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5HT-Kinetics and Sensitivity of Human Blood Platelets: Variations with Age, Gender and Platelet Number

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Key words

5HT kinetics – 5HT sensitivity – Platelet function – Age – Sex

Summary

Platelet shape change and aggregation as well as serotonin (5-hydroxytryptamine; 5HT) content in platelets, spontaneous release of 5HT, 5HT plasma levels, urinary excretion of 5-hydroxyindoleacetic acid (5HIAA) and plasma β-thromboglobulin (β-TG) were investigated in 45 healthy subjects (27 men, 18 women). Platelets from women were more susceptible to aggregation (both by 5HT and ADP) than platelets from men. However, in men, 5HT-induced shape change and aggregation as well as plasma β-TG concentration increased with age. In men, 5HT-induced platelet aggregation correlated positively with 5HT plasma levels. An inverse relationship was found in men between platelet number on the one hand and platelet 5HT content, 5HT release, 5HT plasma levels and urinary 5HIAA excretion rates on the other hand. In all subjects 5HT-induced platelet aggregation correlated negatively with platelet number.

These results indicate that age and gender must be considered in designing clinical studies on 5HT and in evaluating human platelet 5HT kinetics. The platelet number seems to be related to 5HT kinetics of platelets and their reaction to 5HT in men. An age-dependent change in the reactivity of platelets to 5HT (rather than their absolute 5HT sensitivity) may contribute to the enhanced platelet turnover and higher incidence of cardiac events in older men.

Introduction

Blood platelets exhibit membrane receptors for 5-hydroxytryptamine (5HT) which belong to the $5HT_2$ subclass; in blood vessels both $5HT_1$ (endothelium) and $5HT_2$ receptors (vascular smooth muscle cells) have been shown to occur. Stimulation of $5HT_2$ receptors by 5HT causes vasoconstriction and activation of blood platelets, i. e. a shape change reaction and aggregation, whereas stimulation of $5HT_1$ receptors causes vasodilation (1). Blood platelets also contain endogenous 5HT and various subcellular elements involved in the regulation of the cellular dynamics of 5HT, e. g. a specific uptake system at the plasma membrane and specific intracellular organelles (dense bodies) storing 5HT, and monoamine oxidase (MAO), an enzyme responsible for the oxidative deamination of amines like 5HT.

In pathological conditions, e. g. cardiovascular and neuropsychiatric disorders, 5HT kinetics and activation of platelets sometimes seem to be altered (2, 3). However, the results of such studies have often been ambiguous. For this reason, a better understanding of the factors affecting physiological variability of platelet sensitivity and 5HT kinetics in healthy subjects would be desirable. Only a few such factors are known with respect to 5HT; for instance, the specific binding of imipramine, a marker for the 5HT uptake carrier, has recently been shown to exhibit circadian rhythmicity (4).

This study investigates whether there are other factors besides the known physiological ones which might influence the functional state of platelets. We present evidence that the activating effect of 5HT on human platelets is age- and sex-dependent, and that platelet number is a determining factor for 5HT kinetics and sensitivity in platelets.

Subjects, Materials, and Methods

Subjects

45 normotensive healthy volunteers (blood pressure ≤ 145/85 mmHg; 18 women and 27 men) aged 30–73 years participated in the study after having given their informed consent. Blood and urine samples were taken at 8 a. m. following overnight fasting. Sampling in women was performed between the tenth and eighteenth day of the menstrual cycle, if present. Subjects were asked to avoid products rich in tryptophan (nuts, tomatoes, bananas, chocolate etc.) for at least 48 hours prior to sampling and to take no drugs for at least 2 weeks beforehand.

