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Effect of Resveratrol on Some Activities of Isolated and in Whole Blood Human Neutrophils

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Summary

Resveratrol, which is a polyphenol present in red wines and vegetables included in human diets, exerts many biological effects. The aim of the present study was to investigate its effect on some activities of polymorphonuclear leukocytes, particularly the generation of superoxide anion (O_2^-) in whole blood, hypochlorous acid (HOCl) and nitric oxide (NO) production by isolated cells, and chemotaxis. Resveratrol showed significant dose-dependent inhibitory effect on all these activities. In particular, it inhibited O_2^- generation in stimulated but not in resting neutrophils, decreased HOCl much more than O_2^- production indicating an effect on myeloperoxidase secretion since HOCl production is directly and proportionally dependent on O_2^- generation and reduced cell motility. The small dose of resveratrol (4.38 nM) used is attainable with a diet including red wine and vegetables confirming its protective role against some pathological processes such as inflammation, coronary heart disease, and cancer.

Key words

Resveratrol • Neutrophils • Reactive oxygen species • Chemotaxis

Introduction

The phytoalexin resveratrol (trans-3,4',5-trihydroxystilbene) is a non-flavonoid polyphenol isolated from at least 72 species of spermatocytes. The products of many of these plants are included in human diets as consumable fruits, vegetables or beverages (principally grapes, mulberries, peanuts, red wine and grape juices).

The observations that resveratrol has antiinflammatory and anticarcinogenetic effect (Jang *et al.* 1997, 1999, Fremont 2000) and exerts chemopreventive activities on cancer and cardiovascular diseases (Bertelli *et al.* 1999, Jang and Pezzuto 1999, Lin and Tsai 1999) induced researchers to study the biological and pharmacological activities of this substance. The results of these studies showed that anti-inflammatory effect is due to its inhibitory activity on reactive oxygen species (ROS) and prostaglandin production (Kimura *et al.* 1985, Martinez and Moreno 2000). The anticancer effect is due to its ability to arrest proliferation (Jang *et al.* 1997, Raglone *et al.* 1998), to induce differentiation (Mgbonyebi *et al.* 1998) or apoptosis in tumor cells (Clement *et al.* 1998, Tsan *et al.* 2000) and to inhibit ribonucleotide reductase and DNA synthesis (Fontecave *et al.* 1998). The preventive effect on cardiovascular diseases is due to estrogenic actions of resveratrol (Gehm *et al.* 1997), to its effect on platelets

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(Bertelli *et al.* 1995, Pace-Asciak *et al.* 1996), plasma lipoproteins (Belguendouz *et al.* 1998, Frankel *et al.* 1993), and vascular smooth muscle cells (Mizutani *et al.* 2000).

This variety of effects presupposes a complex molecular activity of resveratrol revealed by its ability to change enzymes (Jang and Pezzuto 1998, Garcia-Garcia *et al.* 1999, Hsieh *et al.* 1999) and gene actions (Hsieh *et al.* 1999, Jang and Pezzuto 1999, Mitchell *et al.* 1999).
The effects of resveratrol on phagocytes were considered mainly for their involvement in phlogosis, cancer and cardiovascular diseases (Rotondo *et al.* 1998, Bertelli *et al.* 1999, Martinez and Moreno 2000), and consequently not on all functions of these cells. The aim of this paper was to study the effect of resveratrol on neutrophil activities not yet studied.

Methods

All the chemicals were purchased from Sigma (Milan, Italy) and plastic disposable Boyden chambers from FAR (Division Diagnostic, Verona, Italy).

Isolation of neutrophils

Human venous blood from healthy adult volunteers was collected with disposable plastic syringes and anticoagulated with 10 units of heparin per ml. Peripheral blood was diluted 1:2 in Hank's balanced salt solution (HBSS), layered on Hystopaque-1077 and centrifuged at 400 x*g* for 45 min at room temperature. The bottom washed twice in PBS was resuspended in 0.6 % dextran, then left for 45 min to allow erythrocytes to sediment; the clear erythrocyte shore was recovered, centrifuged at 1800 rpm for 15 min and residual red cells were removed by hypotonic lysis as described by Haslett *et al.* (1985). Cells were found to be >98 % pure and >95 % viable as measured by trypan blue exclusion. When a large quantity of cells was required, platelet-free buffy coat supplied by the local Transfusion Centre (University Policlinic, Messina, Italy) was used as the source and treated as above.

