5 μ M ADP in the absence (E) or in presence of 1,000 μ g/l prolactin (F).

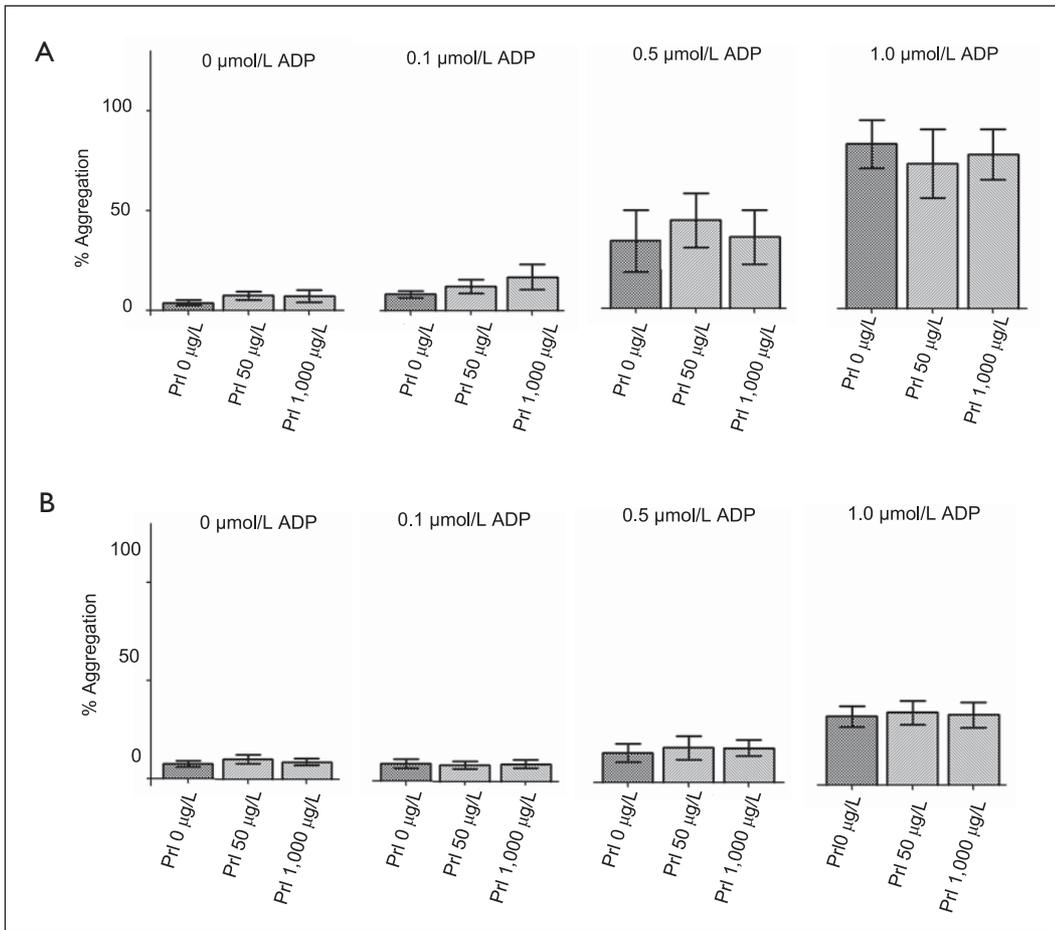


Figure 4: Effect of prolactin on platelet aggregation (optical aggregation and whole blood aggregation, respectively). Data from all donors analysed. Overall data obtained with optical (A) and whole blood platelet aggregation (B) are summarised. Addition of either 50 or 1,000 μg/l prolactin alone, i.e. in the absence of ADP, induced no platelet activation. Upon incubation with increasing concentrations of ADP, aggregation was increased, but this exposure was not further enhanced upon co-incubation with prolactin. Per ADP concentration, all p-values were > 0.05 for the detected difference between bars.

shows ADP-induced platelet aggregation in the presence of ADP alone (0.5 μM), i.e. in the absence of prolactin, and in the presence of 1,000 μg/l prolactin (Fig. 3F), showing no additional platelet aggregation.

