Nitric Oxide Production and Endothelium-Dependent Vasorelaxation Ameliorated by \(N^1\)-Methylnicotinamide in Human Blood Vessels

Teresa B. Domagala, Agata Szeffler, Lawrence W. Dobrucki, Jerzy Dropinski, Stanisław Polanski, Magdalena Leszczynska-Wilocz, Katarzyna Kotula-Horowitz, Jacek Wojciechowski, Leszek Wojnowski, Andrzej Szczeklik, Leszek Kalinowski

Abstract—\(N^1\)-methylnicotinamide (MNA\(^+\)) has until recently been thought to be a biologically inactive product of nicotinamide metabolism in the pyridine nucleotides pathway. However, the latest observations imply that MNA\(^+\) may exert antithrombotic and anti-inflammatory effects through direct action on the endothelium. We examined both in vivo and in vitro whether the compound might induce vasorelaxation in human blood vessels through the improvement of nitric oxide (NO) bioavailability and a reduction of oxidative stress mediated by endothelial NO synthase (eNOS) function. MNA\(^+\) treatment (100 mg/m\(^2\) orally) in healthy normocholesterolemic and hypercholesterolemic subjects increased the l-arginine (l-NMMA)-inhibitable flow-mediated dilation (FMD) of brachial artery responses that also positively correlated with MNA\(^+\) plasma concentrations \((r=0.73\) for normocholesterolemics and \(r=0.78\) for hypercholesterolemics; \(P<0.0001\)). MNA\(^+\) increased FMD at the same concentration range at which it enhanced NO release from cultured human endothelial cells after stimulation with either the receptor-dependent (acetylcholine) or the receptor-independent endothelial NO synthase agonists (calcium ionophore A23187). MNA\(^+\) restored the endothelial NO synthase agonist-stimulated NO release after the exposure of the cells to oxidized low-density lipoprotein. This effect was also associated with the normalization of the [NO]/[superoxide] balance in the endothelial cells. Taken together, the increased NO bioavailability in the endothelium contributes to the vasorelaxing properties of MNA\(^+\). Targeting eNOS with MNA\(^+\) might be therapeutically relevant for functional disorders of the endothelium, such as hypercholesterolemia and atherosclerosis. (Hypertension. 2012;59:825-832.)  ● Online Data Supplement

Key Words: \(N^1\)-methylnicotinamide ■ endothelial cells ■ nitric oxide ■ superoxide ■ endothelial nitric oxide synthase ■ oxidized low-density lipoprotein ■ flow-mediated dilation

\(N^1\)-methylnicotinamide (MNA\(^+\)) is an endogenous organic cation that is biosynthesized in the liver from exogenous niacin and tryptophan via nicotinamide (NA) in a reaction catalyzed by NA N-methyltransferase.\(^1\) In contrast to NA, MNA\(^+\) has until recently been construed to be biologically inactive.\(^2\) However, the most recent studies suggest an antithrombotic activity of MNA\(^+\) related to both the inhibition of platelet aggregation and the activation of fibrinolysis, offering certain advantages over the use of NA.\(^3\)–\(^6\) These observations have been provisionally attributed to the release of endothelial mediators such as prostacyclin, which boosts antiaggregatory and profibrinolytic properties. However, the actual mechanism of MNA\(^+\) effects still begs comprehensive clarification.

An improvement in the dysfunctional endothelial NO synthase (eNOS)/NO pathway is an attractive strategy in preventing and treating cardiovascular diseases.\(^7\)–\(^10\) Although NO bioavailability is decreased in dysfunctional endothelium, the levels of eNOS mRNA and protein are maintained or even enhanced but associated with the increased NO synthase-dependent superoxide (O\(_2^-\)) formation because of the enzymatic “uncoupling” of NO synthase; electron flow through the eNOS enzyme is then diverted to molecular oxygen rather than to l-arginine, which facilitates the production of O\(_2^-\) rather than NO.\(^7\)\(^9\)–\(^11\) This consequently leads to an O\(_2^-\) reaction with NO, resulting in the formation of highly reactive and cytotoxic peroxynitrite and the loss of NO bioavailability.

