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Low Concentrations of Resveratrol Potentiate the Antiplatelet Effect of Prostaglandins

Abstract

Resveratrol, a polyphenolic compound found in grapes and other fruits, is thought to contribute to the cardioprotective effect of red wine. While resveratrol exhibits some antiplatelet effect *in vitro*, the concentrations needed are much higher than those in plasma after consumption of red wine. In the present study, we investigate if resveratrol is able to potentiate the effect of endogenous antiplatelet substances – prostaglandin (PG) I_2 and PGE_1. In human platelet suspension resveratrol at relatively low concentrations (2 or 5 μM), which did not affect platelet function, significantly enhanced the inhibitory activity of PGs on platelet aggregation caused by collagen. The mechanisms underlying this effect may be associated with the inhibition of protein kinase C activation and protein tyrosine phosphorylation, but not with cyclic nucleotide levels and intracellular calcium mobilization in platelets. Our results might provide a possible explanation for the *in vivo* antiplatelet effect of resveratrol despite the poor bioavailability and the weak *in vitro* activity.

Key words
Resveratrol · prostaglandins · platelet aggregation · protein kinase C · protein tyrosine phosphorylation

Introduction

Regular consumption of red wine is often credited as the explanation for the "French paradox" – a term coined to describe the observation that the French enjoy a relatively low risk of cardiovascular disease despite a diet that is high in saturated fat [1]. Resveratrol, a constituent of red wine, is thought to be the major contributor to such an effect [2], [3]. Several *in vitro* and *in vivo* studies showed that resveratrol inhibits platelet aggregation, which is a critical step in arterial thrombosis [4], [5]. Moreover, systemic administration of resveratrol reduces the atherosclerotic area and the size of the thrombus generated by laser-induced damage to the endothelium in hypercholesterolemic mice [6]. On the other hand, however, results from pharmacokinetic studies indicate that circulating resveratrol is rapidly metabolized, and cast doubt on the physiological relevance of the high concentrations typically used for *in vitro* experiments [7]. To explain the discrepancy, we wanted to investigate if resveratrol is able to potentiate the antiplatelet effect of prostaglandins which are important antiplatelet substrates released from vascular endothelium or peripheral blood cells [8].

Materials and Methods

Materials

Resveratrol, PGE_1, collagen (type I, bovine Achilles tendon), and fura-2/AM were obtained from Sigma Chem. Co. (St. Louis, MO, USA). PGI_2 was purchased from Cayman Co. (Ann Arbor, MI, USA); cAMP and cGMP enzyme immunoassay kits were obtained from Amersham Co. (Piscataway, NJ, USA). Anti-phosphotyrosine monoclonal antibody PY20 and phospho-MARCKS-specific poly-

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clonal antibody were purchased from Santa Cruz Biotechnology (St. Cruz, CA, USA). Anti-phospho-Src (Tyr416) antibody was obtained from Cell Signaling Technology, USA (Beverly, MA, USA). All other chemicals were purchased from Sigma Chem. Co.

Preparation of washed human platelets

Human blood anticoagulated with acid citrate dextrose was obtained from healthy human volunteers who had not taken any drugs within the last two weeks. The platelet suspension was then prepared according to the washing procedure described previously [9]. Platelets were finally suspended in Tyrode's solution containing Ca^{2+} (2 mM), glucose (11.1 mM) and bovine serum albumin (3.5 mg/mL) at a concentration of 3×10^8 platelets/mL.

Measurement of platelet aggregation

Platelet aggregation was measured turbidimetrically with a light-transmission aggregometer (Chrono-Log Co.; Havertown, PA, USA). The platelet suspension was incubated with dimethyl sulfoxide (DMSO, vehicle) or resveratrol at 37 °C for 3 min under stirring (1200 rpm) prior to the addition of collagen. The extent of platelet aggregation was measured as the maximal increase of light transmission within 5 min after the addition of stimulators. In all experiments, the final concentration of DMSO was fixed at 0.5 % in the samples – a concentration that has no effect on platelet aggregation.

