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Acetylcholinesterase Conformational States Influence Nitric Oxide Mobilization in the Erythrocyte

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Abstract In the human erythrocyte, band 3 protein mediates nitric oxide (NO) translocation and its effects are strongly related to phosphorylated/dephosphorylated intracellular states. The metabolism of NO could change in the presence of acetylcholinesterase (AChE). Therefore, the present study was designed to assess the effect of conformational changes in AChE (via N-19 and C-16 antibodies) and enzymatic inhibition/activation of protein kinase C (PKC) in erythrocyte NO mobilization in vitro. Our results show that by inhibiting PKC with cheletrine, impaired erythrocyte NO efflux and s-nitrosoglutathione (GSNO) levels were verified, while PKC's activation by Phorbol 12-myristate 13-acetate had the opposite effect. Those results demonstrate the influence of 4.1R complex and band 3 protein level of phosphorylation on NO efflux and GSNO concentration mediated by PKC inhibition/activation. In addition, the present study shows evidence that conformational changes in AChE promoted by incubation with N-19 and C-16 antibodies alter the enzyme's functional connection to acetylcholine (ACh) (AChE-ACh complex) in an irreversible manner, resulting in impaired GSNO concentration and NO efflux from the erythrocyte. Novel insight into NO metabolism in the erythrocyte is brought with the presented findings allowing new possibilities of modulating NO delivery, possibly involving PKC and AChE conformational alterations in combination.

Keywords Acetylcholinesterase · Erythrocyte · Protein kinase C · Nitric oxide

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Introduction

Acetylcholine (ACh) is synthesized by endothelial cells and T-lymphocytes before release into blood circulation (Wessler et al. 1999) and can be found in several inflammatory conditions (Kawashima and Fujii 2004). In the intact endothelium, ACh acts as a vasodilator in a process mediated by nitric oxide (NO) (Furchgott and Zawadzki 1980). In the human erythrocyte, internal and external stimuli such as ACh can stimulate variations in NO derivatives levels, resulting from the internal NO metabolism (Lopes de Almeida et al. 2012). Shear stress can also lead to the release of NO (Ulker et al. 2013). In these blood cells, band 3 protein mediates NO translocation (Carvalho et al. 2008) and its effects are strongly related to phosphorylated/dephosphorylated intracellular states (Bordin et al. 1995). Association between band 3 and Gi proteins with subunits Giα1/2 and Gβ occurs and an acetylcholinesterase (AChE) involvement in a conformational linkage between Gi and band 3 proteins was proposed, associated with AChE's activity dependence on protein band 3 phosphorylation status (Carvalho et al. 2009). Alternative splicing in the 3' region of the primary transcripts originates different AChE variants, exhibiting the same catalytic domain but different C-terminal peptides, which determine their post-translational maturation and oligomeric assembly (Massoulié and Bon 2006). Erythrocytes contain the highest AChE concentrations among blood elements (Wright and Plummer 1973). The structure of this enzyme includes a globular core, at the bottom of which the active site is located, the latter including several functional subsites like a secondary substrate-binding site (Grisaru et al. 1999). Erythrocytary AChE is a glycophosphatidylinositol-linked dimer to be found in the plasma membrane (Bon et al. 1991).

Cellular enzyme protein kinase C (PKC)—a family of serine–threonine kinases isoforms—has been widely

studied and related to a broad range of important biological processes. PKC transduces post-ligand-binding events, resulting in other kinases activation, hence regulating intracellular processes, cell metabolism, cell behavior, and DNA transcription (Rosse et al. 2010; Cosentino-Gomes et al. 2012). In animal models, it was recently suggested that PKCδ can modulate NO machinery in cerebral vasculature (Lin et al. 2014). PKC plays a key role in regulation of membrane deformability and stability in erythrocytes (de Oliveira et al. 2008), and in certain forms of hypertension, PKC inhibitors modulate vascular function (Khalil 2013). Also, PKC has been reported as a potential therapeutic agent in vascular diabetes disorders (Kizub et al. 2014). Chelerythrine (Che), a benzophenanthridine alkaloid, which is a substrate-competitive active-site inhibitor of PKC both in vitro (Herbert et al. 1990; Lee et al. 1998; Davies et al. 2000; Anastassiadis et al. 2011) and in cells (Gould et al. 2011). Phorbol 12-myristate 13-acetate (PMA) is a PKC activator (Emerit and Cerutti 1981). As previously described by us (Carvalho et al. 2009), band 3 protein phosphorylation by protein tyrosine kinase (PTK) activates AChE, while band 3 protein dephosphorylation inhibits AChE. Hence, PTK can be activated through phosphorylation by PKC, and band 3 protein phosphorylation occurs. Also, protein tyrosine phosphatase (PTP) phosphorylation by PKC occurs leading to dephosphorylation of protein band 3.

