Resveratrol Reduces Oxidative Stress Induced by Platinum Compounds in Blood Platelets

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Abstract. The effects of resveratrol (*trans*-3,4′,5-trihydroxystilbene) on the oxidative stress in blood platelets induced by platinum compounds [cisplatin and selenium-cisplatin conjugate] were studied *in vitro*. The production of thiobarbituric acid reactive substances (TBARS), the level of conjugate diene, the generation of superoxide anion radicals ($O_2^{-\bullet}$) and other reactive oxygen species ($O_2^{-\bullet}$, H_2O_2, singlet oxygen and organic radicals) were measured by chemiluminescence in blood platelets treated with platinum compounds. Cisplatin at the concentration of 10 μg/ml, as well as selenium-cisplatin conjugate (10 μg/ml) induced oxidative stress in blood platelets: an increase in TBARS, conjugate diene, chemiluminescence and generation of $O_2^{-\bullet}$. In the presence of resveratrol (a natural compound with antioxidant activity) at the concentrations of 1–25 μg/ml, the chemiluminescence, the levels of $O_2^{-\bullet}$, conjugate diene and TBARS were reduced ($p < 0.05$). We showed that resveratrol at different concentrations (1–25 μg/ml) had a protective effect against oxidative stress in platelets caused by platinum compounds (10 μg/ml) and it diminished platelet lipid peroxidation and reactive oxygen species generation induced by platinum compounds.

Key words: Resveratrol — Platinum compounds — Reactive oxygen species — Lipid peroxidation — Blood platelets

Abbreviations: ACD, acid citrate-dextrose; cis-Pt, cisplatin; ROS, reactive oxygen species; Se-Pt, selenium-cisplatin conjugate; TBARS, thiobarbituric acid-reactive substances.

Introduction

Cisplatin (*cis*-diamminedichloroplatinum II; cis-Pt) belongs to the most effective anticancer compounds among all platinum-based drugs. The antitumor activity of cis-Pt has mainly been attributed to its ability to form adducts with DNA (Keppler 1993; Lindauer and Holler 1996). On the other hand, a variety of adverse effects

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may accompany the use of this drug. cis-Pt causes haematological toxicity inducing oxidative stress and the changes of biological function of blood cells. It has an inhibitory effect on blood platelet activation (Olas and Wachowicz 1996; Wachowicz and Olas 1995, 1997), induces platelet lipid peroxidation and causes the generation of reactive oxygen species (ROS) in these cells (Olas et al. 1999a, 2000b). Our earlier results showed that selenium-cisplatin conjugate ($[(NH_3)_2Pt(SeO_3)]$; Se-Pt) had less toxic effect on blood platelet functions (Wachowicz and Olas 1997). There is a growing interest in compounds present in human diet with antioxidative properties and with different biological activity, including antiplatelet and anticancer action. Among them, polyphenols and vitamins could be considered as a tool in prevention of cancer. Therefore, the aim of our study was to assess the action of resveratrol – antioxidant with carcinostatic and antiplatelet activity on the oxidative stress in pig blood platelets induced by platinum compounds (cis-Pt and Se-Pt) at the therapeutic dose (10 μg/ml) *in vitro*. Pig blood platelet is a convenient and very good model due to its morphological and functional similarities to human platelet.

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a natural molecule with antioxidant, antifungal, antiinflammatory, antiplatelet and anticancer action (Daniel et al. 1999; Fremont et al. 1999). Our previous results showed that resveratrol exerted a powerful antioxidant effect on generation of reactive oxygen species and lipid peroxidation in resting and stimulated blood platelets (Olas et al. 1999b; Żbikowska et al. 1999). The purpose of this study was to assess the effect of resveratrol on oxidative stress in blood platelets induced by platinum compounds. We studied also the effects of resveratrol with platinum compounds on platelets activated by thrombin, that is a strong platelet activator. The procoagulant activity of different tumors depends on thrombin generation and thrombin may induce the expression of platelet binding sites on tumor cells (Goad and Gralnick 1996).

