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PRL as a Novel Potent Cofactor for Platelet Aggregation

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Pregnancy (including puerperium) is a period of hypercoagulability and seems to be an independent major risk factor for venous thromboembolism (VTE). However, the basis of the increased risk of VTE in pregnancy and around delivery is unknown. We hypothesized that changes in PRL, which is a prominently increased hormone during pregnancy and lactation, might be involved in the activation of platelets.

To investigate platelet functional abnormalities in pregnancy, we assessed the ADP-stimulated and nonstimulated P-selectin expression of platelets in 42 consecutive pregnant women, 22 normo- and hyperprolactinemic patients with pituitary tumors, and controls. In addition, the aggregation of platelets by human PRL *in vitro* was studied. We found a significant correlation between PRL values and ADP stimulation of platelets in pregnant women ($r = 0.56$; $P < 0.0001$) and patients with pituitary tumors ($r = 0.57$; $P = 0.006$). Hyperprolactinemic pregnant women or hyperprolactinemic patients with pituitary tumors revealed significantly higher ADP stimulation of platelets ($P < 0.0001$) than healthy controls or normoprolactinemic patients with pituitary tumors. These results were reconciled by increased *in vitro* stimulation and aggregation of platelets using human PRL.

Our novel findings demonstrate that hyperprolactinemia causes increased platelet aggregation via ADP stimulation both *in vitro* and *in vivo*. Moreover, our data indicate that PRL may be a physiological cofactor of the delicate coagulation balance during pregnancy and puerperium that might explain the increased risk of VTE in pregnant women around delivery. Further studies of the interaction between PRL and platelets will clarify the clinical relevance of hyperprolactinemia as a potential risk factor for VTE. (*J Clin Endocrinol Metab* 86: 5912–5919, 2001)

VENOUS THROMBEMBOLISM (VTE) is the leading cause of obstetric morbidity and mortality in the United Kingdom and other countries even in the absence of known congenital risk factors (1–5). Reproducible increases in procoagulant factors (von Willebrand factor, factors V and VIII, and fibrinogen), decreases in protein S, an acquired resistance to protein C, and increases in plasminogen activator inhibitors 1 and 2 produced by the placenta have all been found to occur during pregnancy and probably represent physiological preparation for delivery (2–5). However, pregnancy (including puerperium) is an extended period of the hypercoagulable state and seems itself to be an independent major risk factor for VTE. Therefore, additional and as yet unknown acquired risk factors must increase the risk of VTE in pregnancy and around delivery (2, 6).

Beyond the well known plasmatic factors, the pathogenesis of thrombosis involves complex platelet-leukocyte interaction, the details of which are not fully elucidated (6). P-Selectin mediates rolling of platelets and leukocytes on activated endothelial cells. Recent data indicate that P-selectin interaction with a ligand stabilizes initial glycoprotein IIb/IIIa-fibrinogen interactions, thus allowing the formation of large stable platelet aggregates (7). These studies suggest that the activation of platelets is an initial step in the development of venous thrombosis. Until now the significance of activated platelets for the development of VTE has not been systematically investigated, whereas the influence of platelet activation on arterial thrombosis is undisputed.

We hypothesized that changes in endocrine factors related to pregnancy might contribute to the hypercoagulable state by causing the activation of platelets. PRL is one of the prominent hormones that increase during pregnancy and lactation (8, 9), and expression of both PRL and PRL receptor (PRLR) has recently been identified in hemopoietic tissue (10). Platelets derived from megakaryocytes (11) play a critical role in normal hemostasis (12). In an attempt to investigate possible platelet functional abnormalities in pregnancy, we assessed the P-selectin expression of ADP- and thrombin receptor activator 6 (TRAP-6)-stimulated and nonstimulated platelets of 42 consecutive pregnant women. To further investigate the influence of hyperprolactinemia on platelet activation we determined nonstimulated and stimulated P-selectin expression in 22 normo- or hyperprolactinemic patients with pituitary tumors, in 9 consecutive patients during TRH stimulation test, and in 4 consecutive patients with prolactinomas during dopamine agonist therapy. In addition, the *in vitro* ADP stimulation and aggregation of platelets by human PRL (hPRL) was studied. We report in this study that hyperprolactinemia is a potent costimulator of platelet aggregation and, therefore, may be the cause of the hypercoagulable state observed in pregnancy and the puerperium or other hyperprolactinemic states.

Subjects and Methods

Subjects

Forty-four consecutive healthy pregnant women (mean age, 27 ± 12 yr; first trimester, n = 7; second trimester, n = 13; third trimester, n =

Abbreviations: hPRL-pit, Human pituitary PRL; hPRL-plas, human plasma PRL; PRLR, PRL receptor; PRP, platelet-rich plasma; TRAP-6, thrombin receptor activator 6; VTE, venous thromboembolism.

