H. Wallaschofski [1]
A. Kobsar [4]
M. Koksch [1]
A. Siegemund [1]
B. Hentschel [2]
U. Tuschy [3]
T. Lohmann [1]
O. Sokolova [4]
M. Eigenthaler [4]

Prolactin Receptor Signaling during Platelet Activation

Abstract

Prolactin is a newly recognized platelet coactivator that functions through potentiation of ADP-induced platelet activation. However, the possible association between hyperprolactinemia and venous thromboembolism (VTE) has not been systematically investigated up to now; prolactin signaling mechanisms in platelets still need to be elucidated. In this study, plasma prolactin levels in healthy subjects and patients with VTE were determined, demonstrating that patients with VTE and no other congenital risk factors had significantly increased plasma prolactin levels. Moreover, prolactinoma patients demonstrated a higher incidence of VTE than the general population. To elucidate the molecular mechanisms for the development of venous thrombosis, prolactin receptor signaling during platelet activation was investigated with a focus on ADP-stimulated G-protein-regulated signaling pathways. The short isoform of prolactin receptors was detected on platelets. Signaling through this receptor, although not directly linked to Gq-proteins, substitutes for Gq-protein regulated signaling pathways involved in platelet activation. We identified protein kinase C, a well-established signaling molecule in platelet activation, as a target molecule for prolactin signaling pathways in human platelets. Our findings indicate that hyperprolactinemia may be an important novel risk factor for VTE, suggesting that its thrombogenic effect may be mediated through enhanced platelet reactivity. Revealing the molecular mechanisms of prolactin signaling will allow the design of new antithrombotic therapies.

Key words
Prolactin · Thrombosis · Platelet activation · G proteins · Protein kinase C · Pituitary tumor

Abbreviations

JAK, janus kinases; PG-E_1, prostaglandin E_1; PRL, prolactin; PKC, protein kinase C; PLC, phospholipase C; STAT, signal transducer and activator of transcription; VASP, vasodilator-stimulated phosphoprotein; VTE, venous thromboembolism

Introduction

Venous thromboembolism (VTE) is a multifactorial disease with an incidence of 1 per 1000 and a high mortality of about 30% within 30 days [1]. The manifestation of VTE depends on a complex balance between possible congenital or/and acquired risk factors such as anti-phospholipid antibody syndrome, hyperhomocysteinemia due to vitamin deficiency, activated protein C resistance without factor V Leiden mutation and increased factor

Affiliation
[1] Department of Internal Medicine I, University of Erlangen, Germany
[2] Institute for Medical Informatics, Statistics and Epidemiology, University of Leipzig, Germany
[3] Department of Internal Medicine II, Hospital of Erfurt, Germany
[4] Institute of Clinical Biochemistry and Pathobiochemistry, University of Würzburg, Germany

Participation
T. Lohmann, M. Eigenthaler, H. Wallaschofski and A. Kobsar contributed equally.

Correspondence
T. Lohmann, MD · University of Erlangen · Department of Internal Medicine I ·
Krankenhausstraße 12 · 91054 Erlangen · Germany ·
Phone: +49 (913) 1 8536970 · Fax: +49 (913) 1 8533428 · E-Mail: Tobias.Lohmann@med1.imed.uni-erlangen.de

Received 2 July 2002 · **Accepted after revision** 10 September 2002

Bibliography
Horm Metab Res 2003; 35: 228–235 © Georg Thieme Verlag Stuttgart · New York · ISSN 0018-5043

VIII [2,3]. The known congenital risk factors for VTE include antithrombin, protein C, protein S deficiencies and especially the activated protein C resistance phenomenon attributable to the presence of the factor V Leiden mutation. The prothrombin G20210A mutation results in hyperprothrombinemia and hyperhomocysteinemia. Further conditions known to raise the risk for VTE are pregnancy [4,5] as well as tumors, increasing age, male gender, surgery, and trauma.

In a recent study, we demonstrated that hyperprolactinemia causes increased platelet aggregation via potentiation of ADP effects both *in vitro* and *in vivo*[6]. Moreover, our data indicated that prolactin may be a physiological cofactor for platelet activation in the delicate coagulation balance during pregnancy and puerperium. Hyperprolactinemia is part of the physiological preparation for delivery and breast-feeding in pregnancy or puerperium where an increased risk for VTE has been reported [4,5]. In another situation also associated with hyperprolactinemia, treatment with antipsychotic drugs [7], an increased incidence of VTE has been documented [8]. Therefore, we hypothesized that changes of prolactin might contribute to the hypercoagulable state in patients with VTE by causing the activation of platelets. However, possible associations between hyperprolactinemia and VTE has not been systematically investigated up to now, and epidemiological data of VTE in other hyperprolactinemic states such as prolactinoma still need evaluated.

In an attempt to investigate the possible influence of prolactin on VTE, we compared prolactin levels in healthy subjects, patients with VTE and congenital risk factors and patients with VTE but neither congenital nor acquired risk factors for VTE. Furthermore, we investigated the incidence of VTE in prolactinoma patients. The present study demonstrates that patients with VTE without congenital or acquired risk factors have higher levels of prolactin than those with congenital risk factors or healthy controls. This indicates that prolactin may be a candidate for an acquired VTE risk factor. This hypothesis is supported by the demonstration of higher VTE incidence in prolactinoma patients than in the general population. Furthermore, we will demonstrate the expression of the short form of the prolactin receptor on platelets and characterize the molecular mechanism of prolactin receptor signaling during platelet activation.

