EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 3
Issue: 4
28-34
Year: 2016

FIBRINOGEN SIGNALLING IN ERYTHROCYTE NITRIC OXIDE MOBILIZATION IN PRESENCE OF PI3-K AND ADENYLYL CYCLASE INHIBITORS

Ana S. Silva-Herdade, Teresa Freitas, José P. Almeida and Carlota Saldanha*

Instituto de Bioquímica, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa. Av. Prof Egas Moniz Lisboa,1649-028. Portugal.

***Author for Correspondence: Carlota Saldanha**
Instituto de Bioquímica, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa. Av. Prof Egas Moniz Lisboa,1649-028. Portugal.

Article Received on 24/01/2016 **Article Revised on 15/02/2016** **Article Accepted on 07/03/2016**

ABSTRACT
Soluble form of fibrinogen (Fib) and the peptide 4N1K are ligands of erythrocyte membrane CD47. Fibrinogen reinforces the ability of erythrocyte to scavenger nitric oxide (NO). Hiperfibrinogenemia increased NO efflux from erythrocyte in dependence of band 3 phosphorylation which is abolished by the presence of 4N1K. Herein we study *in vitro* the effect of high fibrinogen levels, on the NO efflux from erythrocytes and on its mobilization under influence of phosphoinositide-3 kinase (PI3-K) and adenylyl cyclase (AC) inhibitors in presence of 4N1K. Erythrocyte NO efflux, peroxynitrite, nitrite, nitrate and S-nitrosoglutathione (GSNO) were determined in blood samples in presence of 4N1K, wortmannin (WORT, PI3-K inhibitor) and MDL (AC inhibitor) under high fibrinogen concentrations. 4N1K with WORT and high fibrinogen levels induce, in relation to Fib plus WORT samples no variations on the erythrocyte NO efflux, decreased peroxynitrite, increased of nitrite, nitrate and GSNO concentrations. When 4N1K is present with MDL and high fibrinogen levels show, in relation to fibrinogen plus MDL samples increased erythrocyte NO efflux and nitrite, nitrate and GSNO concentrations. In conclusion, under high Fib levels and 4N1K the erythrocytes show: preservation of NO and impaired peroxynitrite in presence of PI3K inhibition; increased efflux of NO at lower levels of cAMP resulting from adenylyl cyclase inhibition.

KEYWORDS: Nitric oxide; S-nitrosoglutathione; human erythrocyte; phosphoinositide -3 kinase; adenylyl cyclase; fibrinogen; wortmannin ; peroxynitrite.

1. INTRODUCTION
The endothelial nitric oxide (NO) liberated to the lumen of the vessel passes through the erythrocyte membrane band 3 protein and is fixed by hemoglobin molecules with generation of S-nitrosohemoglobin (SNO-Hb) (Stamler et al.1997; Huang et al. 2001). Inside erythrocytes the glutathione reacts with NO originating S-nitrosoglutathione (GSNO) (Gali et al. 2002). Also NO can react with superoxide anion forming peroxynitrite that decomposes in nitrites and nitrates (Murphy and Sies 1991; Huie and Padmaja 1993; May et al. 2000). The efflux of NO from erythrocytes occurs through a trans-nitrosylation process involving the thiol group of the band 3 protein that receives NO from SNO-Hb (Gross, 2001; Pawloski et al. 2005). We have documented that band 3 is directly involved in NO efflux and mobilization, by modulation of its phosphorylation degree (Carvalho et al 2008). Band 3 phosphorylation is promoted by tyrosine-kinases (PTK) and dephosphorylation by tyrosine-phosphatases (PTP) (Bordin et al. 2005; Brunati et al. 2005; Brunati et al. 1996). The enzymes PTP and PTK when are phosphorylated in serine/threonine residues by protein kinase C (PKC) become, respectively, into inactive and active forms (Zipser et al. 2012). The PKC enzyme activity increases in presence of adenylyl cyclase inhibitor (MDL) (Almeida et al. 2008). The cell amount of cyclic adenosine monophosphate (cAMP) decreases by the action of phospodiesterase-3 (PDE3) (Hanson et al. 2008). PDE3 enzyme activity is activated by phosphoinositide-3 kinase (PI3-K) in a directly dependent way or indirectly via protein kinase B (PKB) (Shakur et al. 2001). There is a need to clarify the participation of AC and PI3-K in the erythrocyte NO signaling pathway.

