Preeclampsia is not associated with altered platelet vasopressin binding and cytosolic Ca^{++} concentration

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OBJECTIVES: Preeclampsia is an important cause of fetal and maternal morbidity and mortality. Recently it was described that platelet cytosolic Ca^{++} levels could be used to screen for preeclampsia. The current study investigated platelet arginine vasopressin receptor characteristics, platelet cytosolic Ca^{++} levels, plasma- and platelet-bound arginine vasopressin in white pregnant women.

STUDY DESIGN: In a cross-sectional study nine third-trimester nulliparous pregnant women with gestational hypertension (seven with proteinuria, two with excessive weight gain without proteinuria) were compared with nine healthy nulliparous pregnant women matched for gestation length and age and 10 healthy age-matched nonpregnant women. Determined were (1) platelet arginine vasopressin receptor number and affinity, (2) platelet cytosolic Ca^{++} levels, both basal and on arginine vasopressin or thrombin stimulation, and (3) plasma- and platelet-bound arginine vasopressin levels.

RESULTS: None of the measured parameters differed significantly among the three groups studied. Mean arginine vasopressin receptor number and affinity ranged from 108 to 143 receptors per platelet and 0.35 to 0.40 nmol/L, respectively. A single population of binding sites was found (Hill number 0.96). Basal Ca^{++} levels ranged from 113.4 to 133.3 nmol/L, on arginine vasopressin stimulation from 199 to 250 nmol/L. Median arginine vasopressin levels in platelet-poor plasma were between 1.2 and 2.4 pg/ml, with circulating platelets being estimated to possess two to five molecules of arginine vasopressin per platelet. A significant correlation was found between platelet cytosolic Ca^{++} levels before and after arginine vasopressin stimulation ($r = 0.69$, $p < 0.001$) and a weak correlation between platelet receptor density and arginine vasopressin-stimulated platelet cytosolic Ca^{++} levels ($r = 0.38$, $p < 0.05$).

CONCLUSIONS: The studied parameters, platelet cytosolic Ca^{++} levels, whether basal or after stimulation with arginine vasopressin and vasopressin platelet receptor density and affinity, do not discriminate already hypertensive or preeclamptic white women from nondiseased subjects. A valuable test to screen for preeclampsia awaits further prospective studies. (AM J OBSTET GYNECOL 1993;169:1169-78.)

Key words: Preeclampsia, vasopressin, platelets, calcium

Preeclampsia is a trophoblast-dependent disease and a major cause of maternal and neonatal mortality and morbidity. Platelet dysfunction may play an important role in its cause.¹ Increases in platelet turnover,^{2,3} basal cytosolic free Ca^{++} levels,⁴ sensitivity to angiotensin II,⁴ and responsiveness to 8-arginine-vasopressin⁵ and a decreased responsiveness of platelets to serotonin⁶ have

been found in preeclampsia. Although currently delivery remains the only effective cure for preeclampsia, early administration of prostaglandin synthetase blockers like aspirin⁷ seems promising in inhibiting the development of preeclampsia. This effect may be because of the blocking of the formation of the arachidonate metabolite thromboxane by platelets.⁸

Recently Zemel et al.³ claimed that increased platelet cytosolic Ca^{++} levels after arginine vasopressin, but not after thrombin stimulation in vitro, would predict the development of preeclampsia in urban black women, among whom a high incidence of preeclampsia is found. Such findings would not only be useful as a screening test, facilitating therapeutic intervention, but also enable studies on pathophysiologic mechanisms of preeclampsia (e.g., on circulating plasma and platelet-bound arginine vasopressin levels and on platelet arginine vasopressin receptors).¹ Incubation of platelets with high concentrations of arginine vasopressin (0.01

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Table I. Characteristics of the study groups

	<i>Normal pregnant controls</i>	<i>Preeclamptic pregnant women</i>	<i>Nonpregnant control group</i>
No. of individuals	9	9	10
Age (yr)	28.3 (1.2)	28.6 (1.1)	28.0 (1.2)
Amenorrhea (days)	257 (11)	235 (14.2)	
Proteinuria (gm/24 hr)	Neg.	1.67 (0.58)	
Whole-blood platelet count ($10^9/L$)	257 (24.7)	239 (16)	275 (15.8)
Diastolic blood pressure			
At first antenatal visit	73 (1.7)	77 (1.2)	
At entry	75 (4.0)	98 (4.9)*	76 (3.0)
Maximal level measured at least twice during pregnancy	81 (1.6)	111 (4.9)†	

Values are presented as mean and SEM at entry in the study unless stated otherwise. Blood pressure level is Korotkoff phase 4 in millimeters of mercury, measured by standard mercury sphygmomanometer. *Neg.*, Negative result with dipstick throughout pregnancy.

*Significance between normal pregnant controls and preeclamptic pregnant women, $p = 0.007$.

