Changes in platelet vasopressin after smoking in relation to platelet function

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(Received for publication February 15, 1999)

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Key words: nicotine, platelet, vasopressin, receptor, aggregation, desensitization

Summary

We examined the effects of smoking on plasma and platelet arginine vasopressin (AVP) levels, platelet aggregation with AVP, and AVP binding to platelets in normal subjects. Plasma and platelet AVP levels increased to the peak level at 5 to 10 min after the start of smoking, and decreased thereafter in parallel with plasma nicotine levels. A good correlation was found between plasma nicotine levels and plasma AVP levels, and also between plasma and platelet AVP levels. These results suggest that platelets rapidly take up endogenously released AVP from plasma. We also observed a decreased platelet aggregation with AVP *ex vivo* when the plasma and platelet AVP levels were elevated *in vivo* after smoking. Binding experiments of washed platelets did not demonstrate any change in receptor number or affinity before and after smoking. These findings indicate that endogenously released AVP taken up by platelets desensitizes platelets to AVP without loss of AVP receptor number or modification of receptor affinity for AVP.

Introduction

The neurohypophyseal hormone arginine vasopressin (AVP) has multiple actions, including antidiuresis in the kidney, contraction of smooth muscle cells, stimulation of liver glycogenolysis, modulation of adrenocorticotrophic hormone release from the pituitary. These actions of AVP are mediated through specific G protein-coupled receptors. AVP receptors have been classified into at least three subtypes, V1a (vascular hepatic), V1b (anterior pituitary), and V2 (kidney) AVP receptors [1]. In addition to many other actions, AVP has been shown to induce shape changes and aggregation of human platelets [2,3]. We and others [3,4,5,6] have described the characteristics of specific receptor belonging to the V1a vascular subtype on intact human platelets. About 90% of the circulating immunoreactive AVP in normal humans is found in platelets [3,7,8,9], and is associated with the

membrane component of platelets [10]. However, it remains controversial whether platelets take up AVP from the plasma and discharge it with the change in plasma AVP levels. Provided that platelets take up the endogenously released AVP, it would be of interest to know its pathophysiological significance. Although cigarette smoking is known to stimulate the release of AVP, it remains controversial whether or not the smoking-induced increase in plasma AVP is caused by nicotine stimulation.

In the present study, we estimated plasma nicotine levels and AVP levels in platelet-free plasma (PFP) and platelets after stimulation of endogenous AVP release by acute cigarette smoking in healthy subjects. We also performed a platelet aggregation study with AVP, as well as binding assay of AVP to intact platelets.

Subjects

Twelve men (mean age, 28.8 ± 0.9 years) from the medical school staffs, who were in good general health and had not taken any medications for at least 10 days, participated in this study. They were all habitual smokers, and smoked an average of 20 cigarettes per day. They gave informed consent before the study and were abstained from food, water and cigarettes overnight prior to the study.

Methods

Venous blood samples were obtained from the antecubital vein using an indwelling vein needle to measure the plasma nicotine levels and AVP levels in the PFP and platelets, and to carry out the aggregation study and the AVP binding assay following 30 min rest in the sitting position. After baseline blood samples was obtained, the subjects smoked one commercial filter cigarette (containing 2.2 mg nicotine per cigarette) with deep inhalation over a period of 5 min. Blood samples were obtained at 5, 10 and 30 minutes after the start of smoking. Plasma nicotine levels and AVP levels in PFP and platelets were estimated. Platelet aggregation studies were performed at 0, 5, 10 and 30 min, and binding assays were carried out at 0 and 30 min.

Radioimmunoassay of PFP and platelet AVP

For radioimmunoassay of the AVP, 10 ml of venous blood was taken into a plastic syringe containing 50 ml heparin and centrifuged at 140 g for 10 min at 25°C to obtain platelet-rich plasma (PRP). Then, 1 ml of PRP was centrifuged at 25000 g for 15 min at 4°C to obtain platelets and platelet-free-plasma (PFP). The platelet count in the PRP was $5.21 \pm 0.41 \times 10^5/\text{ml}$, which was less than $5 \times 10^3/\text{ml}$ in the PFP. The platelet pellet from 1 ml of PRP was suspended in isotonic saline containing 0.1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Mo, USA). PFP and platelet AVP levels were measured by specific radioimmunoassay after extraction by the acetone-petroleum ether method as reported previously [11]. Highly specific antibody to AVP was a kind gift from Dr. T. Saito (Jichi Medical College, Tochigi, Japan). It did not cross-react with dDAVP and oxytocin, but 4.2% cross-reactivity was observed with lysine vasopressin. The intra-assay and inter-assay coefficients

of variation were 6.0% and 7.4%, respectively, with a detection limit of 0.7 fmol/ml. The platelet AVP levels were expressed as AVP from 1 ml of PRP, in which the platelet number was corrected to $5 \times 10^4/\text{ml}$.

