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Platelet Aggregation and Vasopressin Receptors in Patients with Diabetes Mellitus (42720)

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Abstract. Plasma vasopressin, vasopressin-induced platelet aggregation, and platelet vasopressin receptors were investigated in 10 normal subjects and 14 diabetic patients free of microangioapthy. Basal plasma vasopressin concentration was identical in the two groups. Platelet aggregation induced by vasopressin as well as by epinephrine was not significantly altered in the diabetic patients. However, exploration of platelet V1-vasopressin receptors revealed in the diabetic group a dramatic reduction in the number of binding sites without alteration of the receptor affinity for tritiated vasopressin. Thus, vasopressin-induced platelet aggregation in uncomplicated diabetes mellitus remains normal despite a decrease in the number of vasopressin receptors presumably due to alterations of the platelet membrane structure.

There is considerable evidence arising from both animal studies and clinical observations that blood platelets are instrumental in the development of atherosclerosis and thromboembolic disorders. Several studies have reported an increased sensitivity of platelets from diabetic patients to aggregating agents such as collagen, ADP, and epinephrine (1). Such abnormality was identified especially in diabetic patients with microangiopathy, therefore suggesting a key role of platelets in the accelerated development of atherosclerosis in this disease (1, 2). Vasopressin not only is a potent vasoconstrictor and water-retaining hormone but also activates platelet release reaction and aggregation, stimulates the production of several coagulation factors, and enhances fibroblast proliferation (3, 4). We and others have characterized specific V1-vasopressin receptors of human platelets which mediate vasopressin-induced platelet aggregation (5–7). More recently, we have shown that these specific vasopressin receptors of human platelets stimulate the formation of 1,4,5-inositol triphosphate and 1,2-diacylglycerol (8). In addition, we have solubilized the platelet vasopressin receptor in a functional state (9) and evaluated the molecular weight of this specific receptor by gel filtration, sucrose gradient ultracentrifugation, and gel electrophoresis (10). In this study, we explored platelet vasopressin receptors and vasopressin-induced platelet aggregation in diabetic patients and control subjects.

Subjects and Methods. Venous blood was obtained after overnight fasting from 10 normal subjects and 14 diabetic patients free of microangiopathic complications at the kidney and retina levels. None of the patients smoked and all drugs that interfere with platelet aggregation (i.e., nonsteroidal anti-inflammatory drugs, beta blockers) were prohibited for 2 weeks prior to the study. Blood was drawn on heparin (100 IU/ml) in three separate test tubes processed respectively for measurement of

—plasma glucose, osmolality, and vasopressin (11),

—affinity and capacity of platelet vasopressin receptors (5), and

—platelet aggregation induced by vasopressin (10^{-5} to 10^{-10} *M*) or epinephrine (10^{-6} *M*) (5).

For the vasopressin radioreceptor assay, platelet particulates were prepared in 50 m*M* Tris–HCl + 5 m*M* EDTA buffer, pH 7.4, as already described (5) and were thawed and diluted (0.4 to 1 mg/ml final concentration) in binding assay buffer (50 m*M* Tris–HCl + 4 m*M* $MgCl_2$, pH 7.8). Duplicate samples were incubated at 30°C for 30 min in 5 ml polypropylene plastic tubes in a final volume of 250 μl containing 1 mg/ml gelatin and seven different concentrations of tritiated vasopressin (NEN, sp act 64 Ci/m*M*) ranging from 0.3 to 15 n*M*. Incubation was terminated by adding 5 ml of ice-cold buffer

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and free from bound tritiated vasopressin was separated by rapid filtration with a Brandel filtration unit through Whatman GF/B glass fiber filters previously soaked in assay buffer plus 1 mg/ml gelatin. The filters were rinsed with 15 ml of buffer and transferred to plastic minivials containing 4 ml of scintillation liquid (Beckman solution HP/b) and the radioactivity was determined in a Beckman scintillation counter at an efficiency of 59%. Data from binding saturation experiments were analyzed using an iterative nonlinear least-squares curve-fitting program and the equation (5)

$$[PL] = \frac{[P_t] * L_f}{L_f + K_d} + NSB * L_f,$$

where [PL] is the concentration of bound tracer, $[P_t]$ is the total concentration of receptors, L_f is the concentration of unbound tracer, K_d is the dissociation constant, and NSB is the nonspecific binding.

