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Simvastatin treatment increases nitrite levels in obese women: Modulation by T^{-786}C polymorphism of *eNOS*

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ABSTRACT

Objective and Subjects: Evidence indicates an impairment of nitric oxide (NO) in obesity. Statins present pleiotropic effects independently of cholesterol-lowering, including increasing of eNOS expression and antioxidant effects. We evaluated the effects of simvastatin treatment at 45 days on circulating nitrite (NO marker) and TBARS-MDA levels in obese women without comorbidities (hypertension, diabetes and dyslipidemia). Moreover, we verified whether obese women carrying the C variant of T^{-786}C polymorphism located in eNOS may have increased levels of nitrite after treatment compared to TT genotype. *Results:* After simvastatin treatment, while the plasma nitrite levels increased 42% ($P = 0.0008$), the TBARS-MDA levels reduced 58% ($P = 0.0069$). We observed increased levels of nitrite in both groups of genotypes (TT vs. TC + CC); however, rise in C-allele carriers was 60% comparing with 44% in TT. *Conclusion:* Our results demonstrated a restoration of nitrite levels in obese women treated with simvastatin, which is modulated by T^{-786}C polymorphism.

Introduction

Nitric oxide (NO) is a crucial regulator of the cardiovascular system, which controls the vasodilator tone and promotes vascular homeostasis [19]. NO bioavailability is determined by balance between NO biosynthesis by the endothelial NO synthase (eNOS) and its degradation by reactions with cell-free hemoglobin and reactive oxygen species (ROS) [10,32].

Several evidence indicate that diseases characterized by endothelial cell dysfunction, including obesity [33], present unbalanced circulating levels of NO metabolites due to increased oxidative stress [4] and decreased NO production [9]. In fact, NADPH oxidases [2] and uncoupled eNOS [7] contribute to a higher generation of ROS. Additionally, studies have shown that a single nucleotide polymorphism (SNP) clinically relevant located in promoter region of the eNOS gene (T-786C, rs2070744) reduces gene promoter activity by approximately 50% [21], besides it was also associated with reduced eNOS expression on myocardial tissue [5].

Statins block the conversion of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate inhibiting cholesterol synthesis in the liver. However, it is well documented that the pleiotropic effects of statins are independent of cholesterol-lowering responses. Some of these effects include the increase of eNOS expression and activity and antioxidant effects [17]. Moreover, statins might increase the expression of endoglin (CD105), a molecule associated with eNOS in caveolae, which regulates its activity and local vascular tone [23]. Other regulator of eNOS is its endogenous inhibitor ADMA (asymmetric dimethylarginine). Some studies evaluated the effect of simvastatin on ADMA levels, however, none addressed this effect in obese women [30,31].

Although these observations provide strong evidence that statins can increase endogenous NO production and reduce oxidative stress, no previous study has examined whether these pleiotropic effects may increase the bioavailability of NO (measured by nitrite levels) in obese women, especially those that carry the C allele of T-786C polymorphism.

Material and methods

Subjects

The Institutional Review Board of Santa Casa de Belo Horizonte, Brazil, approved the use of human subjects, and the subjects gave informed consent. The procedures followed were in accordance with institutional guidelines.

We recruited 25 obese women (BMI $\geqslant$ 30 kg/m^2) aged 19–58 years from the local community. All women were Caucasians. Obesity was defined according to the guidelines of the World Health Organization. The body mass index (BMI) was calculated

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as the weight in kilograms divided by the squared height in meters. The blood pressure (BP) measurement was taken three times from all volunteers with subject in the seated position with the use of an automatic blood pressure device.

The exclusion criteria included hypertension, dyslipidemia, heart disease, diabetes mellitus, thyroid and renal diseases, obstructive sleep apnea, pregnancy or breast-feeding, cigarette smoking and chronic alcohol consumption.