Tissues present lower partial oxygen pressure (PaO2) are prone to receive NO from erythrocytes while the opposite is observed it tissues with higher PaO2 where NO is scavenged by erythrocytes (Sonveaux et al.2007). The erythrocyte bioavailability in NO is perturbed by endogenous and exogenous stimuli (Gross 2001; Lopes de Almeida et al 2009a , b; Saldanha et al. 2013). Present in plasma there is acetylcholine that signaling the NO pathway in erythrocytes through the formation of an enzyme complex with the membrane

acetylcholinesterase, associated to Gi protein and band 3 protein (Gross 2001) Fibrinogen (Fib) is known as an acute phase protein due to its high plasma levels in inflammatory response and mediate erythrocyte aggregation (Rampling 1998; Saldanha 2013). At physiological concentrations fibrinogen modulates erythrocyte NO mobilization by decreasing NO efflux and enhancing the formation of GSNO, nitrites and nitrates (Lopes de Almeida et al 2009a). When hyperfibrinogenemia was simulated *in vitro* erythrocyte NO efflux was reinforced when band 3 was phosphorylated (Lopes de Almeida et al 2011). However both high Fib levels and acetylcholine rescue NO inside erythrocyte (Saldanha et al. 2012). Soluble Fib binds erythrocyte membrane CD47 (de Oliveira et al. 2012). Erythrocyte membrane CD47 in the complex Rh establishes contact with protein 4.2 which in turn interacts with band 3 (Dahl et al. 2004). The agonist peptide 4N1K binds to CD47 and induced mobilization of NO in the erythrocyte in dependence of the status of phosphorylation of band 3 protein in an *in vitro* model of hiperfibrinogenemia (Saldanha et al. 2014). The manipulation of human blood samples by adding fibrinogen has done before and mimics the pathological condition of hyperfibrinogenemia (Lopes de Almeida eta al. 2011; Saldanha et al. 2012). Using this same model herein, we reported the study of its effect on the erythrocyte NO efflux and mobilization under influence of PI3-K and adenylyl cyclase inhibitors in absence and presence of CD47 agonist peptide, 4N1K

2. Experimental Section

2.1 Materials and Reagents

General reagents were purchased by Sigma- Aldrich Co., Wortmannin (WORT), a PI3-K inhibitor and MDL hydrochloride (MDL) an adenylyl cyclase inhibitor were purchased from Sigma-Aldrich Co. Inhibitors were prepared as recommended in the information sheet of the products: WORT is prepared in a DMSO solution at 10−3 M concentration; MDL was prepared in distilled water at the same concentration; nitrate reductase from Aspergillus Niger, NADPH (tetra sodium salt), sodium nitrate, sodium nitrite and atropine were all 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 tubes BD VacuntainerTM with Lithium heparin (17UI/mL) as an anticoagulant. This “in vitro” study was performed under the protocol established with the Portuguese Institute of Blood in Lisbon. All males donors (N=10; aged between 30 and 40 years old) were duly informed and signed their agreement. The chosen concentrations for fibrinogen were based on its physiological levels and previous studies (Sargento et al. 2005).The human fibrinogen was purchased from Sigma (Poole, UK).

2.2 *Experimental model*

The experimental model that we here describe was done for each blood sample taken from ten donors. So, each blood sample was divided into six aliquots of 1 mL centrifuged at 11,000rpm (Biofuge 15 Centrifuge, Heraus) during 1 min at room temperature. In 3 aliquots 60µL of plasma was replaced with the 50 µL of fibrinogen NaCL isotonic solutions pH 7.4 (30 mg/dL) plus 10 µL of MDL or 10 µL of WORT or 10 µL of 4N1K to achieve the 10µM final concentration. The same procedure for the control sample has been performed with the difference that, 50 µL of plasma was replaced with the same volume of isotonic NaCl. From the others 2 samples, 70 µL of plasma was taken and replaced by 50 µL of Fib solution plus 10 µL of MDL plus 10 µL of 4N1K or 50 µL of Fib solution plus 10 µL of WORT plus 10 µL of 4N1K to achieve the 10µM final concentration. Blood samples were then incubated during 15 min with slight agitation. To avoid changes on blood samples due to possible temperature fluctuations during incubation all measurements of the NO and its derivatives molecules were performed at room temperature. At the end of incubation samples were centrifuged and plasma removed for fibrinogen concentration assessment. Plasma fibrinogen concentrations were evaluated using the Fibritimer BFT* Analyser (Dade Behring, Marburg GmbH, Germany) based on the Clot technology.

