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Resveratrol attenuates thromboxane A_2 receptor agonist-induced platelet activation by reducing phospholipase C activity

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Abstract

Resveratrol (3,5,4′-trihydroxystilbene) is a naturally occurring compound shown to decrease the incidence of thromboembolic disease. Although considerable data are available as to the inhibitory effect of resveratrol on the platelet aggregation and thrombopoiesis in human, its underlying mechanism, at the cellular level, has not been rigorously studied. In this experiment, we studied the effect of resveratrol and 1-[6-[[17-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione, a phospholipase C inhibitor (U-73122) on the thromboxane A_2 receptor agonist (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$, U46619)-induced platelet aggregation, platelet P-selectin expression, and the activity of phospho-phospholipase C β3 (P-PLC β3) and total-phospholipase C β3 (T-PLC β3), which play key roles in the signal transduction system of platelet in human. It was found that resveratrol blocked platelet aggregation and platelet P-selectin expression induced by U46619 in a concentration-dependent manner. U-73122 and resveratrol had additive effect in inhibiting platelet aggregation and platelet P-selectin expression. Resveratrol (final concentration was 50 μM) could reduce the ratio of P-PLC β3 to T-PLC β3. Taken together, these results show that resveratrol suppresses U46619-induced platelet aggregation and P-selectin expression partly through the decrease of the activity of phospholipase C β of platelets.

Keywords: Resveratrol; Platelet; Thromboxane A_2 receptor agonist; Phospholipase C β; Phospholipase C β inhibitor

1. Introduction

Coronary heart disease and its consequences such as myocardial infarction and heart failure remain the leading cause of death in developed countries. Epidemiological studies have shown that the French population has lower morbidity and mortality from coronary heart disease than other Western populations despite their high-fat and high-cholesterol diet (Renaud and de Lorgeril, 1992; Tunstall-Pedoe et al., 1999). Recently, it has been shown that this paradoxical finding, termed "French paradox", may be attributed to regular consumption of red wine and that the unique antiatherogenic effects of red wine reside in the action of resveratrol (Leger et al., 2005; Grønbaek,, 2002; Vogel, 2002). Numerous pharmacological evidences have demonstrated that resveratrol has various biological activities including antiplatelet activity, anti-fibrotic, antioxidant, increased insulin sensitivity, decreased circulating free fatty acids, decreased insulin-like growth factor, increased activity of the energy-sensing enzyme, AMP-activated protein kinase, and increased mitochondrial number (Opie and Lecour, 2007). Resveratrol, a phytoalexin present in a wide variety of plant species, including mulberries, peanuts and grapes, has gradually become a hot spot in experimental and clinical cardiovascular research (El-Mowafy, 2002). Shen et al. (2007) demonstrate for the first time that resveratrol inhibits platelet aggregation via inhibition of p38 mitogen-activated protein kinase phosphorylation and an increase in NO/cyclic GMP formation in washed human platelets, and Stef et al. (2006) show from clinical study that resveratrol inhibits aggregation of

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platelets from high-risk cardiac patients with aspirin resistance, which is significantly related to myocardial infarction and cerebrovascular accident in patients with stable cardiovascular disease (Gum et al., 2001, 2003). However, the detailed mechanisms by which resveratrol inhibits platelet activation are not completely understood. Further research may provide convincing and exact evidence that resveratrol would be invaluable for medical treatment of cardiovascular diseases.

