Long-Term *N*-Acetylcysteine and L-Arginine Administration Reduces Endothelial Activation and Systolic Blood Pressure in Hypertensive Patients With Type 2 Diabetes

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OBJECTIVE — Reactive oxygen and nitric oxide (NO) have recently been considered to be involved in the cardiovascular complications of patients with type 2 diabetes, as NO is thought to lose its beneficial physiological effects in the presence of oxygen radicals. For this reason, we tested the effects of L-arginine (ARG) and *N*-acetylcysteine (NAC) administration in increasing NO bioavailability by reducing free radical formation.

RESEARCH DESIGN AND METHODS — A double-blind study was performed on 24 male patients with type 2 diabetes and hypertension divided into two groups of 12 patients that randomly received either an oral supplementation of placebo or NAC + ARG for 6 months.

RESULTS — The NAC + ARG treatment caused a reduction of both systolic ($P < 0.05$) and diastolic ($P < 0.05$) mean arterial blood pressure, total cholesterol ($P < 0.01$), LDL cholesterol ($P < 0.005$), oxidized LDL ($P < 0.05$), high-sensitive C-reactive protein ($P < 0.05$), intracellular adhesion molecule ($P < 0.05$), vascular cell adhesion molecule ($P < 0.01$), nitrotyrosine ($P < 0.01$), fibrinogen ($P < 0.01$), and plasminogen activator inhibitor-1 ($P < 0.05$), and an improvement of the intima-media thickness during endothelial postischemic vasodilation ($P < 0.02$). HDL cholesterol increased ($P < 0.05$). No changes in other parameters studied were observed.

CONCLUSIONS — NAC + ARG administration seems to be a potential well-tolerated antiatherogenic therapy because it improves endothelial function in hypertensive patients with type 2 diabetes by improving NO bioavailability via reduction of oxidative stress and increase of NO production. Our study's results give prominence to its potential use in primary and secondary cardiovascular prevention in these patients.

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Abbreviations: ABP, arterial blood pressure; ABPM, ambulatory blood pressure monitoring; ADMA, asymmetrical dimethyl-arginine; ARG, L-arginine; hs-CRP, high-sensitive C-reactive protein; sGC, soluble guanylyl cyclase; cGMP, intracellular guanosine 3′,5′-cyclic monophosphate; GSH, glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; ICAM, intercellular adhesion molecule; IL-6, interleukin-6; IMT, intima-media thickness; ox-LDL, oxidized LDL; NAC, *N*-acetylcysteine; cNOS, constitutive NO synthase; PAI-1, plasminogen activator inhibitor-1; ROS, reactive oxygen species; SDMA, symmetrical dimethyl-arginine; TNF-α, tumor necrosis factor-α; VCAM, vascular cell adhesion molecule.

Cardiovascular complications represent 80% of the causes of death in patients with type 2 diabetes. Several causes may explain this mortality excess. Among these, the decreased availability of nitric oxide (NO) has increasingly gained credit. In fact, reduced NO availability has been demonstrated not only in diabetes (1) but also in other diseases, such as atherosclerosis and hypertension, known to be associated with increased mortality due to cardiovascular causes (2,3). NO is crucial for regulating the vascular tone and maintaining the intrinsic thromboresistant and atheroprotective properties of the vascular wall (4).

NO production by constitutive NO synthase (cNOS) is mainly dependent on the availability of L-arginine (ARG) (5). Several studies have demonstrated that ARG infusion is able to improve endothelial function in normal subjects and patients with coronary heart disease and hypertension (6), but the results, although encouraging, are not conclusive, probably because of the short-term effects of ARG intravenous administration. Oral ARG has a longer half-life and longer-term effects than ARG given intra-arterially or intravenously so that in long-term health maintenance or symptoms management the oral route would be preferred. Unfortunately, studies in diabetic patients are not so widely positive, since oral ARG does not improve endothelial function (7).

It is well known that thiols are active intermediates in the NO pathway (8). Existing data document an increased free radical production in diabetes and hypertension with a consequent decrease in thiol levels. In this situation, NO reacts with the superoxide anion and to a lesser extent with thiols so that the beneficial effects of NO are lost. We might suppose that in patients with diabetes, diminished NO availability and the failure to ameliorate NO availability after oral ARG supplementation could be attributed to this

mechanism. Accordingly, we previously demonstrated that, in type 2 diabetic patients, a short-term intramuscular administration of glutathione (GSH) is able to increase NO availability (9).

