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A new role of red wine in modulating erythrocytes antioxidant defense

Stefania Moccia [1*§], Idolo Tedesco [1§], Carmela Spagnuolo [1], Maria Russo [1], Carmen Cervellera [1], Gian Luigi Russo [1]

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[1] National Research Council, Institute of Food Sciences, 83100 Avellino, Italy; idolo.tedesco@isa.cnr.it; carmela.spagnuolo@gmail.com; mrusso@isa.cnr.it; carmencervellera@libero.it; gianluigi.russo@gmail.com
* Correspondence: : stefania.moccia@isa.cnr.it; Tel.: +39 0825299 261
§ Equal contribution

Abstract: Dealcoholated red wine has been shown to exert protective effects, reducing the risk of cardiovascular events by improving endothelium function and inhibiting platelet aggregation. These biological activities have been associated with the polyphenolic component of red wine, suggesting that the pool of polyphenols, as flavonoids and anthocyanins, could be responsible for its functional effects. Here, we hypothesize a new role of red wine polyphenols (RWp) in modulating antioxidant potential of erythrocytes, protecting against oxidative stress. We previously demonstrated that RWp activated an important enzymatic system involved in neutralizing plasma free radicals, namely Plasma Membrane Redox System (PMRS). The present work investigates the underlying mechanism triggered by RWp in the activation of PMRS via the involvement of intracellular GSH. Hence, the increase of GSH intracellular concentration results from the activation of GSH-dependent enzymes, namely glutathione reductase (GR) of about 30% in the presence of RWp (73 μg/ml Gallic Acid Equivalents). Changes in GSH pathway induced by RW were associated with a slight but significant increase of ROS (reactive oxygen species) concentration. We conclude that the pro-oxidant effect of RWp promotes an adaptive stress response in human erythrocytes, which improves their antioxidant defense protecting them from oxidative stress.

Keywords: red wine polyphenols; PMRS; erythrocytes; antioxidant; adaptive response

1. Introduction

The protective role of red wine against oxidative stress in red blood cells (RBCs) has been widely reported by *in vivo* and *in vitro* evidence [1-5]. One of the main mechanisms involved in red wine protective effect involves the antioxidant response, since its high content of antioxidant compounds, namely polyphenols. In our previous study, we demonstrated for the first time that RWp activated PMRS, an important regulator of homeostasis and redox state of RBCs [6]. This transmembrane enzymatic system is also involved in reducing oxidative stress by neutralizing plasma free radicals through electron transfer. PMRS activity is mediated by GSH, one of the most important intracellular donors of electrons to this system. Here, we investigated the related-mechanism induced by RWp to protect human RBCs from oxidative stress through a potential activation of PMRS, suggesting a new role of RWp in regulating antioxidant defense.

1. Materials and Methods

2.1 Red wine polyphenols (RWp)

We tested an experimental red wine made from "Aglianico'' grapes and obtained by a microvinification process, as we previously reported [6]. Red wine samples were dried and suspended in 0.01 N HCl for the biological assays. RWp was measured by Folin-Ciocalteu's assay and quantified as μg/ml gallic acid equivalent (GAE), whose presence is significant in red wine [7]. The anthocyanin content was determined using a pH shift method and results were expressed as μM of malvidin-3-glucoside (M3GE), the main anthocyanin of red wine [8].

2.2 Preparation of red blood cells (RBCs)

Human blood samples were isolated from healthy donors, who have provided their informed consent for this study, which are kept in the Blood Donation Centre at the Division of Onco-Hematology of "San Giuseppe Moscati" hospital in Avellino. The participants were not-smokers, males and females with an average age of 41.2. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-treated tubes and RBCs were isolated through consecutive centrifugations and washing in phosphate buffer saline (PBS) to remove plasma, platelets, and buffy coat.

2.3 Determination of Plasma Membrane Redox System (PMRS)

To measure PMRS activity, RBCs ($8x10^5$ RBCs/μl) were diluted with PBS and incubated at 37 °C with RWp (73 μg/ml GAE) for 10′. RBCs were washed with PBS and treated with a mixture containing PBS, 5 mM glucose and 1 mM $K_3Fe(CN)_6$ at 37 °C for 30′. After centrifugation at 1800x*g*, the supernatants were collected for PMRS assay as we previously reported [6,9]. Absorbance was measured at 540 nm and results were expressed as picomoles ferrocyanide/10^6 RBCs/min.

2.4 Measurement of GSH

RBCs ($8x10^5$ RBCs/μl) were diluted with PBS and incubated at 37 °C with RWp (73 μg/ml GAE) for 10′. Samples were washed with PBS, and solubilized with trichloroacetic acid (TCA) solution (5% v/v in 0.1 M HCl and 10 mM EDTA). Samples were treated with phtaldialdehyde (0.5 mg/ml) and 10 mM EDTA and fluorescence of supernatants was measured at 340 nm (excitation wavelength) and 460 nm (emission wavelength) [10]. The micromolar concentration of GSH was calculated from a standard curve of pure GSH.

