Platelet Activity and Phosphoinositide Turnover Increase with Advancing Age

EDWARD J. BASTYR, III, M.D., MARK M. KADROFSKE, B.S., AARON I. VINIK, M.D., *Ann Arbor, Michigan*

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 [^{32}P]-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) and plasma β-TBG (r = 0.381, p <0.055). There was a time-dependent increase of [^{32}P]orthophosphate ($^{32}P_i$) incorporation into platelet phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 4-phosphate (PIP), and isotopic equilibrium was reached by 120 minutes at 37°C. A positive correlation was found between age and basal ^{32}P-PIP_2 (r = 0.640, p <0.001) and ^{32}P-PIP (r = 0.676, p <0.0005). Basal $^{32}P_i$ incorporation into PIP_2 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 $^{32}P_i$ 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 ^{32}P-PIP_2 (r = 0.768, p <0.005) and ^{32}P-PIP (r = 0.505, p <0.066). Finally, increasing age correlated with thrombin (4 U/mL)-stimulated ^{32}P-PIP_2 hydrolysis (r = 0.694, 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.**

From the Department of Internal Medicine, Division of Endocrinology and Metabolism, and the Department of Surgery, University of Michigan, Ann Arbor, Michigan. This work was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health (1F32DK08108-01), the Michigan Diabetes Research and Training Center (2P60DK20572-11), and Roerig, Division of Pfizer Inc. This work was presented in part at the meeting of the American Federation of Clinical Research in Washington, D.C., on May 2, 1988. Requests for reprints should be addressed to Aaron I. Vinik, M.D., Department of Internal Medicine and Surgery, 2922B Taubman Center, University of Michigan Medical Center, Ann Arbor, Michigan 48109-0331. Manuscript submitted October 2, 1989, and accepted in revised form February 23, 1990.

Dr. Bastyr's present address is 4.174 Old John Sealy Hospital, University of Texas Medical Branch—Galveston, Galveston, Texas 77550.

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 1,4,5-triphosphate (IP_3) from hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) [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, PIP_2 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 Diabetes Research and Training Center at 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 glucose, 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 (1×10^9 platelets) were incubated with 20 μCi/mL of carrier-free [^{32}P]orthophosphate ($^{32}P_i$). After 180 minutes, the incubation was terminated with the addition of phosphate-washing buffer (3.2 mM dipotassium phosphate, 24.4 mM monosodium potassium, 4.2 mM disodium phosphate, 150 mM sodium chloride, 5.5 mM glucose, 0.025% bovine serum albumin, pH 7.4) and then centrifuged at 1,000 × G for 12 minutes. The wash was repeated, the pellet was resuspended in 500 μL TRIS-HCl pH 7.4 buffer, and the platelet membrane phospholipids were extracted using methanol/chloroform/hydrochloric acid. The extract was then dried under nitrogen and dissolved in chloroform/methanol. The extracts were applied to heat-activated oxalate-coated silica plates, and phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP), PIP_2, and PA separated by thin-layer chromatography (TLC) using a Jolles solution [15]. Following TLC and autoradiography, bands were identified and scraped, and the radiolabeled phospholipids were counted by liquid scintillation spectroscopy.

Phosphoinositide turnover and PA formation were also assessed after the addition of thrombin. Aliquots of washed platelets (1×10^9 platelets) were incubated with 20 μCi/mL of carrier-free $^{32}P_i$ for 120 minutes at 37°C. The incubation was terminated with the aforementioned phosphate-washing buffer and then centrifuged at 1,000 × G for 12 minutes. The platelets were suspended in 500 μL of the aforementioned TRIS-HCl pH 7.4 buffer and stimulated with 4 U/mL human thrombin. The reaction was terminated with 1.0 mL of cold methanol and chloroform (2:1, v:v). The radiolabeled phospholipids were extracted, isolated by TLC, and quantitated as just described.