Measurement of plasma nicotine levels

Separate samples of PRP were centrifuged at 1400 g to obtain supernatant for measurement of the plasma nicotine levels and plasma osmolalities.

The plasma nicotine levels were determined by the FTD-gas chromatographic technique according to Feyerebant and Russel [12] after microextraction (using 100 ml plasma) with isopropyl ether. A Hitachi gas chromatograph (Model 153, Hitachi Co., Tokyo, Japan) equipped with an FTD detector was used. The glass columns (2m $\times$ 3mm id) were packed with 80-100 mesh chromosorb W-AW, coated with 10% Apiezon L and 10% KOH. The flow rate of carrier gas (helium) was 50 ml/min, and the column temperature, injection port temperature and detector temperature were 190°C, 300°C, and 300°C, respectively. With this method, the calibration curve was linear over a working range of 20 to 100 ng/ml. The recovery of added nicotine at between 2 to 10 ng to 100 ml of plasma was about 100%, and the intra-assay coefficients of variation were 12.9, 3.4 and 4.2%, respectively, when 2, 5 and 10 ng of nicotine was added.

Platelet AVP binding assays

Binding assays were performed as reported previously [3], with slight modifications. Briefly, 17 ml of whole blood was collected into a plastic syringe containing 3 ml of citrate-phosphate-dextrose solution, and immediately transferred to a plastic tube containing EDTA-2Na (2mg/ml). The blood was centrifuged at 140g for 10min at 25°C to separate the PRP was washed with Tris-HCl Buffer-A (Tris-HCl 50mM, NaCl 138mM, EDTA-2Na 5mM, glucose 2mg/ml, pH 7.4) and centrifuged at 1500g for 10min at 4°C. The platelet pellet was washed twice with the same buffer and resuspended in Tris-HCl Buffer-B (Tris-HCl 50mM, NaCl 138mM, MgCl₂ 10mM, glucose 2mg/ml, pH 7.4), and adjusted to 10^6 platelets/ml. For the binding assay, 150ml of platelet suspension, 1 nM of [³H] AVP (New England Nuclear, Boston, MA, USA) and 20ml of various concentrations of unlabelled AVP (Grade VI, Sigma Chemical Co., St. Louis,

Table 1. PFP and platelet AVP levels, plasma nicotine levels and maximal percentage aggregation with AVP before and after smoking.

		Before	Peak	30 min after
Plasma nicotine (ng/ml)	A	4.9 ± 1.7	24.7 ± 4.2 ***	11.6 ± 3.0 *
	B	4.5 ± 3.4	79.9 ± 23.9 *	23.9 ± 4.9
PFP AVP (f mol/ml)	A	2.8 ± 0.5	34.8 ± 12.6 *	7.0 ± 1.6
	B	8.4 ± 1.3	107.8 ± 30.3 *	27.6 ± 2.8
Platelet AVP (f mol/ml)	A	14.9 ± 2.2	26.1 ± 5.4 *	20.3 ± 2.9
	B	23.6 ± 2.5	135.1 ± 22.3 *	49.3 ± 6.8
Maximal aggregation (%)				
with 500 nM AVP	A	32.4 ± 7.1	22.8 ± 7.0 *	32.8 ± 8.3
	B	50.3 ± 14.7	4.0 ± 1.5 *	38.3 ± 12.3
with 250 nM AVP	A	33.4 ± 8.0	21.3 ± 6.1 *	29.9 ± 7.6
	B	52.3 ± 15.7	4.0 ± 1.0 *	32.0 ± 9.7
with 100 nM AVP	A	33.4 ± 7.7	22.8 ± 6.9 *	31.6 ± 9.0
	B	50.3 ± 14.5	4.3 ± 0.9 *	33.7 ± 10.4
with 50 nM AVP	A	29.6 ± 7.0	20.4 ± 5.9 *	28.6 ± 7.6
	B	45.0 ± 13.1	3.7 ± 1.2 *	29.0 ± 8.5
Maximal aggregation (% of baseline)				
with 500 nM AVP	A	100	65.5 ± 12.1 **	94.7 ± 10.4
	B	100	8.2 ± 1.7 ***	74.2 ± 5.9 **
with 250 nM AVP	A	100	62.9 ± 11.0 **	89.0 ± 8.6
	B	100	9.2 ± 3.5 ***	61.5 ± 4.0 **
with 100 nM AVP	A	100	64.0 ± 11.0 **	90.6 ± 9.2
	B	100	10.6 ± 3.9 ***	65.4 ± 4.0 **
With 50 nM AVP	A	100	65.6 ± 11.6 **	90.6 ± 6.7
	B	100	9.7 ± 3.5 ***	64.1 ± 0.6 **

