

Original Paper

Deoxygenation Affects Composition of Membrane-Bound Proteins in Human Erythrocytes

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Key Words

Erythrocytes • Deoxygenation • ATP release • Hemolysis • Membrane bound proteins

Abstract

Background/Aims: ATP release from erythrocyte plays a key role in hypoxia-induced elevation of blood flow in systematic circulation. We have previously shown that hemolysis contributes to erythrocyte ATP release triggered by several stimuli, including hypoxia, but the molecular mechanisms of hypoxia-increased membrane fragility remain unknown. **Methods:** In this study, we compared the action of hypoxia on hemolysis, ATP release and the composition of membrane-bound proteins in human erythrocytes. **Results:** Twenty minutes incubation of human erythrocytes in the oxygen-free environment increased the content of extracellular hemoglobin by ~1.5 fold. Paired measurements of hemoglobin and ATP content in the same samples, showed a positive correlation between hemolysis and ATP release. Comparative analysis of SDS-PAGE electrophoresis of erythrocyte ghosts obtained under control and deoxygenated conditions revealed a ~2-fold elevation of the content of membrane-bound protein with Mr of ~60 kDa. **Conclusion:** Deoxygenation of human erythrocytes affects composition of membrane-bound proteins. Additional experiments should be performed to identify the molecular origin of 60 kDa protein and its role in the attenuation of erythrocyte integrity and ATP release in hypoxic conditions.

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Introduction

Regulation of vascular tone facilitates oxygenation and supply of tissues with glucose and other substances required for synthesis of high-energy compounds in order to balance energy consumption. The regulation in lumen diameter of blood vessels in response to

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decline in partial oxygen pressure (pO_2) is one of the most intriguing and intensively studied phenomena [1]. Dietrich and co-workers were the first to report that the presence of erythrocytes is obligatory for elevation of lumen diameter seen in small vessels isolated from systemic circulation and subjected to hypoxic conditions [2]. Gaseous transmitters (NO, CO, H_2S) and purinergic signaling system are considered among the main tentative mechanisms involving erythrocytes in regulation of blood vessel tone [3]. A key role of purinergic signaling system in regulation of vascular tone under hypoxic conditions is supported by several observations. *First*, since the seminal paper of Burnstock and Kennedy [4], numerous research teams observed that luminal application of ATP leads to relaxation in different vascular beds (for comprehensive review, see [5, 6]). *Second*, attenuation of partial oxygen pressure (pO_2) leads to release ATP from human erythrocytes [7, 8] as well as erythrocytes isolated from other mammals studied so far [9]. *Third*, ATP content in the effluent blood from exercising thigh increased during intense hypoxic exercise as compared to normoxic ones [10]. Several studies demonstrated attenuated ATP release in erythrocytes from older adults [11] and in patients with type 2 diabetes mellitus [12, 13], cystic fibrosis [14] and idiopathic pulmonary hypertension [15] thus suggesting pathophysiological implications of this phenomenon.

In spite of extensive number of observations showing ATP release from erythrocytes subjected to attenuated pO_2 as well as other physiological and pathophysiological stimuli including hypercapnia [7], modest acidification [8], elevated temperature [16], deformation [14] and shear stress [17], molecular mechanisms underlying this phenomenon remain poorly understood [3]. Indeed, exocytosis as a major mechanism of hormone and neurotransmitter secretion in nucleated cells should be excluded in mature mammalian erythrocytes lacking endoplasmic reticulum. Keeping this in mind, several non-vesicular mechanisms, including conductive transport via pannexin-1, voltage- and volume-dependent anion channels, and cystic fibrosis transmembrane conductance regulator (CFTR) were proposed to be involved in hypoxia-induced ATP release [9, 18, 19].