Materials and Methods

Chemicals

Thrombin, luminol, cisplatin, thiobarbituric acid, resveratrol and cytochrome c were purchased from Sigma (St. Louis, MO, USA). Conjugate of selenium with cisplatin $[(NH_3)_2Pt(SeO_3)]$, synthesised in the Institute of Pur Chemicals, Lachema Brno (Czech Republic) (Batch No. 290592) was a gift obtained from Prof. V. Kleinwachter (Institute of Biophysics, Czech Academy of Sciences, Brno). Stock solution of resveratrol was made in 50 % dimethylsulfoxide (DMSO) at the concentration of 25 mg/ml and kept frozen (the final concentration of DMSO in platelet suspension was 0.05 %). All other reagents were of analytical grade and were provided by commercial suppliers.

Isolation of blood platelets

Pig blood obtained from slaughter house was collected into ACD solution (citric acid/citrate/dextrose, 5 : 1, v/v, blood/ACD) and platelets were isolated by

differential centrifugation of blood (20 min at $200 \times g$). Platelet-rich plasma was separated and centrifuged for 20 min at $1000 \times g$ to sediment platelets. The resulting pellet was gently resuspended in Ca^{2+}/Mg^{2+} free modified Tyrode's buffer (140 mmol/l NaCl, 10 mmol/l glucose and 15 mmol/l Tris/HCl, pH 7.4), and the platelets were subsequently washed three times with the same buffer. The entire washing procedure was performed in plastic tubes and carried out at room temperature. Blood platelets were suspended in Ca^{2+}/Mg^{2+} free Tyrode's buffer at the final concentration of 5×10^8 platelets/ml. Platelets suspensions were incubated with resveratrol at the final concentrations of 1, 2.5, 5, 10 and 25 μg/ml (2 or 30 min, 37℃) and with platinum compounds (10 μg/ml, 2 or 30 min, 37℃). In some experiments platelets after preincubation with resveratrol and platinum compounds were stimulated with thrombin.

The platelets were counted by the photometric method as described by Walkowiak et al. (1989). Total platelet protein was determined by modified Lowry method (Vatassary and Smitch 1987).

Chemiluminescence measurements

The level of reactive oxygen species ($O_2^{-\bullet}$, H_2O_2, singlet oxygen and organic radicals) in control blood platelets and platelets incubated with different compounds (resveratrol and platinum compounds) was recorded using the chemiluminescence method as described by Król et al. (1990). The chemiluminescence signals were evaluated by means of a Berthold LB950 automatic luminescence analyser after the addition of 20 μl of 2 mmol/l luminol solution in buffered saline. The final concentration of luminol was 40 μmol/l in a sample. Results were presented as % of control values obtained for control platelets.

$O_2^{-\bullet}$ generation in blood platelets

Generation of superoxide anion radicals ($O_2^{-\bullet}$) in control platelets and in platelets incubated with resveratrol and platinum compounds was measured by cytochrome c reduction, as described earlier (Olas et al. 2000c). For that an equal volume of Ca^{2+}/Mg^{2+} free Tyrode's buffer, containing cytochrome c (160 μmol/l), was added to a 1 ml suspension of platelets. After incubation, the platelets were sedimented by centrifugation at $2000 \times g$ for 5 min and the supernatants were transferred to cuvettes. Reduction of cytochrome c was measured spectrophotometrically at 550 nm and results were expressed as nmoles of $O_2^{-\bullet}$ (Jahn and Hansch 1990).

TBARS production in resting blood platelets

Incubation of blood platelets suspensions (control and incubated with resveratrol and platinum compounds) was stopped by cooling the samples in an ice-bath. Samples of platelets were transferred to an equal volume of 20% (v/v) cold trichloroacetic acid in 0.6 mol/l HCl and centrifuged at $1200 \times g$ for 15 min. One volume of clear supernatant was mixed with 0.2 volume of 0.12 mol/l thiobarbituric acid in 0.26 mol/l Tris at pH 7.0 and immersed in a boiling water bath for 15 min. Absorbance at 532 nm was measured and results were expressed as nmoles of TBARS (Wachowicz 1984).