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

Following incubation, blood samples were centrifuged and sodium chloride 0.9 % at pH 7.0 was added on to compose a hematocrit of 0.05%. The suspension was mixed by gently inversion of tubes. For amperometric NO quantification we used the amino-IV sensor (Innovative Instruments Inc. FL, USA), according to the method described previously (Carvalho et al. 2004). 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 it was continuously monitored with a computerized inNOTM system (with a software version 1.9, Innovative Instruments Inc., Tampa, FL, USA) and connected to a computer. Calibration of the NO sensor was performed daily. For each experiment, the NO sensor was immersed vertically in the erythrocyte suspension vials and allowed to stabilize for 30 min to achieve NO basal levels. 30 µl of acetylcholine (ACh) was added to erythrocyte suspension samples in order to achieve the final concentrations of 10µM of ACh and NO. Data were recorded from constantly stirred suspensions at room temperature. The measurement of nitrite/nitrate concentration was done using the spectrophotometric Griess method as described previously (Guevara et al. 1998), 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 (Cook et al. 1996). For measurement of S-nitrosoglutathione (GSNO) 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 HgCl2 (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 gentile 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 described [39]. For determinations of peroxinitrite levels the erythrocyte suspensions (1mL) were incubated with 2,7-dichlorofluorescein diacetate (DCFC-DA) 15µM, in 3mL buffer (Pi 155mM, pH 7.4) during 30 min, at room temperature. Suspensions were rinsed several times and diluted in the working solution with 1.8mL of the same buffer. The pellets were rinsed and used for fluorescence measurement with a Hitachi F-300 fluorospectrophotometer (Hitachi, Japan) with excitation and emission wavelengths at 503 and 523nm, respectively. The concentration of peroxynitrite was finally calculated through a calibration graph (Possel et al. 1997).

2.4 *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 analysis 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 hyperfibrinogenemia effects on erythrocyte nitric oxide efflux, S-nitrosoglutathione, peroxynitrite, nitrite and nitrate levels in presence of 4N1K and PI3-K inhibitor (WORT), Table 1.

Our results showed that in high fibrinogen concentration (510 mg/dL), the efflux of NO from erythrocyte is not changed significantly by the presence of wortmannin (WORT, PI3-K inhibitor), neither by 4N1K nor by the presence of both effectors, Table 1. Significant increased levels of GSNO were obtained in samples with Fib plus WORT plus 4N1K in relation to Fib plus WORT ($p< 0.0001$) and the control ($p< 0.001$). Regarding the sample Fib plus WORT plus 4N1K and comparing them with Fib plus WORT and with control samples significantly decreased values of peroxinitrite were verified respectively $p<0.05$ and $p< 0.001$, Table 1. In Fib plus WORT plus 4N1K samples significantly augmented values of nitrite ($p<0.001$; $p<0.0001$) and nitrate ($p<0.001$; $p<0.001$) were obtained when compared with Fib plus WORT samples and control samples, Table 1.

Table 1. Values (Mean±SD) of erythrocyte nitric oxide (NO) efflux, GSNO, peroxynitrite, nitrite and nitrate

Blood samples	NO	GSNO	Peroxinitrite	Nitrite	Nitrate
Control	1.50±0.89	8.05±0.34	199.10±46.77	7.95±0.75	8.85±0.68
Fib+MDL	1.26±0.18	8.81±0.76	196.04±55.21	9.65±0.86*	9.95±0.97 $^{\#}$
Fib+WORT	1.47±0.13	9.56±0.72*	184.71±60.67	9.40±0.71**	10.15±0.76 $^{\#}$
Fib+4N1K	1.33±0.26	10.52±0.86*	193.18±27.07	10.40±0.59*	10.70±0.74**
Fib+MDL+4NIK	1.64±0.36 $^{\partial}$	12.28±0.70$^{*, \&}$	187.71±34.45	10.90±1.06$^{*,\&}$	11.55±0.69$^{*, \&}$
Fib+WORT+4N1K	1.40±0.361	11.73±1.17$^{**, \times}$	145.30±42.99$^{\#, @}$	11.35±0.35$^{*,+}$	11.75±0.47$^{**, +}$

