RESEARCH ARTICLE

Effects of indomethacin on erythrocyte nitric oxide metabolism and erythrocyte deformability

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Abstract

Increased erythrocyte filtration index (decreased erythrocyte deformability; ED) was observed in patients with Behçet disease. When patients with Behçet took indomethacin, decreased values of erythrocyte acetylcholinesterase enzyme activity were obtained. An inverse association between nitric oxide (NO) efflux from erythrocytes and ED values was verified in blood samples of patients with vascular inflammatory diseases.

The aim of this *in vitro* study was to verify the effect of indomethacin on ED, NO efflux, nitrite nitrate, peroxynitrite and nitroso- glutathione (GSNO) in the presence of protein tyrosine kinase (PTK Syk), protein tyrosine phosphatase (PTP), adenylyl cyclase (AC) and phosphatidylinositol 3 kinase (PI3K) enzyme inhibitors respectively Syk inhibitor, calpeptin, MDL and wortmannin (WORT). Blood samples from ten healthy donors were divided and aliquots were performed in absence (control aliquot) and presence of indomethacin or indomethacin plus each one of the inhibitors. Further ED and NO efflux and its metabolites were evaluated by the usual methodology.

A significant increase in ED values was noted in the presence of indomethacin plus WORT. No significant differences where verified in NO efflux from erythrocytes in all blood aliquots. Significantly increase in GSNO concentration in the presence of indomethacin plus calpeptin, MDL or Wort were observed. Nitrate and peroxynitrite concentrations increased or decreased significantly respectively in all aliquots in relation to the control one. The nitrite levels increased in the aliquots with indomethacin and indomethacin plus MDL, or indomethacin plus Syk.

In conclusion indomethacin act as antioxidant decreasing peroxynitrite, not changing NO efflux values and increasing nitrate levels independent of band 3 phosphorylation degree or cyclic adenosine monophosphate (cAMP) concentration. Indomethacin mobilizes nitrite and GSNO in dependant of band 3 phosphorylation degree and cAMP. Indomethacin did not change erythrocyte deformability, unless in combination with wortmannin which increased it.

Keywords: Indomethacin; erythrocyte deformability; nitric oxide; antioxidant.

1. Introduction

Indomethacin (indo) is one of the nonsteroidal anti-inflammatory drugs (NSAIDs) with analgesic and antipyretic actions. Beyond those therapeutics effects also micro molecular actions are attributes to indo. Indomethacin is highly amphiphilic and associates strongly with lipid membranes enhancing immiscibility of saturated and unsaturated lipids as observed using large uni lamellar vesicles experiments performed *in vitro* (1, 2). Indo induces the formation of gel-phase domains in lyposomes as a mixed model membrane bilayers (1). Later, it was evidenced that indo generates in artificial and in plasma cell membrane micro environment modifications in protein localization and function (3). Erythrocytes or red blood cells (RBCs) undergo irreversible normal discocyte shape to echinocyte one, when they are exposed to high indo concentrations (4). Lower dose of indo is necessary not to be exceeded in blood circulation to avoid the absence of erythrocyte reversible shape changes or disturbances in the ability to deform. The erythrocyte deformability (ED) is indispensable for travel through the microcirculatory vessels network with diameter lower than RBCs.

Increased values of RBC filtration index (decreased RBC deformability, ED), plasma viscosity, and red blood cell (RBCs) membrane enzyme acetylcholinesterase (AChE) activity were observed in patients with Behçet disease (5). This disease is a chronic, inflammatory, vascular disorder with a wide spectrum of clinical presentations. It has been noted that, when Behçet`s patients were in active phase and took indomethacin, there was decreased plasma viscosity and AChE activity. This effect was maintained over a 4 month follow-up (5).

