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Activation of the erythrocyte plasma membrane redox system by resveratrol: a possible mechanism for antioxidant properties

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Abstract:
Resveratrol is one of the most widely studied of all the plant-produced polyphenols and has diverse, beneficial health effects including anti-cancer and cardio-protective effects. Many of the biological actions of this polyphenol have been attributed to its antioxidant properties. Erythrocytes contain a plasma membrane redox system (PMRS), which transfers electrons from intracellular donors (NADH and/or ascorbate (ASC)) to extracellular acceptors. There is evidence that the intracellular ASC donates electrons to extracellular ascorbate free radicals (AFRs) *via* the PMRS, which encompasses an AFR reductase; such a redox system enables the cells to effectively counteract oxidative processes. We present evidence to show that human erythrocytes take up resveratrol, and once inside the cell, resveratrol can donate electrons to extracellular electron acceptors through the erythrocyte PMRS and AFR reductase. Incubating human erythrocytes with resveratrol (10 μM) caused a significant activation of the PMRS (41%) and AFR reductase (30%) over (basal level) the control; the effect of resveratrol was concentration-dependent. The electron donating ability of resveratrol is slightly less than that observed with quercetin. The role of resveratrol in activating the erythrocyte PMRS and AFR reductase may assume significance in all disease conditions in which there is a decrease in plasma antioxidant potential.

Key words:
erythrocyte, resveratrol, PMRS, AFR reductase, antioxidant

Introduction

Resveratrol is a natural phytoalexin polyphenolic compound found largely in the skin of grapes and is well known for its presence in red wine [16]. Its stilbene-based structure has two phenolic rings linked by a styrene double bond, which allows the *cis* and *trans* orientations to generate 3,4',5-trihydroxystilbene (Fig. 1). Growing evidence suggests that resveratrol may play an important role in prevention of human diseases such as cancer, cardiovascular disease, diabetes and aging [32]. Many of the biological actions of this polyphenol have been attributed to its antioxidant properties [18, 22].

Studies show that human erythrocytes contain a plasma membrane redox system (PMRS), which transfers electrons from intracellular donors (NADH and/or ascorbate (ASC)) to extracellular acceptors, although the physiological acceptor is still unclear [20]. There is evidence that the intracellular ASC donates electrons to extracellular ascorbate free radicals (AFRs) *via* the PMRS, which encompasses an AFR reductase; such a redox system enables the cells to ef-

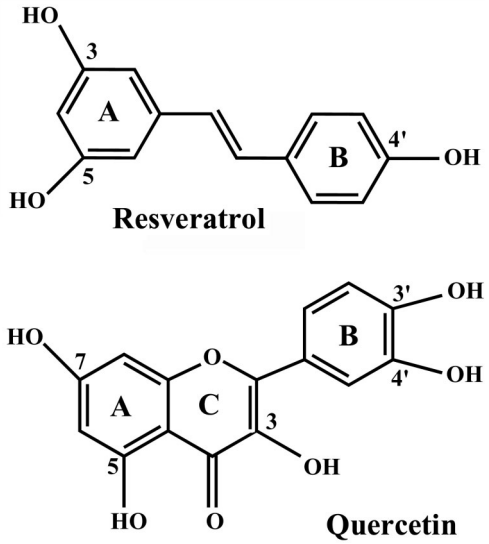

Fig. 1. Chemical structures of resveratrol and quercetin. Resveratrol (3,4',5-trihydroxystilbene) belongs to the stilbene class of the polyphenolic compounds. It consists of two rings, A and B, and three hydroxyl groups. Quercetin (3,3',4',5,7-pentahydroxyflavone) is a flavonoid and contains three rings, A, B and C, and five hydroxyl groups

fectively counteract oxidative processes and thereby prevent depletion of extracellular ASC [35]. Recently, we have shown that the PMRS, along with AFR reductase, is a compensatory/protective mechanism that operates to maintain the ASC level in plasma and thereby minimize oxidative stress during aging [30, 31].

It was suggested that the activation of red cell plasma membrane oxidoreductase (PMOR) by some polyphenols constitutes a mechanism whereby these compounds elicit their antioxidant effects and promote beneficial effects [10, 11], but the study was incomplete because it was based on only the activation of PMOR, and it is now clear that PMOR is part of a system of electron carriers collectively referred to as the PMRS [30, 33]. In the PMRS, AFR reductase plays an important role in transferring electrons to extracellular AFRs, the first oxidation product of ASC, and reducing AFRs back to ASC. The proper functioning of the PMRS thus plays an important role in ascorbate recycling [31]. In the present study, we present evidence that erythrocytes take up resveratrol and quercetin, and once inside the cell, these polyphenols can donate electrons to extracellular electron acceptors through the erythrocyte PMRS and AFR reductase. We have used quercetin, a well studied polyphenol [28] as a positive control for comparing the effect of resveratrol on the PMRS.