24) without a history of VTE were investigated for PRL values and ADP stimulation of platelets. Furthermore, 22 patients (mean age, 45 ± 28 yr; 15 women and 7 men) with normo- (n = 11; PRL, <400 mU/liter) or hyperprolactinemic (n = 11; PRL, >1000 mU/liter) pituitary tumors were investigated for PRL values and for flow cytometric platelet function analysis. To investigate the influence of short-term changes in PRL levels on platelet function, PRL values and flow cytometric platelet function were determined in 9 consecutive patients with nonfunctioning pituitary adenomas (mean age, 46 ± 27 yr; 7 women and 2 men) during the standard iv TRH stimulation test. Seven of these 9 patients revealed normoprolactinemic values, whereas 2 showed hyperprolactinemic values (884 and 1289 mU/liter). The standard iv TRH stimulation test (200 μg Relefact, Hoechst, Frankfurt am Main, Germany) was performed as previously reported (13). PRL values and aggregation of platelets were measured basally and 30 and 60 min after iv TRH injection. Furthermore, we investigated the changes in PRL values and flow cytometric platelet function during dopamine agonist therapy in 4 consecutive patients with prolactinomas (mean age, 52 ± 8 yr; 2 women and 2 men). Two patients had interrupted their dopamine agonist therapy; in 1 of them therapy was restarted after a PRL increase. The 2 other patients were started with dopamine agonist therapy, and levels of PRL and P-selectin expression on platelets are determined after 2 wk of therapy.

One hundred healthy, sex- and age-matched subjects (mean age, 30 ± 18 yr; 46 women and 54 men) served as controls for ADP stimulation of platelets. All 100 controls showed normal PRL values. All investigations were accepted by the local institutional ethical committee (registration no. 967/99).

Determination of serum PRL

The serum PRL values of healthy controls, pregnant women, and patients with pituitary tumors were determined with the Axsym PRL assay (Abbott Laboratories, Inc., Chicago, IL). This assay was performed according to the manufacturer's instructions. Serum PRL values were considered normal if they were less than 580 mU/liter (24.2 ng/ml) in women or less than 450 mU/liter (18.77 ng/ml) in men.

Flow cytometric platelet analysis

The determination of ADP- and TRAP-6-stimulated and nonstimulated P- selectin expression of platelets using a flow cytometric method was performed as previously described (14–16). Citrated whole blood from healthy donors, pregnant women, and patients suffering from pituitary tumors was diluted in HBSS (Sigma, Deisenhofen, Germany) containing 1 mg/ml BSA (Sigma). Platelet count was adjusted to approximately 20,000 platelets/μl. Aliquots of the platelet suspension were activated for 10 min at 37 C with 5 μM ADP (final concentration; Sigma) and 10 μM TRAP-6 (final concentration; Bachem, Bubendorf, Switzerland). Native nonactivated whole blood from the same sample served as a control. The aliquots were incubated at 37 C with saturating concentrations of a murine phycoerythrin-labeled antiglycoprotein IIb/IIIa monoclonal antibody (clone P2, CD41, Beckman Coulter, Inc., Krefeld, Germany) and a murine fluorescein isothiocyanate-labeled anti-GMP 140 monoclonal antibody (clone CLB-Thromb/6, CD62P, Beckman Coulter, Inc.). Platelet activation and staining were stopped after 5-min incubation with 2 ml 4 C HBSS buffer. An EPICS XL flow cytometer equipped with standard filters for fluorescein isothiocyanate and phycoerythrin fluorescence analysis, and XL System II software R 2.0 (Beckman Coulter, Inc.) were used for measurements. Platelets were gated in the forward scatter *vs.* fluorescence 2 (CD41-PE) dot plot based on the characteristic forward scatter signal and the high CD41-PE signal. To exclude all CD41-negative events the discriminator was set in fluorescence 2. In the list mode 10,000 platelets were acquired at a flow rate of less than 500 particles/sec and subsequently analyzed. All assays were performed in at least two separate experiments. The normal range for ADP-stimulated P-selectin expression of platelets was determined using blood from 100 healthy blood donors (mean fluorescence intensity in arbitrary fluorescence units, 4.88 ± 1.35; mean ± 2 SD). Results above the 2 SD limit of the mean value of the 100 control subjects (6.23) were considered positive for increased ADP stimulation as previously described (15, 16). For the investigation of PRL effects on ADP stimulation of platelets, citrated whole blood of healthy donors was diluted with different concentrations of plasma hPRL (hPRL-plas; hyperprolactine-