Based on these observations, we carried out a study where ARG and *N*-acetylcysteine (NAC) were administered together in patients with type 2 diabetes and hypertension: ARG was administered to enhance NO production while NAC was administered to ameliorate antioxidant defense and increase intracellular nitrosothiol concentration, thus increasing NO availability.

RESEARCH DESIGN AND METHODS

— The study was approved by the ethics committee at our institution, and written informed consent was obtained from all recruited subjects. It was a randomized, double-blind, placebo-controlled small-scale study of 6 months. Male patients (n = 24, median age 64 years [95% CI 51–74]) with type 2 diabetes and hypertension treated with oral hypoglycemic and antihypertensive drugs were enrolled. All the subjects were recruited from the outpatient clinic of the Diabetic Centre of the Division of Endocrinology of the University of Turin, Italy.

Only male subjects were selected to avoid interferences on NO due to the action of estrogens. All the subjects were nonsmokers, had no secondary hypertension, cancer, or systemic, hepatic, pulmonary, cardiovascular, or renal diseases or psychiatric disorders, and were not being treated with nitrates, NAC, or ARG; furthermore, they were not hypersensitive to NAC and had no contraindications to β-blocker treatment. Subjects were all following a diet for diabetic patients without changing their food habits for the whole duration of the study.

The eligible patients underwent an antihypertensive treatment washout for 15 days. The subjects were enrolled only if, at the end of this period, they presented a systolic mean arterial blood pressure (ABP) between 135 and 180 mmHg and a diastolic mean ABP between 85 and 110 mmHg, measured with ambulatory blood pressure monitoring (ABPM) for 24 h. During the washout time, and for the whole duration of the study, all patients received atenolol (50 mg per os daily) for precautionary reasons.

Patients were randomly assigned to either the placebo group (n = 12, group A) or the NAC + ARG group (n = 12, group B). Subjects in the two groups were overlapping in terms of mean age (62.5 [95% CI 59.3–74.5] vs. 67.0 years [51.0–69.7]) and BMI (27.6 [23.6–38.9] vs. 28.5 kg/m^2 [25.1–38.4]). Group A patients received placebo (compounds identical in appearance to ARG and NAC) for 6 months, while group B patients received NAC (600 mg Acetilcisteina, one tablet twice a day) plus ARG (1,200 mg Zentrum, one vial a day) per os. Compliance was checked by pill and vial counts. No adverse effects were noted during the treatment. There was one dropout in each group, both not for medical reasons.

In all subjects, before and after 6 months of treatment/placebo, blood samples were obtained in order to evaluate A1C, total, HDL, and LDL cholesterol, oxidized LDL (ox-LDL), triglycerides, ratio of reduced GSH to oxidized GSH (GSSG) in erythrocytes, nitrites/nitrates, asymmetrical and symmetrical dimethylarginine (ADMA and SDMA, respectively), nitrotyrosine, arginine, homocysteine, high-sensitive C-reactive protein (hs-CRP), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), intercellular and vascular cell adhesion molecules (ICAM and VCAM, respectively), plasminogen activator inhibitor-1 (PAI-1), and fibrinogen. Moreover, before and after 6 months of treatment, the patients underwent an ABPM for 24 h and an ultrasound assessment of intima-media thickness (IMT) after endothelium-dependent flow-mediated vasodilation of the brachial artery.

The different variables were measured in the laboratories of the Endocrinology Division at the University of Torino, the Department of Biomedical Sciences at the University of Modena and Reggio Emilia, and the Department of Medical and Surgical Sciences at the University of Padova.

Serum levels of A1C were measured using a high-performance liquid chromatography (HPLC) autoanalyzer (Auto-Alc-HA-8121; Menarini, Florence, Italy). Total cholesterol and triglycerides in the plasma were measured with enzymatic methods using the Cobas Mira Plus autoanalyzer (ABX Diagnostic Italia, Rome, Italy). HDL cholesterol was measured after precipitation of lipoproteins containing apolipoprotein B (Lipid Research Clinics Program, 1982). LDL cholesterol was calculated according to the Friedewald equation. Plasma levels of ox-LDL were measured with a competitive ELISA (Alpco Diagnostics). Normal values were 37–1,200 units/l.

Plasma levels of arginine, ADMA, and SDMA were determined using an HPLC assay (Waters, Milford, MA). Normal subjects evaluated in a previous study showed the following mean values for ARG, ADMA, and SDMA: 105 ± 6.1, 1.41 ± 0.2, and 0.68 ± 0.07 μmol/l, respectively (10).

