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Timolol Modulates Erythrocyte Nitric Oxide Bioavailability

Carlota Saldanha*, Pedro Teixeira, Santos-Freitas T and Patrícia Napoleão

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

Abstract

Changes of oxygen partial pressure in tissues are sensed by the erythrocytes that with the efflux or with the maintenance of nitric oxide that contains promote vasodilation or vasoconstriction. Binding of acetylcholine with the acetylcholinesterase of the erythrocyte membrane originates a signal transduction mechanism that involves both the Gi protein as well as band 3 protein what stimulates nitric oxide efflux. The bioavailability of nitric oxide in presence of velnacrine maleate an inhibitor of acetylcholinesterase is preserved what means that there are no changes in the nitric oxide efflux. Timolol maleate is an inhibitor of acetylcholinesterase.

The aim of this study was to assess the role of the timolol maleate in the erythrocyte in respect to bioavailability on nitric oxide and compare it with effect resulting of the presence of acetylcholine. Venous blood samples were collected from the forearm vein of fifteen healthy Caucasian men after informed consent. Each blood sample was divided in three 1 mL samples, centrifuged, and suspensions of erythrocyte were performed in order to achieve 10 μM final concentration either of acetylcholine or of the timolol. Levels of nitric oxide efflux were evaluated by amperometric method. S-nitrosoglutathione, nitrites and nitrates were assessed with the Griess reaction using the spectrophotometric method.

The nitric oxide efflux by the erythrocyte in presence of timolol is like to the control sample but significantly decreased it when compared to the sample with acetylcholine. The presence of timolol induces decrease in the erythrocyte levels of S-nitrosoglutathione significantly in relation with the control and with acetylcholine samples.

In conclusion, *in vitro*, in erythrocyte the nitric oxide content is maintained by timolol maleate. It may be expected the same role for timolol in the ocular microcirculation when being applied as a therapeutic compound.

Keywords: Erythrocyte; Nitric oxide; Timolol maleate; Acetylcholine; S-nitrosoglutathione; Sodium chloride (NaCl)

Introduction

In physiological conditions the tissue oxygen partial pressure (PaO2) is sensed in the erythrocyte that scavenges the oxygen and nitric oxide (NO) when PaO2 is high and liberates them when the value of PaO2 is lower [1]. The capture and donation of both gases is associated with the states of relax and tense of haemoglobin respectively [2,3]. In endothelial cells, NO is synthesised from L-arginine and molecular oxygen by type III isoform of nitric oxide synthase (eNOS) and liberated to the vascular lumen and for the smooth muscle cells (SMC) inducing here vasodilation [2,4].

The increase eNOS immunoreactivity has been reported in the vasculature of the head optic nerve (ONH) and explained as to promote tissue blood flow and vasodilation [5]. Deregulation in the ONH perfusion with lower blood supply is shown in primary open-angle glaucoma (POAG) which show the overexpression of both isoforms of NOS the neuronal and the endothelial [6,7]. It was evidenced local alterations of the L-arginine/NO system in POAG [8].

The nitric oxide signalling in SMC is mediated by guanylate cyclase activation generating cyclic guanosine monophosphate (cGMP) that acts as inhibitor of myosin light chain (MLC) kinase and activator of MLC phosphatase resulting in MLC dephosphorylation and muscle relaxation [9]. Decreased levels of cGMP and of nitrite (NO derivative molecule) were obtained in plasma and in aqueous humour of patients with POAG [10].

The endothelial NO when liberated to the vessel lumen is scavenged by erythrocyte passing through band 3 protein to be fixed by haemoglobin molecules with generation of nitrosohemoglobin [11,12]. Glutathione is an abundant molecule inside erythrocytes that reacts with nitric oxide originates S-nitrosoglutathione (GSNO) [13]. The metabolism of NO inside erythrocyte generates several derivatives. Any alterations in concentrations of those molecules are dependent of internal and external stimuli such as acetylcholine (ACh) [14]. In the presence of ACh the existence of NO inside of erythrocytes has been observed by fluorescence microscopy [15]. It was evidenced by us a signal transduction pathway for the changes in human erythrocyte NO mobilization under the influence of ACh that it is recognised by the membrane acetylcholinesterse (AChE) with increase NO efflux [16,17]. Otherwise when an inactive complex like velnacrine-AChE was formed no changes in erythrocyte NO efflux was verified unless band 3 is being dephosphorylated [16]. Erythrocytes of patients with POAG have higher AChE enzyme activity than the healthy humans [18]. It was evidenced that the increase of the membrane enzyme activity did not result from the therapeutic effect of timolol maleate because it is an inhibitor of AChE [18].