†Significance between normal pregnant controls and preeclamptic pregnant women, $p = 0.0009$.

to 10 $\mu\text{mol/L}$) is known to lead to activation of phospholipase C and thus to the production of diacylglycerol and inositol triphosphate.⁹⁻¹¹ Inositol triphosphate mobilizes Ca^{++} from the platelet internal stores, and diacyl glycerol activates protein kinase C. Shape change and aggregation of platelets and thromboxane A_2 formation ensue. Phospholipase C activation is mediated by a G protein-associated V_1 receptor. Inflow of extracellular Ca^{++} results from the opening of receptor-operated Ca^{++} channels. In nonpregnant women 60% to 90% of the circulating arginine vasopressin is bound to platelets.¹²⁻¹⁴

To extend the observations of Zemel et al.⁵ to our white population of pregnant women, we measured platelet cytosolic Ca^{++} levels both at rest and after stimulation with arginine vasopressin or thrombin in nonpregnant women, normal pregnant women, and pregnant women who had preeclampsia. Preeclampsia is defined as gestational hypertension in combination with proteinuria and/or edema.¹⁵ Concomitantly, to explain differences in Ca^{++} metabolism at the arginine vasopressin receptor level, we determined the number of platelet arginine vasopressin binding sites and their affinity and plasma- and platelet-bound arginine vasopressin levels.

Methods

Subjects. The study population consisted of 28 white women. Nine nulliparous pregnant women had preeclampsia according to criteria of The American College of Obstetricians and Gynecologists (blood pressure $>140/90$ mm Hg and proteinuria [>0.3 gm per 24 hours] or edema or both).¹⁵ Nine nulliparous healthy pregnant and 10 nonpregnant healthy women served as controls. None of the women had a history of hypertension or had taken any medication for at least 3 days before blood collection and did not use aspirin. In-

cluded were all third-trimester hypertensive women attending the obstetric clinic of the Academic Medical Centre of the University of Amsterdam between June and September 1991, who had no history of previous disease, and whose diastolic blood pressure (Korotkoff phase 4) at the first prenatal visit, before a gestational age of 20 weeks was ≤ 80 mm Hg, measured by a standard mercury sphygmomanometer. Healthy pregnant controls were recruited from the outpatient department of the same hospital matched for gestational and maternal age. Nonpregnant women were healthy medical workers at the obstetric and hemostasis departments taking no medication but oral contraceptives. Further patient characteristics are given in Table I. Informed consent was given by all subjects, and the study was approved by the Ethics Committee at Amsterdam Medical Centre, University of Amsterdam.

Platelet cytosolic Ca^{++} . Platelets were isolated according to the procedure described by Hallam et al.¹⁶ Briefly, blood samples (20 ml) were collected in 10 ml Venoject syringes (Terumo, Leuven, Belgium) containing 15 mg of sodium ethylenediaminetetraacetic acid (EDTA) and centrifuged at 700g for 5 minutes at 20° C to obtain platelet-rich plasma. The platelet-rich plasma was transferred to an aluminum foil-jacketed polystyrene tube, incubated for 30 minutes in fura-2-acetoxymethyl ester (Calbiochem, La Jolla, Calif., dissolved in dimethylsulfoxide and kept at -20° C) at a final concentration of 2 nmol/L at 37° C and subsequently centrifuged at 350g for 20 minutes at room temperature. The supernatant was aspirated and the pellet washed carefully with 1 ml of HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]-buffer (10 mmol/L HEPES, 145 mmol/L sodium chloride, 10 mmol/L glucose, 1 mmol/L magnesium sulfate), pH 7.4, without disturbing the pellet. The platelets were finally resuspended in the same buffer and diluted to a

concentration of 4×10^7 platelets per milliliter. Platelet count was determined with a Coulter counter (S-plus STKS, Coulter Electronics, Hialeah, Fla.). Fluorescence of the platelet preparation was measured in an Aminco Bowman spectrophotofluorometer (AIC, Travenol, Silver Spring, Md.) with excitation and emission wavelengths of 340 and 505 nm, respectively. Entry and exit slits were set at 1 mm, corresponding to 5.5 nm band-pass. Measurements were made in a thermoregulated quartz cuvette under continuous stirring at 37° C. In the cuvette 1.2 ml of the platelet suspension was mixed with 1.2 ml HEPES buffer, pH 7.4, containing 2.0 mmol/L calcium chloride. The initial fluorescence of the Ca^{++} fura-2-acetoxymethyl ester complex reflected the basal cytosolic Ca^{++} concentration after stabilization of the signal. Vasopressin (8-arg-vasopressin, Sigma, St. Louis) at a final concentration of 9.1 $\mu\text{mol/L}$ or thrombin (Hofmann La Roche, Basel) at a final concentration of 1 U/mL was added to determine stimulated cytosolic Ca^{++} levels. Maximal and minimal fluorescence were obtained after addition of 0.1% Triton X-100 and 1 mmol/L manganese chloride final concentrations, respectively. Cytosolic Ca^{++} levels were calculated according to the following formula:

$$[\text{Ca}^{++}]_i = K_d \frac{F - F_{\min}}{F_{\max} - F}$$

where K_d is affinity <224 nmol/L at pH 7.4 and F is fluorescence in intact platelet suspension, as discussed by Grynkiewicz et al.¹⁷

In preliminary experiments it was found that basal cytosolic Ca^{++} levels increase if periods extend beyond 2 to 3 hours after blood collection, so care was taken not to exceed this period.