Subjects received simvastatin for 6 weeks, 20 mg/day. Venous blood samples were collected before and after treatment in standard Vacutainer tubes (Becton-Dickinson, Brazil) containing ethylenediaminetetraacetic acid (EDTA), after an overnight fast (8–12 h). These tubes were immediately centrifuged at 2000*g* for 10 min at room temperature, and the plasma and serum samples were stored at −80 °C until assayed.

Laboratory analyses

The glucose concentrations, hepatic enzymes and lipid parameters (total cholesterol, triglycerides, and high-density lipoprotein cholesterol) in plasma and serum, respectively, were measured by routine enzymatic methods with commercial kits (Doles, Brazil). The low-density lipoprotein cholesterol (LDL-C) concentration was calculated according to the Friedewald formula. Insulin and C-reactive protein (CRP) concentrations were determined with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen Corporation). Plasma concentrations of ADMA were measured with commercially available enzyme-linked immunosorbent assay kits (DLD Diagnostika GmbH, Hamburg, Germany) and endoglin levels were measured with commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions.

Measurement of plasma nitrite level and lipid peroxidation

Venous blood samples were collected into standard Vacutainer tubes (Becton-Dickinson) containing heparin and immediately centrifuged at 1000*g* for 3 min. Plasma aliquots were then immediately removed and stored at −70 °C until analyzed in triplicate for their nitrite content using an ozone-based chemiluminescence assay, as previously described [26]. Briefly, 200 μL of plasma samples were injected into a solution of acidified triiodide, purging with nitrogen in-line with a gas-phase chemiluminescence NO analyzer (Sievers Model 280 NO Analyzer). Approximately 8 mL of triiodide solution (2.0 g of potassium iodide and 1.3 g of iodine dissolved in 40 mL of water with 140 mL of acetic acid) were placed in the purge vessel into which plasma samples were injected. The triiodide solution reduces nitrite to NO gas, which is detected by the NO analyzer. The lipid peroxidation was assessed by measurement of thiobarbituric acid-reactive substances (TBARS), using the method described by Buege and Aust (1978) [3].

Genotype determination for the $T^{-786}C$ polymorphism in the 5′flanking region of eNOS

Venous blood samples were collected and genomic DNA was extracted from the cellular component of 1 ml of whole blood by a salting-out method and stored at −20 °C until analyzed. Genotyping was carried out by high-resolution melting assay with HRM primers, Type-It HRM PCR kit (Qiagen, Alameda, CA, USA; cat 206542) and Eco Real-Time PCR System (Illumina, CA, USA). The following primers were used: 5′AAGTGCCTGGAGAGTGCTG GTGTA 3′(sense) and 5′ACCCTGTCATTCAGTGACGCACGCTT 3′ (antisense). To validate the HRM genotyping, we performed in a set of six samples a polymerase chain reaction (PCR) amplification using the primers 5′-GGAGAGTGCTGGTGTACCCCA-3′ (sense) and 5′-GCCTCCACCCCCACCCTGTC-3′ (antisense) and PCR conditions as previously described [24,25]. The amplified products were digested with MspI for at least 4 h, at 37 °C, producing fragments of 140 and 40 bp for the wild-type allele ("T" allele) or 90, 50, and 40 bp in the case of a polymorphic variant ("C" allele). Fragments were separated by electrophoresis in 12% polyacrylamide gels and visualized by silver staining. All the genotypes are concordant in both methods.

Statistical analysis

The clinical characteristics were compared by the Student paired *t* test. Pearson's correlations (r, P) were calculated for associations between plasma nitrite, LDL, PCR and TBARS-MDA. The effects of eNOS polymorphism on nitrite levels were evaluated comparing the plasma nitrite levels before and after treatment in TT genotype or TC + CC genotypes using Student paired t test. A probability value <0.05 was considered the minimum level of statistical significance.