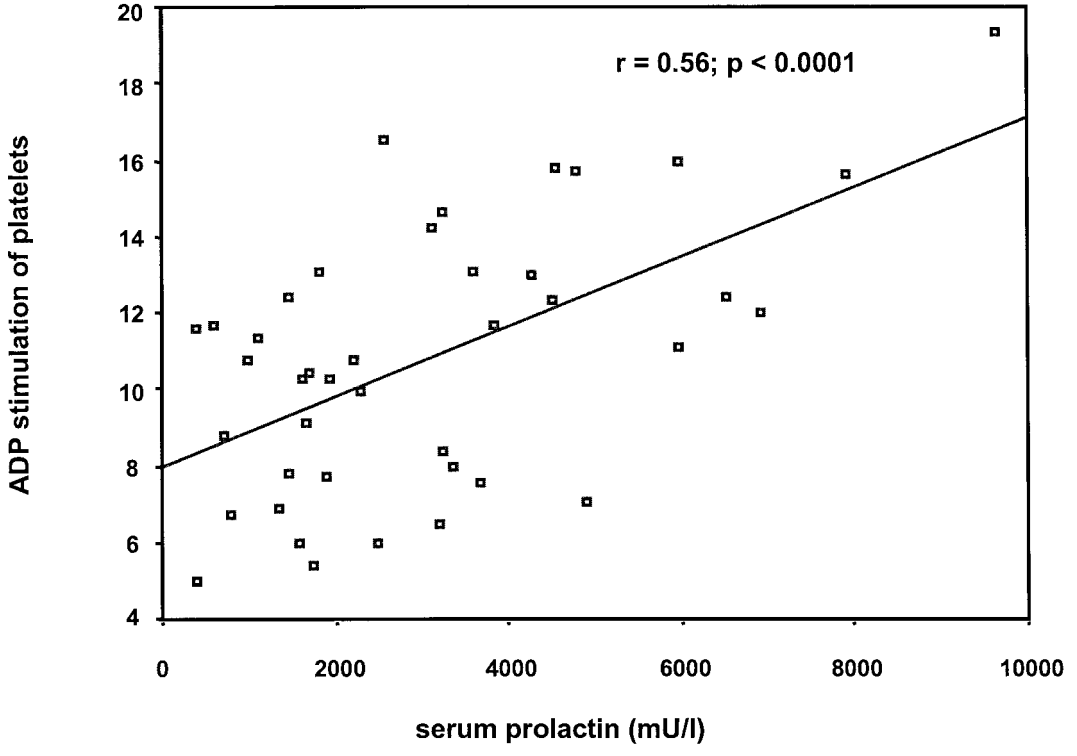

FIG. 1. Correlation between ADP stimulation of platelets and serum PRL in 44 pregnant women. The correlation between ADP stimulation of platelets, presented as P-selectin expression, and serum PRL values in 44 consecutive pregnant women was analyzed and quantified with the Pearson correlation coefficient.

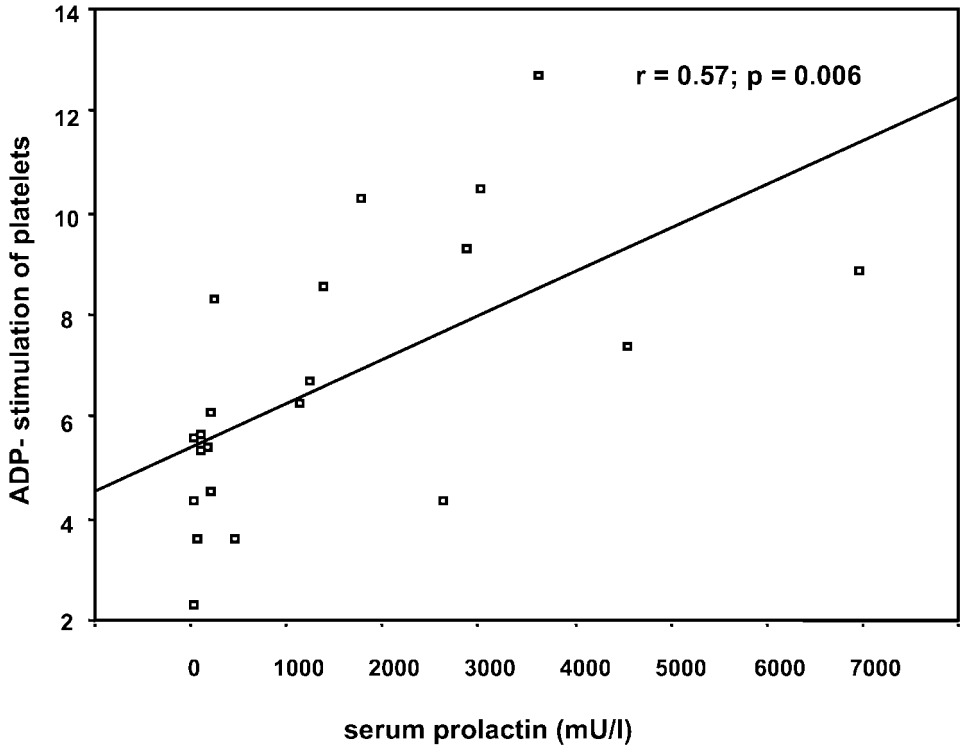

FIG. 2. Correlation between ADP stimulation of platelets and serum PRL in 22 patients with pituitary tumors. The correlation between ADP stimulation of platelets, expressed as P-selectin expression, and serum PRL values in 22 consecutive patients with pituitary tumors was analyzed and quantified with the Pearson correlation coefficient.