Binding of tritiated arginine vasopressin to intact platelets. Platelet suspensions were prepared according to Haslam and Rosson.⁹ Venous blood (40 to 80 ml) from the forearm was collected with a Venoflux infusion set with a 19-gauge needle into 10 ml polystyrene syringes (Sarstedt, Essen, Germany) containing 1.6 mL citrate/glucose buffer (0.09 mol/L sodium citrate, 0.1 mol/L glucose, 0.07 mol/L citric acid), pH 4.4, at room temperature. Blood was centrifuged at 90g for 20 minutes at 20° C, and platelet-rich plasma was collected. The platelet-rich plasma was then centrifuged at 2000g (20 minutes, 20° C) and the plasma aspirated. The pellet was washed twice with phosphate-buffered saline solution (135 mmol/L sodium chloride, 1.47 mmol/L potassium phosphate, 8 mmol/L sodium bicarbonate, 5 mmol/L EDTA, 0.1% bovine serum albumin [Sigma]), pH 7.4, and resuspended in phosphate-buffered saline solution for incubation (see below). Two 250 μL samples of platelet-rich plasma and of the final platelet suspension (1:1 and 1:2 diluted in phosphate-buffered saline solution) were kept for platelet counting.

Binding assays were performed as described by Bichet et al.¹⁴ Triplicate assays, final volume 250 μL , were performed in 5 ml polypropylene tubes incubated for 45 minutes at 30° C in a shaking water bath. Incubation medium was 1 mmol/L calcium chloride, 2.6 mmol/L potassium chloride, 1.47 mmol/L potassium phosphate, 0.5 mmol/L magnesium chloride, 135 mmol/L sodium chloride, 8 mmol/L sodium bicarbonate, 5.5 mmol/L glucose, and 0.1% bovine serum albumin, pH 7.4, and 50×10^6 platelets were used to start the incubation. The volumes contained 50 μL tritiated arginine vasopressin (1983 GBq/mmol, NEN, Boston), prepared in a serial 2:1 dilution to obtain a concentration range of 0.06 to 5 nmol/L. After incubation the reaction was stopped with 3.5 ml of phosphate-buffered saline solution precooled to 4° C, and the mixture was passed through a bovine serum albumin-coated filter (Whatman GF/C, Maudstone, England) and rinsed with 7 ml of phosphate-buffered saline solution of 4° C with a cell harvester (Skatron, Lier, Norway). Controls without platelets were run in each experiment. Filter-bound radioactivity was determined by liquid scintillation (2.5 ml of Ultima Gold MV, Packard) with a 2200 CA β -counter (Packard). Specific binding was defined as the difference between total binding and nonspecific binding (i.e., binding in the additional presence of nonlabeled arginine vasopressin at 1 $\mu\text{mol/L}$), the latter never exceeding 20% of the total binding. Scatchard and Hill analyses were performed with LIGAND software¹⁸ providing the affinity constant and the total number of receptors. In preliminary experiments, all performed with platelets of pregnant women, we checked that there was a linear correlation between an increase in the number of platelets (range 10 to 320×10^6) and specific tritiated arginine vasopressin binding. Binding was time dependent. Equilibrium was reached after 40 minutes and remained constant for at least 80 minutes.

In a group of 11 women, including seven nonpregnant controls, we measured the affinity constant and the number of binding sites in duplicate on the same day and in separate assays. Analyses were established at estimated coefficients of variation of 12.2% and 5.5%, respectively.

Plasma arginine vasopressin and platelet arginine vasopressin content. Special care was taken during sample preparation to inhibit cystyl-amino-peptidase activity with the enzyme inhibitors o-phenanthroline (Sigma) and sodium EDTA, as described by Davison et al.¹⁹ and Burd et al.²⁰ Whole blood was taken from an antecubital vein into silicone-coated vacuum tubes (Venoject) precooled on ice and containing 100 μL of a 125 mmol/L o-phenanthroline solution; these samples were then immediately transferred to identical tubes containing 100 μL of a 1 mol/L ethylenediaminetet-

raacetic acid solution and on melting ice. Centrifugation followed at 90g for 15 minutes at 4° C to obtain platelet-rich plasma and at 2000g for 15 minutes at 4° C to obtain platelet-poor plasma. With the centrifugation scheme platelet number in platelet-poor plasma was <1% of that in platelet-rich plasma.

Extraction of arginine vasopressin from both preparations was performed within 1 hour over octadecasilyl silica packed Sep Pak C18 columns (Waters) with 2 ml aliquots, acidified with 3 ml of 0.1N hydrochloric acid to pH 1 to 2, as described by LaRochelle et al.²¹ Inhibitors were sufficiently removed by a wash with 2 ml of chloroform, and subsequently arginine vasopressin was eluted with 4 ml of 100% methanol. No cystyl-amino-peptidase activity was detectable in this extract. The eluate was dried overnight in a vacuum rotator with a refrigerated condensation trap (Savant Speedvac RH 40-12, RT-100A) and the residue redissolved in 125 µl of assay buffer (0.05 mol/L Tris-hydrochloric acid, 139 mmol/L sodium chloride, 1 mmol/L EDTA, and 1% bovine serum albumin [Sigma, radioimmunoassay grade]), pH 8.

