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# Nonneuronal Cholinergic System in Human Erythrocytes: Biological Role and Clinical Relevance

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**Abstract** Acetylcholine is well known in the medical setting as one of the most exemplary neurotransmitters. Its ubiquity in nature otherwise suggests a theoretically diverse spectrum of action and an extremely early appearance in the evolutionary process. In humans, acetylcholine and its synthesizing enzyme, choline acetyltransferase, have been found in various nonneural tissues such as the epithelium, mesothelium, endothelium, muscle, immune cells and blood cells. The widespread expression of nonneuronal acetylcholine is accompanied by the ubiquitous presence of acetylcholinesterase and nicotinic/muscarinic receptors. Structural and functional dissimilarities are evident between the nonneuronal and neuronal cholinergic systems. An increasing body of evidence throughout the last few years has placed acetylcholine as a major cellular signaling molecule in many pathways. Furthermore, numerous erythrocyte physiological events in the microcirculation are strongly regulated by acetylcholine. Thus, it is time to revise our understanding of the role of vascular acetylcholine in humans. Its biological and pathobiological roles must be evaluated in more detail to eventually achieve novel therapeutical targets. The present article reviews recent findings about nonneuronal acetylcholine in red blood cells, with special regard to (1) red cell rheology, (2) plasma ion concentrations, (3) nitric oxide intracellular translocation and metabolism and (4) band 3 protein phosphorylation.

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## Introduction

Acetylcholine (ACh) is known to act as a neurotransmitter inside the central and peripheral nervous systems in human beings (Wessler and Kirkpatrick 2008). However, recent experiments in humans have documented a wider expression of the cholinergic system in several nonneuronal tissues. The "nonneuronal cholinergic system" (NNCS) is a new concept concerning the extraneuronal effects of ACh. It is becoming progressively evident that the cholinergic system is not confined to the nervous system but is nearly ubiquitous. ACh is far from being exclusively a neurotransmitter, thereby doing extremely more than just mediating rapid communication between neurons and effector cells. It has been shown to be present in human blood circulation, being produced by T lymphocytes and vascular endothelium (Grando et al. 2003, 2007; Wessler et al. 2007; Wessler and Kirkpatrick 2001, 2008). An interesting finding concerns the enhanced levels of circulating ACh observed in inflammatory conditions along with the anti-inflammatory effect of ACh in the rat systemic response to endotoxin (Grando et al. 2007; Klapproth et al. 1997; Wessler and Kirkpatrick 2001).

In fact, the biology of the NNCS in humans has been recognized in the past, in a way to better optimize the cholinergic action for its additional neural role. For instance, vascular endothelial cells together with immune cells (namely, lymphocytes) express the whole components of the cholinergic system independently of any neural innervation. The synthesis and function of ACh in the

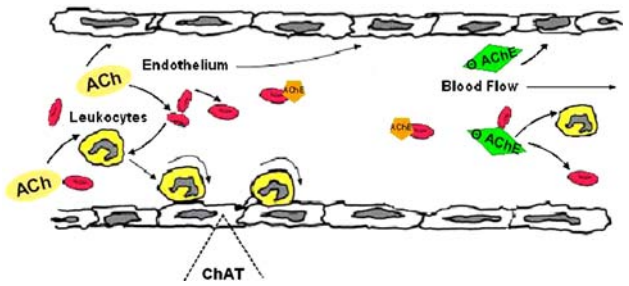


**Fig. 1** Circulating ACh as a broad vascular modulator

nonneuronal system (e.g., blood cells) has been object of many investigations taking into account its vascular action (Fig. 1; Kirkpatrick et al. 2001, 2003; Neumann et al. 2007).

Altogether, ACh properties may reflect a crucial "trophic property." The leading tasks for the decades to come will be to dissect the biological role of nonneuronal ACh in detail and to track pathological conditions in which this system may be up- or downregulated. This issue could provide the basis for the development of further therapeutic weapons aimed at targeting the NNCS. The present review will primarily focus on the expression and function of the NNCS, which we have been studying in human red blood cells, and hypothesize possible implications in the clinical setting.