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From the Departments of Medical Biochemistry (T.B.D.) and Medicine (T.B.D., J.D., S.P., K.K.H., A.S.), Jagiellonian University Medical School, Krakow, Poland; Departments of Medical Laboratory Diagnostics (A.S., M.L.W., L.K.) and Cardiovascular Surgery (J.W.), Medical University of Gdansk, Gdansk, Poland; Division of Cardiovascular Medicine (L.W.D.), Department of Internal Medicine, Yale University School of Medicine, New Haven, CT; Beckman Institute for Advanced Science and Technology (L.W.D.), University of Illinois, Urbana-Champaign, IL; Department of Pharmacology (L.W.), Universitätsmedizin, Johannes Gutenberg University, Mainz, Germany.

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Correspondence to Leszek Kalinowski, Department of Medical Laboratory Diagnostics, Medical University of Gdansk, Debniki 7, 80-211 Gdansk, Poland. E-mail lekal@gumed.edu.pl

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We hypothesized that the aforementioned vascular effects of MNA+ may be mediated by the changes in eNOS-derived NO. We, therefore, investigated the effect of MNA+ on eNOS-dependent endothelial function using the ultramicrosensors for the measurement of biologically active NO with concurrent measurements of O2− in real time in a single endothelial cell. The NO and O2− ultramicrosensors, designed for cell cultures, allow for direct quantification of both of these radical species with high sensitivity. This approach is particularly useful for testing the compounds that potentially target eNOS. In clinical terms, there is a prevalent notion that endothelium-dependent vasodilation is to be regarded as a surrogate for NO bioavailability. This also prompted us to assess endothelial function as an endothelium-dependent, flow-mediated dilation (FMD) of the brachial artery in healthy normocholesterolemic and hypercholesterolemic humans treated with the pharmacological dose of MNA+.

**Materials and Methods**

Details are available in the online-only Data Supplement.

**Study Subjects and Experimental Protocol**

We examined in the double-blind, randomized, placebo-controlled study the effect of oral MNA+ administration on endothelial function in healthy normocholesterolemic (age: 32.4±9.6 years; n=16), and hypercholesterolemic subjects (age: 31.6±8.2 years; n=24) with low-density lipoprotein (LDL) cholesterol >3.4 mmol/L. (Table S1, available in the online-only Data Supplement). Study subjects were randomly assigned to receive either MNA+ (100 mg/m² of body surface area; 2.43±0.28 mg/kg of body weight) or organoleptically identical placebo (tablets of microcrystalline cellulose as a vehicle) with a 1:1 allocation ratio. Endothelium-dependent, FMD of brachial artery in response to reactive hyperemia and endothelium-independent, nitroglycerin-induced dilation (NTG-MD) was evaluated noninvasively by the use of high-resolution ultrasound before, 2 and 4 hours after oral administration of MNA+ or placebo, during infusion (into the brachial artery) of saline (0.9% NaCl vehicle) or Nω-monomethyl-L-arginine (L-NMMA), a selective inhibitor of NO synthase.

The study was approved by the local ethics review committees of the Jagiellonian University School of Medicine and the Medical University of Gdansk.

**Determination of MNA+**

Blood samples were obtained from the antecubital vein before and 2 and 4 hours after oral administration of the compound. Concentrations of MNA+ in the plasma blood samples were measured by high-performance liquid chromatography with fluorescent detection.

**Cell Culture and Treatments**

Human umbilical vein endothelial-derived E.A.hy926 cells after obtaining confluence (4 to 5x10⁵ cells per 35-mm dish) were used for electrochemical measurements of NO and O2−. Before the measurements, the cells were pretreated with different concentrations of MNA+ for 1 to 180 minutes with or without Nω-nitro-L-arginine methyl ester (L-NNAME), a selective eNOS inhibitor, oxidized LDL (ox-LDL), or a combination of ox-LDL and MNA−.

**NO and O2− Measurements**

A 3-electrode system was used for concurrent measurements of NO and O2−, consisting of the NO and O2− ultrasensor working electrodes combined into a single working module, a silver/silver chloride reference electrode, and a platinum-wire counter electrode. A E.A.hy926 culture cluster was placed in a well on the stage of an inverted research microscope (Olympus, IX81) equipped with digital camera. A module of NO/O2− ultramicrosensors was lowered near the surface (5±2 μm) of a single cell membrane with the aid of a computer-controlled micromanipulator. To stimulate NO and O2− releases dependent on eNOS activation, the receptor-dependent acetylcholine (Ach) and the receptor-independent eNOS agonist calcium ionophore (CaI) A23187 were then injected with a microinjector, also positioned by a computer-controlled micromanipulator.