The arginine vasopressin radioimmunoassay was a modified version of the microassay described by Dogterom et al.²² Arginine vasopressin tracer was iodinated by the Chloramine-T method and purified by fractionation on a Sephadex G25 column. Specific activity was >64.8 TBq/mmol. Polyclonal anti-arginine vasopressin antibody (M160480) was raised in a rabbit and was used in a final dilution of 1:100,000. Standard curves ranged from 0.125 to 32 pg of arginine vasopressin and were performed in triplicate. Samples were assayed in duplicate. Total volume in assay tubes was 110 µl and contained 50 µl of antibody solution and 50 µl of sample or standard solution (all in assay buffer). After 3 days of incubation at 4° C 10 µl (2000 counts/min) tracer was added, and after overnight incubation the free label was separated from the bound fraction by adding 50 µl of second antibody-coated cellulose suspension (donkey antirabbit, IDS, Boldon, England) followed by a 5 minute centrifugation at 1700g. The pellet was then counted in a Packard-Cobra A5005 γ-counter. Standard curves and samples were processed with Cobra 5005 software (RIA-SMART, Packard), which includes standard curve fitting with MASS-spline and quality control analysis. The detection limit, defined as $B/B_0 \leq 90\%$ (B is bound fraction of iodine 125-arginine vasopressin; B_0 is bound fraction of ¹²⁵I-arginine vasopressin in absence of cold arginine vasopressin), was 0.25 pg per tube. Intraassay coefficient of variation was 9.7%, interassay coefficient of variation in 10 different assays was 13.4%. Recovery of 3.2 pg/ml of arginine vasopressin to whole blood of 22 pregnant women and nine nonpregnant controls was 57% and 59%, respectively. In plasma from pregnant women, kept at 20° C for several minutes without cystyl-amino-peptidase inhibitors, arginine vasopressin

levels were equal to the detection limit. Platelet-bound arginine vasopressin was defined as the difference between platelet-rich plasma and cystyl-amino-peptidase values corrected for the number of platelets.

Statistical analysis. Overall differences were analyzed by one-way analysis of variance, or Kruskal-Wallis one-way analysis of variance in case of nonnormal distribution or inhomogeneity of variances. Differences between groups were tested with a Student *t* test or a Mann-Whitney *U* test. For overall differences a *p* value <0.05 was considered significant and for differences between groups a *p* value <0.05/*k* (*k* = number of comparisons, according to Bonferroni). Possible relationships between the number of binding sites or affinity constant and Ca^{++} levels were tested as described by Steel and Torrie.²³ In short, for each study group Pearson's correlation (*r*) was determined, and the Fisher transform *z* was calculated. Group *z* values were then tested for homogeneity, and a pooled weighted mean value of *z* was obtained with 95% confidence intervals, after which *z* was backtransformed to *r*.

Results

Characteristics of the patients. The normotensive and hypertensive pregnant women did not differ in blood pressure at the first antenatal visit (Table I). Nor was there a difference in age or in gestational age. However, the groups differed significantly in blood pressure at the entry of the study.

Platelet cytosolic Ca^{++} levels. Neither the basal nor the stimulated cytosolic Ca^{++} levels of the platelets differed significantly among the three groups studied (Table II). Expressing stimulated Ca^{++} levels as percent of increase from baseline did not show differences either (Fig. 1). Basal Ca^{++} levels correlated significantly with arginine vasopressin-stimulated levels ($r = 0.69$, $p < 0.001$, Fig. 2) but not with thrombin-stimulated levels ($r = 0.24$, $p < 0.3$).

Arginine vasopressin binding studies. Binding of arginine vasopressin to the platelets revealed a single population of binding sites, which appeared not to be different in receptor density number of binding sites or receptor affinity among the three study groups. The mean number of receptors per platelet varied between 108 and 143. The affinity constant was 0.35 to 0.40 nmol/L (Table II). A typical Scatchard plot of a normotensive pregnant woman is shown in Fig. 3. Because the results were not different among the groups, we pooled the data. Then, a number of binding sites of 124.5 ± 10.7 sites per platelet and an affinity constant of 0.39 ± 0.02 nmol/L were obtained ($n = 28$). Evidence for the presence of cooperativity, Hill number 0.96 ± 0.026 , was not obtained.

The arginine vasopressin-stimulated cytosolic Ca^{++} levels corrected for nonstimulated levels correlated significantly with the number of arginine vasopressin receptors per platelet ($r = 0.38$, $p < 0.05$, Fig. 4). There

Fig. 1. Stimulation of cytosolic Ca^{++} concentration of platelet. Increase is compared with nonstimulated levels. Mean $\pm$ SEM is indicated. Results were calculated as follows:

$$\frac{\text{Stimulated } \text{Ca}^{++} - \text{Basal } \text{Ca}^{++}}{\text{Basal } \text{Ca}^{++}} \times 100\%$$

Agonists were 8-arginine-vasopressin (AVP) (9 $\mu\text{mol/L}$) and thrombin (1.0 U/ml). Analysis of variance revealed no differences among groups ($p = 0.46$ and $p = 0.28$, respectively).