The mass of the individual phosphoinositides was estimated using a spectrophotometric assay for phosphorus determination, as previously described [16]. Briefly, the individual radiolabeled phospholipids were extracted from the silica and dephosphorylated with hot magnesium nitrate, and aliquots were added to a malachite green/ammonium molybdate dye solution. Absorbance was read at 660 nm in a light spectrophotometer. Recoveries from silica gel were typically as follows: PA 79%, PIP_2 39%, PIP 54%, PI 79%. These are similar to recoveries previously published [17].

Total serum cholesterol, triglycerides, creatinine, and fasting glucose were determined in the Chemical Pathology Laboratory of the University of Michigan Hospital from samples drawn at the time of platelet testing.

All statistical evaluations were performed on the University of Michigan IBM 3090-600E computer. Simple linear regression, multivariate linear regression, and analysis of covariance were performed where appropriate. Coefficients of correlation (r) were determined by the method of least mean squares. All data are expressed as mean ± the standard error of the mean. Statistical significance was accepted at the 95% confidence level ($p < 0.05$).

RESULTS

The platelet aggregatory response to both 1.25 μM and 2.5 μM ADP increased with advancing age (**Fig-**

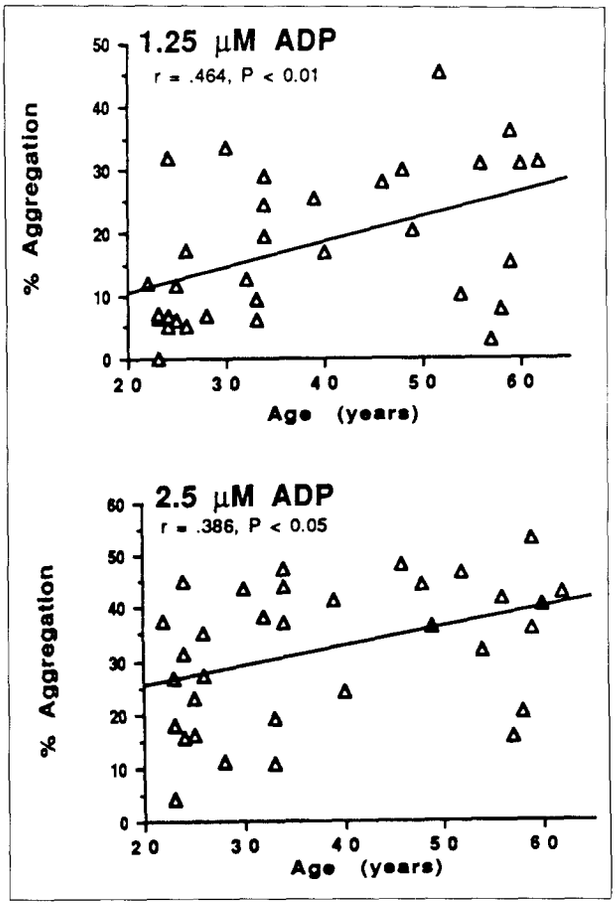

Figure 1. Relationship between *in vitro* platelet aggregation and age in response to 1.25 μM ADP (**top**) and 2.5 μM ADP (**bottom**). Values (n = 33) for aggregation are given as the mean of percent aggregation measured at 30, 60, and 120 seconds after the addition of ADP. The correlation coefficient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

ure 1). 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 $^{32}P_i$ 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 ($^{32}P/\mu g$ phosphorus) of PI and PIP_2 reached equilibrium at 60 to 90 minutes for PIP_2 and between 90 and 120 minutes for PIP (**Figure 2**). Thus, labeling of these polyphosphoinositides with $^{32}P_i$ 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 $^{32}P_i$ incorporation at 120 minutes into PIP and PIP_2 with advancing age (**Figure 3**). In addition, the incorporation of $^{32}P_i$ in both phosphoinositides, PIP and PIP_2, at the equilibrium time of 120 minutes was found to correlate strongly with the ag-

