ALTERATION OF BLOOD COMPONENTS MEMBRANE PROPERTIES IN HIV-1 INFECTED PATIENTS

N.C. Santos[1,*], *F. Antunes*[1,2], *M. Doroana*[1,2], *N. Duarte*[2], *L. Tavares*[1,2]
J. Martins-Silva[1], *C. Saldanha*[1]

FEBS Lecture Course
NEW DEVELOPMENTS IN MEMBRANE BIOLOGY:
RAFTS, PROTEIN SORTING AND SIGNAL TRANSDUCTION
SHERATON HOTEL/TBILISI/REPUBLIC OF GEORGIA
JUNE 28-3 JULY, 2003

1. INTRODUCTION

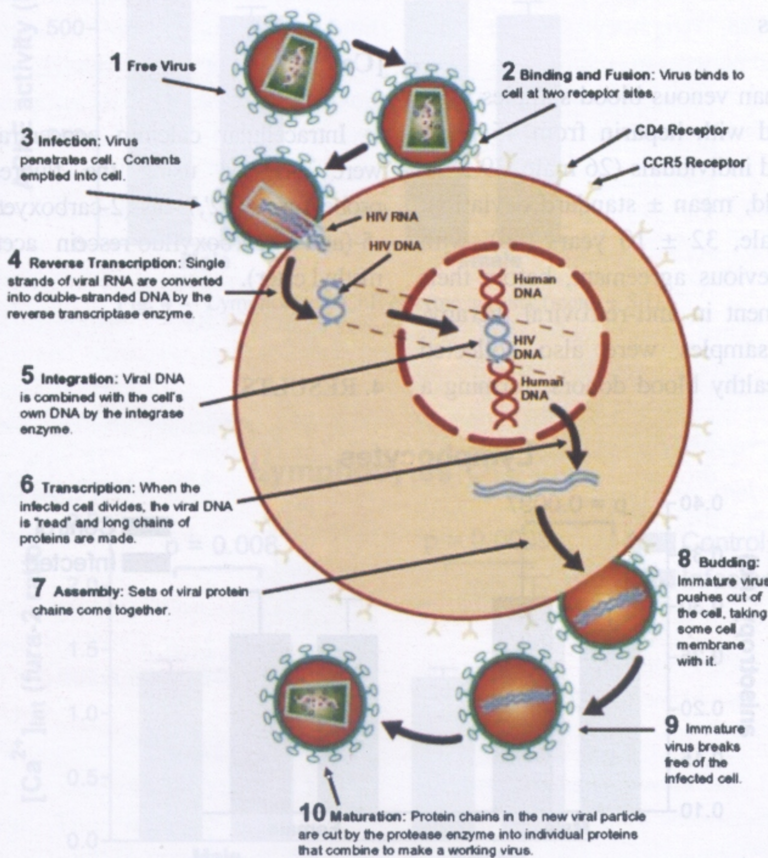


Fig. 1– HIV-1 life cycle (from http://www.aids.org).

[1] Faculdade de Medicina de Lisboa
[2] Hospital de Santa Maria, Lisbon, Portugal.
[*] Mailing address – Instituto de Bioquímica/ /Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal.
E-mail: nsantos@fm.ul.pt.

2. OBJECTIVES

- To evaluate the possible HIV-1 infection-induced changes on cell membrane properties and on electrolyte balance, by assessing membrane fluidity, acetylcholinesterase (AChE, a GPI-anchored protein) activity and intracellular calcium concentration ($[Ca^{2+}]_{int}$), both in human lymphocytes and erythrocytes.
- To compare the values obtained for male and female infected patients (previously to engagement in anti-retroviral therapy).
- To compare the values obtained for infected patients with those from healthy blood donors.