## Nonneuronal ACh

The cholinergic system, both neuronal and nonneuronal, consists of the synthesizing enzyme choline-acetyltransferase (ChAT), the signaling molecule ACh, storing organelles (cholinergic vesicles and transporter proteins), ACh-sensitive nicotinic and muscarinic receptors and the hydrolyzing enzymes, i.e., the specific acetylcholinesterase (AChE) and the nonspecific cholinesterases (pseudocholinesterase, butyrylcholinesterase, plasmacholinesterase; Grando et al. 2003; Sastry and Janson 1994; Wessler et al. 2001, 2003).

ACh, being composed of the three most common atoms (carbon, nitrogen and oxygen), represents a phylogenetically old signaling compound with a locally acting function. One may hypothesize that this purported evolutionary process may have optimized the cholinergic system to possibly utilize ACh as a signaling molecule, through either autocrine or paracrine behaviors, and eventually as a neurotransmitter. Therefore, it is not surprising that the vast majority of human cells contain ACh. The stockpile, transport and release of ACh seem to differ between neuronal and nonneuronal cells. Throughout time, variable regulations of ACh synthesis in all the different cells have been achieved by creating ChAT isoenzymes (Wessler et al. 1999). Yet, the number of distinct isoenzymes expressed in human tissues remains to be elucidated.

The action of ACh is prevented by local inactivating enzymes, predominantly the specific AChE and the nonspecific butyrylcholinesterase (BChE). The ubiquitous expression of these cholinesterases guarantees a functional division between the molecule's dual role—the local hormone/modulator and the neurotransmitter. Nonneuronal ACh appears to be involved in the regulation of elementary cell functions such as cell mitosis, cell–cell interaction, cell automaticity, locomotion, ciliary activity, barrier function, resorption and secretion. In the airways, for instance, the great majority of cells express the components of the NNCSm and it is documented that a substantial increase in ACh levels triggers the release of proinflammatory effectors (Tracey 2002). In addition, the excitability of airway mast cells can be powerfully inhibited by ACh (Grando et al. 2007; Kirkpatrick et al. 2001; Wessler et al. 2003, 2007; Wessler and Kirkpatrick 2001).

Both ChAT and ACh have been described to occur in all epithelial surface cells, submucosal glands and airway smooth muscle fibers. ACh is also found in effector cells of the immune system (lymphocytes, macrophages, and mast cells). Epithelial, endothelial and immune cells are additionally endowed with nicotinic and muscarinic receptors. Thus, the cytomolecule ACh can contribute to the regulation of basic cell functions via auto-/paracrine mechanisms (proliferation; differentiation; secretion of water, ions and mucus; cytoskeletal organization; release of cytokines; proliferation; cell activation; inhibition). With regard to the cholinesterases, red blood cells are in fact the blood elements with the highest content of AChE, whereas BChE is uniformly distributed in the liver, lung, smooth muscle and bloodstream (Kawashima and Fujii 2004; Neumann et al. 2007; Wessler et al. 1998).

Finally, detailed analysis of the expression and function of nonneuronal ACh may help to gain more knowledge about the pathogenesis of chronic inflammatory pathology of several tissues. Upregulated epithelial ACh may cause an impairment of the barrier and immune functions. It will be a major goal for further research to identify the physiopathological conditions in which the system is up- or downregulated. Additionally, innovative compounds, targeting the expression and function of the NNCS by topical application (e.g., in eye, skin, airways, alimentary tract), could be developed.

## The NNCS in Human Erythrocytes

There is much ongoing research about the cholinergic effects on erythrocytes. A similar mechanism to what happens in vascular endothelial cells may occur (Eglen

2006; Furchgott and Zawadzki 1980; Kirkpatrick et al. 2003). The nonneuronal relevance of ACh effects on erythrocyte membranes has been questioned by the presence of the AChE membrane-bound enzyme. In truth, the proper role of erythrocyte AChE is still unclear and the object of many investigations. Erythrocytes are the peripheral blood elements with the highest levels of this membrane-spanning enzyme and, hence, are regarded as markers for their integrity. Despite its well-known function in the neuromuscular junctions, the key role of erythrocyte AChE remains to be explored (Tang 1986; Wright and Plummer 1973). Erythrocytes are very effective scavengers of nonneuronal ACh escaping into the bloodstream. In addition, the specific cholinesterase shows the utmost substrate turnover rate of all enzymes characterized in biological systems hitherto. This high activity limits ACh action within the cell microenvironment, preventing it from working as a hormone for actions remote from its place of synthesis (Wright and Plummer 1973). In parallel, the ACh muscarinic receptors were characterized in erythrocytes as type M1 (Tang 1986).

