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Resveratrol inhibits polyphosphoinositide metabolism in activated platelets

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Abstract

The effects of resveratrol (*trans*-3,4′,5-trihydroxystilbene) on activation responses and the polyphosphoinositide metabolism in human blood platelets have been studied. Resveratrol partially inhibited secretory responses (liberation of dense granule nucleotides and lysosomal acid hydrolases), microparticle formation and protein phosphorylations induced by thrombin. The effects of resveratrol on phosphoinositide metabolites, phosphatidate (PtdOH), phosphatidylinositol (PtdIns), phosphatidylinositol-4-phosphate (PtdIns-4(5)-*P*), phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P_2), phosphatidylinositol-3,4-bisphosphate (PtdIns-3,4-P_2) and phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3) were monitored in blood platelets prelabelled with [^{32}P]P_i. Resveratrol not only inhibited the marked increase in levels of PtdOH in platelets activated by thrombin (0.1 U/ml) but it decreased the steady state levels of the other polyphosphoinositide metabolites. The distribution of ^{32}P in phosphoinositides in activated platelets was consistent with inhibition of CDP-DAG inositol transferase and a weak inhibition of PtdIns-4(5)-*P* kinase. These observations show that resveratrol has a profound effect on phospholipids, particularly on polyphosphoinositide metabolism, and may decrease the amount of PtdIns-4,5-P_2 available for signalling in these cells.

Keywords: Blood platelet; Polyphosphoinositide signalling; Resveratrol

1. Introduction

Resveratrol (*trans*-3,4′,5-trihydroxystilbene), a phytoalexin present in red wine and other constituents of the human diet (grapes, peanuts, mulberries and pine nuts), has been shown to have variable biological activities in different test systems [1–5], but the molecular basis for its activity is not well understood. This polyphenol inhibits cyclooxygenase-1 activity as well as some isoforms of cytochrome P450 and is implicated in chemopreventive action, i.e., inhibition of carcinogen formation [6–8]. A protective effect against coronary heart diseases has been attributed to resveratrol via mechanisms that may include vasorelaxation and inhibition of platelet activation. Resveratrol is also a free radical scavenger that may prevent heart disorders by inhibiting lipoprotein oxidation [9–12] as well as platelet activation [13,14]. The low incidence of ischaemic heart disease observed in the French population was called the “French Paradox” and was attributed in part to a moderate consumption of resveratrol-containing red wine (13). Our previous studies demonstrated that resveratrol in vitro inhibited the first step of platelet activation, i.e., platelet adhesion to collagen and fibrinogen, reduced platelet aggregation and secretion, inhibited synthesis of eicosanoids and the production of reactive oxygen species in platelets [15–21]. In the present study, we show how resveratrol affects agonist induced activation of the polyphosphoinositide (PPI) cycle and correlated this decrease in PPI signalling with inhibition of various normal platelet activation parameters such as dense granule and lysosomal secretion, microparticle formation and protein phosphorylation in thrombin-activated human platelets.

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2. Materials and methods

2.1. Materials

[^{32}P]P_i (carrier-free, Code PBS-11) was obtained from Amersham, Bucks, UK. Bovine serum albumin, fraction V was from ICN Biomedicals, Aurora, OH, USA. [^{3}H]Adenine (NET-350) was from New England Nuclear. Bovine thrombin was obtained from Parke-Davis, Morris Plains, NJ, USA. Thrombin receptor agonist peptide (TRAP) was a gift from Dr. N. O. Solum, University of Oslo. Stock solutions of thrombin (100 U/ml in 0.15 M NaCl) or TRAP (1 mM) were stored at −20 °C and diluted to the desired concentrations just before use, and discarded after one thawing. Resveratrol was purchased from Sigma and CalBiochem Biochemicals. A stock solution of resveratrol was made in 50% dimethylsulfoxide (DMSO) at a concentration of 5 mg/ml and kept frozen in aliquots. (Resveratrol, a polyphenol, was quite labile and became ineffective over time.) We used fresh stock when the activity seemed to decrease. Addition of 1 mM ascorbic acid to the incubation medium partially restored effectivity, but ascorbate was not routinely used in our experiments. (Red wines which contain varying amounts of resveratrol are said to contain several antioxidants which may protect the compound in the flask.) ATP monitoring reagent (firefly luciferin/luciferase kit) was from Bio Orbit Oy, Turku, Finland. FITC anti-P-selectin (CD62) for flow cytometry was from Becton-Dickinson. Phosphotyrosine-specific antibody 4G10 was obtained from Upstate Biotechnology and the horseradish peroxidase (HRP)-conjugated secondary antibody was from Bio-Rad Laboratories. Enhanced chemiluminescent substrate for detection of HRP was obtained from Pierce. All other chemicals were reagent grade material obtained from standard chemical suppliers.