Table II. Platelet cytosolic calcium⁺⁺ levels, arginine vasopressin binding studies, and plasma and platelet vasopressin levels

	Normal pregnant controls	Preeclamptic women	Nonpregnant control group	Significance
Calcium levels (nmol/L)				
No. of analyses	9	9	10	
No stimulation	113 (6.2)	130 (8.9)	133 (10.4)	$p = 0.25$
Arginine vasopressin stimulation	199 (22)	109 (18)	250 (30.4)	$p = 0.31$
Thrombin stimulation	603 (51.3)	543 (53.3)	628 (58.2)	$p = 0.54$
Binding studies				
No of binding sites (sites per platelet)	108 (18.4)	123 (13.9)	143 (24.4)	$p = 0.41$
Affinity constant (nmol/L)	0.40 (0.04)	0.40 (0.03)	0.35 (0.02)	$p = 0.38$
Vasopressin levels				
No. of analyses	9	7	9	
Platelet-poor plasma (pg/ml)	1.2 (0.5-3.7)	2.5 (0.9-6.1)	2.4 (0.5-7.8)	$p = 0.17$
Platelet-bound arginine vasopressin	0.3 (ND-2.2)	0.6 (ND-2.0)	0.9 (ND-5.3)	$p = 0.33$
Arginine vasopressin (molecules per platelet)	1.6	3.4	5.1	

ND, Not detectable. For the calcium⁺⁺, affinity constant, and number of binding site values, means and SEM are provided. Vasopressin values are median and range corrected for recovery. The p values are given for analysis of variance (binding and calcium⁺⁺ studies) or Kruskal-Wallis (plasma arginine vasopressin levels).

was no significant correlation between receptor density and thrombin-induced Ca^{++} levels ($r = 0.28$, $p < 0.3$).

Circulating arginine vasopressin. The number of arginine vasopressin molecules per platelet was calcu-

lated from the difference between the arginine vasopressin levels in platelet-rich and platelet-poor plasma after correction for arginine vasopressin recovery. A measured value of 1 pg/ 10^8 platelets represents 10 and

Fig. 2. Basal platelet cytosolic Ca^{++} levels versus arginine vasopressin (AVP)-stimulated levels. NTP, Normotensive pregnant women; NP, nonpregnant women; solid dots, nonproteinuric preeclamptic women (HTP). Values are highly correlated ($r = 0.69$, $p < 0.001$). For statistics see Methods.

9.5 molecules per platelet, respectively (molecular weight arginine vasopressin 1080), which means that the approximate number of arginine vasopressin molecules per platelet in the groups studied was 1.6 to 5.1 (Table II).

Comment

Prediction of preeclampsia would be of high clinical relevance, because it might enable early therapeutic intervention. We did not attempt to test the findings by Zemel et al.,⁵ that arginine vasopressin stimulation of platelets in early pregnancy results in a significantly increased cytosolic Ca^{++} level in platelets of women who will have preeclampsia, compared with normal nonpreeclamptic women, but chose a cross-sectional design, speculating that the presumed increase would also appear in patients in whom the disease was already clinically overt. Redman¹ recently reviewed ample evidence that gestational hypertension (preeclampsia) is a trophoblastic disease in which platelets play an important role and that platelet characteristics may be associated with preeclampsia. Differences in basal platelet cytosolic Ca^{++} levels, as described by Haller et al.,⁴ could well be in agreement with in vivo platelet activation (e.g., in case of a malfunctioning placenta or by inherent genetic properties). One could conclude from the data of Zemel et al.,⁵ that the same would hold for

the different cytosolic Ca^{++} levels of the platelets after stimulation with arginine vasopressin. The current study shows a highly significant correlation between basal platelet cytosolic Ca^{++} levels and the levels obtained after arginine vasopressin stimulation but not after thrombin stimulation. The absence of correlation for thrombin may be because the Ca^{++} response on thrombin stimulation leads to a maximal response, which is unlikely to be dependent on a variable basal level. Because the degree of in vitro activation is similar among the three study groups involved, basal and arginine vasopressin-stimulated Ca^{++} levels might indeed reflect in vivo activation or genetic characteristics.

However, we were unable to demonstrate differences among groups in either nonstimulated cytosolic Ca^{++} levels or increased levels on stimulation with arginine vasopressin or thrombin. This cannot be readily explained. The basal platelet cytosolic Ca^{++} values we found are twice as high as those reported by Barr et al.,⁶ but they are within the range of values reported by Haller et al.,⁴ Kilby et al.,²⁴ and Dominiczak et al.²⁵ Zemel and Zemel²⁶ corrected their original data in a letter without giving exact intraplatelet Ca^{++} levels, comparison with their data is unfortunately not possible. The difference might depend on the procedure of platelet preparation, type of fluorochrome, or the

Fig. 3. Scatchard plot. Typical plot of arginine vasopressin (AVP) binding data from a normal pregnant woman used for Scatchard analysis is presented. K_d , Receptor affinity; B_{max} , platelet receptor concentration; 49 pmol/L represents 152 sites per platelet.

