Boletim Hemorreologia nº 3/2002
Pista: Bole-4

URETHANE EFFECTS ON RED BLOOD CELLS

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BACKGROUND

Urethane is an anaesthetic agent frequently used for acute animal experimentation because it produces an adequate level of anaesthesia with minimal changes of cardiovascular system and has no effect on the control of breathing. “In vitro” studies demonstrated that urethane increases the fluidity of extracellular leaf of red blood cells membrane. On the other hand, other authors hypothesised that production of nitric oxide results from changes in membrane fluidity.

Although the presence of nitric oxide synthase on erythrocytes is not well characterised, “in vivo” NO-hemoglobin predominant reactions and interactions are a matter of relevant studies.

Aim of the Study

To determine the effect of urethane on NO metabolites (nitrites/nitrates) concentrations in red blood cells suspensions.

MATERIAL AND METHODS

Human blood collection and preparation of erythrocytes suspensions Venous blood samples were collected from a forearm vein of ten caucasian healthy men (30 ± 5 years old) following their informed consent. Blood was anticoagulated with sodium heparin 10Ul/mL. After that blood samples were centrifuged at 1040g during 10 minutes and plasma and buffy coat (leukocytes and platelets) were discarded. Sodium chloride 0,9% pH 7.0 was added in order to reconstitute the initial hematocrit (approximately 45%).

Experimental design

Aliquots of erythrocytes suspension were obtained and incubated in presence of urethane 10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}M and 10^{-4}M.

All erythrocytes suspension aliquots were incubated during 15 minutes at room temperature with slight agitation.

Measurement of nitrite and nitrate (NO metabolites) concentrations in erythrocyte suspensions

Nitrites and nitrates concentration were measured both in supernatant (extra-erythrocyte compartment) and pellet (intra-erythrocyte compartment). The total suspension nitrites or nitrates concentrations was determined on the basis of the haematocrit (Htc). In case of nitrites, Nitrites(suspension) = ($_{pellet}NO_2$- x Htc) + [$_{supematant}NO_2$- x (1- Htc)].

Nitrites concentrations were determined using the Griess reaction method and for nitrate measurement, NO_3- was first reduced to NO_2- in presence of NADPH by *Aspergillus* nitrate reductase according to Guevara et al. (Clin Chem Acta 1998; 274: 177-188).

Data analysis

Data are expressed as means ± SE. Student paired t- tests were used to compare values between different performed with the use of SPSS software, version 10.

RESULTS

CONCLUSION

This “in vitro” study showed that urethane increases nitrites and nitrates concentrations in erythrocyte suspensions.

Fig. 1 – Values of mean ± standard error of nitrites and nitrates concentrations in erythrocytes suspensions aliquots incubated in absence and in presence of urethane10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}M and 10^{-4}M.
(1) significant difference relatively to the control aliquot, 0M of urethane ($p<0.001$).

VELNACRINE MALEATE
– AN ACETYLCHOLINESTERASE INHIBITOR
INCREASE LEUKOCYTE ROLLING IN CREMASTER MUSCLE POST
– CAPILLARY VENULES

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BACKGROUND

Acetylcholinesterase (AChE) is an enzyme (E.C. 31.1.7) localised in the extracellular leaf of plasma membrane of different cells such as leukocytes and endothelium. Although it is an enzyme characteristic of cholinergic nervous cells, its extra-neuronal localisation have supported the hypothesis of having other functions resides the catalytic one.

At level of microcirculation, velnacrine maleate (an ACHE inhibitor) increases the LPS – induced inflammatory response in Wistar rats, increasing the number of adherent leukocytes to endothelium of mesenteric post - capillary venules.

Aim of the Study

To verify if velnacrine maleate interaction with AChE induces an increase of leukocytes -endothelium interactions in normal Wistar rats without previous LPS -induced inflammation.

MATERIAL AND METHODS

Twelve wistar rats of 270 – 340g of body wt were randomised either to Control group (n=4) or the Velnacrine group (n=8).

Animal Preparation

Anaesthesia was induced by an intraperitoneal injection of urethane (1.5g/Kg body wt) and after 20 minutes, ketamine 50mg/Kg body wt was injected intramusculare.

