original paper

In vitro and *in vivo* effects of desmopressin on platelet function

CARLO L. BALDUINI, PATRIZIA NORIS, SIMONA BELLETTI, PIERANGELO SPEDINI, GABRIELLA GAMBA

Institute of Internal Medicine and Medical Oncology, University of Pavia, IRCCS Policlinico San Matteo, Pavia, Italy

ABSTRACT

Background and Objectives. **Desmopressin (DDAVP) may shorten bleeding time in patients with disorders of platelet function, but its mechanism of action in these conditions is still a matter of debate. In particular, contrasting results have been obtained concerning the ability of DDAVP to interact with platelets and to activate them directly. To gain further information on the DDAVP-platelet interaction, we studied the *in vitro* and *ex vivo* effects of DDAVP on platelet function.**

Design and Methods. **Platelet responses to DDAVP both as a single agent and in conjunction with agonists of platelet activation were investigated. For *in vitro* experiments platelets were obtained from healthy adult volunteers, while the *ex vivo* effects of DDAVP were studied in 12 patients with a bleeding disorder receiving a test dose of this drug.**

Results. **DDAVP *in vitro* did not induce either platelet aggregation or surface expression of the activation-dependent antigens; it did, however, greatly inhibit platelet aggregation response to vasopressin (AVP) and increased the maximal extent of platelet aggregation induced by collagen and ADP. DDAVP infusion did not promote the expression of activation antigens, but significantly enhanced *ex vivo* platelet aggregation stimulated by ADP and collagen. This priming effect was observed in patients with von Willebrand's disease, hemophilia A, May-Hegglin anomaly, gray platelet syndrome and Ehlers-Danlos syndrome. In all these patients bleeding time was shortened by DDAVP infusion. In contrast, neither platelet aggregation nor bleeding time was modified in two subjects with Glanzmann's thrombasthenia.**

Interpretation and Conclusions. **Our *in vitro* experiments indicate that DDAVP interacts directly with platelets and facilitates their activation via other agonists. *In vivo* results suggest that this effect occurs and is clinically relevant in patients with platelet dysfunction responding to DDAVP with a shortening of bleeding time.**
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Key words: DDAVP, platelets, platelet activation, disorders of platelet function

Correspondence: Prof. Carlo L. Balduini, Istituto di Medicina Interna e Oncologia Medica, IRCCS San Matteo, p.le Golgi, 27100 Pavia, Italy. Fax: international +39-0382-525222 – E-mail: c.balduini@smatteo.pv.it

Over the last several years, desmopressin (DDAVP), a synthetic analog of arginine vasopressin (AVP) has become the drug of first choice for prevention and treatment of bleeding in patients with mild hemophilia A and von Willebrand's disease (vWD).[1] More recently, it has been proven to reduce bleeding tendency in many congenital and acquired disorders of platelet function.[2] While there is little doubt that the DDAVP effect in hemophilia and vWD results from the increase of factor VIII (FVIII) and von Willebrand's factor (vWF) in blood, its mechanism of action in platelet disorders is still a matter of debate.[3] In particular, contrasting results have been obtained concerning the ability of DDAVP to interact directly with platelets and to induce platelet modifications which may favor the hemostatic process. Thus, we sought to re-examine the question of DDAVP action on human platelets, and studied the *in vitro* and *ex vivo* effects of this molecule both as a single agent and in conjunction with other known agonists of platelet activation. The results reported here suggest that DDAVP interacts directly with platelets and exerts a priming effect on platelet aggregation stimulated by ADP or collagen.

Design and Methods

Study subjects

Blood for *in vitro* experiments was obtained from healthy adult volunteers who had not taken aspirin-containing drugs for at least 10 days before donation.

The *in vivo* effect of DDAVP on platelet function was studied in 12 patients affected by a bleeding disorder who received a test dose of this drug at the time of diagnosis or before an elective surgical treatment. All subjects gave informed consent to participation in the study. Two patients were affected by mild hemophilia A, three by vWD, three by May-Hegglin anomaly, one by gray platelet syndrome, one by Ehlers-Danlos syndrome and two by Glanzmann's thrombasthenia. DDAVP (Minirin®, Ferring Pharmaceutical, Kiel, Germany) was infused at a dosage of 0.3 µg/kg over 30 minutes in 50 mL of saline. A post-infusion increase of factor VIII:C (determined by a coagulometric method and the addition of factor VIII deficient plasma - Behringwerke AG, Mar-

burg, Germany), or vWF:Ag (Assera® - Plate vWF kit-Diagnostica Stago, Asnière sur Seine, France) ≥ 50% was observed in all patients, and a shortening of bleeding time (measured with a modified Ivy's technique using a Surgicutt® device – International Technidyne Corporation, Edison; NJ) ≥ 50% was observed in all patients, with the exception of the two subjects with Glanzmann's thrombasthenia.