Materials and Methods

The protocol of the study conformed to the guidelines of the Allahabad University Ethical Committee. Human venous blood from different healthy volunteers of both sexes between the ages of 21–30 years was obtained by venipuncture in heparin after an overnight fast; the criteria for selecting healthy human volunteers have been defined previously [31]. The blood was centrifuged at 1,800 × g for 10 min at 4°C. After removal of plasma, the buffy coat and approximately upper 15% of the packed red blood cells (PRBCs) were washed twice with cold phosphate buffered saline (PBS; 0.9% NaCl, 10 mM Na_2HPO_4, pH 7.4).

Incubation of human erythrocytes with resveratrol and quercetin

Packed RBCs (10% v/v) were incubated in PBS containing 5 mM glucose in the presence of resveratrol or quercetin (different doses) at 37°C for 30 min. The plasma concentrations of resveratrol are known to range from 0.1 μM to 1 μM [3]. Because *in vitro* incubation can be done for only short durations, we used higher polyphenol concentrations of 1 μM to 100 μM. After incubations, the suspensions were immediately centrifuged at 1,800 × g, and the PRBCs were washed twice with at least 50 volumes of PBS and then subjected to subsequent analyses.

Extracellular and intracellular content of polyphenols

The intracellular and extracellular content of resveratrol and quercetin were measured according to Fiorani et al. [10] by performing ethyl acetate extractions. Briefly, human PRBCs were incubated for 10 and 30 min at 37°C in 10% PBS in the presence of the two polyphenols separately at different final concentrations ranging from 10 μM to 100 μM. The study could not be performed with very low polyphenol concentration because of the limitation of the assay below 10 μM. To measure the extracellular concentration of polyphenols, the supernatant fraction obtained at the end of the incubation period was extracted three times with ethyl acetate.

For estimation of the intracellular content of the polyphenols, the erythrocytes obtained after centrifu-

gation were washed at least two times with 50 vol. of PBS followed by re-suspension of the sample with 3 vol. of cold distilled water and maintenance for 10 min at 4°C. The erythrocyte lysates were extracted three times with ethyl acetate. The absorbance of the clear upper phase was measured spectrophotometrically at the wavelength corresponding to the maximum absorption spectra, 307 nm for resveratrol and 378 nm for quercetin. Concentrations were obtained from calibration curves.

Measurement of erythrocyte PMRS activity

The activity of the erythrocyte PMRS was estimated by the reduction of ferricyanide according to the method of Avron and Shavit [2]. PRBCs (0.2 ml) were suspended in PBS containing 5 mM glucose and 1 mM freshly prepared potassium ferricyanide to a final volume of 2.0 ml. The suspensions were incubated for 30 min at 37°C and then centrifuged at 1,800 × g at 4°C. The supernatant collected was assayed for ferrocyanide content using 4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt and measuring absorption at 535 nm (ε = 20,500 M^{-1} cm^{-1}). The results are expressed in µmol ferrocyanide/ml PRBC/30 min.

Measurement of erythrocyte AFR reductase activity

Erythrocyte AFR reductase activity was assayed following the method described by May et al. [24]. The washed erythrocytes were hemolyzed and diluted 100% (v/v) by addition of water followed by centrifugation for 10 min in the cold. AFRs were generated in diluted hemolysates by incubating the lysates at 37°C in PBS (pH 7.0) containing 1 mM ascorbate, 5 units/ml ascorbate oxidase and 0.1 mM of NADH. The rate of NADH oxidation was measured spectrophotometrically at 340 nm for 3 min at 37°C. The change in NADH concentration was calculated from the slope of the resulting line, using an extinction coefficient ε = 6.22 mM^{-1} cm^{-1}. The values were corrected in each experiment for the rate observed with lysate and reduced nucleotide alone. AFR reductase activity is reported in terms of µM NADH oxidized/min/ml PRBC.

Statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA).

