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Red Wine Inhibits Aggregation and Increases ATP-diphosphohydrolase (CD39) Activity of Rat Platelets *in Vitro*

Elisabetta Caiazzo[1], Idolo Tedesco[2], Carmela Spagnuolo[2], Gian Luigi Russo[2], Armando Ialenti[1] and Carla Cicala[1*]

[1]*Department of Pharmacy*, *University of Naples "Federico II"*, *Naples*, *Italy*
[2]*Institute of Food Sciences*, *National Research Council*, *Avellino*, *Italy*

cicala@unina.it

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Moderate consumption of red wine has been shown to exert a peculiar cardioprotective effect compared with other alcoholic beverages; inhibition of platelet aggregation seems to be one of the mechanisms underlying this beneficial effect. CD39/ATP-diphosphohydrolase is an integral membrane glycoprotein metabolizing ATP and ADP to AMP; in concert with CD73/ecto-5'-nucleotidase, it contributes to extracellular adenosine accumulation. CD39 is considered a key modulator of thrombus formation; it inhibits platelet aggregation by promoting ADP hydrolysis. There is evidence that red wine consumption increases CD39 activity in platelets from streptozotocin-induced diabetic rats. Here we show that two kinds of Aglianico red wines inhibit aggregation and increase ATP – and ADPase activity in rat platelets.

Keywords: ADP, CD39, Platelets, Rat, Red wine, Thrombosis.

Wine is an alcoholic beverage derived by yeast fermentation of grapes harvested from *Vitis vinifera.* Wine, in particular red wine, has been shown to exert a peculiar cardioprotective effect compared with other alcoholic beverages; inhibition of platelet aggregation seems to be one of the mechanisms underlying this beneficial effect. The antiplatelet effect of red wine has been supposed to be due to a reduced prostanoid synthesis, an increased nitric oxide production and/or an enhanced platelet c-AMP level [1,2]; however, the mechanism is still unclear. CD39 (ATP-diphosphohydrolase) is an integral membrane glycoprotein metabolizing ATP and ADP to AMP; in concert with CD73 (ecto-5'-nucleotidase), it contributes to extracellular adenosine accumulation [3]. CD39 is considered a key modulator of thrombus formation, since it may inhibit platelet aggregation promoting ADP hydrolysis and, in combination with CD73, promotes adenosine accumulation that is a vasodilator and a platelet antiaggregating agent [4-7]. Loss of CD39 activity from the endothelium sustains platelet aggregation and thrombogenesis [8-13]. There is evidence that moderate red wine consumption increases CD39 activity in platelets from streptozotocin-induced diabetic rats [14]; furthermore, flavonoids, which are major wine constituents and well known vasoprotective agents, also increase ATP - and ADPase activity in rat serum [15]. Here, we compared two different red wines, "R" (GAE, gallic acid equivalent, 1.09 mg/mL) and "F" (GAE, 2.19 mg/mL), produced from Aglianico grapes in 2008 and 2011 respectively. Concentrations of different classes of compounds present in the two red wines were investigated and their antioxidant activity was evaluated. The wines presented values that were different, according to the different vintage. As expected, anthocyanin content was higher in the youngest one ("F"; 2011) possibly for its limited concentration of anthocyanin polymers. Both FRAP and DPPH assays showed a direct correlation between the high concentration of phenolic compounds in F 2011 and its antioxidant capacity (Table 1).We found that both wines "R" and "F" inhibited ADP-induced platelet aggregation evaluated in PRP; it is worth noting that while "R" was active only at low ADP concentrations, the effect of "F" was evident even at the highest ADP concentration (Figure 1).

Table 1: Total flavonoids, phenols,anthocyanins and antioxidant properties of Aglianico red wines.