The rat was placed in a self-regulated heating pad and the right jugular vein and left carotid artery were cannulated with polyethylene tubing. Intravenous sodium chloride (0.9% pH 7.0) were administered via the internal jugular catheter at the rate of 4ml/hr controlled by a programmable syringe pump (Kent

Scientific Corporation, USA). The cardiac frequency and arterial pressure were monitorised by connecting the arterial catheter to a pressure transducer TRANSPAC (Abbot, Ireland) and registered with the aid of hardware and software system PowerLab/400 (AO Instruments, Australia).

Cremaster preparation

and intravital microscopy

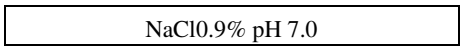
Cremaster muscle was prepared for intravital microscopy on a Plexiglass microscope stage with a five-point fixation as previously described by Hill et al (Microvasc Res 1990, 39: 349-363). The stage was placed on a Leitz® FLUOVERT FU inverted microscope (Leica, Germany), and the tissue imaged with a 40X/0.5 magnification objective. The images were projected on a CCD-IRIS camera DXC-107AP (Sony), displayed on a monitor screen PVM 1440QM (Sony), and recorded for off-line analysis on a videotape AG-MD830 (Panasonic). Randomised venules of about 20 μm diameter were analysed.

Quantification of leukocyte
– endothelial cells interaction

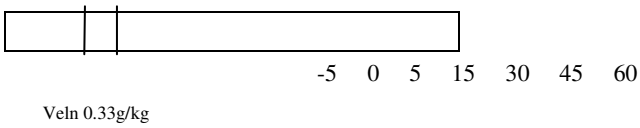
The number of leukocytes rolling was quantified by counting the number of leukocytes moving along the venular wall substantially slower than the surrounding red blood cells and passing an imaginary line perpendicular to the venular axis per minute. The number of adherent leukocytes was determined counting the number of leukocytes remaining stationary for ≥ 30s in a 100 μm segment of venule.

Experimental protocol

Control Group (n=4)



Velnacrine Group (n=8)



Data analysis

To enable comparisons between groups, data were normalized to baseline values. Normalized data are presented as medians with their interquatile ranges (ie. the spread from 25th to 75th percentile).

Nonparametric statistical analysis was performed within study groups using the Wilcoxin test The Mann--witney U test was used to compare data between

Velnacrine and Control groups at each time point. Differences were judged significant for $p < 0.05$.

RESULTS

CONCLUSION

This *in vivo* study demonstrates that velnacrine maleate, an AChE inhibitor, increases leukocyte rolling along the rat cremaster post-capillary venules endothelium. The mechanism of this effect remains to be clarified.

Fig. 1– Fotography of rat cremaste post-capillary venule, showing leukocytes-endothelium interaction obtainde by intravital microscopy
(Total magnification of 100X)

Fig. 2– Median number of leukocytes rolling (normalized to baseline values) in Velnacrine and Control groups Bars represent the interquartile ranges Times shown along the x axis indicate the number of minutes after start of velnacrine infusion
* indicates a significant difference as compared to control group.

ACETYLCHOLINE AND CHOLINE INCREASE NITRIC OXIDE METABOLITES IN ERYTHROCYTES SUSPENSIONS

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BACKGROUND

Acetylcholine, classically considered a neurotransmitter has been detected in the human blood being produced by T lymphocytes and endothelial cells. Additionally acetylcholine receptors and acetylcholinesterase (AChE) are present in lymphocytes, endothelial cells and erythrocytes. Erythrocytes are the peripheral blood cells with more AChE content in membranes and acetylcholine muscarinic receptors were characterised as type M1.

Acetylcholine increases human erythrocyte deformability and decreases erythrocyte aggregation, however the mechanism under these effects is unknown.

Acetylcholine stimulating nitric oxide synthase increases nitric oxide production by vascular endothelial cells, an effect mediated by its interaction with muscarinic receptors. However, on erythrocytes, the presence of nitric oxide synthase is not well characterised and *in vivo* NO- hemoglobin predominant reactions and interactions are a matter of relevant studies. Nevertheless, recent evidence suggests that erythrocytes may release NO bound to hemoglobin in the microcirculation under low oxygen tension and are capable of producing NO from nitrovasodilators such as isosorbide dinitrate.