Mesaurement of superoxide anion release

O_2^- release was estimated using superoxide dismutase-inhibitable reduction of cytochrome c assay described by Bellavite *et al.* (1983). The results were expressed as nmoles of $O_2^-/10^6$ granulocytes, on the basis of the total and differential counts of white blood cells. All assays were carried out in duplicate.

Measurement of hypochlorous acid generation

The generation of HOCl was determined by assay for taurine-chloramine (Weiss *et al.* 1982) using thio-bis(2-nitrobenzoic acid) (TNB) generated by treating 1.0 mM 5,5'-dithio-bis(2-nitrobenzoic acid) with 2.0 mM sodium borohydride for 6 h at 37 °C as oxidant substrate. The results were expressed as nmoles of taurine chloramine/10^6 cells. The assays were carried out in duplicate.

Measurement of nitrite production

Nitrites were measured by the Griess reaction as described by Miles *et al.* (1995). The concentration of nitrite was determined from a standard curve using $NaNO_2$. The assays were carried out in duplicate.

Chemotaxis assay

Neutrophil migration was measured with the Boyden chamber technique, as described by Boyden (1962) and modified by Zigmond and Hirsch (1973) using plastic disposable chambers in which the two compartments were separated by a cellulose nitrate filter with a pore size of 3 μm. Neutrophil migration within the filter was determined under light microscopy by the leading front method, in which the distance was measured from the top of the filter to the farthest plane still containing two cells under a x40 objective. The assays were carried out in duplicate and the extent of migration was determined at 10 different randomly chosen filter sites. In some chambers, the chemoattractant was replaced by resveratrol to better observe the effect on the cell surface. Results were expressed as the distance in μm traveled by cells into the filter.

Experimental protocols

Whole blood was subdivided in aliquot parts of 2 ml and preincubated with 10^{-2}, 10^{-4} or 10^{-6} mg/ml final concentration of resveratrol for times varying from 5 min to 1 h at 37 °C under continuous shaking. Isolated neutrophils were suspended in opportunely supplemented HBSS Ca^{2+} and Mg^{2+}-free without phenol red at the concentration of 10^6 cells/ml for superoxide assay, of $3x10^7$ cells/ml for nitrite assay, of $2x10^6$ cells/ml for hypochlorous and chemotaxis assays; then preincubated as whole blood for one hour with the exception of nitrite assay in which the preincubation time was extended to 4 hours. The controls were treated under the same conditions but without resveratrol preincubation.

Statistical analysis

The data were analyzed by unpaired Student's t-test, significance was considered when $p \leq 0.05$ was attained.

Results

Effect of resveratrol on superoxide anion release

Figure 1 shows the effect of resveratrol on neutrophils in whole blood and on isolated cells. In whole blood the neutrophils treated with 10^{-6}, 10^{-4} or 10^{-2} of the studied agent and stimulated with zymosan or FMLP showed a significant dose-dependent decrease of O_2^- release by 36.9-56.4 % and by 25.6-59.9 %, respectively. Isolated neutrophils, treated as above, showed and inhibition of O_2^- production by 50.3-71.7 % after zymosan stimulation and by 29.3-67.3 % after FMLP stimulation, the decrease was statistically significant and dose-dependent in both cases.

Figure 2 shows the effect of resveratrol on basal production of O_2^- by neutrophils. The upper diagram shows the effect on normal basal production of isolated and in whole blood neutrophils; both isolated cells and that in whole blood, treated as above without stimulation, showed a significant decrease of 46.4 % and 29.7 %, respectively, only at the highest concentration. The lower diagram shows the effect on isolated and in whole blood neutrophils with natural high basal generation; resveratrol inhibited significantly O_2^- generation by 30.5-49.8 % in isolated cells and by 25.7-49 % in whole blood cells.

