Brief Report

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Meldonium Decreases the Diet-Increased Plasma Levels of Trimethylamine N-Oxide, a Metabolite Associated With Atherosclerosis

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Keywords

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Meldonium (mildronate; 3-(2,2,2-trimethylhydrazinium) propionate dehydrate) is an anti-ischemic and anti-atherosclerotic drug that exerts its cardioprotective action through an L-carnitine-lowering effect.[1,2] The long-term administration of mildronate also significantly lowers the L-carnitine plasma concentrations in non-vegetarian, healthy volunteers.[3] Meldonium regulates the bioavailability of L-carnitine by inhibiting its biosynthesis via the gamma-butyrobetaine hydroxylase enzyme[4] and its reabsorption in the kidneys via carnitine/organic cation transporter (OCTN) 2 proteins.[5] In experimental models, meldonium increases the levels of gamma-butyrobetaine, a precursor of L-carnitine biosynthesis.[6] However, the effects of meldonium treatment on other metabolites of L-carnitine have not been studied.

The homeostasis of L-carnitine is maintained through biosynthesis, efficient kidney reabsorption, and food intake, particularly from meat and dairy products.[7] In the intestine, L-carnitine is degraded to trimethylamine (TMA),[8,9] a simple tertiary amine that is subjected to metabolism by flavin-containing monooxygenase (FMO) and oxidized to odorless trimethylamine N-oxide (TMAO). Both TMA and TMAO are common metabolites in animals.[10] TMAO decomposes to TMA, which is the main odorant characteristic of degrading seafood and is an osmolyte found at high concentrations in fish.[11] A recent study has provided strong associations between systemic TMAO levels and both angiographic measures of coronary artery atherosclerosis and cardiac risk.[12] Furthermore, plasma L-carnitine levels in patients with concurrently high TMAO levels predict increased risk for both prevalent cardiovascular disease and incident major adverse cardiac events.[13] Thus far, no pharmacological agent has been shown to lower TMAO concentrations in vivo.

Taking into account the L-carnitine-lowering activity of meldonium, the aim of the present study was to investigate the effects of meldonium on diet-increased TMAO levels in plasma and the urine samples of healthy volunteers. In addition, the effects of meldonium and TMAO on in vitro L-carnitine uptake were studied in human embryonic kidney 293 (HEK293) cells.

Subjects and Methods

Subjects and Study Design

This study was performed with the approval of the Central Medical Ethics Committee of Latvia, and informed consent was obtained from all subjects. This open label, interventional study included eight healthy volunteers: four female and four male. The duration of the study was 3 weeks. All study participants were European descent. The criteria for the volunteers to participate in the study were as follows: good health, as assessed by clinical examination, nonsmoking, not pregnant, and no history of drug or alcohol abuse. The data collected at the study inception included age, medical history, and anthropometric indices.

Before beginning the study, the participants had not included any fish or marine products in their diet for 7 days. The subjects arrived at the trial unit at 8:30 am after overnight fasting, and basal blood samples were collected. For the next 7 days, the participants consumed a TMA-rich lunch that included approximately 150 g of fish and sea

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products (salmon, cod, herring, or shrimps). Plasma samples were collected at the end of the period, and a 7-day meldonium (Mildronate®, Grindeks, Latvia) treatment then began in addition to the TMA-rich diet (the choice of fish products was similar to the previous week). Meldonium was administered perorally at a dosage of 500 mg, twice daily between meals. Plasma samples were collected at the end of the period. Urine samples were collected every morning and evening during the experimental period. Urine aliquots and plasma samples were stored at −20°C prior to analysis.

The Inhibition of OCTN2-Mediated Transport of L-Carnitine

The transport of L-carnitine was measured as L-[N-methyl-^{3}H] carnitine hydrochloride (specific activity, 85 Ci/mmol, Biotrend, Köln, Germany) uptake by HEK293 (ATCC collection code CRL-1573, LGC Standards AB, Boras, Sweden) cells. The cells were grown in 24-well plates in DMEM/F-12 medium (Sigma–Aldrich, Steinheim, Germany) supplemented with 10% fetal bovine serum (FBS, Sigma–Aldrich) until approximately 90% confluence was reached. Prior to the assay, the cells were washed twice with 0.5 mL DMEM/F-12 without FBS; the assay was performed in 300 μL of medium. The cells were pre-incubated with meldonium or TMAO (Sigma–Aldrich; 10, 30, 100, 300, and 1,000 μM) for 15 min at 37°C, and uptake was initiated by the simultaneous addition of unlabeled L-carnitine (10 μM) and L-[*N*-methyl-^{3}H] carnitine (4 nM, 12 kBq/mL). After incubation for 60 min at 37°C, the medium was removed, and the cells were washed three times with 300 μL ice-cold phosphate-buffered saline (PBS). The cells were then lysed directly in the plate with 100 μL 0.1% sodium dodecyl sulfate (SDS) in 1 M NaOH. A 200-μL aliquot of scintillation cocktail was added to 50 μL of the cell lysate, and the radioactivity was measured using Wallac MicroBeta Trilux scintillation counter (PerkinElmer Inc., Waltham, Massachusetts, USA). The data were normalized to the protein content, as determined using the Lowry method. The control measurement of L-carnitine uptake in the absence of additional substances was taken as 100%. The measurements were performed in triplicate for each compound concentration, and the data were analyzed using GraphPad Prism 3.0 software.