From those studies in erythrocytes, we can hypothesize that AChE could influence the signal transduction pathways that regulate the NO metabolism in erythrocytes. Therefore, the present research study was designed to assess the effect of conformational changes in AChE (via N-19 and C-16 antibodies) and enzymatic inhibition/activation of PKC in erythrocyte NO mobilization.

Materials and Methods

Chemicals

Sodium chloride was purchased from AnalaR, BDH Laboratory, Poole, UK, and chloroform and ethanol 95 % from *MER*CK, Darmstadt, Germany. Acetylcholine iodide, choline chloride, nitrate reductase (Aspergillus Niger), NADPH (tetra sodium salt), mercury chloride, sodium nitrate, sodium nitrite, 4,5-diaminofluorescein (DAF-2), 2,7-dichlorofluorescein diacetate (DCF-DA), phorbol myristate acetate (PMA), chelerythrine, and dimethyl sulfoxide (DMSO) were all from Sigma Chemical Co., St Louis, MO, USA. The Griess Reagent kit was purchased from Molecular Probes, Eugene, USA. AChE antibodies (N-19 and C-16) were purchased from Santa Cruz Biotechnology, Inc., USA.

Experimental Design

Human venous blood samples were collected from the peripheral vein of 27 healthy Caucasian men after informed consent. The blood container tubes were prepared with 10 IU ml^{-1} of sodium heparin (anticoagulant). In all blood samples, hematocrit was measured in a Centrifuge 4223 MKII (ALC, Milan Italy). The blood was centrifuged at $1{,}0409 \times g$ for 10 min, and the plasma and buffy-coat were discarded. Erythrocyte suspensions were performed with the addition of sodium chloride (0.9 % at pH 7.4) in order to reconstitute the initial hematocrit (Ht of 45 %). Each blood sample was divided in 1 ml samples and centrifuged at 11,000 rpm for 1 min. Erythrocyte suspension aliquots were incubated for 15 min at room temperature, with either AChE antibodies (N-19 or C-16) or PKC modulators (PMA or chelerythrine), in the absence and presence of ACh, with a 10 μM final concentration for each modulator. ACh concentrations used are based on previous studies (Niday et al. 1980). ACh at 10 μM concentration is below the Km value of AChE and consequently far from the excess of substrate concentration that inhibits this enzyme (Saldanha 1985). Following incubation, samples were centrifuged, the supernatant was rejected, and erythrocytes were used for NO and GSNO determinations.

The experimental procedures started in average 1 h after blood collection and were performed within 2 h.

Measurement of NO Efflux

Following incubation, erythrocyte suspensions were centrifuged and sodium chloride (0.9 % at pH 7.0) was added in order to compose an Ht of 0.05 %. The suspension was mixed by gentle inversion. For amperometric NO quantification, the amino-IV sensor (Innovative Instruments Inc. FL, USA) was used (Carvalho et al. 2004a). NO diffuses through the gas-permeable membrane triple COAT of the sensor probe and is then oxidized at the working platinum electrode, resulting in an electric current. The redox current is proportional to the NO concentration outside the membrane and is continuously monitored with the in NO model T electrochemical detection system (version 1.9, Innovative Instruments) and connected to a computer. Calibration of the NO sensor was performed daily, and, 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. ACh (30 μl) was added to the erythrocyte suspension aliquots in order to achieve a final concentration of 10 μM, and the NO released from erythrocytes was registered. Data were recorded from constantly stirred suspensions at room temperature.

Measurement of S-nitrosoglutathione (GSNO) (Cook et al. 1996)

Colorimetric solutions containing a mixture of sulfanilic acid (component B of Griess reagent) and NEDD (component A of Griess reagent), consisting of 57.7 mM of sulfanilic acid and 1 mg/ml of NEDD, were dissolved in phosphate buffered solution, at pH 7.4 (PBS). To constitute the 10 mM $HgCl_2$, mercury-ion stock solutions were prepared in 0.136 g/50 ml of dimethyl sulfoxide (DMSO). GSNO was diluted to the following desired concentrations: 75, 15, 30, 45, 60, 120, 240, and 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 left to stand for 20 min. A control spectrum was measured by spectrophotometry at 496 nm (Spectronic 20 Genesys) against a solution without mercury ion; erythrocyte suspensions were added to the reaction mixture, and GSNO concentrations inside the erythrocyte were obtained.