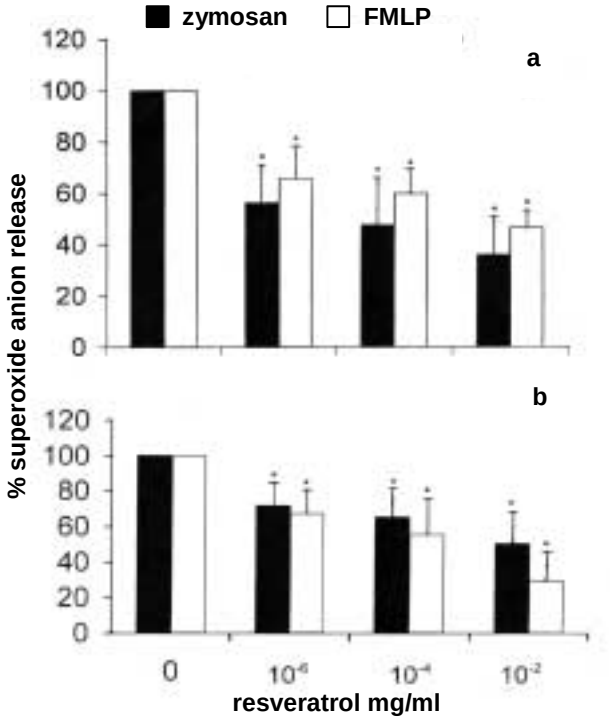

Fig. 1. *Effect of resveratrol on O_2^- production by stimulated human neutrophils:* ***a)*** *in whole blood,* ***b)*** *in isolated cells. The blood samples or isolated cells were preincubated with 10^{-6}, 10^{-4} or 10^{-2} mg/ml of the compound for 1 h followed with zymosan (black columns) or FMLP (white columns) stimulation for 30 min. Values are the means ± S.D. for at least five separate experiments. * Significantly different from controls, $p \leq 0.05$.*

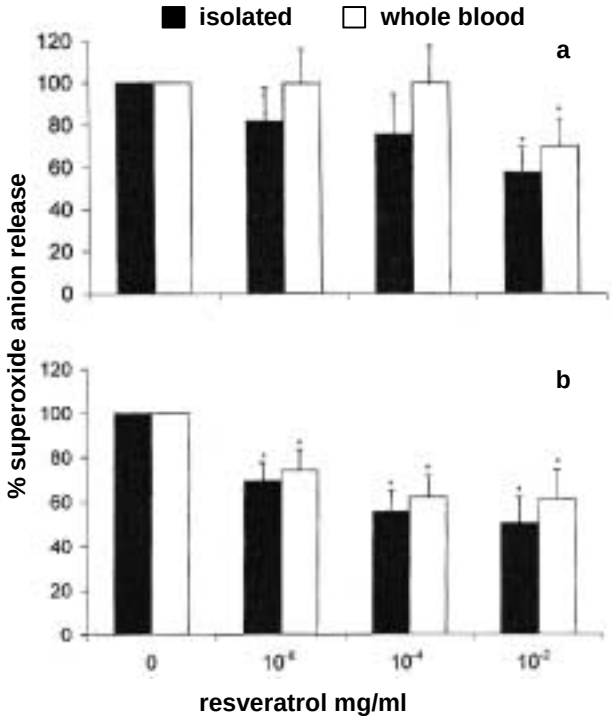

Fig. 2. *Effect of resveratrol on basal production of O_2^- by human neutrophils in whole blood and in isolated cells.* ***a)*** *Neutrophils with normal basal O_2^- generation.* ***b)*** *Neutrophils with natural high basal O_2^- generation. The blood samples or isolated cells were incubated with 10^{-6}, 10^{-4} or 10^{-2} mg/ml of the compound for 1 h. Values are the means ± S.D. for at least five separate experiments. * Significantly different from controls, $p \leq 0.05$.*

Figure 3 shows the time-dependent effect of 10^{-6} mg/ml of resveratrol on O_2^- release by human neutrophils in whole blood stimulated with zymosan. An inhibitory effect of about 14 % was already observed after 5 min and it increased gradually reaching 67 % after 60 min.