Results

As expected, the treatment with simvastatin for 6 weeks reduced the total and LDL cholesterol significantly ($P < 0.05$, Table 1). Importantly, SBP was also decreased with treatment ($P < 0.05$, Table 1). In addition, simvastatin treatment increased significantly the plasma nitrite levels in these obese women (75 ± 28 nM versus 106 ± 31 nM, 42%; $P = 0.0008$, Fig. 1A), and reduced significantly TBARS levels (about 58%; 16 ± 9.8 nM versus 9.5 ± 4.1 nM, $P = 0.0069$, Fig. 1B). Regarding other biomarkers analyzed (hsCRP, ADMA and endoglin), only hsCRP decreased significantly with treatment ($P < 0.05$).

To verify a possible correlation between NO and oxidative stress, we performed a correlation between nitrite and TBARS before and after treatment (Fig. 1C and D). Importantly, we observed a significant negative correlation before and after treatment ($r = -0.41$, $P = 0.03$ and -0.45, $P = 0.03$, respectively). Next, we performed correlation among nitrite and those biomarkers that have changed with treatment (hsCRP and LDL) towards identify possible mechanism related to NO restoration. We correlated the LDL and nitrite levels before and after treatment and lack of correlations were found. Moreover, to evaluate the impact of inflammatory status on nitrite levels, we performed a correlation among C-reactive protein and nitrite levels and none correlation was found.

Finally, we evaluated whether the T-786C polymorphism of eNOS may modulate the simvastatin effect on nitrite levels

Table 1
Clinical and biochemical parameters of subjects.

Parameters	Treatment	
	Before	After
BMI (kg/m^2)	34.4 ± 1.0	34.6 ± 1.0
WC (cm)	104.6 ± 8.2	102.8 ± 7.0
WHR	0.88 ± 0.07	0.87 ± 0.08
TC (mg/dL)	178.4 ± 36.6	146.5 ± 47.7*
HDL C (mg/dL)	44.9 ± 13.2	47.8 ± 14.2
LDL C (mg/dL)	115.2 ± 35.2	83.63 ± 44.3*
SBP (mmHg)	120.4 ± 9.8	115.6 ± 11.9*
DBP (mmHg)	69.2 ± 8.1	66.4 ± 6.4
Triglycerides (mg/dL)	91.3 ± 29.4	75.2 ± 28.7*
Fasting glucose (mg/dL)	86.8 ± 8.9	89.0 ± 9.0
Insulin (μLU/ml)	14.4 ± 6.1	15.5 ± 7.1
hsCRP (mg/dL)	0.16 ± 0.03	0.10 ± 0.06
ADMA (μM)	1.46 ± 0.16	1.49 ± 0.15
Endoglin (ng/mL)	19.3 ± 3.3	19.1 ± 2.1
BMI (kg/m^2)	34.4 ± 1.0	34.6 ± 1.0

