Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 537-543

NON-AGE RELATED DIFFERENCES IN THROMBIN RESPONSES BY PLATELETS FROM MALE PATIENTS WITH ADVANCED ALZHEIMER'S DISEASE

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Received May 21, 1993

Alzheimer's Disease(AD), characterized by a deposition of ß-amyloid peptide(ß/A4) in the brain and in the cerebral microvasculature of affected individuals, is derived from its precursor protein(ßAPP) via proteolytic processing by enzyme(s) which have not yet been characterized or localized. Since platelets carry APP in one of their granules, they have been implicated as a source of the ß/A4 deposits in the microvasculature of AD patients, attributable to either an abnormality in the platelets' stimulus response, in the quantity or nature of the APP they release upon activation and/or in the processing of that protein. We show here that platelets from patients with severe AD have abnormal stimulus responses to α-thrombin. Specifically, these cells hyperacidify. While it is not clear why this abnormality occurs, it may contribute to aberrant granule secretion since we have demonstrated earlier that release of platelet granule contents is partially controlled by the cytoplasmic pH.

Alzheimer's Disease (AD), the most common cause of dementia in the elderly, is a neurodegenerative disease of the central nervous system (CNS), presently affecting an estimated 4 million people in the United States (1-3) and is definitively diagnosed on autopsy by the presence in the brain of mature senile plaques with a central core containing the 4 kDa amyloid ß–peptide (ß/A4) proteolytic product of ßAPP (1-8). Soluble ß/A4 has been detected in normal biological fluids, including cerebral spinal fluid and plasma (9-11), sometimes in a truncated 3kD form (12), as have other proteolytic fragments of ßAPP (13-18).

The ßAPP products found in plasma may arise from the release of α-granule contents by activated platelets, and/or from the endothelial cells with which these platelets can interact. A cleavage product of one of the forms of ßAPP, Protease Nexin II (PNII), is normally present in blood (16) and may play an important regulatory role in normal coagulation, especially in view of recent findings that PNII inhibits factor XIa *in vitro* (1,13,16). The protease inhibitory

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0006-291X/93 $4.00

activity of ßAPP appears to be a normal biological function (19-21) and may account for its ubiquitous presence in so many different cell types.

Platelet activation in response to physiologic stimuli such as α-thrombin (<4.5nM) is accompanied by an increase in cytoplasmic $[Ca^{++}]$ ($[Ca^{++}]_{in}$) (22-24), a membrane depolarization ($\Delta\psi$) (25-28), a rapid transient acidification followed by an alkalinization of the cytoplasm (29-35), and an eventual degranulation (23,26,27,36,37), releasing the contents of the dense granules(e.g. Ca^{++}, ADP, serotonin) within 1 sec., those from the α-granules (e.g. ß-thromboglobulin, platelet factor 4, platelet-derived growth factor, fibrinogen, ßAPP (13,36-39) within 5-30 sec., and those from the lysosomal granules (e.g. ß-glucuronidase) last, after 45-60 sec. The extent to which this process proceeds depends upon the stimulus, thrombin being one which sequentially initiates all three, in, as we have shown, a cytoplasmic pH and $[K^+]$ concentration modulated manner (26,27,35).

Although the platelet ßAPP in non-familial AD patients has exactly the same sequence as that in normal controls (3), some abnormalities in platelets from AD patients have been reported; these include an altered membrane fluidity (40-42) specific to platelets and attributed to altered internal membranes (43) but not to abnormal phospholipid synthesis (44), as well as abnormal $[Ca^{++}]_{in}$ changes (43). In contrast to the latter, we here find that calcium homeostasis is equal in platelets from advanced AD patients and in those from non-demented volunteers, whether of comparable age or much younger.

We studied the thrombin responses of platelets from male Veterans' Administration Hospital patients with advanced AD, in comparison to those from non-demented individuals of comparable age (AM) and to those from younger volunteers (C). We report here that the platelets from AD patients exhibited an abnormal cytoplasmic acidification at thrombin doses ranging from 0.9 to 9.0 nM (saturation~ 4.5nM). It is not yet clear whether this an epiphenomenon or whether this defect is responsible for abnormal release or processing of the platelet α-granules' ßAPP in the cerebral blood vessels of affected patients.