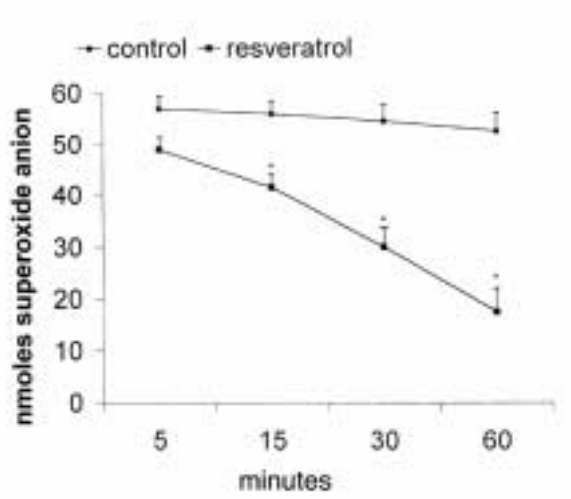

Fig. 3. *Effect of resveratrol on O_2^- production by zymosan-stimulated neutrophils in whole blood as a function of the time. Values are the means ± S.D. for three separate experiments.* Significantly different from controls, $p \leq 0.05$.*

Effect of resveratrol on hypochlorous acid release

Figure 4 shows the effect of resveratrol on isolated neutrophils treated with 10^{-6}, 10^{-4} or 10^{-2} mg/ml and stimulated with zymosan. All concentrations used significantly inhibited HOCl production by 60-85 % in a dose-dependent manner.

Effect of resveratrol on nitrite production

Figure 5 shows the effect of resveratrol (10^{-6}, 10^{-4} or 10^{-2} mg/ml) on isolated neutrophils stimulated with FMLP 100 nM. Resveratrol dose-dependently inhibited the nitrite production by 15-52 %, but the inhibition was significant only at the highest concentration. Since neutrophils produced low quantity of NO, we repeated the experiments on RAW 246.7 cells stimulated with LPS obtaining similarly significant results at all concentrations used (data not showed).

Effect of resveratrol on neutrophil chemotaxis

Figure 6A shows the effect of 10^{-6} and 10^{-4} mg/ml of resveratrol on spontaneous neutrophil motility, both concentrations exerted inhibiting activity of 12.95 % and 22.85 %, respectively.

Figure 6B shows the effect of resveratrol on FMLP-induced chemotaxis. The pretreatment of cells with 10^{-6} mg/ml of resveratrol inhibited the motility by 21.8 % and that with 10^{-4} mg/ml by 34.3 %.

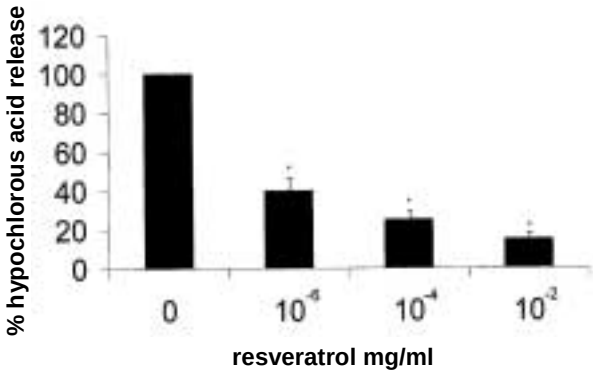

Fig. 4. *Effect of resveratrol on HOCl production by stimulated human neutrophils.The cells were preincubated with 10^{-6}, 10^{-4} or 10^{-2} mg/ml of the compound for 1 hour followed with zymosan stimulation for 30 min. Values are the means ± S.D. for three separate experiments. * Significantly different from controls, $p \leq 0.05$.*

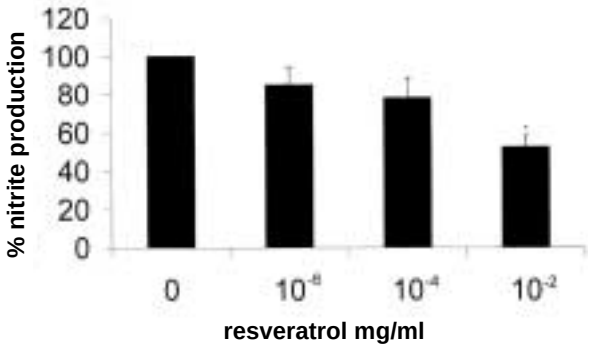

Fig. 5. *Effect of resveratrol on nitrite production by stimulated human neutrophils. The cells were preincubated with 10^{-6}, 10^{-4} or 10^{-2} mg/ml of the compound for 1 h followed with FMLP stimulation for 4 hours. Values are the means ± S.D. for five separate experiments. * Significantly different from controls, $p \leq 0.05$.*