Conjugate diene production in blood platelets

Generation of conjugate dienes in control platelets and in platelets after incubation with resveratrol and platinum compounds was measured by method described by Noguchi et al. (1992). Briefly, samples of platelets (0.5 ml) were transferred to 2.5 ml of solution (ether/ ethanol, 1 : 3, v/v) and vortex (1 min). Then, the samples were centrifuged at $6000 \times g$ for 5 min and the supernatants were transferred to cuvettes. Level of conjugate dienes was measured spectrophotometrically at 234 nm.

Statistical analysis

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

Results

The obtained results indicate that resveratrol (1–25 μg/ml) inhibits the oxidative stress in blood platelets induced by platinum compounds (cis-Pt and Se-Pt) at a concentration of 10 μg/ml (Figs. 1–4). Incubation of the platelets with resveratrol at the highest concentration (25 μg/ml) causes the strongest inhibition of $O_2^{-\bullet}$ production measured by the superoxide dismutase-inhibitable reduction of cytochrome c in blood platelets treated with platinum compounds. However, we did not observe a dose-dependent action of resveratrol on $O_2^{-\bullet}$ generation in platelets ($p > 0.05$) (Fig. 1). Resveratrol also reduces the luminol-dependent chemiluminescence – an indicator of reactive oxygen species generation ($O_2^{-\bullet}$, H_2O_2, singlet oxygen and organic radicals) in platinum compounds-stimulated platelets. In the presence of resveratrol (25 μg/ml), chemiluminescence induced by cis-Pt (10 μg/ml) was reduced by 92.5% ($p < 0.05$) (Fig. 2).

Our results show that resveratrol has an inhibitory action on platelet lipid peroxidation stimulated by platinum compounds, measured by the thiobarbituric acid technique (expressed as TBARS). The inhibitory effect on TBARS production was observed after 30 min action of resveratrol (Fig. 3). Resveratrol slightly reduces platelet lipid peroxidation (estimated as the level of conjugate dienes) induced by platinum compounds ($p < 0.05$) (Fig. 4).

Thrombin (at a dose of 2.5 U/ml) used as an effective inducer of platelet activation caused an increase of chemiluminescence and production of $O_2^{-\bullet}$ in platelets ($p < 0.05$). After 2 min pre-incubation of platelets with platinum compounds (10 μg/ml), the amount of different reactive oxygen species ($p < 0.05$) in thrombin stimulated platelets increasing; however, in the presence of resveratrol this effect was diminished ($p < 0.05$). In the presence of resveratrol (1–25 μg/ml), the amount of $O_2^{-\bullet}$ and generation of reactive oxygen species in platelets treated with platinum compounds and then activated by thrombin were decreased ($p < 0.05$) (Tab. 1).

