Platelet Activity and Phosphoinositide Turnover
Increase with Advancing Age
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PURPOSE: Blood platelet activity increases with advancing age. This study was designed to determine if changes in a key signal-transducing mechanism in the platelet, phosphoinositide turnover, are associated with the enhanced platelet activity seen in aging.

PATIENTS AND METHODS: Platelets were harvested from a total of 40 healthy, non-obese, 22- to 62-year-old individuals, free of any clinical evidence of atherosclerotic vascular disease, and having normal serum laboratory lipid levels. Studies of platelet activity included measurement of in vitro platelet aggregation and plasma β-thromboglobulin (β-TBG), a marker of in vivo platelet secretion. Basal and thrombin-stimulated phosphoinositide turnover was measured following [32P]-orthophosphate incorporation into the various phospholipids, isolation of the phosphoinositides and phosphatidic acid by thin-layer chromatography and autoradiography, and quantification by liquid scintillation spectroscopy of these radiolabeled phospholipids.

RESULTS: There was a positive correlation with age for both adenosine diphosphate (ADP)-induced aggregation (1.25 μM, r = 0.464, p <0.001; 2.5 μM, r = 0.386, p <0.05). There was a time-dependent increase of [32P]orthophosphate (32P) incorporation into phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylglycerol 4-phosphate (PIP) and isotopic equilibrium was reached by 120 minutes at 37°C. A positive correlation was found between age and basal 32P-PIP2 (r = 0.640, p <0.001) and 32P-PIP (r = 0.676, p <0.0005). Basal 32P incorporation into PIP2 correlated positively with in vitro aggregation (1.25 μM ADP, r = 0.795, p <0.0001; 2.5 μM ADP, r = 0.755, p <0.0005) as did 32P incorporation into PIP (1.25 μM ADP, r = 0.815, p <0.0001; 2.5 μM ADP, r = 0.795, p <0.0001). There was also a positive correlation between plasma β-TBG levels and basal 32P-PIP2 (r = 0.768, p <0.005) and 32P-PIP (r = 0.505, p <0.066). Finally, increasing age correlated with thrombin (4 U/mL)-stimulated 32P-PIP2 hydrolysis (r = 0.894, p <0.01) and phosphatidic acid formation (r = 0.556, p <0.05).

CONCLUSION: Advancing age is associated with an increase in in vitro platelet aggregation, elevated β-TBG levels, and enhanced phosphoinositide turnover. Because phosphoinositide turnover is associated with stimulus-coupled platelet activation, the increased basal and stimulated phosphoinositide turnover may mediate the increase in platelet activity with aging.

An increase in platelet aggregability has been proposed as a contributing factor to the development of atherosclerotic vascular disease [1]. Advancing age is associated with both atherosclerosis and platelet hyperaggregation [2]. Increased in vitro platelet aggregatory responses to adenosine diphosphate (ADP) with increasing age have been reported [3]. Plasma levels of β-thromboglobulin (β-TBG) (a platelet-specific protein secreted upon activation) increase with age and have been correlated with platelet hyperaggregation [4]. Biochemical mechanisms associated with the in vitro and in vivo increases in platelet activity with aging are not known.

Platelet activation is associated with phosphoinositide turnover and formation of second messengers 1,2-diacylglycerol (DAG) and inositol 4,5-bisphosphate (PIP2) from hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) [5-9]. DAG is rapidly phosphorylated by a DAG kinase to form phosphatidic acid (PA). Calcium mobilization, protein kinase C activation, and platelet alpha and dense granule secretion are linked to formation of these second messengers [9,10]. The impact of aging on phosphoinositide turnover is not known.

In the present study, we have examined the hypothesis that advancing age enhances ADP-induced aggregation and raises plasma β-TBG as a function of platelet phosphoinositide content and turnover. We confirm here the previous findings that in vitro ADP-induced aggregation and in vivo plasma β-TBG increase with advancing age. We demonstrate that platelet polyphosphoinositide content correlates with both age and ADP-induced aggregation. Furthermore, upon platelet stimulation with thrombin, PIP2 hydrolysis and PA generation increase as a function of increasing age. We suggest that the increase in in vitro platelet aggregation and raised plasma β-TBG levels that occur with advancing age are due to an increase in basal and stimulated platelet phosphoinositide turnover.