Subjects and Methods

Experimental subjects

194 consecutive VTE patients with a congenital risk (group A, see below) and VTE patients without a congenital or acquired risk (group B, see below) for VTE were investigated for prolactin values. Patients with VTE were assigned to group A or B retrospectively. Group A had 96 patients (51 female, 45-male, age: 50 ± 16 years) with VTE and heterozygous (n = 61) or homozygous (n = 5) factor V Leiden mutations and prothrombin G20210A mutations (n = 34). Group B had 98 patients (66 female, 32 male, age: 54 ± 13 years) with VTE without congenital or acquired risk factor for VTE. Factor V Leiden mutation, prothrombin G20210A mutation, antithrombin-, protein C-, protein- S deficiency, activated protein C resistance phenomenon, hyperhomocysteinemia, anti-phospholipid antibodies and increased factor VIII

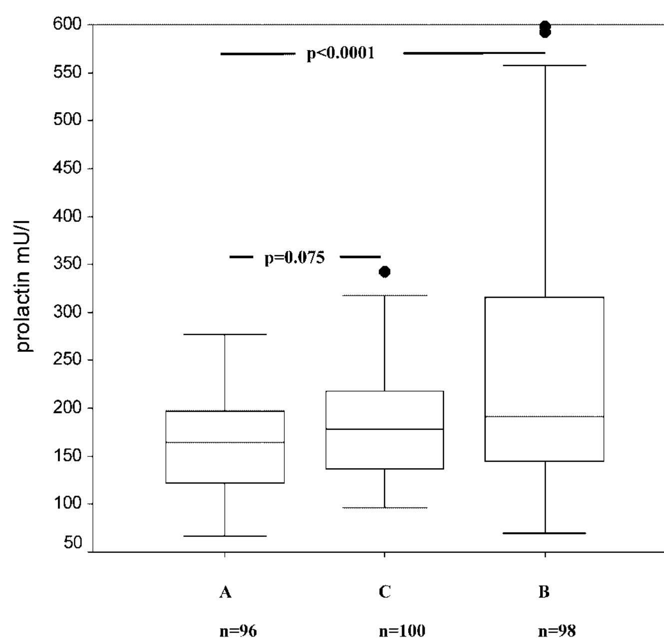

Fig. 1 Prolactin levels in patients with VTE without congenital or aquired risk factors are significantly higher than in patients with VTE and congenital risk factors or healthy controls. The PRL values of VTE patients with congenital risk factors (**a**) were compared to VTE patients without congenital or acquired risk factors (**b**) and healthy controls (**c**) by the Mann-Whitney U-test. Box plots represent 25th and 75th percentiles, error bars minimum or maximum of PRL values, the dots represent values above the maximum of PRL values of the group. P values are given above the plots.

levels were excluded as previously reported [9]. Plasma aliquots of VTE patients were stored after initial investigation at – 80 °C until prolactin determination. 100 healthy, sex and age matched people without history of VTE served as healthy controls (Group C in Fig. **1**) as previously reported [6].

In a retrospective study, we evaluated the documented incidence of VTE in 136 consecutive prolactinoma patients (79 female, 57-male; age: 39 ± 13 years). The diagnosis of prolactinoma based on histological examination or on prolactin levels above 10-fold of the upper limit of the PRL assay at diagnosis and mostly tumor shrinkage in magnetic resonance imaging by dopamine agonist therapies. After screening our 136 patients, 62 cases (38 female, 24 male, age: 37 ± 12.2 years) were identified that had completely documented case histories and database for diagnostics tests during the time of hyperprolactinemia and observation. We evaluated the period of hyperprolactinemia in these 62 patients, which was defined as time in years between first symptoms of hyperprolactinemia or tumor size and documented normalization of PRL by therapy. The summarized period of hyperprolactinemia was 359 years for the 62 patients (5.8 ± 4.6 years per patient). Three of the 62 patients suffered from VTE (1 pulmonary embolism and 2 deep vein thrombosis) during the period of hyperprolactinemia.

Materials and Methods

Monoclonal antibody recognizing the short 43kDa isoform of the prolactin receptor family was obtained from Alexis Biochemicals. Monoclonal antibody recognizing the α-, β- and γ-isoforms of protein kinase C (pan-PKC) was obtained from Abcam Ltd. Polyclonal antibody to identify phosphorylation of α, β-I, β-II, γ and δ PKC isoforms at the C-terminal phosphorylation site corresponding to serine 660 in PKC β-II was obtained from New England BioLabs. Serotonin and purified recombinant human prolactin were purchased from Sigma.

Determination of serum prolactin

The plasma prolactin values of healthy controls and patients with VTE were analyzed by Axsym Prolactin Assay (Abbott, USA). Serum prolactin values were considered normal if they were < 580 mU/l (24.2 ng/ml) in females and < 450 mU/l (18.77 ng/ml) in males. This assay was performed according to the manufacturer's instructions as previously reported [6].

Isolation and stimulation of human platelets

Blood collection from informed healthy human volunteers and preparation of platelet-rich plasma and washed platelets were prepared as described [10]. Washed platelets (1×10^9 platelets/ml) were resuspended in PBS with 1 mM EDTA and 5.5 mM D-glucose. $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM) were added to platelet suspension before preincubation with buffer, 1000 mU/l or 5000 mU/l of recombinant PRL (final concentration) for 5 min at 37 °C, followed by activation with 4 µM ADP. At various time points, aliquots of the cell suspension were removed and reaction was stopped by addition of SDS stop solution for Western blot analysis.

Platelet aggregation assay

Platelet aggregation was determined in a PAP-4 aggregometer (Biodata) as described [11]. Briefly, aggregation was measured in 300 µl samples of PRP that were preincubated with buffer, 1000 mU/l or 5000 mU/l PRL (final concentration) for 5 min at 37 °C and stimulated with ADP (2 µM), Epinephrine (0.7 µM), serotonin (5 µM) or a combination of these substances. The degree of platelet aggregation was measured as percent of change in light transmission of PRP in relation to the light transmission of platelet poor plasma (PPP).

Western blotting and quantification

Western blotting was performed as described [10]. Samples were separated on 9 % SDS-polyacrylamide gels, blotted onto polyvinylidine fluoride (PVDF) membranes that were blocked by 6 % phosphatase-free milk powder in Tris-buffered saline with 0.05 % Tween-20 (TBS/Tween). Proteins were stained with anti-PRL-R antibody (1 : 500 dilution), anti-phospho-PKC (1 : 1000 dilution) or anti-PKC pan (phosphorylation state independent) (1 : 1000) antibodies overnight at 4 °C. Immunoreactivity was determined using peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1 : 3000 dilution for both of these antibodies) and the ECL chemiluminescence reaction. Intensity of Western blot bands was quantified as described previously [10].