A: subjects without nausea (n=9), B: subjects with nausea (n=3)

*** P<0.001, ** p<0.01, * p<0.05 compared with the baseline level

MO, USA), and 12.5ml of 4% BSA, adjusted a final volume to 250ml with Tris-HCl buffer-B, were incubated at 25°C for 180min in duplicate. After incubation, the reaction was stopped by adding 4ml of ice-cold buffer-B, and the samples were rapidly filtered using Whatman GF/C filter to separate free from bound [^3H] AVP. After the filters had been rinsed twice with 4ml of the buffer and dried, the radioactivity was counted in a Packard liquid scintillation counter (United Technologies Packard, IL, USA). The specific binding was defined as the difference between the total and non-specific binding. The non-specific binding was defined as the radioactivity bound to the platelets in the presence of 2mM of unlabelled AVP. Scatchard analysis of competition curves was employed to estimate the receptor number and affinity, and the slopes and intercepts were calculated by the method of least squares.

Platelet aggregation studies

Platelet aggregation studies were carried out by the turbidometric methods of Born [6] using an NKK Hematracer I (Niko Bioscience, Tokyo, Japan) which was calibrated with PRP and PFP to 0 and 100% light transmission, respectively. Employing 200ml of PRP from heparinized blood, which was adjusted to contain 4×10^5 platelets/ml, the platelet aggregation was recorded as the increase in light transmission occurring after the addition of AVP at 37°C. The maximum increase in light transmittance obtained within 5min after the addition of AVP was defined as the maximal percentage aggregation.

The effects of preincubation with nicotine on the AVP-induced aggregation of platelets *in vitro* were studied. The final concentration of nicotine added to the PRP was 100 ng/ml, which was the highest level to be reached *in vivo* after smoking. Following incubation with nicotine for 20min at 25°C and then for 10min at 37°C, aggregation studies with fixed 4 concentrations (500, 250, 100 and 50nM) of AVP were performed. The effects of nicotine on the AVP binding characteristics were also examined. Binding experiments were reperformed after overnight incubation with 100 ng/ml of nicotine.

Statistical analysis. All values are expressed as the mean $\pm$ SEM, and analyzed by two-way analysis of variance followed by Scheffe-type multiple comparison. Correlation coefficients were calculated by the least

squares method.

Results

The subjects were divided into 2 subgroups according to the presence (3 subjects) or absence (9 subjects) of nausea during the experiments, since nausea is an important stimulating factor of AVP release.

Plasma nicotine levels increased to the peak level at 5 to 10min after the beginning of smoking, and decreased thereafter in all subjects (Table. 1). However, there was a wide variation in the peak nicotine levels after smoking (4.1 to 125.2 ng/ml), probably due to the technique of inhaling. Subjects with nausea achieved higher peak plasma nicotine levels after smoking than those without nausea (79.9 ± 23.9 ng/ml and 24.7 ± 4.2 ng/ml respectively). The PFP and platelet AVP levels increased to the peak level at 5 to 10min after the beginning of smoking, and decreased in parallel with plasma nicotine level. The subjects with nausea achieved much higher peak levels of PFP and platelet AVP than those without nausea (PFP-AVP; 107.8 ± 30.3 f mol/ml vs. 34.8 ± 12.6 f mol/ml, platelet AVP; 135.1 ± 22.3 f mol/ml vs. 26.1 ± 5.6 f mol/ml, respectively).

