

Inhibitory effect of resveratrol on free radical generation in blood platelets[⊙]

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Resveratrol (3,4',5-trihydroxystilbene), a compound found in many plants, has been shown to prevent coronary heart diseases and to exert a variety of antiinflammatory and anticancerogenic effects. It is effective in lowering the level of serum lipids and in inhibiting platelet aggregation. We evaluated the effect of *trans*-resveratrol on the production of free radicals in pig blood platelets and showed that resveratrol inhibited the production of different reactive oxygen species ($O_2^{\cdot-}$, H_2O_2 , singlet oxygen and organic radicals) measured by the luminol-dependent chemiluminescence in resting platelets ($P < 0.05$). Resveratrol inhibited also the generation of radicals in platelets activated by thrombin ($P < 0.05$). Treatment of platelets with resveratrol at concentrations of 6.25 and 12.5 $\mu\text{g/ml}$ caused a statistically insignificant increase in the production of $O_2^{\cdot-}$ in these cells, as measured by reduction of cytochrome *c*; however, at higher doses (25, 50 and 100 $\mu\text{g/ml}$) resveratrol distinctly reduced the generation of $O_2^{\cdot-}$ in platelets ($P < 0.05$). We suggest that free radicals play an important role in the reduced reactivity of blood platelets induced by resveratrol.

Resveratrol (3,4',5-trihydroxystilbene) is a phytoalexin found in many plants, mainly in grapes. Significant amounts of resveratrol are present in wine in a *trans*- or *cis*-isoform. Resveratrol, a natural product consumed with red wine, has been suggested to play a role in the prevention or even reduction of incidence of coronary heart diseases because it could in-

hibit blood platelet functions, and modulate lipid or lipoprotein metabolism [1-6]. Coronary heart diseases in France are less frequent than in other countries with similar risk factors (smoking, fat content in the diet, lack of exercise) but high consumption of wine. This phenomenon of the low incidence of coronary artery disease in French people has been

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called the "French paradox". It has been hypothesised that this reduction of the incidence of coronary heart diseases was related partly to the pharmacological properties of resveratrol [6]. Resveratrol exerts also anti-cancer and anti-inflammatory effects [7, 8].

Our preliminary results showed that *trans*-resveratrol *in vitro* inhibited activation of blood platelets. It reduced the first step of blood platelet activation, platelet adhesion to type I collagen. Resveratrol also inhibited thrombin-induced platelet aggregation and synthesis of eicosanoids in activated blood platelets [9]. The molecular mechanism involved in the inhibition of platelet functions by resveratrol is still unclear. We suggest that free radicals generated in activated blood platelets may be responsible for the changes of platelet reactivity induced by resveratrol.

Blood platelets respond to several agonists (thrombin, ADP or collagen) which stimulate platelets by binding to their surface receptors and eliciting events of intracellular second messengers. Multiple intracellular signal transduction pathways are responsible for the physiological responses of blood platelets: shape change, aggregation and secretion of the granule contents [10, 11]. During activation of blood platelets stimulated by different agonists (thrombin or collagen) free radicals are formed. They can behave as second messengers during platelet activation and participate in signalling pathways [10-17].

The purpose of this study was to determine the effects of resveratrol on chemiluminescence as an indicator of production of different reactive oxygen species ($O_2^{\cdot-}$, H_2O_2 , singlet oxygen and organic radicals) and on superoxide anion ($O_2^{\cdot-}$) generation in blood platelets measured by reduction of cytochrome *c*. The level of free radicals in blood platelets was estimated after incubation of platelets with *trans*-resveratrol and/or after action of thrombin as a strong platelet agonist on platelets preincubated with this compound.

MATERIALS AND METHODS

Materials. Thrombin, luminol, *trans*-resveratrol and cytochrome *c* were purchased from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical grade and were provided by commercial suppliers.

Isolation of blood platelets. Pig blood (5 volumes) was collected into ACD solution (65 mM citric acid + 85 mM citrate + 111 mM dextrose; 1 volume) 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 resuspended in Ca^{2+}/Mg^{2+} free Tyrode's buffer (140 mM NaCl, 10 mM glucose, 15 mM 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 at room temperature. Blood platelets were suspended in Ca^{2+}/Mg^{2+} free Tyrode's buffer at a final concentration of 5×10^8 platelets/ml and platelet suspensions were incubated at 37°C for 5, 15 or 30 min with resveratrol at final concentrations ranging from 6.25 to 100 $\mu g/ml$ or with thrombin (1 U/ml). In some experiments, platelets after preincubation with resveratrol (50 $\mu g/ml$, 5 min) were treated with thrombin. The blood platelets were counted by the photometric method as described by Walkowiak *et al.* [18]. Platelet protein concentration was estimated according to Vatasery & Smith [19] (a modified Lowry method). The content of leukocytes in blood platelets was about 0.01%.