**Calculation and Statistical Analysis**

All of the results are reported as mean±SD. Statistical evaluation was pursued with the aid of ANOVA, followed by the Student t test. The Spearman rank correlation test was used to calculate the correlation coefficient between plasma MNA+ concentration and FMD. The value of P<0.05 was considered statistically significant.

**Results**

**Endothelium-Dependent FMD and MNA+ Plasma Concentration**

Endothelium-dependent FMD (Figure 1A and 1B), but not endothelium-independent NTG-MD (Figure 1C and 1D), was significantly lowered in the hypercholesterolemic, as compared with the normocholesterolemic controls, before the treatment with both MNA+ (Figure 1A and 1C; 3.84±1.01% versus 6.52±1.87%, respectively; P<0.001) and placebo (Figure 1B and 1D). In either group, infusion of L-NMMA during pretreatment with MNA+ almost completely abolished the FMD response (6.52±1.87% without L-NMMA versus 0.19±0.86% with L-NMMA for normocholesterolemic and 3.84±1.01% without L-NMMA versus 0.14±0.85% with L-NMMA for hypercholesterolemic subjects; P<0.0001 for each group). In contrast, L-NMMA infusion did not affect the NTG-MD response, thus confirming that the differences in FMD between hypercholesterolemic and normocholesterolemic subjects were associated with the changes in eNOS activity and NO generation. No differences in blood pressure or heart rate were observed during the study between the MNA+ and the placebo-treated groups.

As shown in Figure 1A, FMD of the brachial artery was significantly elevated 2 and 4 hours after MNA+ administration in both studied groups (6.5±1.9% at baseline versus 10.7±3.1% at 2 hours and 10.5±1.5% at 4 hours after MNA+ in normocholesterolemic and 3.8±1.0% at baseline versus 10.7±2.1% at 2 hours and 10.6±1.8% at 4 hours after MNA+ in hypercholesterolemic; P<0.001 for each time point versus baseline in both groups). The increase in FMD after the administration of the compound was more pronounced in hypercholesterolemic subjects than in normocholesterolemic subjects (190±79% versus 68±14% at 2 hours after MNA+ and 187±59% versus 75±18% at 4 hours after MNA+ in normocholesterolemic and hypercholesterolemic subjects, respectively; P<0.01). In both groups, L-NMMA almost completely inhibited FMD, with no difference between previous and subsequent administration of MNA+.

Essentially, NTG-MD did not change within 4 hours after administration of MNA+ and was not affected by L-NMMA (Figure 1C). No differences were detected between either FMD (Figure 1B) or NTG-MD (Figure 1D) before and 2 and 4 hours after placebo administration.
Plasma MNA⁺ concentrations increased significantly after an oral administration of MNA⁺, from 0.019±0.008 µmol/L at baseline to 0.122±0.075 µmol/L at 2 hours and 0.090±0.051 µmol/L at 4 hours in normocholesterolemic subjects and from 0.021±0.006 µmol/L at baseline to 0.141±0.043 µmol/L at 2 hours and 0.118±0.042 µmol/L at 4 hours in hypercholesterolemic subjects (P<0.01 for each time point versus baseline in both groups; Figure 1E). The concentration of MNA⁺ was affected by neither L-NMMA nor placebo (Figure 1E and 1F).

Regression analyses were carried out to further evaluate the relationship between MNA⁺ and endothelial function. A highly significant correlation was found between plasma MNA⁺ concentrations and FMD in both studied groups (r=0.73 in normocholesterolemic subjects and r=0.78 in hypercholesterolemic subjects; P<0.0001 for each group; Figure 2). No difference in association between these 2 parameters was observed in either group in the data set obtained either 2 hours or 4 hours after MNA⁺ administration (P<0.0001 for each time point).

