An in vitro study of adrenaline effect on human erythrocyte properties in both gender

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Abstract. The possibility that erythrocytes may function as a reservoir for noradrenaline and adrenaline and as a modulator of circulating catecholamine concentrations had been suggested. The aim of this work was to study the adrenaline effect on erythrocyte membrane fluidity, acetylcholinesterase (AChE) enzyme activity, P50 and erythrocyte deformability and also to verify if the role of adrenaline on erythrocyte properties is sex-dependent. Blood samples from 42 healthy donors were obtained, and its aliquots incubated 30 min without (control) and with 10^{-5} M concentrations of adrenaline alone (A1) and adrenaline with an α and an β-blocker (A2). Results demonstrate that initial AChE values in female are higher (p ≤ 0.01) than male values. In female, adrenaline decreases AChE activity either when α and β-adrenergic receptors are blocked (p ≤ 0.01) or when they are not. In male, adrenaline increases AChE activity when none of adrenergic receptors are blocked. Control values of male and female erythrocyte membrane fluidity are very similar but behaviour became differently (p ≤ 0.05) when adrenaline is present because it decreases male and increases female values. Gender differences in erythrocyte deformability are verified at high shear stress values (p ≤ 0.02). In female we have also registered the existence of an inverse significant correlation (r = -0.62) between membrane rigidity and AChE activity in A2 values. Adrenaline increases p50 values (p ≤ 0.03) in both sexes. Peripheral blood film has shown echinocytes when adrenaline 10^{-5} M is present. We conclude that in this in vitro study sex-related differences in erythrocyte acetylcholinesterase enzyme activity, membrane fluidity and erythrocyte deformability under adrenaline influence were found.

Keywords: Adrenaline, erythrocyte, erythrocyte membrane fluidity, acetylcholinesterase enzyme activity, P50, erythrocyte deformability, gender

1. Introduction

Several authors have suggested that erythrocytes are involved in the sequestration and inactivation of circulating catecholamines and thus in the modulation of adrenaline concentrations [29] which may have implications on the blood vessels tonus regulation of microcirculation. When incubated “in vitro” at 37°C these blood cells accumulate catecholamines against a concentration gradient. The uptake process was temperature dependent and saturable, both phenomena characterising an active transport system [1,4].

The possibility that erythrocytes may function as a reservoir for adrenaline had also been suggested [4]. Others have proposed a direct influence of catecholamines on erythrocyte functions and properties [29]. A considerable fraction of circulating catecholamines is bound to a specific high-affinity serum protein and a small fraction is also taken up by platelets [29].

We have also based our work on taking into account the presence on the erythrocyte membrane of functional β and α adrenergic receptors with which adrenaline can interact [10,14,16,30]. Besides, in the outer leaflet of the erythrocyte membrane is registered the existence of an important protein that
contribute for the membrane integrity called acetylcholinesterase (AChE). Anchored to the erythrocyte membrane through a covalently linked glycolipid residue, this enzyme is reported by several investigators as been modulated by lipid environment and other membrane surface phenomena [26].

Another important property of erythrocyte, considered as a hemorheologic factor, is its deformability required for normal passage of erythrocytes through the capillaries [7]. The deformability of the normal human erythrocyte is a consequence of its low cytoplasmatic viscosity, its excess of surface membrane area in relation to cell volume, and its viscoelastic cell membrane [11]. The erythrocyte membrane cytoskeleton has also a valorous role on erythrocyte deformability. Haemoglobin is able to bind to those cytoskeletal elements and is responsible for the transport of oxygen to tissues. The haemoglobin affinity to oxygen is experimental measured and referred as P50 values.

Even though all the erythrocyte studies it is hard to believe that only a little is known about eventual membrane erythrocyte properties and functions differences between gender.

The aim of this work was to study the effect of adrenaline $10^{-5}$ M on red blood cell properties namely erythrocyte membrane fluidity, erythrocyte acetylcholinesterase (AChE) enzyme activity, P50 values and erythrocyte deformability and to verify if the role of adrenaline on red blood cell properties is sex-dependent.

2. Material and methods

2.1. Subjects

In order to achieve the aim of our work, we have selected 42 Caucasians healthy donors (21 males and 21 females) after their voluntary approval. The average age was of 24 ± 2 years old and the mean haemoglobin values was of 16.40 ± 0.8 g/dl in males and 13.10 ± 0.5 g/dl in females.

We have considered the following exclusion parameters: the existence of diseases or clinical syndromes related to blood or metabolism, the existence of present medication, more precisely β-agonists and β-blockers or even alcohol intake.