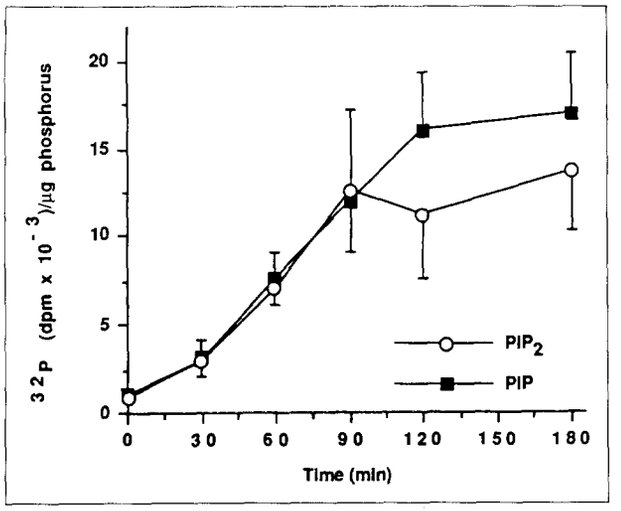

Figure 2. Specific activity of PIP and PIP_2 over 180 minutes. Data are represented as $^{32}P_i$ incorporated per μg phosphorus of the individual polyphosphoinositide. Values are expressed as means ± standard error from six subjects (one from each decade) determined in duplicate.

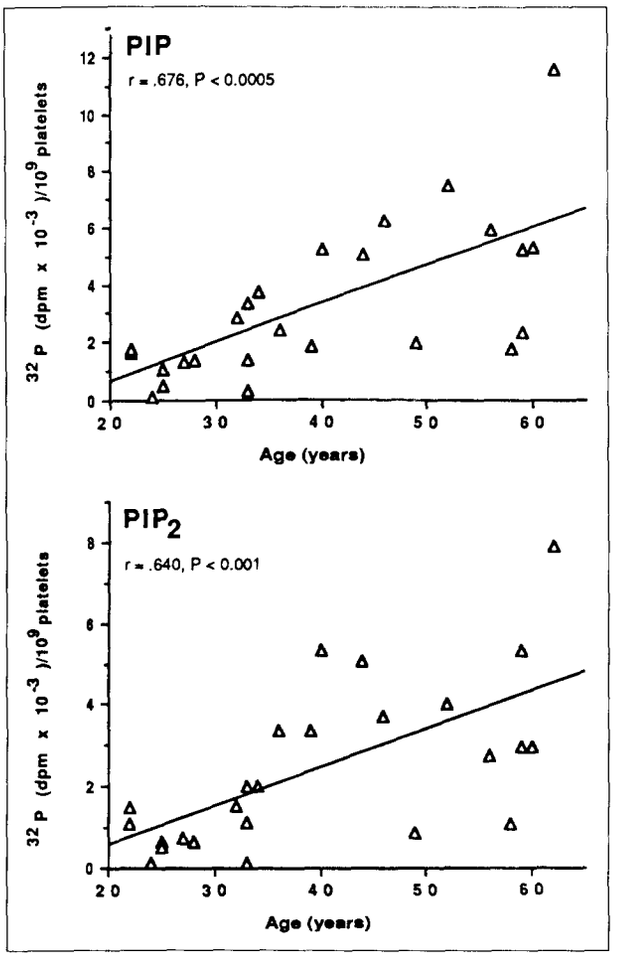

Figure 3. Relationship between $^{32}P_i$ incorporation into the platelet polyphosphoinositides and age for each individual study subject (n = 25). **Top**, ^{32}P-PIP versus age, and **bottom**, ^{32}P-PIP_2 versus age. $^{32}P_i$ incorporation into the polyphosphoinositides at 37°C was measured at 120 minutes (see text). The correlation coefficient for linear regression analysis (r) and significance value (p) are given.