Phosphoinositide metabolism contributes an important intracellular signaling system that is involved in a variety of cell functions such as hormone secretion, neurotransmitter signal transduction, cell growth, membrane trafficking, ion channel activity, and regulation of the cytoskeleton (Fukami, 2002; Martin, 2001; Rhee, 2001; Irvine, 2003). Phospholipase C (PLC), a key enzyme in this system, hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two second messengers, inositol 1,4,5-trisphosphate (IP_3) inducing Ca^{2+} releases from intracellular stores and diacylglycerol (DAG) mediating the activation of protein kinase C (PKC) (Berridge and Irvine, 1984; Nishizuka, 1988). In platelets, both Ca^{2+} and PKC stimulate platelet aggregation and also elicit synergism in platelet aggregation (Crabos et al., 1992). PLC can be categorized into four classes—β, γ, δ, and ε—each containing various isoforms (Rebecchi and Pentyala, 2000; Rhee, 2001). All four PLC β isoforms (β1, β2, β3, and β4) are activated to varying extent by a subunit of a class of G proteins known as G_q (Wu et al., 1992; Park et al., 1993; Jhon et al., 1993; Lee et al., 1994). Experimental studies provide evidence that PLC β3 and PLC β2 play vital roles in platelet cytoskeletal dynamic (Lian et al., 2005). The U-73122 (1-[6-[[17-3-methoxyestra-1,3,5 (10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione) is widely used as a tool to investigate the involvement of the PLC in signal transduction, particularly in studies attempting to characterize pathways leading to intracellular Ca^{2+} mobilization upon agonist challenge (Bleasdale et al., 1989, 1990; Smith et al., 1990). Detailed analysis of U-73122 actions confirmed that the compound directly blocked PLC isoenzymes in vivo and in vitro (Bleasdale et al., 1990; Smith et al., 1990; Hou et al., 2004).

Upon exposure to activating agonists (e.g. thrombin, adenosine diphosphate, and collagen), platelets release arachidonic acid stored as phospholipid in the platelet plasma membrane, which is then converted into thromboxane A_2 (TXA_2) by sequential oxygenation by cycloxygenase and TXA_2 synthase (Samuelsson et al., 1978). The released TXA_2 acts as a positive feedback mediator in the activation and recruits of more platelets to the primary hemostatic plug (Hourani and Cusack, 1991). Either as a potent platelet agonist (Hourani and Cusack, 1991; Shen and Tai, 1998) or as a weak agonist (FitzGerald, 1991), TXA_2 exerts its actions via specific G protein-coupled receptors and plays an important role in amplifying the response of platelets to more potent agonists (FitzGerald, 1991). In the present study, the effects of resveratrol and U-73122 on U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$, a stable TXA_2 analog)-induced platelet aggregation, platelet P-selectin expression, and the activity of PLC β3 were studied with platelet aggregometer, flow cytometry and Western blotting. The aim of this study was to investigate whether the molecular mechanism of resveratrol in exerting cardiovascular protective effect is through inhibiting the activity of PLC β of platelet.

2. Materials and methods

2.1. Chemicals

Resveratrol, apyrase (glade VII), U-73122, and U46619 were purchased from Sigma (St. Louis, MO, USA). R-Phycoerythrin (RPE)-conjugated mouse anti-human CD62P monoclonal antibody was purchased from BD Biosciences. Rabbit anti-human P-PLC β3 and T-PLC β3 were purchased from Cell Signaling Technology (Danvers, MA, USA). HRP-conjugated mouse anti-rabbit IgG secondary antibody was from Amersham. Stock solutions of resveratrol (12.5 mM) were prepared in dimethyl sulfoxide (DMSO) and kept frozen at −20 °C. Unless otherwise indicated, all reagents used in this study were dissolved in DMSO. All the concentrations expressed in this study were final concentrations.

2.2. Platelet preparations

Platelet-rich plasma and platelet-poor plasma were prepared as described previously (Dogné et al., 2000, 2001). Whole blood was obtained by venous-puncture from healthy non-smoking human volunteers (ages 23–38 years) who had not consumed any medication known to affect platelet function for at least 2 weeks, using standard blood drawing procedures (normal blood flow and no pressure). Informed consent was signed by all subjects. Blood was drawn into tubes containing one-sixth volume of ACD (2.5 g sodium citrate, 1.5 g citric acid, and 2 g glucose in 100 ml deionized water) and then centrifuged (Optima L-XP centrifuge, Beckman Coulter, USA) at 230 g for 20 min at room temperature to obtain platelet-rich plasma. Platelet-poor plasma was prepared from the remaining volume of blood by centrifugation at 1600 g for 10 min. Platelet in platelet-rich plasma was adjusted to 2.5 and 3.0×10^8 cell/ml (Shah and Saeed, 1995) by dilution platelet-poor plasma with F-820 automatic blood analysator (Sysmex, Japan). Both platelet-rich plasma and platelet-poor plasma were used within 1.5 h of blood collection.