Aim of the Study

To examine whether acety1choline modulate nitric oxide melabolism in human erythrocytes depending of its interaction with muscarinic receptors and acetylcholineslerase.

To test the effect of choline – the metabolite of acetylcholine hydrolysis by acetylcholinesterase – on NO metabolites concentrations.

Material and Methods

Human blood collection and preparation of erythrocytes suspensions.

Venous blood was collected from a forearm vein of ten caucasian healthy men (30 ± 5 years old) following their informed consent. Blood was anticoagulated with sodium heparin 10UI/ml After that blood was centrifuged at 1040g during 10 minutes plasma and buffy coat (leukocytes and platelets) was discarded. Sodium chloride 0,9% pH 7.0 was added in order to reconstitute the initial hematocrit (approximately 45%).

Experimental design

Aliquots of erythrocytes suspension were obtained and incubated in presence of acetylcholine 10^{-6}M, acetylcholine 10^{-5}M, choline 10^{-6}M and choline 10^{-5}M (final concentrations) in presence and absence of atropine 10^{-5}M or velnacrine maleate (an AChE inhibitor) 10^{-5}M.

All erythrocytes suspension aliquots were incubated during 15 minutes at room temperature with slight agitation.

Measurement of nitrite and nitrate (NO metabolites) concentrations in erythrocyte suspensions

Nitrites and nitrates concentrations were measured both in supematant (extra-erythrocyte compartment) and pellet (intra-erythrocyte compartment) The total suspension nitrites or nitrates concentrations was determined on the basis of the haematocrit (Htc). In case of nitrites, Nitrites (suspension)= ($_{pelet}NO_2$- x Htc) + [$_{supernatant}NO_2$- x (1- Htc)].

Nitrites concentrations were determined using the Griess reaction method and for nitrate measurement, NO_3- was first reduced to NO_2- in presence of NADPH by *Aspergillus* nitrate reductase according to Guevara et al (Clin Chem Acta 1998;,274: 177-188).

Data analysis

Data are expressed as means ± SE. Student paired t-tests were used to compare values between different erythrocytes suspension aliquots. All statistics were performed with the use of SPSS software, version 10.

RESULTS

CONCLUSION

This study showed that acetylcholine and choline, increase nitrites and nitrates concentrations in erythrocyte suspensions and that these effects are mediated by their interaction with the muscarinic receptor and acetylcholinesterase.

Fig. 1– Values of mean ± standard error of nitrites and nitrates concentrations in erythrocytes suspensions aliquots incubated with acetylcholine (ACh) in presence or absence of atropine (Atr) or velnacrine maleate (Vel). (1) significant difference relatively to the control aliquot ($p<0.001$), (2) significant difference relatively to the respective ACh aliquot ($p<0.001$), (3) significant difference relatively to the respective aliquot incubated with atropine ($p<0.0l$)]

Fig. 2– Values of mean ± standard error of nitrites and nitrates concentrations in erythrocytes suspensions aliquots incubated with choline (Ch) in presence or absence of atropine (Atr) or velnacrine maleate (Vel) (1) significant difference relatively to the control aliquot ($p<0.001$), (2) significant dif- ference relatively to the respective Ch aliquot ($p<0.05$), (3) significant difference relatively to the respective aliquot incubated with atropine ($p<0.001$)

URETHANE EFFECT ON HUMAN UMBILICAL VENOUS ENDOTHELIAL CELL

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ABSTRACT

The purpose of this study was to evaluate the "*in vivo*"effect of urethane an intravenous anaesthetic, upon the membrane of human umbilical venous endothelial cells (HUVECs). The amounts of nitrites/nitrates released after the exposure to this anaesthetic were also measured.

Cultured HUVECs (n=10) were incubated 30 min., 37ºC with urethane final concentrations of 0 (control), 10^{-8}M to 10^{-4}M. After that the supernatants were collected for quantification of nitrites (*Griess* method) and nitrates (*Griess* method after enzymatically converted to nitrites, with use of the nitrate reductase).

The HUVECs membrane fluidity is determined by means of fluorescence polarisation with 1,6-biphenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammoniumphenyl)-6 phenyl-1,3,5-hexa-triene (TMA-DPH) and 4-heptadecyl-7-hidroxycumarine (HC) fluorescence probes incorporated in the membrane. Urethane increase nitrite/nitrate levels. Urethane decreases significantly ($p<0.05$) HUVECs membrane fluidity assessed using HC probe, although no changes are observed using DPH and TMA-DPH probes.