Processing of Samples

Blood from the antecubital vein was mixed with 3.8% natrium citrate (9:1) and immediately centrifuged (at room temperature for 10 min at 160 × g) to obtain platelet rich plasma (PRP). Part of the PRP was taken for the platelet aggregation studies and shape change reactions, and the rest was further centrifuged with Na_2EDTA (final concentration 3 mM) at room temperature for another 10 min at 600 × g to obtain platelet poor plasma (PPP) and a platelet pellet. Part of the PPP was used to adjust the platelet number for the aggregation and shape change studies and the rest was centrifuged at 10,000 × g for 5 min to obtain platelet free plasma (PFP), which served as a reference in aggregation studies. PFP was also taken for the determination of free serotonin (5HT) and 5-hydroxyindoleacetic acid (5HIAA). The platelet pellet was resuspended in calcium-free Tyrode buffer and 3 mM EGTA and centrifuged for 10 min at 600 × g. The sediment was resuspended to give a stock suspension of 10^9 platelets/ml, which was used the determine to content, uptake and release of 5HT in platelets.

For the determination of β-thromboglobulin (β-TG) and platelet factor 4 (PF4), 4 ml of blood was drawn into an ice-cold syringe containing 1 ml of anticoagulant solution (A.C.D., EDTA, adenosine, prostaglandin E1). PPP and PFP were obtained as described above; the latter was stored at −20° C (up to a maximum of 3 months) for β-TG and PF4 measurement.

Samples of 24-hour urine (collected 7 a. m. onwards into 10 ml 6 N HCl) were deproteinised and passed through a purification filter containing silicagel RP C18 (5). The eluate was injected into an HPLC system for the determination of 5HIAA.

Measurements

Shape change reactions of platelets were measured according to the method described previously (6). Shape change reaction was induced by 5HT ($10^{-8} - 5 \times 10^{-6}$ M), ADP ($10^{-7} - 5 \times 10^{-6}$ M) and LDL (1–40 μg/ml).

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Results are expressed as the threshold concentration of the activating substance inducing half of the maximal shape change response (EC_{50}).

Platelet aggregation was performed according to Born with slight modifications (7, 8). Thus, platelets were flushed with a mixture of CO_2 5% and O_2 95% to keep to optimal pH (7.6–7.8) (8), platelet number was adjusted to 2.5×10^8/ml and tris buffer was not added because of its interaction with platelet function (9). All aggregation experiments were completed within 120 min after blood sampling; the schedule was identical for all subjects (5HT-induced aggregation during the first 60–70 min, ADP-induced during the next 25–30 min and collagen-induced during the remaining 20–30 min). Platelet response induced by 5 µM 5HT was expressed as the slope of aggregation (% change of light transmission/min). ADP-induced aggregation was carried out by adding increasing amounts of ADP (usually ranging from 1.0 to 5.0 µM f. c.) and determining the threshold concentration inducing biphasic irreversible aggregation. Concentration of collagen needed to induce aggregation were in the range 0.16–20.0 µg/ml; platelet response was expressed as EC_{50} (concentration of collagen inducing half of the maximal aggregation of platelets).

^{14}C-5HT uptake was measured in samples of platelet stock suspension (50 µl) diluted with 450 µl of buffer containing various concentrations of ^{14}C-5HT. After 2 min of incubation at 37° C the reaction was stopped by addition of ice-cold buffer (3 ml) and the sample was immediately filtered under vacuum through Whatman GF/C glass fiber filters. The latter were then rinsed twice with 3 ml of ice-cold buffer and transferred to plastic vials. The radioactivity on the filter was extracted with 10 ml Quickszint (Zinsser Analytic, Frankfurt, FRG) and counted by liquid scintillation spectrometry. Nonspecific uptake was determined by parallel incubation with 10 µM paroxetine. Maximal uptake velocity (V_{max}) and affinity constant (K_M) were calculated as uptake parameters.