2.2. Methods

2.2.1. Platelet isolation

Fresh blood (450 ml), drawn into 72 ml of acid citrate dextrose (ACD), was obtained from medication-free, regular donors and hemochromatosis patients at the blood bank (Haukeland Hospital, Bergen, Norway). [Blood from hemochromatosis patients is generally not used for transfusion purposes and is therefore readily available to us; the platelets appear to be completely normal in our screening and according to the literature.] The blood was centrifuged at 535×*g* for 6 min at ambient temperature to sediment red and white cells. Platelet-rich plasma (PRP) was collected and the platelets were sedimented at 3000×*g* for 10 min. The supernatant plasma was removed and the platelets resuspended in 10 ml of the same plasma (concentrated PRP). This suspension was incubated for 1 h at 37 °C with [^{32}P]P_i (0.1–0.2 mCi/ml). The platelets were then transferred by gel filtration into a calcium- and phosphate-free Tyrode's buffer containing 5 mM glucose and 0.2% bovine serum albumin at pH 7.3 [22]. The platelet concentration, determined by a Coulter Counter, was adjusted to 3.5×10^8 platelets/ml. For [^{32}P]P_i uptake studies, aliquots were taken from the incubation with radioisotope at various times and the platelets were centrifuged in the presence of EDTA and washed once by centrifugation before scintillation counting.

2.2.2. Incubations with resveratrol

Resveratrol was added to suspensions of gel-filtered platelets (GFP) during incubation at 37 °C under non-stirring conditions for 5 min before thrombin was added, mixed and allowed to stand without further stirring; samples were then withdrawn at the times given for measurement of the various parameters. A time course study of shorter incubation times with resveratrol showed that full inhibition took longer than 5 s preincubation time but less than 15 s (not shown).

2.2.3. Extraction, thin layer chromatography and determination of radioactivity of phospholipids

PPI analysis was carried out essentially as described by Tysnes et al. [23]. Briefly, aliquots of the incubations were mixed with 4 volumes of methanol/chloroform/HCl (20:40:1), phase separation was induced as described by Bligh and Dyer [24], the organic layer was saved and the solvent evaporated by a jet of argon (g) and finally chromatographed on silica plates (Silica gel 60, Merck, Darmstadt, Germany) with chloroform/methanol/20% methylamine in water (60:35:10). The radioactive PPI spots were visualized and determined by Instant Imager analysis (Packard Instrument Co., CT, USA). The areas with radiolabelled PtdIns-4,5-P_2 and below to the application zone, which also contains much smaller amounts of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 (less than 2–4% of the PtdIns-4,5-P_2), were scraped off the plates. The silica powder was heated with 1 ml of deacylating reagent [26.8 ml methylamine (40% in water), 45.7 ml methanol, 11.4 ml n-butanol, 16 ml H_2O] at 53 °C for 50 min before drying under an argon stream. The silica powder was then suspended in 1.2 ml water, centrifuged and filtered through a Millipore HV 0.45 μm filter and the filtrate analyzed by HPLC eluted with a 0 to 2 M gradient of ammonium phosphate (pH 3.8) according to Auger et al. [25]. The deacylated PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 radioactive peaks separated from deacylated PtdIns-4,5-P_2 and were quantitated in the eluate by Cerenkov scintillation with a flow radioactive monitor (Ramona D, Raytest, Munster, Germany). The 3-phosphorylated products were not measurable in resting platelets with our on-line assay system which has a relatively high background, but has been routinely under 1000 cpm under similar experimental conditions when fractions of column products have been collected and the radioactivity quantified by scintillation counting.

2.2.4. Measurement of secreted ADP+ ATP and β-hexosaminidase

Samples of GFP with and without resveratrol incubated with or without thrombin were pipetted into Eppendorf tubes in ice containing 0.14 vol. of 1 M formaldehyde/50 mM EDTA in order to stop secretion. The mixture was centrifuged at 4 °C for 2 min at 7000×*g*, and one volume of the supernatant was mixed with one volume of freshly prepared EDTA/ethanol (1 volume of 100 mM EDTA and 9 volumes of 96% ethanol). ADP was converted to ATP with phosphoenol pyruvate and pyruvate kinase and measured by a firefly luciferase method [26]. Another 100 μl aliquot of the formaldehyde-stopped supernatants was mixed with 0.1% Triton X-100 for determination of β-hexosaminidase with 4-methyl-umbelliferyl *N*-acetyl-β-D-glucosaminidase as a substrate [27]. The total activities of the acid hydrolase were determined in Triton X-100 extracts of non-centrifuged platelets.

2.2.5. Protein phosphorylation

GFP was incubated with and without resveratrol (5 min) and with or without thrombin (2 min). The reactions were stopped by adding 1 vol. of incubate to 0.3 vol. of 4× concentrated SDS sample buffer (280 mM Tris pH 7.2, 40% glycerol, 0.01% bromphenol blue, 8% SDS, 4% β-mercaptoethanol). Samples were sonicated for 2 min, heated to 95 °C for 5 min and then centrifuged for 5 min at 7000×*g* to remove debris. The solubilized proteins were separated on a linear 5–15%-gradient, reducing SDS polyacrylamide gel and blotted on to a 0.2-μm nitrocellulose filter (Schleicher and Schuell, Dassel, Germany) and probed with anti-tyrosine phosphate antibody. The ^{32}P phosphorylated pleckstrin bands were studied in [^{32}P]P_i-labelled platelets and were visualized directly on dried gels and quantified on the Packard Instant Imager. Two gels from each experiment were run and the duplicates were averaged.