The erythrocyte membrane AChE has two biological functions acting as enzyme and as a membrane receptor (6, 7). So, binding to acetylcholine (ACh) originates AChE-ACh active complex state which associates to Gi protein, adenylyl cyclase (AC) and band 3 protein protein (8,9). The AChE conformations states are influent in the amount of nitric oxide (NO) efflux from erythrocytes as evidenced by us (9).The RBCs band 3, known as the anion exchanger 1 is a transmembrane protein containing tyrosine residues able to become phosphorylated by protein tyrosine kinase (PTK), namely kinases $p72^{syk}$ and $p53/56^{lyn}$ and dephosphorylated by protein tyrosine phosphatase (PTP) (8,10). Several *in vitro* experiments using spectro-photometric, spectrofluorometric, photoly-sis, and chemiluminescence, immunopreci-pitation methods and compounds that block the anion transport property of band 3 was shown that the influx and the efflux of NO into or from erythrocytes occurs through band 3 protein (11, 12). AChE- ACh active state is a positive modulator of the PKC enzyme activity which in turn activates by phosphorylation phosphatidy-linositol 3 kinase (PI3K) which activates phosphodiesterase 3 with decrease of cyclic adenosine monophosphate (cAMP) as reviewed by us (6).

We have observed that the ED values increase in the presence of AChE-ACh active complex form (13). In patients with vascular disease showing lower ED values, an inverse association between ED and NO efflux has been demonstrated (14).

The aim of this *in vitro* study was to verify the effect of indomethacin in ED, NO efflux, nitrite nitrate, peroxynitrite and nitroso- glutathione (GSNO) in the presence of PTK Syk, PTP, AC and PI3K inhibitors, namely Syk inhibitor, calpeptin, MDL and wortmannin (WORT).

2. Material and Methods

2.1. Reagents

p72syk inhibitor, adenylyl cyclase inhibitor (MDL hydrochloride) were purchased from Sigma (St Louis, MO, USA). Protein tyrosine kinase (PTK) inhibition Syk, a PTK p72syk inhibitor and calpeptin (PTP inhibitor) was purchased from Calbiochem (Darmstadt, Germany). Nitrate reductase from Aspergillus Niger, NADPH (tetra sodium salt), sodium nitrate, sodium nitrite and atropine were all purchased from Sigma Chemical Co., St Louis, MO, USA. The Griess Reagent kit was purchased from Molecular Probes, Eugene, USA. Sodium chloride was purchased from AnalaR (UK) and chloroform and ethanol 95% from MERCK, Darmstadt, Germany. Blood samples were collected into BD Vacutainer TM tubes with Lithium heparin (17UI/mL) as an anticoagulant. This in vitro study was performed according to the protocol established by the Portuguese Institute of Blood in Lisbon. All male donors (N=10; aged between 30 and 40 years old) were duly informed and signed their agreement. Written, informed consent was obtained by all donors (n=10; all male; aged 30-40 years old).

2.2. Blood sampling and experimental model

Blood was supplied, according to protocol, by the Portuguese Institute of Blood, Lisbon. Blood samples were collected into tubes with lithium heparin (17 IU/ml) as anticoagulant. Each blood sample was divided into nine aliquots of 1 ml each and centrifuged at 11 000 rpm (Biofuge 15 centrifuge, Heraeus) for one minute at room temperature. Thereafter, 10 μL of plasma were replaced by the same volume of either physiological serum (control aliquot) or indomethacin 10^{-5} M or indomethacin plus

each inhibitor, so that the final concentration of the inhibitor in the whole blood aliquots was 10^{-5} M. Besides, other concentrations (5×10^{-5}, 10^{-6} and 5×10^{-6} M) were tested, and no significant alterations were observed with these concentrations in relation to 10^{-5} M. The blood samples were then homogenized by gentle inversion and incubated for 15 minutes at room temperature. Deformability in all aliquots was assessed and thereafter the NO efflux from RBC and the other NO were determined following using the methods described in 2.3.

2.3. Measurement of erythrocyte NO efflux, nitrite, nitrate, GSNO and peroxynitrite

Following incubation, blood samples were centrifuged and sodium chloride 0.9 % with pH 7.0 was added to establish a hematocrit of 0.05%. The suspension was mixed by gentle inversion.