PATIENTS AND METHODS
A total of 40 healthy 22- to 62-year-old volunteers (21 men and 19 women) free of any clinical disease, including evidence of atherosclerotic vascular disease, were recruited for the study. None of the volunteers...
were obese, and all had serum cholesterol, triglyceride, creatinine, and fasting glucose levels within the normal laboratory range. The subjects were instructed to take no medications specifically known to affect platelet function for at least 1 week before the study. Blood sampling was performed at the same early morning hour (8 A.M.) after at least a 12-hour fast. Informed written consent was obtained from each volunteer. The study was approved by the Institutional Review Board Human Use Committee at the University of Michigan Medical Center.

*In vitro* platelet aggregation was performed by a turbidometric technique similar to that described by Born and Cross [11]. Forty milliliters of whole blood were gently dispersed into a polypropylene tube containing 3.5% trisodium citrate anticoagulant (9:1, volume:volume [v:v]) and centrifuged in a swinging bucket rotor at 125 × G for 12 minutes at 22°C. The platelet-rich plasma (PRP) was removed to within 1 cm of the buffy coat. The remaining blood was centrifuged at 5,000 × G for 12 minutes at 22°C and the platelet-poor plasma (PPP) removed (less than 100 platelets per μL). The platelet count was adjusted to 300,000/μL with autologous PPP using an electronic particle counter. Aliquots (450 μL) of PRP were incubated at 37°C for 2 minutes with constant stirring at 1,200 revolutions/minute and the light transmittance was monitored using an aggregometer. Aggregation was measured on a strip chart recorder in mV and expressed as a percentage of light transmittance of the PPP. For each aggregation curve, percent aggregation was measured with the light transmittance of PPP representing 0% and the light transmittance of the PRP representing 100%. Aggregation studies were completed within 3 hours of obtaining samples. Percent aggregation was measured during primary aggregation at 30, 60, and 120 seconds following the addition of 1.25 and 2.5 μM ADP. To verify that the platelet release reaction did not occur, adenosine triphosphate (ATP) release from platelet dense-granules was monitored with the luciferin-luciferase reaction by a modification of methods previously described [12].

*In vivo* platelet activity was determined by measuring plasma levels of β-TBG, a protein secreted from platelet alpha-granules upon activation [13]. Triplicate 2.5-mL samples of whole blood were placed in plastic tubes on ice containing 150 μL of 134 mM EDTA and 15 mM theophylline, and β-TBG was assayed by radioimmunoassay in the Ligand Laboratory of the University of Michigan Medical Center.

Platelet phosphoinositide and PA determinations were carried out by slight modifications of methods established in the literature [14]. Two hundred fifty milliliters of PRP were obtained by plasmapheresis from whole blood collected in an acid/citrate/dextrose anticoagulant buffer (71 mM citric acid, 85 mM trisodium citrate, 111 mM dextrose, pH 5.5, 5:1 v:v). The PRP was centrifuged twice in a swinging bucket rotor at 100 × G for 12 minutes at 22°C to remove any remaining red blood cell contamination. Platelets were pelleted at 1,200 × G for 10 minutes and suspended in a small volume of incubating buffer (20 mM TRIS-HCl, 150 mM sodium chloride, 5 mM magnesium chloride, 0.025% bovine serum albumin, pH 7.4) and brought to 2 × 10^9 platelets/mL with autologous PPP. The final volume of plasma added was 7.5 ± 0.4 mL (range of 7.1 to 7.7 mL) with a final phosphorus concentration of 0.8 ± 0.3 mM (range 0.6 to 1.0 mM). Aliquots of 500 μL of 1% bovine serum albumin, pH 7.4) and TRIS-HCl, 150 mM sodium chloride, 5 mM glucose, were pelleted at 1,200 × G for 10 minutes and suspended in a small volume of incubating buffer (20 mM TRIS-HCl, 150 mM sodium chloride, 5 mM glucose, 0.025% bovine serum albumin, pH 7.4) and brought to 2 × 10^9 platelets/mL with autologous PPP. The final

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Figure 1. Relationship between in vitro platelet aggregation and age in response to 1.25 μM ADP (top) and 2.5 μM ADP (bottom). Values (n = 33) for aggregation are given as the mean of percent aggregation measured at 30, 60, and 120 seconds after the addition of ADP. The correlation coefficient for linear regression analysis (r) and significance value (p) are given.