In conclusion, urethane interferes with membrane fluidity and increases nitric oxide (NO) production. Accor- ding, with these results may hypothesise that either NO metabolism is sensible to changes on membrane fluidity or that classically membrane actions of anaesthetics are attributable to NO.

1. INTRODUCTION

- Urethane (ethyl carbarmate) has been widely used as an intravenous anaesthetics in animal experiments.
- It is known in many scientific literatures that anaesthetics act on endothelial cells.
- More specifically anaesthetics changes the cell membrane fluidity and disturbs cellular membrane proteins functions.
- Nitric oxide synthase is a endothelial membrane protein which changes on activity could alter the production of nitrites and/or nitrates by endothelial cells.

2. OBJECTIVE

The aim of this study was to evaluate the urethane effect upon the HUVECs membranes fluidity and quantify the nitrites and nitrates produced in presence of this anaesthetic.

3. MATERIALS AND METHODS

a) Materials

- Urethane obtained from *Sigma. Aldrich* (St. Louis, 99% purity);
- *Griess* Reagent Kit from *Molecular Probs, Inc.*(Eugene, USA);
- Fluorescent probes:
  - 1.6-biphenyl-1,3,5-hexatriene (DPH);
  - 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-exatriene (TMA-DPH);
  - -4-heptadecyl-7-hidroxycumarine (HC).

  (all from *Molecular Probes, Inc.*(Eugene, USA)

b) Biological sample

- Endothelial cells isolated from human umbilical cords obtained from St. Maria's Hospital Obstetrics Service.

c) Methods

- *Griess* Method – spectrophotometric method for nitrites and nitrates quantification (nitrates are enzymatically converted to nitrites with nitrate reductase);
- Endothelial membrane fluorescence polarization – DPH (inner membrane layers), TMA-DPH e HC (external membrane layers) fluorescence probes.

4. EXPERIMENTAL MODEL

Cultured HUVECs (from 10 umbilical cords) were incubated in PBS + Glucose 5mM for 30 minutes, 37ºC (in humidity air of 5% CO_2 in presence of urethane at final concentrations of zero (control), and 10^{-8}M to 10^{-4}M.

After incubation, the supernatants were collected for quantification of nitrites and nitrates. The nitrates are enzymatically reduced to nitrites with nitrate reductase. The HUVECs are trypsinised and collected before counted and adjusted to $4x10^5$ cells/sample. The HUVECs membrane fluidity was determined by fluorescence polarization with DPH, TMA-DPH e HC fluorescence probes.

Lower values or fluorescence anisotropy means higher membrane fluidity.

5. RESULTS

FLUIDITY OF HUVECs MEMBRANE

- Using HC fluorescent probe, the fluorescence anisotropy increase with the increase or urethane concentration so the membrane flui-dity decreases significantly for the outer membrane layer ($P<0.05$).
- In relation with the control sample, no significantively changes of the samples with urethane are observed using DPH and TMA-DPH fluorescence probes.

NITRITES AND NITRATES PRODUCTION ON HUVECS

- The Urethane increases the nitrites and/or nitrates production on HUVECs, comparing with the control sample.

6. CONCLUSIONS

- Urethane "*in vivo*" interferes with HUVECs membrane fluidity which effects were only observed when the HC fluorescent probe are used.
- HUVECs membrane fluidity or the outer face or external layer measured by fluorescence anisotropy with HC fluorescent probe decrease in presence of different concentrations of urethane.
- HUVECs produced higher nitrites and nitrates levels in presence of urethane at different concentrations than in its absence.

NO metabolites are sensible to changes on membrane fluidity or classically membrane action of anaesthetics may be attributable to NO is an open question. I

ACKNOWLEDGMENS

I would like to thank to St. 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 vivo*" study.