Measurement of intracellular Ca^{2+} mobilization

Platelets pelleted from platelet-rich plasma were resuspended in Ca^{2+}-free Tyrode's solution, then incubated with fura-2/AM (4 μM) at 37 °C for 30 min. After washing twice, the fura-2-loaded platelets were finally suspended in Ca^{2+}-free Tyrode's solution at a concentration of 3×10^8 platelets/mL. The fura-2-loaded platelets were preincubated with test compounds in the presence of 1 mM extracellular calcium at 37 °C for 3 min prior to the addition of collagen. Fluorescence (Ex 340 nm, Em 380 nm) was measured with a fluorescence spectrophotometer (Model F4000; Hitachi; Tokyo, Japan). Cytosolic free calcium concentration was calculated by the method of Grynkiewicz et al. [10].

Estimation of platelet cyclic nucleotide contents

Platelet cyclic nucleotide contents were measured as described previously [11]. In brief, the platelet suspension was incubated with test compounds at 37 °C for indicated periods under stirring (1200 rpm) in a light-transmission aggregometer. The reaction was stopped by adding EDTA (10 mM) followed immediately by boiling for 2 min. Upon cooling to 4 °C, cell debris was removed by centrifugation at 10,000 × *g* for 5 min. The supernatant was then used to assay for cAMP and cGMP using enzyme immunoassay kits.

Platelet lysis and Western blotting

To prepare whole platelet lysates, the reaction was terminated at the indicated time points by addition of 5 × SDS sample buffer (1 ×, 2 % SDS, 62.5 mM Tris-HCl, pH 6.8, 10 % glycerol, 5 % β-mercaptoethanol). The samples were boiled for 5 min and subjected to immunoblotting analysis.

Platelet lysates were electrophoresed on an SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Trans-blot; Bio-Rad; Hercules, CA, USA). The membranes were blocked overnight in 5 % non-fat dry milk in TBST (Tris-buffered saline supplemented with 0.1 % Tween 20), washed three times in TBST, and incubated for 1 h in the primary antibody solution of interest in TBST with 1 % bovine serum albumin. After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. After washing with TBST, protein bands on the membrane were visualized by an enhanced chemiluminescence Western blotting detection system (Western Lightning®; Perkin Elmer; Boston, MA, USA).

Statistics

Results are expressed as the mean ± standard error of the mean (SEM). Statistical significance was calculated by analysis of variance (ANOVA) followed by Dunnett's test or Student's t-test. $P < 0.05$ was considered statistically significant.

Results

In washed human platelets, resveratrol, PGE_1, and PGI_2 inhibited collagen (10 μg/mL)-induced platelet aggregation with IC_{50} values of 14 μM, 0.4 μM, and 7.5 μM, respectively.

By using low concentrations of resveratrol (2 and 5 μM), which had no or little effect collagen-induced platelet aggregation, the interaction between resveratrol and prostaglandins was investigated. When platelets were incubated with PGE_1 in the presence of 2 μM or 5 μM resveratrol, the concentration-response curve of PGE_1 was significantly shifted to the left with IC_{50} values of 0.036 μM and 0.006 μM, respectively (Fig. **1**). In a similar but less pronounced manner, the antiaggregatory activity of PGI_2 was also enhanced by resveratrol (Fig. **1**). Because PGE_1 is a more potent and stable antiplatelet agent than PGI_2, it was chosen in the subsequent experiments for investigating the mechanism of synergism between resveratrol and prostaglandins.

To assess whether the action of resveratrol was due to elevation of intracellular levels of cAMP and/or cGMP, two major inhibitory messengers in regulating platelet aggregation [12], the effect of resveratrol on cyclic nucleotide levels in platelets was examined. In washed human platelets, we found that resveratrol alone affected neither cAMP levels nor cGMP levels (Table **1**). In addition, resveratrol did not affect the cyclic nucleotide-elevating activity of PGE_1. The combination of resveratrol and the phosphodiesterase inhibitor IBMX also did not further increase the levels of cyclic nucleotides in platelets (Table **1**).