Overall data obtained with optical and whole blood platelet aggregation are summarised in Figures 4A and 4B, respectively (mean ± SEM of column).



Figure 5: Detection of prolactin receptor by Western blot. Lane 1 (MCF7) and lane 2 (HEK293 transfected with the long isoforms of the prolactin receptor) display a strong band at the expected molecular mass (95–100 kDa), corresponding to the prolactin receptor. None of the platelet lysates obtained from seven different healthy individuals (lanes 1–7) contained any detectable form of the prolactin receptor.

In conclusion, these data indicate that prolactin by itself or in the presence of increasing concentrations of ADP, does not induce or enhance platelet aggregation. These data are in line with our finding that prolactin did not induce or enhance the binding of PAC-1 (Fig. 2B).

Detection of the prolactin receptor on human platelets

The presence of the prolactin receptor on human platelets was studied by Western blot and by flow cytometric analysis. As shown on Figure 5A, lysates from both the human breast cancer cell line MCF7 and transfected human embryonic kidney cells (HEK293), both stably expressing the long isoform of the human prolactin receptor (16, 17), displayed a strong band at the expected molecular mass of 95–100 kDa, corresponding to the prolactin receptor. Otherwise, none of the platelet lysates obtained from seven different healthy individuals (lanes 1–7) contained any detectable form of the prolactin receptor.

In addition, we evaluated whether prolactin receptors could be identified on the surface of human platelets by flow cytometry. First, the ability of the Zymed antibody to detect the prolactin receptor on MCF7 cells was tested (Fig. 6A and B). Arbitrarily, a fluorescent threshold was set to distinguish cells exposing the prolactin receptor from cells not or hardly exposing the prolactin receptor. Figure 6B shows that at least a large subpopulation of MCF7 cells (50–60%) exposed detectable amounts of the prolactin receptor. As shown in Figure 6, neither resting pla-