In relation to the control *p<0.0001; **p<0.001; # < 0.05
In relation to Fib+MDL $^{\&}$ p<0.0001; ∂ p<0.05
In relation to Fib+Wort $^{\times}$ p<0.0001; $^{@}$ p<0.05; + p<0.001

3.2 Effects of *in vitro* hyperfibrinogenemia, on erythrocyte nitric oxide efflux, S-nitrosoglutathione, peroxynitrite, nitrite and nitrate levels in presence of 4N1K and adenylyl cyclase inhibithor. Table 1.

We observed that in high fibrinogen concentration (510 mg/dL) the erythrocyte efflux of NO is increased significantly by the presence of MDL (adenylyl cyclase inhibitor) plus 4N1K ($p< 0.05$) in relation to the sample of Fib plus MDL, Table 1. However there is no variation in NO efflux when compared to the control or with Fib plus 4N1K samples.

Significantly increased values of NO derivatives molecules namely GSNO ($p< 0.0001$; $p<0.0001$), nitrite ($p< 0.0001$; $p<0.0001$), and nitrate ($p< 0.0001$; $p<0.0001$), were obtained in presence of MDL plus 4N1K under high Fib levels in relation to control or to

high Fib plus MDL blood samples, Table 1. However no variations on peroxynitrite concentrations were observed in all manipulated blood samples, Table 1.

DISCUSSION

Previously was verified that in high Fib levels and presence of 4N1K, the equilibrium existing between the phosphorylated and dephosphorylated state of band 3 inside the erythrocyte of healthy humans is maintained (Carvalho et al. 2008). Under high Fib concentrations with and without 4N1K similar profile of the NO derivatives molecules content inside the erythrocytes has been obtained in dependence of band 3 protein phosphorylation (Lopes de Almeida et al. 2011). It was observed that WORT plus 4N1K are unable to increase the band 3 protein phosphorylation that mediates the NO efflux from erythrocytes under high fibrinogen levels (Lopes de Almeida et al. 2011). In the present work we confirm that the NO efflux from erythrocyte do not change in samples under high Fib plus WORT plus 4N1K in relation to control or to Fib plus WORT, Table 1.

Peroxynitrite is a reactive nitrogen species (RNS) and an index of auto-oxidation of oxyhemoglobin (Balagopalakrishna et al.1996). In the present work the samples containing WORT plus 4N1K with high Fib concentration are able to decrease the levels of erythrocyte peroxynitrite in relation to control and to Fib plus WORT samples, Table 1. The inhibition of PI3-K by WORT prevents the activation of phosphodiesterase 3 (PDE3) and consequently there is no decrease in the levels of cAMP allowing the activation of the cAMP-dependent kinase (Jindal et al. 1996). Pyruvate kinase (PK) turns to an inactive form after phosphorylation by cAMP- protein kinase dependent (Jindal, 1980). It is expected inhibition of glycolysis and promotion of the pentose phosphate pathway with decreases of oxidative stress environment in erythrocytes. This means less expected peroxinitrite formation which is confirmed in those samples referred above namely Fib plus WORT plus 4N1K, in comparison with Fib plus WORT and with control samples (Table 1). It is known that decomposition of peroxynitrite leads to nitrite and nitrate molecules and contributes to decrease oxidation in glutathione leaving it prone to become a reservoir of NO inside erythrocytes in the form of GSNO (May 2000; Pfeiffer and Mayer 1998; Soszynski and Bartosz 1996). So, we have observed increase of nitrite and nitrate concentrations inside the erythrocyte under high Fib plus WORT plus 4N1K Table 1. We cannot exclude the possible reaction of peroxynitrite with haemoglobin generating SNOHb, which could in a reductive erythrocyte environment liberate NO to thiol group of glutathione generating GSNO and nitrate (Gladwin et al. 2002). These could explain the increased levels of GSNO and nitrate found in those samples, Table 1. Another possible explanation for GSNO levels could come from the remaining NO inside erythrocyte resulting from the slight not significantly decrease of NO efflux observed in the blood samples with high Fib, WORT and 4N1K. The NO may reduce oxyhaemoglobin to methaemoglobin (MetHb) along with the formation of nitrate instead to combine with superoxide anion (Mesquita et al 2001). This will be another explanation for the lower peroxynitrite level inside erythrocyte of samples under high Fib plus WORT plus 4N1K.