5HT content of platelets, as well as 5HT and 5HIAA in PRP and PFP, were determined by HPLC according to Honegger et al. (10) slightly modified. The plasma samples were deproteinized by diluting (10:1) with 4 M perchloric acid containing 4 µM 5-hydroxyindolecarbonic acid (HICA) as an internal standard and centrifuged (10 min, 4° C, 9000 × g). The supernatant (20 µl) was injected into the HPLC system. Platelet samples were frozen at −80° C before treatment in the same way. For the determinations a solvent delivery pump (LC410, Kontron Analytic, Zürich, Switzerland) was used in conjunction with a cooled (2° C) automatic sampler (655A-40, Merck-Hitachi, Zürich, Switzerland). Pulseless solvent delivery was achieved with two membrane-type dampers (Portmann, Therwil, Switzerland). The column system consisted of a NewGard RP-18 and a 22 cm analytical cartridge RP-18 Spheri-5 with ID 4.6 mm (G18-013 and OD-224, Brownlee Labs., Santa Clara, CA, USA). The electrochemical detector (656 with 641 VA Detector, Metrohm, Herisau, Switzerland) with a glassy carbon electrode was set at a potential of 0.8 V (versus Ag/AgCl reference electrode) and a sensitivity of 5 nA. To cut off the first peak a Gynkotek Column Switching Module (Labomatik, Allschwil, Switzerland) was inserted between the column and the detector. The whole system was controlled by an integrator (HP 3390 A, Hewlett-Packard, Avondale, PA, USA) and an intermediate sampler/event control module (19400 A, Hewlett-Packard). The mobile phase contained NaH_2PO_4 0.1 M, EDTA 0.08 mM, n-octyl $NaHSO_4$ 0.025 mM and acetonitrile 8.5%. The pH was adjusted to 3.75 with H_3PO_4. The flow rate was 1.1 ml/min, pressure 110 bars.

Spontaneous release of 5HT was measured using platelets suspended in calcium-free Tyrode buffer (10^8 pl/ml) containing 5 µM paroxetine as 5HT-uptake inhibitor. Platelets were incubated at 37° C for 5 or 60 min. After the incubation the samples were cooled in ice and centrifuged in a Hereaus swing out centrifuge (10 min, 4° C, 1500 × g). The supernatants were discarded and the platelet pellets were washed twice with buffer. Platelets were lysed by adding a buffer containing 0.4 M perchloric acid and 0.4 µM 5HICA to the pellets. After centrifugation the supernatants were injected (20 µl) into the HPLC system. Spontaneous release of 5HT was calculated as the difference between the amount of 5HT remaining in the platelets after 5 and 60 min of incubation.

To assess platelet turnover β-TG in PFP was determined with a commercial radioimmunoassay kit (The Radiochemical Centre, Amersdam, UK) according to the method of Ludlam et al. (11). To exclude artefacts caused by *ex vivo* platelet destruction β-TG was not evaluated in those samples with PF4 > 10 ng/ml (12). PF4 was determined by radioimmunoassay (Abbott Laboratories, Chicago, IL, USA).

Urinary 5HIAA was measured using the HPLC method of Koch and Kissinger (5, 13) slightly modified.

Statistical Analysis

Results are expressed as mean ± sem. Student's 2-tailed t-test for unpaired samples was used for comparisons between two groups. Correlation coefficients were computed by the method of least squares. The null hypothesis was rejected when the P-value was less than 0.05.

Chemicals

Human LDL isolated by ultracentrifugation was a gift from Laboratoire des Lipides, Hôpital Cantonal, Geneva, Switzerland. 5HT-creatinine sulphate, 5HIAA, 5HICA were purchased from Sigma, St. Louis, MO, USA; ^{14}C-5HT from Amersham Int., Amersham, UK; ADP from Fluka AG, Buchs, Switzerland, collagen from Collagenreagent Horm, Hormon-Chemie GmbH, München, FRG; paroxetine from Hoffmann-La Roche AG, Basel, Switzerland. All other chemicals were obtained from commercial sources.