For platelet aggregation measurement, platelet-rich plasma (300,000 platelets/mm^3) was prepared as described earlier (5) and platelet aggregation was measured within 2 hr after blood draw at 37°C in a Payton aggregometer connected to a Fisher Recordall recorder. The maximal change in light transmittance after addition of vasopressin (10^{-10} to 10^{-5} M) or epinephrine (10^{-6} M) was determined. Calibrations were made by assuming that the platelet-poor plasma blank represents 100% aggregation and that the platelet-rich plasma represents 0% aggregation. This protocol was approved by the Committee on Human Research of the Medical College of Ohio. Values are expressed as the mean ± SE of the mean and data were analyzed by Wilcoxon rank sum test and two-factor analysis of variance. A two-tailed P value less than 0.05 was considered statistically significant.

Results. The normal subjects and diabetic patients were comparable in terms of age (44 ± 5 vs 48 ± 4 years), sex ratio (3 males/7 females vs 4 males/10 females), and weight (81 ± 6 versus 74 ± 5 kg). Six diabetics were insulin dependent and preliminary analysis of data revealed no difference between the insulin-dependent and the insulin-independent patients. They were consequently pooled into a single group for comparison with the normal subjects.

Plasma glucose (238 ± 29 vs 91 ± 3 mg%, $P < 0.001$) and plasma osmolality (292 ± 1 vs 289 ± 1 mOsm/kg, $P < 0.05$) were greater in the diabetic patients than in the normal subjects. Plasma vasopressin concentrations were identical in both groups (1.56 ± 0.06 and 1.58 ± 0.12 pg/ml). Platelet aggregation induced by 10^{-6} M epinephrine was slightly but not significantly increased in the diabetic patients compared to the controls (74 ± 6 vs 57 ± 4%, respectively). For vasopressin-induced platelet aggregation, there was no significant difference between the two groups, in terms of both maximum aggregation (61 ± 7 vs 55 ± 4%) and effective dose 50 (36 ± 3 vs 28 ± 2 nM; Fig. 1). The analysis of aggregation data by a two-way analysis of variance revealed no interaction between the factor category (volunteers versus diabetics) and the factor vasopressin concentration used to induce platelet aggregation ($P = 0.8854$).

Analysis of saturation binding experiments with platelet particulates and tritiated vasopressin revealed no alteration of the equilibrium dissociation constant in the diabetic group ($K_d = 1.29 \pm 0.19$ nM) compared to the normal subjects ($K_d = 1.30 \pm 0.17$ nM). However, the maximum number of binding sites was dramatically reduced by 58% in the diabetic group (51 ± 7 vs 122 ± 13 fmole/mg protein, $P < 0.002$; Fig. 2). Nonspecific binding was identical in both groups (0.0063 ± 0.0003 vs 0.0063 ± 0.0003).

To test the influence of glucose on the vasopressin platelet receptor's characteristics,

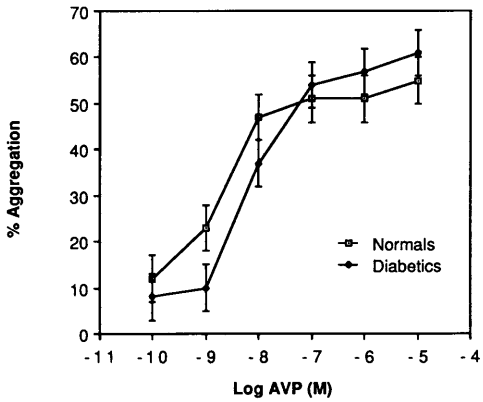

FIG. 1. Aggregation of platelets by vasopressin in normal subjects and diabetic patients.

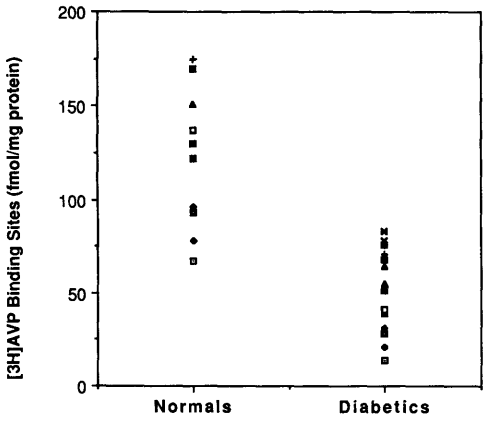

FIG. 2. Maximum number of platelet vasopressin binding sites in normal subjects and diabetic patients.

saturation experiments were carried out with platelet particulates prepared as described above from outdated platelet-rich plasma units obtained from the local blood bank. Addition of α-D-glucose (2g/liter) to the radioreceptor assay buffer did not modify the equilibrium dissociation constant of tritiated vasopressin for the receptor (K_d = 0.87 ± 0.09 vs 1.05 ± 0.05 n*M*) nor the density of vasopressin receptors (B_{max} = 76 ± 5 vs 87 ± 3 fmole/mg of protein, n = 6).