For the isolation of washed platelets, apyrases (1 U/ml) were added to whole blood to obtain the platelet-rich plasma. After diluted 3-fold by ACD containing apyrases, the platelet-rich plasma was then centrifuged for 10 min at 980 g at room temperature to pellet the platelets. Platelets were resuspended in Tyrode Buffer (138 mM NaCl, 2.7 mM KCl, 1 mM MgCl, 3 mM NaH_2PO_4, 5 mM glucose, 10 mM HEPES, pH 7.4, 0.2% bovine serum albumin) containing 0.01 U/ml apyrase and adjusted to 2.5 and 3.0×10^8 cell/ml. Washed platelets were used for PKC activity studies within 3 h after isolation.

2.3. Platelet aggregations

Platelet aggregation was studied as described previously by Born (1962) in a four-channel platelet aggregometer (Chronolog, Chicago, USA). Briefly, samples (250 μl) of platelet-rich plasma as prepared above in cuvettes were mixed respectively with 5 μl of HEPES Buffer (Unstimulated Group and Control Group), with resveratrol at 25, 50 and 100 μM (Resveratrol 25, Resveratrol 50, and Resveratrol 100 Group), with 1 μM of U-73122 (U-73122

Group), with 1 μM of U-73122 and 50 μM of resveratrol (U-73122+Resveratrol 50 Group), or with DMSO (DMSO Group). The final concentration of DMSO was 2% (v/v) in all groups except Unstimulated Group and Control Group. After reacting for 6 min at 37 °C, aggregation in all groups except Unstimulated Group was started by U46619 (1 μM, dissolved in HEPES Buffer). Aggregation was allowed to proceed with constant stirring at 1000 rpm. Results were evaluated and calculated using maximal aggregation ratios, which expressed as the percent increase in light transmission after 6 min. The ratio of inhibition of platelet aggregation was calculated by using the following equation:

$$\text{inhibition}(\%) = (1 - B/A) \times 100$$

where A is the maximal aggregation ratio of cuvette of DMSO (resveratrol at 0 μM) and B is the maximal aggregation ratio of cuvettes of Resveratrol 25, Resveratrol 50, Resveratrol 100, U-73122 or U-73122+Resveratrol 50.

2.4. Flow cytometry

Except specifically stated, all reagents used in flow cytometry were dissolved in 20% DMSO (DMSO:HEPES=1:4). The flow cytometric analysis of platelets in whole blood has been described previously (Chronos et al., 1994). RPE-CD62P fluorescence was monitored to obtain the percentage of P-selectin-positive platelets. Within 3 min of collection, 5 μl of whole blood was respectively added to prewarmed cuvettes containing 5 μl of RPE-CD62P, and incubated in the dark at room temperature for 10 min with HEPES Buffer (Unstimulated Group and Control Group), with resveratrol at six different final concentrations (6.25, 12.5, 25, 50, 100, and 200 μM), with 1 μM of U-73122 (U-73122 Group), with 1 μM of U-73122 and 50 μM of resveratrol (U-73122+Resveratrol 50 Group), or with 20% DMSO (DMSO Group). Total volume was 45 μl by added HEPES in all cuvettes except Unstimulated Group cuvettes which was 50 μl, and final concentration of DMSO was 2% (v/v) in all groups except Unstimulated Group and Control Group. Afterwards, U46619 (1 μM, dissolved in HEPES Buffer) was added in all cuvettes except Unstimulated Group cuvette, and samples were further incubated in the dark at room temperature for 10 min without stirring. Thereafter, the blood samples were labeled for flow cytometric analyses.