Results

We carried out studies to determine the ability of resveratrol to donate electrons to the erythrocyte PMRS and to reduce extracellular AFR to ASC *via* AFR reductase. It has been reported that quercetin can activate erythrocyte PMOR [10], thus we used quercetin as a control to evaluate the efficacy of resveratrol. Table 1 shows the uptake of resveratrol and quercetin by erythrocytes after the cells were loaded with these compounds. The cells accumulated resveratrol (89%) and quercetin (80%) above the initial loading concentration (10 µM). The percent accumulation of resveratrol inside the erythrocyte did not significantly change after a 30-min incubation (data not shown). Our observations compare well with another report [11] in which an 86% accumulation of quercetin in erythrocytes was reported. There was no significant difference in the percent accumulation of resveratrol inside the erythrocytes when incubated at concentrations of 10, 50 and 100 µM.

Tab. 1. Uptake of resveratrol and quercetin by human erythrocytes

Treatment	Intracellular concentration	
	(µmole)	(%*)
10 µM		
Resveratrol	6.54 ± 0.79	89 ± 7
Quercetin	5.39 ± 0.95	80 ± 5
50 µM		
Resveratrol	38.51 ± 2.06	91 ± 5
Quercetin	33.38 ± 2.69	81 ± 6
100 µM		
Resveratrol	82.31 ± 7.33	88 ± 8
Quercetin	76.11 ± 6.76	82 ± 7

* Erythrocytes were incubated for 10 min at 37°C with each of the two compounds with final concentrations of 10 µM, 50 µM and 100 µM. Experiments were also conducted after a 30-min incubation, and the pattern of results was the same as shown here for the 10-min incubation (data not shown). After washing and extraction with ethyl acetate (as described in the Materials and Methods section), the amount of polyphenols was measured in the intracellular and extracellular compartments. The data are the percentage of compound recovered in the cytosol with respect to the total compound content (intra + extracellular concentrations)

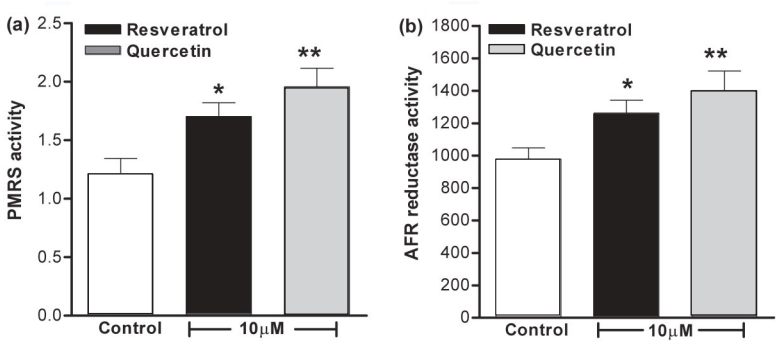

Fig. 2. Effect of resveratrol and quercetin on the activity of the erythrocyte (**a**) PMRS and (**b**) AFR reductase. Packed RBCs (10% v/v) were incubated in PBS containing 5 mM glucose in the presence of resveratrol or quercetin (final conc. 10 μM) at 37°C for 30 min. After incubation, the suspensions were immediately centrifuged at 1,800 × g, and the RBCs were washed twice with at least 50 volumes of PBS. Subsequently, the PMRS and AFR reductase activities were determined as described in the Materials and Methods. PMRS activity is expressed in μmol ferrocyanide/ml PRBC/30 min. AFR reductase activity is reported in terms of μM NADH oxidized/min/ml PRBC. Values are expressed as the mean ± SEM of 10–12 independent experiments; * $p < 0.01$, ** $p < 0.001$ compared to normal (control)

Figure 2a shows the activation of erythrocyte PMRS activity by resveratrol and quercetin. The results show that resveratrol (10 μM) activated erythrocyte PMRS activity 41% over (basal level) control (1.207 ± 0.135 *vs*. 1.700 ± 0.118), and under similar conditions quercetin caused activation of the PMRS 62% above control levels (1.207 ± 0.135 *vs*. 1.956 ± 0.160). Figure 2b shows the activation of erythrocyte AFR reductase activity by resveratrol (30% above control; 967.500 ± 69.689 *vs*. 1,262.109 ± 80.560) and quercetin (44% above control; 967.500 ± 69.689 *vs*. 1,399.833 ± 121.292) under the same conditions. The activation of the PMRS and AFR reductase activities by resveratrol and quercetin were concentration-dependent between 1 μM and 100 μM (Fig. 3a and 3b).

