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Effect of resveratrol on platelet aggregation by fibrinogen protection

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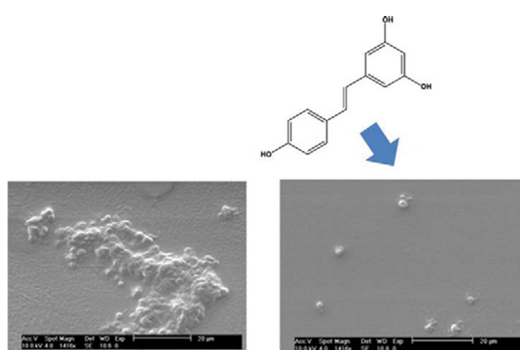
HIGHLIGHTS

- This study shows the ability of resveratrol to preserve fibrinogen from denaturation and platelets from aggregation.
- SEM analysis revealed that the presence of resveratrol completely inhibited the platelet aggregation.
- The degree of platelet adhesion decreased by increasing the resveratrol concentration.
- The protective properties of resveratrol can be explained on the basis its ability to bind the catecholamine.

GRAPHICAL ABSTRACT



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ABSTRACT

The effect of resveratrol (RSV) in inhibiting platelet adhesion and aggregation, as well as fibrinogen (FBG) conformational changes promoted by epinephrine (EP), were studied, by using complementary experimental techniques.
NMR and IR spectroscopies were used to investigate possible protective effects by RSV towards FBG, in presence of EP. The protective effect of RSV towards FBG was highlighted by spin nuclear relaxation experiments that were interpreted for determining the thermodynamic equilibrium constants of FBG-EP interaction, and by infrared measurements, that showed EP-induced conformational changes of FBG.
The ability of RSV in inhibiting platelet adhesion and aggregation promoted by EP was evaluated by scanning electron microscopy (SEM), measuring the platelet adhesion and aggregation degree, in comparison to data obtained for platelet aggregation in platelet rich plasma (PRP).
The experimental combined approach pointed out that RSV is able to protect both FBG and platelets from the denaturant and aggregating action of EP at stress level concentration.

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1. Introduction

Blood platelet activation plays a crucial role in many important physiological and pathological processes, such as several arterial disorders, including strokes and myocardial infarction [1]. The activation of

Abbreviations: RSV, 3,5,4′-trihydroxy-*trans*-stilbene; FBG, fibrinogen; EP, epinephrine; SEM, scanning electron microscopy; PS, polystyrene; PRP, platelet rich plasma.

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platelets is also considered to be an important step in inflammatory processes, i.e. synthesis and release of inflammatory substances like cytokines and prostanoids. It is well known, that blood platelets also participate in allergic and non-allergic inflammation and tumor progression [2].

Natural products having antioxidant properties are useful for understanding the mechanism of pathologies onset. Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, RSV) exhibits a wide range of biological activities, including anti-inflammatory and anti-tumoral effects [3]. RSV may be responsible for the protection against coronary heart diseases [4,5]. The mechanism of RSV action has not been totally elucidated, but it seems to be related to its antioxidant activity, its ability to inhibit ribonucleotide reductase, DNA polymerase and cyclooxygenase 2 (COX-2) transcription, and its action as agonist for the estrogen receptor [6]. RSV inhibits the biological activity of blood platelets [3,7], causing reduction of thrombin-induced aggregation, decreasing platelet adhesion to type I collagen [8] and fibrinogen (FBG) [9] and inhibiting platelet aggregation induced by ADP, collagen, and thrombin [7,9]. RSV also acts as an antioxidant against free radical generation in blood platelets [10] and may be responsible for modulating signaling pathway.

Several studies relevant to platelets morphology and ultrastructure of platelets interacting with other species, i.e. fibrin networks in animals and humans, via microscopy techniques (TEM and SEM) are reported [11]. The studies allow to verify the modification of animals platelets morphology (including humans, from which they are originated) and the aggregation stage induced by the presence of coagulant and/or anti-aggregation species like epinephrine (EP) and/or resveratrol (RSV), respectively.

FTIR studies on protein conformational changes induced by several substances, surfaces or physical parameters, have also been reported [12,13,14], shedding light on the relation between proteins structure and their bioactivity.

Thus, it is interesting to underline the power of an integrated characterization approach based on spectroscopic/microscopic studies and biological tests. On these reasoning, the RSV effect towards platelet adhesion and aggregation induced by EP was evaluated together with the antioxidant ability to preserve the FBG native conformation in the presence of EP with the aim of understanding the potential effects of antioxidant compounds in regulating physiological or pathological processes. Moreover, platelet aggregation and protein denaturation processes were studied by using relaxation and infrared spectroscopy measurements, compared with biological data also of exploiting chemical-physical methods in biological approaches.