Statistical Analysis

Data were summarized as mean and standard deviation (SD). Comparison between the different treatments of erythrocyte suspensions was made using repeated measures ANOVA and Bonferroni post hoc test. Statistical significance was set at a $p < 0.05$ level. Statistical analysis was conducted using the SPSS 20.0 and GraphPad Prism 5.0.

Results

Effect of AChE Conformational Alteration with N-19 and C-16 Antibodies

NO efflux from erythrocytes and GSNO levels decreased in the presence of N-19 antibody relative to untreated erythrocyte suspensions. In the presence of ACh, N-19 antibody addition (AChE N-19 + ACh 10 μM) caused a decrease in NO efflux levels, relative to ACh and untreated erythrocyte suspensions; therefore, the NO efflux decrease caused by the blockage of AChE N-terminal domain was not reversed by ACh. Also, ACh and N-19 antibody (AChE N-19 + ACh 10 μM) treatment caused a decrease in GSNO levels relative to ACh treatment and untreated suspensions. These results suggest that the decrease in the GSNO concentrations originated by blockage of AChE N-terminal domain was not reversed by adding ACh (Table 1).

Lower levels of NO efflux from erythrocytes and GSNO concentrations were determined in the presence of C-16 antibody relative to control and ACh treatments. Presence of ACh and C-16 antibody (AChE C-16 + ACh 10 μM) caused a decrease in NO levels relative to ACh treatment, suggesting weakening of the AChE C-terminal domain blockage by the presence of ACh. Regarding GSNO concentrations, ACh and C-16 antibody (AChE C-16 + ACh 10 μM) treatment triggered a decrease relative to ACh treatment but with no significant variations when compared to control samples, which suggests that the GSNO concentration decrease caused by the blockage of AChE N-terminal domain was reversed by the presence of ACh (Table 2).

Effect of Enzymatic PKC Inhibition/Activation

NO and GSNO concentrations were reduced in presence of Che in comparison to untreated erythrocyte and ACh suspensions. Regarding erythrocyte suspensions treated with PKC inhibitor and ACh (Che + ACh 10 μM), higher levels of NO were registered in relation to Che treatment but with no significant variations when compared to control samples, suggesting a reversal in PKC inhibition. However, the same was not observed for GSNO concentrations (Table 3).

In PMA- and ACh-incubated erythrocyte suspensions, a greater NO efflux from erythrocytes was observed, relative to its absence. A similar effect was observed in the presence of PMA + ACh, although the simultaneous presence of both effectors does not suggest a cumulative effect. GSNO concentrations were increased in PMA treatment in comparison to ACh and control samples. In suspensions incubated with PMA + ACh, increased GSNO levels were registered relative to controls (Table 4).

Discussion

The present study demonstrated that, by inhibiting PKC, Che impaired NO efflux and GSNO levels in the erythrocyte, while PKC's activation by PMA had the opposite effect. In erythrocytes, PKC has a dual role. On one hand, PKC phosphorylates 4.1R protein which decreases spectrin–actin interactions (Nunomura et al. 2000). On the other hand, PKC induces protein tyrosine phosphatases (PTP) phosphorylation in the serine/threonine amino acid residues (Mohamed and Steck 1986), promoting PTP inactivation and consequent PTK activation (Yannoukakos et al. 1991), hence inducing indirect band 3 protein phosphorylation (Zhaowei and Cohen 1993). Enzymatic PKC inhibition caused by Che causes a less-efficient phosphorylation in 4.1R complex, increasing the bonds between spectrin and actin and promoting a stronger association between membrane and cytoskeleton proteins. In addition, an indirect

Table 1 Effect of N-terminal AChE antibody, in the presence and absence of ACh, on NO and GSNO levels

	Treatments			
	Control	ACh 10 μM	AChE N-19 10 μM	AChE N-19 + ACh 10 μM
NO efflux (nM)	1.84 ± 0.25	2.06 ± 0.33[a]	1.49 ± 0.15[a,b]	1.60 ± 0.19[a,b,c]
GSNO (μM)	7.68 ± 2.11	8.44 ± 3.19	6.07 ± 1.08[a,b]	6.35 ± 1.67[a,b]