Sample preparation

For the *in vitro* studies, blood from healthy volunteers was collected in sodium heparin. Sodium heparin was chosen as the anticoagulant because AVP requires extracellular magnesium for its interaction with the V1 receptor[4] and because citrate significantly blunts the platelet aggregation response to AVP.[5]

Blood obtained from patients before and after DDAVP infusion was anticoagulated with sodium heparin for aggregation studies, with EDTA (1 mM, final concentration) for flow cytometry and with 3.8% (w/v) sodium citrate for vWF: Ag and factor VIII:C assays.

Platelet rich plasma (PRP) and platelet poor plasma (PPP) were obtained by standard methods as previously reported.[6] When required, after PRP collection, the remaining whole blood was mixed 1:1 with NaCl 0.9% and layered onto Ficoll (Pharmacia, Uppsala, Sweden). After centrifugation at 400×g for 20 minutes, mononuclear cells were harvested from the band at the interface between the sample layer and the Ficoll solution. Cells were then washed once in NaCl 0.9% before being used.

Platelet aggregation

Platelet aggregation was studied in heparinized PRP containing 250×10^9 platelets/L by Born's method[7] with a Lumi-Aggregometer (Chrono-Log Corporation, Havertown, PA, USA). In some experiments PRP was enriched with mononuclear cells prepared as described above in order to contain 250, 500 or 4,000 mononuclear cells $\times 10^6$/L. Platelet aggregation was stimulated by AVP, ADP (Sigma Chemical Company, St. Louis, MO, USA) or collagen (Mascia Brunelli, Milan, Italy), and the maximal extent of aggregation was recorded. When required, DDAVP or $(CH_2)_5$ Tyr(Me)AVP (Sigma) were added to the stirring sample immediately prior or three minutes before agonist addition. Preliminary experiments showed that the excipients of Minirin® had no effect of platelet aggregation induced by AVP, ADP or collagen.

Flow cytometry studies

For the *in vitro* studies, heparinized PRP containing 50×10^9 platelets/L was incubated for 5 min at 37°C with DDAVP (1, 25 and 250 nM). PRP was also incubated with PBS as the negative control or with 25 uM thrombin receptor activating peptide (TRAP)(gift from Hoffman-La Roche, Basel, Switzerland) for a positive control. At the end of incubation, platelets

were fixed with 0.2% paraformaldehyde. For the *ex vivo* experiments, PRP obtained from blood anticoagulated with EDTA was immediately diluted with PBS, pH 7.2, containing EDTA (17.5 mM Na_2HPO_4, 17.5 mM Na_2HPO_4, 150 mM NaCl, 0.3 mM EDTA) and fixed with 0.2% paraformaldehyde. Then, for both the *in vivo* and *ex vivo* experiments, fixed platelets were incubated for 30 min in the dark, at room temperature, with saturating concentrations of FITC-bound monoclonal antibodies against the activation-dependent antigens CD62 (GMP 140) or CD63 (GP 53)(Immunotech SA, Luminy, France) or with a FITC-conjugated unrelated monoclonal antibody (anti-CD14-MO2, Coulter Corporation, Miami, FL, USA) for non-specific binding. Binding of monoclonal antibodies to platelets was analyzed in a Coulter Epic XL (Coulter Corporation). The platelet population was identified on a FSC/SSC plot and a gate including platelets of all sizes and excluding debris was analyzed for green fluorescence: twenty thousand events were collected. It was shown in preliminary experiments that the excipients of Minirin® do not interfere with platelet expression of activation-dependent antigens.

Data analysis

Statistical comparisons were performed with StatView software (Abacus Concepts, Berkeley, CA, USA) and ANOVA or Scheffé tests were used.