Discussion

Oxidative stress plays an active role in alteration of normal physiological processes and development/progression of several diseases [1, 15]. Numerous studies have demonstrated that the polyphenolic compounds found in wine have an antioxidant capacity and free radical scavenging activity [9, 34]. In support of the above observations, administration of exogenous antioxidative agents, including resveratrol, has been shown to exert protective effects on oxidative cardiovascular injury [12]. Resveratrol has been an effective scavenger of hydroxyl, superoxide, and metal-induced radicals, as well as showing antioxidant abilities in cells producing reactive oxygen species (ROS)

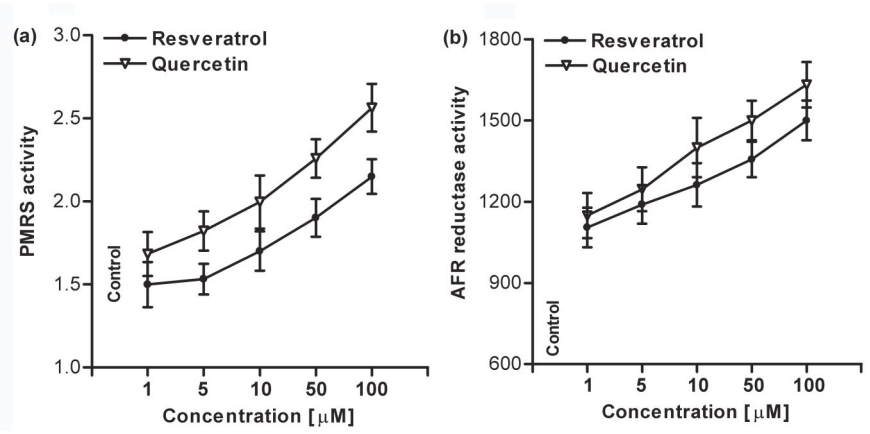

Fig. 3. Dose-dependent effect of resveratrol and quercetin on the erythrocyte (**a**) PMRS and (**b**) AFR reductase activities. Packed RBCs (10% v/v) were incubated in PBS containing 5 mM glucose in the presence of resveratrol or quercetin (indicated final concentrations) at 37°C for 30 min. After incubation, the suspensions were immediately centrifuged at 1,800 × g, and the RBCs were washed twice with at least 50 volumes of PBS. Subsequently, the PMRS and AFR reductase activities were determined as described in the Materials and Methods. PMRS activity is expressed in μmol ferrocyanide/ml PRBC/30 min. AFR reductase activity is reported in terms of μM NADH oxidized/min/ml PRBC. Values are expressed as the mean ± SEM of 10–12 independent experiments

[21]. It has also been found to exhibit a protective effect against lipid peroxidation in cell membranes. Several of the biological effects of resveratrol have been attributed to its antioxidant property although the exact mechanism in most cases is not clear [13].

A critical question concerns the concentrations of resveratrol that can be achieved in target organs of animals/humans upon oral administration. The plasma concentrations of resveratrol range between 100 nM and 1 μM [3], concentrations which are insufficient for any known biological activity, with only a few exceptions [4]; this has given rise to a paradoxical situation in which epidemiological observations cannot be substantiated by *in vitro* direct study. Our results provide a possible explanation to this paradox. We show that resveratrol can enter into erythrocytes and accumulate in much higher concentrations compared to plasma levels. The exact mechanism of transport of resveratrol across erythrocyte membranes is not known; however, resveratrol uptake by hepatic cells involves two processes – a passive one and a carrier-mediated one [19]. Gusman et al. [14] reported that resveratrol could accumulate in tissues such as heart, liver, and kidney in rats after oral administration. Accumulation of a significant amount of resveratrol has also been reported in cardiac tissue [6].

Human erythrocytes are constantly exposed to sources of ROS and thus are highly susceptible to oxidative damage [8]. The presence of the PMRS in the erythrocyte provides the cell with an extra level of defense against extracellular oxidants and enables the cell to respond to changes in both intra- and extracellular redox environments. The electron acceptor used to investigate erythrocyte PMRS activity is the membrane impermeable oxidant ferricyanide. The reduction of ferricyanide to ferrocyanide occurs at the cell membrane and is measured spectrophotometrically.

The reduction of extracellular ferricyanide by cells was originally attributed to the transfer of reducing equivalents from the intracellular NADH [6]; however, NMR experiments [17] and investigations into the role of ascorbate in PMRS activity performed by May et al. [23–27] have indicated that the main source of reducing equivalents for ferricyanide reduction is ascorbate [26]. Transmembrane AFR reductase activity has also been identified in the erythrocyte membrane [24, 25, 35, 36]. The intracellular ascorbate supplies an electron that reduces extracellular AFRs to extracellular ascorbate. The role of AFR reductase is thus to recycle extracellular ascorbate from its oxidized AFR; this has been suggested to represent a physiologic function of the erythrocyte PMRS [37].