Effect of MNA⁺ on NO Production via eNOS

We then evaluated the effect of MNA⁺ on endothelium-dependent NO release from human culture endothelial cells as a function of dosage and incubation time. MNA⁺, per se, did not stimulate NO release from endothelial cells. After Ach or CaI administration, a rapid release of NO was observed from both the control and the MNA⁺-treated cells. The incubation of cells for 30 minutes with increasing MNA⁺ concentrations resulted in a dose-dependent increase of the peak NO production after stimulation of eNOS by either CaI or Ach (Figure 3A).

The curves reflecting the production of the species reached a semiplateau at ~10 µmol/L of MNA⁺ for each of the eNOS activators (539±28 nmol/L after Ach and 875±40 nmol/L after CaI). Subsequently, the peak NO concentrations did not change significantly at MNA⁺ concentrations ≤100 µmol/L (the highest MNA⁺ concentration tested). An analysis of the time-dependent effect of the constant MNA⁺ concentration on the stimulated peak NO release showed a rapid rise of NO production for 30 minutes after
At concentrations of 0.5 to 10.0 μmol/L, MNA⁺ had a concentration-dependent potentiating effect on both Ach- and CaI-stimulated NO release from endothelial cells, shifted the eNOS agonists-concentration response curves to the left, and increased the maximal NO releasing response (Figure 4A for Ach and Figure 4B for CaI). To verify the potential involvement of eNOS on the effect of MNA⁺ on Ach- and CaI-stimulated NO release, endothelial cells were incubated with different concentrations of MNA⁺ in combination with 300 μmol/L of L-NAME before the administration of the eNOS agonists. L-NAME significantly decreased the release of NO throughout the concentration response to both Ach and CaI.

**Effect of MNA⁺ on eNOS Coupling/Uncoupling**

To directly assess the effect of MNA⁺ on NO bioavailability and eNOS functional state, the kinetics of NO release were determined with concurrent kinetics of O₂⁻ release after the stimulation of eNOS with CaI in the single endothelial cells using tandem NO/O₂⁻-ultramicrosensors. The effect of MNA⁺ was examined in the nonox-LDL–treated cells, as well as in the ox-LDL–treated cells that were used as a model of endothelial dysfunction (Figure 5A for NO and Figure 5B for O₂⁻). Both concentration profiles changed with time, and the maximal concentrations changed appreciably after MNA⁺ incubation with either the nonox-LDL–treated or the ox-LDL–treated cells. The presence of MNA⁺ potentiated the increase in the rate of NO release, as well as the peaks of NO concentrations achieved after the stimulation with CaI in both cell treatment models. Furthermore, the incubation of the cells with MNA⁺ resulted in the kinetics of O₂⁻ release in ox-LDL–treated cells being similar to those in the nonox-LDL–treated cells.

Because the changes in kinetics of NO and O₂⁻ releases could be related to O₂⁻ scavenging properties of MNA⁺, we also examined the effect of MNA⁺ on O₂⁻ released in the xanthine/xanthine oxidase system (Figure S1, available in the online-only Data Supplement). MNA⁺ did not reveal O₂⁻ scavenging properties in the concentrations that affected...
the kinetics of NO and O$_2^-$ releases in the cells after eNOS stimulation.

The analysis of Cal-stimulated peak responses of NO and O$_2^-$ concentrations after cell incubation with MNA$^+$ or L-NAME in the nonox-LDL–treated and the ox-LDL–treated cells is shown in Figure 6. The set of experiments with L-NAME clearly confirmed that the release of NO and O$_2^-$ observed after adding Cal was attributed to eNOS activation, because eNOS inhibition of the enzyme by L-NAME significantly blocked the release of both of the detected species. In comparison with the nontreated control cells, the cells treated with ox-LDL significantly revealed lowered Cal-stimulated peak NO concentration with an accompanying elevation of the maximal concentration of O$_2^-$.

Figure 4. Concentration-response curves for the stimulatory effect of acetylcholine (Ach; A) or calcium ionophore (Cal; B) on the NO release from the endothelial cells in the presence of a range of N$^\prime$-methylnicotinamide (MNA$^+$) concentrations. Cells were exposed to MNA$^+$ concentrations from 0.5 to 10.0 μmol/L for 30 minutes and then treated with various concentrations of the endothelial NO synthase (eNOS) agonists. For the maximal tested MNA$^+$ concentration of 10 μmol/L, the cells were also coincubated without or with 300 μmol/L of N$^\prime$-nitro-L-arginine methyl ester (L-NAME). n=6 for each point. *P<0.05, **P<0.01 vs with MNA$^+$.