The aim of this study was to assess the role of timolol maleate in erythrocyte nitric oxide bioavailability and compare it with acetylcholine effect.

***Corresponding author:** Carlota Saldanha, Instituto de Bioquímica, Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Edifício Egas Moniz, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal, E-mail: carlotasaldanha@fm.ul.pt

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Material and Methods

Chemicals

Sodium chloride was purchased from AnalaR, BDH Laboratory, Poole, UK) and chloroform and ethanol 95% from *MERCK, Darmstadt, Germany*. Acetylcholine iodide, choline chloride, 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*. Timolol maleate was purchased from Laboratórios Químico-farmacêuticos Chibret, Lda.

Experimental design

Human venous blood samples were collected from the forearm vein of fifteen 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 haematocrit was measured in a Centrifuge 4223 MKII (ALC, Milan Italy). Each blood sample was divided in three 1 mL samples and centrifuged at 11,000 rpm for 1 minute in Biofuge 15 centrifuge (Haraeus, Sepatech). Afterwards, the plasma and buffy-coat (leucocytes and platelets) were discarded. Erythrocyte suspensions were performed with the addition of sodium chloride (0,9% at pH 7,4) to reconstitute the initial hematocrit (Ht of 45%). Erythrocyte suspension aliquots were then incubated during 30 min at room temperature, in the absence and presence of either acetylcholine 10 μM or timolol maleate 10 μM.

Measurement of NO

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 of tubes.

For amperometric NO quantification we used the *amino-IV* sensor (Innovative Instruments Inc. FL, USA), a method previously described by us [19]. Briefly, after stabilization of the NO sensor immersed in erythrocyte suspensions we stimulated the erythrocytes with ACh 10 μM and registered changes in the electric current. This alteration is proportional to the amount of NO mobilized by ACh-stimulated erythrocytes.

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, pH 7.4 (PBS). To constitute the 10 mM $HgCl_2$ (Aldrich) mercury ion stock solutions were prepared in 0.136 g/50 mL of dimethyl sulfoxide (DMSO) (Aldrich). GSNO was diluted to the wanted concentration 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 twenty minutes. A control spectrum was measured by spectrophotometry at 496 nm against a solution without mercury ion. Erythrocyte suspensions (300 μl) were added to the reaction mixture and GSNO concentrations were obtained [20].

Measurement of erythrocyte nitrite and nitrate

Colorimetric method has been described for us since 2004 [21].

Statistical analysis

Data are expressed as means ± SD. One-way analysis of variance was applied to assess statistical significance between the different treatments of erythrocyte suspensions. Turkey post-hoc tests were conducted when appropriate. Statistical significance was set at a $p<0.05$ level. Statistical analysis was conducted using the SPSS 20.0.

Results and Discussion

The concentrations of acetylcholine and timolol were selected based on previous studies [18,22]. The ACh 10 μM concentration is below the Km value of AChe and consequently very far from the excess of substrate concentration that inhibit the enzyme [20].

In control erythrocyte suspensions, we verified an average NO concentration of 1.16 ± 0.34 nM. The presence of acetylcholine increased those levels, statistically significantly (2.04 ± 0.41 nM; $P<0.05$). On the other side, the suspensions treated with timolol showed decreased levels of NO (1.48 ± 0.45 nM; $P< 0,05$) in relation to the suspensions with ACh , but variation in the NO values in relation to the control suspensions were not verified Table 1.

Higher levels of GSNO in erythrocyte suspensions were obtained in presence of ACh in relation to the control (7.4 ± 1.6 μM versus 6.3 ± 1.6 μM; $p<0.05$) and in relation to the erythrocyte suspension with timolol (1.8 ± 1.0 μM; $p<0.05$) Table1. The relation between the GSNO levels obtained in erythrocyte suspensions treated with timolol and the control suspensions is also statistically significant (1.8 ± 1.0 μM versus 6.3 ± 1.6 μM; $P<0.05$) Table 1.