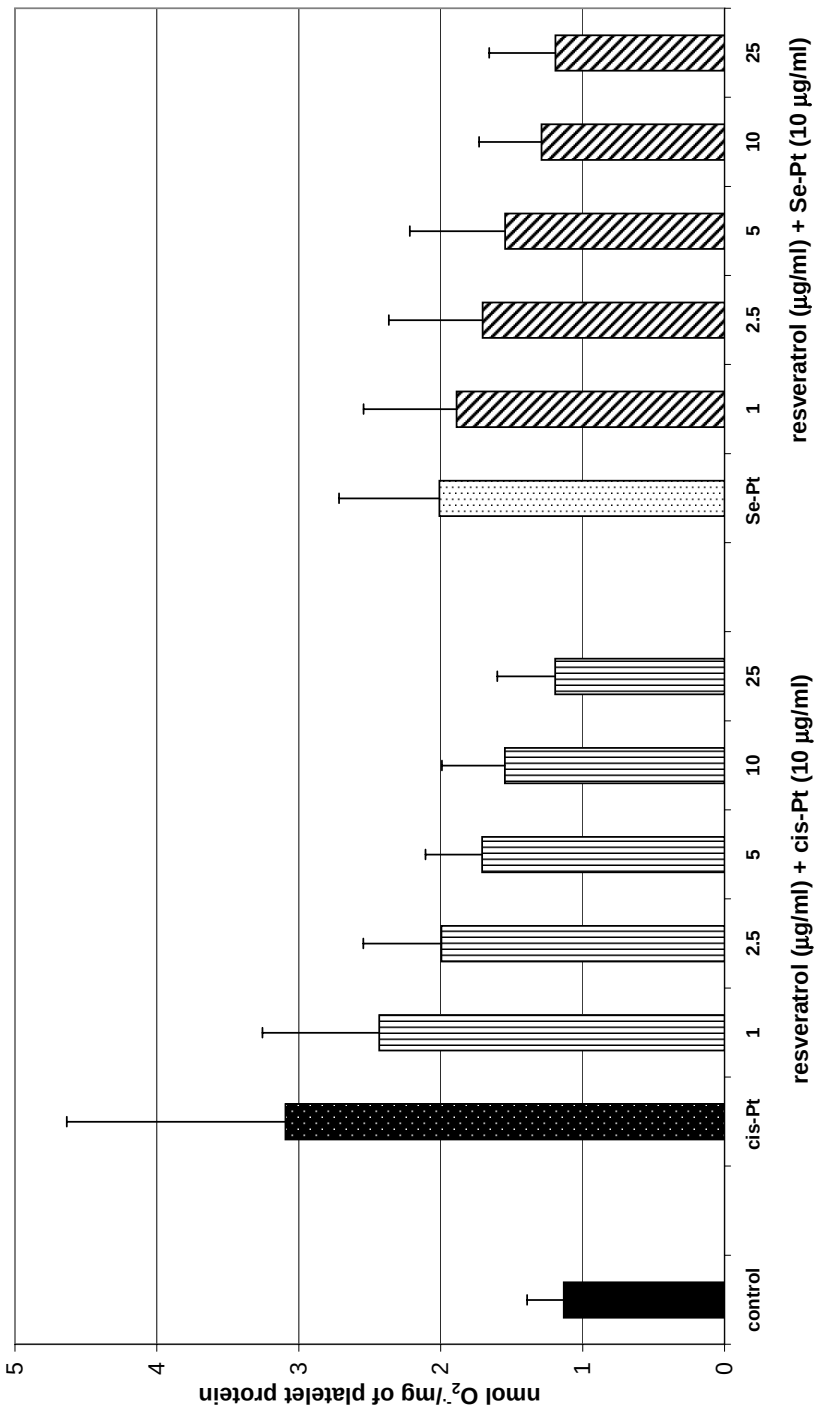

Figure 1. The effects of resveratrol (30 min, 37 °C) and platinum compounds (2 min, 37 °C) on the level of $O_2^{-\bullet}$ in resting blood platelets. Each experiment was carried out in five independent measurements. cis-Pt or Se-Pt treated platelets *versus* control platelets, ($p < 0.05$); resveratrol and platinum compounds (cis-Pt or Se-Pt) treated platelets *versus* platinum compounds treated platelets, ($p < 0.05$).