Results

In vitro experiments

In vitro experiments failed to show any significant effect of DDAVP as a single platelet agonist, in that at concentrations of 1.0 to 250 nM it did not induce either platelet aggregation or surface expression of the activation-dependent antigens CD62 and CD63 (data not shown). However, platelet pre-incubation with DDAVP greatly reduced platelet aggregation response to AVP. The inhibitory effect of DDAVP was quite similar to that of $(CH_2)_5$ Tyr(Me)AVP, a well known antagonist of platelet V1-receptor for AVP[8] (Table 1). DDAVP pre-treatment also increased

Table 1. Effect of platelet pre-incubation with DDAVP and $(CH_2)_5$ Tyr(Me)AVP on maximal extent of aggregation induced by AVP. Mean of 10 experiments ± SD.

	platelet aggregation (%)
AVP 10 U/mL	55±20
DDAVP 25 nM + AVP 10 U/mL	35±26*
DDAVP 250 nM + AVP 10 U/mL	10±8*
$(CH_2)_5$ Tyr(Me)AVP 25 nM + AVP 10 U/mL	33±11*
$(CH_2)_5$ Tyr(Me)AVP 250 nM + AVP 10 U/mL	13±11*

*$p \leq 0.01$ with respect to AVP.

Table 2. Effect of a three minute platelet pre-incubation in the presence of DDAVP on maximal extent of aggregation induced by collagen and ADP. Similar results were obtained when DDAVP was added to PRP immediately prior to the agonist. Different collagen and ADP concentrations were used in each donor in order to induce a 30-50% control aggregation (ADP concentration: mean 0.69 µM, range 0.25-1; collagen concentration: mean 0.53 µg/mL, range 0.25-1). Mean of 13 experiments ± SD.

Pre-incubation	ADP-induced aggregation (%)	Collagen-induced aggregation (%)
Saline	42±7	39±11
DDAVP 1 nM	47±18	47±12*
DDAVP 25 nM	53±22*	57±23°
DDAVP 250 nM	42±18	14±10°

$p \leq 0.05$; °$p \leq 0.01$ with respect to saline.

platelet aggregation response to ADP and collagen, although the extent of this modification was different in individual healthy donors (Table 2). A significant increase of collagen-stimulated aggregation was observed with both 1 nM (a pharmacologic concentration) and 25 nM DDAVP, while 25 nM were necessary to potentiate ADP aggregation significantly.

When the concentration of DDAVP was raised to 250 nM, ADP-induced aggregation was no longer potentiated and collagen-induced aggregation was strongly inhibited. In three experiments, the priming effect of DDAVP on platelet aggregation induced by collagen or ADP was similar in PRP samples containing 250, 500 or 4,000 mononuclear cells ×10⁹/L (data not shown).

Ex vivo experiments

Platelet modifications induced *in vivo* by DDAVP infusions were quite similar to those observed during the *in vitro* experiments, in that the drug did not induce expression of activation-dependent antigens, but had a priming effect on platelet aggregation stimulated *in vitro* by collagen and ADP. Figure 1 shows that neither CD62 nor CD63 expression after DDAVP infusion increased over baseline values at any of the investigated time intervals. Figure 2 shows that the maximal extent of platelet aggregation induced by ADP and collagen was significantly greater at the end of DDAVP infusion and one hour later than at baseline. This figure does not take into account the two patients with Glanzmann's thrombasthenia, whose platelets failed to aggregate before, during or after DDAVP infusion.
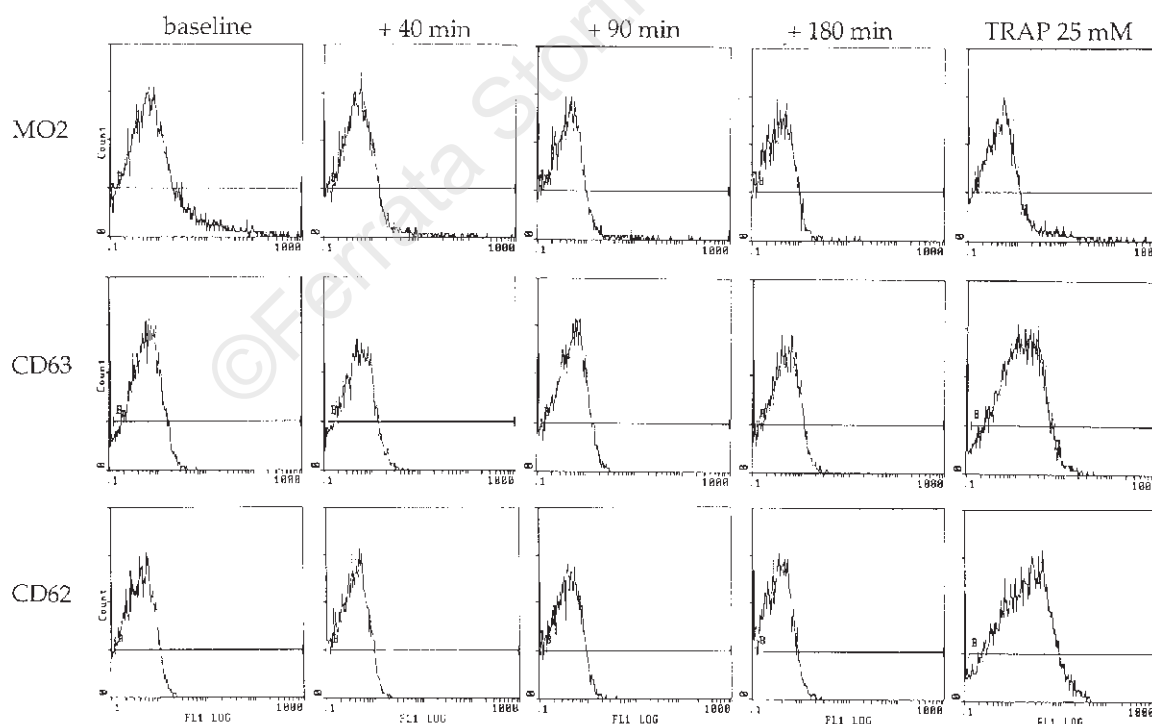