On the one hand, nonneuronal ACh is capable of modulating the hemorheological and oxygen-carrying properties of human erythrocytes (Fig. 2; Mesquita et al. 2001, 2002; Santos et al. 2003). Red cell rheology in particular remains a focus of interest, given the fact that the presence and/or prognosis of cardiovascular diseases may be associated with abnormalities of one or more blood properties. According to prior studies from our research group, ACh induces changes in erythrocyte aggregation, erythrocyte deformability and lipid membrane fluidity. Aggregation and deformability are two closely associated properties on which blood viscosity is for the most part dependent. Under normal blood flow, red cells mostly aggregate in postcapillary venules, where there is a shear stress decrease; also, the ability to deform is a property of red cells related to their rigidity, essential for flowing through narrowed capillaries with lower diameters than their own. In a former in vitro study, we demonstrated that ACh decreases erythrocyte aggregation and increases deformability (at lower shear stress) when present in blood samples from healthy donors. Moreover, ACh increases lipid fluidity, which is an index of order and rate of phospholipid movement in the bilayer. Changes in the erythrocytic hemorheological properties have physiological relevance since they trigger changes in blood viscosity and modulate tissue oxygenation and the distribution of blood in the several vascular territories (Mesquita et al. 2001, 2002; Santos et al. 2003).

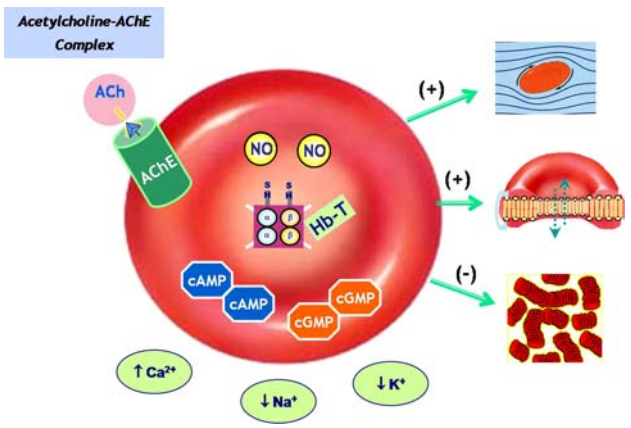


**Fig. 2** ACh-mediated effects in human erythrocytes: *1* increased NO and cyclic nucleotide levels, *2* increased P50 and lower Hb–oxygen affinity, *3* enhanced red cell deformability and membrane fluidity and impaired aggregation and *4* changes in plasma ions

Additionally, significant decreases in plasma pH as well as $K^+$ and $Na^+$ concentrations have been detected in the presence of ACh, plus an increase in $Ca^{2+}$ concentration and P50 values. Further evidence disclosed that, depending on the nitric oxide (NO) levels, there are different erythrocyte structural and functional properties. Increasing NO concentrations stimulate lipid membrane fluidity and P50 reduction, unlike deformability and methemoglobin levels, which are enhanced. In the presence of spermineNONOate, a NO donor, the same results are observed along with an increase in plasma pH and a decrease in $Na^+$ and $Ca^{2+}$ concentrations (Mesquita et al. 2001, 2002).

From a different angle, erythrocytes also have the ability to release their NO stores, previously bound to hemoglobin (Hb), thereby being considered a chariot for NO bioactivity. A parallel study of what occurs in the endothelium has illustrated significant alterations in the efflux of erythrocytic NO and its oxidative metabolites, nitrites and nitrates ($NO_x$), in ACh-treated erythrocytes (Carvalho et al. 2004, 2005). This issue will be discussed later in this review.

In this scenario, our research group has raised a few hypotheses aimed at explaining the rationale behind the aforementioned results (Fig. 3), some of which will be dissected in detail further. The action of nonneuronal ACh on erythrocytes could occur by (1) binding to the AChE enzyme with activation of a G protein, which could modulate band 3 protein activity, an interaction that could be the basis of NO translocation among nitrosylated molecules and phosphorylated/dephosphorylated band 3 protein, respectively, by protein-tyrosine kinases and phosphotyrosine phosphatases; (2) binding to muscarinic receptors with consequent activation of G protein-dependent phospholipase C enzyme and then protein kinase C activation with cytoplasmatic calcium uptake; (3) inner "cross-talk" processes involving cAMP and/or cGMP formation by adenylyl cyclase and/or guanylate cyclase activation, respectively.