Beyond the contribution of PI3-K inhibition by WORT when associated to 4N1K and high Fib levels in erythrocytes protection from RNS effects, it was published that when WORT is present in blood samples induce decreased erythrocyte aggregation without modification on erythrocyte deformability (Saldanha et al 2007). The inhibitor of adenylyl cyclase MDL do not induces variations in the erythrocyte deformability (Saldanha et al 2007) and promotes decrease in cAMP levels which impair the enzyme activity of the cAMP kinase dependent (Almeida et al. 2008). Consequently pyruvate kinase it will be in the dephosphorylated active state allowing glycolysis to function. For that the glycolytic enzymes are release from band 3 protein which becomes able to be phosphorylated. MDL activates PKC which will inhibit PTK and activate PTP by phosphorylation (Carvalho et al. 2008; Almeida et al. 2008). Probably the band 3 phosphorylation do not result only from the enzymatic action of PTK (p72 ^{syk}) but either by p59/61 ^{hck} or by casein kinase I (Carvalho et al. 2008; Wang et al. 1997). Another possibility is through PKC itself that when activated moves from cytoplasm to membrane coupled receptor protein G promoting phosphorylation of band 3 (Escribá et al.2003). So, the presence of 4N1K and Fib (both ligands of CD47 in erythrocyte of membrane) stimulate NO efflux from erythrocytes under MDL (or lower levels of cAMP) may be through the band 3 phosphorylation. All together, these stimuli contribute to the active conformation state of AChE necessary to the signaling pathway coupled with band 3 protein phosphorylation (Carvalho et al. 2009; Teixeira et al. 2015). SNOHb is a reservoir of NO inside erythrocyte and responsible for the NO mobilization inside the erythrocyte, as well as, for its efflux (Carvalho et al 2008; Carvalho et al 2004). The NO could be transferred from SNOHb to glutathione originate GSNO and nitrate or react with anion superoxide forming peroxynitrite (Carvalho et al 2004). In the present study the first reaction seems to occur and originate higher GSNO concentrations as obtained in samples with high Fib plus MDL plus 4 N1K in relation to control and also to Fib plus MDL. Any significant changes of peroxynitrite levels were observed in all samples in relation to the control or between each other, beside their lower levels. Otherwise, NO could also reduce oxyhaemoglobin to methaemoglobin along with the formation of nitrate (Mesquita et al. 2001). Increased nitrate concentrations were obtained. Besides the absence of measurement of MetHb in this work, it was expect no variation in MetHb concentration due the presence of haemoglobin reductase coupled with the NADH produced in the glycolytic pathway (Inal and

Egüz 2004). In the previous work no changes in MetHb concentrations were obtained in presence of each enzyme inhibitors WORT and MDL respectively of PI3K and AC (Saldanha et al 2007).

4. CONCLUSIONS

In conclusion the inhibition of PI3-K enzyme activity (by WORT) when associated to 4N1K and high Fib levels, in erythrocytes, unchanged the NO efflux and contribute to lower the peroxynitrite concentration which protect the erythrocytes from the RNS effects avoiding oxidation of glutathione and promoting inactivation of AChE (Soszynski and Bartosz 1996). The inactive state of the enzyme contributes to the unchanged normal values of NO efflux from erythrocytes (Carvalho et al 2008; Teixeira et al 2015). Also the inhibition of PI3-K became unable to activate PDE3 leaving the cAMP levels dependent of PKB. However the lower levels of cAMP provoked by AC inhibition in presence of the 4N1K and Fib (in high concentration) generates proper band 3 protein conditions to transfer to outside increased levels of NO. Three major messages were taken from the present work under high Fib concentration and 4N1K associated with the erythrocyte NO availability based on signaling pathway: dependence on PI3-K and adenylyl cyclase enzymes activity; lower values of cAMP promote increased efflux of NO from erythrocytes and left unchanged peroxynitrite levels; direct inhibition of PD3 by PI3K induce unchanged efflux of NO from erythrocytes and decreased peroxynitrite concentration lowering RNS.