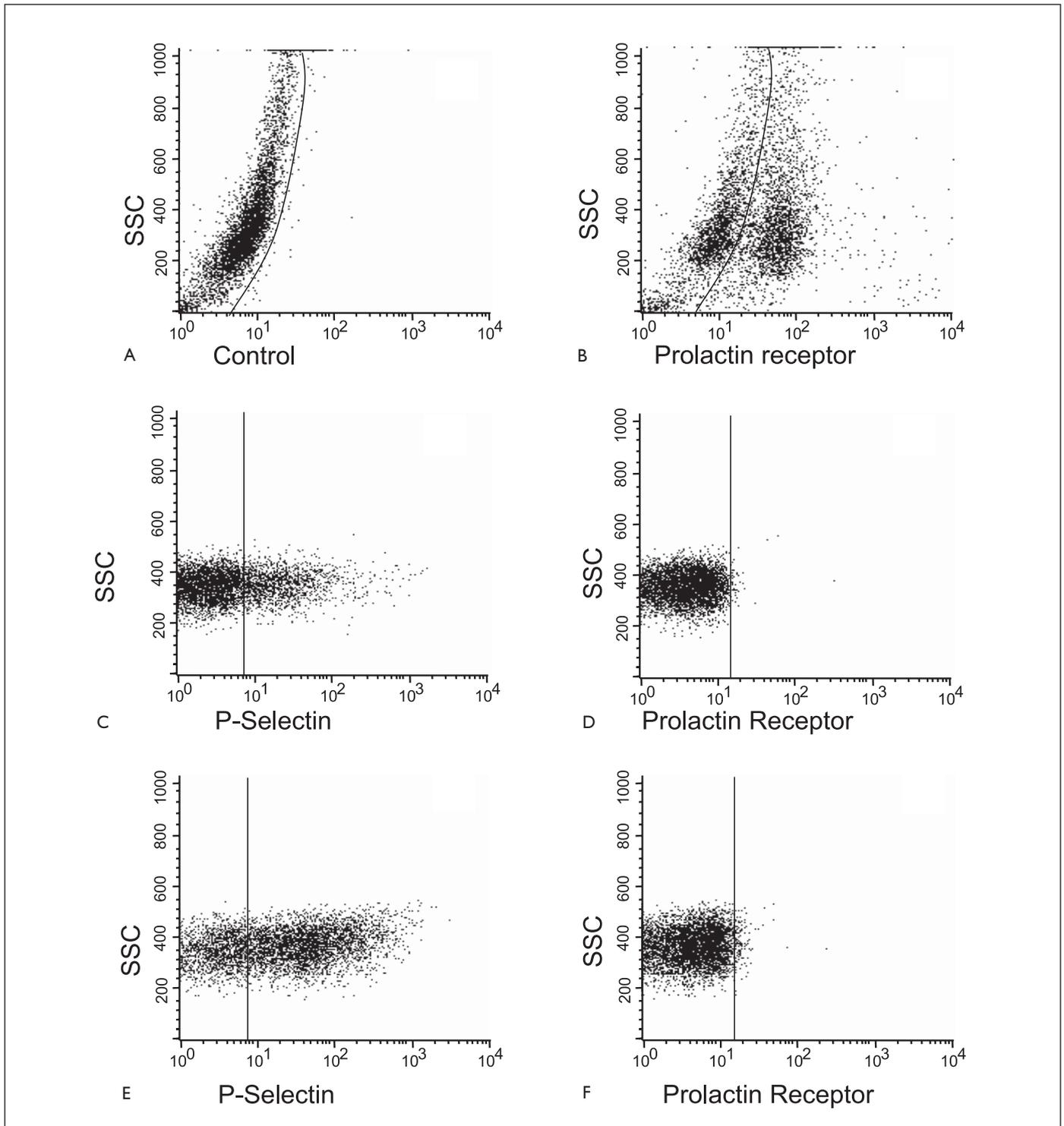


Figure 6: Detection of prolactin receptor by flow cytometry. MCF7 cells with isotype control (A) and MCF7 cells with prolactin receptor antibody (B). Resting platelets (C) do not expose the prolactin receptor (D). Upon activation (E), platelets do neither expose the prolactin receptor (F).

telets (Fig. 6C and D), nor maximally activated platelets (with TRAP; Fig. 6E and F, respectively) did expose the prolactin receptor.

Taken together, it is highly unlikely that platelets have a functional prolactin receptor, and that prolactin affects platelet activation or aggregation.

Discussion

Our data indicate that prolactin, by itself or in combination with ADP, does not affect platelet activation or aggregation in an ex-vivo model. In the present experiments we used prolactin from two different origins, functionality of which was found to be

What is known about this topic?

- There is obscurity about the effect of prolactin on platelet function.
- Platelets may have a prolactin receptor.
- Patients with acute coronary syndrome and ischaemic stroke have higher prolactin levels.

What does this paper add?

- Prolactin does not increase platelet activation or aggregation.
- There is no detectable prolactin receptor on platelets.

equivalent using a sensitive cell-based assay involving proliferation of lymphoid Ba/F3 cells stably expressing the human prolactin receptor (18) (data not shown). The absence of any detectable effect of prolactin was correlated to the fact that we did not find any evidence for the existence of a platelet prolactin receptor.