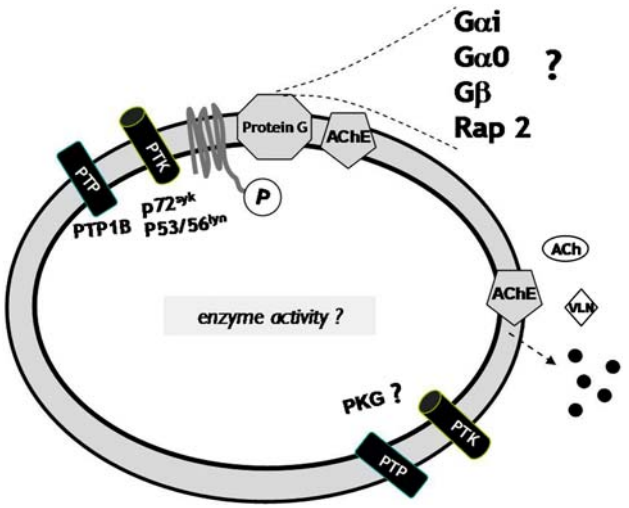


**Fig. 3** Possible erythrocytic mechanisms to explain ACh-dependent modulation

## ACh and NO Bioactivity

A variety of physiopathological processes in microcirculation disease feature overt alterations in the NO intracellular or plasma metabolism. NO is an endothelium-derived decisive mediator of microvascular homeostasis since it is a key molecule in the respiratory cycle and sustains a targeted delivery to red blood cells, thereby modulating the vasoconstriction/vasodilation balance in blood flow (Gross 2001; Gross and Wolin 1995). Characteristically, erythrocytes are known to be involved in various hemorheological phenomena. Following NO passage into red cells by simple random diffusion, it may be either stored or returned to the bloodstream as an active *S*-nitrosothiol molecule. Particularly, it interacts with the erythrocyte membrane molecules along with Hb, yielding *S*-nitrosohemoglobin (SNO-Hb), wherein its major intravascular stores are bound. The main biological metabolites of NO metabolism are represented by $NO_x$ and include both nitrates ($NO_3^-$) and nitrites ($NO_2^-$) (Pawloski et al. 2001).

Despite hard research, the machinery of release of NO-bound intermediaries in the erythrocyte cytoplasm is poorly settled. Provided that the erythrocyte works as an NO scavenger, it allows an important NO-mediated blood flow regulation mediated by responses to changing oxygen levels (Pawloski et al. 2001). This principle should result in capture of NO in highly oxygenated tissues and release in relatively hypoxic tissues. In fact, in high oxygen saturation vessels Hb remains in the R structure and NO is foraged to $Fe^{2+}$ and, afterward, to Cys $\beta$-93; on the contrary, low oxygen saturation blood vessels prompt the T structure to allow SNO-Hb binding to band 3 protein and, consequently, yield SNO-band 3.

With regard to the ACh vasodilating action, universally addressed to the vascular endothelium, it is well established that ACh stimulates NO synthase. As a result, increased NO levels occur from its natural substrate L-arginine, which will next activate guanylate cyclise enzyme (Furchgott and Zawadzki 1980; Kirkpatrick et al. 2001, 2003). As to this, we have formerly proposed a similar mechanism occurring inside red blood cells, anticipating that band 3 protein would be involved in the signal transduction mechanisms associated with nitrite/nitrate production (European Society for Microcirculation 2006). Band 3 is a multifunctional protein containing four tyrosine residues, the phosphorylation level of which is able to regulate physiological processes such as glycolysis, cell shape and membrane transport. Regardless of the higher erythrocyte content of AChE shed vesicles, when compared with band 3 molecules, we hypothesized that changes in band 3 conformation could occur when ACh binds to AChE (Fig. 4; European Society for Microcirculation 2006).

Concerning the second messenger levels within the erythrocytes, ACh was proved to be a trigger of both cGMP and cAMP cyclic nucleotides, which together with NO values can hypothetically lead us to a cross-talk mechanism (unpublished). Increased NO will activate the guanylate cyclase responsible for cGMP production, via GTP. Either a hypothetical stimulation of cAMP or the well-known inhibition of phosphodiesterase III could be the basis of its intracellular boost (Fig. 5).