ACKNOWLEDGMENTS

This study was supported by grants from the FCT—Fundação para a Ciência e a Tecnologia (project reference PTDC/SAU-OSM/73449/2006). The authors also thank Emília Alves for all the help with the typewriting.

Author Contributions

CS conceived, designed and managed the project; MTS performed the assays; ASH leads the experimental work and performed statistical analysis : JPA performed assays.

Conflicts of Interest

The authors declare no conflict of interest.

REFERENCES

1. Stamler, J.; Jia, L.; Eu ,J,:P.; McMahon ,T,J.; Demchenko ,J.; Bonaventura, C.; Gernert, K.; Piantadosi, C.;A. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. *Science.*, 1997; *276:* 2034- 47.
2. Huang, K.T., Han, T.H. Hyduke, D.R.; Vaughn, M.W,.;Van Herle ,H.; Hein, T.W.; Zhang, C.; Kuo, L.; Liao, J.;C. Modulation of nitric oxide bioavailability by erythrocytes. *Proc Natl Acad Sci U S A.*, 2001; *98*: 11771-11776.
3. Galli, F., R.; Rossi, P.;D.; Simplicio, A.; Floridi, Canestrari, F. Protein thiols and glutathione influence the nitric oxide-dependent regulation of the red blood cell metabolism. *Nitric Oxide.*, 2002; *6*: 186-199.
4. Murphy, M.: Sies, H. Reversible conversion of nitroxyl anion to nitric oxide by superoxide dismutase. *Proc. Natl. Acad. Sci.* USA., 1991; *88*: 10860-10864.
5. Huie, R.; Padmaja S. The reaction of NO with superoxide. *Free Radic. Res. Commun*. 1993, *18*, 195 -199.
6. May, J.M., Qu Z.; C.; Xia L.; Cobb, C.;E. Nitrite uptake and metabolism and oxidant stress in human erythrocytes. *Am. J. Physiol.Cell Physiol*., 2000; *279*: C1946-C1954.
7. Gross, S.;S. Target delivery of nitric oxide. *Nature.*, 2001; *409*: 577-588.
8. Pawloski, R.; Hess, D.;T.; Stamler, J.;S. Impaired vasodilation by red blood cells in sickle cell disease. *Proc. Natl. Acad.Sci.USA.*, 2005; *102*: 2531-2536.
9. Carvalho, F.;A.; Almeida, J.P.; Fernandes, I.;O.; Freitas-Santos, T.; Saldanha ,C. Non-neuronal cholinergic system and signal transduction pathways mediated by band 3 in red blood cells. *Clin Hemorheol Microcirc.*, 2008; *40*: 207-227.
10. Bordin, L.; Ion-Popa F.; Brunati, A.;M.; Clari G.; Low, P.;S. Effector-induced Syk-mediated phosphorylation in human erythrocytes. *Biochim. Biophys. Acta.*, 2005; *1745*: 20-28.
11. Brunati, A.;M.; Bordin L.; Clari, G.; James, P.; Quadroni, M.; Baritono, E.;Pinna L.;A.; Donella-Deana, A. Sequential phosphorylation of protein band 3 by Syk and Lyn tyrosine kinases in intact human erythrocytes: identification of primary and secondary phosphorylation sites, *Blood*., 2000; *96*: 1550-1557.
12. Brunati, A.;M.; Bordin L.; Clari, G.; Moret, V. The Lyn-catalyzed Tyr phosphorylation of the transmembrane band-3 protein of human erythrocytes. *Eur. J. Biochem.*, 1996; *240*: 394-399.
13. Zipser, Y.; Piedade, A.; Barbul, A.; Korenstein, R.; Kosower, N.; S. Ca+ promotes erythrocyte band 3 tyrosine phosphorylation via dissociation of phosphotyrosine phosphatase from band 3. *Biochem J.*, 2002; *368*: 137-144.
14. Almeida, J.P.; Carvalho, F.A.; Martins-Silva, J.; Saldanha, C. The modulation of cyclic nucleotide levels and PKC activity by acetylcholinesterase effectors in human erythrocytes. *Actas Bioquímica*., 2008; *9*: 111-114.
15. Hanson ,M.S.; Stephenson, A.H.; Bowles, E.A.; Sridharan. M.; Adderley, S.; Sprague, R.; S. Phosphodiesterase 3 is present in rabbit and human erythrocytes and its inhibition potentiates iloprost-induced increases in cAMP. *Am J Physiol Heart Circ Physiol*., 2008; *295*: H786–H793.
16. Shakur, Y.; Holst, L.; S.; Landstrom, T. R.; Movsesian, M.; Degerman, E.; Manganiello ,V. Regulation and function of the cyclic nucleotide