UPLC/MS/MS Analysis

The TMAO concentrations in both the plasma and urine samples were determined using a UPLC/MS/MS method and a Quattro Micro triple–quadrupole mass spectrometer (Micromass, Waters, Milford, MA, USA) using electrospray ionization in the positive ion mode. The plasma sample to be tested (25 μL) was mixed with 500 μL internal standard (3-(2,2-dimethyl-2-prop-1-yl-hydrazinium)propionate, 200 ng/mL, synthesized in-house) solution in acetonitrile (gradient grade, Sigma–Aldrich)/methanol (gradient grade, Merck, Darmstadt, Germany) mixture (3/1). The urine sample was diluted with deionized water (1/50), and 100 μL was mixed with 700 μL internal standard (3-(2,2-dimethyl-2-prop-1-yl-hydrazinium)propionate, 200 ng/mL) solution in an acetonitrile/methanol mixture (3/1). The samples were centrifuged for 10 min at 13,000 rpm, and the clear supernatants were separated and used for the analysis. The chromatographic separation of analytes was achieved using an Acquity UPLC system (Waters, Milford, MA, USA). The analytes were separated on an HILIC BEH column (2.1 mm × 100 mm, 1.7 μm, Waters) using a gradient elution from 75% to 55% acetonitrile in 10 mM aqueous ammonium acetate (pH 4) at a flow rate of 0.25 mL/min. The TMAO analyses were performed in the multiple reaction mode (MRM). Two precursors to product ion transitions (m/z 75.8 > 58.3 for TMAO and m/z 175.4 > 86.0 for the internal standard) were monitored. Data acquisition and processing were performed using MassLynx V4.1 and QuanLynx V4.1 software (Waters).

Statistical Analyses and Calculations

The results are expressed as the mean ± standard error means (SEM). Statistically significant differences in the mean values were tested by a repeated measures ANOVA and Tukey's post-test. The differences were considered significant when $P < .05$. The data were analyzed using GraphPad Prism 3.0 statistical software (GraphPad Inc., La Jolla, CA, USA). The amount of TMAO excreted in the urine was calculated as the area under the curve after taking into account the average creatinine excretion rate determined by the Jaffe method, as described previously.[3]

Results and Discussion

Because fish products are rich in TMA and it has been shown that the consumption of fish products increases the urinary concentrations of TMAO,[14,15] we used fish as part of a TMA-rich diet in the present study. The average age and body-mass index of the study participants did not differ significantly between the genders and was 30 ± 2 years and 23 ± 1 kg/m^2, respectively. No adverse events were observed during the study. As shown in Figure 1A, the average plasma concentration of TMAO was 4.9 ± 1.3 μM before the start of the study, and the plasma TMAO concentration increased 16-fold (81.5 ± 8.6 μM) after 7 days of the TMA-rich diet. The addition of meldonium to the TMA-rich diet increased the plasma concentration of TMAO only ninefold (43.0 ± 3.8 μM; Figure 1A). The excretion of TMAO in the urine followed the plasma analysis patterns: the concentration of TMAO was 2.8 ± 0.5 μmol/mg creatinine × 7 days at the beginning of experiment, with a sixfold increase (18.2 ± 2.2 μmol/mg creatinine × 7