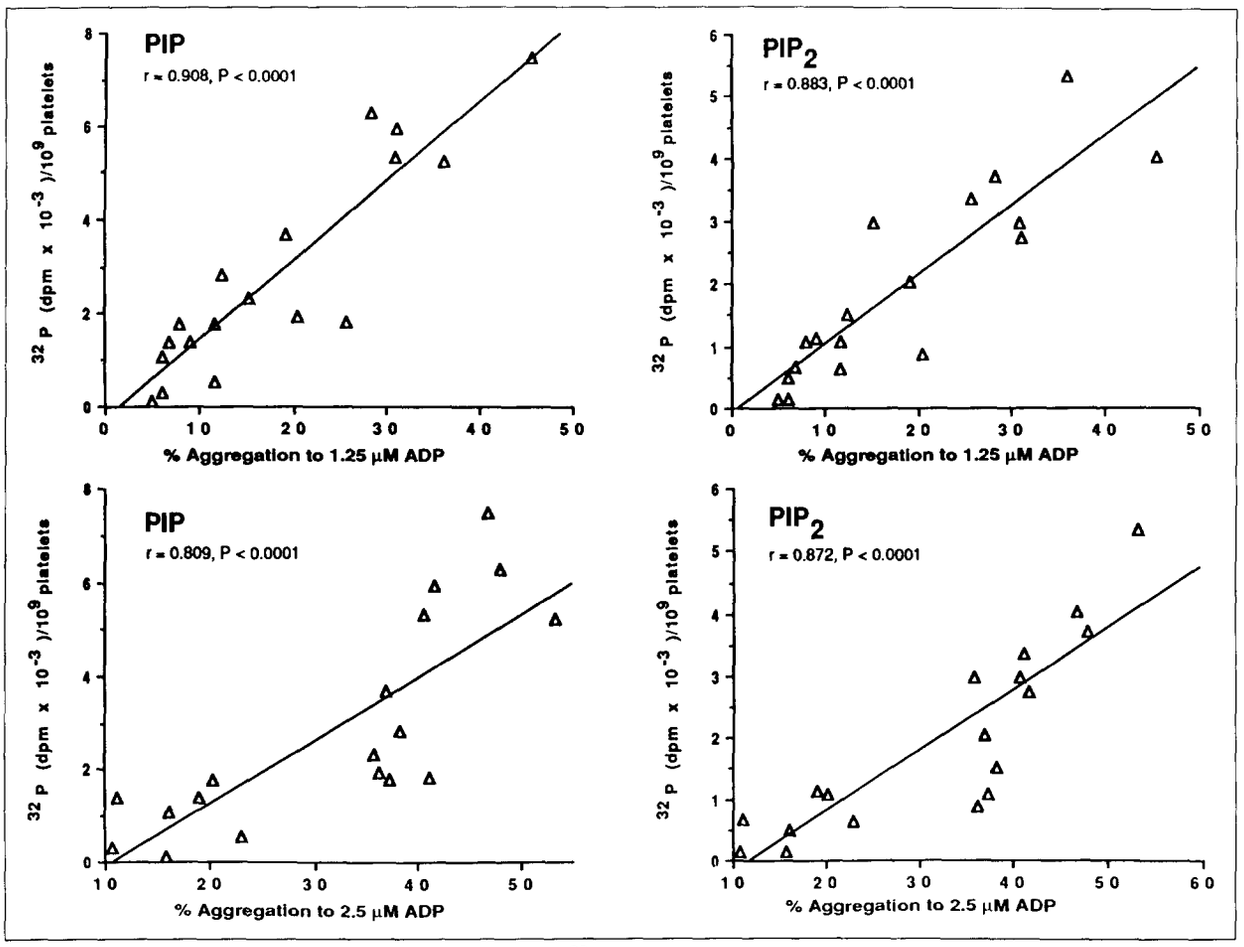

Figure 4. Relationship between platelet aggregation and $^{32}P_i$ incorporation into the platelet polyphosphoinositides (n = 19). ^{32}P-PIP versus percent aggregation to 1.25 μM ADP (**top left**) and 2.5 μM ADP (**bottom left**), and ^{32}P-PIP$_2$ versus percent aggregation to 1.25 μM ADP (**top right**) and 2.5 μM ADP (**bottom right**). The correlation coefficient for linear regression analysis (r) and significance value (p) are given.

gregatory response to both 1.25 μM and 2.5 μM ADP with correlation coefficients of 0.8 or more and significant levels of p <0.0001 (**Figure 4**). It therefore appeared that *in vitro* aggregation was a function of phosphoinositide turnover.