Nitrites/nitrates were measured simultaneously: nitrates were first converted to nitrites by means of enzymatic conversion, then total nitrites were measured in the sample using the Griess reagent. The concentration of nitrites in samples was calculated by comparison with a standard curve of sodium nitrite (Sigma-Aldrich, Milan, Italy). Normal value ± SEM was 20.1 ± 4.3 mmol/l.

GSH and GSSG in erythrocytes were determined by HPLC after a derivatisation of hemolysed samples using a fluorescence detector (Varian 9070). GSSG was calculated as the difference between total and reduced GSH.

Plasma levels of nitrotyrosine were assessed by ELISA (HyCult Biotechnology); the normal range was 0–120 μmol/l. Plasma levels of homocysteine were determined by EIA (Axis-Shield Diagnostics, Dundee, U.K.); the normal range was 3–37 μmol/l.

Serum levels of hs-CRP (ng/ml) were measured with a high-sensitive ELISA (Bender MedSystems); the normal range was 136–800 ng/ml. Serum levels of cytokines (IL-6 and TNF-α) and adhesion molecules (ICAM-1 and VCAM-1) were measured with ELISA (R&D Systems); the normal range was 0.43–8.87 pg/ml for IL-6, 0–4.22 pg/ml for TNF-α, 115–306 ng/ml for ICAM-1, and 341–897 ng/ml for VCAM. Fibrinogen was measured with ELISA (GENTAUR). Normal plasma concentration was 2.26–3.30 mg/ml. Human PAI-1 Ag (endothelial type) was measured with ELISA (GENTAUR). Normal poor-platelet plasma concentration was 5–40 ng/ml.

The ABPM was performed using an instrument of AND INTERMED s.r.l. (TM-2430). ABP was measured every 15 min between 7 A.M. and 11 P.M. and every 20 min between 11 P.M. and 7 P.M.; mean systolic and diastolic ABP values were calculated as the media of the 24-h registrations.

The ultrasound assessment of the endothelial-dependent flow-mediated vasodilation of the brachial artery was used to measure the IMT and performed using B-mode ultrasonography with a 7-MHz linear transducer (ATL-APOGEE 800 PLUS) as follows. The subject was positioned supine with the arm in a comfortable posi-

Table 1—*Baseline characteristics of the study groups*

	Group A	Group B	*P* (A vs. B)
n	12	12	
Age (years)	67.0 (51.0–69.7)	62.5 (59.3–74.5)	NS
Height (cm)	165.0 (160.5–175.5)	170.0 (161.3–179.5)	NS
Weight (kg)	77.0 (70.8–103.4)	84.0 (65.5–107.7)	NS
BMI (kg/m^2)	28.5 (25.1–38.4)	27.6 (23.6–38.9)	NS
Mean systolic ABP (mmHg)	140.5 (136.0–170.7)	148.5 (137.3–175.2)	NS
Mean diastolic ABP (mmHg)	87.0 (86.0–94.7)	87.5 (86.0–92.6)	NS

Data are median (95% CI).

tion for imaging the brachial artery. After acquiring a baseline rest image, a sphygmomanometric cuff was placed above the antecubital fossa to create a flow stimulus. Arterial occlusion was created by cuff inflation to at least 50 mmHg above systolic pressure for 5 min, causing ischemia. Subsequent cuff deflation induced a brief high-flow state (reactive hyperemia); the resulting increase in shear stress caused the brachial artery to dilate (endothelial postischemic vasodilation) and, subsequently, the intima to become thinner (11).

Data analysis

All data in text and figures are provided as medians and 95% CIs. Statistical significance was tested using Wilcoxon's signed-rank test or the Mann-Whitney rank-sum test when appropriate; the level selected for statistical significance was set at $P < 0.05$. The analysis was performed using Statistica '98 edition software (StatSoft, Tulsa, OK).

RESULTS — The baseline characteristics of the two groups are outlined in Table 1; no differences existed in the measured parameters. Basal values were superimposable in the two groups except for HDL cholesterol and fibrinogen (Table 2). After 6 months of NAC + ARG administration, in comparison with baseline, the following results were obtained (Table 2): *1*) reduction of systolic and diastolic medium ABP, total cholesterol, LDL cholesterol, ox-LDL, hs-CRP, ICAM, VCAM, nitrotyrosine, fibrinogen, PAI-1, and IMT; *2*) increase of HDL cholesterol and nitrites/nitrates; and *3*) no change in A1C, triglycerides, IL-6, TNF-α, GSH-to-GSSG ratio, arginine, ADMA, SDMA, and homocysteine levels.