Side-by-side with ATP-conducting pathways listed above, elevation of extracellular ATP concentration may be caused by hemolysis, i.e. disruption of erythrocyte membrane integrity. Indeed, it was shown that intensive hypoxic exercise leads to intravascular hemolysis correlated with elevation of plasma ATP content [20, 21]. Recently, we compared ATP and hemoglobin release from human erythrocytes in response to hyposmotic swelling, hypoxia and mechanical stimuli. In these experiments we revealed positive correlation between extracellular content of ATP and hemoglobin [22]. In the case of hypotonic shock, a key role of hemolysis in elevation of extracellular ATP concentration was confirmed by simultaneous luminescence ATP imaging and infrared differential interference contrast images of substrate-attached erythrocytes [22]. It should be noted that Berfeld and Forrester did not find any effect of 50-s hypoxia/hypercapnia on human erythrocyte hemolysis [7]. Negative results were also obtained in comparative analysis of hemoglobin and ATP release evoked by several toxins and activators of cAMP-signaling (for recent review, see [3, 23]). In this study, we performed a correlation analysis of hypoxia-induced ATP and hemoglobin release from human erythrocytes of young volunteers and the action of hypoxia on the composition of membrane-bound proteins as a factor determining erythrocyte integrity.

Materials and Methods

Erythrocytes

Blood samples from young volunteers (9 men and 8 women, 26 ± 4 and 28 ± 6 years old, respectively) were collected in tubes containing heparin (5U/ml). Samples were centrifuged 1500 g for 10 min at $+4^\circ C$, blood plasma and white blood cells were removed by aspiration and erythrocytes were washed three times in buffer A, containing 145 mM NaCl, 5 mM KCl, 4 mM Na_2HPO_4 , 1 mM NaH_2PO_4 , 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose (pH 7.4). Then, erythrocyte suspension was adjusted to 30% hematocrit, stored at $+4^\circ C$ and used for experiments within 3 hours.

Oxygen depletion

Oxygen depletion was performed at room temperature with erythrocyte suspension mixed at 30 rpm in an airtight flow-through chamber with a continuously injected gas mixture containing N_2 and 0.04% CO_2 . To control oxygen depletion we employed Resonance Raman spectroscopy of hemoglobin with near-infrared laser excitation [24]. Raman spectra were obtained by means of Raman spectrometer utilizing laser 473 nm (Ciel, Eurolase, Russia) and registration system MORS 1/3648 (Troitsk, Russia) based on CCD TCD1304DG (Toshiba, Japan) with the edge filter LPO2-473RS-50 (Shemrock, USA). Considering the laser wavelength and hemoglobin absorption at 473 nm we worked with non-resonance Raman scattering. Excitation of Raman scattering was done with laser power of 17 mW per laser spot of 2 mm in diameter. In preliminary experiments, we observed that in our conditions half-maximal attenuation of the content of oxygenated hemoglobin in erythrocytes occurs in 5-7 min of their exposure to oxygen-free environment. Considering this we extended the incubation time up to 20 min. Figure 1 shows that at this time point oxygen-free gas mixture endorsed complete oxygen depletion of hemoglobin indicated by the absence of 1375 cm^{-1} band in the Raman scattering spectra. In the control samples, erythrocyte suspension was injected with air at the same flow intensity. To obtain erythrocyte ghosts, we removed oxygen from the buffer used for erythrocyte lysis and further washout of the erythrocyte ghosts by bubbling it with the N_2/CO_2 gas mixture. Oxygen content in the chamber, measured with KE25 oxygen sensor (Figaro, Japan), did not exceed 1%.

Erythrocyte hemolysis

Erythrocyte hemolysis was estimated as a percentage of extracellular hemoglobin to the total hemoglobin content in erythrocyte. Degree of hemolysis was estimated by optical density of hemoglobin in supernatant fluid at 415 nm after sedimentation of erythrocytes at 500 g for 10 min at room temperature. The presented values are corrected for the background degree of hemolysis, which was obtained before the experimental treatments.