METHODS

Materials: Sepharose, apyrase, N-2-hydroxyethyl piperazine N-2-ethane sulfonic acid(Hepes), ethylenediamine tetraacetic acid (EDTA),4-methylumbelliferyl ß-D-glucuronide(MUG) and di-methylsulfoxide (DMSO) were purchased from Sigma Chemical, St. Louis MO. The fluorescent compounds Indo-1-acetoxymethyl ester,2',7'-bis-(carboxyethyl)-5(6)-carboxyfluorescein-acetoxy- methylester (BCECF), and 3,3'-dipropylthiodicarbocyanine ($diSC_3(5)$) were obtained from Molecular Probes, Eugene, OR. 5-N,N-dimethyl amiloride was the kind gift of Merck, Sharp and Dohme, Rahway, NJ. All other chemicals were of reagent grade.

Subjects: Blood for platelet separation was obtained from 3 groups of donors: healthy young controls (C)(age range 20-47 yrs., mean age± standard deviation, 28.7±4.9, male/female ratio 13/12, n=25), non-demented age-matched volunteers(AM) (age range 50-79 yrs., mean 60.8±8.2, male/female ratio 10/7, n=17), and Alzheimer Disease(AD) patients from the Veterans' Administration Hospital, Bedford, MA, diagnosed as having probable AD by the Diagnostic and Statistical Manual of Mental Disorders, third edition revised, and by McKhann et al. (45) criteria (age range 61-84 yrs, mean 70.5±5.4, n=21). The latter were all males in the advanced stages of AD, mean duration 11 ±3.6 years, with a zero score on the Mini-mental State Examination score(46,47); their sole medication, if any, was a low dose of Haldol or Dilantin. Both of the latter were incubated with gel purified platelets at 4ng/ml and 20mg/ml, respectively, for 30 min. prior to stimulation with thrombin in the presence and absence of dimethyl amiloride. These doses would result in drug serum levels similar to those seen in the patient population. The

drugs had no effect on our measured parameters. The data are presented, for males only , in Table 1 and for the much larger male/female population, in Figure 1. It should be noted that although we found no significant difference between males and females for any of the activation parameters measured we performed statistical analysis on the males only in Table 1 due to the predominantly male AD population

Platelet, Neutrophil and Thrombin Preparation: Platelets, neutrophils and α-thrombin were prepared as previously described (26,27,30,48-50).

Membrane Potential, Serotonin Release, Cytosolic pH and $[Ca^{++}]_{in}$ Measurements: These measurements were also made as previously described (22,23,25-27,29-31,34,51). The rate of serotonin release was measured 15 seconds after stimulation rather than after 5 min. as previously described (26). For acidification studies, platelets were pretreated for 1 minute with dimethyl amiloride (10^{-4}M) prior to thrombin stimulation.

Lysosomal Degranulation : ß-Glucuronidase release was measured using the fluorescent substrate 4-methylumbelliferyl ß-D-glucuronide (MUG) as described by Mandell (52).

Statistical Analysis : Each parameter was collected in duplicate or triplicate for each donor and the mean ±standard deviation (SD) calculated. A given donor may have been tested up to 12 times for a resting value and up to 3 times for each dose response. Analysis of variance and standard error of the mean (ANOVA), using one-way analysis with StatView™ for the Macintosh, was used to determine the variance between donors, and the statistical significance of the observed response differences.

RESULTS

In order to determine whether abnormal ß/A4 amyloid deposits in the microvasculature might originate from inappropriately released or processed ßAPP from platelets, we have compared several thrombin initiated responses (ΔpH, $\Delta[Ca^{++}]_{in}$, Δφ, degranulation) by platelets from the three donor groups, AD, AM and C. As internal controls, the chemotactic peptide responses by neutrophils isolated from the same blood sample were measured for each donor, in terms of membrane potential changes, peroxide formation and elastase release, and found to be comparable for all three groups of samples (data not shown). Thus, any deviations found in platelets from AD patients are not attributable to any artifact in the patient's blood *per se*. We have examined the platelets of younger as well as older control subjects from both sexes and found their parameters and responses identical; however, the data presented in Table 1 refer only to males since virtually all patients in the Veterans' Administration Hospital are male.

The resting (pre-thrombin exposure) parameters of platelets from all three donor groups were virtually identical (pH_{in}=6.98±.038(n=15), 6.94±.033(n=18), and 6.89±.021(n=15) and $[Ca^{++}]_{in}$=75.7±6.3(n=17), 76.7±2.5(n=18), and 75.5±5.7nM(n=14) for C, AM and AD platelets, respectively). Thrombin induced changes in membrane depolarization, the ensuant alkalinization, and lysosomal granule release, which we have shown previously to be interdependent, and to be separable from the acidification (27-30,34), did not differ significantly in the 3 groups of platelets (Table 1). In contrast, we found serotonin secretion and extent of cytoplasmic acidification to be significantly different from controls in the platelets from AD patients. While the differences in serotonin secretion are correlatable with age, those in extent of acidification were observed only in the platelets from AD patients (Fig.1). The data presented in this figure include not only the male AD population data but also the data from the much larger male/female control groups.