After 6 months of placebo administration, PAI-1, total cholesterol, LDL cholesterol, and ox-LDL levels were significantly higher than those observed at the baseline; however, the ratio of ox-LDL to LDL cholesterol remained unchanged, as did the other evaluated parameters (Table 2).

CONCLUSIONS — NO has several effects realized through different pathways; the most important physiological one being its interaction with soluble guanylyl cyclase (sGC) leading to an accumulation of intracellular guanosine 3′,5′-cyclic monophosphate (cGMP) (12), which mediates its antiatherosclerotic effect. NO has a half-life of only 5–15 s as it rapidly reacts with other molecules depending on the redox state of the cell. In the presence of a reduced intracellular state, NO reacts with thiols, producing s-nitrosothiols, which are relatively long-lived reservoirs of NO that transport it on the heme group of the sGC, inducing cGMP synthesis (8). On the other hand, when large amounts of reactive oxygen species (ROS) are generated, burning the antioxidant defenses (i.e., oxidative stress), the s-thiols decrease, leading to a reduced concentration of nitrosothiols. This could represent the first step of the endothelial damage, since it causes a reduced production of cGMP. In the meantime, superoxide anions react with NO, producing a strong pro-oxidant represented by peroxynitrite. In brief, when cellular conditions are favorable (i.e., during the reductive state), NO produces its antiatherosclerotic effects; whereas when NO is synthesized under unfavorable conditions (i.e., during oxidative stress), it can be harmful. Recently, it has been demonstrated that the metabolism of every molecule of glucose yields a superoxide anion, so that chronic hyperglycemia leads to oxidative stress (13).

This study was designed to induce NO antiatherosclerotic effects in diabetic patients by increasing NO production, induced by ARG supplementation, in the presence of a reductive state obtained through supplementation of NAC, which acts as an antioxidant and GSH precursor (Fig. 1). As estrogens increase NO production, in the young female individuals NO changes during the cycle represent a confounding factor. Therefore, only male subjects were chosen for this study, and consequently the results can only be applied to the male sex. The study was carried out in a 6-month period, and as that time was not enough for adequate evaluation of hard end points, the study assesses only intermediate end points.

As in vivo NO measurement is very difficult, its production is usually esti-

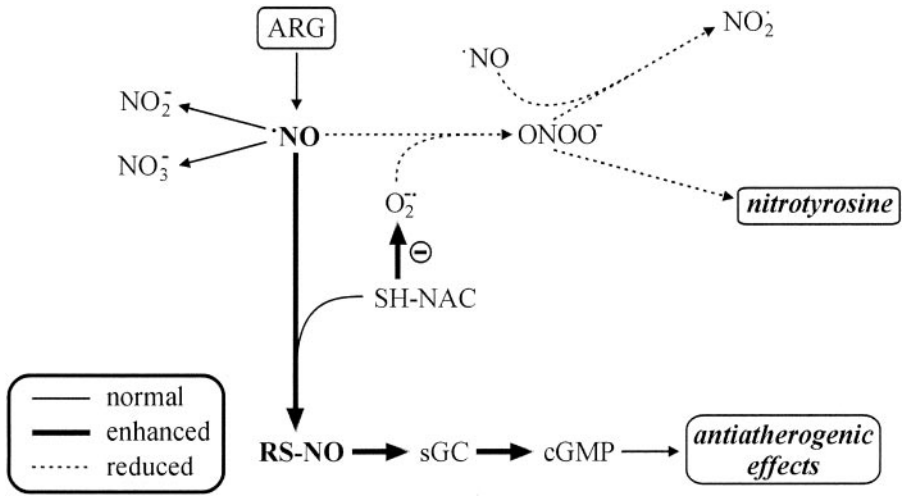

Figure 1—*NO pathways involved in NAC + ARG supplementation. Arginine improves NO generation, leading to nitrosothiol formation through the increased thiol (SH) groups availability, furnished by NAC supplementation; nitrosothiols transport NO to the sGC, inducing cGMP synthesis. NAC inhibits superoxide formation and, as a consequence, peroxynitrite, nitrogen dioxide, and nitrotyrosine formation. •NO, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate; $O_2^•$, superoxide; ONOO-, peroxynitrite; $NO_2^•$, nitrogen dioxide; RS-NO, nitrosothiol; SH-NAC, thiol group of NAC.*

mated by measuring nitrites and nitrates, which represent stable end products of its metabolism. In group B, the observed increase of plasma nitrites and nitrates and the improvement of IMT, a good index that directly correlates with NO bioavailability, suggest a gain in NO production, with the decrease of ABP representing the clinical effect of these changes.