Furthermore, fresh intact platelets obtained from the local blood bank were incubated for 24 hr with or without addition of 2 g/liter α-D-glucose. They were kept at room temperature in a slow-rotating shaker and then washed twice in 50 m*M* Tris–HCl, 5 m*M* EDTA, 138 m*M* NaCl, buffer pH 7.4 and saturation experiments were performed as described earlier (5). In the absence of glucose preincubation, the affinity (K_d) and the capacity (B_{max}) of washed intact platelets vasopressin receptors were 0.91 ± 0.29 n*M* and 176 ± 9 fmole/mg of protein, respectively. Preincubation with glucose did not alter these parameters; affinity and capacity were 0.74 ± 0.25 n*M* and 172 ± 8 fmole/mg of protein, respectively.

Discussion. It is now clearly established that vasopressin, the antidiuretic hormone, is actively involved in the maintenance of blood volume and blood pressure (12). Recent evidence indicates that vasopressin triggers platelet aggregation as well as the release of several coagulation factors (3, 13). Moreover, it has been suggested that vasopressin is an important mediator of stress-induced hemostatic alterations and might contribute to the thrombotic risk associated with surgical operations (14). In the present study, we investigated specific platelet vasopressin receptors and vasopressin-induced platelet aggregation in diabetes mellitus, a disease characterized by accelerated atherosclerosis and enhanced platelet aggregability.

Normal subjects and diabetic patients free of microangiopathic complications and thromboembolic events were selected for this initial investigation. In agreement with other investigators, we observed that epinephrine-induced platelet aggregation was slightly enhanced in our patients who were free of microangiopathic lesions and acute metabolic complications (2, 15, 16). This abnormality was more marked in other studies using greater doses of epinephrine or high doses of ADP and collagen (15, 17) or in patients with deteriorating microangiopathy (2, 16). The extent of platelet aggregation induced by vasopressin was not significantly enhanced in the diabetic group compared with that of control subjects in terms of both maximum effect and sensitivity (assessed by ED_{50}).

Exploration of specific vasopressin receptors of human platelets in our study revealed in the diabetic group a normal affinity and a 58% reduction of the maximum number of binding sites. This pattern cannot be related to the classical down-regulation of receptors by increased circulating level of the hormone since basal plasma vasopressin was normal in our patients. However, this possibility cannot be completely ruled out since we do not know the integrated plasma vasopressin profile throughout the circadian rhythm nor vasopressin response to osmotic and nonosmotic stimuli in these patients. Despite this significant decrease in the number of vasopressin receptors, vasopressin-induced platelet aggregation was not significantly altered in the diabetic group. Similarly, in their review of β-adrenergic receptors, Stiles *et al* (18) quoted two studies where a reduction in the number of β-adrenergic receptors was found in experimental diabetes mellitus while there was no alteration in basal-, isoproterenol-, epinephrine-, or sodium fluoride-stimulated adenylate cyclase activities. This discrepancy could be reconciled by the theory of spare receptors.

The dramatic reduction in the number of vasopressin receptors in diabetic patients could be explained by an increased nonenzymatic glycosylation of membrane proteins in the presence of excessive glucose concentration (1, 19). One may hypothesize that vasopressin receptors of platelets from diabetic patients are among the proteins undergoing glycosylation with subsequent interference with binding of tritiated vasopressin to the receptor. To test this hypothesis, we first ruled out any effect of glucose by itself on the characteristics of the platelet vasopressin receptors during the radioreceptor assay. Second, incubation of intact normal platelets with 2 g/liter glucose for 1 day did not significantly reduce the number of platelet vasopressin binding sites. Therefore, if the above mechanism were correct, then a chronic exposure of platelets to a high glucose environment ought to be required.

In conclusion, diabetic patients free of acute metabolic events, thromboembolic complications, and microangiopathic lesions are characterized by normal basal plasma vasopressin levels and no increased sensitivity of blood platelets to vasopressin *in vitro*. The affinity of platelet receptors for vasopressin was not altered while a dramatic reduction of the number of binding sites was observed. We plan to extend our study to the severe and complicated forms of the disease and look for a possible time-dependent relationship between the number of platelet vasopressin receptors and hemoglobin A1C levels.

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