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Original Contribution

Antioxidants change platelet responses to various stimulating events

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ABSTRACT

The role of platelets in hemostasis may be influenced by alteration of the platelet redox state—the presence of antioxidants and the formation of reactive oxygen and nitrogen species. We investigated the effects of two antioxidants, resveratrol and trolox, on platelet activation. Trolox and resveratrol inhibited aggregation of washed platelets and platelet-rich plasma activated by ADP, collagen, and thrombin receptor-activating peptide. Resveratrol was a more effective agent in reducing platelet static and dynamic adhesion in comparison with trolox. The antioxidant capacity of resveratrol was, however, the same as that of trolox. After incubation of platelets with antioxidants, the resveratrol intraplatelet concentration was about five times lower than the intracellular concentration of trolox. Although both antioxidants comparably lowered hydroxyl radical and malondialdehyde production in platelets stimulated with collagen, TxB_2 levels were decreased by resveratrol much more effectively than by trolox. Cyclooxygenase 1 was inhibited by resveratrol and not by trolox. Our data indicate that antioxidants, apart from nonspecific redox or radical-quenching mechanisms, inhibit platelet activation also by specific interaction with target proteins. The results also show the importance of studying platelet activation under conditions of real blood flow in contact with reactive surfaces, e.g., using dynamic adhesion experiments.

Platelets exert a crucial function in hemostasis, wound repair, and the formation of vascular plugs, underlying thrombotic diseases such as stroke and myocardial infarction. Exposure to the subendothelial matrix induces rapid platelet activation giving rise to formation of a vascular plug and release of stimulatory molecules that initiate the repair process [1,2]. The activation of platelets is regulated and modulated by numerous relatively well-characterized factors, including ADP, serotonin, and thromboxane A_2 (TxA_2)[1], which are released from activated platelets and further potentiate platelet aggregation [3]. Because platelets perform their functions mainly at high shear, the availability of the cone and plate(let) analyzer makes it possible to measure platelet adhesion in whole blood, under physiologic blood flow conditions [4–6].

Intracellular signaling, which is necessary for platelet cytoskeletal reorganization and granule secretion, includes the phosphoinositide hydrolysis pathway [7,8], the eicosanoid synthesis pathway [9], etc. The formation of eicosanoids from arachidonic acid catalyzes cyclooxygenase 1 (COX-1) and COX-2. COX-1 is involved in platelet functions; COX-2 is primarily present at sites of inflammation [9]. Several publications have suggested that reactive oxygen species (ROS) represent a new modulator of platelet activity. It has been shown that either exogenous or platelet-derived ROS have influences on platelet function [10]. The release of several ROS, including $O_2^{\bullet -}$, $HO^{\bullet}$, and H_2O_2, from platelets was reported [11], both from resting platelets and after platelet stimulation with agonists such as collagen [12,13] or thrombin [14].

There is mounting evidence that antioxidants modulate platelet activation [15]. The effects of antioxidant supplementation on platelet function in vivo are still controversial. Nevertheless in vitro-added antioxidants, such as vitamin C, vitamin E, resveratrol, flavonoids, and others, generally attenuate platelet activation either by ROS quenching or by substance-specific mechanisms [16–18].

In this study, we concentrated our experimental effort on two antioxidants: trolox, a vitamin E analogue, and resveratrol. Resveratrol (*trans*-3,4′,5-trihydroxystilbene), a naturally occurring hydroxystilbene, is contained in red wine and possesses chemopreventive properties and also cytostatic activities. It has been published that resveratrol inhibits platelet activation [19–21]. The inhibitory effects of resveratrol possibly involve inhibition of the p38 MAP kinase,

Abbreviations: ABTS, 2,2′-azinobis(3-ethylbezothiazoline-6-sulfonic acid); COX, cyclooxygenase; DHB, dihydroxybenzoic acid; ELISA, enzyme-linked immunosorbent assay; $HO^{\bullet}$, hydroxyl radical; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; $O_2^{\bullet -}$, superoxide; PBS, phosphate-buffered saline; PRP, platelet-rich plasma; ROS, reactive oxygen species; TEAC, trolox equivalent antioxidant capacity; TRAP, thrombin receptor activating peptide; TxA_2, thromboxane A_2; TxB_2, thromboxane B_2; WP, washed platelets.