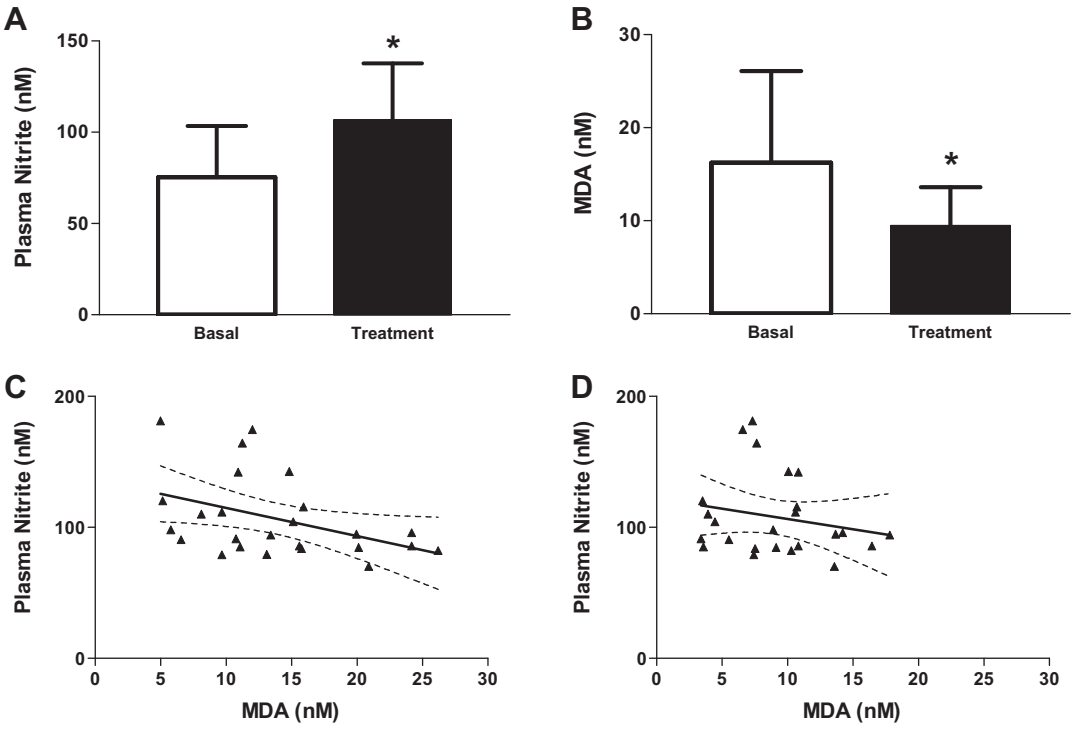

Fig. 1. Plasma nitrite (A) and plasma MDA (B) levels in obese women treated with simvastatin before (basal) and after 45 days. Correlation between plasma nitrite and plasma MDA in simvastatin-treated group before (basal, C) and after treatment (D). The bars indicate the means ± SDM. *P < 0.05 vs. basal.

(Fig. 2). We observed increases in the levels in both groups of genotypes (TT, $n = 10$ and TC/CC, $n = 15$), however, the raise in C-allele carrier was 60% while in TT, 44%. Interestingly, this increase was 2.16 folds when only the CC genotype was considered.

Discussion

The main novel findings reported here are: (1) simvastatin treatment increases 42% the nitrite levels in obese women without comorbidities; (2) simvastatin treatment decreases 58% the TBARS-MDA levels in obese women without comorbidities, and these levels are negatively correlated with nitrite concentrations; (3) the T-786C polymorphism of eNOS modulates the nitrite levels in response to simvastatin in these women.

NO is a major player in the regulation of the cardiovascular system, and reduced NO bioavailability has been linked to cardiovascular disorders [34]. In obesity, an impairment of NO endothelium-dependent relaxation is observed and it seems that the association of decreased NO production and increased NO scavenging might compromise NO bioavailability. In fact, a reduction of eNOS phosphorylation (ser 1177) via phosphatidylinositol 3-kinase/Akt results in decreased of eNOS activation. Moreover, in obesity a systemic inflammatory state is found and it has been demonstrated that some molecules, such as TNF-α, may impair the endothelial function through inhibition of eNOS gene expression in endothelial cells [13].

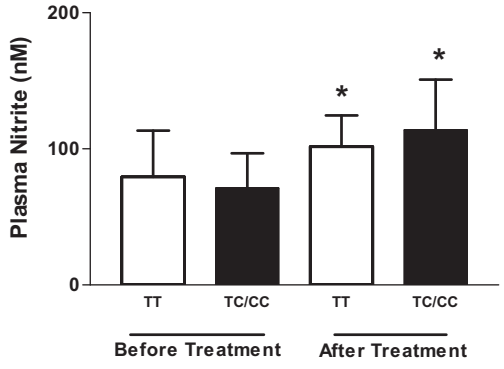

Fig. 2. Plasma nitrite concentrations in TT and TC + CC genotype groups before and after treatment with simvastatin. The bars indicate the means ± SDM. *$p < 0.05$ vs. TT.

In addition, uncoupled eNOS has been shown to generate superoxide anion which rapidly reacts with NO producing peroxynitrite, a much more powerful oxidant. Indeed, many reports support the idea that oxidative stress is involved in the pathophysiology of cardiovascular diseases by reducing NO bioavailability and consequently the levels of NO [12,18,35].