2.2.6. Flow cytometry for detection of P-selectin expression and microparticle formation

Flow cytometric analysis was carried out as previously described [28] with a FACSort flow cytometer (Becton Dickinson, BDIS, San Jose, CA). The platelets were fixed in 0.2% formaldehyde in PBS buffer 2 min after TRAP addition; and labelled with R-PE conjugated anti CD-62P and chicken anti-human platelet-FITC (Biopool, Umea, Sweden). Gating on both R-PE, FITC and light scatter profiles identified platelets and microparticles, 5000 events per analysis in triplicate.

2.2.7. Statistics

Statistical analysis was done by several tests. In order to eliminate uncertain data, both the Q-Dixon and Grubbs tests were performed. All the values in this study were expressed as means ± SEM. The statistically significant differences between variations were found (Snedecor-Fisher test) so the differences between means were assessed by applying the Cochran-Cox test or Student's *t* test.

3. Results

3.1. Resveratrol inhibition of platelet activation parameters

Inhibition of various platelet activation parameters is shown in Fig. 1. Dense granule and lysosomal secretion are shown in Fig. 1A together with inhibition of pleckstrin phosphorylation. The degree of inhibition for these parameters was not more than 30–40% except for dense granule secretion which was greater than 50% at the highest concentration of resveratrol tested, 50 μg/ml. Platelet activation is associated with expression of P-selectin on the platelet surface and microparticle formation. Both of these phenomena were inhibited by increasing concentrations of resveratrol (Fig. 1B), although a significant increase in P-selectin exposure and apparently of microparticle formation were seen at 0.625 μg/ml.

Platelet activation by thrombin is also accompanied by an increase in tyrosine phosphorylation of many cellular proteins due to autocrine stimulation by released substances [29]. Fig. 1C shows the pattern of platelet tyrosine phosphorylation induced by thrombin and the marked inhibitory effect of resveratrol at the concentration of 2.5 μg/ml and higher added to platelet suspensions. The inhibition is particularly evident on the protein bands of about 120, 105, 101, 72 (Syk) and 60 kDa after the thrombin-induced phosphorylation.

3.2. Resveratrol inhibition of PPI metabolism—concentration effects

[^{32}P]P_i-labelled platelets in GFP were incubated for 2 min with and without 0.1 U/ml of thrombin in the presence of different concentrations of resveratrol. The levels of [^{32}P]PPI metabolites in control resting platelets are shown in the first set of columns (far left) in Fig. 2. In the absence of thrombin, resveratrol caused no significant changes in the [^{32}P]-labelled phospholipids (not shown). After 2 min incubation with thrombin alone, the level of [^{32}P]-labelled PPI metabolites increased markedly (Fig. 2, second set of columns). In thrombin incubations with resveratrol present, the levels of [^{32}P]-labelled PPIs resembled more the resting state than the thrombin-stimulated state (Fig. 2, column sets 3–6). The most outstanding effect of thrombin on the [^{32}P]PPI metabolites was the 8 times increase in PtdOH (Fig. 2, second set of columns compared to the first set of columns); this increase was completely abolished with the lowest concentration of resveratrol used (Fig. 2. column sets 3). In the presence of the highest dose of resveratrol, 50 μg/ml (not shown), the levels of [^{32}P]PPI metabolites were further reduced by about 10% compared to 5 μg/ml.

3.3. Time course of changes in radioactive PPIs in thrombin-stimulated platelets in the presence of resveratrol

We investigated the time course of resveratrol effects on PPI metabolism (as an early signalling event) in platelets stimulated with thrombin. Fig. 3 shows the time course of the changes in the levels of [^{32}P]PPI metabolites. In the absence of resveratrol, the levels of [^{32}P]PtdOH, [^{32}P]PtdIns, [^{32}P]PtdIns-4(5)-*P* and [^{32}P]PtdIns-4,5-P_2 increased rapidly and stabilized 30 to 60 s after addition of thrombin. In the presence of resveratrol, the [^{32}P] levels of these phospholipids also increased initially as shown in Fig. 3, but after about 5 s leveled off and the steady states returned to control levels by 15 s instead of remaining elevated as in thrombin controls. The extent of these changes were more or less the same at 0.625, 5 and 50 μg/ml resveratrol for all the phosphoinositides except PtdIns