Our observation of the activation of the erythrocyte PMRS and AFR reductase activity by resveratrol suggests that resveratrol can also donate electrons to the PMRS for reduction of extracellular oxidants and for recycling of ascorbate. The PMRS/AFR reductase-driven reduction of extracellular AFRs has been shown to be an electrogenic process, indicating that vectorial electron transport is involved in the reduction of extracellular AFRs [36]. The electron donating ability of resveratrol is slightly less than that observed with quercetin. The ability of resveratrol to activate the erythrocyte PMRS and AFR reductase may provide protection to the plasma membrane against oxidative changes in addition to helping to maintain plasma reduced ascorbate levels (Fig. 4). Wright et al. [37] demonstrated hydrogen ion transfer to be the dominant mechanism of action for phenolic antioxidants; however, the mechanisms of action of resveratrol are not completely understood despite its wide range of biological activities. It has been established that the electron donating ability of polyphenols depends on the position and degree of hydroxylation

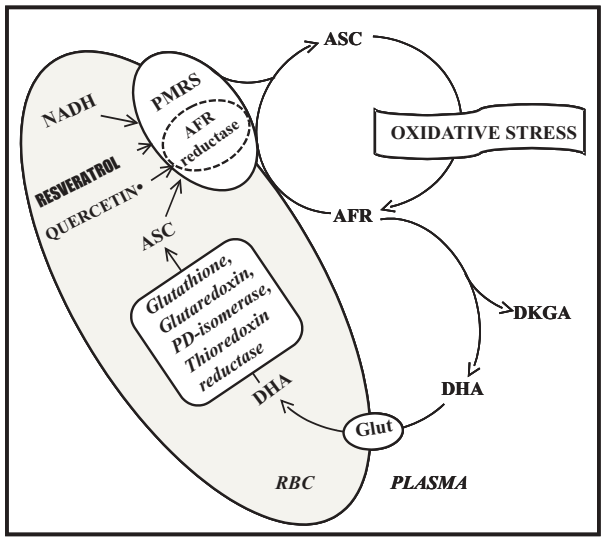

Fig. 4. Schematic diagram of the involvement of the erythrocyte PMRS and AFR reductase in maintaining intracellular and extracellular redox state. In addition to ASC and NADH, it is now known that quercetin and resveratrol can also donate electrons to the PMRS, which in turn transports electrons outside the cell. AFR reductase reduces extracellular AFR to ASC through a one-electron transfer. This scheme provides a mechanism for recycling ASC between erythrocytes and plasma. ASC – ascorbate, AFR – ascorbate free radical, DHA – dehydroascorbate, DKGA – diketogulonic acid, Glut – glucose transporter, RBC – red blood cell

[29], the reducing ability being enhanced by the presence of hydroxyl groups at the three and five positions. Resveratrol has three hydroxyl groups that participate in an extensive three-dimensional hydrogen-bonding network. The hydrogen bonding due to the molecular packing in the crystal structure demonstrates the ready mobility of up to three hydrogen atoms per resveratrol molecule. These hydrogen atoms may be transferred to reactive cell species to neutralize their harmful effects [7]. It has also been shown that the *para*-4-hydroxy group in resveratrol is more acidic than the two *meta*-hydroxy groups and therefore, any chemical or biological features that modify the acidity of resveratrol will probably increase its activity [5, 7].

Because activation of the erythrocyte PMRS and AFR reductase has been considered as a compensatory mechanism operating to counterbalance increased oxidative stress [30, 31], the role of resveratrol in activating the erythrocyte PMRS and AFR reductase may assume significance in all disease conditions in which there is a decrease in plasma antioxidant potential. This may, in part, explain the efficacy of the antioxidant effect of resveratrol in conditions/diseases that are accompanied by increased oxidative stress.

In conclusion, we provide evidence to show that resveratrol can enter human erythrocytes and accumulate in higher concentrations compared to plasma. At this higher concentration, resveratrol plays an important role in activating the erythrocyte PMRS and AFR reductase activity, which results in transfer of reducing equivalents to be transported to the extracellular compartment. This property provides resveratrol a novel mechanism to exert its antioxidant effect at concentrations that are achievable in plasma.

Conflict of interest: None.

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