In a previous study, Wallaschowski et al. demonstrated by Western blot that human platelets expressed the short isoform of the prolactin receptor (12). The specificity of the signal detected can be questioned, however, since these authors used an antibody raised against the ligand-binding domain of the *rat* prolactin receptor, and this antibody does not cross-react with prolactin receptors from other species (Alexis Biochemicals; ALX-803-003-C100, clone T6 product specification sheet). Kawaguchi et al. recently showed that human monocytes stained for the prolactin receptor with flow cytometry (19). They used another anti-rat prolactin receptor monoclonal antibody, namely U5, which, according to the manufacturer (Affinity Bioreagents; MAI-610), recognizes the prolactin receptor from other species, including human. Therefore, we compared the U5 clone and the human receptor-specific 1A2B1 clone for their ability to detect the human prolactin receptor by flow cytometry. We first used HEK293 cells as an experimental model, as these cells are known not to endogenously express the prolactin receptor, but to express high amounts after transfection with an expression plasmid encoding the human prolactin receptor. Accordingly, less than 1.0% of parental (untransfected) cells stained with either U5 or 1A2B1 antibody, while after transfection, the number of positive cells raised up to 6.9% and 21.3% using U5 or 1A2B1 antibodies, respectively (data not shown). In addition, we tested the ability of 1A2B1 antibody to detect the prolactin receptor on the human breast cancer MCF7 cell line, which constitutively expresses the prolactin receptor (16). As expected, over 50% of the cells stained positively using this antibody (Fig. 6A and B). These observations are in analogy with a recent study, which identified 1A2B1 antibody as the more specific and reliable to detect the human prolactin receptor in breast cancer cell lines using immunohistochemistry (20). Although it is well known that antibodies can be very appropriate for some technical approaches but not for others (e.g. immunohistochemistry, flow cytometry, Western blot, etc), the poor cross-reactivity of U5 with the prolactin receptor of human origin reported by Galsgaard et al. may question the specificity of the signal reported by Kawaguchi et al. for monocytes (see above). Based on our preliminary

data and on the literature, we therefore selected 1A2B1 antibody as the most appropriate antibody to detect the prolactin receptor in human platelets by flow cytometry.

Previously, elevated levels of prolactin have been reported in patients suffering from atherothrombotic disease, such as ischaemic stroke, coronary syndromes or venous thromboembolism (1, 2). To which extent prolactin is associated with the underlying pathology leading to these disorders, however, is unclear. Wallaschowski et al. showed that prolactin may modulate platelet activation *in vitro* and *ex vivo* (1, 2, 12, 13). Their results, however, are contradicted by our present data, thus leaving the role of platelets as a link between prolactin and atherothrombosis unresolved.

In order to increase plasma concentrations of prolactin, Wallaschowski et al. mixed citrate whole blood of human healthy individuals with hyperprolactinemic plasma samples of pregnant women (13). The pregnant state is characterised by many changes, including substantial hormonal alterations such as enhanced prolactin levels. Thus, since plasma samples of hyperprolactinaemic individuals have many different levels of other hormones, cytokines, growth factors and various circulating factors as well, such a procedure does not allow concluding that the effects observed are only due to increased levels of prolactin.

Our present ex-vivo data are in agreement with the study of Atmaca et al., who assessed the possible effects of prolactin on platelets by comparing pregnant- and non-pregnant women (14). Despite higher levels of prolactin in pregnant (median 132 µg/l) versus non-pregnant (9 µg/l) women, the platelet function – as measured by the Platelet Function Analyser-100 – did not differ between the two populations.

An alternative mechanism through which prolactin may affect atherothrombosis is inflammation (21). Several other cell types involved in atherogenesis express functional prolactin receptors. These cell types may all differentiate into a more pronounced inflammatory phenotype in the presence of higher levels of prolactin (1, 5, 6, 8, 22). Against this background, we hypothesise that prolactin affects atherothrombosis by stimulating pro-inflammatory cells.

In conclusion, it is unlikely that prolactin directly affects platelet function. Recently, platelets have been implicated to play not only a role in thrombo-embolic disease, but also in the *development* of atherothrombosis through interaction with, for instance, inflammatory cells (23). With regard to the reported association between enhanced levels of prolactin and atherothrombosis, we postulate that platelets and prolactin are indeed both involved in this disease. Their involvement, however, is not necessarily via a direct prolactin-platelet interaction, but more likely to be mediated by components of the immune system (24).

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