2.5. Western blot analysis of P-PLC β3 and T-PLC β3

For immunoblotting with P-PLC β3 and T-PLC β3 antibodies, four samples (250 μl) of washed platelets as prepared above in cuvettes were mixed with HEPES Buffer (Unstimulated Group and Control Group), with resveratrol (50 μM, Resveratrol 50 Group), and with DMSO (DMSO Group). The final concentration of DMSO was 2% (v/v) in RESV 50 Group and DMSO Group. After reacting for 5 min at 37 °C, aggregation in all groups except Unstimulated Group was started by U46619 (1 μM, dissolved in HEPES Buffer). To measure the activation state of PLC β3, aggregation reactions that make use of washed platelets were directly quenched by the addition of 1044 μl of 4×Platelet Lysates Buffer containing 400 μl of 4×SDS Sample Loading Buffer (1 M pH: 6.8, Tris–HCl, 2.5 ml, SDS, 0.8 g, bromophenol blue, 0.004 g, glycerol, 4 ml, dd H_2O were added to 10 ml), 400 μl of 1 M DTT, 4 μl of 1 M NaF, 40 μl of 0.1 M $NaVO_3$ and 200 μl of Protease inhibitor and immediately boiled prior to Western analysis. Proteins containing in 40 μl of samples were separated on SDS–polyacrylamide gels containing 5% polyacrylamide, transferred onto nitrocellulose membranes for 1 h at 100 V by Bio-Rad gel analysis system (Bio-Rad Corporation, USA). The membranes were blocked with TBS (Tris-Buffered Saline) containing 5% milk and incubated for 1 h with 5% milk (in a Tween containing TBS Buffer) containing antibody (1:1000) that recognize active phosphorylated form of PLC β3 (P-PLC β3). The membranes were subsequently incubated with HRP-coupled secondary antibody (mouse anti-rabbit IgG). Antigen–antibody complexes were detected with the chemiluminescence (ECL) method (Amersham Corp.). The intensities of the bands were quantitated by densitometry (ATTO Densitograph AE-6900M). Thereafter, P-PLC β3 proteins containing in the membrane were eluted by adding 50 ml of Stripping Buffer (10% SDS, 10 ml, Beta-mercaptoethanol, 350 μl, 1 M pH: 6.7, Tris–HCl, 3.125 ml, dd H_2O, 36.525 ml), and the membranes were re-incubated with antibody (1:1000) that recognize inactivated form of PLC β3 (T-PLC β3) in order to quantitate the T-PLC β3 proteins containing in the membrane. The ratio of P-PLC β3 to T-PLC β3 was determined as the quantities of P-PLC β3 relative to T-PLC β3 in order to avoid the difference of protein containing in each samples. The data presented in each gel figure are from a single experiment and representative of four independent experiments.

2.6. Statistical analyses

Chi square tests were employed to compare categorical variables, while Mann Whitney U tests were used, where appropriate, in the univariate analysis. Platelet aggregation changes were evaluated by repeated-measures analysis of variance for intra-group comparisons. Values of $P<0.05$ were considered significant. SPSS 10.0 Statistical software was used in the statistical analysis.

3. Results

3.1. Effect of resveratrol on platelets aggregation

Compared with Unstimulated Group, U46619-induced platelet aggregation ratio was significantly higher in Control Group (80.81±8.63% vs 7.37±3.29%, $P<0.01$). Because resveratrol is almost insoluble in saline that is normally used as a solvent in the present assay method, we dissolved resveratrol in DMSO. As shown in Fig. 1A, stimulation of DMSO-treated platelet-rich plasma (DMSO Group, resveratrol at 0 μM, 66.30±7.03) with U46619 resulted in slightly decreased aggregation in comparison with no-treated platelet-rich plasma induced by U46619 ($P>0.05$), whereas pretreatment of platelet-rich plasma with resveratrol at 25, 50 and 100 μM (60.90±9.40%, 49.45±8.47%,