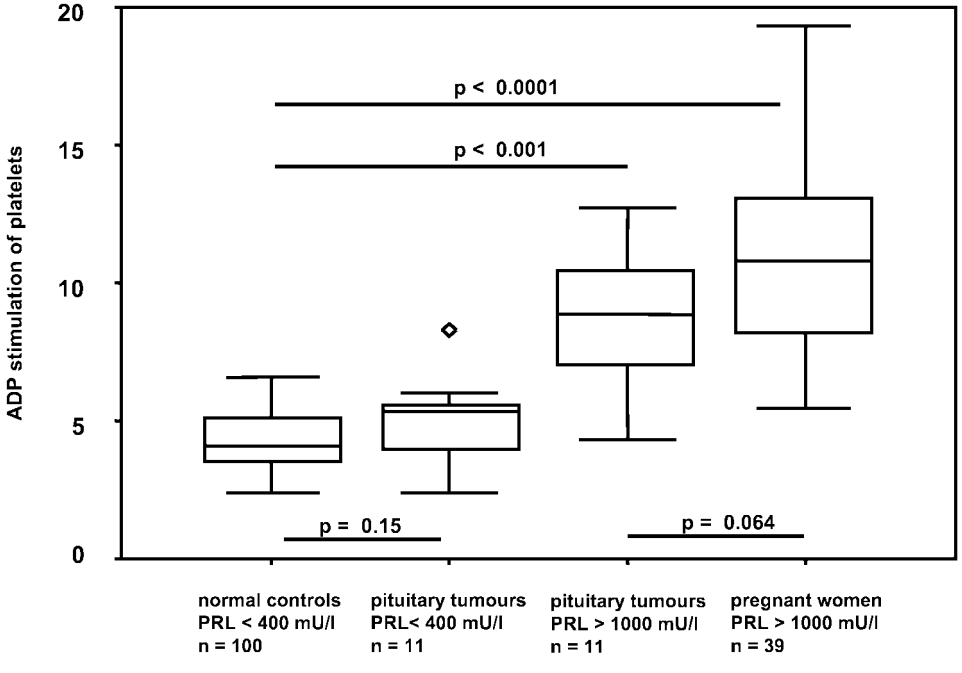

FIG. 3. ADP stimulation of platelets in healthy controls, normo- or hyperprolactinemic patients with pituitary tumors, and pregnant women. Comparison of ADP stimulation of platelets between 39 hyperprolactinemic pregnant women and 11 hyperprolactinemic patients with pituitary tumors with 100 healthy controls or 11 normoprolactinemic patients with pituitary tumors by the Mann- Whitney *U* test. Box plots represent 25th and 75th percentiles, *error bars* represent the 10th and 90th percentiles, and the *diamond* shows a single value above the 90th percentile. *P* values are given above and below the plots.

mic plasma of a healthy pregnant women) or pituitary hPRL (hPRL-pit; Sigma) *in vitro*. Flow cytometric platelet analysis was performed in duplicate in at least two separate experiments.

Platelet aggregometry

Freshly drawn venous blood was collected into 0.1 vol 3.8% trisodium citrate. Platelet-rich plasma (PRP) was obtained after centrifugation of the citrated whole blood ($150 \times g$, 15 min). The aggregation studies were performed using a PAP-4C aggregometer (Bio/Data, Horsham, PA) in 250 μl PRP adjusted to 200,000 platelets/μl with platelet-poor plasma. PRP was preincubated 5 min at 37 C with different concentrations of plasma hPRL and activated using 5 μM ADP. The OD of platelet-poor plasma was set at 100%, and that of PRP was set at 0% before addition of activators as previously reported (14, 15). Platelet aggregometry was performed in duplicate in at least two separate experiments.

Statistical analysis

The Pearson correlation coefficient was calculated to analyze the correlation between the PRL values and ADP stimulation of platelets in pregnant women and patients with pituitary tumors. A multiple re-

gression model was performed to assess the independent contribution of PRL to the ADP stimulation with adjustment for gestational age. The Mann-Whitney *U* test was used to compare the ADP stimulation of platelets among healthy controls, normo- or hyperprolactinemic patients with pituitary tumors, and hyperprolactinemic pregnant women. For multiple comparisons, Bonferroni correction was used. The Wilcoxon signed-rank test was performed to analyze the changes in PRL values and ADP stimulation of platelets 30 min after the TRH stimulation test. To quantify the agreement between the changes in PRL and ADP stimulation of platelets during the TRH stimulation test, the κ coefficient was calculated. The κ coefficient has a maximum of 1 if the agreement is perfect; a value of zero indicates no agreement (17). $P < 0.05$ was considered statistically significant. Data were analyzed with SPSS for Windows (realized 9.0.1, SPSS, Inc., Chicago, IL).