Flow cytometry

Flow cytometric analysis of intracellular VASP phosphorylation in platelets was performed as previously described [10]. Briefly, platelet-rich plasma was incubated for 3 min with 0.5 µM PG-E1 with or without 20 µM ADP, 1 µM epinephrine, 1000 mU/l or 5000 mU/l prolactin or 5 µM serotonin. The reaction was stopped by addition of 3 % final concentration of methanol-free formaldehyde followed by permeabilization by 0.2 % Triton X-100 in phosphate-buffered saline. Staining was performed using the VASP-Serine-157 phosphorylation-specific antibody (dilution) and fluorescein isothiocyanate (FITC)-conjugated, affinity-purified goat anti-mouse IgG (Sigma, St. Louis,MO). Data were analyzed at a low flow rate on a Becton Dickinson FACSCalibur with the settings described earlier [10].

Statistical analysis

The Mann-Whitney U-test was used to compare the prolactin values between healthy controls, patients with VTE with congenital (group A) and without congenital and acquired risk factors (group B). Bonferroni correction was used for multiple comparisons. The incidence rate of VTE was calculated as the number of cases of thromboembolism during the period of hyperprolactinemia divided by the number of person-years. The risk ratios for thromboembolism during the period of hyperprolactinemia in comparison with the risk for thromboembolism of two populations [12,13] were estimated at 95 % confidential intervals. The increase in PKC phosphorylation was tested by Student's *t*-test; p-values less than 0.05 were considered statistically significant. Data were analyzed using SPSS for Windows (release 9.0.1).

Results

Prolactin levels in patients with VTE without congenital or acquired risk factors are significantly higher than in patients with VTE and congenital risk factors or healthy controls

The mean values of PRL were 185 ± 60 mU/l and 165 ± 50 mU/l in the healthy controls and patients with congenital risk for VTE, respectively. These levels were not significantly different after Bonferroni correction (Fig. **1**, p = 0.075). In contrast, in patients with VTE but without congenital or acquired risk factors, the mean PRL value was 285 ± 351 mU/l, therefore significantly higher than in those patients with congenital risk factors (p < 0.0001. Fig. **1** shows median, 25th and 75th percentile, maximum and minimum of prolactin values). Furthermore, the PRL values in 6 of the patients with VTE without risk factors were above the maximum in the group (PRL levels between 635 and 3028 mU/l; dots in Fig. **1**).

Increased incidence of VTE in patients with prolactinoma

We found 6 patients with documented VTE in our series of 136 prolactinoma patients; three of them had also documented pulmonary embolism. A further 18/136 patients had indication for previous VTE, such as post-thrombotic syndrome. Complete documentation of case history and PRL levels was available in 62 of the 136 patients during the period of hyperprolactinemia. Three cases of VTE were documented in this subgroup with a total hyperprolactinemia history of 359 years (mean 5.8 ± 4.6 years per patient). The incidence of 3 VTE during 359 years of hyperprolactinemia is significantly elevated compared to the documented

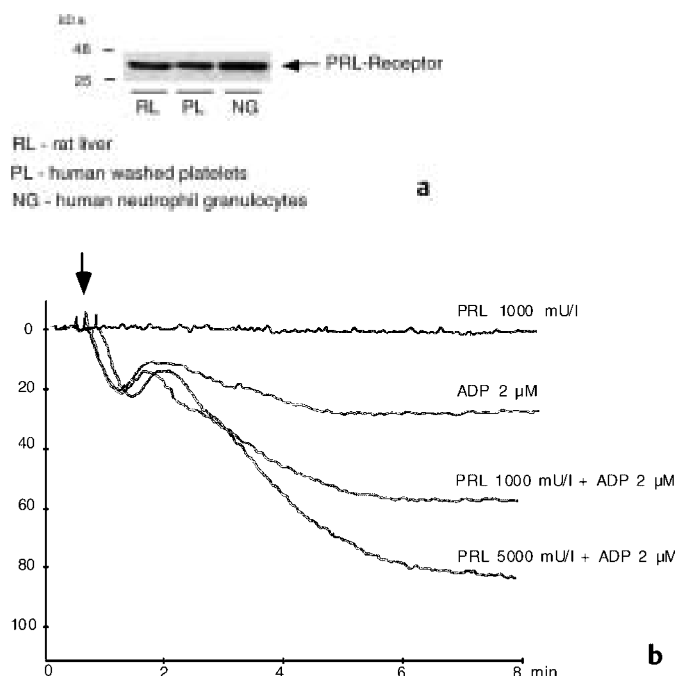

Fig. 2 Detection of the short isoform of prolactin receptors on human platelets (**a**) and potentiation of ADP-induced platelet aggregation by purified, recombinant human prolactin (**b**). (**a**) Autoradiograph showing the presense of the short isoform of prolactin receptors in rat liver (24 µg homogenate), washed human platelets (15 µg homogenate) and human neutrophil granulocytes (10 µg homogenate). Western blot analysis was performed as described in Methods. (**b**) Effect of recombinant human prolactin on ADP-induced platelet aggregation. PRP was preincubated with buffer, 1000 mU/l or 5000 mU/l prolactin as described in Methods. Platelet aggregation was induced by addition of 2 µM ADP as indicated by the arrow. Aggregation curves shown are representative for at least three independent experiments.

annual incidence of VTE (627/342 000, RR = 4.7, 95 % CI 1.5 – 14.5) or idiopathic VTE (230/1 000 000, RR = 36.5 95 % CI 11.7 – 114.1) in Caucasoid persons older than 18 years [12,13]. Therefore, hyperprolactinemia in patients with prolactinoma is associated with an in increased risk for VTE.