Figure 1. Flow cytometric analysis of the binding of monoclonal antibodies against platelet activation-dependent antigens (CD62 and CD63) to platelets obtained from a patient with a bleeding disorder before and at different times after DDAVP infusion. Histograms with the log of fluorescence intensity on the abscissa and cell number on the ordinate are shown. Histograms obtained in this patient are representative of those obtained in all other patients.
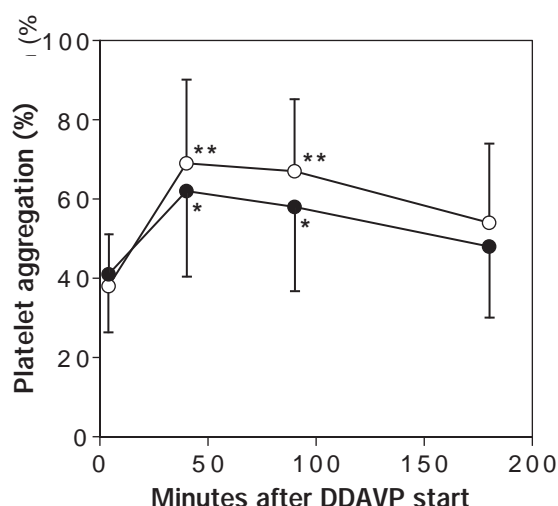
Figure 2. Maximal extent of *in vitro* platelet aggregation induced by ADP (close circles) or collagen (open circles) in samples obtained from 10 patients with a bleeding disorder receiving a 30 minute infusion of DDAVP. *$p \leq 0.05$, **$p \leq 0.01$ with respect to pre-DDAVP value.

Discussion

The cellular effects of AVP are mediated by interactions of the peptide with two principal types of receptors: V1 (located on smooth muscle, renal medullary interstitial cells and platelets) and V2 (located on the cells of the kidney collecting system and monocytes). The biological effects of AVP-V1 interaction include vasoconstriction, vascular smooth muscle cell growth, glycogenolysis, ACTH release and platelet activation, while AVP binding to V2 greatly increases permeability of the renal collecting tubules to water and urea.[3] DDAVP is a synthetic analog of AVP with augmented antidiuretic (V2) activity and absent (or nearly absent) vasopressor (V1) effects,[9-11] which was primarily used in the treatment of conditions such as diabetes insipidus and enuresis. Subsequently, it was shown to induce the release of FVIII and vWF from endothelial cells by stimulating monocytes to secrete platelet-activating factor (PAF),[12] and due to this property it is currently used to treat mild hemophilia A and vWD.[13] More recently, it has been reported that DDAVP infusion results in a shortening of bleeding time in patients with primary (congenital or acquired) disorders of platelet function,[14-16] but the mechanism for this has not been clarified. In particular, contrasting results have been obtained on the ability of DDAVP to promote platelet activation. On the basis of *in vitro* experiments some authors concluded that DDAVP does not induce human platelet aggregation or favor platelet aggregation stimulated by other agonists[17] and that it does not have any direct effect on platelet adhesion to collagen,[18] platelet secretion, cal-