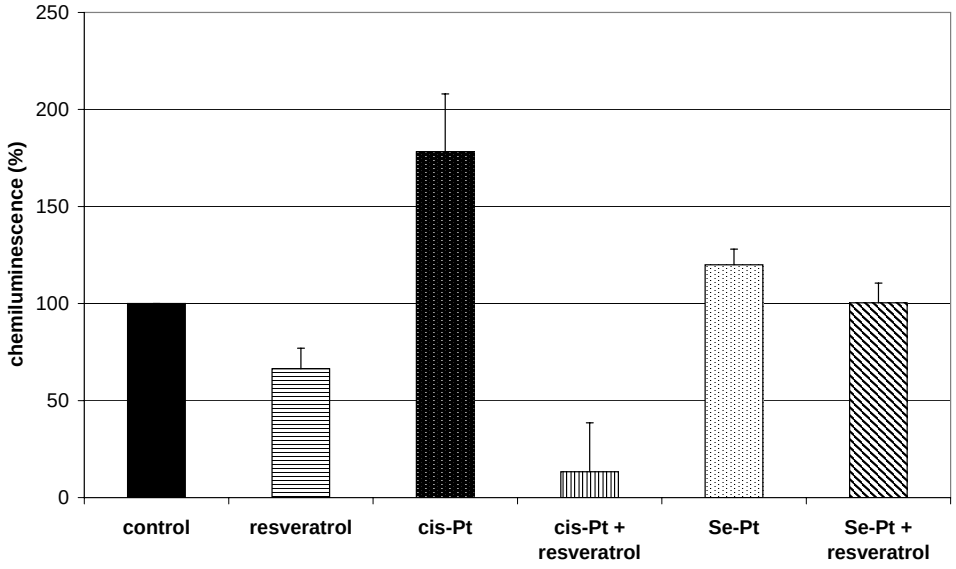

Figure 2. The effects of resveratrol (2 min) and platinum compounds (2 min) on the chemiluminescence in resting blood platelets. The experimental conditions as in Fig. 1.

Table 1. The effects of resveratrol (2 min, 37°C) and platinum compounds (10 μg/ml, 2 min, 37°C) on the level of $O_2^{-\bullet}$ and the chemiluminescence in blood platelets activated by thrombin (2.5 U/ml). Chemiluminescence was measured at the highest concentration of resveratrol (25 μg/ml). Each experiment was carried out in five independent measurements ($n = 5$, $p < 0.05$).

Thrombin-activated blood platelets	nmol $O_2^{-\bullet}$/mg of platelet protein	Chemiluminescence (%)
(–) cis-Pt (–) resveratrol [control]	1.987 ± 0.549	100
(+) cis-Pt	2.506 ± 0.594	155.4 ± 23.7
(+) resveratrol (1 μg/ml) (+) cis-Pt	2.458 ± 0.824	—
(+) resveratrol (2.5 μg/ml) (+) cis-Pt	2.182 ± 0.868	—
(+) resveratrol (5 μg/ml) (+) cis-Pt	1.708 ± 0.810	—
(+) resveratrol (10 μg/ml) (+) cis-Pt	1.707 ± 0.809	—
(+) resveratrol (25 μg/ml) (+) cis-Pt	1.140 ± 0.604	104.4 ± 8.3
(+) Se-Pt	2.504 ± 0.611	115.9 ± 15.1
(+) resveratrol (1 μg/ml) (+) Se-Pt	2.206 ± 0.678	—
(+) resveratrol (2.5 μg/ml) (+) Se-Pt	2.043 ± 0.721	—
(+) resveratrol (5 μg/ml) (+) Se-Pt	2.070 ± 0.871	—
(+) resveratrol (10 μg/ml) (+) Se-Pt	1.933 ± 0.850	—
(+) resveratrol (25 μg/ml) (+) Se-Pt	1.706 ± 0.797	95.6 ± 10.0