A short isoform of prolactin receptors is present on human platelets

Several prolactin receptors have been detected in extracts from various tissues and cell types; however, whether a receptor for prolactin is present on human platelets has not been elucidated. Using a monoclonal antibody recognizing a 43 kDa short isoform of the prolactin receptor family, we could demonstrate the presence of this receptor on human platelets (Fig. **2a**). As a positive control, we used extracts from rat liver and human neutrophil granulocytes, both known to contain this prolactin receptor isoform [14,15].

ADP-induced platelet aggregation is potentiated by purified, recombinant human prolactin

We have demonstrated the potentiating effect of plasma from patients with high prolactin levels on ADP-induced platelet activation [6]. They exclude other substances in the plasma that could contribute to this effect; we incubated human platelets with purified, recombinant human prolactin. While low concentrations of ADP induced weak and reversible platelet aggregation, preincubation with 1000 mU/l or 5000 mU/l prolactin induced a dose-dependent potentiation of ADP-induced platelet aggregation leading to full platelet aggregation in the presence of 5000 mU/l prolactin (Fig. **2b**). Incubation with prolactin alone did not induce platelet aggregation (Fig. **2b**). No prolactin-mediated potentiation was detectable on thrombin- or collagen-induced platelet aggregation (data not shown).

Prolactin can substitute for Gq-protein-regulated signaling pathways necessary for human platelet aggregation

Signaling events from both the platelet $P2Y_1$ ADP receptor (Gq-protein coupled) and the $P2Y_{12}$ ADP receptor (Gi-protein coupled) are essential for complete ADP-induced platelet aggregation [16,17]. Analogous to $P2Y_{12}$ receptor activation, epinephrine acts on platelets via its α2a-adrenergic receptor that is coupled to Gi, whereas serotonin through the 5-hydroxytryptamine receptor subtype 2A stimulates Gq-regulated signaling pathways analogous to the $P2Y_1$ receptor [16,18]. To characterize the molecular mechanism by which prolactin potentiates the platelet ADP response, we co-stimulated human platelets with prolactin and epinephrine or serotonin. While prolactin and epinephrine alone did not induce platelet aggregation, the combination of both substances resulted in complete platelet aggregation (Fig. **3a**). Incubation of platelets with serotonin resulted in very weak platelet aggregation (less than 20 %, Fig. **3b**), and the combination of serotonin and prolactin did not increase platelet aggregation any further compared to serotonin alone (Fig. **3b**). As a control, platelets were stimulated with both epinephrine and serotonin resulting in complete aggregation (Fig. **3c**). These experiments indicate that prolactin-signaling pathways can substitute Gq-regulated signaling pathways.

Platelet shape change is known to be induced by Gq-regulated signaling pathways ([16] and model in Fig. **6**). Analysis of shape change in our experiments revealed that only serotonin induced platelet shape change, whereas prolactin did not (Fig. **3d**), indicating that prolactin receptors might not be directly linked to the Gq-protein but might signal through mechanisms downstream of Gq. A potential site of prolactin receptor signaling is protein kinase C, known to be one of the key enzymes in Gq-regulated signaling cascades (Fig. **6**).

ADP and prolactin synergistically activate protein kinase C

To test this hypothesis, washed human platelets were incubated with buffer, prolactin (PRL), ADP, or both ADP and prolactin. Aliquots were removed at 1 and 3 minutes and analyzed by Western blot technique for phosphorylation of protein kinase C at the site corresponding to serine 660 in PKCβ-II. Phosphorylation of that site correlates with PKC activity [19]. While ADP induced only a slight increase in PKC phosphorylation under these conditions, prolactin induced a significant stronger increase in PKC phosphorylation than ADP (Fig. **4**). Incubation with both prolactin and ADP resulted in an additive effect on PKC phosphorylation increasing PKC phosphorylation more than 50 % compared to ADP alone ($p < 0.006$, Fig. **4**).