## ACh and Vascular Disease

The ACh vasomotor response is NO-dependent, and the loss of its biological activity and biosynthesis is one of the known mechanisms responsible for vascular disorders. Atherosclerosis, hypertension, diabetes, reperfusion injury and vasculopathy in general, as well as angioplasty, bypass

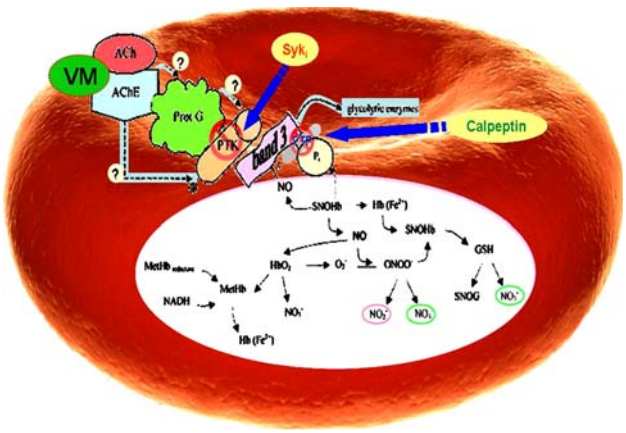


**Fig. 4** Hypothesis for the role of AChE signaling mechanism on red cell nitrite/nitrate levels

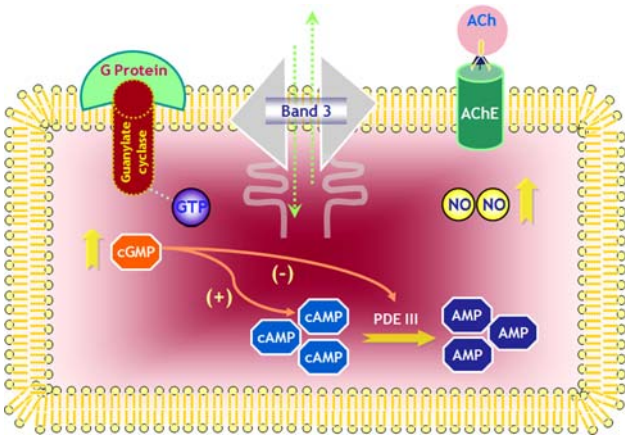


**Fig. 5** ACh influence on cyclic nucleotides (cAMP/cGMP)

surgery and transplantation, are examples of diseases/procedures in which NO deficiency triggers endothelial dysfunction. An increasing body of evidence has shown significant associations between hemorheological properties and hypertension-changed parameters (Gross and Wolin 1995; Lowe et al. 2000). In a recent experiment of ours (Santos et al. 2003), erythrocytes from subjects diagnosed with hypercholesterolemia, renal transplantation and hypertension were stimulated with ACh and afterward compared with healthy subjects on the following measured items: NO levels, Hb and hematocrit values, erythrocyte aggregation, erythrocyte deformability, plasma viscosity and fibrinogen concentration. In each of those dysfunctions, increased NO production was observed after ACh stimulation, albeit statistical significance was only seen in the hypercholesterolemic group (Table 1). In accordance with the literature, recent studies are suggesting that oxidized LDL forms specifically impair NO-dependent arterial relaxation through different mechanisms (McHedlishvili 2000). Patients with hypercholesterolemia showed significantly enhanced erythrocyte aggregation, plasma viscosity and fibrinogen concentration, with impaired erythrocyte deformability. Previous reports documented the enrichment of cholesterol/phospholipids to prompt elevated plasma viscosity and reduced deformation capacity (Wever et al. 1998). We can predict that the physiological repercussions of either higher or lower aggregability are attenuated with changes in NO mobilization. Kidney transplant recipients also revealed augmented erythrocyte aggregation and plasma viscosity values, along with impaired erythrocyte deformability. Both plasma viscosity and fibrinogen levels were significantly increased in patients with vascular disorders ($P < 0.05$).