2.2. Blood sampling aliquots and incubation procedure

The experimental approach of blood sample aliquots is presented in the Fig. 1. Venous blood (3 ml) was obtained after 15 minutes in the recumbent position and collected in tubes with heparin (10 U/ml) as anticoagulant [13]. Total blood was divided in 3 aliquots of 1 ml each and then we centrifuged at 12,000 rpm (5414 Centrifuge Eppendorf®, Sotel) during a short period time of 1 minute. After this procedure 10 µl of plasma were replaced by the same amount of α and β-agonist or α and β-blockading as represented in Table 1, so that the final plasma concentration of the effectors was $10^{-5}$ M. All aliquots were incubated 30 minutes at room temperature.

We have chosen adrenaline for α and β-agonist because it is an ubiquitous physiological molecule. Nadolol was used as β-blockading and tolazoline as α-blockading. Both these latter substances do not have intrinsic adrenergic activity, are competitive and specific blockading and have a rather low lipid partition coefficient.

2.3. Measurements

Erythrocyte acetylcholinesterase (AChE) enzyme activity – Determined by Ellman’ spectrophotometric method modified by Kaplan et al. [5]. This enzyme could be an index expresses of membrane integrity.
Fig. 1. Schematic representation of blood samples aliquots manipulation in absence and presence of effectors (adrenaline $10^{-5}$ M, tolazoline $10^{-5}$ M and nadolol $10^{-5}$ M) in order to further biochemical parameters determinations (erythrocyte acetylcholinesterase enzymatic activity, erythrocyte membrane fluidity, erythrocyte deformability) – see text.

**Erythrocyte membrane lipid fluidity** – Membrane fluidity is usually defined as the mobility status of the membrane lipids. In this work we want to study only hydrophobic membrane fluidity, determined by the method of fluorescence polarization with a probe (1,6-biphenyl-1,3,5-hexatriene or DPH) to hydrophobic region of membrane. Increased values of fluorescence polarization obtained with DPH means decreased membrane lipid fluidity ability (or the inverse which means increased rigidity) [6]. All the fluorescence spectroscopy measurements were carried out in a Hitachi F-3000 fluorescence spectrophotometer (Tokyo, Japan) and the fluorescence polarization intensities recorded at excitation wavelength of 352 nm and emission wavelength of 430 nm.

**Erythrocyte deformability** – Depends mainly on three variables: cytoplasm viscosity, membrane stiffness and the favourable surface to volume ratio. The results were reading in a Myrenne gmbh rheodyn SSD shear stress diffractometer [27,28].

P$_{50}$ is the partial pressure of oxygen that half-saturates haemoglobin and was determined in Hemox$^\text{TM}$-Analyser.

**M.C.H.C. (mean cell haemoglobin concentration)** – Obtained from the quotient between haemoglobin concentration values and the haematocrit ones. Haemoglobin was measured in Osm$^\text{TM}$ 3 Hemoximeter$^\text{TM}$ [17]. Haematocrit was performed by mycrohaematocrit method in ALC 4223 Centrifuge [17].

**Peripheral blood films** were performed from all aliquots, using May–Grunwald–Giemsa coloration.

2.4. **Statistical analysis**

Results are expressed as mean value ± standard deviation.

The unpaired Student $t$-test was performed to estimate the statistical significance of differences between the values of parameters measured of male and female blood. The null hypothesis was rejected for a significance level of $p = 0.05$.

Correlation among all the variables considered was done.
Table 1

Values (mean value ± standard deviation) of the erythrocyte acetylcholinesterase (AChE), fluorescence polarization (determined with DPH), erythrocyte deformability, CMHG, P50, obtained in blood sample aliquots of both sexes in absence (control) and presence of adrenaline 10^{-5} M alone (A1) or together with tolazoline and nadolol (A2).