In fura-2-loaded platelets, collagen caused a marked increase in levels of intracellular free calcium. As shown in Fig. **2**, in the presence of 1 mM of extracellular calcium, pretreatment of platelets with resveratrol (5 μM) did not affect the calcium signal elicited by collagen. In contrast, PGE_1 (0.03 – 1 μM) concentration-dependently inhibited the collagen-induced increase in $[Ca^{2+}]_i$. Interestingly, resveratrol failed to enhance the action of PGE_1 on intracellular calcium levels.

In addition to calcium signaling, agonist-induced protein kinase C (PKC) activation may also contribute to the regulation of platelet aggregation [13]. We thus examined the effect of resveratrol and PGE_1 on PKC activation by measuring the phosphorylation

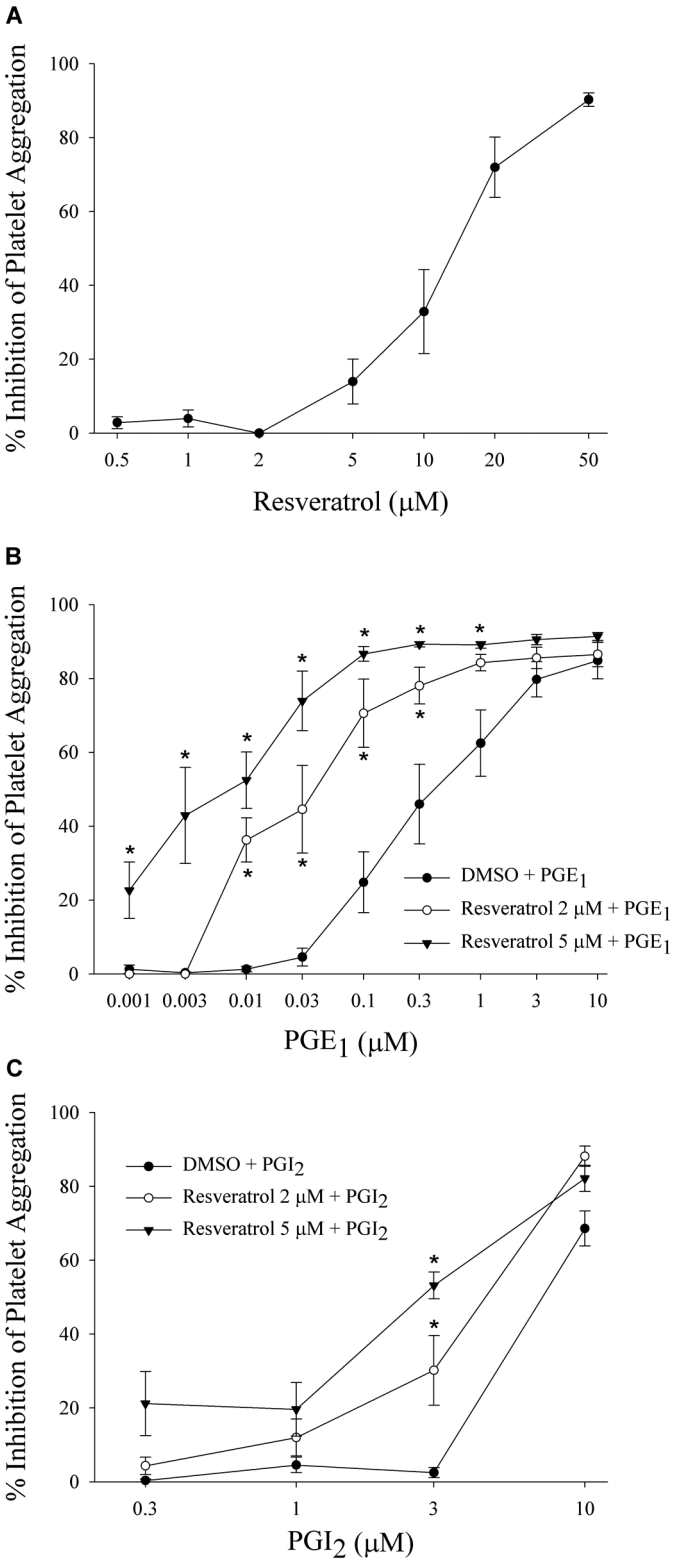

Fig. **1** Effect of resveratrol and prostaglandins on collagen-induced platelet aggregation. (**A**) Washed human platelets were incubated with various concentrations of resveratrol at 37 °C for 3 min, then collagen (10 μg/mL) was added to induce platelet aggregation. Washed platelets were incubated with PGE_1 (**B**) or PGI_2 (**C**) in the absence or presence of resveratrol (2 or 5 μM). Then, platelet aggregation was induced by collagen (10 μg/mL). Values are presented as mean ± SEM (n = 4). * P < 0.05 as compared with the control group.

Table **1** Interactions of resveratrol, PGE_1, and IBMX on platelet cyclic nucleotide levels.

	cAMP (pmol/mL)	*cGMP (pmol/mL)*
Control (DMSO)	2.45 ± 0.32	0.81 ± 0.09
Resveratrol	2.61 ± 0.48	0.81 ± 0.06
PGE_1	4.61 ± 0.35*	0.84 ± 0.01
IBMX	18.29 ± 5.08*	1.79 ± 0.06[a]
Resveratrol + PGE_1	6.42 ± 0.83*	0.87 ± 0.04
Resveratrol + IBMX	20.14 ± 7.61*	1.98 ± 0.15[a]
IBMX + PGE_1	137.80 ± 25.51*#	–