Nitrotyrosine, synthesized by the interaction of tyrosine residues and peroxynitrite, is considered indirect evidence of oxidative stress (14). Accordingly, the diminished nitrotyrosine levels found demonstrate an improvement of the redox state of the cells (i.e., reduction of oxidative stress). Indeed, being a thiol, NAC both in its own form and by conversion in glutathione has a potent antioxidant effect that opposes the activity of ROS by neutralizing them. In a wider view, the reduction of oxidative stress led to increased NO bioavailability. Although an increased GSH-to-GSSG ratio after 6 months of NAC administration would be expected, no statistical change in glutathione was observed. Though in contrast with most data in the literature, this finding agrees with that of Patriarca et al. (15), who also showed no change in GSH content after NAC administration. The failure to find an increased GSH-to-GSSG ratio after thiol administration could be explained by the antioxidant properties of NAC that probably, in oxidized states of the cell, more easily act by directly neutralizing ROS than by participating in GSH production (16).

Besides the vasodilator effect, NO exerts additional antiatherosclerotic effects by inhibiting several signaling pathways including vascular smooth muscle cell migration and monocyte activation, chemotaxis, adhesion, and migration. Such effects are mediated via inhibition of nuclear factor-κB, which plays an important role in the regulation of redox-sensitive inflammation-related genes (17) responsible for the expression of several proteins, including adhesion molecules (ICAM-1 and VCAM-1) and PAI-1. On the other hand, nuclear factor-κB is activated by oxygen free radicals, promoting endothelial dysfunction. Therefore, the observed decrease of both adhesion molecules and PAI-1 might be the result of the same mechanisms, an increase in bioactive NO, and/or a reduction of ROS.

It has been demonstrated that high levels of CRP are a risk factor for cardiovascular morbidity and death. High levels of CRP have been found in diabetes (18)

Table 2—*Evaluated parameters*

	Group A		Group B		*P*		
	A1: Basal	A2: 6 months	B1: Basal	B2: 6 months	A2 vs. A1	B2 vs. B1	B1 vs. A1
Mean systolic ABP (mmHg)	140.0 (136.0–168.3)	141.0 (128.0–173.0)	149.0 (138.5–175.5)	144.0 (128.3–161.5)	NS	<0.05	NS
Mean diastolic ABP (mmHg)	87.0 (86.0–94.8)	89.0 (76.5–98.8)	88.0 (86.0–92.8)	83.0 (78.0–96.3)	NS	<0.05	NS
IMT (Δmm)	0.05 (−0.05 to 0.23)	−0.02 (−0.09 to 0.12)	0.06 (0.00–0.11)	−0.05 (−0.11 to 0.12)	NS	<0.02	NS
Nitrotyrosine (μmol/l)	1.7 (1.2–2.2)	1.6 (1.2–2.2)	1.2 (0.4–2.6)	1.0 (0.3–2.3)	NS	< 0.01	NS
Nitrites and nitrates (mmol/l)	27.3 (23.1–47.2)	28.3 (17.8–64.5)	24.8 (13.9–65.7)	38.2 (18.3–70.9)	NS	<0.05	NS
Arginine (μmol/l)	126.4 (82.0–143.0)	95.5 (84.7–146.4)	99.4 (56.6–192.5)	105.8 (47.5–157.2)	NS	NS	NS
ADMA (μmol/l)	1.8 (1.5–3.0)	1.8 (1.5–2.1)	1.8 (1.5–2.8)	1.7 (1.3–2.8)	NS	NS	NS
SDMA (μmol/l)	1.1 (0.2–1.6)	0.9 (0.8–1.1)	0.8 (0.5–1.9)	0.9 (0.1–1.6)	NS	NS	NS
PAI-1 (ng/ml)	46.4 (32.0–90.5)	47.4 (35.9–89.0)	38.2 (20.6–70.7)	30.5 (20.6–62.0)	<0.05	<0.05	NS
ICAM (ng/ml)	1.3 (0.8–1.7)	1.3 (0.8–1.6)	1.3 (0.7–1.9)	1.1 (0.5–1.2)	NS	<0.05	NS
VCAM (μg/ml)	1.6 (1.3–1.8)	1.6 (1.3–1.8)	1.3 (0.7–1.9)	1.0 (0.6–1.3)	NS	<0.01	NS
Triglycerides (mmol/l)	0.9 (0.6–2.1)	1.2 (0.7–2.2)	1.3 (0.8–2.7)	1.5 (0.6–3.8)	NS	NS	NS
Total cholesterol (mmol/l)	4.4 (3.9–5.7)	4.8 (3.8–6.5)	4.5 (3.5–5.8)	4.5 (3.1–4.9)	<0.05	<0.01	NS
HDL cholesterol (mmol/l)	1.1 (0.9–1.5)	1.2 (1.0–1.5)	0.9 (0.6–1.2)	1.0 (0.6–1.4)	NS	<0.02	<0.02
LDL cholesterol (mmol/l)	2.9 (2.3–4.0)	3.2 (2.2–4.6)	2.8 (2.1–4.4)	2.7 (1.5–3.3)	<0.05	<0.005	NS
ox-LDL (units/l)	49.0 (35.2–58.1)	55.6 (34.3–66.2)	50.0 (32.4–61.9)	47.7 (27.2–58.9)	<0.05	<0.05	NS
hs-CRP (ng/ml)	135.5 (102.1–152.8)	135.5 (67.2–172.2)	141.3 (69.0–224.5)	119.7 (36.1–168.4)	NS	<0.05	NS
TNF-α (pg/ml)	3.2 (1.7–8.9)	2.9 (1.1–8.3)	3.4 (2.1–6.9)	3.4 (2.0–5.1)	NS	NS	NS
IL-6 (pg/ml)	5.7 (3.0–8.6)	5.7 (3.0–10.5)	4.5 (3.2–6.4)	5.4 (3.8–7.2)	NS	NS	NS
Fibrinogen (μmol/l)	15.3 (5.1–17.0)	14.7 (6.1–17.6)	16.8 (14.5–19.0)	13.3 (5.8–16.4)	NS	<0.01	<0.05
GSH-to-GSSG ratio	5.6 (3.7–13.2)	6.4 (5.5–12.3)	8.7 (5.6–19.4)	10.0 (5.9–33.1)	NS	NS	NS
A1C (%)	7.2 (5.9–9.8)	7.5 (6.0–9.6)	6.8 (6.1–10.1)	7.4 (5.8–12.3)	NS	NS	NS
Homocysteine (μmol/l)	25.2 (22.9–35.4)	23.1 (12.3–37.8)	26.8 (20.6–33.5)	25.9 (21.2–34.3)	NS	NS	NS