2.5 Reactive oxygen species (ROS) measurement

RBCs ($8x10^5$ RBCs/μl) were treated for 30′ with 20 μM DCFDA, a non-fluorescent compound that can cross the cellular membrane. Once inside the cell, DCFDA is hydrolyzed to dichlorofluorescein, which reacts with the intracellular peroxide and gives rise to 2′,7′-dichlorofluorescin (DCF), detected spectrofluorimetrically. After incubation, RBCs were washed with PBS and treated for 1′ with RWp (73 μg/ml GAE). ROS production was measured fluorometrically with excitation and emission settings at 495 and 530 nm, respectively [11].

2.6 Activity of glutathione reductase (GR)

RBCs ($8x10^5$ RBCs/μl) were diluted with PBS and treated with RWp (73 μg/ml GAE) for 2′. After incubation, samples were centrifuged at 1800x*g*, washed with PBS and lysed with 5 mM phosphate buffer, pH 8.0 containing phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 11000x*g*, supernatants were used for enzymatic assays. GR activity was measured according to [12]. The

oxidation of NADPH to $NADP^+$ during the reaction of GSSG reduction, was measured at 340 nm. After 5′ of incubation at 37 °C with 5.1 μM FAD 0.16 mM NADPH 0.49 mM EDTA, the reaction was started with the addition of 1.95 mM GSSG. The absorbance was measured after 30′ and the specific enzymatic activity was expressed as nmol/min/ml RBCs.

2.7 *Statistical analysis*

Data are presented as mean values±standard error (SE) and the significance was measured by the use of Student's test of at least five determinations.

3. Results

3.1 Role of RWp in regulating PMRS activity

In our experiments, we employed experimental "Aglianico" red wine and we firstly verified if volatiles compounds and ethanolic components did not harm RBSs through measurement of hemolysis (data not shown). We characterized the total polyphenol content and anthocyanin contents, resulting in 2190±0.05 μg/ml GAE and 109.7±0.8 μM M3GE, respectively. This polyphenol content falls within the range reported in the literature for red wines [13]. Hence, the role of red wine in modulating RBCs antioxidant system was observed through the increase of PMRS activity, which represents one of the key defense mechanisms of RBCs against oxidative stress [14]. In our previous study, we performed a dose-response experiment to select the minimum effective concentration of red wine to be tested in the assays [6]. Here, we evaluated the PMRS activity after RBCs treatment with RWp (73 μg/ml GAE), observing a significant increase in PMRS of about 50% with respect to untreated RBCs (CTRL) (Figure 1, a). We hypothesized that the increase of PMRS activity induced by RWp could be related to the increase of GSH intracellular concentration since GSH is involved in the function of this system as an intracellular donor of electrons to the transmembrane enzyme complex [15]. Indeed, at the same time of treatment with RWp (73 μg/ml GAE), we detected the increase of GSH intracellular levels, as shown in Figure 1, b. We previously confirmed that PMRS activity was mediated by GSH and we also demonstrated that the role of red wine in increasing PMRS is GSH-like, by employing two GSH modulators, IAC, inducing depletion of GSH, and NAC, a synthetic precursor involved in *de novo* synthesis of GSH [6].

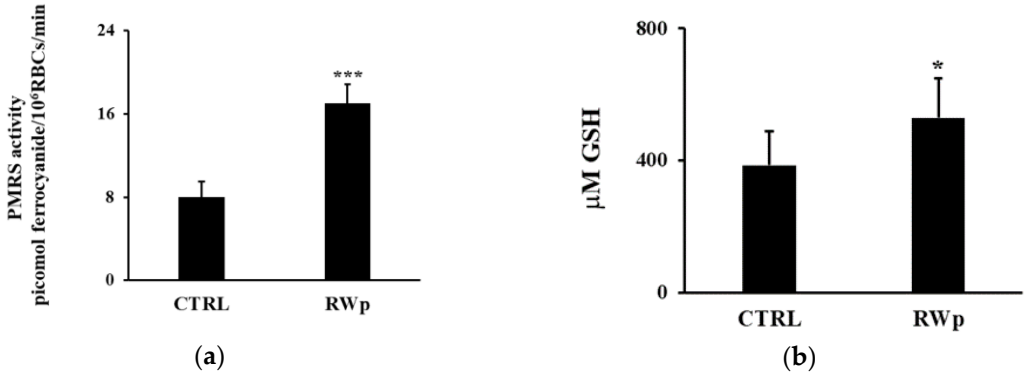
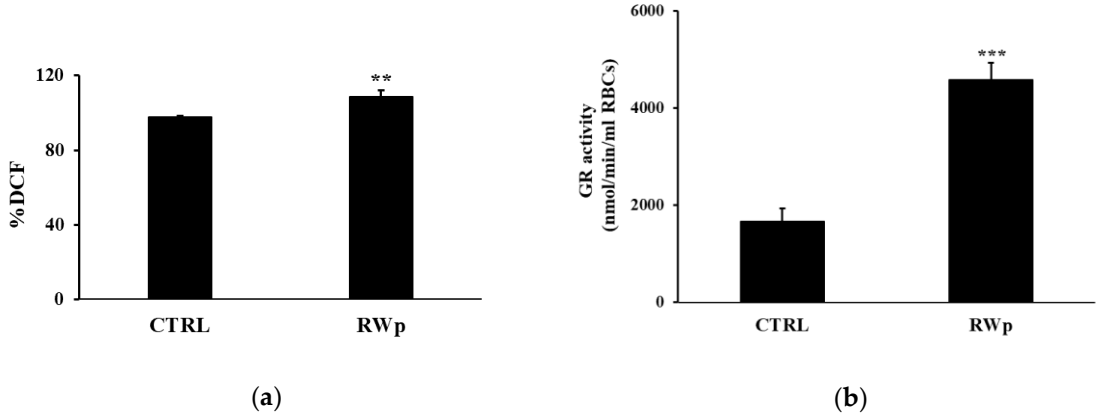