Wine	Total phenol content (mg/mL GAE)	Total flavonoids content (mM QE)	Total anthocyanin content (μM M3GE)	FRAP (mM Fe^{2+} equivalent)	DPPH (% quenching)
F 2011	2.19 ± 0.1	26.92 ±0.77	109.71 ± 10.18	7.60 ± 1.04	80.05 ±1.29
R 2008	1.09 ±0.002	15.17 ± 0.19	37.69 ± 2.88	4.94 ±0.67	53.60±2.9

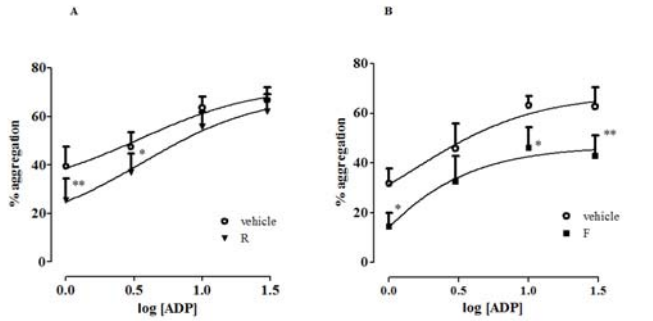

Figure 1: The effect of wines "R" (panel A) and "F" (panel B) on concentration – response curve to ADP. PRP was incubated with either wine (R or F, 10 μL) or with the vehicle (ethanol 12 %) and aggregation in response to ADP (1-30 μM) was evaluated. Each point represents the mean ± standard error of n = 5. * p< 0.05 and ** <0.01 vs. vehicle (two ways ANOVA followed by Bonferroni's test).

Both wines also inhibited washed platelet aggregation induced by THR (0.2 U/ mL); the biological activity was still present following wine dilution; also in this case "F", containing higher levels of flavonoids, anthocyanins, and phenols, and also increased antioxidant activity compared with "R", was more active than "R" (Figure 2).

Both wines increased ATP and ADPase activity evaluated in platelet lysates (Figure 3); this increase had a functional significance since CD39 inhibitor, ARL67156, reversed the inhibitory effect of wines on platelet aggregation without modifying vehicle –treated platelet aggregation (Figure 4).

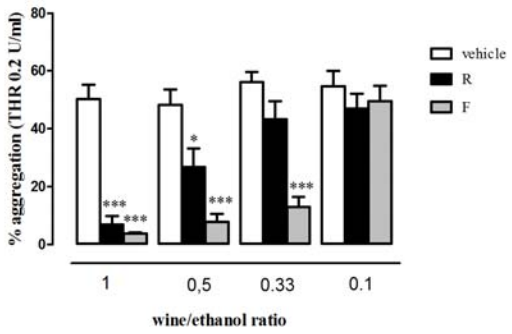

Figure 2: The effect of different dilutions of wine "R" and of wine "F" on THR – induced aggregation of washed platelets. * p <0.05 and p< 0.001 (one way ANOVA followed by Dunnett's test, n =6) vs. vehicle (ethanol, 12 %).

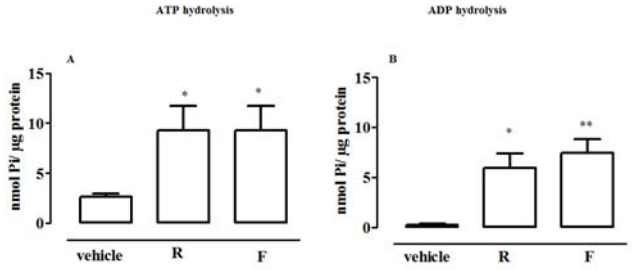

Figure 3: Effect of wine "R" (panel A) and wine "F" (panel B) on ATP – (panel A) and ADP (panel B) – hydrolysis mediated by CD39 by washed platelets. Platelet ATP – and ADPase activity was evaluated by measuring Pi released as described in the experimental section. * p<0.05 and ** p<0.01 vs. vehicle (one way ANOVA followed by Dunnett's test; n =5).