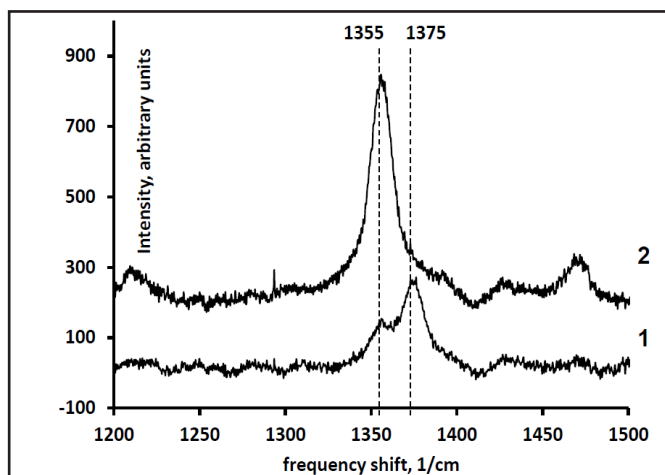
Extracellular ATP assay

ATP was quantified by a luciferin-luciferase bioluminescence assay. Both extracellular ATP and free hemoglobin were always measured in the same supernatant samples as described in detail elsewhere [22].

Erythrocyte ghosts

Erythrocyte ghosts were obtained by hypotonic hemolysis in 20 volumes of ice-cold phosphate buffer (4.7 mM Na_2HPO_4 , 0.3 mM NaH_2PO_4 , pH=7.4) followed by three washing and centrifugations at 3500g for 40 min at +4°C. Then, suspension of erythrocyte ghosts was concentrated by centrifugation in phosphate buffer at 13000 g for 40 min at +4°C. All procedures required for preparation of erythrocyte ghosts in the media with low oxygen content were performed in the airtight flow-through chamber with a continuous injection of gas mixture, which differed from the air. Erythrocyte ghosts were stored at -80°C. Protein concentration was determined by the modified Lowry's method [25] with bovine serum albumin as a standard.

Fig. 1. Raman spectra of erythrocyte suspensions measured after 20-min exposure to air (1) or oxygen-free gas mixture (2).



One-dimensional SDS-polyacrylamide gel electrophoresis

One-dimensional SDS-polyacrylamide gel electrophoresis of erythrocyte ghosts was performed in polyacrylamide gel by the Laemmli method [26] using 6% concentrating gel in 0.5 M Tris-HCl (pH 6.8), 0.4% SDS and 10% separating gel in 1.5 M Tris-HCl (pH 8.8), 0.4% SDS. The electrophoresis cell was filled with buffer containing 1.92 M glycine, 250 mM Tris-HCl (pH 8.6) and 1% SDS. The stock of polyacrylamide contained 30% acrylamide and 0.8% N,N-methylenebisacrylamide. The protein samples for electrophoresis were prepared in a 4-fold buffer (250 mM Tris-HCl, pH 6.8, 8% SDS, 40% sucrose, 0.05% bromophenol blue, 5% β -mercaptoethanol) and incubated for 5 min at 95°C. To control protein molecular weight, we loaded molecular weight marker and prepared protein samples in the wells, using a micropipettor with gel loading tips. Electrophoresis was performed in Mini-Protean III cell (Bio-Rad, USA). When the bromophenol blue has run to the bottom of the gel the electrophoresis was terminated by 1 hr incubation at room temperature in solution containing 25% isopropanol and 10% acetic acid. Then, proteins on the gel were stained for 1 hr with Coomassie R-250 (0.5% Coomassie blue R-250, 10% isopropanol, 10% acetic acid) and incubated overnight with a solution, containing 25% ethanol and 7.5% acetic acid. Then, gel was washed two times in the distilled water. Electrophoregrams were analyzed using *One-Dscan* software. Protein band densities were normalized to the density of band 3 protein.