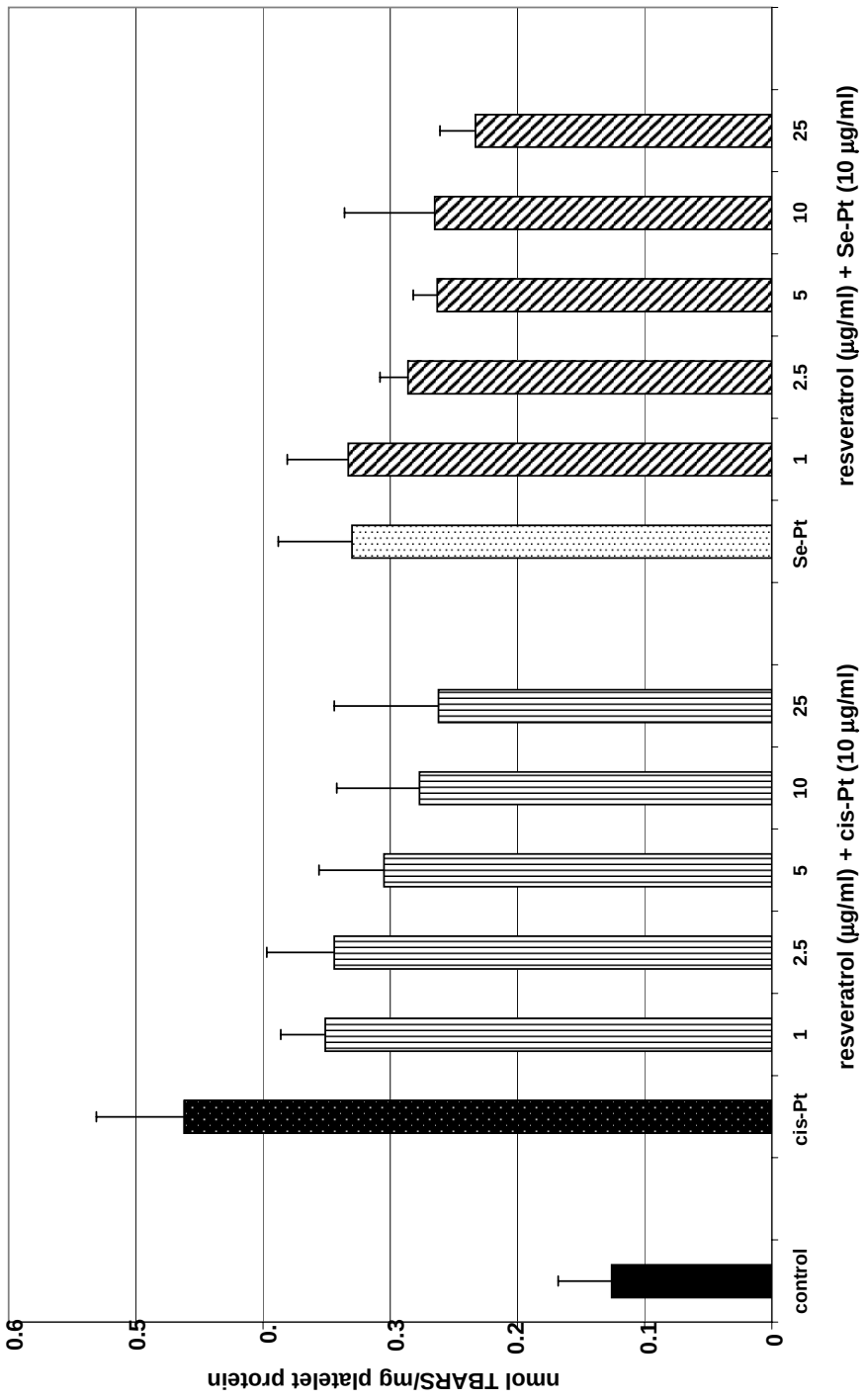

Figure 3. The effects of resveratrol (30 min, 37 °C) and platinum compounds (30 min, 37°C) on the level of TBARS in resting blood platelets. The experimental conditions as in Fig. 1.