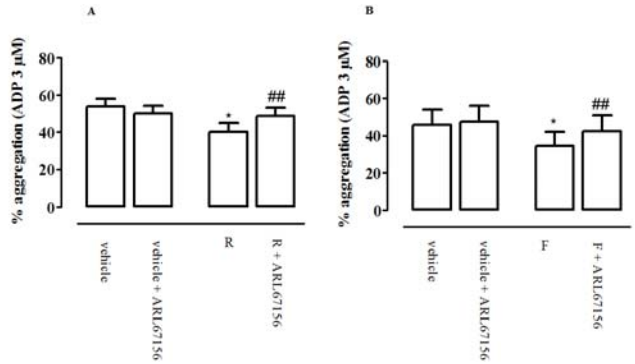

Figure 4: The effect of wine "R" (panel A) and wine "F" (panel B), or of the vehicle (ethanol 12%), on platelet aggregation induced by ADP (3 µM) in presence of CD39 inhibitor (ARL67156, 100 µM). * p<0.05 vs. vehicle; ## p<0.01 vs. R (A) and vs. F (B); n= 5.

In the end, following incubation with "F" wine, there was increased platelet expression of CD39. Conversely, wine "R" did not modify platelet CD39 expression (Figure 5).

Thus, we show that two different kinds of Aglianico red wines inhibit platelet aggregation, and concomitantly increase platelet ATP and ADPase activity; of particular interest is the finding that these effects may be obtained with the wine *in toto*. Furthermore, the youngest wine, "F", was more active than "R", in accordance with its content of total flavonoids, phenols and anthocyanin and its increased antioxidant activity compared with "R". Thus, we suggest that a moderate wine consumption through increasing ATP-diphosphohydrolase (CD39) activity of platelets and avoiding ADP accumulation may offer protection from thrombosis.

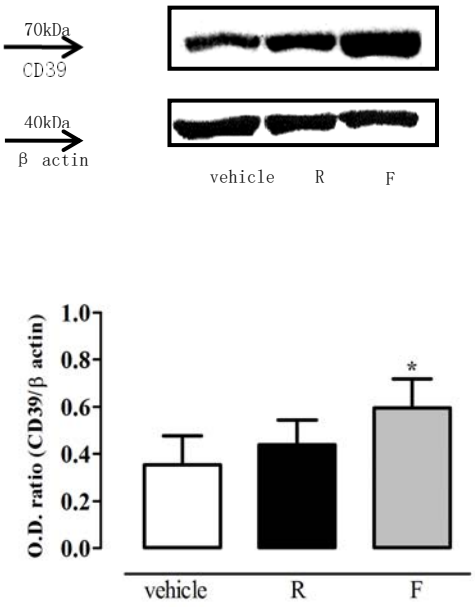

Figure 5: Representative results of Western blot analysis of CD39 expression on platelets following incubation with wine "R" or "F" or with the vehicle (ethanol 12 %). Graph bar represents optical density (O.D.) relative to β actin . * p<0.05 vs. vehicle (repeated measures ANOVA followed by Dunnett's test, n = 5).

Experimental

Preparation of red wines: Two different "Aglianico" red wines were employed in the present study: one from the 2010 vintage (indicated with the letter F 2011) and the other from the 2007 vintage (named R 2008). Both wines were obtained following controlled microvinification procedures. Shortly, grapes from *Vitis vinifera* cv. were harvested from vineyards located in the area surrounding the town of Avellino (Campania, Italy). After grape destemming and crushing, must was treated with potassium metabisulfite (100 mg/L of must) and enzymes were added (2 g/hLLafazym CL; Polsinelli, Frosinone, Italy). Subsequently, must was fermented in stainless steel tanks (100 L) at 22-23°C in the presence of 20 g/hL of *Saccharomyces cerevisiae* strain D254 (Lallemand Inc., Castel d'Azzano, Italy). Maceration of the pomace lasted 10 days; subsequently, the must was pressed to obtain the final wine. After 24 h, wines were racked from gross lees, 100 mg/L of potassium metabisulfitewas added, and the wine bottled. No malolactic fermentation occurred. The wines were analyzed at bottling time. After opening, samples were divided into aliquots, stored in the dark at room temperature and used once for all experiments.