Thus, the enhancement of the extent of acidification, upon thrombin stimulation in the presence of amiloride, a Na^+/H^+ antiport blocking agent, previously shown to affect only the

TABLE 1: Platelet Activation in Male Control (C) (20-47 yrs of age), Alzheimer's Disease Patients (AD), and Age-Matched Controls (AM) (>50 years of age) in Response to Thrombin (0.9 - 9.0 nM). Rate of depolarization, rate of cytoplasmic alkalinization, rate and extent of cytoplasmic acidification (after treatment with 10^{-4}M dimethylamiloride), and secretion of ß-glucuronidase and serotonin after thrombin stimulation. Data are presented as mean±SEM(n). Each (n) represents a single donor and includes ≤3 samplings/experiment. Parameters which are highly significantly ($.01<p\leq .025$), significantly ($.025<p\leq .05$), and nearly significantly ($.05<p\leq 0.1$) different using ANOVA analysis (One-way using StatView for the Macintosh) are indicated by an ***, **, and *, respectively.

PARAMETERS	nM THROMBIN		
	0.9	1.8	9.0
Serotonin Secretion			
Control	32.2±.03(8)***	52.9±6.3(8)*	80.6±2.8(8)***
AD	55.2±4.3(19)**	68.4±4.4(19)*	86.7±2.0(18)**
AM	62.9+1.9(3)***	73.6+1.2(3)*	97.4+4.8(3)***
ß-Glucuronidase Secretion			
Control	.054±.01(5)	.071±.03(5)	.221±.05(6)
AD	.120±.03(15)	.122±.02(16)	.177±.02(24)
AM	.103+.02(6)	.097+.02(5)	.265+.059(9)
Rate of Membrane Depolarization			
Control	.188±.02(13)	.223±.029(14)	.285±.02(14)
AD	.187±.12(24)	.216±.02(24)	.265±.02(24)
AM	.141+.02(10)	.171+.02(10)	.221+.03(10)
Rate of Cytoplasmic Alkalinization			
Control	.106±.01(10)	.198±.02(8)	.327±.04(11)
AD	.128±.02(27)	.218±.02(27)	.381±.03(27)
AM	.135+.02(11)	.233+.04(11)	.329+.04(11)
Rate of Cytoplasmic Acidification			
Control	.046±.01(5)	.051±.01(5)	.077±.01(5)
AD	.046±.01(18)	.069±.01(18)	.070±.01(11)
AM	.038+.01(5)	.043+.01(4)	.065+.01(5)
Extent of Cytoplasmic Acidification			
Control	.035±.01(5)**	.051±.01(5)**	.073±.02(5)***
AD	.064±.01(18)**	.079±.01(17)**	.115±.01(18)***
AM	.049+.01(5)**	.056+.01(5)**	.088+.01(5)***

alkalinization (30,34) is an AD-correlatable difference; this is an important finding since we showed a number of years ago (26,27,35) that platelet degranulation is modulated by the cytoplasmic pH.

DISCUSSION

Platelet secretion is partially controlled by both pH_{in} (27,35) and the transmembrane $[K^+]$ gradient (26,35), and is independent of changes in $[Ca^{++}]_{in}$ (23). The cytoplasmic pH is controlled by a number of factors within the activated cells as well as external platelet membrane receptor-mediated events occurring in response to products released from other platelets, endothelial cells, etc. Some of these products, specifically growth factors, have been reported to initiate changes in their target cells' pH_{in} (53).