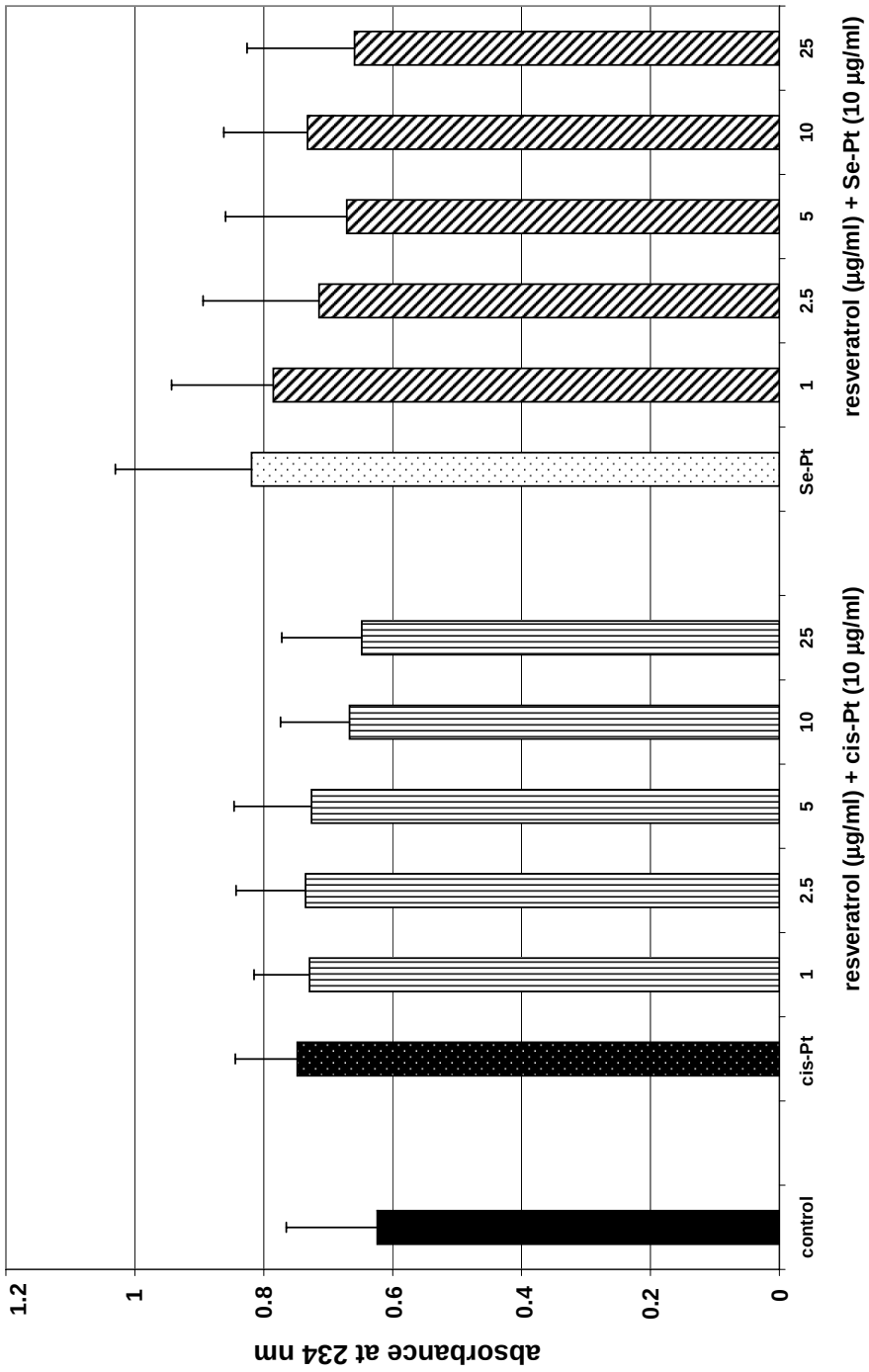

Figure 4. The effects of resveratrol (30 min, 37°C) and platinum compounds (30 min, 37°C) on the level of conjugate diene in resting blood platelets. The experimental conditions as in Fig. 1.