<table>
<thead>
<tr>
<th></th>
<th>AChE (units/min/mgHb)</th>
<th>DPH (nd)</th>
<th>Erythrocyte deformability 6 Pa</th>
<th>Erythrocyte deformability 30 Pa</th>
<th>CMHG</th>
<th>P50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control aliquot</td>
<td>285.7 ± 15.1^{a,b}</td>
<td>0.298 ± 0.03</td>
<td>5.12 ± 1.7</td>
<td>49.9 ± 4.4^c</td>
<td>35.2 ± 1.6</td>
<td>32.07 ± 2.012^a</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>276.1 ± 17.4</td>
<td>0.317 ± 0.03^a</td>
<td>5.56 ± 1.9</td>
<td>50.2 ± 4.5^a</td>
<td>36.1 ± 1.7</td>
<td>34.9 ± 3.53^a</td>
</tr>
<tr>
<td>A2</td>
<td>247.6 ± 17.1^b</td>
<td>0.284 ± 0.06</td>
<td>4.65 ± 1.7</td>
<td>49.3 ± 4.3^b</td>
<td>34.7 ± 1.7</td>
<td>–</td>
</tr>
<tr>
<td>Control</td>
<td>230.8 ± 16.2^a</td>
<td>0.298 ± 0.03</td>
<td>4.59 ± 1.8</td>
<td>45.6 ± 6.2^c</td>
<td>36.1 ± 1.06</td>
<td>30.8 ± 1.29^b</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>260.1 ± 15.2</td>
<td>0.278 ± 0.04^a</td>
<td>4.57 ± 1.8</td>
<td>45.4 ± 5.6^a</td>
<td>35.1 ± 1.3</td>
<td>32.04 ± 5.39^b</td>
</tr>
<tr>
<td>A2</td>
<td>236.2 ± 16.5</td>
<td>0.288 ± 0.03</td>
<td>3.29 ± 1.1</td>
<td>43.5 ± 5.8^b</td>
<td>35 ± 1.06</td>
<td>–</td>
</tr>
</tbody>
</table>

*p* 0.01 0.05 ns 0.02 ns 0.05

3. Results

The summary of the results obtained after blood aliquots manipulation as described above in material and methods is shown in Table 1.

3.1. Erythrocyte AChE enzyme activity

The results obtained show that initial erythrocyte AChE activity female values (285.7 ± 15.1 u/min/mg Hb) were significantly higher ($p \leq 0.01$) than male values (230.8 ± 16.2 u/min/mg Hb). In female, in comparison to control, adrenaline $10^{-5}$ M decreases significantly ($p \leq 0.01$) AChE activity when $\alpha$ and $\beta$-adrenergic receptors are blocked (247.6 ± 17.1 u/min/mg Hb vs 285.7 ± 15.1 u/min/mg Hb) but the difference have no statistical significance when they are not blocked (276.1 ± 17.4 u/min/mg Hb vs 285.7 ± 15.1 u/min/mg Hb). In male, adrenaline seems to increase (but not significantly) AChE when none of adrenergic receptors are blocked (260.1 ± 15.2 u/min/mg Hb vs 230.8 ± 16.2 u/min/mg Hb; Fig. 2).

3.2. Erythrocyte membrane lipid fluidity

Control values of male and female membrane fluidity are very similar (male: 0.2982 ± 0.03 nd; female: 0.2986 ± 0.03 nd). These values are different ($p \leq 0.05$) after adding adrenaline because it increases female (0.317 ± 0.03 nd) and decreases male DPH values (0.278 ± 0.04 nd; Fig. 3). It means that adrenaline $10^{-5}$ M increases female erythrocyte membrane rigidity and, in other hand, increases male erythrocyte membrane fluidity.

In female we have also registered the existence of an inverse significant correlation ($r = -0.62$) between membrane rigidity and AChE activity in A2, which means that in female with an increase of membrane hydrophobic region rigidity we may expect a decrease of that enzyme activity if both adrenergic receptors were blocked.

![Fig. 2. Mean values and standard deviation of erythrocyte acetylcholinesterase activity in 42 blood samples (male: 21; female: 21) in absence (control) and presence of adrenaline [10^{-3} M] alone (A1) or together with tolazoline [10^{-3} M] + nadolol [10^{-5} M] (A2). Statistically significantly to $p \leq 0.01$ (*).](image-url)
Fig. 3. Mean values and standard deviation of fluorescence polarization obtained with the probe DPH in 42 blood samples (male: 21; female: 21) in absence (control) and presence of adrenaline [10\(^{-5}\) M] alone (A1) or together with tolazoline [10\(^{-5}\) M] + nadolol [10\(^{-5}\) M] (A2). Statistically significantly to \(p \leq 0.05\) (*).

Fig. 4. Mean values of erythrocyte deformability to 0.6; 6 and 30 shear stress in 30 blood samples (male: 15; female: 15) in absence (control) and presence of adrenaline [10\(^{-5}\) M] alone (A1) or together with tolazoline [10\(^{-5}\) M] + nadolol [10\(^{-5}\) M] (A2). Statistically significantly to \(p \leq 0.05\) (*) and to \(p \leq 0.01\) (#).

3.3. Erythrocyte deformability

As we can see in Fig. 4, erythrocyte deformability gender differences \((p \leq 0.02)\) are found, although at high shear stress values only. This fact is verified in absence (Control) and presence of adrenaline alone (A1) or together with adrenergic blockers (A2).