In young subjects the percentage aggregation was 10% and 25%, respectively, and increased to 30% and 40% for the two doses of ADP. There was a significant positive correlation between aggregation and age for both 1.25 μM ADP (r = 0.46, p <0.01) and 2.5 μM ADP (r = 0.386, p <0.05). Plasma β-TBG levels also increased with advancing age. The correlation between plasma β-TBG and age approached statistical significance (r = 0.381, p <0.055). These studies provide evidence for increased in vitro and in vivo platelet activity.

Basal 32P incorporation into the phosphoinositides and PA reached isotopic equilibrium by 120 minutes in every subject tested. In the absence of exogenous agonist, the specific activity (32P/μg phosphorus) of PI and PIP2 reached equilibrium at 60 to 90 minutes for PIP2 and between 90 and 120 minutes for PIP (Figure 2). Thus, labeling of these polyphosphoinositides with 32P for 120 minutes reflects their respective phospholipid mass. Therefore, it was possible to examine the relationship between age and mass of the phosphoinositides. There was a significant positive correlation between basal 32P incorporation at 120 minutes into PIP and PIP2 with advancing age (Figure 3). In addition, the incorporation of 32P in both phosphoinositides, PIP and PIP2, at the equilibrium time of 120 minutes was found to correlate strongly with the age.
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Figure 4. Relationship between platelet aggregation and $^{32}$P incorporation into the platelet polyphosphoinositides (n = 19). $^{32}$P-PIP versus percent aggregation to 1.25 $\mu$M ADP (top left) and 2.5 $\mu$M ADP (bottom left), and $^{32}$P-PIP$_2$ versus percent aggregation to 1.25 $\mu$M ADP (top right) and 2.5 $\mu$M ADP (bottom right). The correlation coefficient for linear regression analysis (r) and significance value (p) are given.

Figure 5. Relationship between platelet aggregation and risk factors for vascular disease were sought. No relationship, however, was found between platelet activation and body mass index, total cholesterol, triglycerides, serum creatinine, or fasting glucose.

COMMENTS

Previous studies have shown that enhanced platelet function is associated with aging in healthy individuals [2-4]. In the present study, we examined the relationship between aging and platelet function and phosphoinositide turnover, a key regulatory mechanism involved in platelet activation. The present data confirm our preliminary findings [18], and those of others [19-21], of increased in vitro platelet aggregation and increased in vivo platelet activity with aging. Furthermore, we demonstrate that the increased platelet activity with aging is associated with increased basal platelet polyphosphoinositide content and increased thrombin-stimulated platelet phosphoinositide turnover. The increased platelet activity with advancing age is independent of metabolic and hemodynamic perturbations such as those seen in diabetes mellitus and hypertension [22-24].

There was a strong positive correlation between age and basal $^{32}$P incorporation at 120 minutes into PIP$_2$ and PIP. In addition, $^{32}$P-labeled polyphosphoinositide
content correlated positively with in vitro platelet aggregation and plasma β-TBG release in these same patients. Since $^{32}$P labeling was found to reach equi- 
librium in 120 minutes, tracer was taken to reflect mass, suggesting that polyphosphoinositide content 
was a major factor in the increased in vitro aggregation and the increased in vivo platelet activity associated 
with aging.

When platelets were stimulated with thrombin, we found a significant positive correlation between age 
and $^{32}$P-PIP$_2$ hydrolysis and $^{32}$P-PA formation. This provides evidence that age-related increased platelet 
activity may also be associated with an altered trans-
membrane signaling mechanism involved in the for-
mation of key intraplatelet second messengers. Be-
cause basal levels of PIP and PIP$_2$ correlated with 
thrombin-stimulated levels of $^{32}$P-PIP$_2$ and $^{32}$P-PA 
(data not shown), the stimulated hydrolysis of PIP$_2$ 
may be a function of basal polyphosphoinositide levels.

