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## ANTIOXIDANTS WITH CARCINOSTATIC ACTIVITY (RESVERATROL, VITAMIN E AND SELENIUM) IN MODULATION OF BLOOD PLATELET ADHESION

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Compounds with potential antiplatelet activity can be used in the therapy of cardiovascular disorders. We investigated the effects of three different antioxidants with carcinostatic property: *trans*-resveratrol, Trolox — a water-soluble analog of vitamin E, and inorganic selenocompounds (sodium selenite and selenate) on blood platelet adhesion to fibrinogen (Fg). Adhesion, the initial step of platelet activation, was estimated by the colorimetric method with BCA (bicinchoninic acid) solution in 96-well Fg-coated microtiter dishes. It was shown that resveratrol significantly inhibited adhesion of both thrombin- and ADP-activated platelets to Fg. After incubation of platelets for 30 min. at 37°C with resveratrol at the concentration of 100 µg/ml above 40% inhibition of adhesion was achieved. The inhibition of platelet adhesion of Fg caused by Trolox was lower than by resveratrol and at higher concentration (1 mM) reached maximum 12%. We also demonstrated that neither sodium selenite nor selenate significantly altered platelet adhesion to Fg. We conclude that changed adhesion of blood platelets to Fg in the presence of resveratrol and Trolox, but not selenium may be the result of different antioxidative activities of tested compounds.

**Key words:** *blood platelet adhesion, sodium selenite, sodium selenate, Trolox, resveratrol.*

### INTRODUCTION

In response to the blood vessel wall injury circulating platelets initiate hemostasis at the site of the damage by adhering and spreading on extracellular matrix components i.e. collagen and fibronectin and/or fibrinogen (Fg). Adhesive events itself and action of agonists such as ADP or thrombin induce then platelet aggregation. It has been hypothesized that hyperaggregability of platelets and increased platelet activation is one of the risk factors in pathogenesis of atherosclerosis and thrombosis (1). Blood platelets are also involved in the pathomechanisms of hemostasis in cancer (2). Many antiplatelet

drugs that reduce platelet activation, including the most known aspirin, have been used in the therapy of cardiovascular disorders. Nowadays there is growing interest in new compounds in human diet with antioxidative properties and with potential antiplatelet activity. Among them polyphenols, vitamins and minerals could be considered as a tool in prevention of cardiovascular diseases and cancer. Therefore, the aim of our preliminary study was to assess the action of resveratrol, vitamin E and selenium, antioxidants with carcinostatic activity, on the modulation of pig blood platelet adhesion to homologous fibrinogen. Adhesion of blood platelets is the first crucial step in platelet activation involved in thrombosis and carcinogenesis. Pig platelets are a good model for preliminary investigations due to their morphological and functional similarities to human platelets.

Resveratrol (3, 4',5-trihydroxystilbene) is a phenolic antioxidant found in grapes and other food products. It occurs naturally in a *trans*- or *cis*-isoform. Some evidence suggests that the presence of resveratrol in wines may partly explain the reduced risk of coronary heart disease associated with moderate wine consumption — the event known as a 'French paradox' (3). This effect has been attributed to the inhibition of platelet aggregation, in addition to the anti-inflammatory activity of resveratrol. Moreover, resveratrol has been shown to prevent carcinogenesis in murine models (4). Vitamin E, the most effective chain-breaking lipid-soluble antioxidant in the biological membrane, includes eight naturally occurring compounds in two classes designated as tocopherols with different biological activities (5). A water-soluble analog of  $\alpha$ -tocopherol is Trolox that protects mammalian cells from oxidative damage both *in vitro* and *in vivo* (6). Selenium (Se) as a component of glutathione peroxidases (GPXs) family is a part of the body's antioxidant defence mechanism (7). Evidence has been accumulated that organic and inorganic forms of Se are an important class of chemopreventive compounds (8). Although less toxic synthetic organoselenium compounds may have a great promise in the area of chemoprevention (9) most studies that have examined the role of Se as a chemopreventive agent in laboratory animals have utilized inorganic forms of the element. Some vitamins, e.g. vitamin A and E can modify the chemopreventive effects of selenium (9).