Results

Sex and Age Differences

Platelet Aggregation

The slope of aggregation caused by 5HT (Fig. 1a) was lower (7.33 ± 1.16 vs. 12.42 ± 1.3% light transmission/min; $p < 0.01$) and threshold for biphasic ADP-induced irreversible aggregation (Fig. 1b) was higher in men than in women (5.13 ± 1.18 vs. 1.71 ± 0.23 µM ADP; $p < 0.05$). However, in men only, 5HT- but not ADP-induced aggregation increased with age ($p < 0.01$, $r = 0.507$, $n = 27$, $y = 0.262x - 6.938$). The slope of 5HT-induced aggregation was greater in platelets of men above 60 years than in those of men below 50 (Fig. 2b) (12.1 ± 1.57 vs. 4.22 ± 1.91% light transmission/min; $p < 0.005$) and correlated with plasma levels of 5HT (Fig. 3) ($p < 0.002$, $r = 0.59$, $n = 26$, $y = 142.135x + 4.599$). Aggregation induced by collagen showed no differences related to age and sex.

Shape Change Reaction

The EC_{50} of 5HT for inducing a shape change reaction in platelets was lower in men over 60 years than in those below 50 years (76.8 ± 11.1 vs. 128 ± 17.7 nM 5 HT; $p < 0.05$) (Fig. 2a). This difference was not seen in women. The correlation with age in men was significant and negative ($p < 0.01$, $r = 0.536$, $n = 27$, $y = -2.064x + 208.24$) indicating that platelet reactivity and platelet aggregation to 5HT both increase with age. The shape change caused by other agents (such as LDL and ADP) did not show any dependence on age or sex.

5HT-Release

Spontaneous 5HT release from platelets was lower in the older than in the younger age group, but only in men (0.112 ± 0.038 vs. 0.303 ± 0.084 nmol 5HT from 10^9 platelets per 55 min; $p < 0.05$) (Fig. 2c).

β-Thromboglobulin

Plasma concentration of β-TG was the same in men and women. In men, however, an age-dependent increase in β-TG concentration was found. Mean values of β-TG in men over 60 years were significantly higher then those of men below 50 (19.0 ± 2.39 vs. 12.81 ± 1.18 ng/ml; $p < 0.05$), whereas no significant changes were seen in the corresponding groups of women (14.74 ± 3.3 vs. 12.75 ± 3.3 ng/ml) (Fig. 2d).

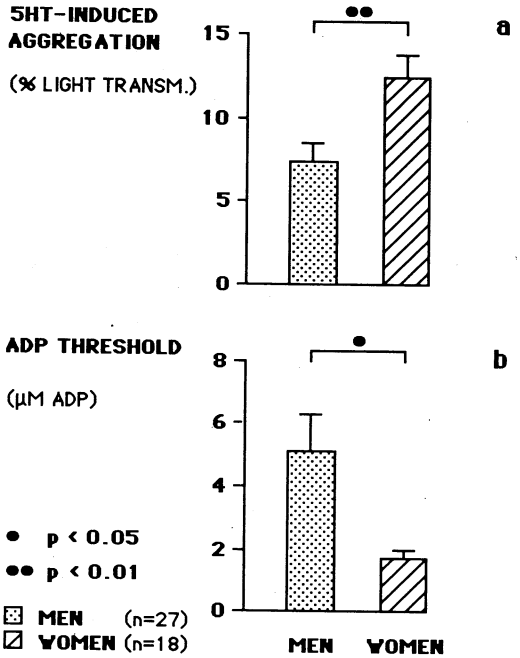

Fig. 1 a) Platelet aggregation induced by 5μM 5HT and b) threshold for biphasic irreversible aggregation induced by ADP in 27 men and 18 women. Mean with sem

5HIAA Excretion

Urinary excretion of 5HIAA in the whole group (men and women together) was higher in subjects under 50 than in those over 60 years (16.1 ± 1.6 vs 12.1 ± 0.9 μmol/24 h; $p < 0.05$). It was also negatively correlated with age ($p < 0.05$, $r = 0.34$, $n = 43$, $y = -0.149x + 21.693$). However, this decrease did not reach significance ($p > 0.05$) either within or between subgroups of men and women.