method to establish minimal and maximal fluorescence in the Ca^{++} measurement. In agreement with the results of Zemel et al.^{5, 26} (for third-trimester pregnant women) and Barr et al.,⁶ but not with those of Haller et al.,⁴ we found no differences in basal Ca^{++} values among the investigated patient groups. It is unlikely that our Ca^{++} assay might be the cause of such discrepancies. Extensive preliminary experiments were performed to control and optimize the platelet cytosolic Ca^{++} measurement. We compared methods of platelet isolation by centrifugation with gel filtration. In addition, we tested the use of digitonin versus saponin to lyse the platelets and the use of manganese chloride versus ethylene glycol bis (β -aminoethyl ether) to establish minimal fluorescence. Because the Ca^{++} response is also dependent on the presence of extracellular divalent cations,^{9-11, 13, 27} we tested whether removal of extracellular Ca^{++} with a 5 mmol/L excess ethylene glycol bis (β -aminoethyl ether) would blunt the response. Indeed, only half the response was subsequently obtained compared with the response in the presence of extracellular Ca^{++} . Also, measurements were performed with different extracellular Ca^{++} or Mg^{++} concentrations and during stimulation with different arginine vasopressin and thrombin concentrations. No evidence was obtained from all these experiments for suboptimal conditions in our Ca^{++} assay, and we therefore consider the assay properly validated. The discrepancy in results, however, could also be caused by differences in study design or patient selection. One

may, for instance, conclude from the data of Zemel and Zemel²⁶ that differences in arginine vasopressin-evoked platelet cytosolic Ca^{++} increase would disappear when the disease becomes clinically overt. We do not rule out this possibility in our study, but differences in Ca^{++} metabolism have been described in diseased patients both in basal levels and in angiotensin II response.⁴ Second, one may hypothesize that observations in urban black women do not hold for a group of white women. Therefore we also performed a pilot study on a small group of urban black women, two with preeclampsia, two normotensive, and one nonpregnant control, and measured Ca^{++} levels in the same range as in the white women (basal 131, arginine vasopressin-stimulated 219, thrombin-stimulated 628 nmol/L; data pooled). These data do not support such an explanation. A third point that should be considered is the fact that oral contraceptives might increase platelet activity. Although literature is in variance, as discussed by Kunz et al.,²⁸ this might mask possible differences between nonpregnant and pregnant women in our study.

We initiated the study of platelet arginine vasopressin binding and plasma arginine vasopressin levels for two reasons. First, we investigated whether we could anticipate a higher platelet cytosolic Ca^{++} level on stimulation by arginine vasopressin on the basis of an increased number or increased affinity of arginine vasopressin receptors on platelets of preeclamptic women, as has been suggested earlier.¹ Because arginine vasopressin receptor characteristics can also be determined on

Fig. 4. Platelet receptor density versus cytosolic Ca^{++} level after arginine vasopressin (AVP) stimulation. Stimulated values are expressed as percent increase from baseline as described (see Fig. 1). NTP, Normotensive pregnant women; NP, nonpregnant women; solid dots, nonproteinuric (HTP). Values are significantly correlated, $r = 0.38$, $p < 0.05$. For statistics see Methods.

platelet membrane fractions stored at -70°C ,²⁹ subsequently a practical screening test might be developed. Second, as Launay et al.³⁰ have reported, down-regulation of arginine vasopressin receptors on platelets by free circulating arginine vasopressin could be expected. However, we could not establish any substantial differences in arginine vasopressin receptor characteristics nor in arginine vasopressin plasma levels among the three study groups. The arginine vasopressin receptor data are well in agreement with those reported for nonpregnant women by Bichet et al.,¹⁴ Thibonnier and Roberts,²⁹ Vittet et al.,³¹ and Inaba et al.³² for number of binding sites (95 to 170 binding sites/platelet), but slightly lower for affinity constant values (0.42 to 2 nmol/L). To our knowledge, no values have been reported so far for pregnant women. We could establish a significant correlation between Ca^{++} response to arginine vasopressin and number of binding sites of arginine vasopressin receptors ($r = 0.38$, $p < 0.05$), whereas no such correlation existed between the response to thrombin and number of binding sites. This, in our opinion, also validates both the Ca^{++} measurement technique and the arginine vasopressin binding studies.

In contrast to Launay et al.,³⁰ we did not obtain evidence for down-regulation of the platelet arginine vasopressin receptor number by plasma free arginine vasopressin levels, because these values were not signif-

icantly correlated ($r = 0.25$, $p < 0.3$). If, however, down-regulation can only be determined after exposure of the platelets to a certain (threshold) level of arginine vasopressin, the absence of such an effect may be readily explained by the low arginine vasopressin levels present in our pregnant subjects, whereas in the nonpregnant or male subjects of Launay et al. high levels were found as well.