phosphodiesterase (PDE3) gene family. *Prog Nucleic Acid Res Mol Biol*., 2001; *66*: 241–277.
17. Sonveaux, P.; Lobysheva, I.;I.; Feron O.; McMahon, T.;J. Transport and peripheral bioactivities of nitrogen oxides carried by red blood cell hemoglobin: role in oxygen delivery. *Physiology*., 2007; *22:* 97-112.
18. Lopes de Almeida, J.;P.; Freitas-Santos, T.; Saldanha C. Fibrinogen-dependent signalling in microvascular erythrocyte function: Implications on nitric oxide efflux. *J Memb Biol.*, 2009; *231*: 47-53.
19. Lopes de Almeida, J.;P.; Carvalho, F.;A.; Silva-Herdade, A.;S.; Santos-Freitas, T.; Saldanha C. Redox thiol status plays a central role in the mobilization and metabolism of nitric oxide in human red blood cells. *Cell Biol Int.*, 2009; *33*: 268-275.
20. Saldanha, C.; Teixeira, P.; Santos-Freitas, T.; Napoleão, P. Timolol modulates erythrocyte nitric oxide bioavailability. *J Clin Exp Opthalmol* . 2013; *4*: 285.
21. Rampling, M.;W. *Clin. Hemorheol. Microcirc*., 1998; 19: 129-32.
22. Saldanha C. Fibrinogen interaction with the red blood cell membrane. *Clin Hemorheol Microcirc*., 2013; *53*: 39-44.
23. Lopes de Almeida, J.;P.; Freitas-Santos, T.; Saldanha C. Evidence that the degree of band 3 phosphorylation modulates human nitric oxide efflux-in vitro model of hyperfibrinogenemia. *Clin Hemorheol Microc.*, 2011; *49*: 407-416.
24. Saldanha, C.; Freitas, T.; Almeida J.; P. Fibrinogen effects on erythrocyte nitric oxide mobilization in presence of acetylcholine. *Life Sci*., 2012; *91*: 1017-1022.
25. de Oliveira, S.; Vitorino de Almeida, V.; Calado, A.; Rosário, H.;S.; Saldanha, C. Integrin-associated protein (CD47) is a putative mediator for soluble fibrinogen interaction with human red blood cells membrane. *Biochim Biophys Acta*. 2012; *1818*: 481-490.
26. Dahl, K.; Parthasarathy, N.; R,; Westhoff, M.; Layton, D.;M.; Discher, D.;E. 2004, *Blood 1*03, 1131-1136.
27. Saldanha, C.; Freitas, T.; Lopes de Almeida J.;P.; Silva-Herdade A. Signal transduction pathways in erythrocyte nitric oxide metabolism under high fibrinogen levels. *KARJ*., 2014; *26*: 217-223.
28. Pfeiffer, S., Mayer, B. Lack of tyrosine nitration by peroxynitrite generated at physiological pH. *J. Biol. Chem*., 1998; *273*: 27280–27285.
29. Soszynski, M,; Bartosz .G. Effect of peroxynitrite on erythrocytes. *Bioch Bioph Acta*. 1996; *1291*: 107-114.
30. Balagopalakrishna, C.; Manoharan, P.;T.; Abugo, O.;O.; Rifkind, J,;M. Production of superoxide from hemoglobin-bound oxygen under hypoxic conditions. Biochemestry., 1996; *35*: 6393-6398.
31. Jindal, H.; K.; Ai, Z.; Gascard P.; Horton, C.; Cohen, C.; M. Specific Loss of Protein Kinase Activities in Senescent Erythrocytes. Blood., 1996; *88*: 1479-1487.
32. Kiener, P.;A.; Westhead, E.;W. Dephosphorylation and reactivation of phosphorylated pyruvate kinase by a cytosolic phosphoprotein phosphatases from human erythrocytes. *Biochem Biophys Res Comm.*, 1980; *96*: 551-557.