Regarding the concentrations of nitrites and nitrates obtained in erythrocytes suspensions treated with ACh or with timolol, all of them have significantly higher values when compared with the control erythrocyte suspensions. Namely for nitrite the values were: ACh 10 μM in relation to the control (12.35 ± 1.87 μM versus 7.85 ± 1.44 μM; $p<0.001$); timolol in relation to control (10.95 ± 0.58 μM versus 7.85 ± 1.1.44 μM; $p<0.001$); and for nitrate the values obtained in the erythrocyte suspensions were: Ach 10 μM in relation to the control (11.25 ± 1.32 μM versus 8.55 ± 1.32 μM; $p<0.001$); timolol 10 μM in relation to control (12.90 ± 2.14 μM versus 8.55 ± 1.32 μM; $p<0.001$) Table 2.

In the present work we have showed no variation in the erythrocyte nitric oxide efflux when in presence of timolol in relation to its absence. However lower levels were obtained when compared to the observed in the erythrocyte ACh suspensions. Since timolol is an erythrocyte AChE inhibitor [18] this result is in accordance with those obtained with the AChE inhibitor velnacrine [16]. The less bioavailability of erythrocyte to liberate nitric oxide induced by timolol may be a local compensatory mechanism in the vasculature when occurs high production of nitric

Erythrocyte Suspensions	NO nM	GSNO μM
Control	1.16 ± 0.34	6.3 ± 1.6
ACh 10 μM	2.04 ± 0.41	7.4 ± 1.6
Timolol 10 μM	1.48 ± 0.45	1.8 ± 1.0

Table 1: Mean ± sd values of NO and GSNO concentration obtained in untreated (Control) and treated blood samples with acetylcholine (ACh) 10 μM and timolol 10 μM.

	Nitrite μM	Nitrate μM
Control	7.85 ± 1.44	8.55 ± 1.32
ACh 10 μM	12.35 ± 1.87	11.25 ± 1.32
Timolol 10 μM	10.95 ± 0.58	12.90 ± 2.14

Table 2: Mean ± sd values of nitrite and nitrate concentration obtained in untreated (Control) and treated blood samples with acetylcholine (ACh) 10 μM and timolol 10 μM.

oxide. For example it can be useful in the vasculature of the optic nerve head of patients with POAG with overexpression of both NOS isoforms the neuronal and endothelial [6,7].

The lower GSNO concentrations observed in the erythrocyte suspensions incubated with timolol in relation to both control and acetylcholine are not in accordance with the higher GSNO values that were obtained with velnacrine [23]. The lower GSNO concentration obtained suggest that it was consumed in the irreversible reaction with deoxygenated haemoglobin to originate glutathione, methahemoglobin and NO [24]. In turn the nitric oxide reduces oxyhemoglobin molecules and originates nitrate and metahemoglobin [25]. The low GSNO concentration is a benefit because it does not inhibits the haemoglobin reductase responsible in normalizing the metahemoglobin levels [25].

The higher nitrite and nitrate levels observed in the presence of timolol are in opposition to those previous obtained with velnacrine a strong AChE inhibitor [21]. The lower percentage of AChE inhibition induced by timolol [18] originates a less strong inactive complex than that produced by velnacrine in the acetylcholinesterase. The difference in the complex status of inactivation in the enzyme - inhibitor may explain the different variations in NO derivatives molecules concentration formed under the presence of timolol and velnacrine. However both inhibitors induce nitric oxide mobilization inside the erythrocyte. The results of this work showed that timolol like acetylcholine induce NO mobilization inside erythrocyte by changing the levels of nitrite, nitrate, and S-nitrosoglutathione in relation to its absence. However timolol preserves NO inside the erythrocyte will not favoured the formation of peroxinitrite in an oxygen reactive stress external environment. The antioxidant ability of timolol was evidenced *in vitro* in endothelial cells and neutrophils, as well *in vivo* in newborn rats under oxygen induced retinopathy [26-28].

From the present work we conclude that timolol retained the nitric oxide inside the erythrocyte preserving its efflux and favouring its mobilization inside. The results obtained in the erythrocyte do reinforce the signal transduction mechanism described for nitric oxide in dependence of the degree of activation or inactivation of the AChE enzyme complexes. It may be expected the same role in the ocular microcirculation when applied as a therapeutic compound.

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