Statistical analysis

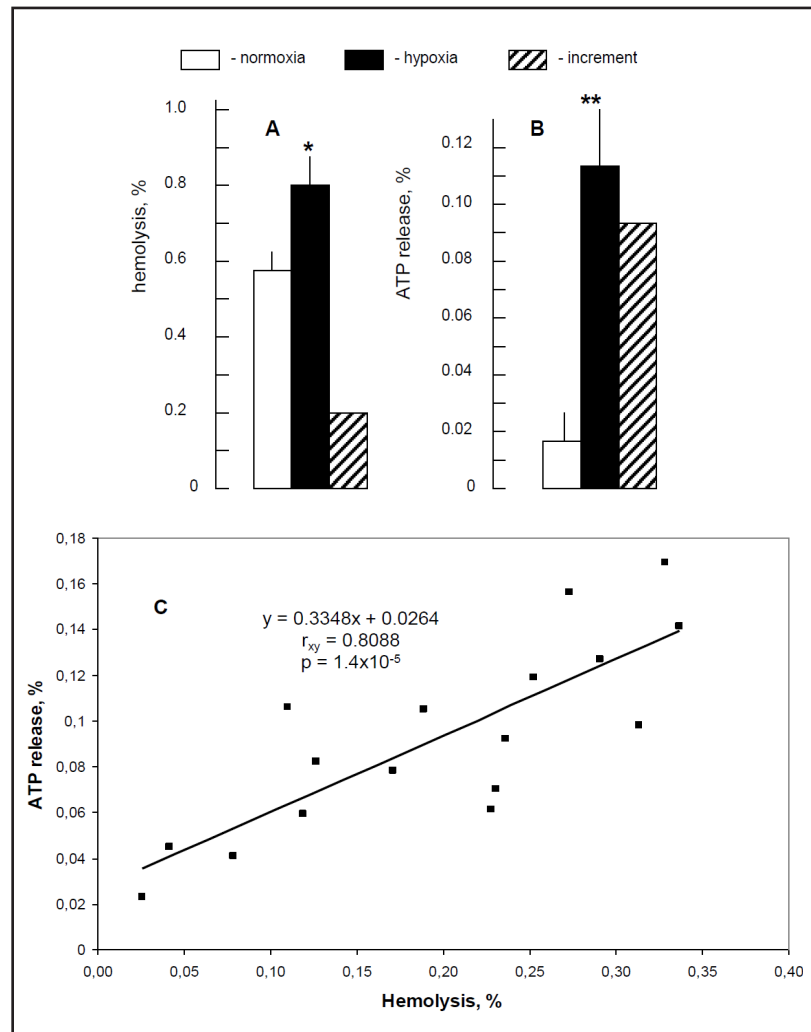
Statistical analysis was performed using Mann-Whitney U-test in *Statistica 8.0* software package.

Results and Discussion

In the initial experiments, we compared hemoglobin and ATP release in control and deoxygenated erythrocytes isolated from 17 volunteers. Sprague and co-workers reported the negligible hypoxia-induced ATP release in erythrocytes from patients with type 2 diabetes mellitus [12, 13], cystic fibrosis [14] and idiopathic pulmonary hypertension [15]. More recently, Kirby et al. did not detect any actions of low pO_2 on ATP release from erythrocytes of aged humans [11]. Considering these data, we limited our correlation analysis of hemolysis and ATP release to young adults. Figures 2A and 2B show that 20-min hypoxia resulted in elevation of extracellular hemoglobin and ATP content by ~ 1.4 and 6-fold, respectively. Importantly, we found a positive correlation between increments of hemolysis and extracellular ATP content evoked by hypoxia with the Pearson product-moment correlation coefficient $r_{xy} = 0.8088$ and $p = 1.4 \times 10^{-5}$ (Fig. 2C). Our results are consistent with data obtained in human erythrocytes subjected to 30-min low-oxygen conditions [22] but contradict to negative results in human erythrocytes exposed to 50-s hypoxia/hypercapnia [7] and 20-min hypoxia in the presence of 20% Dextran 40 [21]. It should be underlined that in our experiments hypoxic conditions were applied at room temperature whereas in other studies erythrocytes were incubated at 37°C [7-9, 11, 15]. This comment become important because elevation of temperature in the range from 33 to 39°C increased baseline ATP release from human erythrocytes by 3-fold [16]. Considering the methodological differences and experimental protocols of free hemoglobin and ATP determinations, i.e. paired vs. unpaired measurements, additional experiments should be performed to clarify the origin of the divergent results obtained in the cited studies as well as the effect hypoxia on the hemolysis of erythrocytes from persons with above-listed pathologies.