Chemiluminescence measurements. The level of free radicals in control blood platelets and platelets incubated with resveratrol or thrombin was recorded using the chemiluminescence method. The chemiluminescence signals were evaluated by means of a Berthold LB950 automatic luminescence analyser after the addition of 20 μl of 2 mM luminol solution in buffered saline to 1 ml of blood platelets. Results were expressed as the integral over

the total measurement time (15 min) and presented as percent of chemiluminescence.

$O_2^{\cdot -}$ Generation in blood platelets. Generation of superoxide anion ($O_2^{\cdot -}$) in control platelets and in platelets incubated with resveratrol or thrombin was measured by means of superoxide dismutase ($1 \mu\text{g}/\mu\text{l}$) -inhibitable reduction of cytochrome *c*, as described by Jahn & Hansch [20]. Briefly, an equal volume of $\text{Ca}^{2+}/\text{Mg}^{2+}$ free Tyrode's buffer, containing cytochrome *c* ($160 \mu\text{M}$) was added to 1 ml platelet suspension. 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. To calculate the molar concentration of $O_2^{\cdot -}$ the extinction coefficient for cytochrome *c* of $18700 \text{ M}^{-1} \text{ cm}^{-1}$ was used.

All the values in this study were expressed as means \pm S.D. The statistically significant differences between variations were found (Snedecor-Fisher test) so the differences between means were assessed by applying Cochran-Cox test. Regression lines were calculated by means of the least squares method.

RESULTS AND DISCUSSION

Resveratrol induced changes in the production of $O_2^{\cdot -}$ in blood platelets. Incubation of the platelets with resveratrol at concentrations of 6.25 and 12.5 $\mu\text{g}/\text{ml}$ caused increased production of $O_2^{\cdot -}$ radicals as measured by the superoxide dismutase-inhibitable reduction of cytochrome *c* (Fig. 1) however, the increase in $O_2^{\cdot -}$ level was not statistically significant ($P > 0.05$) (Fig. 1). At higher concentrations (25, 50 and 100 $\mu\text{g}/\text{ml}$) resveratrol added to blood platelet suspensions reduced the level of $O_2^{\cdot -}$ ($P < 0.05$) (Fig. 1). A decrease of $O_2^{\cdot -}$ level was observed as early as after 5 min incubation of platelets with resveratrol (50 $\mu\text{g}/\text{ml}$) and during the next minutes of incubation (15 and 30 min) the level of $O_2^{\cdot -}$ only slightly decreased (Fig. 2).

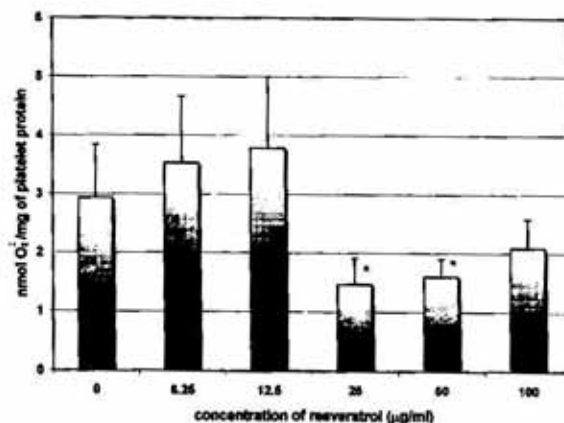


Figure 1. The effects of resveratrol (30 min, 37°C) on the level of $O_2^{\cdot -}$ in blood platelets.

Each experiment was carried out in five independent measurements (* $P < 0.05$ with respect to resveratrol-untreated platelets).

In another set of experiments on blood platelets incubated with resveratrol, we measured the level of different free radicals by the chemiluminescence method. Resveratrol reduced the luminol-dependent chemiluminescence in platelets as soon as after 5 min of incubation and the action of resveratrol was dose-dependent (Fig. 3). Thrombin used (at a dose of 1 U/ml of platelet suspension) as an effective inducer of platelet activation caused an increase of chemiluminescence in platelets (about $37.9\% \pm 10.1$ versus control – resting platelets) ($P < 0.05$) (Table 1). In the presence of resveratrol (50 $\mu\text{g}/\text{ml}$) chemiluminescence of thrombin-activated platelets was lower

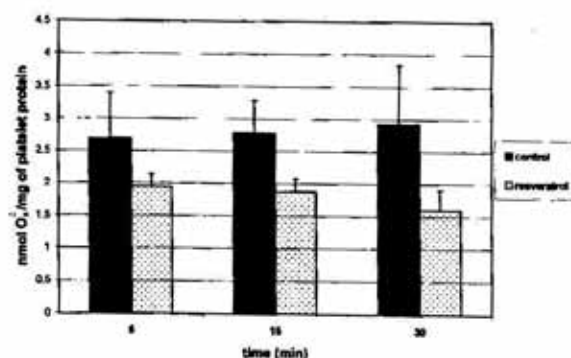


Figure 2. The effect of time of incubation with resveratrol (50 $\mu\text{g}/\text{ml}$, 37°C) on generation of $O_2^{\cdot -}$ in blood platelets ($n = 4$)