No age or sex relationships were found in platelet 5HT content, K_M and V_{max} of 5HT-uptake or plasma levels of 5HT and 5HIAA.

Platelet Number

No differences in platelet number of PRP (which corresponded to the platelet number in whole blood) were found in relation to age and sex. However, in men there were negative correlations between platelet number in PRP on the one hand and platelet 5HT content ($p < 0.002$, $r = 0.455$, $n = 27$, $y = -0.231x + 4.11$) (Fig. 4a), spontaneous release of 5HT ($p < 0.02$, $r = 0.555$, $n = 17$, $y = -1.909x + 3.602$) (Fig. 4b), 5HT levels in PFP ($p < 0.02$, $r = 0.462$, $n = 26$, $y = -11.828x + 3.54$) and the 24-hour urinary excretion of 5HIAA ($p < 0.05$, $r = 0.395$, $n = 26$, $y = -0.057x + 4.115$) on the other hand. These correlations were significant only in men.

In addition, there was a negative correlation between platelet number and slope of aggregation when all subjects were analysed together ($p < 0.01$, $r = 0.384$, $n = 45$, $y = -0.044x + 3.574$) (Fig. 5). Men did not differ from women.

No correlation was found between 5HT content of platelets and K_M or V_{max} of 5HT uptake.

Discussion

The present findings show that there are age- and sex-related differences in the aggregation and shape change responses of platelets. Plasma β-TG increased in men with age (Fig. 2d),