Eryptosis, i.e. the suicidal erythrocyte death characterized by cell shrinkage, membrane blebbing, and phosphatidylserine translocation to the outer membrane leaflet, has been subjected to details investigation by Lang and co-workers [27, 28]. To the best of our knowledge, there is no systematic study on the impact of hemolysis in eryptosis. It should be noted, however, that with a few exceptions [29] eryptosis was not accompanied by any significant accumulation of extracellular hemoglobin. Moreover, both membrane blebbing and phosphatidylserine translocation was detected after 24 hours of the erythrocyte exposure to Ca^{2+} -rising compounds and other canonical eryptotic stimuli [30-33] contrasting to the rapid hemolysis detected in 20 min of hypoxia (Fig. 2A).

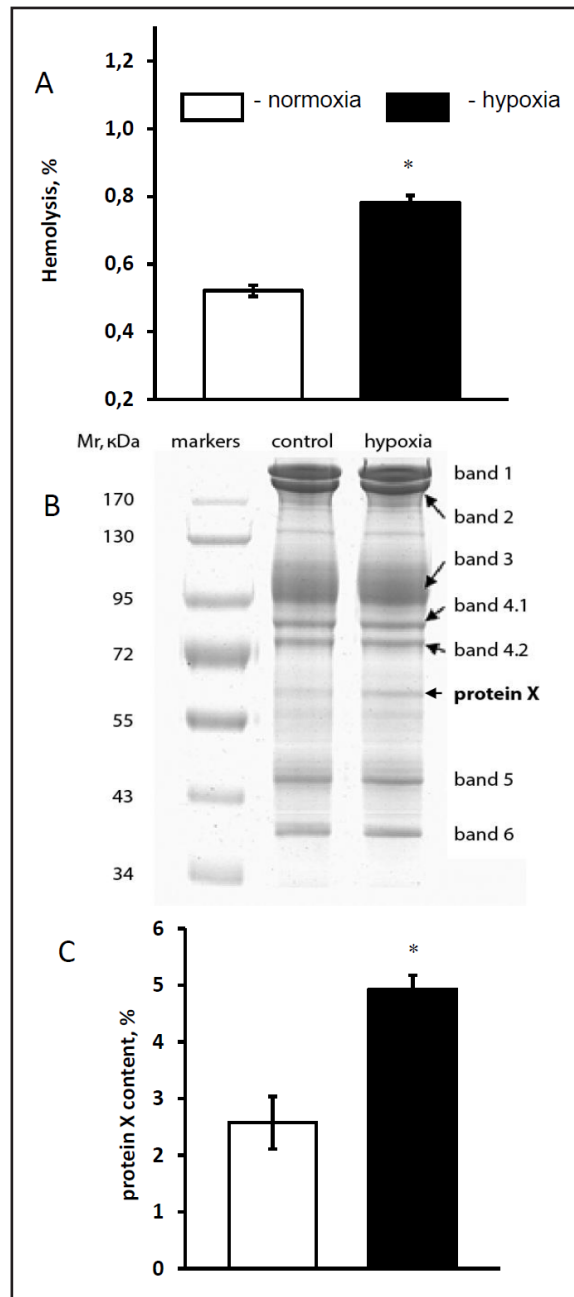
Fig. 2. Relationship between increments of hemolysis and ATP release in human erythrocytes. Accumulation of extracellular hemoglobin (A) and ATP (B) triggered by 20-min incubation of human erythrocytes in normoxic and hypoxic conditions. Means \pm S.E. obtained in 17 experiments with blood from different donors are shown. The total content of hemoglobin and ATP was taken as 100%. *, ** $p < 0.02$ and 0.001 compared to normoxia, respectively. Mean values of increments triggered by hypoxia are shown by hatched columns. C. Linear regression analysis of increments of hemolysis and ATP release triggered by hypoxia. r_{xy} is the Pearson product-moment correlation coefficient and p is the measure of correlation's significance estimated by the Student's t-test.