Figure 5. Representative recordings of NO (A) and superoxide (O$_2^-$; B) concentrations with time on the surface of a single endothelial cell. Release of NO and O$_2^-$ was stimulated by 1.0 μmol/L of calcium ionophore (Cal) at time 0 after 30 minutes of incubation with 100 mg of cholesterol per deciliter of oxidized low-density lipoprotein (ox-LDL) in the presence or absence of 10 μmol/L of N$^\prime$-methylnicotinamide (MNA$^+$).
and endothelial NO synthase (eNOS) was stimulated by 1.0

treated control cells (614

cells were incubated for 30 minutes with the tested substances,

Figure 6. Calcium ionophore (Cal)-stimulated peak NO (●) and

and superoxide (O2●−; ■) releases from endothelial cells after the

incubation with or without 300 μmol/L of Nω-nitro-L-arginine

methyl ester (L-NAME), 10 μmol/L of N′-methyl nicotinamide

(MNA ●), 100 mg/dL of oxidized low-density lipoprotein (ox-LDL)

or 100 mg/dL of ox-LDL together with 10 μmol/L of MNA ●. The

cells were incubated for 30 minutes with the tested substances,

in the presence or absence of 10 μmol/L of N′-methyl nicotinamide

(MNA ●) for 30 minutes, and then NO and O2●− releases were stimulated with

1.0 μmol/L of calcium ionophore (Cal). The ratio of NO:O2●−

concentration after eNOS activation by an agonist illustrates the

status of eNOS coupling/uncoupling and represents the index of

oxidative stress mediated by eNOS; higher [NO]:[O2●−] indicates

stronger eNOS coupling and greater bioavailability of NO within

the cells. Left 2 bars, Prevention of eNOS uncoupling by MNA ●

in the nonox-LDL–treated normal cells: although MNA ●

increased significantly the peak concentrations of both NO and

O2●− after stimulation with Cal, the ratio of [NO]:[O2●−] did not differ when compared with cells without MNA ●. Right 2 bars, Restoration of eNOS coupling by MNA ● in dysfunctional cells (ox-LDL–treated cells): MNA ● significantly increased not only the peak concentrations of both NO and O2●− after stimulation with Cal but also elevated the [NO]:[O2●−] ratio. n = 6 for each point; *P < 0.01 vs control (nontreated cells).

Figure 7. The ratio of peak NO concentrations to peak superoxo-

die (O2●−) concentrations measured in the endothelial cells after

the activation of endothelial NO synthase (eNOS). The endothelial

cells were incubated with 100 mg of cholesterol per deciliter

of oxidized low-density lipoprotein (ox-LDL) in the presence or

absence of 10 μmol/L of N′-methyl nicotinamide (MNA ●) for 30

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Discussion

Endothelial dysfunction construed as a decrease in NO

bioavailability in the vessels is a common and early patho-

genic mechanism by which different cardiovascular risk

factors cause atherosclerotic vascular damage, predisposing

patients to cardiovascular events.7–10 It is, therefore, import-

ant to seek out effective strategies aimed at preventing or

treating endothelial dysfunction via increasing the bioavail-

ability of NO derived from eNOS. Our study reports for the

first time that MNA ●, a primary metabolite of NA, is poten-

t in increasing eNOS-mediated NO release from the endothe-

lial cells. We demonstrated this effect in normal and hyper-

cholesterolemic humans (at the early stages of vascular

disease) in which the abnormality in vasodilator function was

confined to the endothelium- and NO-dependent mecha-

nisms. In contrast to NTG-MD, FMD, after a brief period of

reactive hyperemia fully inhibited by l-NMMA, was signif-

icantly impaired in the hypercholesterolemic, as compared

with the normocholesterolemic, subjects. The reduced portion

of FMD inhibited by l-NMMA in hypercholesterolemia

(compared with normal subjects) implies that the endothelial

release of NO is significantly impaired in the hypercholes-

terolemic subjects.