Figure 1. (**a**) RWp effect on PMRS activity in RBCs. PMRS activity was evaluated as reported in "Materials and Methods" and expressed as pmol ferrocyanide/10^6 RBCs/min. Data represent the means of samples from 5 donors in duplicate ± standard error (SE). Symbols indicate significance: ***$p<0.001$ with respect to untreated control (CTRL). (**b**) Effect of RWp on GSH concentration in RBCs.

GSH (μM) concentration was measured as described in "Materials and Methods". Data represent the means of samples from 5 donors in duplicate ± standard error (SE). Symbols indicate significance: *p<0.05 respect to untreated control (CTRL).

3.2 Modulation of RBCs antioxidant systems by RWp

We reasoned if RWp could increase ROS production, which was responsible for the GSH-related anti-oxidant response. As reported in Figure 2, a when we treated RBCs with RWp (73 μg/ml GAE), we detected a significant increase of about 10 % of ROS concentration. Based on this evidence, we supported that RWp could exert a slight pro-oxidant effect, which could be responsible for the induction of an antioxidant response, as RBC defense mechanism.



(**a**) (**b**)

Figure 2. (**a**) ROS production induced by RWp in RBCs. Intracellular ROS concentration was expressed as % DCF and measured as reported in "Materials and Methods". Data represent the means of samples from 5 donors in duplicate ± standard error (SE). Symbols indicate significance: **p<0.01 with respect to untreated control (CTRL). (**b**) GR activity of RBCs induced by RWp. GR enzymatic activity was measured as described in "Materials and Methods" and expressed as nmol/min/ml RBCs. Data represent the means of samples from 7 donors in duplicate ± standard error (SE). Symbols indicate significance: ***p<0.001 with respect to untreated control (CTRL).

Considering the rapid activation of PMRS, we excluded the possibility that GSH could be due to *de novo* synthesis. Alternatively, we suggested that the conversion from GSSG to GSH could be involved in the activation of PMRS via increased levels of GSH. To explore this possibility, we measured the activity of GR, the enzyme responsible for the conversion of GSSG to GSH. RWp (73 μg/ml GAE) increased GR activity significantly with respect to untreated control (CTRL), indicating the maximum enzymatic activity at 2' of treatment (Figure 2, b).

4. Discussion

Our data suggest the existence of novel mechanisms triggered by RWp leading to the protection of RBCs by plasma oxidizing species. These mechanisms involve the increase of RBCs antioxidant defense by activating PMRS. The importance of this system is closely related to the increased oxidative stress in plasma, that occurs during aging. To support this view, the strengthening of RBCs antioxidant systems, including GSH and GSH-dependent enzymes, exerted by RWp, allow counteracting the oxidative injuries, that constantly damage RBCs due to their biological role of oxygen transporters. An attractive hypothesis suggests that RWp act as pro-oxidant, inducing a slight, but significant increase of intracellular ROS, which is responsible for a cellular adaptive response. Indeed, the presence of ROS at low concentrations like those promoted by RWp seems to be involved in normal cellular function, as well as disease prevention [16]. This circumstantial

evidence supports our hypothesis that RWp generate a low concentration of H_2O_2, which induce cellular stress adaptation to oxidative stress resulting in increasing RBCs antioxidant defense. This aspect highlights the preventive role of polyphenols, which, through this mechanism of cellular adaptation against oxidative stress, can prevent the onset of degenerative and age-depending diseases related to oxidative damage.

Author Contributions: S.M. and I.T. conceived, designed, performed the experiments and wrote the manuscript; C.S. performed the experiments, M.R. and C.C. review; G.L.R. provided ideas and financial support. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:
RWp: Red wine polyphenols
PMRS: Plasma Membrane Redox System
RBCs: Red blood cells
GAE: Gallic acid equivalent
M3GE: Malvidin-3-glucoside
EDTA: Ethylenediaminetetraacetic acid
PBS: Phosphate buffer saline
TCA: Trichloroacetic acid
DCF: 2′,7′-dichlorofluorescin
PMSF: Phenylmethylsulfonyl fluoride

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