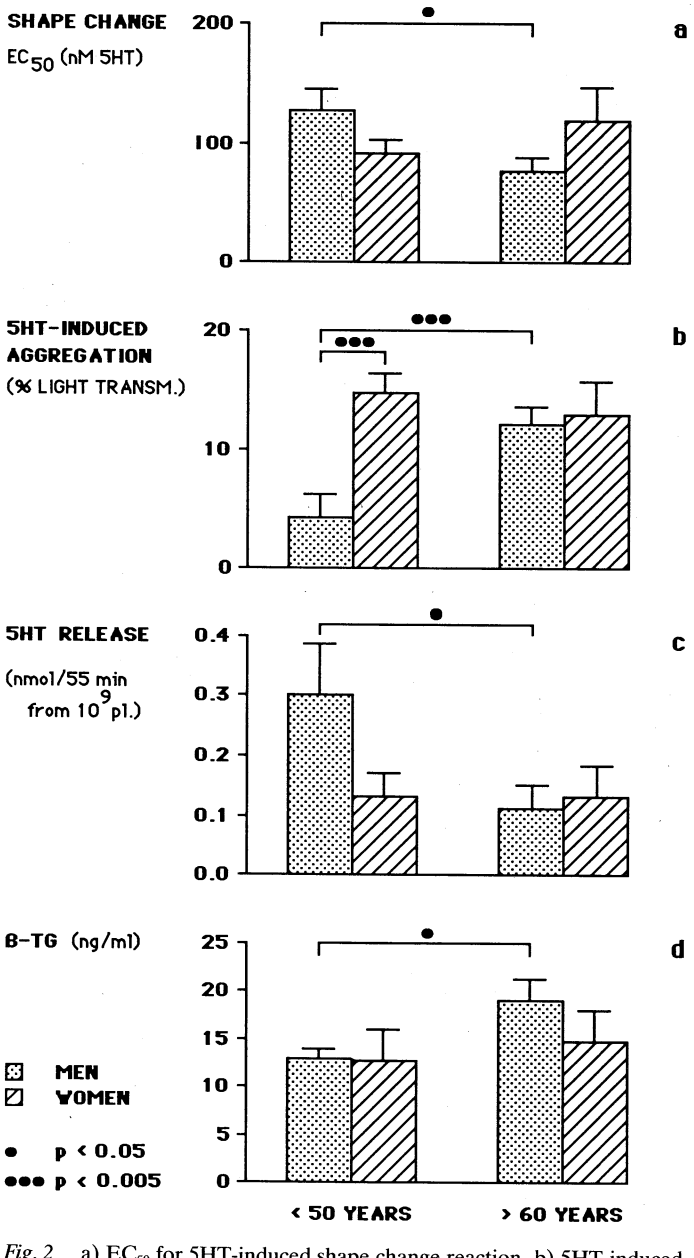

Fig. 2 a) EC_{50} for 5HT-induced shape change reaction, b) 5HT-induced platelet aggregation, c) spontaneous release of 5HT and d) plasma β-thromboglobulin concentration in subjects under 50 and over 60 years. Mean age (with sem) in subgroups of men was 41.6 ± 1.83 and 66.6 ± 1.48 years, in subgroups of women 40.6 ± 2.9 and 63.7 ± 1.8 years. Mean with sem, $n = 8$ in each of 4 subgroups

indicating that there is an enhancement of aggregation *in vivo*, probably brought about by changes in a whole mosaic of different plasma and endothelial factors. We found that the sensitivity of platelets also increased with age, but only in men, and only with 5HT (Fig. 2), not with other platelet activating substances (LDL, collagen and ADP). Apparently, the only platelet response *ex vivo* corresponding to platelet activation *in vivo* is that induced by 5HT. Our results are not in accordance with those of Yamanishi et al. who reported an enhancement of the amplitude of ADP-induced aggregation with age (14). This can probably be explained by differences in methodology (different incubation temperatures and in our case a time lag of 60–70 min between blood withdrawal and the measurement of ADP-induced aggregation due to prior measurement of 5HT-induced aggregation). The age-dependent, male-specific increase in reactivity of platelets to 5HT (accompanied by higher platelet turnover) may be a factor

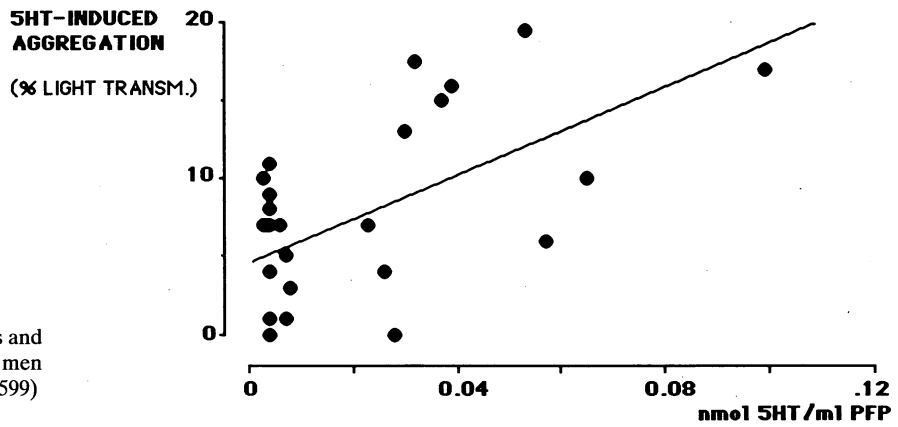

Fig. 3 Correlation between 5HT plasma levels and 5HT-induced platelet aggregation in men ($p < 0.002$, $r = 0.59$, $n = 26$, $y = 142.135x + 4.599$)