A significant correlation was found between plasma nicotine levels and PFP-AVP levels determined in the subjects without nausea ($r = 0.72$, $p < 0.001$) (Fig. 1). However, PFP-AVP levels did not change in those with plasma nicotine level less than 18ng/ml. A significant correlation was observed between PFP-AVP levels and platelet AVP levels determined before and after smoking in all cases ($r = 0.85$, $p < 0.001$) (Fig. 2). No subjects had significant change in plasma osmolality before and after smoking.

In contrast to an increase in PFP and platelet AVP levels, the maximal percentage aggregation with fixed 4 concentrations of AVP (500nM, 250nM, 100nM and 50nM of AVP) was significantly decreased at 5 to 10 min after smoking but recovered at 30 min in both groups. As maximal percentage aggregation with AVP showed wide variation before smoking, we expressed the maximal percentage aggregation as a percent of the pre-smoking value. Reduced platelet aggregation with AVP was observed more prominent in the subjects with nausea.

A significant inverse correlation was found between

Fig. 1 Correlation between plasma nicotine levels and PFP AVP levels determined before and after smoking in the subjects without nausea.
 $Y = -5.80 + 1.31X$ $r = 0.72$, $p < 0.001$

Fig. 2 Correlation between PFP AVP levels and platelet AVP levels determined before and after smoking in all cases.
 $Y = 12.79 + 0.82X$ $r = 0.85$, $p < 0.001$

PFP-AVP levels and the maximal percentage aggregation with 100nM AVP (Fig. 3a and b). (without nausea, $r = -0.80$, $p < 0.001$; with nausea $r = -0.86$, $p < 0.001$). A significant inverse correlation was also found between platelet AVP levels and the maximal percentage aggregation with 100nM AVP in both groups. (Fig. 4a and b). (without nausea, $r = -0.546$, $p < 0.001$; with nausea, $r = -0.87$, $p < 0.001$).

There were no significant differences in the numbers of binding sites (B max: 189.8 ± 24.8 sites/cell at 0 min vs. 177.3 ± 24.7 sites/cell at 30 min;) and in affinity (K_a : $4.9 \pm 1.3 \times 10^8$ M at 0 min vs. $4.8 \pm 1.1 \times 10^8$ M at 30 min) before and after smoking in subjects without nausea. In subjects with nausea, only 10% reduction of binding sites was observed at 10 and 30 min after beginning of smoking, but reduction of maximal percentage aggregation with AVP was 90%. Moreover, there was no effect on the binding characteristics after overnight preincubation of platelets with 100 ng/ml of nicotine in vitro. To test the influence of high level plasma nicotine on platelet aggregation with AVP, we measured the maximal percentage aggregation with fixed 4 concentration of AVP (500nM, 250nM, 100nM and 50nM of AVP) before and after preincubation with 100 ng/ml of nicotine in vitro. Nicotine had no significant effect on

aggregation.

Discussion

Cigarette smoking is thought to stimulate the release of AVP and its carrier protein, neurophysin, by nicotine stimulation [14-17] or by other mechanism [18]. The increase in plasma AVP produced by cigarette smoking is highly variable, because blood nicotine levels after smoking vary according to the degree of inhaling [19].

However, the plasma nicotine level was not determined in most previous studies. In the present study, we measured plasma nicotine and AVP levels in PFP and platelets after smoking. PFP-AVP levels increased to the peak level at 5 to 10 min after the beginning of smoking and decreased in parallel with the plasma nicotine levels. However, PFP-AVP levels did not change at low increase of plasma nicotine levels (plasma nicotine < 18 ng/ml). The results were compatible with the report of Goldsmith et al. [20] who indicated that a plasma nicotine level of 16 ng/ml administered through chewing gum did not stimulate AVP secretion. Although an increase in AVP levels after smoking may be due to some other mechanisms as reported by Rowe et al [18], our results clearly show that smoking with low grade increase in plasma nicotine levels dose not stimulate endogenous