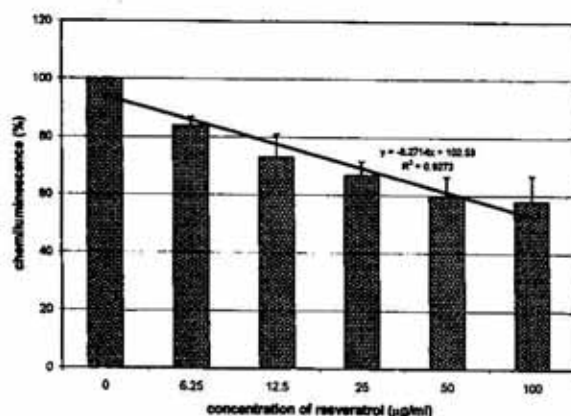


Figure 3. The effects of resveratrol (5 min, 37°C) on the chemiluminescence in blood platelets.

Each experiment was carried out in six independent measurements. Regression lines were calculated by means of the least squares method. The R value was 0.9629 ($P < 0.05$)

than in control platelets stimulated by thrombin ($P < 0.05$) (Table 1).

Oxygen free radicals play an important role in blood platelet activation [15–17]. Our preliminary studies showed that resveratrol reduced activation of blood platelets mainly: at the initial step of this process – adhesion to collagen, platelet aggregation and metabolism of endogenous platelet arachidonate [9]. This study shows that the inhibition of platelet activation induced by resveratrol is associated (in a dose-dependent manner) with the reduced production of free radicals in these cells. The

Table 1. Effect of thrombin and resveratrol on free radical production in blood platelets.

Free radicals were measured by the chemiluminescence method. Platelets were incubated at 37°C for 5 min with thrombin (1 U/ml) or thrombin plus resveratrol (50 µg/ml). The numbers represent luminol chemiluminescence as percentage of that recorded for unactivated platelets (control) and are mean values \pm S.D. for 5–6 experiments. The effects were statistically significant according to the Student's *t*-test, $P < 0.05$.

Additions	Chemiluminescence (% of control)
Thrombin	137.9 \pm 13.3
Thrombin+resveratrol	31.5 \pm 6.7

use of the chemiluminescence method for assessing the level of reactive oxygen species in platelets and the method of reduction of cytochrome *c* for the estimation of the level of $O_2^{\cdot-}$ revealed that resveratrol at higher concentrations (25, 50 and 100 µg/ml) shows antioxidative action. The pathway by which reactive oxygen radicals are generated in blood platelets has not been fully elucidated. The metabolism of endogenous arachidonic acid and the glutathione cycle in activated platelets could be an important source of these species. Jahn & Hansch [20] have demonstrated that the addition of exogenous arachidonic acid to platelets caused $O_2^{\cdot-}$ generation. These radicals could be produced in platelets during enzymatical oxygenation of arachidonate *via* cyclooxygenase or lipoxygenase pathways or during phospholipase-dependent cleavage of platelet phospholipids and release of free arachidonic acid. Platelet agonists may also stimulate the production of $O_2^{\cdot-}$ by NADPH oxidase [21]. Our earlier results showed that resveratrol inhibited eicosanoid synthesis in blood platelets activated by thrombin [9] and in this way it probably decreased the level of free radicals in platelets. In other cells the suppression of arachidonic acid metabolism can be induced by *trans*-resveratrol *via* selective inhibition of cyclooxygenase [22, 23]. Rotondo *et al.* [24] showed that resveratrol had a strong inhibitory effect on radical production in leukocytes, and on the metabolism of arachidonate in these cells. We suggest that the decrease in the level of reactive oxygen species induced by *trans*-resveratrol could be dependent on the inhibition of various steps of platelet activation. Free radicals play also an important role in phosphorylation of platelet proteins on tyrosine, which occurs during the activation of platelets mediated by different agonists (thrombin or collagen) [25–27]. On the other hand, superoxide anions could enhance platelet adhesion and aggregation [27]. It seems that, *in vivo*, resveratrol could affect the haemostatic function of platelets.

Antioxidative action of high concentrations of resveratrol can minimise the risk of various illnesses including cancer diseases, it could also protect against coronary heart diseases. Frankel *et al.* [28, 29] reported that the ability of wines to protect low density lipoproteins (LDL) from oxidation appears to be dependent not only on resveratrol but also on phenolic substances abundant in wine. These compounds as antioxidants seem to be helpful in inhibitions oxidation of low density lipoproteins and in reducing the incidence of coronary heart disease and atherosclerosis [30]. These results, supported by our data, suggest that, in addition to its beneficial effects on platelets, resveratrol merits future investigation as an antioxidant.

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