Since basal ^{32}P incorporation into the polyphosphoinositides would reflect mass of the compounds and not necessarily turnover, the response to activation with thrombin, which is a potent stimulus of platelet phosphoinositide turnover, platelet aggregation, and platelet secretion, was examined. Platelets from 14 subjects were stimulated with 4 U/mL human thrombin, which resulted in a time-dependent increase in ^{32}P-PIP$_2$ hydrolysis and ^{32}P-PA generated when the maximum amount of PIP$_2$ hydrolyzed and PA generated was examined. There was an increase in ^{32}P-PIP$_2$ hydrolysis reflected in decreasing amounts in the PIP$_2$ fraction and increased ^{32}P-PA formation that correlated with advancing age (**Figure 5**).

As a measure of *in vivo* platelet activation, plasma β-TBG levels were determined and found to correlate positively with ^{32}P incorporation into both PIP and PIP$_2$, with correlation coefficients of 0.505 (p = 0.06) and 0.768 (p <0.005), respectively (**Figure 6**).

Because platelet activation could reflect changes within the platelet per se or asymptomatic atherosclerotic vascular disease, correlates between *in vitro* and *in vivo* 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 $^{32}P_i$ incorporation at 120 minutes into PIP$_2$ and PI. In addition, $^{32}P_i$-labeled polyphosphoinositide

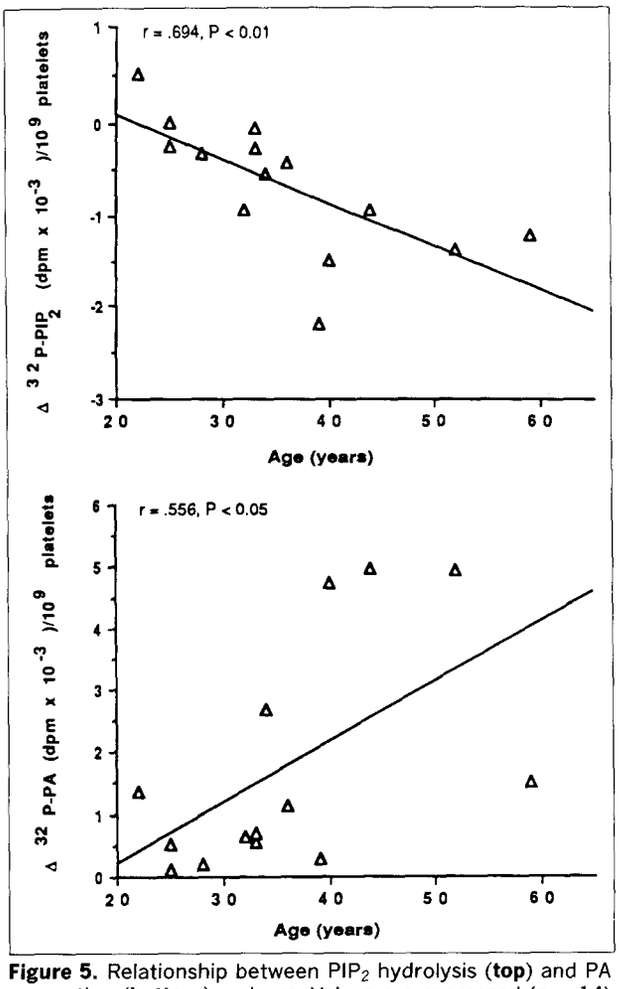

Figure 5. Relationship between PIP_2 hydrolysis (**top**) and PA generation (**bottom**) and age. Values are expressed (n = 14) as change from basal at 15 seconds after platelet stimulation with 4 U/mL human thrombin. The correlation coefficient for linear regression analysis (r) and significance value (p) are given.