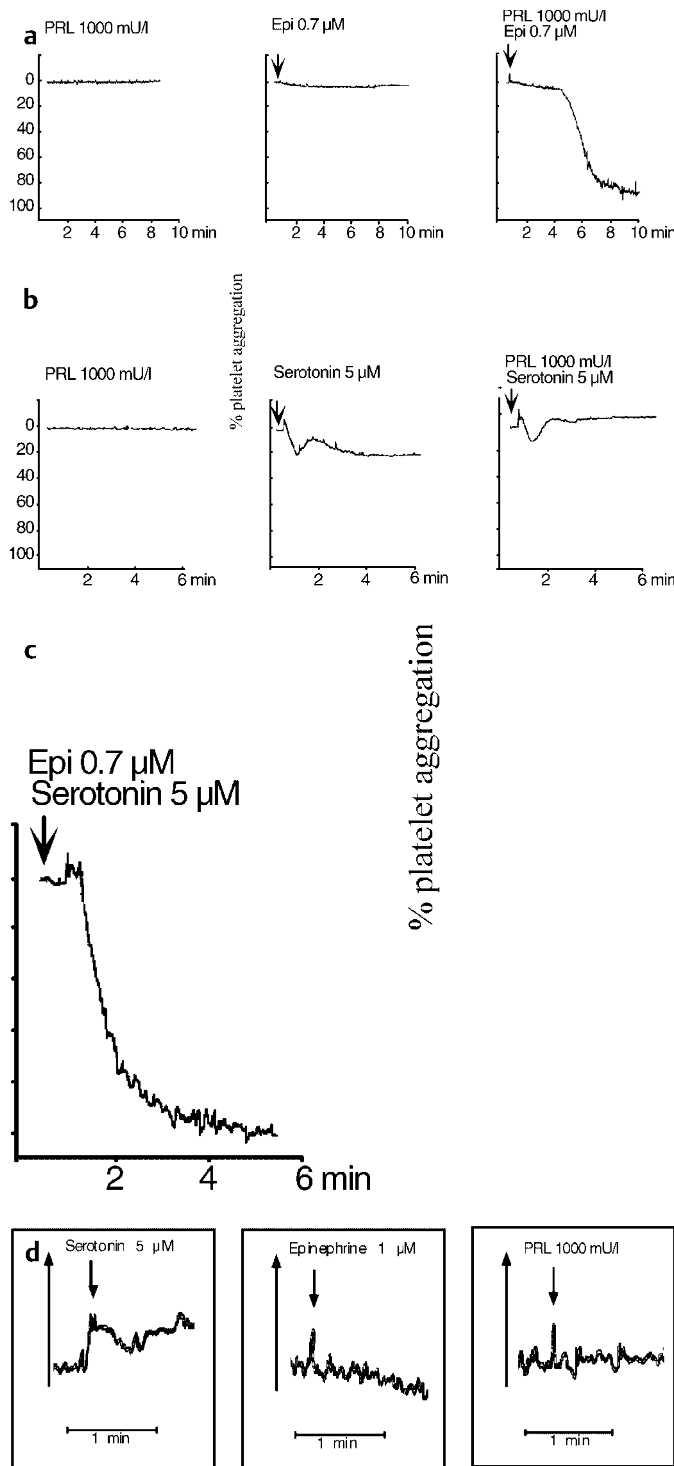

Fig. 3 Effect of prolactin on G-protein-regulated signaling pathways necessary for human platelet aggregation and shape change. (**a**) PRP was preincubated with buffer or 1000 mU/l recombinant human prolactin (PRL) for 5 minutes as indicated. Platelet aggregation was induced by addition of 0.7 μM epinephrine (Epi), 5 μM serotonin or both epinephrine and serotonin as indicated by the arrow. (**b**) Platelet shape change is induced by serotonin, but not by prolactin or epinephrine. Platelets were stimulated with 5 μM serotonin, 1 μM epinephrine (Epi) or 1000 mU/l prolactin. Aggregation curves shown are representative for at least three independent experiments.

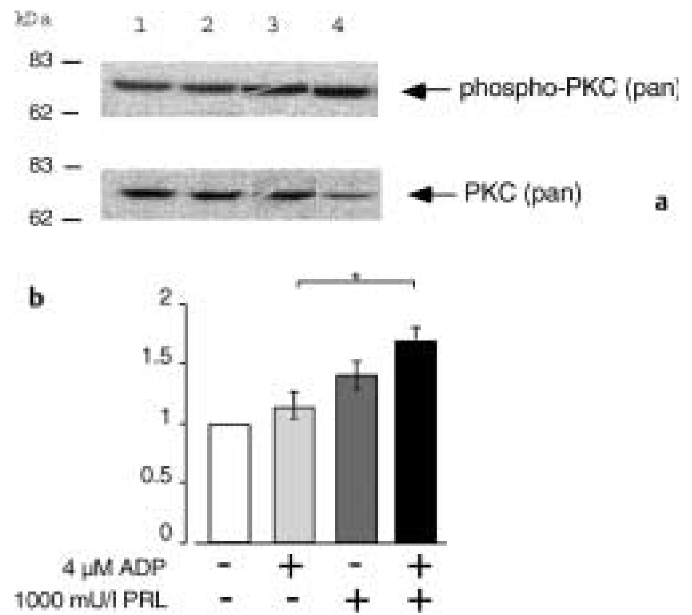

Fig. 4 ADP and prolactin synergistically activate protein kinase C. (**a**) Washed human platelets were preincubated with buffer or 1000 mU/l prolactin (PRL) for 5 minutes. Then, platelets were incubated with buffer or 4 μM ADP, and aliquots of the platelet suspension were removed and stopped at 1 and 3 minutes (the autoradiographs of this figure represent the time point 3 minutes, data for 1 min are not shown). Quantification of protein kinase C phosphorylation (panel A, upper autoradiograph) and total amount of protein kinase C (panel A, lower autoradiograph) was performed as described in Methods. Autoradiographs are representative for at least three independent experiments. (**b**) For quantitative analysis of PKC phosphorylation, image analysis was performed as described in Methods, and the increase in PKC phosphorylation was expressed as fold increase relative to basal PKC phosphorylation in the presence of buffer only. Data shown are mean values ± SEM of three independent experiment. Increase in PKC phosphorylation was tested by student t-test for significance (*p < 0.006).

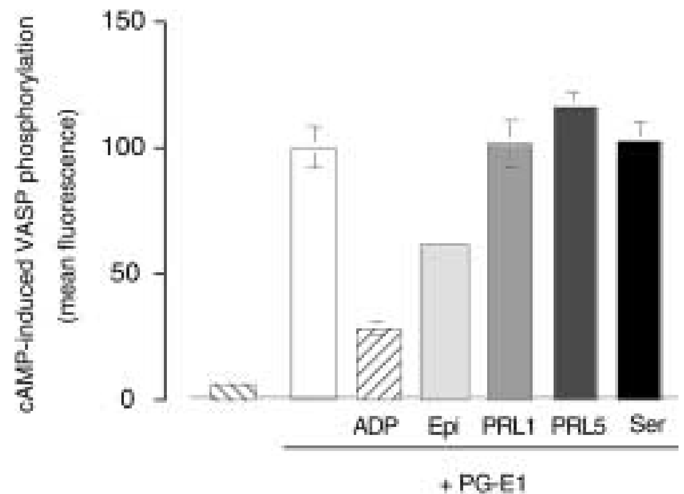

Fig. 5 Prolactin does not stimulate a Gi-coupled signaling pathway in platelets. PG-E_1-induced platelet VASP phosphorylation is inhibited by Gi-regulated signaling pathways. Platelet-rich plasma was incubated for 3 min with 0.5 μM PG-E_1 with or without 20 μM ADP, 1μM epinephrine, 1000 (PRL1) or 5000 (PRL5) mU/l prolactin or 5μM serotonin as indicated in the figure. Analysis of cAMP-induced platelet VASP-phosphorylation using a VASP-Serine-157 phosphorylation-specific antibody was performed as described in Methods. Histograms show mean fluorescence of the binding of this antibody. Data represent means ± SEM of 3 independent experiments.