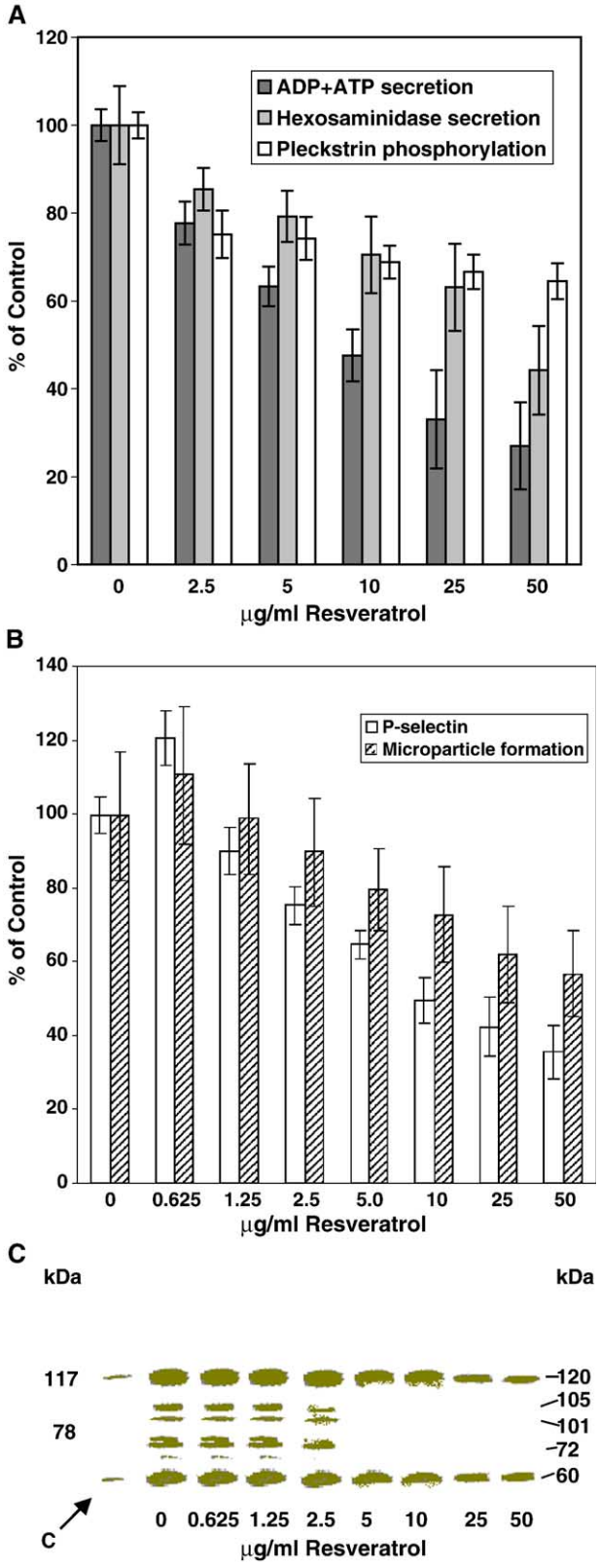

Fig. 1. Effect of resveratrol on thrombin stimulation of dense granule and lysosomal secretion and pleckstrin phosphorylation (A) and P-selectin exposure on the platelet surface and microparticle formation (B). Platelets were preincubated with varying concentrations of resveratrol for 5 min and then stimulated with 0.2 U/ml thrombin for 2 min before reactions were stopped as described under Methods for sample processing. (A) Secretion parameters were compared with secreted amounts induced by 5 U/ml thrombin taken as 100%. Vertical stripes represent dense granule secretion; filled bar, β-hexosaminidase secretion; and open bar, pleckstrin phosphorylation. (B) P-selectin expression (open bar) and microparticle formation (diagonal stripes) was measured by flow cytometry following activation by TRAP as described under Methods. Results are expressed as means ± S.E.M., $n=3$. *P* values were determined by paired samples Student's *t* test, $0.05>P>0.01$ for resveratrol-treated samples. (C) Thrombin-induced protein tyrosine phosphorylation as described in Methods; lane C represents resting platelets. The figure is representative of four separate experiments.

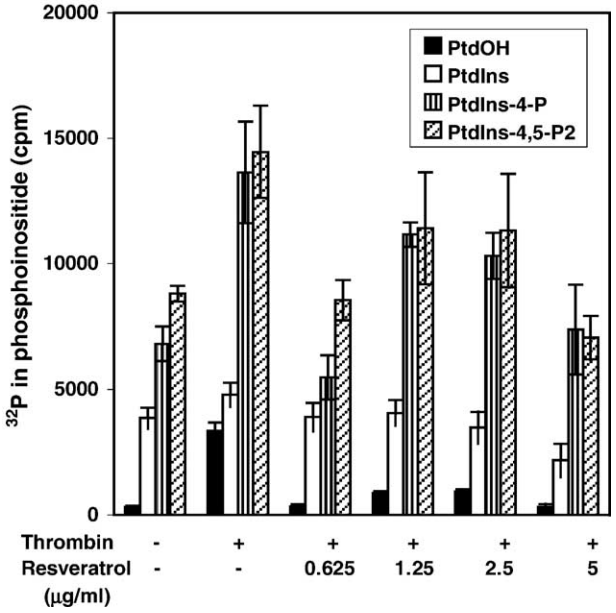

Fig. 2. Effect of resveratrol on resting and thrombin-induced changes in levels of [^{32}P]PPI. metabolites. Platelets were prelabelled with [^{32}P]P_i and incubated for 5 min at 37° with varying concentrations of resveratrol before addition of thrombin 0.1 U/ml; other conditions were as described in Fig. 1. Incubations were stopped with Bligh-Dyer HCl extraction medium and lipids extracted and analyzed as described in Methods. Results are from 4 different donors expressed as the means ± S.E.M., $P>0.05$ for resting platelets and $0.05>P>0.01$ for activated platelets.