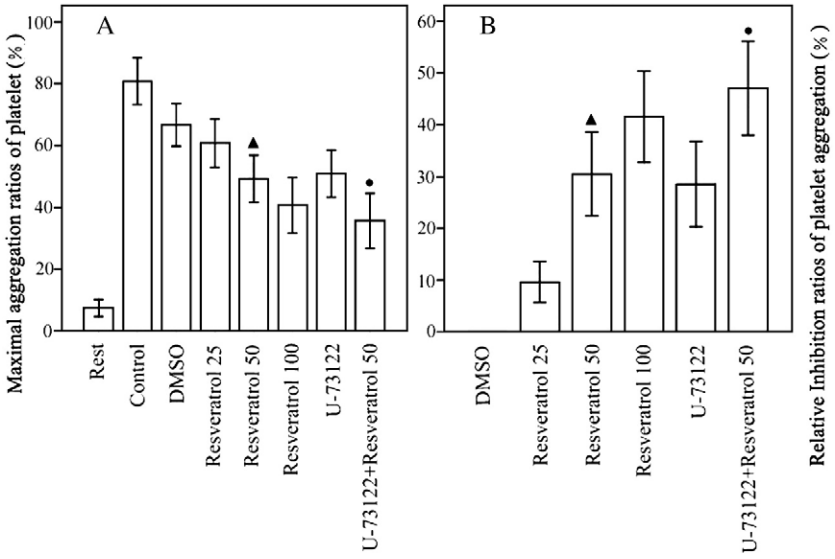

Fig. 1. Resveratrol inhibition of U46619-induced platelet aggregation. Platelet-rich plasma was pretreated in the presence of DMSO (DMSO, resveratrol at 0 μM), 25, 50 and 100 μM of resveratrol (Resveratrol 25, Resveratrol 50, and Resveratrol 100), 1 μM of U-73122 (U-73122) and 1 μM of U-73122 plus 50 μM of resveratrol (U-73122+Resveratrol 50) for 5 min prior to the addition of U46619; PRP was untreated for 5 min prior to the presence (Control) or absence (Unstimulated) of U46619. Aggregation reactions were allowed to proceed and the maximal aggregation ratios of platelet were determined (A). Relative ratios of inhibition of platelet aggregation were also presented (B). Standard error bars were shown. Numerical data were means±S.E.M. of six independent experiments; ▲$P<0.05$ compared to the DMSO and ●$P<0.05$ compared to the Resveratrol 50 and U-73122, respectively.

and 40.62±11.39%, respectively) showed a concentration-dependent increase in inhibiting platelet aggregation and more profound inhibition than DMSO-treated platelet-rich plasma ($P>0.05$, $P<0.05$, $P<0.01$, respectively, Fig. 1A and B). U-73122 (51.81±10.54%) was not a potent inhibitor of aggregation induced by U46619. However, when 1 μM of U-73122 and 50 μM of resveratrol were added together, their effect of inhibiting platelet aggregation was significantly lower as compared with DMSO Group (36.94±12.48%, $P<0.01$). U-73122 and resveratrol showed additive inhibition of U46619-induced platelet aggregation.

When ratios of relative inhibition were calculated, the inhibitory effect of resveratrol on the platelet aggregation induced by U46619 was studied in comparison with that of DMSO Group (resveratrol at 0 μM, ratio of inhibition was 0, Fig. 1B) as the positive control. Approximately 9.62±5.04% of inhibition of platelet aggregation was achieved by 25 μM of resveratrol ($P>0.05$), a higher concentration of resveratrol at 50 and 100 μM markedly increased ratios of inhibition of platelet aggregation from 30.49±9.53% to 41.71±11.27% ($P<0.05$, $P<0.01$, respectively). U-73122 slightly decreased ratio of inhibition of platelet aggregation to 28.56±10.33% ($P<0.05$). However, when 1 μM of U-73122 and 50 μM of resveratrol were added together, their ratios of inhibition of platelet aggregation were significantly higher as compared with DMSO ($P<0.01$, $P<0.05$ with regard to U-73122 and Resveratrol 50, respectively).

3.2. Attenuation of U46619-induced platelet P-selectin expression by resveratrol

U46619-induced platelet P-selectin expression was investigated in the absence or presence of resveratrol. P-selectin expression increased obviously from 1.38±0.46% (Unstimulated Group) to 44.05±10.08% (Control Group, stimulation with U46619, $P<0.01$), while 20% DMSO (DMSO Group, resveratrol at 0 μM) reduced the levels of P-selectin expression to 37.88±9.36% ($P>0.05$ compared with Control Group). Resveratrol at 50 μM did not influence platelet P-selectin expression in unstimulated samples (data not shown), but dose-dependently inhibited U46619-induced platelet P-selectin expression (from 24.41±8.36% to 9.73±3.64% at concentrations of 6.25–200 μM, Fig. 2A). U46619-induced increase in platelet P-selectin expression was slightly reduced by U-73122, a PLC inhibitor (from 37.88±9.36% of 20% DMSO to 20.59±7.83%). However, when 1 μM of U-73122 and 50 μM of resveratrol were added together, their effect of inhibiting platelet P-selectin expression (5.74±1.34%) was significantly lower as compared with 20% DMSO ($P<0.01$). U-73122 and resveratrol showed additive inhibition of U46619-induced platelet P-selectin expression.