By using the NO porphyrinic ultramicrosensor, it was

possible to show that MNA ●-enhanced eNOS agonist-

stimulated NO release in human endothelial cells at potential

therapeutic concentrations effectively improved the l-

NMMA–inhibitable FMD response in healthy normocholes-

terolemic and hypercholesterolemic subjects. MNA ● was

capable of normalizing a functional injury to the endothelium
(before morphological lesions develop) caused by elevated LDL cholesterol levels to the extent observed in the normocholesterolemic subjects. The increase in NO release occurred at MNA+ concentrations that were similar to the plasma concentrations obtained in the subjects after single dosages of 180 to 210 mg of MNA+. Furthermore, a strong positive linear correlation was observed between the plasma concentrations of MNA+ and the extent of FMD response after treatment with the compound, confirming the in vitro observations that MNA+ directly acted on the endothelium through an increase of NO bioavailability. This is consistent with the previous studies in which MNA+ inhibited platelet aggregation in the vasculature of hypertensive animals and the effect was reversed by the eNOS inhibitor L-NAME, whereas MNA+ failed to influence the in vitro platelet aggregation.6

MNA+ has been reported to improve the endothelium- and NO-dependent vasodilation impaired in hypertriglyceridemic and diabetic animals.4 Considering the potential concentrations of MNA+ in blood that are required to reach its therapeutic effect of vasoprotection, it is worth noting that the ionic character of the MNA+ compound, which, when introduced into blood circulation, is capable of interacting with glycosaminoglycans located on the cell surface of vascular endothelium, may lead to increasing its local concentration in the vicinity of the cell membranes.2,16

In our studies, the release of bioactive NO from the endothelial cells after previous exposure to MNA+ was significantly enhanced in response to either Ach or CaI, a receptor-independent and a receptor-dependent eNOS agonist, respectively. This suggests that MNA+ improved NO bioavailability in response to the eNOS agonists by the mechanism unrelated to the muscarinic cell receptors but affected directly by the eNOS function. The release of NO after stimulation with eNOS agonists in the presence of MNA+ was inhibited by L-NAME, and the actual extent of inhibition (by 75% to 90% for each agonist) did not differ from that observed when the cells were pretreated without MNA+ (data not shown). The extent of inhibition is typical for that observed when NO generated by eNOS is being detected close to the surface of an endothelial cell by porphyrinic ultramicrosensor.10,12 This observation corroborates the fact that MNA+ improved the eNOS agonist-stimulated NO release by altering the eNOS function.

eNOS uncoupling accompanies numerous common diseases, for example, hypercholesterolemia, hypertension, and diabetes mellitus.7–10 We have demonstrated recently that eNOS uncoupling occurs not only under specific pathological conditions but also after enzyme activation in the normal endothelium.12 Rapid release of NO by most eNOS agonists is always followed by the release of O2−, for example, CaI and Ach. Therefore, in some of our experiments the release of NO and O2− was measured simultaneously, because O2− generation during NO production is a major determinant of bioavailability of diffusible NO. Real-time measurements of NO and O2− released in a single endothelial cell with tandem ultramicrosensors revealed that a short time exposure to MNA+ of normal, functional cells may lead to an increase in NO bioavailability with no changes in the NO/O2− balance after eNOS stimulation. Furthermore, MNA+ may favorably shift the NO/O2− balance after eNOS stimulation in the highly dysfunctional cells pretreated with ox-LDL. In the dysfunctional cells, MNA+ increased the level of bioactive NO and reduced the level of O2−, the primary component of oxidative stress. This implies that MNA+ may be considered as a useful agent in clinical application for either the prevention of endothelial dysfunction by preserving eNOS coupling (in normal endothelial cells) or the restoration of endothelial function by the reversal of eNOS uncoupling (in the endothelial cells exposed to a risk factor).