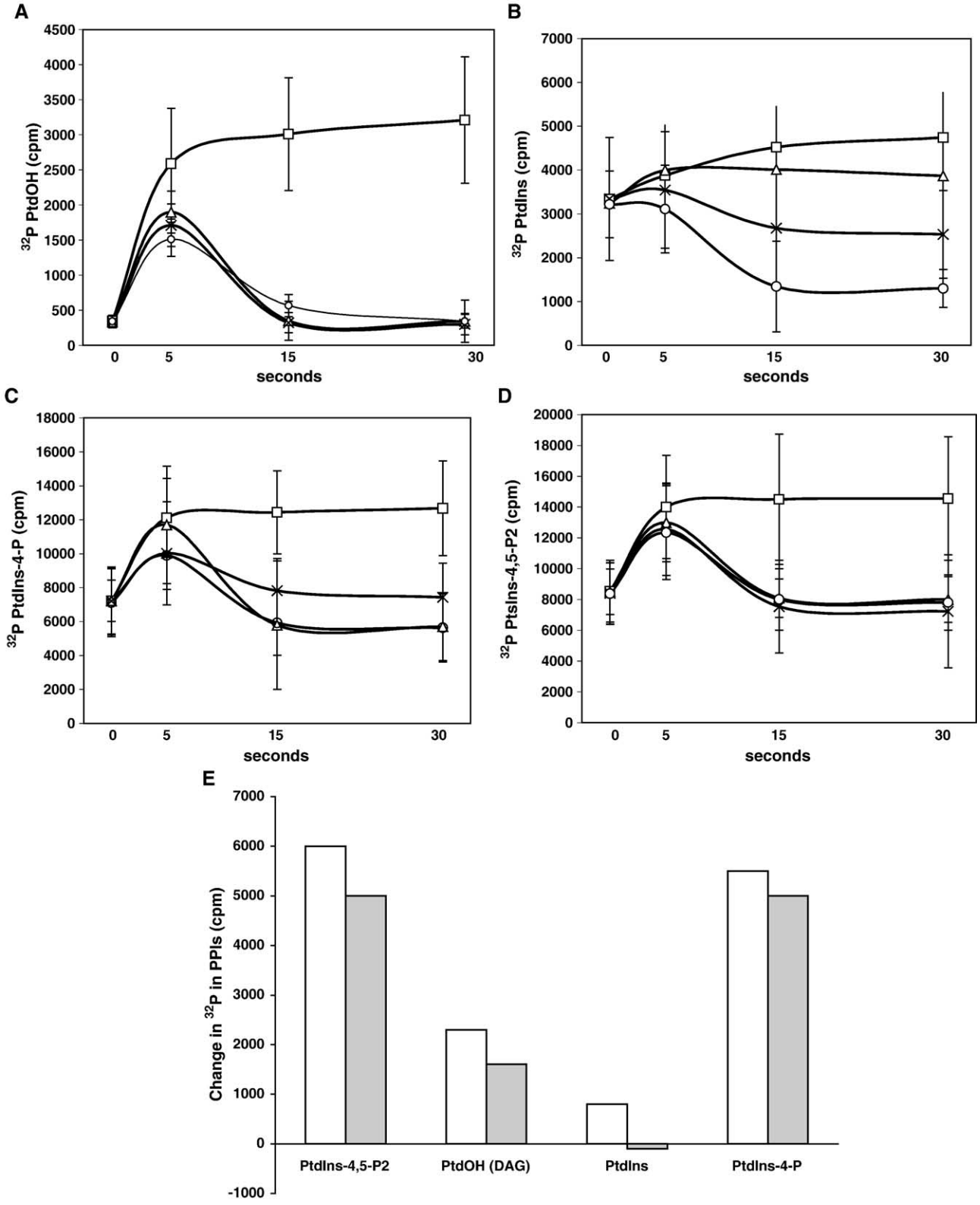

Fig. 3. Time course of changes in levels of [^{32}P]PPI metabolites with thrombin±resveratrol (A–D). The experiments and analyses were carried out as described under Fig. 2 with 0.1 U/ml thrombin. Open squares represent thrombin control; triangles, 0.625 μg/ml resveratrol; X, 5 μg/ml; and circles, 50 μg/ml. Results are from 4 different donors expressed as the means±S.E.M. *P* values were determined by paired samples Student's *t* test $0.05>P>0.01$. (E) Cross-over plot of ^{32}P-PPI metabolism in pre-labelled platelets stimulated for 5 s with thrombin. This cross-over plot is derived from the 5 s point of analysis shown in (A–D) (means only) and is expressed as change in radioactivity from resting baseline levels in the absence (thrombin control, open bar) and presence of 50 μg/ml resveratrol (filled bar). The intermediates are plotted in the sequence of PtdIns-4,5-P_2 hydrolysis and resynthesis.