In order to quantify amperometric NO quantification, we used the amino-IV sensor (Innovative Instruments Inc. FL, USA), according to the method described previously (15). NO diffuses through the gas-permeable membrane tripleCOAT of the sensor probe and it is then oxidized at the working platinum electrode, resulting on an electric current. The redox current is proportional to the NO concentration outside the membrane and was continuously monitored with a computerized inNOTM system, (with a software version 1.9, Innovative Instruments Inc., Tampa, FL, USA). Calibration of the NO sensor was performed daily. For each test, the NO sensor was immersed vertically in the erythrocyte suspension vials and allowed to stabilize for 30 min to achieve basal NO levels. 30 μl of acetylcholine was added to

the erythrocyte suspension samples in order to achieve final concentrations of 10μM of ACh and NO. Data was recorded from the constantly stirred suspensions at room temperature.

The measurement of nitrite/nitrate concentration was performed using the spectrophotometric Griess method as already described (16), after submitting the pellet of each centrifuged blood sample to haemolysis and haemoglobin precipitation. Haemolysis was induced with distilled water and hemoglobin precipitation with a mixture of ethanol and chloroform (5v/3v). The nitrite concentrations were measured with the spectrophotometric Griess reaction, at 548 nm. For nitrate measurement, this compound was first reduced to nitrites in presence of nitrate reductase (17).

S-nitrosoglutathione (GSNO) was measured utilizing colorimetric solutions containing a mixture of sulfanilic acid (B component of Griess reagent) and NEDD (A component of Griess reagent), consisting of 57.7 mM of sulfanilic acid and 1 mg/mL of NEDD were dissolved in phosphate-buffered solution (PBS; pH 7.4). To constitute the 10 mM $HgCl_2$ (Aldrich) mercury ion stock solutions were prepared in 0.136g/50mL of dimethyl sulfoxide (DMSO) (Aldrich). GSNO was diluted to the following desired concentrations: 7,5 μM; 15 μM; 30 μM; 45 μM; 60 μM; 120 μM; 240 μM; 300 μM in the colorimetric analysis solutions. Various concentrations of mercury were then added to a final concentration of 100 μM. Following gentle shaking, the solution was let to stand for twenty minutes. A control spectrum was measured by spectrophotometry at 496 nm against a solution without mercury ion. 300 μL of erythrocyte suspensions were added to the reaction mixture and GSNO concentrations were obtained as already described (18).

For determinations of peroxynitrite levels, the erythrocyte suspensions (1mL) were incubated with 2, 7-dichlorofluorescein diacetate (DCFC-DA) 15μM, in 3 mL buffer (Pi 155 mM, pH 7.4), at room temperature for 30 minutes. Suspensions were rinsed several times and diluted in the working solution with 1.8 mL of the same buffer. The pellets were rinsed in the same buffer and used for fluorescence measurement with a Microplate Reader TECAN Infinite F500 (TECAN Trading AG, Switzerland) with excitation and emission wavelengths at 485 and 535 nm, respectively. The concentration of peroxy-nitrite was finally calculated through a calibration curve previously described (19).

2.4. Erythrocyte deformability

The erythrocyte deformability (ED) for different shear stress (0.30, 0.60, 1.20, 3.00, 12.00, 30.00 and 60.00 Pa) was determined by using the Rheodyn SSD shear stress diffractometer from Myrenne GMBH (Roentgen, Germany), and erythrocyte deformability is expressed as the elongation index (EI) in percentage (20). The Rheodyn SSD diffractometer determines RBC deformability by simulating the shear forces exerted by the blood flow and vascular walls on the erythrocytes. Erythrocytes are suspended in a viscous medium and placed between a rotating optical disk and a stationary disk. A well-defined shear force is exerted upon the suspension which forces the erythrocytes to deform to ellipsoids and align with the fluid shear stresses. If a laser beam is allowed to pass through the erythrocyte suspension a diffraction pattern appears on the opposite end. That diffraction pattern will be circular with resting erythrocytes, but becomes elliptical when the erythrocytes are deformed by shear. The light intensity of the diffraction pattern are measured at two different points

(A and B), equidistant from the center of the image. The erythrocyte elongation index (EEI) is expressed as a percentage, obtained according the following formula: EEI (%) = [(A − B)/ (A + B)] × 100. (20)

2.5. Statistical analysis

Data are expressed as mean values ± SD. Student's paired t-tests were used to compare values between different samples of erythrocyte suspensions. Statistical analysis was conducted using the Statistical Package from the Social Sciences (SPSS; version 16.0). One-way analyses of variance and paired t-tests were applied to assess statistical significance between samples. Bonferroni post-hoc tests were conducted when appropriate. Statistical significance was set at a $p<0.05$ level.