3. MATERIALS AND METHODS

Samples

Human venous blood samples were collected with heparin from 45 HIV-infected individuals (26 male, 39 ± 11 years old, mean ± standard deviation; 19 female, 32 ± 10 years old), with their previous agreement, before their engagement in anti-retroviral therapy. Blood samples were also collected from healthy blood donors, forming a control group with similar characteristics. Erythrocytes were isolated by plasma and buffy-coat removal and lymphocytes by Ficoll-Paque gradient.

Membrane fluidity

The fluorescent membrane probes DPH (diphenylhexatriene) and TMA-DPH (trimethylamino-diphenylhexatriene) were used to assess membrane fluidity by fluorescence anisotropy measurements.

AChE activity

Measured by the colorimetric method of Ellman et al. (*Biochem. Pharmacol.* 1961, **7**, 88-95).

$[Ca^{2+}]_{int}$

Intracellular calcium concentrations were assessed using the fluorescent probe fura-2 (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluo-rescein acetoxymethyl ester).

4. RESULTS

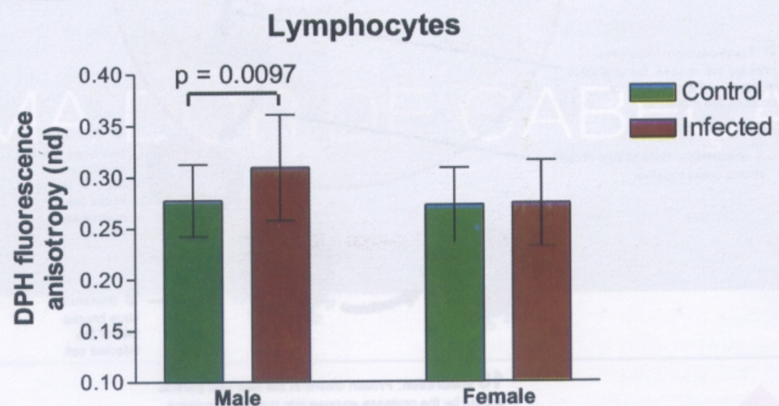


Fig. 2 – Lymphocyte membrane fluidity assessed by DPH fluorescence anisotropy measurements (mean ± S.D.).

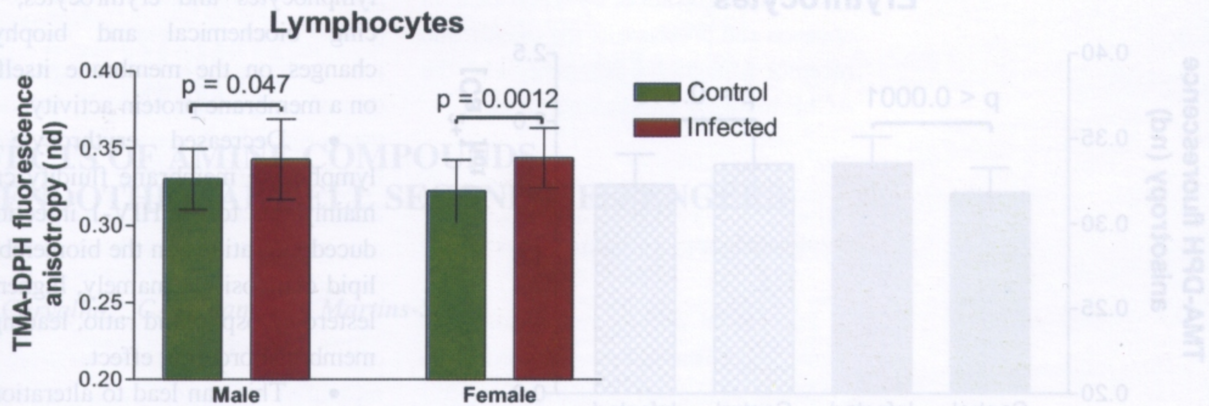


Fig. 3 – Lymphocyte membrane fluidity assessed by TMA-DPH fluorescence anisotropy measurements (mean ± S.D.).