When ratios of relative inhibition were calculated, the inhibitory effects of resveratrol on the P-selectin expression induced by U46619 were studied in comparison with those of 20% DMSO (resveratrol at 0 μM, ratio of inhibition was 0, Fig. 2B) as the positive control. Approximately 39.19±7.89% of inhibition of P-selectin expression was achieved by 6.25 μM of resveratrol ($P<0.05$), a higher concentration of resveratrol from 12.5 to 200 μM markedly increased ratios of inhibition of P-selectin expression from 48.65±8.27% to 75.37±8.62%. U-73122 (1 μM) slightly decreased ratio of inhibition of P-selectin expression to 48.91±9.37%. However, when 1 μM of U-73122 and 50 μM of resveratrol were added together, their ratios of inhibition of P-selectin expression were significantly higher as compared with 20% DMSO ($P<0.01$, $P<0.05$ with regard to U-73122 and Resveratrol 50, respectively).

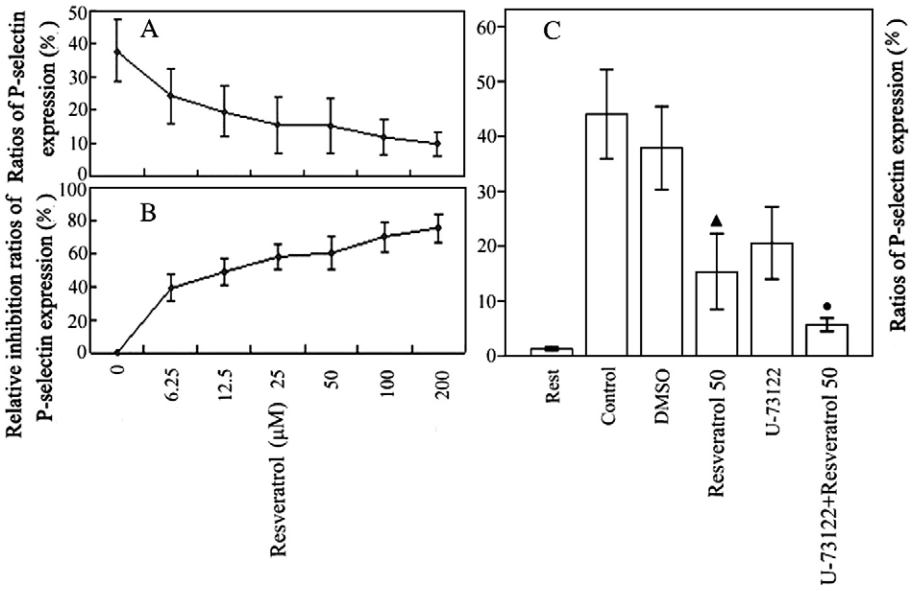

Fig. 2. Resveratrol attenuated U46619-induced platelet P-selectin expression. Whole blood was preincubated at 37 °C without stirring for 10 min in the presence of various concentrations of resveratrol. Samples were then further incubated for 10 min in the presence of U46619. Platelet P-selectin expression was measured by whole blood flow cytometry (A). Relative inhibition ratios of P-selectin expression by resveratrol were also presented (B). Values were means±S.E.M. of six independent experiments. Whole blood was pretreated respectively in the presence of 20% DMSO (DMSO, resveratrol at 0 μM), 50 μM of resveratrol (Resveratrol 50), 1 μM of U-73122 (U-73122), and 1 μM of U-73122 plus 50 μM of resveratrol (U-73122+Resveratrol 50) for 10 min prior to the presence of 1 μM of U46619; Whole blood was untreated for 10 min prior to the presence (Control) or absence (Unstimulated) U46619. Standard error bars were shown. Numerical data were means±S.E.M. of six independent experiments; $^{\blacktriangle}P<0.05$ compared to the DMSO and $^{\bullet}P<0.05$ compared to the Resveratrol 50 and U-73122, respectively.

3.3. Effect of resveratrol on phospholipase Cβ3 activity of platelets

Western blotting was used to study the effect of resveratrol on the activation state of PLC β3 of platelets in healthy volunteers. As shown in Fig. 3, there was no significant difference of T-PLC β3 activity among four groups ($P>0.05$), whereas the ratio of P-PLC β3 to T-PLC β3 was significantly lower in Unstimulated Group (17.23±6.67%) than those in Control Group, DMSO Group and Resveratrol 50 Group (84.73±8.08%, 71.42±9.43%, and 47.66±8.57%, $P<0.01$, $P<0.05$, $P<0.05$, respectively). When Control Group, DMSO Group and Resveratrol 50 Group were compared, the ratio of P-PLC β3 to T-PLC β3 was significantly lower in Resveratrol 50 Group than in Control Group and DMSO Group ($P<0.05$, respectively). There was no statistical difference between Control Group and DMSO Group in PLC β3 activity of platelets.