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Fig. 1 – Fluorescence anisotropy or HUVECs membrane ($4x10^5$ cells/sample) with HC fluorescence probe (n =5) in function of urethane concentration

Fig. 2 – Comparison of nitrites and nitrates produced by cultured HUVECs in function of the Urethane Concentration (n=10) obtained with the *Griess* method

PANCREATIC TRYPSIN INCREASES NEUTROPHIL-RELEASED MMP-9 ACTIVITY IN INTESTINAL ISCHEMIA-REPERFUSION

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ABSTRACT

The role of intraluminal pancreatic trypsin in the pathophysiology of intestinal ischemia-reperfusion (I-R) is being progressively uncovered. We have previously identified that trypsin is involved in leukocyte infiltration following intestinal I-R. Trypsin is also powerful *in vitro* activator of neutrophil-released matrix metaloprotease-9 (MMP-9). The objective of this experiment is to analize the role played by intraluminal trypsin in activating MMP-9 released by infiltrating neutrophils in intestinal I-R. In mature Sprague-Dawley rats a single jejunal loop was isolated and connected to an intraluminal perfusion system with a physiological buffer (3 mL/min.). The animals were divided in two groups: an ischemia-reperfusion group, by clamping the vascular supply to the loop for 45 minutes and then reperfusing it for 90 minutes; and a control group. In each group the perfusion buffer was supplemented with trypsin (1 mg/mL) in half of the animals. At the end of the experiment, the jejunal loop was either collected for histology (hematoxylin-eosin staining for morphological injury; in situ zymography for gelatinase activity) or homogenized for zymographic detection of gelatinase activity following electrophoresis. Our results show that intraluminal trypsin causes increased gelatinase activity in the intestinal wall (most1y associated to infiltrating neutrophils) following I-R. The gelalinase activity is mostly caused by neutrophil-released MMP-9 that presents predominantly in its activated form. These results support a role for intraluminal trypsin in activiting neutrophil-derived MMP-9 in intestinal I-R, and lead the way to further experiments that may uncover the pathophysiological role of intraluminal trypsin in this setting. (Supported by HL 67825, FLAD 470/2000 and the Beckman Foundation).

INTRODUCTION

Intraluminal pancreatic enzymes, namely trypsin, have been linked for long to morphological injury to the intestine during ischemia-reperfusion. However, its potential role as a proinflammatory stimuli has been progressively disclosed.

We have previously identified that trypsin is associated with increased rolling and adhesion of neutrophils to

the microcirculation of the intestine following ischemia-reperfusion.

On the other hand, tlypsin is also a powerfull activator of metalloprotease-9 (MMP-9), which is the main protein in the terciary granules of the neutrophil. We have also shown an increase in MMP-9 in intestinal ischemia-reperfusion, most likely of neutrophil origin.

OBJECTIVE

To identify a possible link between intraluminal trypsin and the infiltration of the intestinal wall by MMP-9, and its potential activation.

METHODS

Experimental Model

Sprague-Dawley rat, under Nembutal anesthesia (50 mg/kg)

in Situ intraluminal and extracorporal perfusion of a single jejunal loop

- Krebs-Henseleit buffer (ph 7.4; 37ºC)
- With or without pancreatic trypsin (1 mg/mL)

45 minutes of ischemia and 90 minutes of reperfusion

Techniques used:

- Histology
- *In situ* zymography
- zymography

RESULTS

CONCLUSIONS

- Pancreatic trypsin plays a role In the immediate potentiation of intestinal ischemia-reperfusion.
- At least in part, this role is associated to an increase of the inflammatory response and of the release of gelatinolytic enzymes, such as MMP-9, by neutrophils.
- We also show for the first time in vivo a pathophyslological mechanism for acute M M P-9 activation by trypsin, which is its most potent in vitro activator.

Histology – Ischemia-reperfusion originates morphological damage which is potentiated by trypsin. The inflammatory infiltrate is also more prominent in this later group

***In situ* zymography –** Ischemia-reperfusion In the presence of trypsin is associated to a major increase In gelatinase activity In the damaged wall of the intestine. This activity is abolished by EDTA, confirming metalloproteases as its source.

Zymography – This electrophoretic technique shows that the previously detected gelatinase activity is originated from metaloprotease-9 (MMP-9). This increase of MMP-9 only happens In ischemia-reperfusion in the presence of trypsin, and the presence of higher molecular weight forms (dimers) suggests that neutrophils are a significant source of this enzyme.

It is of note that MMP-9 appears mostly as the activated form and not as pro-MMP-9 (activatable by trypsin).