Results

Correlation between ADP stimulation of platelets and serum PRL levels in pregnant women

In pregnant women, PRL values were well correlated with gestational age in a multivariate regression analysis (r = 0.6; $P < 0.0001$; data not shown) In these 44 pregnant women, PRL values were also correlated with the ADP stimulation of platelets (r = 0.56; $P < 0.0001$; Fig. 1). After adjustment for gestational age the PRL values revealed an independent influence on ADP stimulation using a multiple regression model (PRL: regression coefficient = 0.001; 95% confidence

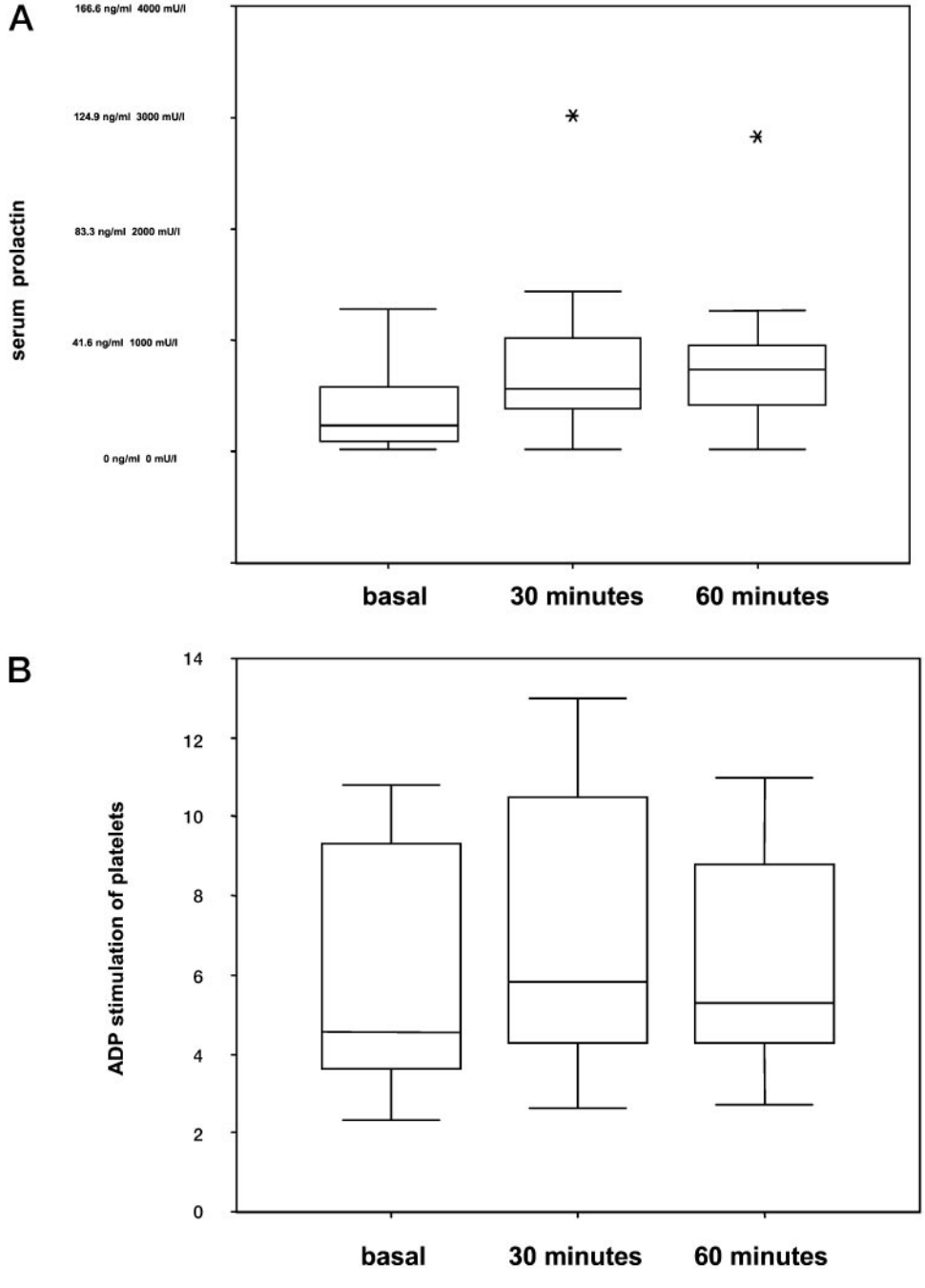

FIG. 4. A and B, PRL response and changes in platelet stimulation in nine patients with pituitary tumors during the TRH stimulation test. The increases in PRL (A) and ADP stimulation of platelets, expressed as P-selectin expression (B), are shown during a standard TRH stimulation test. The box plots are explained in Fig. 3. The Wilcoxon signed-rank test was performed to analyze the changes in PRL values and ADP stimulation of platelets 30 min after TRH stimulation. The *asterisks* show single values above the 90th percentile.

interval, 0.0005, 0.0015; $P < 0.001$; gestational age: regression coefficient = −0.052; 95% confidence interval, −0.159; 0.055; $P = 0.329$). P-Selectin expression of TRAP-6-stimulated and nonstimulated platelets was not influenced by PRL values (data not shown).

Correlation between ADP stimulation of platelets and serum PRL in patients with pituitary tumors

Furthermore, a cohort of 22 patients with pituitary tumors was investigated for PRL values and ADP stimulation of platelets. In these 22 patients, the PRL values were again correlated with the ADP stimulation of platelets (r = 0.57; $P = 0.006$; Fig. 2). Pregnant women and patients with hyperprolactinemia associated with pituitary tumors revealed significantly higher ADP stimulation of platelets than the 100 healthy controls ($P < 0.0001$ and $P < 0.001$, respectively; Fig. 3). The 100 healthy controls and normoprolactinemic patients with pituitary tumors showed no difference ($P = 0.15$) in ADP stimulation of platelets (Fig. 3). P-Selectin expression of TRAP-6-stimulated and nonstimulated platelets was not influenced by PRL values (data not shown).