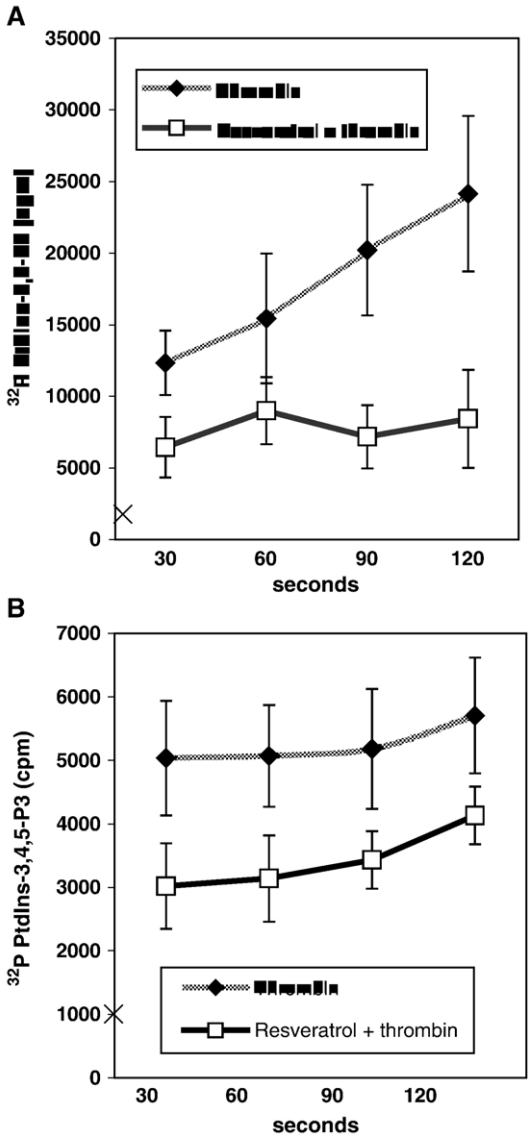

Fig. 4. Inhibition of Ptd-3,4-P_2 (A) and Ptd-3,4,5-P_3 (B) formation by resveratrol (50 μg/ml, 5 min). Platelets were incubated as described in Fig. 2, the lipids were extracted, separated, deacylated and analyzed by HPLC as described under Methods. Resting platelets had levels under 1000 cpm (see text). Representative of 3 different donors, means±S.E.M., P values were determined by paired samples Student's t test ($0.05>P>0.01$). An X marks the control levels of labelled intermediates as less than 1000 cpm or below the sensitivity of the on-line detector as established in separate experiments by collection and counting of individual fractions.

which alone showed a dose-dependent effect and no elevation was seen at 5 s with 50 μg/ml resveratrol (Fig. 3B). Activation by the highest concentration of thrombin tested, 1 U/ml, was just as effectively inhibited by 50 μg/ml resveratrol as was 0.1 U/ml thrombin which was the lowest concentration that gave a maximal activation response in control platelets as measured by PtdOH formation (not shown). The response of platelets to 0.05 U/ml thrombin as measured by increased PtdOH levels was only about 10–20% of that seen with 0.1 U/ml thrombin, and this increase was inhibited by resveratrol (not shown).

3.4. Inhibition of formation of PtdIns-3-kinase products

The incorporation of ^{32}P into PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 usually seen in [^{32}P]P_i labelled platelets treated with thrombin was about 50% inhibited by resveratrol (Fig. 4A and B). The resting levels of these metabolites were too low to be measurable on the Ramona flow-through detector, but they tend to run under 1000 cpm when they are collected and counted in a scintillation teller.

3.5. Effect of resveratrol on ^{32}P-uptake into platelets and incorporation into PPIs

Since thrombin activation of platelets is associated with a 5- to 10-fold increase in net phosphate influx [30] and is inhibited by anion transport blockers structurally similar to resveratrol [31], the effect of resveratrol on phosphate transport was investigated as uptake of [^{32}P]P_i (Fig. 5). Concentrated PRP to which 0.1 mCi/ml of [^{32}P]P_i had been added (in the labelling procedure described in Methods) were divided into two equal portions and 50 μg/ml resveratrol was added to one of the portions within 5 min of [^{32}P]P_i addition. After various incubation times, EDTA was added to aliquots of the platelet suspension at a final concentration of 5 mM and the platelets were pelleted by centrifugation at 1000×g for 10 min to remove excess [^{32}P]P_i. The pellets were washed in 0.9% sodium chloride containing 1 mM EDTA, recentrifuged and aliquots taken for scintillation counting. The effect of resveratrol on