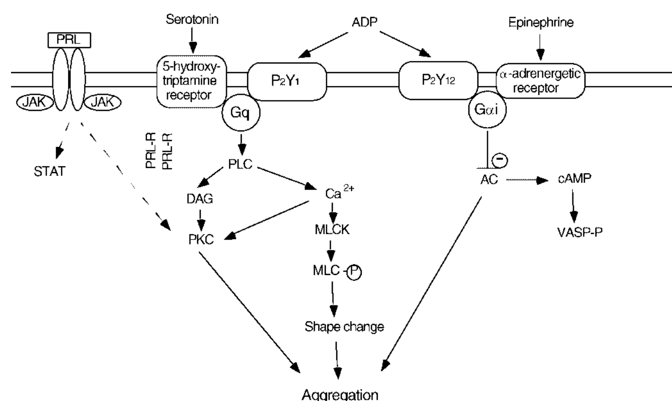

Fig. 6 Model depicting the potential signal transduction mechanisms and physiological effects of prolactin in human platelets. Abbreviations: DAG, 1,2-diacylglycerol; JAK, janus kinases; MLC, myosin light chain; MLCK, MLC kinase; PLC, phospholipase C; STAT, signal transducer and activator of transcription, VASP, vasodilator-stimulated phosphoprotein.

Prolactin does not stimulate Gi-regulated signaling pathways that inhibit adenylyl cyclase/cAMP-regulated pathways in human platelets

Flow cytometry analysis of intracellular phosphorylation of the Vasodilator-stimulated phosphoprotein (VASP) is a useful tool to characterize the coupling of platelet receptors to Gi-proteins [10]. Incubation with PG-E_1 induced strong VASP phosphorylation at serine-157 in platelets. However, co-stimulation of platelets with PG-E_1 and ADP or epinephrine almost completely inhibited PG-E_1/cAMP-induced VASP phosphorylation due to a well-characterized Gi-mediated inhibitory effect on adenylyl cyclase (Fig. **5** and [10,11]). In contrast, prolactin failed to inhibit PG-E_1/cAMP-induced VASP phosphorylation – again indicating that prolactin is not linked to Gi-regulated signaling pathways (Fig. **5**). As expected and known, serotonin did not stimulate the Gi-regulated pathway, either ([20] and Fig. **5**).

Discussion

Beyond the well-known plasmatic factors, the pathogenesis of thrombosis involves complex platelet-leukocyte interactions whose details have not fully been elucidated [21]. P-selectin mediates rolling of platelets and leukocytes on activated endothelial cells. Recent data indicate that P-selectin interaction with a ligand stabilizes initial GP IIb/IIIa- fibrinogen interactions, thus allowing the formation of large stable platelet aggregates [22]. Furthermore, in the Pulmonary Embolism Prevention Trial (PEP), it was been demonstrated that aspirin could reduce VTE by at least a third throughout a period of increased risk [23]. These results, along with those of previous meta-analysis [24,25], suggest that the activation of platelets is an important initial step in the development of venous thrombosis. Up to now, the significance of activated platelets for the development of VTE has not been investigated systematically, whereas the influence of platelet activation on arterial thrombosis is undisputed.

In a recent study, we demonstrated a correlation between PRL levels and platelet activation in pregnant women and patients with hyperprolactinemic pituitary tumors [6]. The possible association between hyperprolactinemia and VTE has not been systematically investigated up to now; epidemiological data of VTE in patients with pituitary tumors are lacking. Now, we have found significantly higher PRL levels in VTE patients without congenital or acquired risk factors compared to patients with such risk factors or healthy controls. Most patients with idiopathic VTE showed PRL values that were still in the upper normal range. However, this finding corresponds to the dose-dependent increased platelet activation and aggregation by human PRL *in vitro* that already occurs within the normal plasma prolactin range (Fig. **2 b** and [6]).

Moreover, hyperprolactinemic prolactinoma patients have a significantly higher incidence of VTE and of idiopathic VTE than the general population. We have used a very strict approach, counting only well-documented, safely diagnosed cases of VTE. All cases we have included were based on objective tests: duplex sonography or venography for deep vein thrombosis, pulmonary angiography, and helicoidal CT scan for pulmonary embolism. We excluded the 18 patients with some indication of previous VTE as post-thrombotic syndrome in our statistical analysis, which may have resulted in an underestimation of cases. Altogether, our epidemiological data suggest that hyperprolactinemia is a candidate for a novel acquired risk factor for VTE, especially idiopathic VTE, which might explain the increased risk for VTE in hyperprolactinemic states. Currently, we are investigating the influence of hyperprolactinemia in prolactinoma patients on the risk of VTE in a prospective trial. If we can confirm our hypothesis, we would conclude that the objective of therapy for hyperprolactinemia especially in prolactinoma patients should be the suppression of PRL levels in the lower normal range, not only a restoration of PRL levels to the upper normal range [26] to minimize the risk of VTE.

PRL-R is widely distributed in mammalian tissues and cells [27]. Multiple isoforms of membrane-bound PRL-R resulting from alternative splicing of the primary transcript have been identified. They differ in the length and composition in their cytoplasmic tails, and are referred to as short, intermediate or long forms of PRL-R with respect to their molecular weight, which ranges from 39 to 110 kDa depending on the cell type and the species investigated. In addition to membrane-anchored PRL-R, soluble isoforms have been identified, but whether they result from alternative splicing of the primary mRNA or proteolytic cleavage of membrane-bound PRL-R is yet unknown [28]. A 43 kDa short isoform of this receptor has been identified [14,29] In human hematopoietic progenitor cells. Using an antibody directed against the short form of the PRL-R present in rat liver that cross-reacts with the human receptor, we have now demonstrated the presence of a short 43 kDa isoform of the PRL-R in human platelets (Fig. **2 a**).