Short-term changes in PRL and ADP stimulation of platelets during TRH stimulation test

The TRH stimulation test causes a significant increase in all individual PRL values (Fig. 4A; $P = 0.012$) as previously reported (13). In parallel, we measured a significant increase in the ADP stimulation of platelets 30 min after TRH stimulation (Fig. 4B; $P = 0.008$). The changes in individual PRL values were correlated to the changes in ADP stimulation of platelets 30 min after TRH stimulation (r = 0.67; $P = 0.05$). Furthermore, the changes in intraindividual PRL values were in parallel to the ADP stimulation of platelets in all patients between the two time intervals (basal to 30 min and 30–60 min) after TRH stimulation (Table 1). A maximum of agreement between the changes in PRL and ADP stimulation was calculated using the κ coefficient ($\kappa = 1$; Table 1). P-Selectin expression of TRAP-6-stimulated and nonstimulated platelets was not influenced by PRL values (data not shown).

Changes in PRL and ADP stimulation of platelets during dopamine agonist therapy

Next, we investigated the changes in intraindividual PRL- and ADP-stimulated P-selectin expression in four patients with prolactinomas during dopamine agonist therapy. The changes in PRL values were parallel to the ADP stimulation of platelets in all patients between the time intervals with or without dopamine agonist therapy (Fig. 5, A and B).

In vitro stimulation and aggregation of platelets by preincubation with human plasma or pituitary PRL

We also tested the ADP stimulation of platelets by direct incubation with hPRL-plas and hPRL-pit *in vitro*. Both hPRL-plas and hPRL-pit caused increased activation of platelets in a dose-dependent manner between 24–24,000 mU/liter (or 1–100 ng/ml) hPRL (Fig. 5A). These results were reconciled by an increased ADP stimulated aggregation of platelets by preincubation with hPRL *in vitro* (Fig. 5B).

TABLE 1. Changes in PRL values and ADP stimulation of platelets in nine patients with pituitary tumour during standard TRH stimulation test

ADP stimulation of platelets	0–30 min		30–60 min	
	PRL ↑	PRL ↓	PRL ↑	PRL ↓
↑	9	0	5	0
↓	0	0	0	4

Discussion

To date, there have been no reports of interactions between hemostasis and PRL via ADP-stimulated P-selectin expression on platelets. Impaired hemostasis has not been reported in hypophysectomized rats with or without neutralized circulating PRL or in PRL or PRLR knockout mouse models (18–20). An association between hyperprolactinemia and VTE has not been systematically investigated, and epidemi-

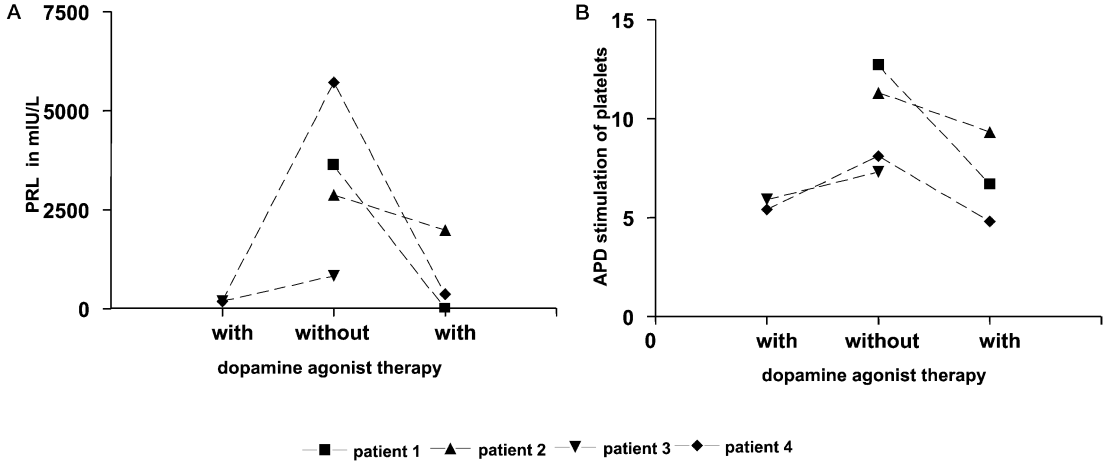

FIG. 5. A and B, Changes in PRL and ADP stimulation of platelets during dopamine agonist therapy. The changes in intraindividual PRL and ADP-stimulated P-selectin expression in four patients with prolactinomas during dopamine agonist therapy are shown. Two patients had interrupted their dopamine agonist therapy; one of them was restarted after a PRL increase. The other two patients were started with dopamine agonist therapy, and levels of PRL and P-selectin expression on platelets are shown after 2 wk of therapy.