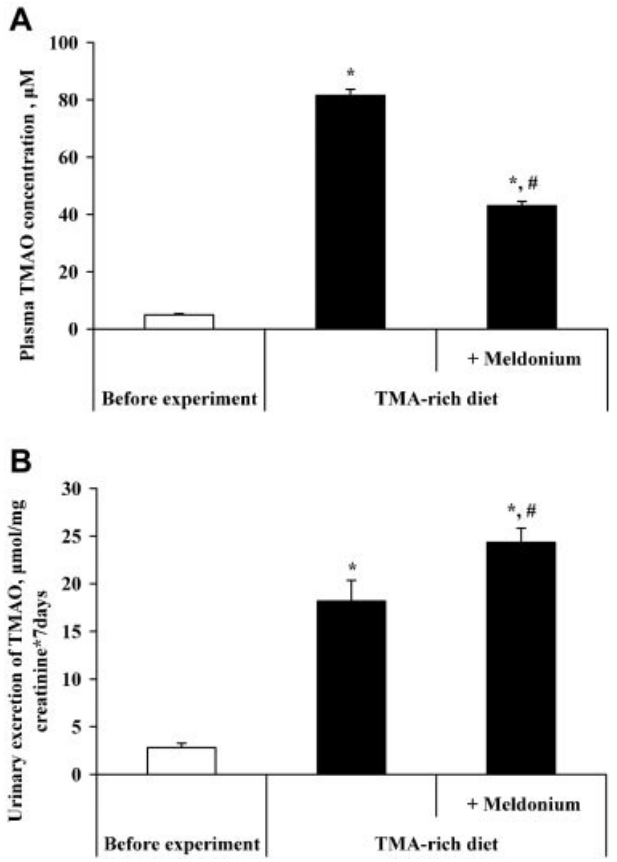

Figure 1. Concentration of TMAO in blood plasma (A) and urine (B) samples. TMAO was measured in blood plasma and urine samples prior to the initiation of a TMA-rich diet, prior to the meldonium treatment, and after 7 days of meldonium treatment (500 mg, twice daily between meals). The data are presented as the mean ± SEM ($n = 8$). *Significantly different from the baseline (before the experiment). #Significantly different from the value obtained after a 7-day TMA-rich diet (repeated measures ANOVA, Tukey's post-test, $P < .05$).

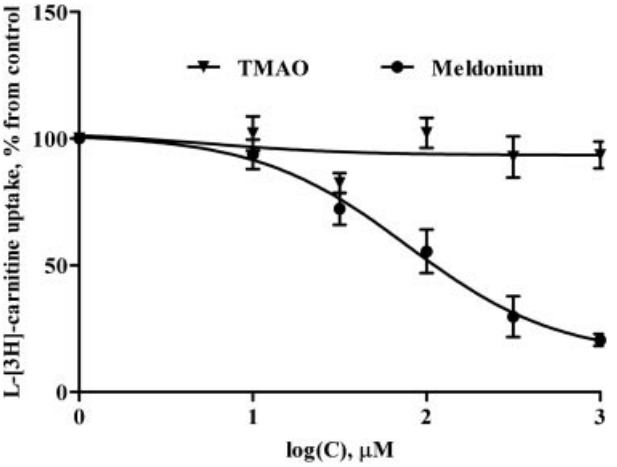

Figure 2. The inhibition of the OCTN2-mediated uptake of L-carnitine by meldonium and TMAO. The inhibition of L-[^{3}H] carnitine was measured for 60 min at 37°C. The data were calculated as the mean ± SEM of three determinations.

days) after 7 days of the TMA-rich diet (Figure 1B). The meldonium treatment increased the urine excretion of TMAO by 34% (24.3 ± 1.5 μmol/mg creatinine × 7 days; Figure 1B). Therefore, meldonium is the first pharmacological agent shown to decrease the concentration of TMAO in human plasma through increased urinary excretion.

To study the possible mechanism for this activity, we performed in vitro OCTN2-related transport experiments using kidney cell cultures. As observed in Figure 2, meldonium (as an inhibitor of OCTN2) inhibited the uptake of labeled L-carnitine in HEK293 cells. However, TMAO at concentrations up to 1 mM did not influence the uptake of L-[^{3}H] carnitine (Figure 2). Since urine is the major route of elimination of TMAO, with 95% of the administered dose being voided in the first 24 hours,[16] we conclude that meldonium probably enhances the renal excretion of TMAO, but the activity of meldonium is not related to its inhibitory effect on OCTN2.

Recently, a link was reported between gut flora-dependent metabolism and atherosclerosis risk through the generation of pro-atherosclerotic metabolites, including TMAO.[12,13] TMAO has been shown to produce a pro-atherogenic macrophage phenotype[12] and affect cholesterol and sterol metabolism in macrophages, liver, and intestine.[13] Thus far, only an observational study has provided evidence for a correlation of TMAO plasma concentrations and atherosclerosis risk in humans.[12] In addition to the proatherosclerotic effects of TMAO, it has been shown that both TMA and TMAO accumulate in patients with kidney failure.[17] Thus, TMAO-lowering pharmacological agents might be useful for the maintenance of cardiovascular functionality in patients with high TMAO and L-carnitine plasma concentrations. Meldonium administration results in L-carnitine-lowering and anti-atherosclerotic effects in experimental and clinical studies,[18,19] and the mechanism behind these effects could be related to the reduction of the plasma TMAO concentration described in the present study.

Further studies with meldonium might help to evaluate the clinical relevance of anti-TMAO agents and the contribution of the TMAO-lowering effect to the clinical effectiveness of meldonium.

Conclusion

The results of the present study provide evidence for the pharmacological regulation of plasma levels of TMAO by meldonium, which increases urinary excretion of TMAO and thus decreases its plasma concentration. In addition, our data suggest that OCTN2 is not a TMAO transporter.

Declaration of Competing Interests and Financial Disclosure

The authors declare that there are no conflicts of interest.

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