To the best of our knowledge, functional effects and mechanisms of PRL action in platelets are unknown. In a recent study, we demonstrated that increased plasma PRL levels potentiate ADP effects on platelet P-Selectin expression and platelet aggregation [6]. There is very little in the way of data regarding functional effects and mechanisms of PRL action in platelets. In mammalian cells, PRL receptors are non-kinase receptors – their activation requires participation of receptor associated kinases known as

janus kinases (JAK) [30, 31]. Dimerization of the receptor induces tyrosine phosphorylation and activation of the JAKs followed by phosphorylation of the receptor. JAK activation results also in phosphorylation of the cytoplasmic factor complex termed signal transducer and activator of transcription (STAT), which translocates into the nucleus and activates transcription of specific genes in mammalian cells [32, 33]. Important signaling molecules involved in platelet activation are regulated in other cell systems by PRL receptors, including the Ras/Raf/MAP kinase pathways [34 – 36] as well as signaling molecules like PI-3 kinase, PLC gamma and PKC [37 – 39].

In platelets, the molecular mechanisms of the PRL action still have to be resolved. Several signaling molecules of PRL receptor-regulated pathways in mammalian cells are also present in human platelets. Since there is clear evidence that PRL can influence signal transduction through G-protein-coupled receptors [40 – 42], we investigated effects of PRL on G-protein-regulated signaling pathways in platelet activation. ADP effects on platelets are mediated by at least two different G-protein-coupled receptors – $P2Y_1$ and $P2Y_{12}$ (formerly termed $P2Y_{AC}$) [16, 20, 43]. $P2Y_1$ is coupled to stimulatory Gq-proteins and activates phospholipase C, whereas $P2Y_{12}$ signals via a Gi protein that inhibits platelet adenylate cyclase function and lowers platelet cAMP [43]. Both receptor pathways are necessary for complete ADP-induced platelet aggregation [16,17,43]. ADP action on these receptors can be mimicked by receptors coupling to functional identical G-proteins. Serotonin, a weak platelet agonist, activates Gq-coupled signaling pathways, but not Gi ([16,17,43] and Fig. **5**). In contrast, epinephrine activates platelets through a Gi-protein, but not through Gq [16,17]. Co-stimulation of platelets with prolactin and these substances demonstrates that prolactin does not signal through Gi-regulated pathways, but is able to substitute for Gq-regulated signaling pathways (Fig. **3**). The complex signaling mechanisms of prolactin need further investigation. Phosphorylation of PKC could be induced by activation of janus kinases, which induces a phosphorylation cascade involving PKC, thereby promoting platelet activation. It has been shown that thrombin activation of JAK3 in platelets is followed by tyrosine phosphorylation of STAT-1 and STAT-3. Inhibition of JAK3 prevented thrombin-induced platelet shape change, serotonin release and platelet aggregation [44]. It is very likely that the PRL receptor is not directly linked to a Gq-protein since incubation with PRL alone did not induce platelet shape change (Fig. **3d**). Although it does not induce platelet aggregation, prolactin by itself induced PKC activation in platelets and increased ADP-induced activation of PKC in an additive way (Fig. **4b**). Similar mechanisms of signaling have been shown for luteinized rat granulosa cells, in which PKC clearly is a downstream signaling molecule of the prolactin receptor [37]. Revealing the molecular mechanisms of prolactin signaling in human platelets will contribute to a better understanding of the thrombogenic effect of this hormone, and may allow new therapeutic strategies for the anti-thrombotic treatment in certain risk situations and thrombotic diseases.

Acknowledgement

A. K. was supported by a grant from the BMBF (IZKF Würzburg, TP E15). O.S was supported by the *Ernst und Hedda Wollheim Stiftung* and the *Deutsche Forschungsgemeinschaft* (DFG GR 1952/1 – 1).

References

1 Heit JA, Silverstein MD, Mohr DN, Petterson TM, Lohse CM, O'Fallon WM, Melton LJ 3rd. The epidemiology of venous thromboembolism in the community. Thromb Haemost 2001; 86: 452 – 463
2 Rosendaal FR. Venous thrombosis: a multicausal disease. Lancet 1999; 353: 1167 – 1173
3 Rosendaal FR. Venous thrombosis: prevalence and interaction of risk factors. Haemostasis 1999; 29 Suppl S1: 1 – 9
4 Bates SM, Ginsberg JS. Thrombosis in pregnancy. Curr Opin Hematol 1997; 4: 335 – 343
5 Greer IA. Thrombosis in pregnancy: maternal and fetal issues. Lancet 1999; 353: 1258 – 1265
6 Wallaschofski H, Donné M, Eigenthaler M, Hentschel B, Faber R, Stepan H, Koksch M, Lohmann T. Prolactin as a novel potent cofactor for platelet aggregation. J Clin Endocrinol Metab 2001; 86: 5912 – 5919
7 David SR, Taylor CC, Kinon BJ, Breier A. The effects of olanzapine, risperidone, and haloperidol on plasma prolactin levels in patients with schizophrenia. Clin Ther 2000; 22: 1085 – 1096
8 Zornberg GL, Jick H. Antipsychotic drug use and risk of first-time idiopathic venous thromboembolism: a case-control study. Lancet 2000; 356: 1219 – 1223
9 Tripodi A, Mannucci PM. Laboratory investigation of thrombophilia. Clin Chem 2001; 47: 1597 – 1606
10 Schwarz UR, Geiger J, Walter U, Eigenthaler M. Flow cytometry analysis of intracellular VASP phosphorylation for the assessment of activating and inhibitory signal transduction pathways in human platelets–definition and detection of ticlopidine/clopidogrel effects. Thromb Haemost 1999; 82: 1145 – 1152
11 Geiger J, Honig-Liedl P, Schanzenbacher P, Walter U. Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. Eur J Pharmacol 1998; 351: 235 – 246
12 Oger E. Incidence of venous thromboembolism: a community-based study in Western France. EPI-GETBP Study Group. Groupe d'Etude de la Thrombose de Bretagne Occidentale. Thromb Haemost 2000; 83: 657 – 660
13 White RH, Zhou H, Romano PS. Incidence of idiopathic deep venous thrombosis and secondary thromboembolism among ethnic groups in California. Ann Intern Med 1998; 128: 737 – 740
14 Bellone G, Geuna M, Carbone A, Silvestri S, Foa R, Emanuelli G, Matera L. Regulatory action of prolactin on the *in vitro* growth of CD34+ human hemopoietic progenitor cells. J Cell Physiol 1995; 163: 221 – 231
15 Draca S. Prolactin as an immunoreactive agent. Immunol Cell Biol 1995; 73: 481 – 483
16 Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc Natl Acad Sci U S A 1998; 95: 8070 – 8074
17 Savi P, Beauverger P, Labouret C, Delfaud M, Salel V, Kaghad M, Herbert JM. Role of P2Y1 purinoceptor in ADP-induced platelet activation. FEBS Lett 1998; 422: 291 – 295
18 Daniel JL, Dangelmaier C, Jin J, Kim YB, Kunapuli SP. Role of intracellular signaling events in ADP-induced platelet aggregation. Thromb Haemost 1999; 82: 1322 – 1326
19 Keranen LM, Dutil EM, Newton AC. Protein kinase C is regulated *in vivo* by three functionally distinct phosphorylations. Curr Biol 1995; 5: 1394 – 1403
20 Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. J Biol Chem 1998; 273: 2030 – 2034
21 Celi A, Lorenzet R, Furie B, Furie BC. Platelet-leukocyte-endothelial cell interaction on the blood vessel wall. Semin Hematol 1997; 34: 327 – 335
22 Merten M, Thiagarajan P. P-selectin expression on platelets determines size and stability of platelet aggregates. Circulation 2000; 102: 1931 – 1936
23 Pulmonary Embolism Prevention trial . Prevention of pulmonary embolism and deep vein thrombosis with low dose aspirin: Pulmonary Embolism Prevention (PEP) trial. Lancet 2000; 355: 1295 – 1302