Washed human platelets (1×10^9 platelets/mL) were incubated with PGE_1 (1 μM) at 37 °C for 1 min in the presence of DMSO, IBMX (300 μM) or resveratrol (5 μM). The reaction was stopped, platelets were then pelleted and supernatants were used to assay for cAMP and cGMP using enzyme immunoassay kits. Values are presented as mean ± SEM (n = 3–4). * P < 0.001 as compared with the control. # P < 0.001 as compared with PGE_1 alone.

of myristoylated alanine-rich C kinase substrate (MARCKS), which is a major substrate of PKC [14]. As shown in Fig. **3**, the PKC inhibitor GF109203X (10 μM) abolished MARCKS phosphorylation caused by collagen, whereas resveratrol (5 μM) alone had no significant effect. The combination of resveratrol and PGE_1 led to a synergistic and complete inhibition of collagen-induced PKC activation. Fig. **4A** shows that collagen stimulation of platelets leads to tyrosine phosphorylation of a number of proteins. Pretreatment of platelets with resveratrol (5 μM) alone did not significantly inhibit protein tyrosine phosphorylation caused by collagen. However, in the presence of resveratrol, the inhibitory effect of PGE_1 on collagen-induced protein tyrosine phosphorylation was significantly enhanced.

We next examined whether the activation of Src, which is a major tyrosine kinase in platelets, was also affected by resveratrol and PGE_1. We assessed the autophosphorylation of Src in col-

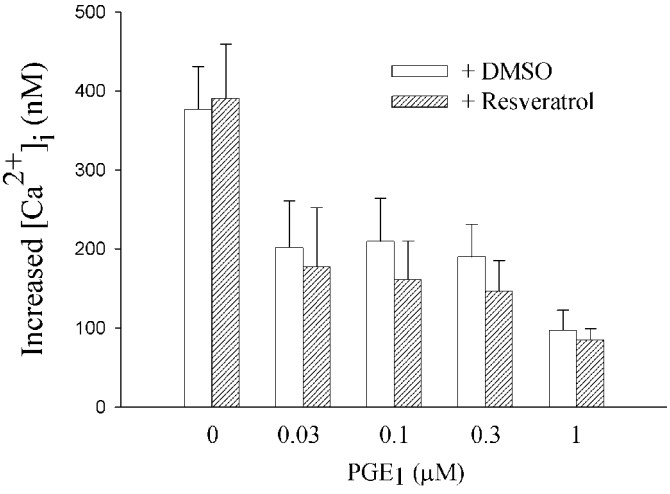

Fig. **2** Effects of resveratrol and PGE_1 on intracellular calcium mobilization in human platelets. Fura-2-loaded platelets were incubated with DMSO or resveratrol (5 μM) at 37 °C for 3 min in the absence or presence of PGE_1, collagen (10 μg/mL) was then added to trigger the increase of $[Ca^{2+}]_i$. Increased cytosolic free calcium concentration was calculated as described in the Methods section. Values are presented as mean ± SEM (n = 3).