The significance of nutritional antioxidants in platelet function is not clear. Thus, the goal of the presented paper was to investigate the effects of different antioxidants, all of which has been reported as chemopreventive agents, on blood platelet adhesion to fibrinogen. The action of *trans*-resveratrol and Trolox as well as two inorganic Se compounds: sodium selenite and selenate was compared.

## MATERIALS AND METHODS

*Materials*

Resveratrol (*trans*-3,4',5-trihydroxystilbene), Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid), sodium selenite and sodium selenate, bovine serum albumin, thrombin were obtained from Sigma Chemical Co (USA). Stock solution of Trolox (50 mM) was prepared in 1 M sodium bicarbonate. The pH of the stock was adjusted to 7.0 with 1 M HCl. Stock solution of resveratrol was made in 50% dimethylsulfoxide (DMSO) at the concentration of 5 mg/ml and kept frozen. Fibrinogen was isolated from pig plasma by the method of Doolittle (10). Prepared protein was electrophoretically homogenous. All other chemicals were of A.R. grade from commercial suppliers.

*Preparation of blood platelets and incubation with antioxidants*

Pig blood platelets were isolated by differential centrifugation of blood collected into ACD (citric acid/citrate/dextrose) solution 5:1 v/v (11). Platelets were washed and resuspended in modified ( $\text{Ca}^{2+}$  free) Tyrode's buffer (0.15 M NaCl, 0.015 M Tris-HCl, 5 mM glucose at pH 7.4) to the concentration of  $1 \times 10^7$  cells/ml. Platelet suspensions (1 ml) were incubated at 37°C with different concentrations of resveratrol or Trolox for 30 min or with sodium selenite or sodium selenate for 60 min, respectively. The control cells were treated under the same conditions without any antioxidant.

*Adhesion assay of blood platelets*

Adhesion assay was performed according to Tuszyński and Murphy with some modifications (12). Wells of a 96-well microtiter dish (CLINIPLATE EB FB PCS/CRS, Labsystems) were incubated overnight with 100  $\mu\text{l}$  of pig fibrinogen (final concentration of 2 mg/ml) dissolved in Tris-buffered saline, pH 7.5 (TBS). The wells were aspirated, washed three times with TBS and treated with 200  $\mu\text{l}$  of 1% bovine serum albumin (BSA) dissolved in TBS and preincubated at 56°C overnight. After 2 hours BSA was discarded and the wells were washed three times with 250  $\mu\text{l}$  of TBST (TBS containing 0.04% Tween). Immediately after washing, the wells were supplemented with 50  $\mu\text{l}$  of the agonist — thrombin or ADP (at final concentration of 0.2 U/ml or 1  $\mu\text{M}$ , respectively). Agonists were resuspended in TBS supplemented with 1 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgSO}_4$ . Then 100  $\mu\text{l}$  of platelet suspension (pretreated with antioxidant or control) was added to each well and the plate was incubated for 1.5 h at 37°C. Nonadherent cells were removed by aspiration and the wells were washed three times with 250  $\mu\text{l}$  of TBST and two more times with TBS. The total cell-associated protein was determined by dissolving the attached blood platelets directly in the microtiter wells with 200  $\mu\text{l}$  of the Sigma BCA working solution. After 60 min. incubation at 37°C plates were allowed to cool to room temperature and the absorbance at 540 nm of each well was determined in a microtiter plate reader (BioRad, Model 550).

*Statistics*

Each adhesion assay was performed in quadruplicate. The data point in figures are the means of  $n = 5$  separate experiments, showing the standard deviation. Mean values between groups were compared by the Student's paired *t* test. A difference between groups of  $p < 0.05$  was considered statistically significant.