The importance of age-related changes in the plate-
let lipid concentration has been studied previously. 
Particular reference to the arachidonic acid pathway 
has been emphasized. Prisco et al [25] have shown that 
platelet lipid composition in healthy 20- to 68-year-old 
subjects had greater concentrations of cholesterol, an 
increase in the saturated fatty acid content of phos-
phatidylcholine, and a decrease in the unsaturated fatty 
acid content with increasing age. These changes 
have been associated with enhanced aggregability pre-
sumably secondary to increased amounts of throm-
boxane A$_2$ formation [26,27].

Alternatively, decreased levels of linoleic acid and 
unsaturated fatty acids in the platelet have been im-
planted as a causal mechanism for increased platelet 
hypersensitivity in the elderly [28]. Prostaglandin syn-
thesis and cyclooxygenase activity are inhibited by 
unsaturated fatty acids [29]. It is known that increased 
thromboxane A$_2$ production and decreased prostacy-
clin formation occur with advancing age. If arachidon-
ate metabolites were primary to the increased platelet 
aggregation in aging, it might be expected that inhibi-
tion of these pathways in vivo would alter clinical out-
come. However, this has not been the case. In the 
Veterans Administration Cooperative Study, the am-
putation rate in a group of individuals with peripheral 
vascular disease receiving aspirin was not different 
from that in patients treated with placebo [30]. Hence, 
formation of arachidonate metabolites may not be the 

Figure 5. Relationship between PIP$_2$ hydrolysis (top) and PA 
generation (bottom) and age. Values are expressed (n = 14) 
as change from basal at 15 seconds after platelet stimulation 
with 4 U/mL human thrombin. The correlation coefficient for 
linear regression analysis (r) and significance value (p) are 
given.

Figure 6. Relationship between in vivo platelet activation (de-
termined as plasma β-TBG) and $^{32}$P incorporation into the 
platelet polyphosphoinositides. Top, $^{32}$P-PIP$_2$ versus plasma β-
TBG (n = 29), and bottom, $^{32}$P-PIP$_2$ versus plasma β-TBG (n 
= 30). Plasma β-TBG was determined from samples collected 
at the time platelets were obtained for phosphoinositide turn-
over and aggregation studies. The correlation coefficient for 
linear regression analysis (r) and significance value (p) are 
given.
primary factor in platelet dysfunction and atherosclerosis.

Age-dependent changes in phosphoinositide content in rat pituitary have been reported [31]. Unlike the rat pituitary in which a decrease was reported [31], we found an increase in platelet membrane PIP and PIP2 with advancing age. Furthermore, we found that upon stimulation with thrombin, hydrolysis of PIP2 and generation of PA increase with advancing years of the individual. Since hydrolysis of PIP2 is known to accompany the initial changes in energy utilization as well as calcium mobilization in the platelet, it would be attractive to speculate that the increase in in vitro and in vivo platelet function accompanying aging might be secondary to increased hydrolysis of PIP2. If this were the case, changes in thromboxane formation or inhibition thereof might not impact platelet function or the clinical outcome, as has been reported [22].

The striking increase in percent aggregatory responses to ADP from 10% to 40% indicates an enormous variation in the normal aging population, and this does not include subjects over 62 years of age. The wide variability in these measurements dictates a need for age-dependent control values to be established before quantitative interpretation of data can be made outside of the research setting. Furthermore, it is not necessarily clear that the platelet changes are intrinsic to the platelet and contribute to vascular disease or simply reflect asymptomatic vascular disease not defined by our rigorous inclusion criteria [32]. Further research into these relationships is clearly necessary.

In summary, we have shown that platelet activity increases with age. Polyphosphoinositide content as measured by basal 32P incorporation into PIP and PIP2 also increases with age. The increase in polyphosphoinositide content correlated positively with both platelet aggregation and β-TBG release and polyphosphoinositide hydrolysis. We conclude that the enhanced platelet activity found in the aging individual might be a consequence of increased platelet polyphosphoinositide turnover.

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REFERENCES