**Table 1** Comparison of hemorheological parameters in patients with different vascular disorders ($P < 0.05$)

| | Hypercholesterolemia | Renal transplantation | Hypertension |
|---|---|---|---|
| NO (nM) | ↑ | ↑ | ↑ |
| Aggregation (nd) | ↑ | ↑ | ↑ |
| Deformability (%) | ↓ | ↓ | ↓ |
| Plasma viscosity (mPa s) | ↑ | ↑ | ↑ |
| Fibrinogen (mg/dl) | ↑ | ↑ | ↑ |

Changes in the hemorheological parameters or NO levels may play an important role in the development of the hypertensive state (Meiselman 1999), which might eventually propose potential targets for vasodilating therapy at the microcirculatory level. In agreement, we may suppose that the erythrocyte aggregation of hypertensive subjects could be related to changes in the NO stockpile since there was a higher tendency for erythrocytes to aggregate and a lower ability to mobilize NO after ACh stimulation in the intervention group. Therefore, the erythrocyte rheological behavior might be compensated by NO production in the presence of physiological ACh. However, the molecular mechanisms underlying erythrocyte aggregation/disaggregation are not yet understood.

## ACh and Redox Thiol Status

New knowledge that NO is ferried throughout thiol groups (–SH) of targeted proteins indicates this mobilization to be on the basis of intracellular $NO_x$ mobilization (Galli et al. 2002; Gladwin 2006; Pawloski et al. 2001). Beyond this fact, the thiol–disulfide interchange is critical in a wide range of biological systems. Dithiothreitol (DTT), an exogenous thio-reducer agent, has special thiol effects in humans: ability to reduce disulfide bonds between cysteines and function as an –SH donor (Ates et al. 2009). Figure 6 illustrates a study in which we treated erythrocytes with DTT, and the results support the idea that the intracellular redox status modulates NO translocation among (nitrosylated) molecules, such as peroxynitrite and glutathione. This study (de Almeida et al. 2009) revealed that NO is mobilized into different derivative species, depending upon the erythrocyte-stimulating effector. Upon redox thiol status stimulation by DTT and ACh, NO is strongly mobilized inside erythrocytes but less is released to the extracellular compartment when compared with the erythrocyte suspensions without ACh. Increasing DTT concentrations significantly increased the levels of nitrite/nitrate. More precisely, ACh-stimulated erythrocytes revealed significantly higher levels of NO, nitrate and methemoglobin but lower oxyhemoglobin. Accordingly, reduced values of peroxynitrite and GSNO obtained in the presence of ACh may be justified by greater erythrocyte NO mobilization. Low oxyhemoglobin levels could

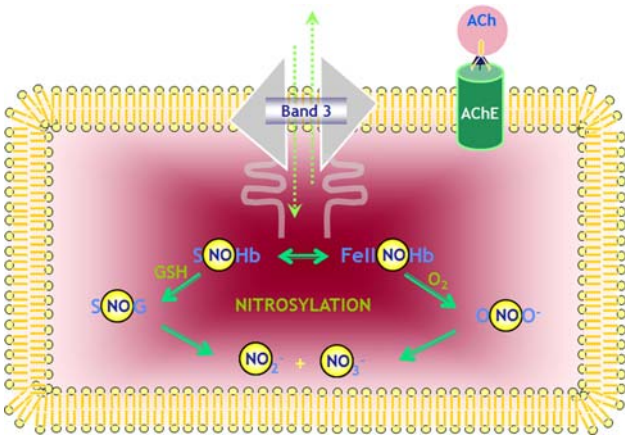


**Fig. 6** Intraglobular translocation of NO in ACh- and DTT-treated erythrocytes

ultimately explain the fewer $O_2^-$ available for peroxynitrite formation. The lower GSNO concentration obtained with ACh may result from its reduction by deoxyhemoglobin to glutathione and NO. No significant changes were seen in SNO-Hb. In view of this, we may speculate that –SH groups are the basis of the purported targeted delivery of NO metabolites.

In a second study (Almeida et al. 2008), we documented for the first time, to the best of our knowledge, that manipulation of the erythrocyte redox thiol status may trigger a tendency to interact with the nonneuronal cholinergic elements. This work revealed that increasing concentrations of a thiol reducing agent (DTT) in red cells do not significantly modify the red cell elongation index, aggregation index and membrane lipid fluidity (hemorheological parameters); but this effect is dependent on nonneuronal AChE modulation. In the presence of ACh, a shift in the erythrocyte discoid shape (observed in blood smears) was reported as well as an impaired erythrocyte aggregation index ($P < 0.05$). Although no influence is exerted on the deformability property, the lower aggregation tendency observed may become valuable in the clinical setting as far as cell-activated thiols are concerned. Determination of intracellular thiol levels may provide interesting data as to why alterations in the hemorheological profile of human erythrocytes may lead to vascular disorders.