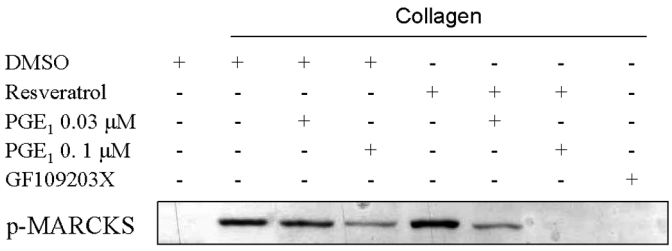

Fig. **3** Effect of resveratrol and PGE_1 on collagen-induced MARCKS phosphorylation. Washed platelets were incubated with DMSO (vehicle), resveratrol (5 μM), or GF109203X (10 μM) at 37 °C for 3 min in the absence or presence of PGE_1. After then, collagen (10 μg/mL) was added for another 1 min. Western blot analysis was performed on platelet lysates using anti-phospho-MARCKS antibody. Similar results were obtained in three separate experiments.

lagen-stimulated platelets by Western blotting using a specific anti-phospho-Src (Y-416) antibody, which specifically detects Src phosphorylated at Tyr-416, a process indicative of Src activation [15]. Fig. **4B** shows that stimulation of platelets with collagen induced Tyr-416 phosphorylation of Src within 1 min. Once again, the inhibition of collagen-induced Src activation by the combination of PGE_1 (0.1 μM) and resveratrol (5 μM) was greater than inhibition by either agent alone.

Discussion

In the present study, we show that although a high concentration (50 μM) of resveratrol was needed to abolish collagen-induced human platelet aggregation, only a low micromolar concentration (2 or 5 μM) of resveratrol was sufficient to potentiate the anti-aggregatory effects of PGE_1 and PGI_2. In the presence of resveratrol, the potency of PGE_1 was greatly enhanced by up to 67-fold. One might speculate that the potentiation of endogenous prostaglandins might take place *in vivo* in blood vessels and this might, in part, explain how resveratrol can exhibit antiplatelet effect in *in vivo* studies regardless of the poor bioavailability and the weak *in vitro* activity.

Because resveratrol neither increased cyclic nucleotide levels in platelets on its own, nor enhanced the cAMP-elevating effect of PGE_1, it was unlikely that cyclic nucleotides are involved in the action of resveratrol. We thus investigated whether resveratrol interferes with the stimulating pathways implicated in regulation of platelet aggregation. Upon platelet stimulation by agonists, phosphatidylinositide-specific phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) which promote activation of PKC and the increase of cytosolic free Ca^{2+}, respectively. Both PKC and Ca^{2+} act either independently or synergistically to induce platelet aggregation [16]. PKC and Ca^{2+} are also involved in platelet shape change, dense and α granule secretion, phospholipase A_2 activation and thromboxane A_2 formation [17], [18]. We show here that resveratrol alone did not inhibit the increase in intracellular Ca^{2+} caused by collagen, and failed to further enhance the inhibitory effect of PGE_1 on Ca^{2+} signaling. These results indicate that resveratrol can potentiate the antiaggregatory activity of PGE_1 without affecting the levels of cytosolic free calcium. We next examined whether resveratrol's action is through interference with the PKC pathway. Resveratrol has been reported to inhibit PKC activity and prevent PKC-mediated signaling in human mammary epithelial cells [19]. At the concentration used in the present experiment, resveratrol alone had no effect on collagen-induced PKC activation; however, a combination of resveratrol and PGE_1 led to a synergistic and complete inhibition on PKC activation caused by collagen. This synergism may be due to the fact that the compounds exert their inhibitory effect at different steps in the PKC signaling pathway; PGE_1 inhibits PLC activation and thus the production of DAG [20], whereas resveratrol directly decreases the enzyme activity of PKC by competing with DAG binding to PKC [19].