Discussion

Physiological level of resveratrol in plasma is very low and depends mainly on grapes and wine consumption. Soleas et al. (2001) have shown that plasma level of resveratrol is about 5 μg/ml after 30-min drank of 100 ml wine containing 25 mg of resveratrol. The presented results indicate that resveratrol, a natural polyphenolic compound at doses 1, 2.5 and 5 μg/ml (corresponding to physiological level in plasma) is capable to lower the toxicity of both, Se-Pt (Figs. 1–4, Tab. 1). The platinum compounds have anticancer activity; however, their toxicity may become an important dose-limiting factor. Blood platelets are one of the key element of human blood. Platelets play not only a central role in the process of thrombogenesis, but they play also an important function in the pathology of haemostasis in cancers (Goad and Gralnick 1996; Olas and Wachowicz 1998a,b). Clinical observations suggest that anticancer drugs could contribute to the thrombotic complications of malignancy in treated patients. During chemotherapy, these drugs, including cis-Pt may change the biological functions of blood platelets (Wachowicz and Olas 1995; Olas and Wachowicz 1996, 1998a,b; Olas et al. 2000a). Although details of biochemical mechanisms responsible for the haematological toxicity of platinum compounds have not yet been known, the involvement of oxidative stress has been indicated by several lines of evidence (Keppler 1993; Olas et al. 1999a). After incubation of platelets with cis-Pt, platelet lipid peroxidation and production of ROS in these cells were demonstrated (Olas et al. 1999a). cis-Pt reduces also the activities of antioxidative enzymes in blood platelets such as superoxide dismutase, glutathione peroxidase and catalase (Olas et al. 2000b).

Different antioxidants present in human diet may be used to protect against cis-Pt-induced cytotoxicity. Fruits, vegetables, teas and red wines are rich in antioxidants such selenium compounds, ascorbic acid, tocopherols, flavonoids and other phenols. We observed that various components of plants – vitamin C (Olas et al. 2000b), vitamin E (Żbikowska and Olas 2000), inorganic forms of selenium (Olas et al. 1999a) and resveratrol – a phenolic compound (Olas et al. 1999b; Żbikowska et al. 1999) modulated the biological activity of blood platelets *in vitro*. Our earlier results indicate that a Se-Pt may minimise the toxicity and side effects of the chemotherapeutic agent without affecting antitumor activity (Olas et al. 1999a). Here, we showed that resveratrol suppresses also the platinum compounds toxicity expressed as oxidative stress in blood platelets. Resveratrol is present in about 70 plant species especially in grapes and wine. It has been the focus of a number of studies investigating its biological attributes which include antioxidant activity (Olas et al. 1999b; Martinez and Moreno 2000), antiplatelet effect (Bertelli et al. 1995, 1996; Rotondo et al. 1996; Żbikowska et al. 1999; Żbikowska and Olas 2000) and chemoprevention (Jang et al. 1997; Daniel et al. 1999). Resveratrol prevents tumorigenesis through an inhibitory effect on tumor initiation, promotion, and progression. We noticed that tested doses of resveratrol decrease the level of different oxidative stress markers in unstimulated blood platelets; DMSO at the highest concentration (0.05 %) did not change chemiluminescence (data are not presented). In

this report, our results suggest that resveratrol may play an important role in the protecting of blood platelets against platinum compounds-induced oxidative stress. In platelets treated with resveratrol (1–25 μg/ml), the oxidative stress induced by cis-Pt or a Se-Pt was distinctly diminished.

Platelet activation induced by some activators, including thrombin, collagen or ADP, is associated with an increase in ROS, which may be produced by different ways (arachidonic acid pathway, glutathione cycle, membrane oxidases and phosphoinositides metabolism) (Blockmans et al. 1995; Pignatelli et al. 1998; Wachowicz et al. 2002). Togna et al. (2000) have reported that cis-Pt increases platelet reactivity in the presence of agonists (collagen and sodium arachidonate) transducing a signal mediated by activation of arachidonic acid metabolism, and direct or indirect activation of platelet phospholipase A_2 appears to be implicated in this process. Our previous studies showed that cis-Pt and Se-Pt stimulates and later inhibits production of arachidonate metabolites and ROS in thrombin activated platelets. Resveratrol may modulate arachidonic acid cascade in various cells (cancer cells, blood platelets, macrophages). Resveratrol not only reduces cyclooxygenase activity, but inhibits also other enzymes (lipoxygenases). The present study shows that in the presence of platinum compounds, resveratrol reduces the production of ROS in blood platelets activated by thrombin (Tab. 1). Concentrations of platinum compounds used in our experiments are consistent with those recorded in plasma of patients. It seems that resveratrol as an antioxidant present in human diet may decrease or eliminate negative consequences of toxic side effects of platinum compounds on blood platelets and their haemostatic function.

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