## ACh and Band 3 Protein

Comprising up to 25% of the erythrocyte membrane, band 3 protein participates in a number of erythrocyte events regulated by the degree of phosphorylation of its tyrosine residues. Band 3 phosphorylation is promoted by protein-tyrosine kinases (PTKs; e.g., syk and lyn) and dephosphorylation by protein-tyrosine phosphatases (PTPs). We have studied several signal transduction pathways mediated by band 3 degree of phosphorylation and ACh in red cells (Carvalho et al. 2008), with special regard to the NO efflux and oxygen-carrying properties. We treated erythrocytes with PTK/PTP inhibitors, thereby inducing phosphorylated and dephosphorylated states on band 3, via activation of specific PTKs and PTPs (p72syk or p53/56lyn inhibitors and calpeptin, respectively). We observed an association between band 3 and AChE substrate (ACh). The presence of an active complex (ACh–AChE) in red blood cells is able to trigger band 3 protein phosphorylation when PTP is inhibited (Western blot analysis), with a higher mobilization of NO-derived metabolites (nitrite/nitrate). In contrast, ACh is unable to induce band 3 phosphorylation upon p53/56lyn and p72syk inhibition, providing a lower degree of $NO_x$ mobilization. Parallel results were obtained as to NO levels, thereby providing information on the intracellular lower/higher mobilization and consequent release of molecular NO toward the plasma (supernatants).

Levels of oxyhemoglobin, glyceraldehyde 3-phosphate dehydrogenase (G-3-PD) and glucose-6-phosphodehydrogenase were found to significantly decrease with ACh regardless of the band 3 phosphorylation status, whereas P50, lactate and both cGMP and cAMP increased ($P < 0.05$). We hypothesized that downregulation of G-3-PD activity upon ACh stimulation might happen via ACh-induced mobilization of NO, previously reported by us, and due to the fact that NO is able to inhibit G-3-PD by nitrosylation. Despite being involved in the regulation of both hemoglobin–oxygen affinity and glycolytic flux, this trend was expected since ACh increases red cell pH, thereby modulating several enzymes required for 2,3-diphosphoglycerate activity.

In a different study (Carvalho et al. 2009), we further investigated whether AChE enzyme activity in erythrocytes is altered under the influence of band 3 degree of phosphorylation, as well as screening for an unidentified G protein purportedly in these mechanisms. We reported for the first time, to the best of our knowledge, that the highest values of AChE activity were found in the presence of ACh without manipulation of the band 3 degree of phosphorylation. In addition, changes in band 3 protein phosphorylation/dephosphorylation status induce a statistically significant decrease in AChE enzyme activity. We then hypothesized that the degree of band 3 phosphorylation could explain those findings concerning AChE enzyme activity, as a consequence of a conformational modulation between the two proteins. In the same study, we therefore proceeded to Western blot analysis and found that among the well-known erythrocyte G proteins there is an association between protein $G_{\alpha i1/\alpha i/2}$ and band 3 at either the N or the C terminus, which is independent of band 3

phosphorylation status. Furthermore, we identified a potential linkage of protein subunits $G_{\alpha i1/2}$ and $G_{\beta}$ with band 3 protein. $G_{\alpha i1/2}$ is associated with the band 3 N-terminal domain, except for ACh aliquots. $G_{\beta}$ is associated with both phosphorylated and dephosphorylated band 3 in the presence of AChE inhibitor. We concluded that AChE enzyme activity is dependent on band 3 protein phosphorylation status and may be involved in a conformational linkage between a G protein and band 3.

## Summary

To review, the NNCS, widely expressed in human cells independent of a nervous function, represents a local regulatory system contributing to cell and organ homeostasis. The ubiquitous expression indicates ACh might act as a global player in nature. These findings should be considered when the therapeutic role of ACh is discussed. There is an array of drugs acting on ACh production, the mechanism of which could be better explored at the circulatory level.

Based on the ubiquitous expression of nonneuronal ACh and its role in maintaining cellular phenotype, impaired expression or function of the NNCS should result in impaired cell/organ homeostasis. It is an essential task to clarify the pathophysiological role of the NNCS in more detail to develop new drugs which can target the synthesis, release, inactivation and cellular activity of nonneuronal ACh. All things considered, these observations will open a new avenue of investigation of the old molecule ACh and its biological functions in nature.

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