Data expressed as mean ± SD, $n = 27$

[a] $p < 0.05$ versus control

[b] $p < 0.05$ versus ACh treatment

[c] $p < 0.05$ versus AChE N-19 treatment

Table 2 Effect of C-terminal AChE antibody, in the presence and absence of ACh, on NO and GSNO levels

	Treatments			
	Control	ACh 10 μM	AChE C-16 10 μM	AChE C-16 + ACh 10 μM
NO efflux (nM)	1.75 ± 0.21	1.99 ± 0.25[a]	1.52 ± 0.24[a,b]	1.65 ± 0.21[a,b]
GSNO (μM)	7.07 ± 0.00	8.00 ± 0.86[a]	5.99 ± 0.00[a,b]	6.35 ± 0.63[b,c]

Data expressed as mean ± SD, $n = 21$

[a] $p < 0.05$ versus control

[b] $p < 0.05$ versus ACh treatment

[c] $p < 0.05$ versus AChE C-16 treatment

Table 3 Effect of Che, in the presence and absence of ACh, on NO and GSNO

	Treatments			
	Control	Che 10 μM	ACh 10 μM	Che + ACh 10 μM
NO efflux (nM)	1.60 ± 0.09	1.48 ± 0.14[a]	1.79 ± 0.11[a,b]	1.55 ± 0.11[b]
GSNO (μM)	7.07 ± 0.00	6.12 ± 0.53[a]	7.78 ± 0.54[a,b]	6.37 ± 0.66[a,b]

Data expressed as mean ± SD, $n = 17$

[a] $p < 0.05$ versus control

[b] $p < 0.05$ versus Che treatment

[c] $p < 0.05$ versus ACh treatment

Table 4 Effect of PMA in the presence and absence of ACh on NO and GSNO

	Treatments			
	Control	PMA 10 μM	ACh 10 μM	PMA + ACh 10 μM
NO efflux (nM)	1.56 ± 0.06	1.81 ± 0.10[a]	1.77 ± 0.08[a]	1.84 ± 0.16[a]
GSNO (μM)	7.07 ± 0.00	8.27 ± 0.34[a]	7.73 ± 0.56	8.16 ± 0.00[a]

Data expressed as mean ± SD, $n = 17$

[a] $p < 0.05$ versus control

[b] $p < 0.05$ versus PMA treatment

[c] $p < 0.05$ versus ACh treatment

dephosphorylation occurs in band 3 protein, impairing NO efflux in the erythrocyte (Carvalho et al. 2009). The unaltered NO efflux levels observed with the simultaneous presence of ACh and Che can therefore be explained through the opposite effects of both modulators, suggesting preservation of phosphorylation levels in band 3 protein. As a result of the mechanisms described previously, PKC enzymatic activation by PMA induced indirect phosphorylation in band 3 protein. The simultaneous presence of PMA and ACh caused an increase in NO efflux and GSNO, although the obtained results suggest this effect not to be cumulative.

Conclusions

Our results demonstrate the influence of 4.1R complex and band 3 protein level of phosphorylation on NO efflux and GSNO concentration mediated by PKC inhibition/activation. Increase in NO efflux and GSNO concentrations in the erythrocyte caused by ACh was previously addressed (Niday et al. 1980; Carvalho et al. 2004b; Almeida et al. 2009) and can be explained by conformational alterations in band 3 e G proteins association, in Gαi1/Gαi2 and Gβ subunits, resulting from the formation of the AChE–ACh complex through the addition of ACh. With these changes, an increase in erythrocytary membrane protein tyrosine kinases activity occurs, resulting in protein band 3 phosphorylation and therefore in an increase of NO efflux and NO metabolites concentrations.

Taking a step further, the present study shows evidence that conformational changes in AChE (promoted by incubation with N-19 and C-16 antibodies) alter the enzyme's functional connection to ACh (AChE-ACh complex) in a reversible manner, resulting in impaired GSNO concentration and NO efflux from the erythrocyte. These findings support the dependence of activation/inactivation status of AChE complex states in the NO signal transduction mechanism. Specific AChE antibodies N-19 and C-16 display the same efficiency level in conformational AChE alteration, and it can be concluded that both can be used for blockage of AChE–ACh complex formation, which may contribute to the modulation of NO delivery in target cells. Consequently, novel insight into NO metabolism in the erythrocyte is brought with the presented findings allowing new possibilities of modulating NO delivery, possibly involving PKC and AChE conformational alterations in combination.

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