As the only known O_2 -binding molecules in red blood cells, hemoglobin has been implicated in cellular responses triggered by hypoxia [9]. Indeed, it was shown that skeletal muscle perfusion and plasma ATP in exercising humans exposed to hypoxia, hyperoxia, carbon monoxide inhalation and anemia are closely related to the amount of oxygenated hemoglobin rather than to plasma pO_2 and the number of hemoglobin molecules [10, 34-36]. Recently Jagger and co-workers demonstrated the negative correlation of ATP release and the content of oxygenated relaxed form of hemoglobin (R-Hb) and suppression of hypoxia-induced ATP release by CO fixing hemoglobin in R-Hb conformation [35]. These data allowed us to propose that deoxygenated tense form of hemoglobin (T-Hb) interacts with unknown membrane-bound protein thus triggering downstream signaling cascade terminated by hemolysis and ATP release. To examine the role of membrane-bound proteins in pO_2 -dependent signaling, we performed comparative analysis of SDS-PAGE electrophoresis of erythrocyte ghosts obtained in control and deoxygenated conditions. Figure 3A shows that 20-min incubation of human erythrocytes in the oxygen-free environment increased the accumulation of hemoglobin in extracellular medium by 1.5-fold ($p < 0.01$). We found that in these red blood cells deoxygenation resulted in ~ 2 -fold elevation of the content of membrane-bound protein with a Mr of ~ 60 kD and shown as protein X (Fig. 4).

Band 3 protein, i.e. the major integral protein of erythrocytes membrane, plays a key role in the organization of membrane cytoskeleton as well as in anion transport (anion exchanger 1, AE1) [37]. It was shown that both in humans and mice the affinity of band 3 fragment with M_r of 43 kDa (CDB3) for T-Hb is much higher than for oxygenated hemoglobin [38, 39]. These

Fig. 3. Effect of hypoxia on hemolysis and the content of membrane bound proteins in human erythrocytes. A - Hemolysis of erythrocytes under conditions of normoxia and hypoxia. Means \pm S.E. from 4 independent experiments are shown. The total content of hemoglobin was taken as 100%. * $p < 0.01$. B - Representative PAGE of erythrocyte ghost obtained in normoxic and hypoxic conditions. C - the relative protein X content in erythrocyte membranes obtained in control (open bars) and hypoxic (black bars) conditions. Means \pm S.E. from 4 independent experiments are shown. The content of band 3 protein was taken as 100%. * $p < 0.01$.



data allowed researcher to assume that T-Hb/band3 interaction is crucial for triggering the downstream pO_2 -dependent signals including altered cytoskeleton assembly which role in the maintenance of erythrocyte membrane integrity is well-documented [40-43]. Indeed, using inside-out vesicles it was shown that addition of T-Hb leads to displacement of ankyrin from band 3 thus weakening membrane-cytoskeleton interactions and promoting plasma membrane vesiculation and rupture [44]. We clear understand, however, that at this stage of knowledge such model is purely speculative. Additional experiments should be performed to identify molecular origin of protein X and its role in pO_2 -sensitive, band-3-mediated cytoskeleton network rearrangement, maintenance of plasma membrane integrity and erythrocyte hemolysis in the modest, *in vivo*-like hypoxic conditions.

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Disclosure Statement

All authors have none to declare.