The correlation with NO bioavailability (nitrite) has never been evaluated in obese women, and our results indicate an important restoration of NO availability with simvastatin treatment. We expected to find correlation among nitrite and LDL or hsCPR. However, although we have biological mechanisms to support these associations, as mentioned in introduction, these hypotheses have failed suggesting that in vivo and in the current experimental conditions (normolipidemic, normotensive, normoglycemic obese women, limited number of subjects, method to evaluate the biomarkers, among other) the effect of simvastatin on nitrite levels is not related to these mechanisms. On the other hand, the antioxidant contribution to increase of nitrite levels is observed through negative correlation between nitrite and TBARS-MDA, suggesting that the increase of NO levels after simvastatin treatment may be related partially with decrease of oxidant environment. Our results confirm the well-known antioxidant effects of statins which involve an inhibition of ROS-generating enzymes, such as NAD(P)H oxidase [29].

To our knowledge, this is the first study to quantify nitrite in adult obesity, and evaluated its response to statin. By the fact that the NO is rapidly oxidized, a reliable quantification of its basal production has been relatively challenging. One alternative to study

its role on the cardiovascular system is by measuring its oxidation products, nitrate and nitrite, as index of endogenous NO formation [6]. The nitrate measurement may suffer the interference of NO synthase independent factors, such as diet, fasting, clinical condition and smoking. On the other hand, it was demonstrated that approximately 70% of plasma nitrite levels reflect NO synthase activity in the endothelium and that the inhibition of NO synthase activity was associated with corresponding decreases in circulation nitrite concentrations [14], indicating the that nitrite might better reflect NO bioavailability. The values found here indicate lower bioavailability of NO in obese women compared with concentrations found in healthy subjects (average of 200 nM [20]), supporting the correlation among obesity and cardiovascular diseases.

In accordance with our findings, different groups have found an increase on circulating nitric oxide biomarker levels (nitrite and nitrate) after administration of statins to hypertensive patients [16] and hypercholesterolemic patients [22]. Additionally, timing and potency of statin treatment during myocardial infarction was shown to attenuate oxidative stress, inflammatory activity, and endothelial dysfunction [15]. However, these studies measured NOx or Nitrite using the conventional Griess method, finding nitrite levels approximately 100 fold higher when compared to the chemiluminescence method used in our study. Additionally, the limit of detection for nitrite analyzed in plasma and blood by chemiluminescence is approximately 1 nM while the limit of detection for biological samples in conventional ultraviolet spectrophotometry ranges from 1 to 5 mM, depending on the method used [27]. This difference between methods (sensibility) allows that small differences, such as polymorphism effect, may be revealed. For this reason, the conventional Griess method does not seem adequate for nitrite analyses, unless combined to Flow Injection Analysis (FIA-Griess) which has a lower nitrite limit of detection (5 nM), but it is still less sensitive than chemiluminescence (1 nM) [8,11].

The biological explanation of how the C-allele carrier respond differently to simvastatin compared with subjects with the TT genotype remains to be elucidated. However, a study showed that fluvastatin inhibited the expression of replication protein A1 (RPA1) more strongly in the CC genotype compared with the TT genotype [1]. This protein acts as a repressor of the transcriptional activity of the eNOS gene when the C allele is present, therefore it is possible that some statins may produce stronger increases in eNOS gene transcriptional activity by inhibiting the expression of RPA1 more strongly in the CC genotype compared with the TT genotype. Moreover, other studies performed with healthy subjects treated with atorvastatin demonstrated the T-786C polymorphism modulation on whole blood nitrite and inflammatory markers [20,28].

In conclusion, our results demonstrated that treatment with simvastatin in obese women without comorbidities associated increase the plasma nitrite and reduce plasma TBARS-MDA concentration, presenting a negative correlation between these levels. Moreover, we show that obese women carrying the C-786 variant of eNOS present higher increases in nitrite levels compared to with women presenting TT genotype, suggesting that those women could benefit more with the simvastatin therapy as regards endothelial function.

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