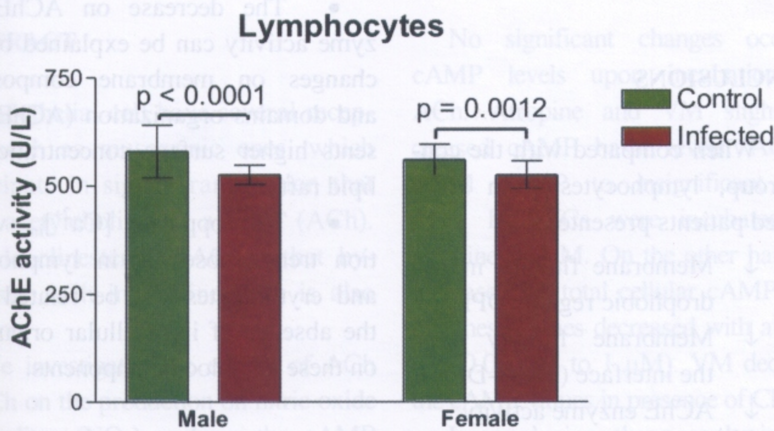


Fig. 4 – Lymphocyte AChE enzyme activity (mean ± S.D.)

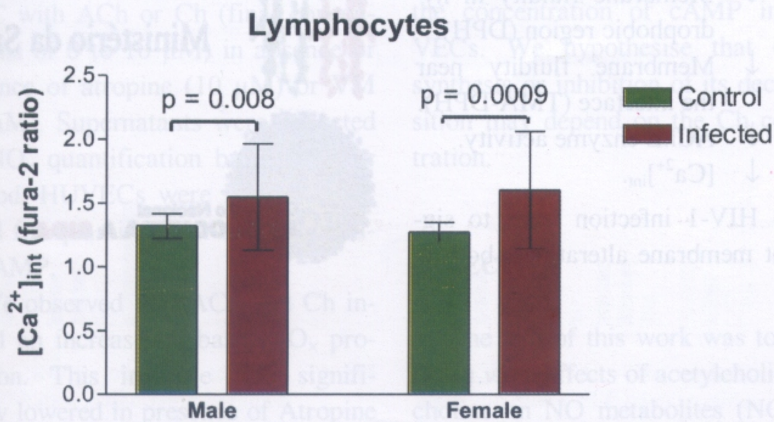


Fig. 5 – Lymphocyte intracellular Ca^{2+} concentrations (mean ± S.D.)

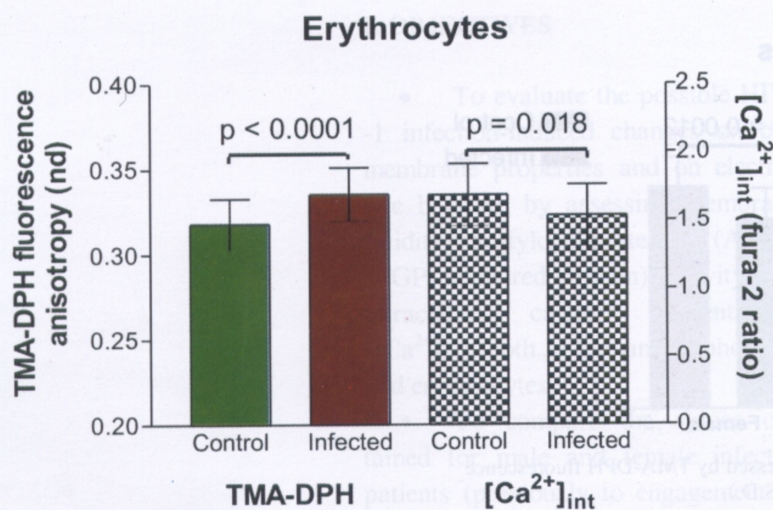


Fig. 6 – Male subjects erythrocyte membrane fluidity, assessed by TMA-DPH fluorescence anisotropy measurements, and intracellular Ca^{2+} concentrations (mean ± S.D.)