References

- 1 Jensen FB: The dual roles of red blood cells in tissue oxygen delivery: oxygen carriers and regulator of local blood flow. *J Exp Biol* 2009; 212:3387-3393.
- 2 Dietrich HH, Ellsworth ML, Sprague RS, Dacey RG: Red blood cell regulation of microvascular tone through adenosine triphosphate. *Am J Physiol Heart Circ Physiol* 2000; 278:H1294-H1298.
- 3 Luneva OG, Sidorenko SV, Maksimov GV, Grygorczyk R, Orlov SN: Erythrocytes as regulators of blood vessel tone. *Biochemistry (Moscow)Supplement Series A: Membrane and Cell Biology* 2015; 9:161-171.
- 4 Burnstock G, Kennedy C: A dual function for adenosine 5'-triphosphate in the regulation of vascular tone. Excitatory cotransmitter with noradrenaline from perivascular nerves and locally released inhibitory intravascular agent. *Circ Res* 1986; 58:319-330.
- 5 Erlinge D, Burnstock G: P2 receptors in cardiovascular regulation and disease. *Purinergic Signal* 2008;4:1-20
- 6 Ralevic V, Dunn WR: Purinergic transmission in blood vessels. *Auton Neurosci* 2015;191:48-66.
- 7 Bergfeld GR, Forrester T: Release of ATP from human erythrocytes in response to brief period of hypoxia and hypercapnia. *Cardiovasc Res* 1992;26:40-47.
- 8 Ellsworth ML, Forrester T, Ellis CG, Dietrich HH: The erythrocyte as a regulator of vascular tone. *Am J Physiol* 1995;269:H2155-H2161.
- 9 Ellsworth ML, Ellis CG, Goldman D, Stephenson AH, Dietrich HH, Sprague RS: Erythrocytes: oxygen sensors and modulators of vascular tone. *Physiology* 2008;24:107-116.
- 10 Gonzalez-Alonso J, Olsen DB, Saltin B: Erythrocyte and regulation of human skeletal muscle blood flow and oxygen delivery. Role of circulating ATP. *Circ Res* 2002;91:1046-1055.
- 11 Kirby BS, Crecelius AR, Voyles WF, Dinunno FA: Impaired skeletal muscle blood flow control with advancing age in humans. Attenuated ATP release and local vasodilatation during erythrocyte deoxygenation. *Circ Res* 2012;111:220-230.
- 12 Sprague RS, Stephenson AH, Bowles EA, Stumpf MS, Lonigro AJ: Reduced expression of Gi in erythrocytes of humans with diabetes type 2 is associated with impairment of both cAMP generation and ATP release. *Diabetes* 2006;55:3588-3593.
- 13 Sprague RS, Goldman D, Bowles EA, Achilleus D, Stephenson AH, Ellis CG, Ellsworth ML: Divergent effects of low O₂ tension and iloprost on ATP release from erythrocytes of humans with type 2 diabetes: implications for O₂ supply to skeletal muscle. *Am J Physiol Heart Circ Physiol* 2010;299:H566-H573.
- 14 Sprague RS, Ellsworth ML, Stephenson AH, Kleinhenz ME, Lonigro AJ: Deformation-induced ATP release from red blood cells requires CFTR activity. *Am J Physiol Heart Circ Physiol* 1998;275:H1726-H1732.
- 15 Sprague RS, Stephenson AH, Ellsworth ML, Keller C, Lonigro AJ: Impaired release of ATP from red blood cells of humans with primary pulmonary hypertension. *Exp Biol Med* 2001;226:434-439.
- 16 Kalsi KK, Gonzalez-Alonso J: Temperature-dependent release of ATP from human erythrocytes: mechanism for the control of local tissue perfusion. *Exp Physiol* 2012;97:419-432.
- 17 Wan J, Ristenpart WD, Stone HA: Dynamics of shear-induced ATP release from red blood cells. *Proc Natl Acad Sci USA* 2008;105:16432-16437.
- 18 Locovei S, Bao L, Dahl G: Pannexin 1 in erythrocytes: function without a gap. *Proc Natl Acad Sci USA* 2006;103:7655-7659.
- 19 Burnstock G: Blood cells: an historical account of the roles of purinergic signalling. *Purinergic Signaling*

2015;1:411-434.