3.4. \(P_{50}\) values

Figure 5 compares male and female \(P_{50}\) blood values in presence of adrenaline to those without that catecholamine. The difference between these values is statistically significant \((p \leq 0.01)\) in both sexes. MCHC values were not statistically different between male and female blood aliquots.
3.5. Peripheral blood films

In the control peripheral blood film we can see normal shapes of erythrocytes or a very little number of echinocytes but when adrenaline $10^{-5}$ M is present the peripheral blood film have shown a large number of echinocytes (Figs 6 and 7).

4. Discussion

In our *in vitro* study we were able to show that adrenaline $10^{-5}$ M increases erythrocyte $P_{50}$ values (Table 1) which means a decrease of haemoglobin affinity to oxygen. This fact may be understood in accordance with results of other study that had shown an increase of 2,3-biphosphoglycerate (2,3-BPG) in presence of adrenaline [24]. It is well known that 2,3-BPG is a negative modulator of oxygen haemoglobin affinity thus the experimental increased $p_{50}$ values confirms this metabolic effect of adrenaline.
Adrenaline $10^{-5}$ M seems to be responsible for the existence of echinocytes in peripheral blood film in both sexes (Fig. 6) which could result from alterations of adenosine triphosphate (ATP) content or pH modifications (work in study).

The gender differences observed in erythrocyte deformability at high shear stress values may not be explained by different internal viscosity because MCHC is similar between women and men. Thus, other erythrocyte properties must be studied (membrane stiffness and surface to volume ratio) to explain these results. On the other hand, attending to the erythrocyte deformation values obtained in both sexes at low shear stress, we think that erythrocyte membrane flexibility in female is near close of male one.

In this work we also found sex-related differences in AChE enzymatic activity and membrane fluidity under adrenaline $10^{-5}$ M influence. Besides the increase of male AChE enzymatic activity values in presence of adrenaline or the decrease of that values in female, the true is that if both β and α adrenergic receptors are blocked we find the same profile of decreasing AChE enzymatic activity under adrenaline influence in both sexes. These results are in accordance with purified AChE studies described of our laboratory (not published) who stated a decrease in purified AChE activity in presence of adrenaline. If these phenomena have any relation with a direct binding of adrenaline to the native form (erythrocyte intact) of the enzyme is a matter of more sophisticated methodological approach.

The profile of erythrocyte membrane fluidity under adrenaline influence is the opposite of that one observed in AChE activity either in male or in female (Fig. 3). This fact forces us to think that this catecholamine may interact with erythrocyte membrane and then interfering with AChE activity independently of sexes. According with this theory is the external membrane localization of AChE that makes it vulnerable not only to the effects of some molecules in the blood circulation but also that ones that could interfere with the structure molecular organisation state of the membrane.

The discovery of sex-related differences in erythrocyte AChE activity and in the membrane hydrophobic region fluidity under adrenaline influence only if β and α adrenergic receptors are free is a fact never described in the literature, as far as we know. These molecular and functional differences obtained with human erythrocyte in this study could contribute to understand different responses, attitudes and behaviours to stress situations verified usually between sexes. The existence of sexually determined characteristics, at the cellular level, have important implications that may influence how we are affected by disease and medication, the respective tissues responses to these situations and even how long we live. It is known that drugs side effects are of different severity depending on gender, besides some drugs are more effective in women [22].
It has become increasingly clear that to improve medicine for both sexes, more studies need to be done to know molecular differences between male and female and to determine how sex influences susceptibility to disease and functioning of organs and organ systems. Agree to that is Pardue, who says that being male or female encompasses far more than reproductive systems and hormones [22].

Sex appears to play a role in the severity and incidence of several diseases, including heart disease [18], obesity, and rheumatoid arthritis. Some authors registered differences between pre-menopausal women and age-matched men in morbidity from cardiac diseases and especially from myocardial infarction [21]. To this fact may also contribute differences in haemorheological properties like erythrocyte aggregation [3,21]. Other data points to more pronounced haemorheological and humoral abnormalities in men than in women with essential hypertension that may also contribute to the increased incidence of cardiovascular events in men [8].

Animal and humans studies demonstrated that also some vascular responses are affected by the sex of the study subject [2,12,17–20], like contraction of carotid arteries to serotonin (that seems to be enhanced in female mice) or even endothelium-derived NO-dependent vasodilatation enhanced by oestrogen in up regulating NO synthase [21,25,31].

These kinds of studies must be continued in order to improve therapies, even that means the use of different molecules to treat the same pathology depending of gender. Is known from literature that adrenaline infusion and exercise induce activation of haemostasis [31] but it would be more interesting to study possible different biochemical responses to stress between women and men that may explain apparent social different behaviours.

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References