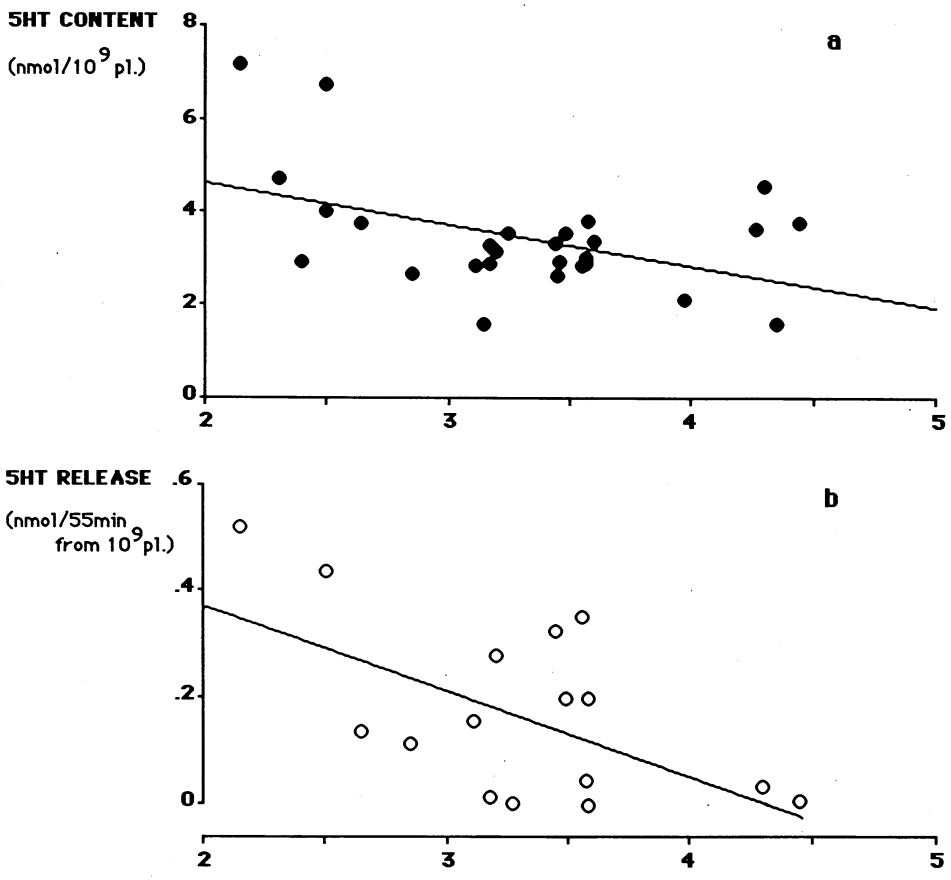

Fig. 4 Correlation between platelet number and a) 5HT content in platelets ($p < 0.002$, $r = 0.455$, $n = 27$, $y = -0.231x + 4.11$) and b) spontaneous 5HT release ($p < 0.02$, $r = 0.555$, $n = 17$, $y = -1.909x + 3.602$) in men

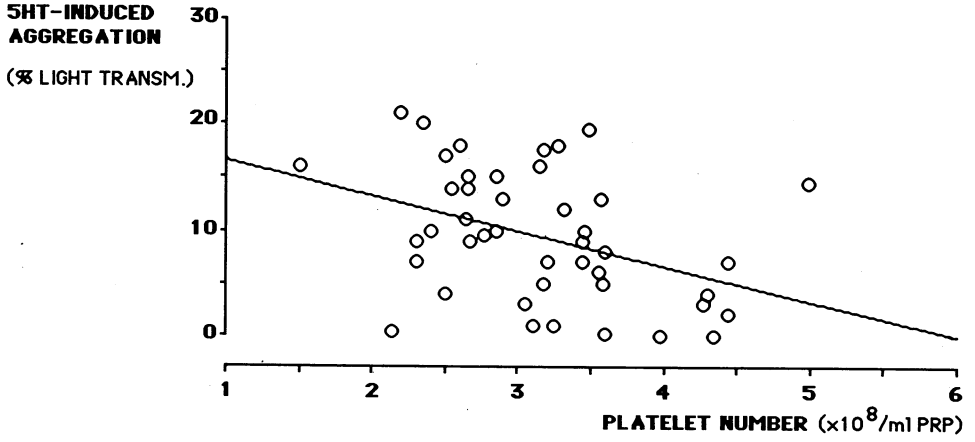

Fig. 5 Correlation between platelet number and 5HT-induced platelet aggregation in all normotensive subjects ($p < 0.01$, $r = 0.384$, $n = 45$, $y = -0.044x + 3.574$)

