ONLINE SUPPLEMENT

NITRIC OXIDE PRODUCTION AND ENDOTHELIUM-DEPENDENT VASORELAXATION AMELIORATED BY N³-METHYLNICOTINAMIDE IN HUMAN BLOOD VESSELS

Teresa B. Domagala¹,², Agata Szeffler³, Lawrence W. Dobrucki⁴, Jerzy Dropinski², Stanisław Polanski², Magdalena Leszczyńska-Wilochn³, Katarzyna Kotula-Horowitzh², Jacek Wojciechowski⁵, Leszek Wojnowski⁶, Andrzej Szczeklik², Leszek Kalinowski³

Department of ¹Medical Biochemistry, ²Medicine, Jagiellonian University School of Medicine, Krakow, Poland; ³Department of Medical Laboratory Diagnostics, Chair of Clinical Chemistry and Biochemistry, Medical University of Gdansk, Gdansk, Poland; ⁴Division of Cardiovascular Medicine, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut, USA and Beckman Institute For Advanced Science and Technology, University of Illinois, Urbana-Champaign, Illinois, USA; ⁵Department of Cardiovascular Surgery, Medical University of Gdansk, Gdansk, Poland; ⁶Department of Pharmacology, Universitätsmedizin, Johannes Gutenberg University, Mainz, Germany

Corresponding Author:
Prof. Leszek Kalinowski, M.D., Ph.D.
Department of Medical Laboratory Diagnostics
Chair of Clinical Chemistry and Biochemistry
Medical University of Gdansk
Debinki 7, 80-211 Gdansk, Poland
Tel.: +48-58-3492791
Tel./Fax: +48-58-3492792
E-mail: lekal@gumed.edu.pl
EXPANDED MATERIALS AND METHODS

Study subjects and experimental protocol

The clinical characteristics of the study populations are presented in Table S1. All participants (male volunteers) gave written informed consent. Exclusion criteria were arterial hypertension ($\geq 140/90$ mmHg), diabetes mellitus, smoking, history of cardiovascular disease, cancer, liver disease, all drugs and vitamin supplementation. The 100 mg/m$^2$ dose of MNA$^+$ given p.o. was selected as it corresponded with the dosages applied in recently completed clinical trials (please see: http://clinicaltrials.gov/ct2/show/NCT00685737?term=mna&rank=2), as well as the recent pre-clinical studies in which it had been suggested that MNA$^+$ may act by way of modulating endothelial function.1-4

Endothelium-dependent, FMD of brachial artery in response to reactive hyperemia and endothelium-independent, nitroglycerin-induced dilation (NTG-MD) was evaluated non-invasively by the use of high-resolution ultrasound before, 2 and 4 hours after oral administration of MNA$^+$ or placebo. Under local anesthesia (lidocaine 1%), an 18-gauge catheter was inserted into the right brachial artery for continuous measurement of arterial pressure and infusion of vehicle (0.9% NaCl) or L-NMMA ($N^G$-monomethyl-L-arginine), a selective inhibitor of NO-synthase. First, ultrasound measurements were taken during saline infusion (at a constant rate of 0.5 mL/min). The measurements were taken in compliance with the method described by Celermajer et al.5 Briefly, all measurements were made on the right brachial artery 2-3 centimeters above antecubital fossa after a patient had stayed in the supine position for 5 min. Reactive hyperemia was induced by the inflation of sphygmomanometer cuff around the forearm to 200 mmHg for 5 min. The 5 min occlusion period was based on the current knowledge of NO dependency of the brachial artery FMD.6 The parameters were taken at baseline (before inflation) and between 60 and 120 seconds after cuff deflation. Endothelium-dependent response was construed as the dilation of the brachial artery induced by an increased flow. Subsequently, sublingual nitroglycerin (0.4 mg) was administered, and brachial artery measurements were obtained after 5 minutes, as described further above. Thirty minutes after cuff release, baseline measurements were repeated during the infusion of saline in order to verify that haemodynamic parameters had returned to the pre-occlusion levels. Subsequently, L-NMMA (Calbiochem) was infused into the brachial artery at a dose of 1.5 mg/min/L (8 $\mu$mol/min/L) for 7 min. This dose of L-NMMA has been shown to induce prolonged inhibition of NOS for at least 40 min and to block an increase in forearm blood flow in response to acetylcholine administered at the doses producing maximal regional effects without systemic haemodynamic changes.7 At 5 min after L-NMMA infusion, all parameters were measured again at baseline and the same FMD and NTG-MD protocol was repeated. The extents of relaxing responses to FMD and NTG-MD recorded in our studies were typical for the vasoreactivities previously observed in the arterial conduit vessels in the subjects with normo- and hypercholesterolemia.5-7