3. Results

3.1. In vitro effect of Indomethacin on erythrocyte deformability under the presence of, WORT, MDL, Calpeptin and Syk inhibitor

Table 1 shows that the values of erythrocyte elongation indexes measured at different shear stresses did not change in the presence of indomethacin or indomethacin plus MDL or plus calpeptin when comparing with the blood control aliquot. When the indomethacin and inhibitor of PTK Syk are present at lower shear stress of 0.3 Pa and 0,6 Pa a significant increase ($p<0.05$) of ED values in relation to their absence namely 3.74± 1.61 versus 2.28 ± 1.82 and 6.54 ± 0.02 versus 5.14± 1.52 respectively were observed. The indomethacin and wortmannin combination showed increase in shear stress and ED. Table 1.

Table 1: Values (Mean±SD) of erythrocyte deformability index (%) obtained in absence and presence of Indomethacin (Indo) and Indo plus phosphoinositol 3 phosphate kinase (WORT), or plus adenylyl cyclase (MDL), or plus PTP (calpeptin; Calp) and plus PTK (Syk inib) inhibitors

Shear stress (Pa)	Samples					
	Control	**Indo**	**Indo+Wort**	**Indo+MDL**	**Indo+Calp**	**Indo+ Syk$_i$**
0.3	2.28 ± 1.82	2.61 ± 1.37	3.77 ± 1.31*	3.39 ± 1.70	3.38 ± 1.74	3.74 ± 1.61*
0.6	5.14 ± 1.52	6.22 ± 2.00	6.66 ± 1.83*	6.02± 2.11	6.82 ± 2.36	6.54 ± 0.02*
1.2	13.91± 2.11	14.05 ±2.95	15.90 ± 3.03*	14.35 ± 3.41	15.44 ±4.04	14.97 ± 3.58
3	28.23 ± 4.50	28.86 ±4.13	29.98 ± 4.59*	28.63 ± 4.51	29.02 ±4.95	28.96 ± 4.42
6	338.05 ± 4.69	38.35±2.36	39.49 ± 4.42*	38.41 ± 4.38	38.66±15.10	38.51 ± 4.95
12	44.52 ± 4.42	44.99± 4.47	46.21 ± 3.91*	45.31 ± 3.93	45.30 ±4.72	4507 ± 4.57
30	47.64 ± 5.04	47.90 ±4.42	49.91 ± 4.21*	48.63 ± 4.26	48.15 ± 4.71	48.16 ± 4.89
60	47.22 ± 4.63	47.87 ±4.61	49.13 ± 4.79*	48.74 ± 4.78	48.24 ± 4.89	48.07 ± 44.89

*p< 0.05

3.2. Effect of indomethacin on erythrocyte, NO, GSNO, peroxynitrite, nitrite and nitrate in the presence of wortmannin (WORT), MDL, calpetin and Syk inhibitor,

Table 2 shows that the values of NO efflux from erythrocyte concentrations obtained in presence of indomethacin or indomethacin and each one of the inhibitors. These did not show significant changes in relation to the control blood aliquot.

When considering the GSNO concentration values inside the erythrocyte these are unchanged in indomethacin and indomethacin plus band 3 dephosphorylated (Syk inhibitor) aliquots, Table 2. The exceptions are those aliquots containing indomethacin plus high cAMP levels (WORT) or plus band 3 protein phosphorylated (calpeptin), or plus lower cAMP (MDL) in which significantly higher values of GSNO inside erythrocytes where quantified (Table 2).

Decreased values of peroxynitrite concentration in relation to control aliquot were observed in all blood aliquots in the presence of indomethacin or indomethacin plus each one of the PI3K, AC, PTP and PTK enzymes inhibitors (Table 2, Figure 1). We must highlight the great decrease of peroxynitrite noted when indomethacin is in presence of band 3 protein dephosphorylated (Syk inb) in relation to indomethacin aliquot and indomethacin plus WORT aliquot (Table 2 and Figure 1).