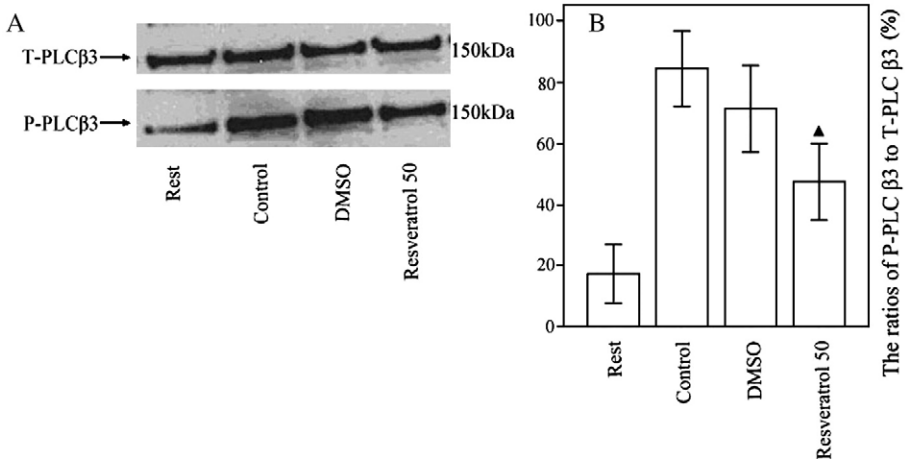

Fig. 3. Effects of resveratrol on activity of phospholipase C β3 (PLC β3) of platelets. Washed platelets were preincubated at 37 °C without stirring for 10 min in the presence of 50 μM of resveratrol (Resveratrol 50) or DMSO (DMSO, resveratrol at 0 μM) prior to the addition of U46619; washed platelets were untreated for 10 min prior to the presence (Control) or absence (Unstimulated) of U46619. Western blotting was used to study the effect of resveratrol on the activation state of phospho-phospholipase C β3 (P-PLC β3) and total-phospholipase C β3 (T-PLC β3) of platelets as described in Materials and methods (A). The ratios of P-PLC β3 to T-PLC β3 were also presented (B). Data were means±S.E.M. of four independent experiments; $^{\blacktriangle}P<0.05$ compared to the DMSO.

4. Discussion

One of the pivotal factors that contribute to cardiovascular diseases is an increased tendency of platelets aggregation. Increased platelet aggregation and atherosclerosis are two principal contributors to the onset and development of cardiovascular diseases, by far the leading cause of death in developed countries (Rahman, 2001). Platelet aggregation is a complex process, and it is generally considered that platelet activation is mainly mediated through adhesiveness of platelets to the site of injury and through the action of endogenous agonists such as adenosine diphosphate, collagen, and thrombin, followed by the release of TXA_2 which acts as an amplifying factor in the platelet aggregation (Jackson et al., 2003; Farndale et al., 2004; Allison et al., 2006). The important role of TXA_2 has been demonstrated by the clinical effectiveness of aspirin in the prevention of cardiovascular diseases such as acute coronary syndromes (Awtry and Loscalzo, 2000; Catella-Lawson et al., 2001; Jneid et al., 2003). In the present study, we showed that resveratrol potently inhibited human platelet aggregation in response to TXA_2 receptor agonist U46619 in a concentration-dependent manner. These results clearly indicated that resveratrol was an effective inhibitor of platelet function. Consequently, resveratrol for therapeutic interventions direct against platelet aggregation may be beneficial in the treatment of arterial thrombosis.