Fig. 3 Correlation between PFP AVP levels and the maximal percentage aggregation with 100nM AVP expressed as a percent of the pre-smoking value after smoking in the subjects without nausea (3-a) and in the subjects with nausea (3-b).
 $Y = 102.04 - 1.01X$ $r = -0.80$, $p < 0.001$ (3-a)
 $Y = 89.46 - 0.69X$ $r = -0.86$, $p < 0.001$ (3-b)

Fig. 4 Correlation between platelet AVP levels and the maximal percentage aggregation with 100nM AVP expressed as a percent of the pre-smoking value after smoking in the subjects without nausea (4-a) and in the subjects with nausea (4-b).
 $Y = 115.91 - 1.44X$ $r = -0.55$, $p < 0.001$ (4-a)
 $Y = 101.79 - 0.68X$ $r = -0.87$, $p < 0.001$ (4-b)

release of AVP, but smoking with high grade increase in plasma nicotine levels stimulates AVP release in proportion to the plasma nicotine levels.

It remains controversial whether AVP is taken up by platelets when plasma AVP level is increased exogenously or endogenously in humans. Bichet et al. reported that changes in AVP levels by acute osmotic stimulation or suppression exerted little effect on platelet AVP, and small intravenous dose of AVP did not bind, but large dose of exogenous AVP could associate with the platelet fraction [9]. On the other hand, Nussay et al. found elevated platelet AVP levels after osmotic stimulation or intravenous infusion of a relatively small amount of AVP [8]. Preibisz et al. reported that human platelets could take up small amounts of AVP when the plasma AVP levels increased in response to tilt [7]. In a previous study, we failed to observe any increase in platelet AVP levels after osmotic stimulation, despite the increase in PFP AVP levels [3]. The present study clearly showed that platelets rapidly take up endogenously released AVP from the plasma, when plasma AVP levels become quite high level, but platelets discharge AVP thereafter. These data suggest that platelets may play a role in the rapid clearance of AVP from the circulation when the plasma AVP level becomes extremely high. Considering that platelets are in close contact with injured or atherosclerotic vascular wall, it is also possible that platelets provide a high concentration of AVP to the local vascular lesion.

AVP induces shape changes and aggregation of human platelets through V1a receptors, which are coupled to G protein belonging to Gq11 family [21], and mediate phosphoinositide hydrolysis and elevation of the cytosolic free calcium [22]. We have shown that maximal percentage aggregation with AVP is directly related to the number of AVP receptors on platelets in healthy humans [3]. An enhanced platelet aggregation due to cigarette smoking or to nicotine has been reported [23,24,25]. On the other hand, reduced platelet sensitivity to AVP with increase in plasma AVP after smoking was found [28]. In the present study, we found reduced platelet aggregation

with AVP in contrast to elevated both PFP and platelet AVP levels after smoking. The finding suggests that endogenously released AVP by smoking after taken up by platelets rapidly desensitizes platelets to exogenously challenged AVP. To clarify whether reduced platelet aggregation with AVP was due to the change in AVP receptor, we performed binding assays before and after smoking. B max of AVP receptors on the washed intact platelets and receptor affinity did not change significantly before and after smoking. Resynthesis and recycling of receptors are also possible mechanisms after the desensitization process as demonstrated in rat smooth muscle cells [26], but recycling to the surface was not observed in human platelet AVP receptors after undergoing internalization and degradation in vitro [27].

The human AVP V1a receptor was cloned and characterized [31]. The precise mechanism of rapid desensitization of AVP V1a receptor was studied in vitro using vascular smooth muscle cells [29], or other cells expressing the cloned receptor [30]. The study using vascular smooth muscle cells showed that hormone receptor occupancy, but not hormone-receptor complex internalization or conformational changes, was incriminated in this AVP homologous desensitization phenomenon. The study also showed that an uncoupling of the hormone-receptor complex and the post-receptor signal transduction mechanisms was important, and that protein kinase C activation was implicated in AVP-induced desensitization of Ca^{2+} mobilization in smooth muscle cells.

Homologous rapid desensitization to AVP in human platelets in vitro was also reported [22]. Subsequent addition of AVP to platelets pre-exposed to a quite high concentration (500mM) of AVP for a short period did not induce Ca^{2+} mobilization, phosphatidic acid formation or a platelet function response. Our results clearly demonstrate that endogenously released AVP after taken up by platelets desensitize the platelet to AVP in vivo without loss of AVP receptor number or modification of receptor affinity for AVP.

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