33. Gladwin, M.; T.; Wang, X.; Reiter, C.; D. SNitrosohemoglobin is unstable in the reductive erythrocyte environment and lacks O2/NO-linked allosteric function. *J. Biol. Chem*., 2002; *277*: 27818–27828.
34. Mesquita, R.; Pires, I.; Saldanha, C. Martins-Silva, J. Effects of acetylcholine and spermineNONOate on erythrocyte hemorheologic and oxygen carrying properties. *Clin.Hemorheol. Microcirc.*, 2001; *25*: 153–163.
35. Saldanha, C.; Silva, A.;S.; Gonçalves, S.; Martins-Silva, J. Modulation of erythrocyte hemorheological properties by band 3 phosphorylation and dephosphorylation. *Clin Hemorheol Microcir*., 2007; *36*: 183-194.
36. Wang, C., C; Tao, M.; Wei, T.; Low, P.; S. Identification of the major casein kinase I phosphorylation sites on erythrocyte band 3. *Blood* ., 1999; *89:* 3019-3024.
37. Escribá, P.; V.; Sanchez-Dominguez, J.; M.; Alemany, R.; Perona, J.; S.; Ruiz- Gutiérrz, V.Alterations of lipids. G protein, and PKC in Cell membranes of elderly hypertensives. Hypertension, 2003; *41*: 176-182.
38. Carvalho, F.; A.; Almeida, J.; P.; Fernandes, I.;O.; Freitas-Santos, T.; Saldanha C. Modulation of erythrocyte acetylcholinesterase activity and its association with G Protein Band 3 interactions. *J Membr Biol.*, 2009; *228*: 89-97
39. Teixeira, P.; Duro, N.; Napoleão, P.; Saldanha C. Acetylcholinesterase Conformational States Influence Nitric Oxide Mobilization in the Erythrocyte. *J Membr Biol*. 2015; *248*: 349-354.
40. Carvalho, F.;A.; Mesquita R.; Martins-Silva J.; Saldanha, C. Acetylcholine and choline effects on erythrocyte nitrite and nitrate levels, J. Appl. Toxicol., 2004; *24*: 419–427.
41. Inal, M.;E.; Egüz, A.;M. The effects of isosorbide dinitrate on methemoglobin reductase enzyme activity and antioxidant states. Cell Biochem. Funct., 2004; *22*: 129–133.
42. Sargento, L.; Saldanha, C.; Monteiro, J.; Perdigão, C.; Silva, J.M. Long-term prognostic value of protein C activity, erythrocyte aggregation and membrane fluidity in transmural myocardial infarction. Thromb. Haemost . 2005, *94*, 380-388.
43. Carvalho, F.A.; Martins-Silva, J.; Saldanha, C. Amperometric measurements of nitric oxide in erythrocytes. Biosens Bioelectron., 2004; *20:* 505-508.
44. Guevara, I.; Iwanejko, J.; Dembińska-Kieć, A.; Pankiewicz, J.; Wanat, A.; Anna, P.; Gołabek, I.; Bartuś, S.; Malczewska-Malec, M.; Szczudlik, A.

Determination of nitrite/nitrate in human biological material by the simple Griess reaction. Clin Chim Acta., 1998; *274:* 177-188.

45. Cook, J.; A.; Kim, S.;Y.; Teague, D.; Krishna, M. C.; Pacelli, R.; Mitchell, J.B.; Vodovotz, Y.; Nims, R.; W.; Christodoulou, D.; Miles, A.M.; Grisham, M.B.; Wink, D.A. Convenient colorimetric and fluorometric assays for S-nitrosothiols. *Anal Biochem.*, 1996; *238*: 150-158.
46. Possel, H.; Noack, H.; Augustin, W.; Keilhoff, G.; Wolf, G. 2,7-dichlorofluorescein diacetate as a fluorescent marker for peroxinitrite formation. FEBS Letters., 1997; *416:* 175-178.