The parameters were measured by external vascular ultrasound Acuson Sequoia 512® (Aquson, Mountain View, CA, USA) with 6.0 MHz transducers. Artery diameters were measured from B-mode ultrasound images and arterial blood (peak systolic velocity, PSV; end diastolic velocity, EDV) was measured using the pulsed wave Doppler signals at a 70º angle to the vessel. All subjects were studied in the fasting state (between 7.00 a.m. and 8.00 a.m.); exposure to caffeine and smoking being prohibited prior to the imaging study.
Determination of MNA$^+$

Concentrations of MNA$^+$ in the plasma blood samples (collected into 10 mL vacutainers containing sodium heparin) were measured by high-performance liquid chromatography (HPLC) and fluorescent detection with excitation and emission wavelengths set at 366 and 418 nm, respectively, as described elsewhere.$^8$ Before chromatographic separations, MNA$^+$ and $N^1$-ethylnicotinamide (internal standard) were reacted with acetophenone in a strong base at 0 degrees C, formic acid was then added, and the reaction mixture was heated in a boiling water bath, resulting in the formation of fluorescent derivatives. Precision and accuracy were generally greater than 90%, interfering peaks did not co-chromatograph, and the limit of quantification was 14 pmol/mL in plasma.

Cell culture and treatments

Human umbilical vein endothelial cells-derived E.A.hy 926 cells were grown under 10% CO$_2$ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1 mmol/L L-arginine, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 x HAT (hypoxanthine, aminopterin and thymidine; Life Technologies Corporation, Warsaw, Poland). Confluent cells (4-5x10$^5$ cells/35-mm dish) were used for electrochemical measurements of NO and O$_2^\cdot$.

Fabrication of ultra-microsensors for NO and O$_2^\cdot$ detection

Concurrent measurements of NO and O$_2^\cdot$ were taken with two electrochemical ultra-microsensors, their design based on the previously developed and well-characterized, chemically modified carbon-fiber technology.$^9$-$^{10}$ Briefly, the NO and O$_2^\cdot$ sensors were made up by threading a carbon fiber through a pulled end of an L-shape glass capillary with 1 cm left protruding. The glass-fiber electrode interface was sealed with a non-conductive epoxy; a copper contact lead was inserted in the opposite end of the glass capillary and sealed off with conductive silver epoxy cement. The single-fiber electrode was thermally sharpened using a propane microburner, then coated with molten beewax-rosin mixture, and sharpened again. The active surface had the length of 2 to 5 µm with a fiber diameter of 0.2 - 0.6 µm. A conductive polymeric film was then deposited on the surface of the carbon fibers from a 0.25 mM solution of nickel (II) tetrakis (3-methoxy-4-hydroxyphenyl)porphyrin in 0.1 M NaOH for the NO sensor and from the mixture of an immobilized osmium(bpy)$_2$Cl/horseradish peroxidase complexes with poly(ethylene)diglycidyl ether (PEGDE) (ratio 1:1) for the O$_2^\cdot$ sensor.$^{11}$-$^{12}$ After drying, the active tip of the NO sensor was additionally immersed in 1% (wt) Nafion solution in alcohol for 15 s and then allowed to dry again.