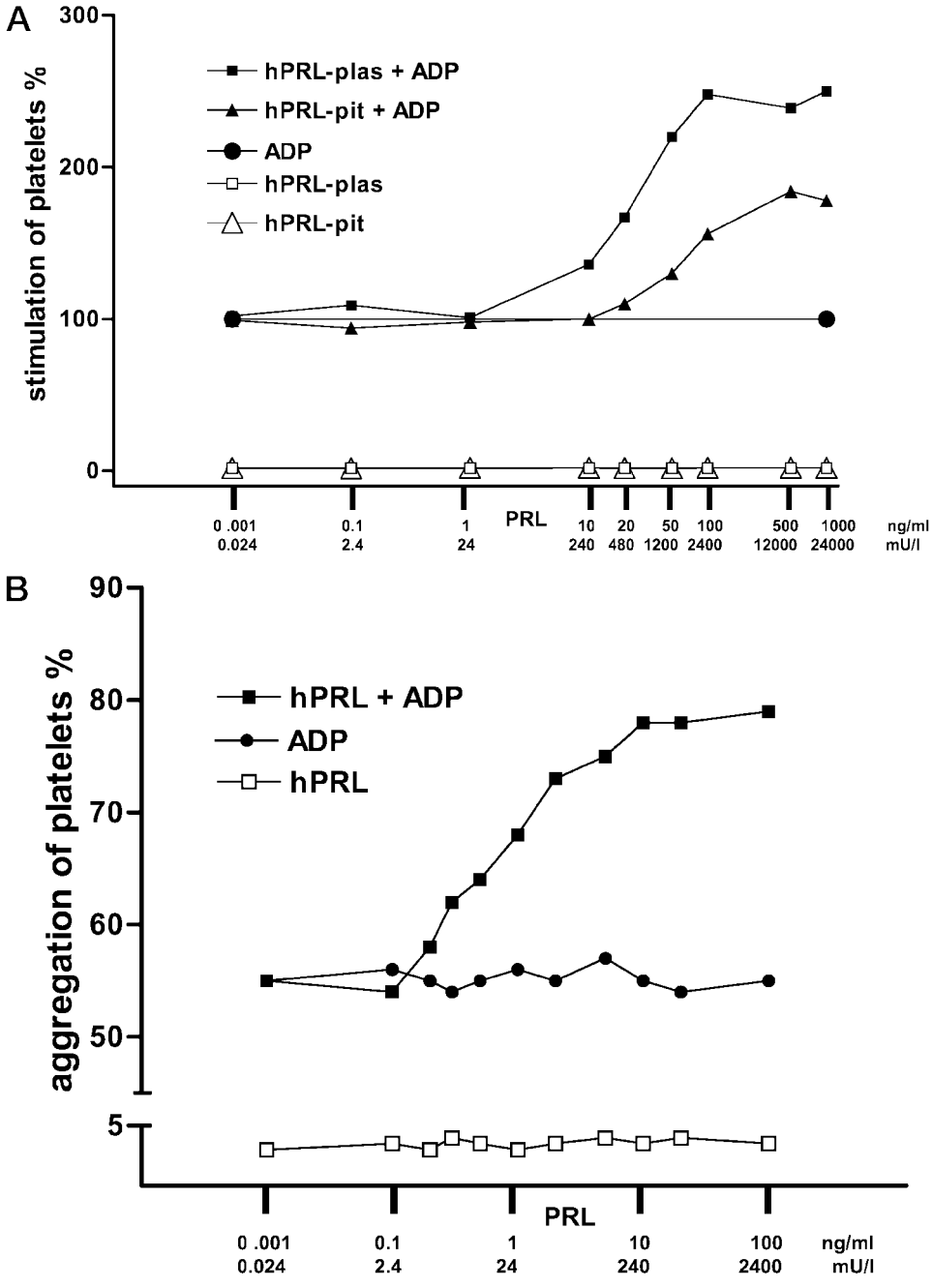

FIG. 6. A and B, *In vitro* stimulation and aggregation of platelets by preincubation with hPRL. Flow cytometric analysis of platelets from a healthy normoprolactinemic blood donor after preincubation with 0.024–24,000 mU/liter hPRL-plas (hyperprolactinaemic plasma of a healthy pregnant women) or hPRL-pit (Sigma) was performed as described in *Subjects and Methods*. ADP stimulation of platelets without hPRL was set at 100%. The *in vitro* aggregation of platelets from a healthy normoprolactinemic blood donor is shown after preincubation with 0.024–2400 mU/liter hPRL using 5 μM ADP. PRP was obtained after centrifugation of citrated whole blood (150 $\times$ *g*, 15 min) and adjusted to 200,000 platelets/μl using platelet poor-plasma. The OD of platelet-poor plasma was set at 100%, and that of platelet-rich plasma was set at 0% before addition of activators.

ological data for VTE in patients with pituitary tumors are lacking. On the other hand, hyperprolactinemia is part of the physiological preparation for delivery and breastfeeding in pregnancy or the puerperium (8, 9), when an increased risk for VTE has been reported (1, 2, 5). In another situation also associated with hyperprolactinemia, the treatment of patients with antipsychotic drugs (21), an increased incidence of VTE has been documented (22). Our data indicate that hyperprolactinemia may be a novel potent cofactor for platelet aggregation, which might explain the increased risk for VTE in hyperprolactinemic states.