## RESULTS

We have used fibrinogen-coated microtitre wells to test the ability of three different antioxidants to mediate platelet adhesion. Our study showed that these compounds modulated platelet adhesion in different ways. Strong inhibitory effect of resveratrol on thrombin-stimulated platelet adhesion to Fg was observed. This effect was dose-dependent (Fig. 1A). The inhibition of

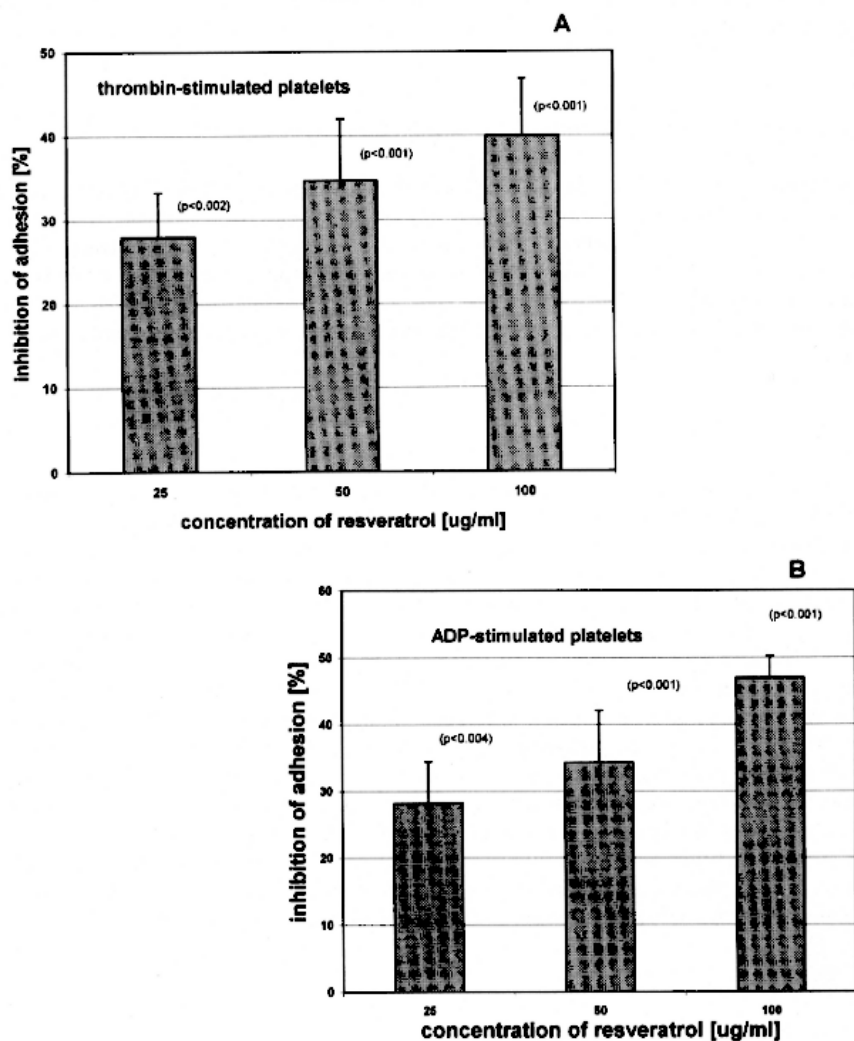


Fig. 1. Inhibition of thrombin- (A) and ADP-stimulated (B) blood platelet adhesion to fibrinogen by *trans*-resveratrol; (n = 5).

platelet adhesion caused by resveratrol was similar and also dose-dependent when ADP was used as an agonist. (Fig. 1 B). The highest concentration of resveratrol used in the experiments (100  $\mu\text{g}/\text{ml}$ ) inhibited adhesion by 47% and 40% in ADP- and thrombin-stimulated platelets, respectively. Trolox, like resveratrol showed inhibitory effect on platelet adhesion to Fg, but its action was much weaker. As was shown in Fig. 2 A and 2 B both in thrombin- and in ADP-stimulated platelets 1 mM Trolox decreased platelet adhesion to Fg by