- 20 Shaskey DJ, Green GA: Sports haematology. *Sports Med* 2000;29:27-38.
- 21 Mairbaurl H, Ruppe FA, Bartsch P: Role of hemolysis in red cell adenosine triphosphate release in simulated exercise conditions in vitro. *Med Sci Sports Exerc* 2013;10:1941-1947.
- 22 Sikora J, Orlov SN, Furuya K, Grygorczyk R: Hemolysis is a primary ATP-release mechanism in human erythrocytes. *Blood* 2014;124:2150-2157.
- 23 Sikora J, Orlov SN, Furuya K, Grygorczyk R: Hemolysis is a primary and physiological relevant ATP release mechanism in human erythrocytes. *Blood* 2015;125:1845-1846.
- 24 Wood BR, Caspers P, Puppels GJ, Pandiancherri S, McNaughton D: Resonance Raman spectroscopy of red blood cells using near-infrared laser excitation. *Anal Bioanal Chem* 2007;387:1691-1703.
- 25 Lowry OH, Rosebrough NJ, Farr AJ, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-275.
- 26 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-685.
- 27 Lang F, Qadri SM: Mechanisms and significance of eryptosis, the suicidal death of erythrocytes. *Blood Purif* 2012;23:125-130.
- 28 Lang E, Lang F: Mechanisms and pathophysiological significance of eryptosis, the suicidal erythrocyte death. *Semin Cell Dev Biol* 2015;39:35-45.
- 29 Voelkl J, Alzoubi K, Mamar A-K, Ahmed MSE, Abed M, Lang F: Stimulation of suicidal erythrocyte death by increased extracellular phosphate concentrations. *Kidney Blood Press Res* 2013;38:42-51.
- 30 Lang KS, Duranton C, Poehlmann H, Myssina S, Bauer C, Lang F, Wieder T, Huber SM: Cation channels trigger apoptotic death of erythrocytes. *Cell Death Differ* 2003;10:249-256.
- 31 Schneider J, Nicolay JP, Foller M, Wieder T, Lang F: Suicidal erythrocyte death following cellular K⁺ loss. *Cell Physiol Biochem* 2007;20:35-44.
- 32 Lang F, Gullbins E, Lang PA, Zappulla D, Foller M: Ceramide in suicidal death of erythrocytes. *Cell Physiol Biochem* 2010;26:21-28.
- 33 Malik A, Bissinger R, Liu G, Liu G, Lang F: Enhanced eryptosis following gramicidin exposure. *Toxins* 2015;7:1396-1410.
- 34 Hanada A, Sander M, Gonzalez-Alonso J: Human skeletal muscle sympathetic nerve activity, heart rate and limb haemodynamic with reduced blood oxygenation and exercise. *J Physiol* 2001;524:603-615.
- 35 Jagger JE, Bateman RM, Ellsworth ML, Ellis CG: Role of erythrocyte in regulating local O₂ delivery mediated by hemoglobin oxygenation. *Am J Physiol Heart Circ Physiol* 2015; 280:H2833-22839.
- 36 Gonzalez-Alonso J, Mortensen SP, Dawson EA, Secher NH, Damsgaard R: Erythrocytes and the regulation of human skeletal muscle blood flow and oxygenation delivery: role of erythrocyte count and oxygenation state of haemoglobin. *J Physiol* 2006;572:295-305.
- 37 Low PS, Westfall MA, Allen DP, Appel KC: Characterization of the reversible conformational equilibrium of the cytoplasmic domain of erythrocyte membrane band 3. *J Biol Chem* 1984;259:13070-13076.
- 38 Walder JA, Chatterjee R, Steck TL, Low PS, Musso GF, Kaiser ET, Rogers PH, Arnone A: The interaction of hemoglobin with the cytoplasmic domain of band 3 of the human erythrocyte membrane. *J Biol Chem* 1984;259:10238-10246.
- 39 Sega MF, Chu H, Christian J, Low PS: Interaction of deoxyhemoglobin with the cytoplasmic domain of murine erythrocyte band 3. *Biochemistry* 2012;51:3264-3272.
- 40 Chasis JA, Agre PA, Mohandas N: Decreased membrane mechanical stability and in vivo loss of surface area reflect spectrin deficiencies in hereditary spherocytosis. *J Clin Invest* 1988;82:617-623.
- 41 Peters LL, Shivdasani RA, Liu S-C, Hanspal M, John KM, Gonzalez JM, Brugnara C, Gwynn B, Mohandas N, Alper SL, Orkin SH, Lux SE: Anion exchanger 1 (Band 3) is required to prevent erythrocyte membrane surface loss but not to form the membrane skeleton. *Cell* 1996;86:917-927.
- 42 Cruz Silva MM, Madeira VMC, Almeida LM, Custodio JBA: Hemolysis of human erythrocytes induced by tamoxifen is related to disruption of membrane structure. *Biochim Biophys Acta* 2000;1464:49-61.
- 43 Barvitenko NN, Adragna N, Weber RE: Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance. *Cell Physiol Biochem* 2005;15:1-18.
- 44 Stefanovic M, Puchulu-Campanella E, Kodippili G, Low PS: Oxygen regulates the band 3-ankyrin bridge in the human erythrocyte membrane. *Biochem J* 2013;449:143-150.