In pregnant women, PRL values were well correlated in a multivariate regression analysis with gestational age in accordance to previous findings (8, 9). However, in these 44 pregnant women, PRL values were also correlated with ADP stimulation of platelets. After adjustment for gestational age, the PRL values revealed an independent influence on ADP stimulation by using a multiple regression model. Therefore, the correlation of PRL values and ADP stimulation of platelets suggests a direct influence of PRL on activation of platelets. In patients with pituitary tumors, the PRL values were again correlated with the ADP stimulation of platelets. The finding of increased ADP stimulation of platelets in hyperprolactinemic patients without pregnancy leads further support to the suggestion that hyperprolactinemia directly increases ADP stimulation of platelets, independently of

pregnancy. Pregnant women and patients with hyperprolactinemia associated with pituitary tumors revealed significantly higher ADP stimulation of platelets than the 100 healthy controls. The 100 healthy controls and normoprolactinemic patients with pituitary tumors showed no difference in ADP stimulation of platelets, excluding an influence of pituitary tumor *per se* on platelet activation.

The TRH stimulation test causes a significant increase in all individual PRL values as previously reported (23). In parallel, we measured a significant increase in the ADP stimulation of platelets 30 min after TRH stimulation. Therefore, our data indicate a short-term stimulation of platelet aggregation by PRL within 30 min. This fast activation of platelet aggregation is most likely explained by direct stimulation of platelets via the PRLR, which is expressed on hemopoietic tissue (10). Moreover, the significant parallel changes in PRL values and ADP stimulation of platelets suggest that the increase or decrease in PRL during TRH stimulation test directly causes increased or decreased ADP stimulation of platelets. This short-term influence of changes in PRL on ADP stimulation has been reconciled with the parallel changes in intraindividual PRL values and ADP stimulation in four patients with prolactinomas during dopamine agonist therapy. It has been demonstrated that one of the major signaling pathways of PRL via PRLR involves the phosphorylation of signal transducer and activator of transcription proteins or insulin receptor substrate-1 via pathways including PI3K, PKC, and intracellular Ca^{2+} (24). As these fast pathways are also included in platelet activation (25–29), it may indicate a basis for the short-term direct influence of PRL via PRLR on platelets, although this interaction remains speculative at moment. We are currently investigating changes in MAPKs by PRL in human platelets *in vitro*. In the first investigation of human platelets, PRL enhances ADP-regulated p38 MAPK activity (Wallaschofski, H., T. Lohmann, and M. Eigenthaler, manuscript in preparation).

We have also tested the influence of hPRL on ADP stimulation of platelets (Fig. 6A) and the ADP-stimulated aggregation of platelets (Fig. 6B) *in vitro*. hPRL-plas showed a higher stimulation of platelets than hPRL-pit. Therefore, further unknown costimulatory factors in hPRL-plas (hyperprolactinemic plasma of a healthy pregnant women) cannot be excluded. However, both hPRLs caused increased stimulation of P-selectin on platelets in a dose-dependent manner between 24–2400 mU/liter (or 1–100 ng/ml) hPRL (Fig. 6A). This finding was reconciled by increased ADP-stimulated aggregation of platelets by preincubation with hPRL *in vitro* (Fig. 6B). These data suggest that already physiological concentrations of PRL (upper limit of normal range, 580 mU/liter in females or 450 mU/liter in males) could have a costimulatory effect on ADP-stimulated platelets (Fig. 6, A and B). However, this *in vitro* finding might also explain interindividual differences in ADP stimulation of platelets in normoprolactinemic individuals. Moreover, our *in vitro* data support the concept that there is a short-term interaction between PRL and platelets, as indicated by our results in the TRH stimulation tests.

In conclusion, our results demonstrate a significant correlation between ADP stimulation of platelets and PRL values. We show that hyperprolactinemic pregnant women and hyperprolactinemic patients with pituitary tumors express higher platelet P-selectin levels after ADP stimulation. Furthermore, these results were reconciled by increased *in vitro* activation of platelets by hPRL. These data suggest that hyperprolactinemia is one of the previously unknown additional acquired risk factors that increase the risk of VTE in pregnant women. Hyperprolactinemia *per se* may be a physiological regulator of the delicate coagulant balance in pregnancy and around the time of delivery. Further investigations of the interaction between PRL and platelets in pregnant women with VTE will clarify the possible clinical relevance of hyperprolactinemia as a novel risk factor for VTE.

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Applicants must have completed 2 or more years of Endocrinology fellowship in an accredited fellowship training program and have a current license to practice Medicine in the United States.

Deadline for application submission: March 15, 2002.

For more information and an application, please contact: Mary Lee Vance, M.D., P.O. Box 800601, University of Virginia Health System, Charlottesville, Virginia 22908-0601; 434-924-2284; E-mail: mlv@virginia.edu.

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