The arginine vasopressin values in plasma corrected for recovery are in agreement with those reported by Davison et al.³³ Platelet-bound arginine vasopressin values are somewhat lower than those reported by Bichet et al.¹⁴ and Chesney et al.³⁴ (two to five against nine or ten molecules per platelet), but our study population consisted of (pregnant) women. Evidently in pregnancy, too, only a minor fraction of the total number of arginine vasopressin binding sites on platelets is occupied. The low arginine vasopressin receptor occupancy we measured by directly analyzing platelet-rich and platelet-poor plasma (i.e., two to five sites occupied per platelet) can be anticipated from the binding data. At a plasma arginine vasopressin (AVP) concentration (platelet-poor plasma) of 1.2 to 2.4 pg/ml (molecular weight 1080 [i.e., 1.1 pmol/L]), with 108 to 143 receptors per platelet, an average platelet concentration of $360 \times 10^9/\text{L}$, and an affinity constant of 0.40 nmol/L, analysis by the equilibrium formula

$$\frac{K_d}{[AVP]_{free}} = \frac{[Receptor]_{free}}{[Receptor]_{occupied}}$$

gives a ratio for free/occupied receptors between 160 and 400 (i.e., 0.3% to 0.5% receptor occupancy). The low receptor occupancy is thus confirmed by the independently established binding data, bearing in mind that the platelet-bound arginine vasopressin values (two to five molecules per platelet [i.e., 1% to 3.6% of the total receptor number]) reflect the sum of aspecifically bound, receptor-associated, and intraplatelet arginine vasopressin.

Because smoking cigarettes elevates arginine vasopressin and platelet-bound arginine vasopressin levels,¹³ blood samples were obtained in the morning before smoking was started. However, because the effects of smoking in pregnancy have not yet been reported with regard to arginine vasopressin levels, analysis was also performed without smokers. Two women from the hypertensive group, one from the normotensive group, and two from the nonpregnant group were excluded. Among the groups no significant differences existed for all measured parameters, median platelet-poor plasma values did not differ, and platelet-bound arginine vasopressin levels were 0.5, 0.4, and 0.9 pg/10⁸ platelets, respectively (i.e., only slightly different from values with smokers included). We analyzed our data also for the proteinuric patients alone, which did not alter the results. Preeclampsia according to criteria of the International Society for the Study of Hypertension in Pregnancy is hypertension in combination with proteinuria, not with edema alone, in contrast to criteria of The American College of Obstetricians and Gynecologists. Basal calcium levels and calcium levels after arginine vasopressin stimulation for the proteinuric group were 130 nmol/L (SD 29) and 203 nmol/L (SD 57), whereas for the whole preeclamptic group (American College of Obstetricians and Gynecologists' criteria) levels were 130 nmol/L (SD 26.8) and 210 nmol/L (SD 54). Number of binding sites and affinity constant were 123 sites per platelet (SD 43) and 0.42 nmol/L (SD 0.08) for the proteinuric group and 123 sites per platelet (SD 39) and 0.40 nmol/L (SD 0.08) for the whole group.

We realize that the number of investigated patients is small and the power of the study limited. However, if one, for example, demands a power of 0.80, the sample sizes required to find differences would be between 64 (number of binding sites on platelets) and ± 6000 (arginine vasopressin binding affinity) patients, assuming normal distributions. In vivo arginine vasopressin binding would require even larger sample sizes, which is not feasible.

In conclusion, the current cross-sectional study determined arginine vasopressin binding characteristics of platelets in human pregnancy and measured platelet

cytosolic Ca⁺⁺ response on arginine vasopressin or thrombin stimulation, revealing no statistically significant differences between normal pregnant, preeclamptic pregnant, and nonpregnant women, although nonpregnant women displayed slightly higher values than pregnant women for all parameters, except for affinity constant values. Preeclamptic women gave intermediate results. The significant correlation between total platelet receptor number and arginine vasopressin-stimulated Ca⁺⁺ response may reflect a functional significance of the arginine vasopressin receptor. The usefulness of the measured parameters in screening for preeclampsia awaits further prospective studies.

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REFERENCES

1. Redman CWG. Platelets and the beginnings of preeclampsia. *N Engl J Med* 1990;323:478-80.
2. Redman CWG, Bonnar J, Beilin L. Early platelet consumption in preeclampsia. *BMJ* 1978;1:467-9.
3. Walker JJ, Cameron AD, Bjornsson S, Singer CRJ, Fraser C. Can platelet volume predict progressive hypertensive disease in pregnancy? *Am J Obstet Gynecol* 1989;161:676-9.
4. Haller H, Oeney T, Hauck U, Distler A, Phillip T. Increased intracellular free calcium and sensitivity to angiotensin II in platelets of preeclamptic women. *Am J Hypertens* 1989;2:238-43.
5. Zemel MB, Zemel PC, Berry S, et al. Altered platelet calcium metabolism as an early predictor of increased peripheral vascular resistance and preeclampsia in urban black women. *N Engl J Med* 1990;323:434-8.
6. Barr SM, Lees KR, Butters L, O'Donnell A, Rubin PC. Platelet intracellular free calcium concentration in normotensive and hypertensive pregnancies in the human. *Clin Sci* 1989;76:67-71.
7. Wallenburg HCS, Dekker GA, Makovitz JW, Rotmans P. Low-dose aspirin prevents pregnancy induced hypertension and pre-eclampsia in angiotensin sensitive primigravidae. *Lancet* 1986;1:1-3.
8. Schiff E, Peleg E, Goldenberg M, et al. The use of aspirin to prevent pregnancy-induced hypertension and lower the ratio of thromboxane A₂ to prostacyclin in relatively high risk pregnancies. *N Engl J Med* 1989;321:351-6.
9. Haslam RJ, Rosson GM. Aggregation of human blood platelets by vasopressin. *Am J Physiol* 1972;223:958-69.
10. Pollock WK, MacIntyre DE. Desensitization and antagonism of vasopressin-induced phosphoinositide metabolism and elevation of cytosolic free calcium concentration in human platelets. *Biochem J* 1986;234:67-73.
11. Siess W, Stifel M, Binder H, Weber PC. Activation of V₁-receptors by vasopressin stimulates inositol phospholipid hydrolysis and arachidonate metabolism in human platelets. *Biochem J* 1986;233:83-91.
12. Preibisz JJ, Sealy JE, Laragh JH, Cody RJ, Weksler BB. Plasma and platelet vasopressin in essential hypertension and congestive heart failure. *Hypertension* 1983;5(suppl 1):129-38.
13. Nussey SS, Ang VT, Bevan DH, Jenkins JS. Human platelet arginine vasopressin. *Clin Endocrinol* 1986;24:427-33.
14. Bichet DG, Arthus MF, Barjon JN, Lonergan M, Kortas C. Human platelet fraction arginine-vasopressin. Potential physiological role. *J Clin Invest* 1987;79:881-7.
15. Sibai BM. Pitfalls in diagnosis and management of preeclampsia. *Am J Obstet Gynecol* 1988;159:1-5.
16. Hallam TJ, Thompson NT, Scrutton MC, Rink TJ. The