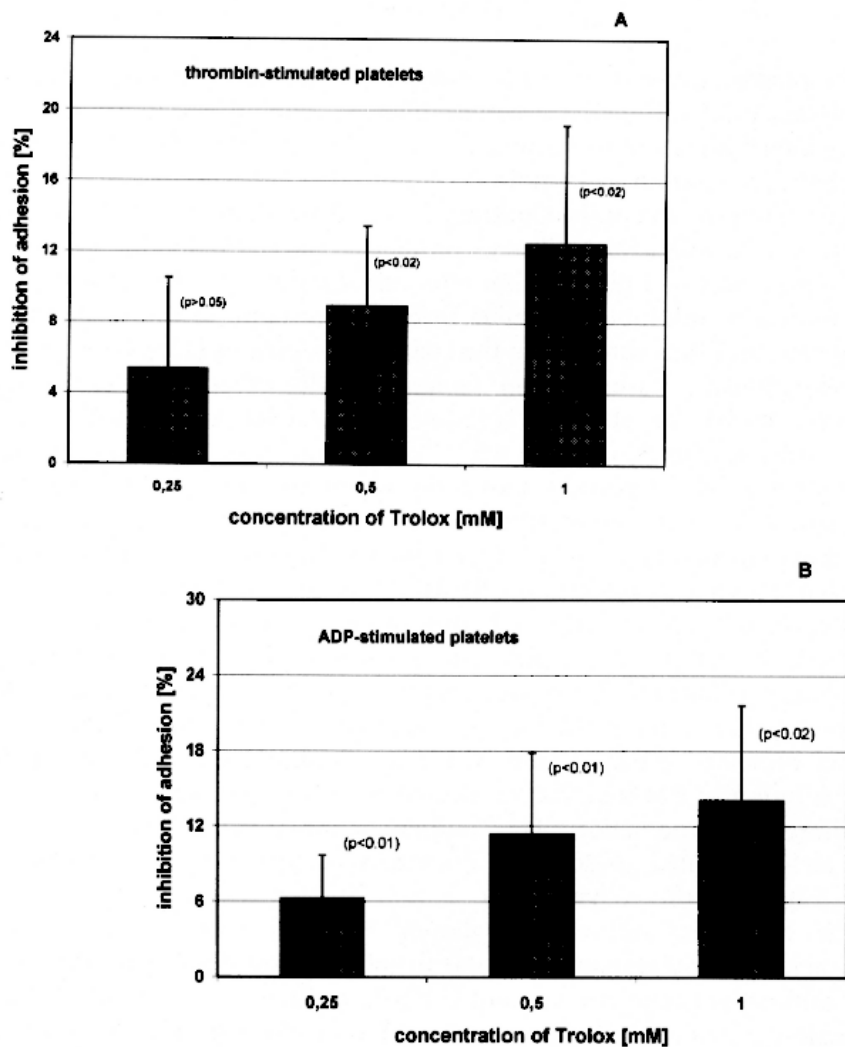


Fig. 2. Inhibition of thrombin- (A) and ADP-stimulated (B) blood platelet adhesion to fibrinogen by Trolox; (n = 5).

approx. 12%. In both cases the inhibition of adhesion caused by Trolox was concentration-dependent (Fig. 2 A and 2 B). Contrary to resveratrol and Trolox action (despite using a wide range of concentrations from 1  $\mu\text{M}$  referred as a physiological dose to 100  $\mu\text{M}$  reported to be toxic) selenium compounds — seldium selenite and selenate did not distinctly affect platelet adhesion to fibrinogen.