cium movement, arachidonate metabolism or inositol phospholipid hydrolysis.[19] However, other *in vitro* experiments showed that DDAVP inhibits platelet response to AVP,[17] enhances aggregation response to subthreshold concentrations of ADP or thrombin,[20] stimulates the expression of activation-dependent platelet antigens[21] and induces formation of platelet microparticles.[22] The results of the *in vitro* study we describe in the present paper support the hypothesis that DDAVP at a pharmacological concentration is not able to activate platelets, but they also suggest that it can potentiate platelet aggregation induced by other agonists. Moreover, they confirm that DDAVP interacts directly with platelets, since it inhibited platelet aggregation induced by AVP and its priming effect on aggregation induced by ADP or collagen was not dependent on the presence of monocytes. On the whole, results from literature and our experience suggest that platelets bind DDAVP and that the result of this interaction may be different according to the experimental setting. This conclusion is not surprising, since it is well known that *in vitro* platelet response to stimulating agents is strongly influenced by the methods used to prepare platelet samples and by the composition of the suspending medium. Therefore, *in vitro* experiments indicate that DDAVP is potentially able to favor platelet activation, but they can not predict if this action really occurs *in vivo*. A solution to this dilemma can be obtained only from *in vivo* studies. Two reports from literature[23,24] and our own experience indicate that both in healthy subjects and patients with bleeding disorders DDAVP administration does not induce expression of the activation-dependent antigens CD62 or CD63. This conclusion is also supported by the study by Jilma *et al.*, who showed that the circulating level of P-selectin (CD62) was not increased by DDAVP infusion.[25] There is, therefore, a general agreement that DDAVP *in vivo* does not induce a release reaction, but two pieces of evidence from literature and our study suggest that it can promote platelet modifications favoring the hemostatic process. In fact, DDAVP infusion has been shown by Horseman *et al.*[22] to promote vesciculation and procoagulant activity of platelets, and by Lethagen *et al.*[26] to increase platelet adhesiveness as measured by a platelet retention test. This latter effect was dependent on glycoprotein (GP) IIb-IIIa, since it was not observed in two patients with Glanzmann's thrombasthenia; moreover, in these two patients DDAVP did not shorten the bleeding time. We also observed a clear effect of DDAVP infusion on platelet function in 10 of 12 patients affected by bleeding disorders, in that the maximal extent of collagen and ADP-induced platelet aggregation was increased by more than 50% with respect to the basal values. Bleeding time was significantly shortened in all the patients with a platelet disorder responding to DDAVP with an increase of platelet aggregation, while it remained unchanged in the two subjects with

Glanzmann's thrombasthenia, whose platelet aggregation was not modified by drug infusion. Therefore, both in our and Lethagen's experience,[26] DDAVP infusion in patients with platelet disorders was able to shorten bleeding time whenever it had a priming effect on platelet aggregation or platelet adhesion as measured by a retention test. Since both tests depend on GPIIb-IIIa mediated platelet clumping we can conclude that the final effect of DDAVP on platelets is to facilitate GPIIb-IIIa-ligand interaction. This conclusion is also supported by previous investigations[27,28] that failed to demonstrate any clinical usefulness of DDAVP in patients with Glanzmann's thrombasthenia, who are genetically lacking in GPIIb-IIIa. Since our *in vitro* studies demonstrated a direct platelet-DDAVP interaction, we are tempted to infer that also *in vivo* the pro-hemostatic effect of DDAVP derived from its direct interaction with platelets. However, it is well known that DDAVP increases levels of vWF with a high multimeric composition and induces changes in circulating catecholamines,[29,30] and therefore we can not exclude that these modifications have a role in the changes of platelet aggregation observed in our patients.

In conclusion, our study demonstrates that DDAVP interacts *in vitro* with platelets facilitating their aggregation response. We hypothesize that this effect is operative and clinically relevant also *in vivo* in patients with disorders of platelet function with normal amounts of GPIIb-IIIa.

Contributions and Acknowledgments

CLB was primarily responsible for the conception of this investigation and writing of the paper. PN, SB and PS performed the experiments and helped CLB with data analysis interpretation. GG was primarily responsible for the clinical assessment of the patients. The order of authorship reflects the significance of each of the author's contribution to the study.

Disclosures

Conflict of interest: none.
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Manuscript processing

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