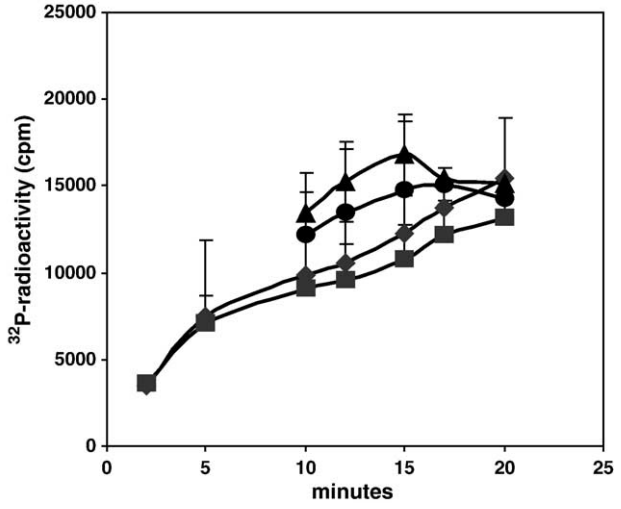

Fig. 5. Effect of resveratrol (50 μg/ml, 2 min) on [^{32}P]P_i uptake in resting and thrombin-stimulated platelets. Symbols represent resting platelets (filled diamonds), resveratrol-treated resting platelets (filled squares), thrombin-stimulated platelets (filled triangles) and resveratrol-treated thrombin-stimulated platelets (filled circles). Thrombin was added to a portion at the concentration of 0.1 U/ml at 10 min after the start of the incubation (filled triangles and filled circles); saline was added to the resting platelets (filled diamonds and filled squares). [^{32}P]P_i was added at time 2 min, and the uptake of radiolabel into the blood platelets was studied. Representative of 3 different donors, means±S.E.M., P values were determined by paired samples Student's t test ($P<0.05$).

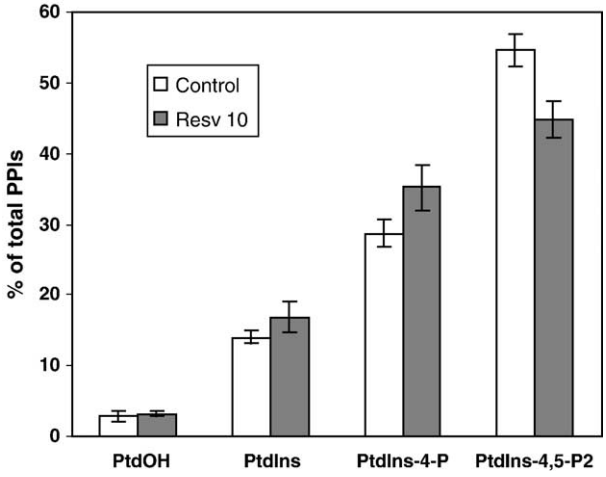

Fig. 6. Cross-over plot of ^{32}P incorporation into PPIs in resting platelets. Platelets were incubated with [^{32}P]P_i for 30 min without (open bar) and with 10 μg/ml resveratrol (filled bar) and then gel-filtered at room temperature. After a further 5 min at 37°, the platelet lipids were extracted and analyzed as described in Methods. The PPI intermediates are plotted in the sequence of synthesis or phosphate incorporation.

phosphate uptake itself appeared to be only slightly inhibitory in both control and thrombin-activated platelets and was not statistically significant.

Resveratrol appeared to have no effects on PPI metabolism in ^{32}P-prelabelled, resting platelets, so the process of ^{32}P incorporation into PPIs itself was studied (Fig. 6). Radio-active phosphate was added to concentrated PRP as described in Methods and the preparation was immediately divided into two portions, to one of which was added 50 μg/ml resveratrol. After 60 min incubation at 37°, the two preparations were gel-filtered in two parallel columns, adjusted for platelet counts and total lipids extracted immediately by the HCl supplemented Bligh-Dyer procedure. The lipid extracts were separated by TLC and the ^{32}P incorporation into platelet phosphoinositides was determined as described in Methods. Resveratrol did not alter the total amount of counts incorporated into phosphoinositides (not shown), but it affected the distribution of label among the PPIs in resting platelets (Fig. 6). Resveratrol increased the level of [^{32}P]-label in PtdIns-4-*P* and PtdIns and decreased the amount of label in PtdIns-4,5-P_2 compared to control platelets. The ratio of PtdIns-4-*P* in control vs. resveratrol-treated resting platelets was 0.8, and the corresponding ratio for PtdIns-4,5-P_2 was 1.2.

4. Discussion

Resveratrol has many effects and the literature is replete with numerous targets of its action in very different types of biological systems (see ref. 5 for review). In platelets, it inhibits partially nearly all measurable activation parameters as shown in Fig. 1, references 1, 13–21 and the current work in which we show that the activation of the PPI cycle is also inhibited (Fig. 2). The apparent discrepancy between the degree of inhibition between platelet responses shown in Fig. 1 and inhibition of the PPI cycle shown in Fig. 2 may be attributable in part to the delay in the onset of latter as shown in the time course study in Fig. 3. Most of the responses shown in Fig. 1 occur within seconds after thrombin addition and are not reversible. We attribute the reproducibly greater effect of 0.625 μg/ml resveratrol compared to higher doses (Fig. 2) to a not uncommon hormetic effect, according to and as described by Calabrese and Baldwin [32]. A similar effect in platelets was previously described for phenothiazines and related compounds [33].