5. CONCLUSIONS

- When compared with the control group, lymphocytes from HIV-infected patients presented:
  - ↓ Membrane fluidity in hydrophobic region (DPH).
  - ↓ Membrane fluidity near the interface (TMA-DPH).
  - ↓ AChE enzyme activity.
  - ↑ $[Ca^{2+}]_{int}$.
- When compared with the control group, erythrocytes from HIV-infected patients presented:
  - ↓ Membrane fluidity in hydrophobic region (DPH).
  - ↓ Membrane fluidity near the interface (TMA-DPH).
  - ↓ AChE enzyme activity.
  - ↓ $[Ca^{2+}]_{int}$.
- HIV-1 infection leads to significant membrane alterations, both in lymphocytes and erythrocytes, inducing biochemical and biophysical changes on the membrane itself and on a membrane protein activity.
- Decreased erythrocyte and lymphocyte membrane fluidity can be mainly due to the HIV-1 infection induced alterations on the biomembranes lipid composition; namely, higher cholesterol/phospholipid ratio, leading to a membrane ordering effect.
- This can lead to alterations on the membrane heterogeneous distribution of components (lipid domains, rafts), which is important for the HIV entrance on a target cell and virus budding.
- The decrease on AChE enzyme activity can be explained by the changes on membrane composition and domains organization (AChE presents higher surface concentration on lipid rafts).
- The opposite $[Ca^{2+}]_{int}$ variation trends observed in lymphocytes and erythrocytes can be related with the absence of intracellular organelles on these last blood components.

SUPPORTED BY

Ministério da Saúde

EFFECTS OF AMINE COMPOUNDS ON ENDOTHELIAL CELL SECOND MESSENGERS

F. A. Carvalho, C. Saldanha, J. Martins-Silva*

Instituto de Bioquímica/Instituto de Medicina Molecular,
Faculdade de Medicina de Lisboa, Portugal
*mena_ist@hotmail.com

ABSTRACT

Endothelial cell have several receptors such as muscarinic ones, which participate in signal transduction that have acetylcholine as agonist (ACh). Acetylcholinesterase (AChE) that hydrolyses ACh in choline (Ch) is also present.

We investigated the effects of ACh and Ch on the production on nitric oxide metabolites (NO_x) and on the cAMP concentration, in absence or presence of atropine and velnacrine maleate (VM) in cultured HUVECs.

Cells were incubated for 30 min at 37° C with ACh or Ch (final concentrations of 0 to 10 μM) in absence or presence of atropine (10 μM) or VM (10 μM). Supernatants were collected for NO_x quantification by the *Griess* method. HUVECs were lysed and assayed for quantification of total cellular cAMP.

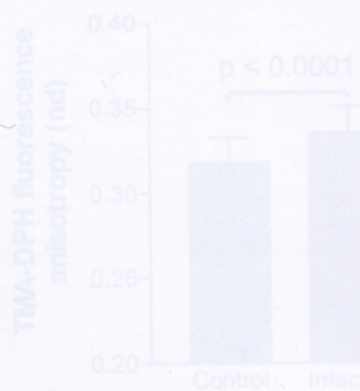
We observed that ACh and Ch induced an increase on basal NO_x production. This increase was significantly lowered in presence of Atropine or VM ($p<0.05$).

No significant changes occur in cAMP levels upon incubation with ACh. Atropine and VM slightly increased cAMP basal levels. ACh reduced cAMP to insignificant levels when HUVECs were incubated with atropine or VM. On the other hand, Ch increased the total cellular cAMP levels but these values decreased with atropine (Ch 0.01 μM to 1 μM). VM decreased the cAMP values in presence of Ch.

In conclusion, these results indicate that ACh and Ch activate the mechanisms of NO_x production in HUVECs. As expected, this was inhibited with atropine and VM. Ch also increased the concentration of cAMP in HUVECs. We hypothesise that cAMP synthesis or inhibition of its decomposition may depend on the Ch concentration.