Linear calibration curves were constructed for each sensor from 1 nmol/L - 10 µmol/L before and after the measurements with aliquots of NO and O$_2^\cdot$ standard solutions. Amperometry was carried out with a computer-based Gamry VFP600 multichannel potentiostat (Gamry Instruments, Warminster, PA, USA). The currents proportional to NO or O$_2^\cdot$ concentrations were monitored simultaneously at constant potentials of 0.650 V and -0.230 V, respectively, (detection limit of 1 nmol/L and the resolution time below 1 ms for each sensor). In the set of experiments with E.A.hy 926 cells, the reference and counter electrodes were positioned in the well adjacent to the culture cluster. The receptor-dependent and the receptor independent releases of NO from these cells were tested using Ach and CaI, respectively. Unlike a receptor-dependent eNOS agonist (e.g. Ach), CaI can produce unlimited intracellular influx of calcium, leading to full activation of eNOS in the endothelial cells through the increased levels of a cofactor - the calcium-calmodulin complex.

To assess whether MNA$^+$ has O$_2^\cdot$ scavenger properties, O$_2^\cdot$ was measured by O$_2^\cdot$ sensor in a xanthine-xanthine oxidase O$_2^\cdot$ generating system in the presence of different
concentrations of MNA+ according to the procedures published previously.13 Briefly, 20 µL of a given concentration of MNA+ was mixed with 5 µL of 0.5 mmol/L xanthine in 2 mL phosphate buffer with pH=7.4. The reaction was initiated by adding 10 µL of 0.02 U xanthine oxidase.

Reagents

$N^\delta$-methylnicotinamide, chloride salt (MNA+) was kindly provided by Dr Jan Adamus (Institute of Applied Radiation Chemistry, Technical University, Lodz, Poland). MNA+ was synthesized by alkylation of the corresponding 3-substituted pyridine derivatives with methyl iodide in methanol solution, as previously described.14 The resulting iodide salt was converted to chloride in aqueous solutions using freshly precipitated silver chloride and then purified by repeated crystallization from acetone–methanol. The purity of the synthesis product was established by HPLC and exceeded 99.8%. Fetal calf serum was purchased from Gibco, Poland; cell culture flasks were obtained from Corning, Poland; oxidized low-density lipoprotein (ox-LDL) from Biomedical Technologies Inc., MA, USA). All other reagents utilized were purchased from Sigma-Aldrich, Poland, unless otherwise specified.

References


Table S1. Clinical characteristics of the study populations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal subjects</th>
<th>Hypercholesterolemic subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 16</td>
<td>n = 24</td>
</tr>
<tr>
<td>Age, y</td>
<td>32.4 ±9.6</td>
<td>31.6 ±8.2</td>
</tr>
<tr>
<td>Gender, male/female</td>
<td>16/0</td>
<td>24/0</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.7 ±3.7</td>
<td>28.1 ±1.7*</td>
</tr>
<tr>
<td>Fasting plasma glucose, mmol/L</td>
<td>5.1 ±0.4</td>
<td>5.3 ±0.2</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>127 ±11</td>
<td>136 ±7</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>79 ±7</td>
<td>82 ±5</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>4.58 ±1.14</td>
<td>7.91 ±0.92†</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.77 ±0.99</td>
<td>5.63 ±0.74†</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.21 ±0.29</td>
<td>1.08 ±0.31</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.29 ±0.65</td>
<td>1.98 ±0.72</td>
</tr>
<tr>
<td>Creatinine, µmol/L</td>
<td>84.7 ±11.3</td>
<td>88.2 ±10.5</td>
</tr>
</tbody>
</table>

Values are mean ±SD. LDL, low-density lipoprotein; HDL high-density lipoprotein.
*P<0.05, †P<0.01 vs. normal subjects.
Figure S1. The effect of MNA\(^+\) on O\(_2^-\) released in the xanthine/xanthine oxidase system. O\(_2^-\) was measured in situ by the O\(_2^-\) ultra-microsensor. There were no statistically significant differences in O\(_2^-\) concentrations in the presence of the tested MNA\(^+\) concentrations.