This work has shown that, in addition to preventing platelet activation and aggregation, resveratrol reduced the expression of P-selectin (CD62P) on the platelet surface in response to U46619-induced platelet activation in whole blood. P-selectin is a member of the selectin family and resides in platelets and endothelial cells. It mediates the adhesion of activated platelets to neutrophils and monocytes via a specific interaction with P-selectin glycoprotein-1 (Wagner and Burger, 2003; Palabrica et al., 1992; Furie et al., 2001) and may trigger multiple intracellular events within leukocytes to promote vascular inflammation and facilitate atherosclerosis and thrombosis (Furie et al., 2001; Burger and Wagner, 2003; Furie and Furie, 2004). In resting platelets, P-selectin is localized on the membranes of platelet-granules (Stenberg et al., 1985). After platelet activation, it is redistributed to the platelet surface, where it initiates adhesion to leukocytes (Hamburger and Mcever, 1990). Under conditions of blood flow and shear stress, this glycoprotein promotes platelet cohesion and stabilizes newly formed aggregates (McEver, 2001). Clinically, an increased expression of platelet P-selectin has been shown in patients with atherothrombotic diseases, including those in the acute phase of ischemic stroke (Marquardt et al., 2002) and with acute coronary syndrome (Ault et al., 1999; Yip et al., 2005). In our study, increasing P-selectin expression in activated platelets was confirmed (Fig. 2). This effect was significantly diminished when platelets were exposed to resveratrol at concentration as low as 6.25 μM (Fig. 2A), resveratrol-treated and U46619-activated platelets decreased their P-selectin expression in a concentration-dependent manner. Observed effects on P-selectin implied that resveratrol inhibited the release of a-granule components in activated platelets, which affected many proaggregatory molecules and also provided new insights into the antithrombotic mechanisms of resveratrol.

It is well known that U46619, a TXA_2 mimic, acts directly on the TXA_2 receptor and then induces G protein-coupled PLC β activation, resulting in an increase of Ca^{2+} and protein kinase C activation (Jackson et al., 2003). Activation of PLC β is essential for agonist-induced physiological responses in platelets. Upon activation of PLC β, PIP_2 in the plasma membrane's inner leaflet is hydrolysed to DAG and IP_3. While DAG serves as a stimulatory cofactor for the activation of PKC, IP_3 induces the release of calcium from the platelet dense tubular system, resulting in a rapid rise in intracellular calcium. Furthermore, DAG from the activation of PLC stimulates several metabolic cascades leading to various effects including protein phosphorylation, granule secretion and release in activated platelets (Ha and Exton, 1993; Hug and Sarre, 1993). In the present study, we found that the results of U46619-stimulated generation of total PLC β3 shown in Fig. 3 were quite similar among Unstimulated Group, Control Group, DMSO Group, and Resveratrol 50 Group. We also found that in platelets pretreated with resveratrol at the dose of 50 μM, phosphorylated PLC β3 was inhibited partially upon activation by U46619 when examined by Western immunoblotting. According to our data, it is highly likely that resveratrol inhibits the PLC-mediated signal transduction pathway in platelets since both PLC β3 and PLC β2 play vital roles in platelet cytoskeletal dynamics (Lian et al., 2005).

The amino steroid U-73122 has been widely used as an inhibitor of PLC β and consequently has been shown in platelets to inhibit DAG and IP_3 formation and increase of the intracellular Ca^{2+} in response to agonists known to stimulate PLC β (Bleasdale et al., 1990). Lockhart and McNicol (1999) have confirmed many of these effects of U-73122 on PLC β-mediated signal transduction in platelets. For example, U-73122 inhibited aggregation, [^{32}P]-phosphatidic acid production (an index of PLC activity), and pleckstrin phosphorylation (a consequence of PKC activity) induced by the stable TXA_2 mimetic U46619 which is known to activate PLC β via a heterotrimeric GTP binding protein, G_q. In the present study, we obtained several lines of evidence indicating that U-73122, as same as resveratrol, inhibited platelet aggregation and attenuated P-selectin expression on platelets of healthy volunteers, and that U-73122 and resveratrol showed additive inhibition of U46619-induced platelet aggregation and P-selectin expression. Furthermore, U46619-induced platelet PLC β3 activity was depressed by pretreated platelet with resveratrol.

In conclusion, our work suggested that observed cardioprotective effects of resveratrol, inhibiting U46619-induced platelet aggregation and attenuating P-selectin expression, could be linked to reducing PLC β activity in platelets. Resveratrol might act as an inhibitor on PLC β activity in platelets and serve as a novel antithrombotic agent.

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