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cytosolic phospholipase A_2, arachidonic acid, Ca^{2+} cascade and activation of NO/cyclic GMP, inhibition of phospholipase C, and protein kinase C activation [22].

There are a lot of papers describing the influence of vitamin E on blood platelets [23–25] but information that trolox (stable analogue of vitamin E) is able to inhibit platelet aggregation is meager [26,27].

The aim of this study was to determine the effects of either resveratrol or trolox on normal washed platelet functions assessed by platelet aggregation induced by collagen and platelet adhesion. In particular, the relationship between the antioxidant capacity of the antioxidants, their bioavailability in platelets, the concentration of hydroxyl radicals produced during platelet activation, malondialdehyde (MDA) and thromboxane B2 (TxB_2) formation, and the tendency for platelets to aggregate and to adhere under static or dynamic conditions were investigated.

Methods

Materials

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 3,4′,5-trihydroxystilbene (resveratrol), adenosine 5′-diphosphate (ADP), thrombin receptor-activating peptide SFLLRNP (TRAP), prostaglandin E1, Triton X-100, *p*-nitrophenyl phosphate, human fibrinogen, acetonitrile, ethanol and methanol (HPLC grade), myoglobin, 2,2′-azinobis(3-ethylbezothiazoline-6-sulfonic acid) (ABTS), sodium salicylate, 2,3-dihydroxybenzoic acid (2,3-DHB), and 2,5-dihydroxybenzoic acid (2,5-DHB) were from Sigma–Aldrich Prague (Czech Republic); collagen was from Bio/Data (USA); Sephadex G-10 was from Pharmacia (Switzerland); and bovine serum albumin (BSA) was from Imuna (Slovakia). Other chemicals were purchased from Lachema (Czech Republic) and were of analytical grade.

Preparation of blood platelets and their incubation with antioxidants

Blood was drawn from healthy volunteers who had not ingested an acetylsalicylic acid-containing drug for at least 2 weeks. All individuals tested agreed to this study at the time of blood collection. The study was approved by the Institute of Hematology and Blood Transfusion Ethics Committee and all samples were obtained in accordance with the regulations of the ethical commission of the institute.

Platelet-rich plasma (PRP) was prepared from blood (9 ml) that was drawn by venipuncture into 1 ml of 3.8% trisodium citrate (w/v). PRP was obtained by centrifugation of blood at 250 *g* at 37°C for 15 min and the platelet count in the PRP was estimated by a blood counter (Coulter Counter Onyx, Coultronix, France). Platelet-poor plasma was obtained by centrifugation of blood or PRP at 1400 *g* at 25°C for 10 min. The platelet count of the PRP was adjusted with Platelet poor plasma (PPP) to 200,000 platelets/μl.

Washed blood platelets were isolated by differential centrifugation of blood collected into citric acid/citrate/dextrose solution at 8.1:1.9 (v/v). Briefly, PRP was obtained by centrifugation of blood at 250 *g* at 37°C for 15 min. The PRP, after the addition of prostaglandin E1 (1 μM, final concentration), was incubated in a water bath at 37°C for 10 min and centrifuged at 1000 *g* at 37°C for 10 min. The platelet pellet was resuspended in modified (Ca^{2+} free) Tyrode's buffer, pH 6.2, in the presence of 1 μM prostaglandin E1 and centrifuged at 600 *g* at 37°C for 10 min. The platelets were finally resuspended in Tyrode's buffer, pH 7.4, with fibrinogen (final concentration 0.1% (w/v)) to a concentration of 200,000 platelets/μl, equilibrated for 30 min at 37°C, and used for experiments within 1 h.