Nitrate values were all increased in aliquots where indomethacin or Indomethacin plus inhibitor were used (Table 2). Increased values of nitrite are observed in aliquots with indomethacin and indomethacin plus MDL (lower cAMP levels) or plus band 3 dephosphorylated (Syk inb) in relation to the control aliquots (Table 2). The exception, where no change in nitrite concentration was evaluated was in indomethacin plus WORT or plus band 3 phosphorylated (calpeptin) in relation to control aliquots (Table 2).

Table 2: Values (mean ± SD) of erythrocyte nitric oxide efflux (nM) GSNO (μM), peroxynitrite (μM), nitrate (μM) and nitrite(μM) obtained in absence [control aliquot) and in presence of indomethacin (Indo) without or plus the addition of phosphoinositol 3 phosphate kinase (WORT), or plus adenylyl cyclase (MDL), or PTP (Calpeptin) and or plus PTK (Syk inib) inhibitors.

Blood samples	Nitric oxide	GSNO	Peroxynitrite	Nitrat	Nitrite
Control	1.48±0.45	8.81±0.56	256.5±20.10	9.45±0.58	9.15±0.37
Indo	1.26±0.36	9.34±0.79	243.60±50.70[#]	10.50±0.57**	10.05±1.04[#]
Indo+WORT	1,28±0.29	9.87±0.75**	231.90±61.20[#]	10.58±0.99[#]	10.05±1.28
Indo +MDL	1.46±0.46	10.64±1.15**	221.10±71.90[#]	11.30±0.75*	10.80±1.03*
Indo+Calpeptin	1.57±0.48	11.40±1.15*	198.20±61.30[#]	11.40±0.65*	10.95±0.97
Indo+Syki	1.55±0.40	10.53±1.12	166.90±42.60[#]	11.85±0.68[#]	11.35±0.63[#]

[#]p<0.05; *P<0.001; **p<0.005 in relation to the control
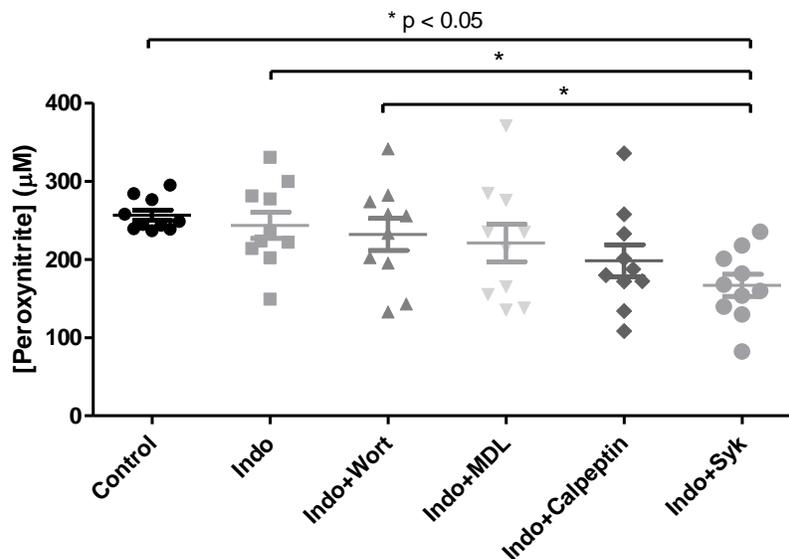
Figure 1. Profile of erythrocyte peroxynitrite values (mean±SD) obtained under the absence (control) and presence of indomethacin (indo) or plus the addition of phosphoinositol 3 phosphate kinase (WORT), or plus adenylyl cyclase (MDL), or PTP (Calpeptin) and or plus PTK (Syk inib) inhibitors.