PtdIns synthesis was inhibited by resveratrol in a concentration-dependent manner in thrombin-activated platelets (Fig. 3B). No increase in PtdIns was seen in the first 5 s after thrombin addition in the presence of 50 μg/ml resveratrol whereas PtdOH did increase transiently. Fig. 3E indicates that DAG kinase, i.e., PtdOH formation, was not severely inhibited at the 5 s time point, but that CDP-diacylglycerol inositol transferase, i.e., PtdIns formation, was completely inhibited. In resting platelets, a slight inhibition of PtdIns-4,5-P_2 synthesis accompanied by a corresponding build-up of PtdIns-4(5)-*P* and PtdIns was detected in resting platelets (Fig. 6) in which the ratio of labelled PtdIns-4,5-P_2 to Ptdins-4-*P* decreased from 1.92±0.25 (S.D., $n=3$) to 1.29±0.21 (S.D., $n=3$) in the presence of resveratrol. However, the PPI cycle appears to be more or less intact in resveratrol-treated, resting platelets in that the intermediates all accumulated ^{32}P from cellular ATP nearly to the same level as control platelets. The inhibition of PtdIns synthesis seen at 5 s in activated platelets (Figs. 3B and E) was probably not seen in resting platelets in which the PPI cycle turnover is relatively slow compared to that in activated platelets. PtdIns 3-kinase product formation was also partially inhibited in non-stirred, thrombin-activated platelets, perhaps due to a lack of elevated levels of PtdIns-4,5-P_2 or a down-stream effect of inhibition of the metabolism of canonical PPIs and protein kinase C (Fig. 4).

The levels of PPIs that remained in resveratrol-inhibited, thrombin-activated platelets are puzzling (Figs. 2 and 3). The actual levels of PtdIns-4,5-P_2 in inhibited platelets are comparable to those in resting platelets (Figs. 2 and 3D). These results suggest that the ability of the cell to increase flux through the PPI cycle so as to attain elevated PtdIns-4,5-P_2 levels is necessary for the activation response to agonist. The residual PtdIns-4,5-P_2 may be bound to various cytoskeletal components [34,35] and not be available for signalling. It may be that only a small portion of resting state PtdIns-4,5-P_2 has to be hydrolyzed in order to initiate a large increase in PPI turnover.

An alternative explanation is that phospholipase Cβ becomes inhibited after the initial lag phase (Fig. 3). This phospholipase appears not to be inhibited in resting platelets, since its basal activity is presumably the mecha-

nism by which PtdIns become labelled with ^{32}P (Fig. 6). However, reactive oxygen species are generated upon platelet activation and these may convert resveratrol to an active metabolite that inhibits phospholipase C. Resveratrol inhibition of phospholipase Cβ as measured by intraplatelet calcium elevation with FURA-2/AM has been reported, but the authors themselves suggest that their finding could be an artifact because of the effects of resveratrol itself on the assay system fluorescence [36].

Inhibition of phosphatidate and diacylglycerol formation by resveratrol has also been reported in human neutrophils [37]. The plant flavonoids, quercetin and catechin, which also have antioxidant properties like resveratrol, were reported to inhibit PtdIns-4,5-P_2 labelling and its resynthesis after activation of blood platelets [38].

Resveratrol has also been reported to inhibit isolated mitochondrial ATP synthase [39], which in turn would inhibit the PPI cycle, that being dependent on constant ATP production. The compound is a polyphenolic aromatic hydrocarbon, not unlike the classical mitochondrial uncoupler, 2,4-dintrophenol. Although ATP limitation was not a problem in resting cells since incorporation of ^{32}P into PPIs did take place in the presence of resveratrol (Fig. 6), the rate of ATP consumption increases some 5-fold upon platelet activation [40]; the possibility that the polyphenol is a partial uncoupler cannot then be excluded. Resveratrol has also been reported to bind to and inhibit several protein kinase C isoforms [41], a relatively upstream signalling event. Our findings of the inhibitory effect of resveratrol on the PPI cycle do not eliminate the possibility that also other enzymes are inhibited.

Many of the pleiotropic effects of resveratrol in nucleated cells in vitro may be attributed to its antioxidant effects [5] and its amphipathic structure. Studies on phospholipid monolayer films using the Langmuir technique showed that resveratrol interacts strongly with negatively charged phospholipids (unpublished observations); control studies with ^{3}H-adenine labelling of cytoplasmic ATP showed that this interaction of resveratrol with membrane lipids did not compromise membrane integrity by causing leakage of radioactive, cytoplasmic ATP in both resting and thrombin-stimulated platelets (not shown), as is the case with a number of amphipathic compounds [33,42]. Its solubility properties due to its aromatic rings and phenolic functional groups undoubtedly enable it to penetrate into diverse cellular compartments and then adsorb to various proteins as well as form DNA adducts, resulting in various degrees of enzyme activation or inhibition or interference with transcription. With regard to the French paradox [13], the question remains as to how much resveratrol consumed in food and drink would be available in vivo, not to mention the additive effects of numerous phytochemicals contained in other foods that have similar effects [43]. Our in vitro platelet studies were carried out in 0.2% bovine serum albumin whereas plasma protein concentrations exceed 5%. Resveratrol may contribute to elevation of the threshold for platelet reactivity in a manner that decreases incidents of spurious activation. The active oxygen species scavenging effect of polyphenols generally would also contribute to minimize vessel and tissue damage that might initiate activation events.

In conclusion, the in vitro studies presented here show that inhibition of the increase in the polyphosphoinositide cycle turnover that accompanies platelet activation can be added to the list of resveratrol's pleiotropic effects on cells. The mechanism of action of resveratrol may be related to its lipophilic character and specific association with negatively charged phospholipids in membranes (unpublished observations).

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