Platelet suspensions were incubated at 37°C with various final concentrations of either resveratrol (0.300, 0.150, 0.075, 0.038, 0.019, 0.009, 0.005, and 0.002 mM) or trolox (4.20, 3.15, 2.10, 1.05, 0.53, 0.26, and 0.13 mM) for 30 min. Antioxidants were diluted in ethanol. The control samples were treated under the same conditions with a final concentration of 0.6% ethanol (v/v) without any antioxidant [23].

Measurement of platelet aggregation

Platelet aggregation was studied in platelet suspensions turbidimetrically [28] using a Chrono-Log aggregometer (Chrono-Log, Havertown, PA, USA). Tests were performed at 37°C in cuvettes stirred at 1000 rpm. Two hundred fifty microliters of sample was stimulated with ADP (0.01 mM final concentration), TRAP (0.01 mM final concentration), or collagen (18.8 μg/ml final concentration). The concentrations of agonists were the lowest ones sufficient to induce a full platelet aggregation response (from five independent experiments). The final percentage of aggregation was recorded at 6 min. Percentage inhibition was calculated using the aggregation response of a sample containing 0.6% ethanol (v/v, final concentration) as a control.

Trolox equivalent antioxidant capacity assay—measurement of resveratrol and trolox antioxidant capacity

The antioxidant activity of vitamins was measured by the trolox equivalent antioxidant capacity assay and the results were expressed in trolox equivalents (TEAC; the millimolar concentration of a trolox solution having the antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation) [29,30]. The TEAC value is based on the ability of the antioxidant to scavenge the blue-green $ABTS^{\bullet+}$ radical cation relative to the $ABTS^{\bullet+}$ radical cation scavenging ability of the water-soluble vitamin E analogue, trolox. The assay was slightly modified and performed in microplates. Briefly, in a separate Eppendorf tube, 8.4 μl of either tested antioxidant (1 mM antioxidant solution in PBS buffer) or trolox solution was thoroughly mixed with 36 μl of 70 μM metmyoglobin and 300 μl of 500 μM ABTS. The solution was transferred in three 70-μl aliquots to microplate wells and brought to 35°C in an ELISA reader for 5 min. The production of $ABTS^{\bullet+}$ was started by the addition of 130 μl of freshly prepared and prewarmed (5 min, 35°C) solution of 100 μM hydrogen peroxide. All substances were dissolved in PBS buffer (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4 \cdot 12\ H_2O$, 1.5 mM KH_2PO_4, pH 7.4). The microplate was read at 15-s intervals with a microplate reader at 750 nm for 8 min. A calibration curve based either on absorbance values or on lag phase was constructed.

Measurement of resveratrol concentration in platelets

The platelet suspension incubated with resveratrol (0.3 mM, final concentration) was briefly washed several times in Tyrode's buffer. Fifty microliters of washed platelet suspension was vortexed with 10 μl of methanol, 25 μl of Na_2HPO_4 (0.25 mM), and 600 μl of ethyl acetate. The organic phase was separated using a centrifuge, dried out, and dissolved in 200 μl of mobile phase (acetonitrile, 25 mM Na_2HPO_4, pH 4.2, 30:70, v/v). Forty microliters was injected onto an SGX C18 column, 150×3 mm, 5 μm (Tessek, Prague, Czech Republic). The chromatography was performed isocratically with a flow rate of 0.5 ml/min at room temperature using UV detection at 310 nm.

Measurement of trolox concentration in platelets

The platelet suspension incubated with trolox (4.2 mM, final concentration) was briefly washed several times in Tyrode's buffer. Two hundred microliters of washed platelet suspension was vortexed with 200 μl of acetonitrile and centrifuged. Five microliters of supernatant was injected onto an SGX C18 column, 150×3 mm, 5 μm (Tessek). The mobile phase consisted of methanol and 30 mM H_3PO_4 adjusted to pH 3 with NaOH (58:42, v/v). The chromatography was performed isocratically with a flow rate of 0.5 ml/min at room temperature using UV detection at 290 nm.