Notably, not only did MNA+ enhance the ratio of bioactive NO versus O2− but also the rate of NO generation, and it reduced the rate of NO fading. The favorable changes of MNA+ in the kinetics of NO release after stimulation of eNOS in the cells exposed to a risk factor (eg, high level of ox-LDL) are essential for maintaining a high gradient of NO concentration between the endothelium and the adjacent tissues that allows for NO-dependent long-distance signaling. Maintaining the adequate NO gradient in vasculature makes its diffusion efficient enough to be reached by NO typical targets, such as vasodilation and the inhibition of platelets. It is also worth noting that the presence of L-NAME significantly blocked the CaI-stimulated release of both NO and O2−, and completely obliterated the differences in the levels of the detected molecules between the cells treated with or without ox-LDL. These observations further confirmed that, in our experimental model, eNOS was an enzymatic source producing simultaneously both radicals and that the pretreatment of the cells with ox-LDL efficiently increased the extent of eNOS activity dysfunction.

**Perspectives**

Although eNOS targeting is an attractive approach for preventing and treating atherosclerosis and other cardiovascular disorders, the phenomenon of eNOS uncoupling hampers the attempts to assess the efficacy of pharmacological interventions in modulating endothelial function. eNOS must be regarded as both an NO- and an O2−-producing enzyme, and, therefore, eNOS may have a dual effect on vascular function, depending on its functional state.7,10,11 Our results provide evidence that the direct action of MNA+ on the endothelium in the healthy normocholesterolemic and hypercholesterolemic subjects improves the endothelium-dependent vasorelaxing effect produced by the enhancement of NO bioavailability and the reduction of eNOS-dependent oxidative stress. MNA+ is capable of improving NO bioavailability within the endothelium by acting toward either the prevention of endothelial dysfunction (prevention of eNOS uncoupling) in normal cells or the restoration of endothelial function (restoration of eNOS coupling) in the cells exposed to a specific cardiovascular risk factor (high level of ox-LDL). This effectively makes it a highly promising compound worth further evaluation in the treatment of hypercholesterolemia and atherosclerosis.

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Disclosures
None.

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NITRIC OXIDE PRODUCTION AND ENDOTHELUM-DEPENDENT VASORELAXATION AMELIORATED BY $N^\circ$-METHYLNICOTINAMIDE IN HUMAN BLOOD VESSELS

Teresa B. Domagala$^{1,2}$, Agata Szeffler$^3$, Lawrence W. Dobrucki$^4$, Jerzy Dropinski$^2$, Stanisław Polanski$^2$, Magdalena Leszczyńska-Wiloch$^3$, Katarzyna Kotula-Horowitz$^2$, Jacek Wojciechowski$^5$, Leszek Wojnowski$^6$, Andrzej Szczeklik$^2$, Leszek Kalinowski$^3$

Department of $^1$Medical Biochemistry, $^2$Medicine, Jagiellonian University School of Medicine, Krakow, Poland; $^3$Department of Medical Laboratory Diagnostics, Chair of Clinical Chemistry and Biochemistry, Medical University of Gdansk, Gdansk, Poland; $^4$Division 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; $^5$Department of Cardiovascular Surgery, Medical University of Gdansk, Gdansk, Poland; $^6$Department 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 (≥140/90 mmHg), diabetes mellitus, smoking, history of cardiovascular disease, cancer, liver disease, all drugs and vitamin supplementation. The 100 mg/m² 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.

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\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 μmol/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.\textsuperscript{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.\textsuperscript{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. 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% CO₂ 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 (4-5x10⁵ cells/35-mm dish) were used for electrochemical measurements of NO and O₂⁻.

Fabrication of ultra-microsensors for NO and O₂⁻ detection 
Concurrent measurements of NO and O₂⁻ were taken with two electrochemical ultra-microsensors, their design based on the previously developed and well-characterized, chemically modified carbon-fiber technology. Briefly, the NO and O₂⁻ 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)₂Cl/horseradish peroxidase complexes with poly(ethylene)diglycidyl ether (PEGDE) (ratio 1:1) for the O₂⁻ sensor. 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 O₂⁻ 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 O₂⁻ 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 O₂⁻ scavenger properties, O₂⁻ was measured by O₂⁻ sensor in a xanthine-xanthine oxidase O₂⁻ generating system in the presence of different
concentrations of MNA$^+$ according to the procedures published previously. 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**

$N^\beta$-methyl nicotinamide, 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. 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 n = 16</th>
<th>Hypercholesterolemic subjects n = 24</th>
</tr>
</thead>
<tbody>
<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>
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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 \textit{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.