- role of cytoplasmic free calcium in the response of quin2-loaded human platelets to vasopressin. *Biochem J* 1984; 221:897-901.
17. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;260:3440-50.
 18. Munson PJ, Rodbard D. LIGAND: a versatile computerized approach for the characterization of ligand binding systems. *Anal Biochem* 1980;107:220-39.
 19. Davison JM, Gilmore EA, Dürr J, Robertson GL, Lindheimer MD. Altered osmotic thresholds for vasopressin secretion and thirst in human pregnancy. *Am J Physiol* 1984;246:F105-9.
 20. Burd JM, Davison J, Weightman DR, Baylis PH. Evaluation of enzyme inhibitors of pregnancy associated oxytocinase: application to the measurement of plasma immunoreactive oxytocin during human labour. *Acta Endocrinol* 1987;114:458-64.
 21. LaRochelle FT, North WG, Stern P. A new extraction of arginine vasopressin from blood: the use of octadecasilylsilica. *Pflugers Arch* 1980;387:79-81.
 22. Dogterom J, Van Wimersma Greidanus TB, Swaab DF. Evidence for the release of vasopressin and oxytocin into cerebrospinal fluid: measurements in plasma and CSF of intact and hypophysectomized rats. *Neuroendocrinology* 1977;24:108-18.
 23. Steel GD, Torrie JH. Principles and procedures of statistics. A biometrical approach. 2nd ed. Singapore: McGraw-Hill, 1984:280.
 24. Kilby MD, Broughton-Pipkin F, Cockbill S, Heptinstall S, Symonds EM. A cross-sectional study of basal platelet intracellular free calcium concentration in normotensive and hypertensive primigravid pregnancies. *Clin Sci* 1990; 78:75-80.
 25. Dominiczak AF, Morton JJ, Murray G, Semple PF. Platelet cytosolic free calcium in essential hypertension: responses to vasopressin. *Clin Sci* 1989;77:183-8.
 26. Zemel MB, Zemel PC. Correction: platelet calcium metabolism in the prediction of preeclampsia. *N Engl J Med* 1992;326:647.
 27. Roos I, Ferracin F, Pletscher A. Interaction of vasopressin with human blood platelets: dependency on Mg^{2+} . *Thromb Haemost* 1986;56:260-2.
 28. Kunz FK, Pechlaner C, Tabarelli M, Soelder E, Zwierzina WD. Influence of oral contraceptives on coagulation tests in native blood and plasma. *Am J OBSTET GYNECOL* 1990; 163:417-20.
 29. Thibonnier M, Roberts JM. Characterization of human platelet vasopressin receptors. *J Clin Invest* 1985;76:1857-64.
 30. Launay JM, Vittet D, Vidaud M, et al. V_{1a} -vasopressin specific receptors on human platelets: potentiation by ADP and epinephrine and evidence for homologous down-regulation. *Thromb Res* 1987;45:323-31.
 31. Vittet D, Rondot A, Cantau B, Launay JM, Chevillard C. Nature and properties of human platelet vasopressin receptors. *Biochem J* 1986;233:631-6.
 32. Inaba K, Umeda Y, Yamane Y, Urakami M, Inada M. Characterization of human platelet vasopressin receptor and the relation between vasopressin-induced platelet aggregation and vasopressin binding to platelets. *Clin Endocrinol* 1988;29:377-86.
 33. Davison J, Shiells EA, Philips PR, Lindheimer MD. Serial evaluation of vasopressin release and thirst in human pregnancy. Role of human chorionic gonadotropin in the osmoregulatory changes of gestation. *J Clin Invest* 1988; 81:798-806.
 34. Chesney CM, Crofton JT, Pifer DD, et al. Subcellular localization of vasopressin-like material in platelets. *J Lab Clin Med* 1985;106:314-8.
 35. Davey DA, MacGillivray I. The classification and definition of the hypertensive disorders in pregnancy. *Am J OBSTET GYNECOL* 1988;158:892-8.