4. Discussion

We have previously shown unchanged ED values at all shear stress in blood samples of healthy donors in presence of Syk, PTP, AC or PI3K inhibitors, with an increase of extracellular potassium concentrations (21). The effects of these inhibitors in ED may change its values due the presence of the external compound indomethacin. In the present study, we found that the presence of indomethacin in blood aliquots did not change erythrocyte deformability values at any shear stress in relation to the control aliquot (Table 1). The same unchanged ED was verified when indomethacin is under the effect of band 3 phosphorylation by PTP inhibition with calpeptin or under low cAMP levels by AC inhibition (Table 1). Nevertheless, indomethacin increases ED when band 3 is dephosphorylate by Syk inhibitor at lower shear stress (Table 1). Also, increased ED resulted from inactive states of PI3K inhibited by wortmannin (Table 1). PDE3 is activated by PI3K in a directly dependent way (22). The inhibition of PI3-K by WORT prevents the activation of phosphodiesterase 3 (PDE3) with increase of the levels of cAMP allowing the activation of the cAMP- protein kinase dependent (cAMPPK) (23, 24). The cAMPPK phosphorylates pyruvate kinase to convert it to an inactive form (24). Inhibition of glycolysis and promotion of the pentose phosphate pathway and decreases of oxidative stress environment in erythrocytes is expected. The present study confirms the impaired peroxynitrite amount inside erythrocyte in presence of indo alone and also independent of band 3 phosphorylation degree by PTK or PTP inhibition and cAMP levels modulation in presence of indo (Table 2). These data demonstrate the antioxidant effect of indomethacin.

The values of NO efflux from erythrocyte do not change in any experimental blood aliquots studied, as seen in Table 2. The lower peroxynitrite levels obtained (Table

2) allow us to assume that the auto-oxidation of hemoglobin with production of peroxide anion to combine with the NO did not occur inside the erythrocytes (25). Our results point to the anti-inflammatory action of indomethacin as previous described (26).

It is known that NO efflux from RBCs increases or decreases according to the AChE active, less active and inactive forms [8]. In the present study with blood samples obtained from healthy humans, the unchanged values of NO efflux from erythrocytes in presence of indomethacin seems that there are no AChE enzyme activity or conformation modifications. Also the same happens in aliquots with indo plus internal modification of band 3 phosphorylation status or cAMP levels changes. However, it is already known that, AChE enzyme activity increases and decreases when band 3 is phosphorylated or dephosphorylated (8,9). So, indo in a certain unknown way avoided the modification of AChE conformations induced by PTK, PTP inhibitors. The indo 10μM used in the present study, unchanged the level of membrane AChE that was seen to be release from human erythrocyte membranes, as acetylcholinesterase-containing vesicles, by the anionic echino-cytogenic compounds like indo 150 mM (27, 28).

Different reactions occurring inside erythrocytes can explain the elevated amounts of nitrate obtained in all aliquots in relation to the control ones, Table 2. The decomposition of peroxynitrite molecules leads to nitrite and nitrates which are designated NO derivatives molecules (NOx) (29). The reaction between peroxynitrite and hemoglobin generates SNOHb, could decompose to nitrosothiol and nitrate (29). However, NO could reduce

oxyhemoglobin to methemoglobin along with the formation of nitrate without any variation in methemoglobin concentration (30). This could be due to the presence of hemoglobin reductase coupled with the NADH produced in the glycolytic pathway (31).

The nitrites concentration showed increase levels in blood aliquots with indo and indo plus band 3 protein dephosphorylation or plus AC inhibitior, Table 2. The erythrocyte NO reservoir molecule GSNO maintain unchanged in presence of indo or indo plus band 3 dephosphorylated but otherwise its levels increase when indo is present plus band 3 protein dephosphorylation or indo plus AC or plus PI3K inhibitors Table 2. So, the present study showed that RBCs GSNO concentration is independent of the presence of indomethacin but dependent of the degree of band 3 protein dephosphory-lation and cAMP content.

5. Conclusions

Indomethacin acts as antioxidant decreasing peroxynitrite, not changing NO efflux values and increasing nitrate levels independent of band 3 phosphorylation degree and cyclic adenosine monophosphate (cAMP) concentration. Indomethacin mobilizes nitrite and GSNO in dependence of band 3 phosphorylation degree and cAMP. Indomethacin increased erythrocyte deformability only when PI3K is irreversibly inhibited by wortmanin meaning high cAMP levels. Further studies are needed to understand how indo maintain unchanged the AChE conformational states induced by band 3 phosphorylation degree.

Acknowledgments

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