## DISCUSSION

The present study showed how treatment with antioxidants that function by different mechanism(s) and within different cellular locations would affect blood platelet adhesion to fibrinogen. Both platelet adhesion and aggregation require the recognition and binding of Fg or other adhesive proteins to platelet specific membrane receptors. Contrary to well defined function of Fg in platelet aggregation its role as an adhesive protein is not clear. Soluble Fg does not bind to non-activated platelets but after adsorption to the site of injury or by deposition as its insoluble derivative, fibrin, it can support irreversible adhesion of platelets, and thus can initiate the coagulation process (13, 14). In presented here experiments resveratrol in comparison to other tested antioxidants appeared to be the strongest inhibitor of platelet adhesion to Fg. The mechanisms responsible for the effects of resveratrol on adhesion, one of the initial steps of blood platelet activation, is not yet known. We have showed elsewhere (15) that resveratrol decreased adhesion of both rested and thrombin-activated platelets to I type collagen. Interaction of platelets with Fg is mediated by  $\alpha_{\text{IIb}}\beta_3$  integrin (GPIIb/IIIa) receptor, and as collagen receptors an integrin  $\alpha_2\beta_1$  (GPIIa-IIa) and glycoproteins GPIV or GPVI have been proposed. In activated platelets an integrin  $\alpha_{\text{IIb}}\beta_3$  serves additionally as a secondary collagen receptor. Our results suggest that resveratrol may interact with both primary and secondary platelet membrane receptors. Rotondo *et al.* (16) has presented similar results with polymorphonuclear leukocytes (PMN). The inhibition of PMN activation caused by resveratrol was not via a specific ligand-receptor interaction. The antiinflammatory properties of resveratrol were also attributed to selective inhibition of cyclooxygenase (COX-1 and COX-2 form of the enzyme) (17).

The lipophilic antioxidant vitamin E is thought to be the major chain-breaking antioxidant in cellular membranes and lipoproteins. In addition to its antioxidant function, vitamin E has also other roles. Vitamin E inhibits the action of vitamin K in promoting blood clotting, and it may reduce thromboxane  $A_2$  synthesis by platelets and promote prostacyclin formation by endothelial cells (18). The data concerning the effects of vitamin E on platelet function are fragmentary and depend on whether they were based on *in vitro* or

*in vitro* studies. Vitamin E has been shown to reduce prostanoid biosynthesis possibly by inhibition of phospholipase A<sub>2</sub> and cyclooxygenase activities (19), it may also modulate platelet adherence and aggregation but results obtained in different laboratories are conflicting (5). One possible explanation of changed platelet aggregation in the presence of vitamin E is that all forms of tocoferol used in experiments were dissolved in alcohol. Ethanol at the concentrations lower than 0.05% inhibits ADP-induced platelet aggregation and MDA/thromboxane A<sub>2</sub> production (data not shown). To avoid these complications we used Trolox — a water-soluble analog of vitamin E. As we showed in this study inhibition of platelet adhesion to Fg caused by Trolox was less than by resveratrol and at higher concentration (1 mM) reached maximum 12%. We also demonstrated that neither sodium selenite nor selenate significantly altered platelet adhesion to Fg. Antioxidative activity of selenium compounds is mediated by Se-dependent enzymes: glutathione peroxidase and 5'-deiodinase. In *in vitro* studies where protein biosynthesis does not occur the incubation of platelets, anucleated cells, with selenocompounds results in the oxidation of glutathione as well as essential — SH groups of proteins. It has been suggested that toxic effects of inorganic and also of some organic forms of Se involve formation of reactive oxygen species since superoxide dismutase and catalase reduced the toxicity of these compounds in different systems (20).

We conclude that changed adhesion of blood platelets to Fg in the presence of resveratrol and Trolox, but not selenium may be the result of different antioxidative activities of tested compounds and free radicals generated in platelets are involved in the modulation of platelet response. Studies on the effects of these antioxidants on the generation of reactive oxygen species in blood platelets are in progress.

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