Total flavonoid, phenol and anthocyanin contents: The total flavonoid content was determined according to Bunea [16]. "F" and "R" wines (10 µL) or standard (quercetin up to 50 µM) were diluted to a final volume of 1 mL with distilled water. After the addition of 5% $NaNO_2$(60 µL), the mixture was incubated at room temperature for 5 min. Then, an equal volume of 10% $AlCl_3$was added and incubated for 6 min before the addition of 1 N NaOH (400 µL). Finally, absorbance was measured against blank at 510 nm. Total flavonoid content was expressed as millimolar of quercetin equivalent (QE). The total phenol contents of the wines were determined using the Folin-Ciocalteau's method of Singleton and Rossi [17]. Briefly, red wines (1 µL) or standard (gallic acid up to 100 µM) were diluted with distilled water to 1 mL. After the addition of 50 µLFolin-Ciocalteau's reagent, the mixture was incubated for 5 min at room temperature. At the end of incubation, 100 µL of 20% $Na_2CO_3$was added and the absorbance measured at

760 nm. Results was expressed as mg/mL of gallic acid equivalents (GAE).The anthocyanin content of wines was estimated using a pH shift method [18]. The results were expressed as micromolar of malvidin-3-glucoside (M3GE), the major anthocyanin present in red wines, using the molar extinction coefficient of 28.000 M^{-1} cm^{-1}.

Antioxidant activity of red wines: Antioxidant power was determined by 2 different methods. The Ferric Reducing Ability Power (FRAP) assay was as reported by Benzie [19] and results expressed in millimolar of a solution of pure $FeSO_4$ (up to 40 μM). Briefly, red wines (2μL) were added to a mixture containing 1.7 mM $FeCl_3$ and 0.85 mM of 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) in 300 mM acetate buffer, pH 3.6 (100 μL final volume) and incubated at 37°C for 6 min. Subsequently, absorbance was measured against blank at 590 nm (Microplate reader, Synergy HT BioTek, Milan, Italy).The second method to determine the antioxidant capacity, the 2,2-diphenyl-1-picrylhydrazyl (free radical DPPH) free radical scavenging assay, was performed according to the Wan-Ibrahim procedure, with some modification [20]. One mL of a methanolic solution of DPPH (100 μM) was incubated with "F" and "R" wines (1 μL), vortexed and incubated at room temperature for 30 min before absorbance measurement at 517 nm. The antiradical activity was defined as the amount of antioxidant necessary to decrease the initial DPPH absorbance. The radical scavenging activity was expressed as percentage of quenching.

Platelet preparation and aggregation: Blood was withdrawn by cardiac puncture from male Wistar rats (Harlan Nossan, 200-250 g) slightly anesthetized with enflurane, and anticoagulated with 3.8 % (w/v) trisodium citrate (1:9, v/v). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described [21]. Platelet count in PRP was performed by a cell counter *(*Beckman Coulter s.r.l., Milano, Italy) and adjusted to 3 x 10^5 platelets / μL with autologous PPP. Platelet aggregation was monitored by a light transmission aggregometer (Chrono-Log, Coorporation, Mod.490, USA) by measuring changes in turbidity of 0.25 mL of re-calcified ($CaCl_2$ 1mM) PRP warmed at 37°C and under continuous stirring. A concentration response curve (1-30 μM) to ADP was evaluated. To evaluate the effect of red wines "R" (1.09 mg/mL GAE) and "F" (2.19 mg/mL GAE) on ADP – induced platelet aggregation, 10 μL of either red wine ("R" or "F") or of ethanol (12 %, v/v) was added to PRP, and after 2 min incubation, a response curve to ADP (1 – 30 μM) was performed. In some cases, the effect of different wine dilutions (ratio wine / ethanol, 0.5; 0.33; 0.10) was evaluated on aggregation induced by ADP (3μM). Experiments were also performed in the presence of CD39 inhibitor, ARL67156 trisodium salt; in brief, following 1 min PRP incubation with either wine or vehicle, ARL67156 (100 μM final concentration) or vehicle (distilled water) was added to the platelet suspension, and, after 1 min, aggregation to ADP (3μM) was evaluated.