Figure 6C shows the effect of 10^{-6} and 10^{-4} mg/ml of resveratrol used as chemoattractant instead of FMLP on human neutrophils. Under these conditions resveratrol also inhibited cell motility by 16.1 % and 20.3 %, respectively.

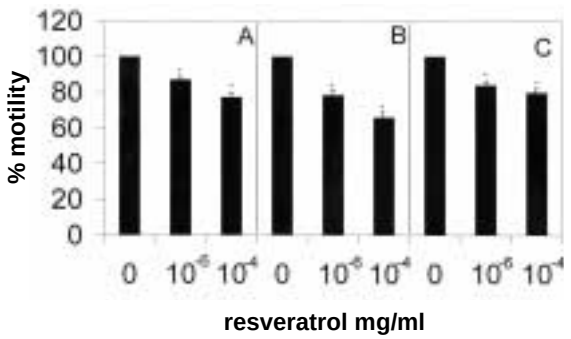

Fig. 6. *Effect of resveratrol on neutrophil chemotaxis* ***A.*** *Cells were preincubated for 1 h with 10^{-6} or 10^{-4} mg/ml of the compound and then tested for chemotaxis without chemo-attractant.* ***B.*** *Cells were preincubated for 1 h with 10^{-6} or 10^{-4} mg/ml of the compound and then tested for chemotaxis in the presence of chemoattractant.* ***C.*** *Untreated cells were tested for chemotaxis applying 10^{-6} or 10^{-4} mg/ml of resveratrol as chemoattractant. Values are the means ± standard deviation for three separate experiments. * Significantly different from the controls, $p \leq 0.05$.*

Discussion

The antioxidant properties of resveratrol are well known, but in human polymorphonuclear leukocytes they were tested only on ROS production by chemiluminescence assay (Rotondo *et al.* 1998, Jang *et al.* 1999) and O_2^- production in isolated cells using micromolar concentrations of the compound (Rotondo *et al.* 1998). In our study, we used a wider range of resveratrol concentrations (from 4.38 nM to 43.8 μM) and we determined the effects on neutrophils in whole blood avoiding stress and/or activation due to manipulations necessary to isolate cells from other blood elements. These results were compared with experiments performed on isolated cells under the same conditions. We also used two different stimulating agents, particulate as zymosan or chemical as FMLP. In whole blood, we observed a dose-dependent inhibitory effect of resveratrol, but the particulate stimulant (Fig. 1a, black columns) was more effective than the chemical one (Fig. 1a, white columns); isolated neutrophils showed similar behavior but the chemical stimulant (Fig. 1b, white columns) was more effective than the particulate one (Fig. 1b, black columns). The higher effect of resveratrol observed in whole blood in inhibiting O_2^- production after particulate stimulation is imputable to opsonization of zymosan that is better in blood than *in vitro*; the lesser effect after chemical stimulation is imputable to the presence of cells and plasmatic components that could react with some FMLP thus reducing its action on neutrophils. This inhibition of O_2^- generation independent of the nature of the stimulus indicates an intracellular action of resveratrol, because an action on cell membrane would inhibit the zymosan effect that triggers all steps leading to O_2^- production, but not the FMLP effect that directly activates protein kinase c bypassing membrane steps. Results observed in isolated cells, using FMLP as stimulant and 49.8 μM of resveratrol, were almost identical to those obtained by Rotondo *et al.* (1998) under the same conditions. We noticed an inhibition of 70.7 % after 30 min of stimulation in comparison with 80 % reported by Rotondo *et al.* (1998) after 40 min of stimulation. This is compatible since the inhibiting effect of resveratrol is time-dependent (Fig. 3). It is particularly remarkable that resveratrol was significantly effective in inhibiting O_2^- production of stimulated neutrophils also at 4.38 nM, the smallest concentration used by us. This concentration of the compound can easily be attained in the plasma and in different tissues by consumption of red wine and other juices or vegetables containing resveratrol, confirming the observation that also a moderate but regular use of red wine may protect against the effect of cream sauces and butter known as "French paradox" (Kopp 1998), and prevent some inflammatory and neoplastic events (Fremont 2000).