Protein tyrosine phosphorylation is another important stimulating pathway involved in platelet aggregation [21]. Tyrosine kinase inhibitors are capable of decreasing the activation of glycoprotein (GP) IIb/IIIa and subsequent fibrinogen binding [22]. Furthermore, loss the function of Syk or Src family kinases impairs the platelet response to collagen [23], [24]. Resveratrol has been reported to inhibit Src kinase activity in HeLa cells and human umbilical endothelial cells [25], [26]. In the present work, we also found that resveratrol partially decreased Src autophosphorylation by its own manner. Moreover, the inhibitory activity of

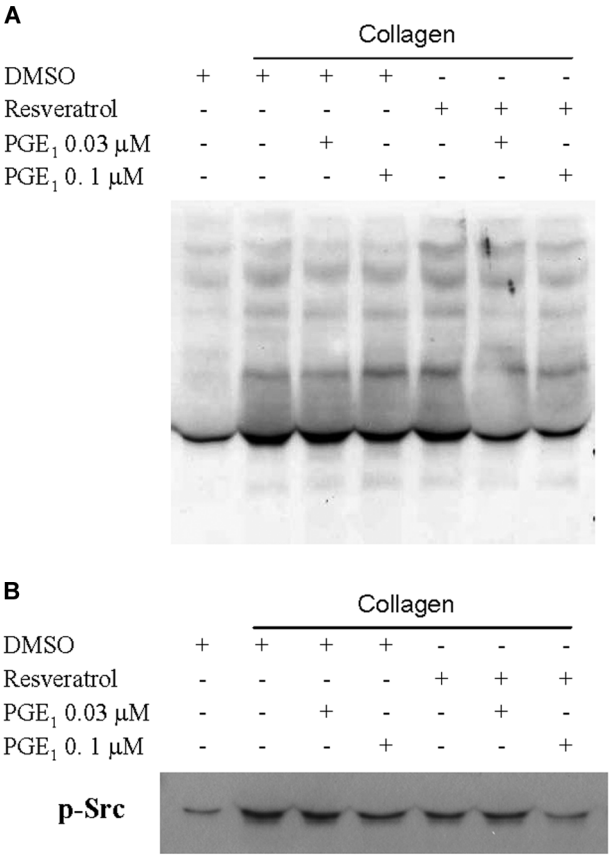

Fig. **4** Effect of resveratrol and PGE_1 on collagen-induced protein tyrosine phosphorylation. Washed platelets were incubated with DMSO (vehicle) or resveratrol (5 μM) at 37 °C for 3 min in the absence or presence of PGE_1. After then, collagen (10 μg/mL) was added for another 3 min (**A**) or 1 min (**B**). Western blot analysis was performed on platelet lysates using anti-phosphotyrosine antibody PY20 (**A**) or anti-phospho-Src (Tyr416) antibody (**B**). Similar results were obtained in three separate experiments.

PGE_1 on both collagen-induced Src activation and protein tyrosine phosphorylation in platelets were significantly potentiated by resveratrol. These results suggest that the enhanced inhibition of Src tyrosine kinase may be involved in the synergism between resveratrol and PGE_1.

There is a complicated cross-talk between tyrosine kinases and PKC signaling pathways. In human platelets, inhibition of PKC resulted in decreased Src activation by thrombin receptor agonist peptide (SFLLRN) [27]; on the other hand, the activity of PKC also can be positively regulated by Src [28]. Therefore, the enhancement of antiplatelet effect of PGE_1 by resveratrol could result from the inhibition of either or both PKC and protein tyrosine kinases, although the exact mechanisms remain to be elucidated.

In conclusion, we have demonstrated that the antiplatelet activity of prostaglandins can be potentiated by low concentrations of resveratrol. The mechanisms underlying this effect may be associated with the inhibition of PKC activation and protein tyrosine phosphorylation. Our results might provide a possible explanation for the *in vivo* antiplatelet effect of resveratrol in spite of its poor bioavailability and weak *in vitro* activity.

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