Washed platelets preparation: Platelets were washed by adding to PRP an equal volume of citric/trisodium citrate buffer (*plus* prostaglandin E_1,1 μM) and centrifuged at 3000 rpm x 15 min. The platelet pellet was re-suspended in calcium - free Tyrode's buffer to the concentration 3 x 10^5/ μL. Platelet suspension was incubated with 10 μL of either wine or ethanol, as described above, and aggregation to thrombin (THR, 0.2 U/mL) was evaluated.

Western blot analysis: Washed platelets were incubated with either wine or ethanol, as described above, and then centrifuged at 2500 rpm at 25°C for 15 min. Platelets were homogenized in the following buffer: 50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% sodium deoxycholate (0.25%); 1 mM phenylmethylsulphonyl fluoride; 10 mg/mLaprotinin; 20 mM leupeptin; 1 mM sodium orthovanadate. Total protein concentration in lysates was determined by Bradford assay, using BSA (bovine serum albumin) as standard. Proteins were separated on 8% SDS polyacrylamide gels and blotted onto nitrocellulose membrane. The membranes were saturated by incubation with 5% non-fat dry milk in PBS supplemented with 0.1% Tween-20 (PBS-T) for 30 min at room temperature and then incubated with anti-CD39 goat antibody (1:200; Santa Cruz Biotechnology, Italy) overnight at 4°C. Successively, membranes were washed and then incubated for 2 h at room temperature with the secondary antibody conjugated with horseradish peroxidase, and anti-goat IgG-HRP (1:2000; Dako, Denmark). Immune reactive proteins were visualized by enhanced chemiluminescence using Image Quant 400 GE Healthcare software (GE Healthcare, Italy). Successively, to confirm the equal protein loading, membranes were stripped and incubated with anti β-actin monoclonal antibody (1:2000; Sigma-Aldrich, Italy) and subsequently with anti-mouse IgG-HRP (1:2000; Dako, Denmark), both for 120 min at room temperature.

Measurement of platelet ATP - and ADPase activity: In another set of experiments, platelet lysates (50 μg) obtained from washed platelets incubated with red wines or vehicle, as described above, were pre-incubated in 200 μL of reaction buffer containing 10 mM NaCl; 5 mMKCl; 60 mM glucose; 5 mM $CaCl_2$ and 50 mM Tris-HCl buffer, pH 7.5, at 37°C for 10 min. The enzyme reaction was started by the addition of either ATP or ADP to a final concentration of 1 mM; after 40 min at 37°C, the reaction was stopped by the addition of 200 μL of trichloroacetic acid (TCA). Following sample centrifugation at 3000 rpm for 10 min, at 37°C, the release of Pi was measured using malachite green as a colorimetric reagent and KH_2PO_4 as standard [22]. To have the net value of Pi produced following enzymatic reaction, aspecific Pi released in the absence of either ATP or ADP in each sample was evaluated and the value obtained was subtracted from the value obtained following incubation with the substrate. Protein concentration was measured by Bradford assay, using bovine serum albumin as standard, and results were expressed as Pi released nmol/μg protein.

Statistical analysis: All results are expressed as mean $\pm$ S.E. (n=4 - 6). Concentration – response curves were represented by non- linear regression and analysed by two way analysis of variance (ANOVA) followed by Bonferroni's test. In other cases, one way ANOVA followed by Bonferroni's or Dunnett's test, as appropriate, was used. All statistical analysis was performed using GraphPad, Prism V5.0 (Graphpad software, California, USA). A value of $p<0.05$ was considered significant.

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