OBJECTIVE

The aim of this work was to study the *in vitro* effects of acetylcholine and choline on NO metabolites (NO_x; nitrites and nitrates) production and



cAMP concentration by HUVECs in absence and presence of the muscarinic receptor antagonist, atropine, and the AChE inhibitor, velnacrine maleate.

EXPERIMENTAL DESIGN

Endothelial cells were isolated from human umbilical cords obtained from Stª Maria's Hospital Obstetrics Service.

Cultured HUVECs were incubated in PBS+Glucose 5 mM for 30 minutes. 37° C in humidity air of 5% CO_2 in presence of ACh or Ch (final concentrations of 0 to 10 μM) in absence or presence of atropine (10 μM) or VM (μM). After incubation, the supernatants were collected for quantification of nitrites and nitrates with use of the spectrophotometric *Griess* Method from *Molecular Probes, Inc.* (Eugene, USA). The nitrates are enzymatically reduced to nitrites with nitrate reductase (Guevara I. et al; Clin. Chem. Acta (1998) 274: 177-188). The total cellular cAMP concentration are measured in supernatants, after proceed to the HUVECs lyses, with a cAMP enzymeimmunoassay kit (*Amersham Pharmacia Biotech* (UK)). Before the lyses of the HUVECs, the cells concentration is 4×10^5 cells/sample.

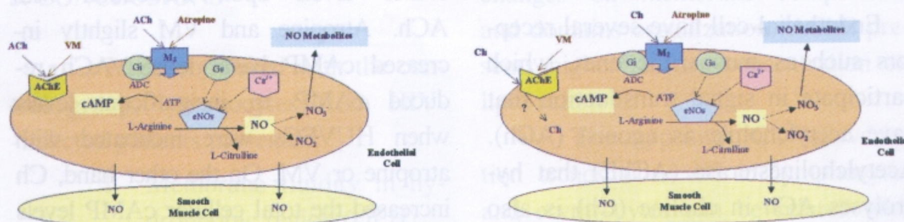


Fig. 1 and 2 – Relationship between the endothelial cell and the vascular smooth muscle cell, and the regulation of the synthesis/release of nitric oxide (NO), nitric oxide metabolites (nitrites and nitrates) and the cyclic adenosine monophosphate (cAMP). Stimulation of the receptor, i.e, M_2-receptor coupled with a G protein (Gi) by acetylcholine (ACh) or choline (Ch) induce several signal transduction mechanisms that leads to the synthesis of cAMP. Also ACh is a natural substrate of acetylcholinesterase (AChE) and Ch its inhibitor. AChE hydrolyses ACh to Ch and acetic acid. The velnacrine maleate (VM) is a inhibitor of the AChE and atropine a muscarinic receptor antagonist. ACh (or Ch) induce NO formation in the endothelial cells when the calcium-calmodulin complex (Ca^{2+}) is formed, NO synthase (eNOs) is stimulated and the natural substrate (L-arginine) are present. The mechanisms of NO formation on the endothelium by the cAMP signal transduction pathway remains to demonstrate

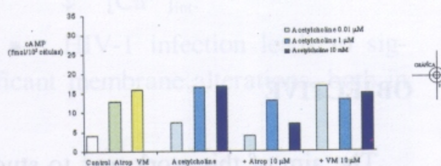


Fig. 3 – Changes on the cAMP values achieved in HUVECs with differents concentrations of ACh in absence or presence of atropine and VM

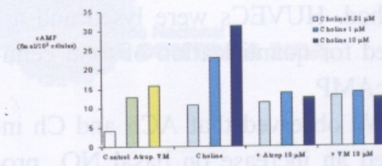


Fig. 4 – Changes on the cAMP values achieved in HUVECs with differents concentrations of Ch in absence or presence of atropine and VM