Improving the safety and functioning of blood-contacting devices, often requires the development of strategies that allow to avoid adverse reactions, particularly platelet adhesion and aggregation at the implants surface. Although, the effect of RSV in reducing platelet aggregation in platelet rich plasma (PRP) was largely demonstrated [15,16], the ability of this molecule to influence platelet adhesion and aggregation at the interface with polymeric surfaces has never been proved. The results obtained and reported in this paper show that RSV is able to reduce platelet aggregation induced by epinephrine not only in PRP but also on polystyrene. The presence of the antioxidant compound really contributes to enhance the hemocompatibility of blood-contacting devices.

2. Experimental section

2.1.1. Materials

Epinephrine (EP) and *trans*-resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, RSV) were purchased from Sigma Aldrich Chemie GmbH (Buchs, Switzerland) and used without further purification. Fibrinogen (FBG, Human Plasma, MW = 341 kDa) was purchased from Calbiochem (San Diego, USA) and used without further purification.

All materials used in platelet adhesion and aggregation studies were supplied by Sigma Aldrich (Switzerland).

2.1.2. NMR measurements

All the ^{1}H spectra were recorded on a Bruker AMX 400. The solutions were obtained by dissolving the appropriate amounts of epinephrine (EP) and resveratrol (RSV) in D_2O.

The spin-lattice relaxation rates were measured using the (180°-τ-90°-t)$_n$ sequence. The τ values used for the selective and non-selective experiments were the following: 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.8, 1, 1.4, 2, 3, 4, 5, 8, 20 s respectively, and the delay time (t) was 20 s. The 180° selective inversion of the proton spin population was obtained by a selective soft Gaussian perturbation pulse (width: 60 ms, attenuation level: 120 dB) [17]. The FID (Free Induction Decay) was processed using an exponential window function with line broadening of 1 Hz. The selective and non-selective spin-lattice relaxation rates refer to the H-6 of epinephrine. Since in general the recovery of proton longitudinal magnetization after a 180° pulse is not a single exponential, due to the sum of different relaxation terms, the selective spin-lattice relaxation rates were calculated using the initial slope approximation and subsequent three parameter exponential regression analysis of the longitudinal recovery curves. The maximum experimental error in the relaxation rate measurements was 5%. The affinity index was calculated by linear regression analysis of the experimental data.

All the spectra were processed using the Bruker Software XWINNMR, version 2.5 on Silicon Graphics O_2 equipped with RISC R5000 processor, working under the IRIX 6.3 operating system.

2.1.3. Calculation of the affinity index

The analysis of non-selective R_1^{NS} and selective R_1^{SE} proton spin-lattice relaxation rates, allows to determine the *affinity index* for the ligand-macromolecule equilibrium, as previously reported in literature [18,19,20].

To remove the effects of different correlation times and different proton densities, and to isolate the effects of restricted motions, due to the interaction of the ligand with the macromolecule, it a *normalized affinity index* should be used [21]:

$$\Delta R_{1N}^{SE} = \frac{K R_{1b}^{SE}[M_0]}{(1 + K[L]) R_{1f}^{SE}} \quad (1)$$

where K is the thermodynamic equilibrium constant, R_{1f}^{SE} and R_{1b}^{SE} are the selective spin-lattice relaxation rates for the free and bound ligand fractions, M_0 and L are the macromolecule and ligand concentrations, respectively.

The dependence of the normalized relaxation rate, enhancements ΔR_{1N}^{SE} by the macromolecule concentration $[M_0]$ is represented by a straight line passing through the origin of the axes with slope:

$$\left[A_l^N\right]_L^T = \frac{K R_{1b}^{SE}}{(1 + K[L]) R_{1f}^{SE}} \quad (2)$$

$[A_l^N]_L^T$ is still a constant at constant temperature and ligand concentration and it is defined as *normalized affinity index* (dm^3 mol^{-1}).

2.1.4. Calculation of the binding constants of epinephrine-fibrinogen complexes

Plotting $1/\Delta R_1^{SE}$ as function of the ligand concentration $[L]$, a linear correlation is obtained, the slope (S) and the intercept (I) of the straight line being:

$$S = \frac{1}{R_{1b}^{SE}[M_0]} \quad (3)$$

and

$$I = \frac{1}{K R_{1b}^{SE}[M_0]} \quad (4)$$

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