content correlated positively with *in vitro* platelet aggregation and plasma β-TBG release in these same patients. Since $^{32}P_i$ labeling was found to reach equilibrium 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 transmembrane signaling mechanism involved in the formation of key intraplatelet second messengers. Because 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 platelet 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 phosphatidylcholine, and a decrease in the unsaturated fatty acid content with increasing age. These changes have been associated with enhanced aggregability presumably secondary to increased amounts of thromboxane A_2 formation [26,27].

Alternatively, decreased levels of linoleic acid and unsaturated fatty acids in the platelet have been implicated as a causal mechanism for increased platelet hypersensitivity in the elderly [28]. Prostaglandin synthesis and cyclooxygenase activity are inhibited by unsaturated fatty acids [29]. It is known that increased thromboxane A_2 production and decreased prostacyclin formation occur with advancing age. If arachidonate metabolites were primary to the increased platelet aggregation in aging, it might be expected that inhibition of these pathways *in vivo* would alter clinical outcome. However, this has not been the case. In the Veterans Administration Cooperative Study, the amputation 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

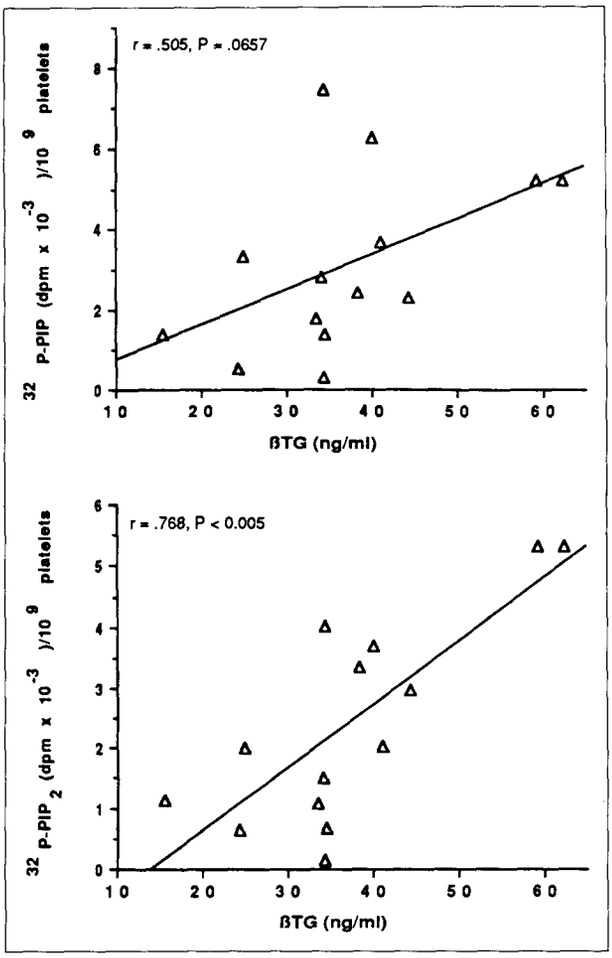

Figure 6. Relationship between *in vivo* platelet activation (determined as plasma β-TBG) and $^{32}P_i$ incorporation into the platelet polyphosphoinositides. **Top**, ^{32}P-PIP versus plasma β-TBG (n = 29), and **bottom**, ^{32}P-PIP_2 versus plasma β-TBG (n = 30). Plasma β-TBG was determined from samples collected at the time platelets were obtained for phosphoinositide turnover 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 PIP_2 with advancing age. Furthermore, we found that upon stimulation with thrombin, hydrolysis of PIP_2 and generation of PA increase with advancing years of the individual. Since hydrolysis of PIP_2 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 PIP_2. If this were the case, changes in thromboxane formation or inhibition thereof might not impact on 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 $^{32}P_i$ incorporation into PIP and PIP_2 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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