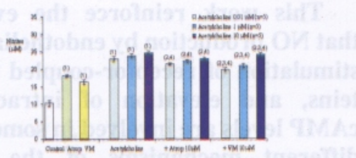


Fig. 5 – Changes on the nitrites values released by HUVECs with differents concentrations of ACh in absence or presence of atropine and VM. Values of mean ± SD

(1) Significant difference relatively to the control aliquot (P<0.001);

(2) significant difference relatively to the respective ACh aliquot (P<0.02);

(3) significant difference relatively to the respective ACh aliquot incubated with atropine (P<0.03);

(4) significant difference of the respective ACh aliquot incubated with atropine or VM to the control aliquot (P<0.005).

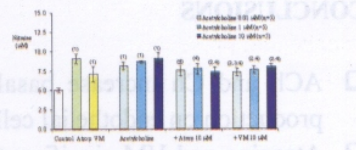


Fig. 6 – Changes on the nitrates values released by HUVECs with differents concentrations of ACh in absence or presence of atropine and VM. Values of mean ± SD

(1) Significant difference relatively to the control aliquot (P<0.001);

(2) significant difference relatively to the respective ACh aliquot (P<0.02);

(3) significant difference relatively to the respective ACh aliquot incubated with atropine (P<0.03);

(4) significant difference of the respective ACh aliquot incubated with atropine or VM to the control aliquot (P<0.005).

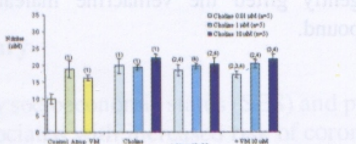


Fig. 7 – Changes on the nitrites values released by HUVECs with differents concentrations of Ch in absence or presence of atropine and VM. Values of mean ± SD

(1) Significant difference relatively to the control aliquot (P<0.001);

(2) significant difference relatively to the respective ACh aliquot (P<0.02);

(3) significant difference relatively to the respective ACh aliquot incubated with atropine (P<0.04);

(4) significant difference of the respective ACh aliquot incubated with atropine or VM to the control aliquot (P<0.002).

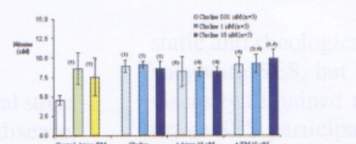


Fig. 8 – Changes on the nitrates values released by HUVECs with differents concentrations of Ch in absence or presence of atropine and VM. Values of mean ± SD

(1) Significant difference relatively to the control aliquot (P<0.001);

(2) significant difference relatively to the respective ACh aliquot (P<0.02);

(3) significant difference relatively to the respective ACh aliquot incubated with atropine (P<0.04);

(4) significant difference of the respective ACh aliquot incubated with atropine or VM to the control aliquot (P<0.002);

(5) significant difference of the respective ACh aliquot incubated with VM to the control aliquot (P<0.002).

CONCLUSIONS

- ACh and Ch increase basal NO_x production on endothelial cells.
- Atropine and VM significantly decrease the NO_x levels of endothelial cells related to those obtained in presence of ACh and Ch.
- ACh did not induced significant changes on HUVECs cAMP concentration.
- The HUVECs cAMP values increase with the Ch concentrations.
- Atropine and VM significantly decreased the cAMP values of HUVECs related to those obtained in presence of Ch.

This work reinforce the evidence that NO production by endothelial cells, stimulation of receptor-coupled G proteins, and elevation of intracellular cAMP levels are involved in some of the different mechanisms of the signal transduction pathway. Either the cAMP synthesis or inhibition of its decomposition on endothelial cells could be dependent of the presence of choline.

ACKNOWLEDGMENTS

We would like to St[a]. Maria's Hospital Obstetrics Service (Prof. Doutor Luis Mendes Graça) who provide the Human umbilical cords for the endothelial cells culture necessary to this experimental "*in vitro*" study. We au also grateful to *Hoechst Marion Roussel Pharmaceutical Inc.* (New Jersey, USA) for gently gifted the velnacrine maleate compound.