[24] Collins R, Baigent C, Sandercock P, Peto R. Antiplatelet therapy for thromboprophylaxis: the need for careful consideration of the evidence from randomised trials. Antiplatelet Trialists' Bmj 1994; 309: 1215 – 1217

[25] Antiplatelet Trialists' Collaboration. Collaborative overview of randomised trials of antiplatelet therapy–III: . Reduction in venous thrombosis and pulmonary embolism by antiplatelet prophylaxis among surgical and medical patients. BMJ 1994; 308: 235 – 246

[26] Molitch ME, Thorner MO, Wilson C. Management of prolactinomas. J Clin Endocrinol Metab 1997; 82: 996 – 1000

[27] Nagano M, Kelly PA. Tissue distribution and regulation of rat prolactin receptor gene expression. Quantitative analysis by polymerase chain reaction. J Biol Chem 1994; 269: 13 337 – 13 345

[28] Postel-Vinay MC. Growth hormone- and prolactin-binding proteins: soluble forms of receptors. Horm Res 1996; 45: 178 – 181

[29] Dardenne M, de Moraes M, Kelly PA, Gagnerault MC. Prolactin receptor expression in human hematopoietic tissues analyzed by flow cytofluorometry. Endocrinology 1994; 134: 2108 – 2114

[30] Rui H, Kirken RA, Farrar WL. Activation of receptor-associated tyrosine kinase JAK2 by prolactin. J Biol Chem 1994; 269: 5364 – 5368

[31] Goffin V, Kelly PA. The prolactin/growth hormone receptor family: structure/function relationships. J Mammary Gland Biol Neoplasia 1997; 2: 7 – 17

[32] Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev 1998; 19: 225 – 268

[33] Frank DA. STAT signaling in the pathogenesis and treatment of cancer. Mol Med 1999; 5: 432 – 456

[34] Rao YP, Buckley DJ, Buckley AR. Rapid activation of mitogen-activated protein kinase and p21ras by prolactin and interleukin 2 in rat Nb2 node lymphoma cells. Cell Growth Differ 1995; 6: 1235 – 1244

[35] Erwin RA, Kirken RA, Malabarba MG, Farrar WL, Rui H. Prolactin activates Ras via signaling proteins SHC, growth factor receptor bound 2, and son of sevenless. Endocrinology 1995; 136: 3512 – 3518

[36] Clevenger CV, Torigoe T, Reed JC. Prolactin induces rapid phosphorylation and activation of prolactin receptor-associated RAF-1 kinase in a T-cell line. J Biol Chem 1994; 269: 5559 – 5565

[37] Peters CA, Maizels ET, Robertson MC, Shiu RP, Soloff MS, Hunzicker-Dunn M. Induction of relaxin messenger RNA expression in response to prolactin receptor activation requires protein kinase C delta signaling. Mol Endocrinol 2000; 14: 576 – 590

[38] Rao YP, Buckley DJ, Olson MD, Buckley AR. Nuclear translocation of prolactin: collaboration of tyrosine kinase and protein kinase C activation in rat Nb2 node lymphoma cells. J Cell Physiol 1995; 163: 266 – 276

[39] Vacher P, Tran VanChuoi M, Paly J, Djiane J, Dufy B. Short term effect of prolactin on intracellular calcium in Chinese hamster ovary cells stably transfected with prolactin receptor complementary deoxyribonucleic acid. Endocrinology 1994; 134: 1213 – 1218

[40] Larrea F, Sanchez-Gonzalez S, Mendez I, Garcia-Becerra R, Cabrera V, Ulloa-Aguirre A. G protein-coupled receptors as targets for prolactin actions. Arch Med Res 1999; 30: 532 – 543

[41] Johansen PW, Lund HW, Gordeladze JO. Specific combinations of G-protein subunits discriminate hormonal signalling in rat pituitary (GH(3)) cells in culture. Cell Signal 2001; 13: 251 – 256

[42] Kline JB, Moore DJ, Clevenger CV. Activation and association of the Tec tyrosine kinase with the human prolactin receptor: mapping of a Tec/Vav1-receptor binding site. Mol Endocrinol 2001; 15: 832 – 841

[43] Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, and KunapuliSP. Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. J Biol Chem 1998; 273: 2024 – 2029

[44] Tibbles HE, Vassilev A, Wendorf H, Schonhoff D, Zhu D, Lorenz D, Waurzyniak B, Liu XP, Uckun FM. Role of a JAK3-dependent biochemical signaling pathway in platelet activation and aggregation. J Biol Chem 2001; 276: 17 815 – 17 822