contributing to the well-known higher incidence of cardiac events in elderly men compared with women of the same age. On the other hand, platelets of women showed a higher absolute sensitivity than those of men to the aggregatory effects of 5HT and ADP, in agreement with previous findings on ADP (15) (Fig. 1). This may indicate that the higher incidence of thromboembolic disorders in men is related to a change in sensitivity rather than to its absolute degree.

Our results point to the possibility that platelet number may be a factor influencing the 5HT kinetics in platelets and in plasma. In fact, in men the plasma 5HT and the spontaneous release of 5HT from platelets (measured in suspensions with the same platelet number) showed a negative correlation with platelet number *in vivo* (Fig. 4b). Platelet number was also negatively correlated with the 5HIAA in the urine, which however probably does not all originate from platelet 5HT, another possible source being the enterochromaffine cells of the gastrointestinal tract. In addition, the 5HT content of platelets decreased with increasing platelet number in PRP (Fig. 4a). This may be due to the fact that the supply of 5HT available to the platelets (which is the net result of release from the enterochromaffine cells and degradation by MAO e. g. in liver and in lungs) is limited. Accordingly, the 5HT content per platelet would be higher in PRP with a low platelet count. The 5HT leakage might possibly also be higher in this case, which would explain the inverse correlation between platelet number and spontaneous 5HT release from platelets *in vitro*. Changes in platelet 5HT uptake are unlikely to be involved since there was no correlation between platelet number and V_{max} or K_M.

The present results also suggest that at least in men there is a relationship between platelet number and platelet sensitivity to 5HT. In fact, there was a negative correlation between aggregation and platelet number (Fig. 5) and a positive correlation between aggregation and plasma 5HT (Fig. 3). It is reasonable to assume that enhanced concentrations of plasma 5HT (which, at least in part, may be due to a decreased removal of 5HT from plasma in subjects with low platelet number) sensitize the platelets to the aggregating effects of added 5HT, i. e. by increasing the $[Ca^{2+}]_i$ concentrations via $5HT_2$ receptors (16).

The influence of platelet number on 5HT kinetics in the blood was more prominent in men than in women. Whether this influence plays a role in the age-dependent increase of platelet sensitivity to 5HT in men cannot be decided on the basis of the present experiments. Thus, there was no correlation between platelet number, spontaneous 5HT release from platelets or the urinary exretion of 5HIAA and platelet sensitivity to 5HT.

Platelet function has been found to correlate with platelet volume and number. In fact, aggregation induced by ADP, collagen and epinephrine was proportional to platelet volume (17). Platelet MAO-activity (which is genetically determined) has also been shown to depend on non-genetic factors such as platelet number, volume and protein density (18, 19, 20). Our experiments show that 5HT-kinetics and sensitivity of platelets to 5HT are related to platelet number, too. Platelet size could therefore play a role, since a negative correlation between platelet number and volume has been found (17). Whether platelet size (which may be an indicator of platelet age) represents a primary cause for the heterogeneity of platelets regarding 5HT-kinetics and sensitivity remains to be further investigated.

Since the platelet is a possible model for other cells such as neurons and vascular smooth muscle cells, there might also be differences with age in the functional state of vascular smooth muscle cells, which also exhibit $5HT_2$-receptors. This view is supported by experiments with rats showing an increasing reactivity of vascular $5HT_2$-receptors in isolated kidney with ageing (21). The present results show that age, sex and platelet number have to be born in mind as factors contributing to the variability of 5HT kinetics and sensitivity of blood platelets to 5HT in normotensive subjects.

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