As yet, the reason for the enhanced change in pH_{in} in thrombin-stimulated platelets from AD patients with an advanced stage of disease is unknown, as is the level of dementia at which the defect occurs. However, it is possible that, since the activity of most enzymes is exquisitely pH sensitive, this defect may affect the processing of the ßAPP and therefore the appearance of its proteolytic fragments, including not only ß/A4 but also the small soluble fragments (3-4kD) which have recently been found in normal biological fluids (9-12). It is clear that one or more

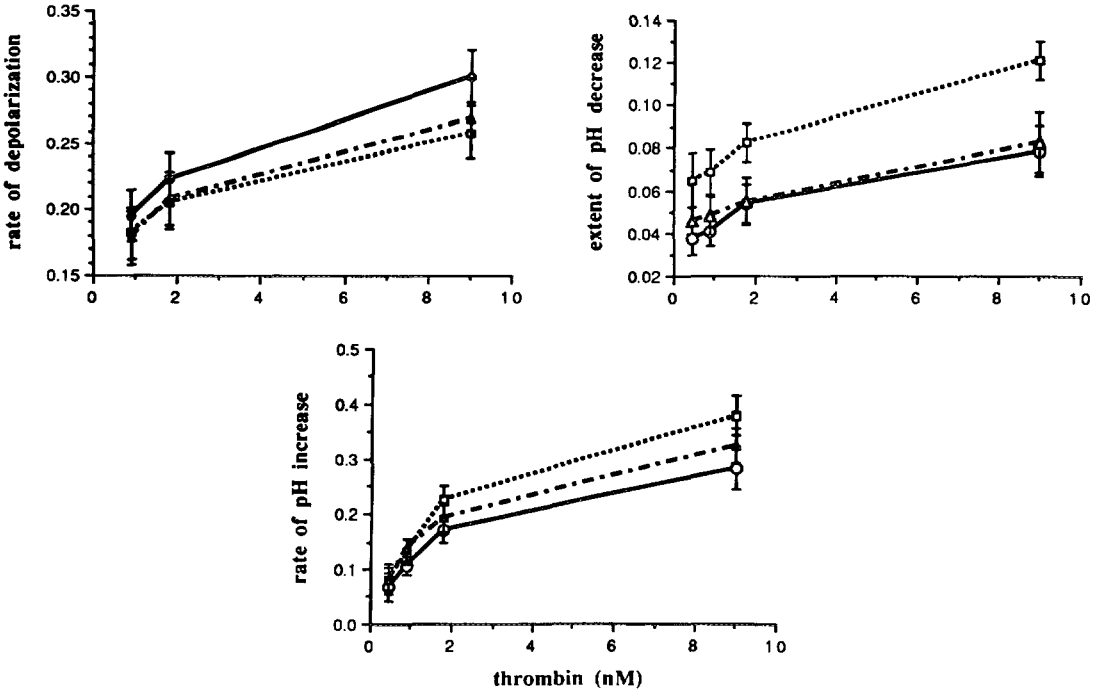

Figure 1. Platelet Response to Thrombin (0.45-9.0nM) Measured by the Rate of Depolarization and Cytoplasmic Alkalinization and the Extent of Acidification in Platelets: Control (C) (20-47 yrs of age) (○), Alzheimer's Disease (AD) (□), and Age-Matched Controls (AM) (>50 years old) (Δ). Gel purified platelets from each subject population [C (n=21), AM (n=12), AD (n=21)] including males and females were stimulated with thrombin and the subsequent $\Delta\varphi$, $+\Delta pH_{in}$ (rate of alkalinization), and $-pH_{extent}$ (extent of acidification following pretreatment with 10^{-4}M dimethyl amiloride) were evaluated. All data are presented as mean±SEM. Each data point represents a single donor and includes 1-3 samplings/ experiment.

proteases capable of splitting ßAPP exist; whether these are present in the fluids or are released by activated cells such as platelets or endothelial cells is unknown. If the level of activity or the specificity of such an enzyme is altered by an abnormally low pH, the production of fragments of ßAPP would clearly also be altered. Our current reasoning therefore suggests that, while ßAPP processing and secretion may be a normal function, in view of the ubiquity of the protein, a processing step concurrent with or after secretion, whose activity is enhanced by acidic pH, may be accountable for the abnormal amyloid fibril formation in the microvasculature.

It is as yet unclear whether the abnormally low pH_{in} attained in stimulated platelets from Ad patients is a cause, an epiphenomenon or a result of the disease, nor have we determined at what stage of the disease it appears. Its origin is also still unknown, though one can speculate that a AD-altered rapid metabolic pathway, proton-driven channel or ATPase may be the culprit. In any case, this abnormality is strongly correlated with advanced AD disease.

ACKNOWLEDGMENTS

We thank the Alzheimer's Association/United Airlines Foundation for a Pilot Research Grant and the NIH for grants AG10684 and AG11526. We are very grateful to the patients'

families for their cooperation and for their permission to study their loved ones' blood cells. These families have been a source of encouragement and moral support in our endeavors.

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