We also took into account the effect of resveratrol on basal O_2^- generation of neutrophils in whole blood. We observed that there are 20 % volunteers that had activated O_2^- generation by some pathological defect or something else than zymosan or FMLP. Therefore, those were considered separately. We observed that in volunteers with normal low basal O_2^- production resveratrol showed an inhibiting effect only at highest concentration used (Fig. 2a). On the contrary, in volunteers with natural high basal O_2^- production, resveratrol inhibited superoxide anion generation at all concentrations used (Fig. 2b). We observed inhibitory effect in isolated neutrophils also in subjects with low

basal O_2^- production. This was due to manipulations to isolate neutrophils from blood, that in some way activate the cells inducing the generation of O_2^- in small quantities but sufficient to permit the appearance of the inhibition.

The hypochlorous production, as expected, was inhibited by resveratrol in a dose-dependent manner (Fig. 4). In fact, hypochlorous generation is directly dependent on superoxide anion production since O_2^- is rapidly converted to secondary toxic oxygen species such hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) that determines HOCl formation in presence of the enzyme myeloperoxidase of the neutrophils. We observed that at all concentrations used the inhibiting effect of resveratrol on HOCl release was strongly higher than that on O_2^- generation, since the hypochlorous generation is directly dependent on that of superoxide anion. It is thus evident that resveratrol exerted inhibiting effect also on myeloperoxidase; this is supported by the demonstration that the compound prevents release and inhibits secretion of elastase and β-glucuronidase (Rotondo *et al.* 1998), two enzymes located in granules of neutrophils as myeloperoxidase.

Resveratrol exerts a strong inhibiting effect on nitric oxide production in macrophages (Kawada *et al.* 1998, Jang and Pezzuto 1999, Matsuda *et al.* 2000) and we showed a similar effect in neutrophils, less evident but evenly dose-dependent and significant at higher concentration (Fig. 5). This smaller effect can be attribuited to the fact that the time of incubation and stimulation granted to neutrophils was only 5 hours compared to 18 hours granted to macrophages, inasmuch as polymorphonuclear cells survive only 8 hours after blood withdrawal in our conditions. Hence, after the manipulation to separate them from other blood cells we have a maximum of 5 hours for incubation and for stimulation to have neutrophils in good metabolic condition. Moreover, since NO release of neutrophils was small, we repeated the experiments on RAW 264.7 cells incubated and stimulated for 18 h after treatment with resveratrol, obtaining a significant dose-dependent inhibiting effect (data not shown).

Concerning neutrophil chemotaxis, resveratrol showed an inhibiting dose-dependent effect on random migration of cells (Fig. 6A), on cells pretreated with the studied compound (Fig. 6B), and on untreated cells when resveratrol was added into the lower compartment of a Boyden chamber instead of FMLP (Fig. 6C). The inhibition of FMLP-induced chemotaxis could be caused either by heterologous desensitization of surface receptors as competition with another chemoattractant compound, or by intracellular interference on enzyme systems. Since the resveratrol used as chemoattractant instead of FMLP inhibited chemotaxis (Fig. 6C), we can affirm that its activity is intracellular and interfere with biological processes that neutrophils use to control their shape and movement.

In conclusion, these studies demonstrate that resveratrol in whole blood also inhibits superoxide anion generation of stimulated human neutrophils, but it is ineffective in basal production of resting cells; it strongly decreases the hypochlorous generation, reduces nitric oxide production and chemotaxis, these effects being dose-dependent. The activity of the compound is strictly intracellular. The dosages attainable by a normal alimentary route are effective on all considered neutrophil activities confirming the protective role of resveratrol against various pathological events such as inflammation, coronary heart disease and cancer.

Acknowledgements

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Reprint requests

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