Data are median (95% CI).

and essential hypertension (19) and, furthermore, have been shown to significantly correlate with both oxidative stress and endothelial activation (20). NAC + ARG treatment has been able to decrease CRP, likely due to both an antioxidant effect and an increased NO availability, which downregulates CRP expression in vivo (21), supporting the hypothesis that strategies meant to lower plasma CRP could include the increase of both NO production and availability.

A growing number of studies has focused attention on fibrinogen in diabetes, suggesting its role as a marker of cardiovascular risk in this disease. In fact, an association between diabetes and fibrinogen levels has been demonstrated (22), and fibrinogen seems to be a major independent predictor of cardiovascular mortality in type 2 diabetes. On the other hand, different evidence (23) indicates that a short-term inhibition of NOS increases fibrinogen levels. The findings of the present study are indirectly in accordance with that assertion.

No changes in IL-6 and TNF-α levels were found in our patients during the ARG + NAC treatment. This is probably due to the fact that the treatment was meant to increase NO production and lower the oxidative state but not to decrease an inflammatory state.

We also evaluated ADMA concentration, which has been considered by some authors as a novel cardiovascular risk factor (24). The structural homology between ARG and ADMA suggests that this compound might regulate NO synthesis through a competition for the active site of NOS. In patients supplemented with ARG + NAC, we did not observe any variation in ADMA levels or in its analog SDMA levels. More importantly, we found that the observed levels of this methylarginine in both groups of patients were lower than the concentration range of 3–15 μmol/l which has been demonstrated to be necessary to significantly inhibit NO formation (25). From these data, we cannot hypothesize an involvement of ADMA in the dysregulation of the NO pathway.

In conclusion, combined NAC and ARG administration seems to be a successful and well-tolerated antiatherogenic therapy, capable of improving endothelial function in hypertensive diabetic male patients because it reduces oxidative stress and, at the same time, promotes NO antiatherosclerotic effects. Our study's results, therefore, give prominence to its potential use in primary and secondary cardiovascular prevention in type 2 diabetic patients. Further clinical studies on a larger scale are needed to support our experimental data.

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