Oxidative-stress-mediated arterial dysfunction in patients with peripheral arterial disease

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KEYWORDS
Peripheral vascular disease; Flow-mediated dilation; Nitric oxide; Oxidative stress; Propionyl carnitine

Aims To investigate the existence of a relationship among flow-mediated dilation (FMD), nitric oxide (NO), and oxidative stress in patients with peripheral arterial disease (PAD), and to assess if the administration of an antioxidant was able to improve arterial dilatation.

Methods and results We performed a cross-sectional study comparing FMD, 8-Hydroxy-2-deoxy-2-deoxyguanosine (8-OHdG), a marker of oxidative stress, and nitrite/nitrate (NOx) serum levels in a population of 25 PAD patients and 40 controls. In the second part of the study, 21 PAD patients were randomly allocated to a treatment sequence of 7 days of i.v. infusion of placebo or 6 g/day propionyl-L-carnitine (PLC) in a cross-over design. Compared with controls, patients with PAD had enhanced 8-OHdG serum levels (2.4 ± 1.2 vs. 4.24 ± 3.11 ng/mL; P < 0.001), reduced NOx (17.02 ± 6.11 vs. 11.28 ± 6.02 μM; P < 0.001), and lowered FMD (10.34 ± 2.14 vs. 6.69 ± 2.95; P < 0.001).

PLC infusion was associated with an increase of FMD [from 6.6 ± 0.6 to 11.1 ± 1.2% (mean ± SE), P = 0.004] and NOx (from 14.5 ± 1.4 to 17.1 ± 1.2 μM; +18%, P = 0.012) and a decrease of 8-OHdG (from 3.62 ± 0.37 to 2.64 ± 0.32 ng/mL; −27%, P < 0.001). No changes were observed after placebo treatment.

Conclusion This study shows that in PAD patients, oxidative stress is implicated in determining reduced FMD.

Introduction

Peripheral arterial disease (PAD) is a clinical setting affecting more than 5% of population older than 60.¹ Despite the low rate of peripheral complications and amputation, PAD is complicated by high rate of coronary and cerebral events. For this reason, PAD is considered a marker for systemic atherosclerosis and its early diagnosis may be helpful for identifying patients at risk for cardiovascular events.²

Among patients with PAD, analysis of ankle/arm pressure ratio may help in identifying those who are at higher risk of cardiovascular events.²,³ More recently endothelial dysfunction has been recognized as another useful marker to stratify the risk of cardiovascular disease not only in patients with coronary heart disease (CHD) but also in PAD patients.⁴–⁶ In particular, Brevetti et al.⁶ demonstrated that PAD patients having low flow-mediated vasodilatation (FMD) were at high risk to develop cardiovascular complications including CHD and leg amputation.

Even if FMD is prevalently dependent upon release of nitric oxide (NO), a potent vasodilator and anti-aggregating molecule, from vascular wall,⁷ the interplay between FMD and NO in PAD has not been fully clarified. In subjects with cardiovascular risk factors, Heiss et al.⁸ demonstrated that plasma nitroso compounds, a marker of NO generation, inversely correlated with FMD. The mechanism accounting for reduced NO generation and, more in particular, the interplay between NO and oxidative stress was not investigated. Thus, oxidative stress plays a major role in modulating NO bioactivity inasmuch as it greatly contributes to its rapid metabolism via NO inactivation by O₂⁻;⁹ such interaction leads to formation of peroxinitrite, a potent oxidant molecule present in the atherosclerotic plaque.⁹ On the basis of the fact that oxidative stress is enhanced¹⁰ and FMD is reduced in PAD,¹¹ we speculated that oxidative stress could be responsible for reduced NO bioactivity and eventually arterial dysfunction. Therefore, the first aim of the study was to investigate the existence of a relationship among oxidative stress, NO, and FMD in patients with PAD. The second aim of the study was to assess if the administration of an antioxidant was able to improve arterial dilatation in PAD patients.

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Oxidative stress in patients with PAD

Methods
The study has been divided in two parts. In the first part, we performed a cross-sectional study comparing FMD, oxidative stress, as assessed by serum levels of 8-Hydroxy-2-deoxy-2-deoxyguanosine (8-OHdG),12 and NO generation, as assessed by serum levels of nitrite/nitrate (NOx), in a population of PAD and controls.

In the second part, we performed an interventional trial in PAD patients to assess if the infusion of an antioxidant, propionyl-L-carnitine (PLC), was able to improve endothelial dysfunction.

Cross-sectional study
The study has been carried out in 25 consecutive patients, aged between 40 and 80, presenting for symptoms of intermittent claudication (for at least 6 months) in the ambulatory of our Division between September 2004 and October 2005. Forty consecutive subjects presenting themselves for metabolic screening, without clinically relevant atherosclerotic disease, were enrolled in the same period as control. These patients had no clinical history of cardiovascular disease, had normal ECG, and echocardiography. Every PAD patient to be enrolled in the study had:

(1) claudication (defined as leg pain on walking, disappearing within 10 min of standing, of presumed atherosclerotic origin) and
(2) ankle/brachial index (ABI) that was assessed as ankle/arm systolic blood pressure ratio by Doppler ultrasonography <0.80 on the worst leg at rest.

All subjects underwent a full medical history, physical examination, 12-lead ECG, laboratory test, and measurement of ABI. Patients had to be in stable conditions without abrupt changes of ABI (>20%) in the last month before the enrolment. Subjects were excluded from the study if they had liver insufficiency, serious renal disorders (serum creatinine >2.8 mg/dL) and myocardial infarction, coronary revascularization, peripheral vascular surgery or percutaneous intervention procedure, unstable angina, acute cerebro-vascular disease or deep venous thrombosis or if they were current smokers. Informed written consent was obtained from all subjects: the study was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee.

To evaluate the presence of atherosclerotic disease in PAD patients and controls, all subjects enrolled in the study underwent ultrasonographic scans of the carotid arteries for intima–media thickness (IMT) evaluation; controls were excluded from the study if they had an IMT >0.9 mm.

The number of patients initially assessed for inclusion into the study was 35; after initial assessments four patients were excluded from the study for an ABI <0.8, one patient for serum creatinine >2.8 mg/dL, four patients for CHD, and one patient for current smoking; four subjects enrolled for cross-sectional study refused to participate in the interventional study.

Interventional study with PLC
Twenty-one of 25 PAD patients accepted to participate in the interventional study that was performed between January and March 2006. They were randomly allocated to a treatment sequence with i.v. infusion of placebo or PLC (6 g/day) in a double-blind crossover design. There was a 1 month-washout between the two phases of the study. Every infusion was carried out over a 12 h period for 7 days. All patients have been hospitalized during each period of the study. FMD, oxidative stress, and NOx were measured at baseline and 12 h after the last infusion of placebo or PLC.

Randomization and blinding
A person, not involved in the study, assigned codes to the study treatments, randomly allocated the selected participants to a treatment sequence with i.v. infusion of placebo or PLC (6 g/day), and kept the key in a sealed envelope. The randomization was carried out by a procedure based on a random numeric sequence. The authors were unaware of treatment allocation.

ABI measurement
ABI was calculated with the patient placed in the supine position, measuring the higher systolic pressure of the anterior or posterior tibial artery in each limb and dividing this pressure with the highest brachial systolic pressure.3 In patients with diabetes, we performed toe pressure determinations.

Measurement of carotid intima–media thickness
Longitudinal ultrasonographic scans of the carotid artery were obtained on the same day as the studies of the brachial artery reactivity and included the evaluation of the right and left common carotid arteries 1 cm proximal to the carotid bulb. In each examination, the same operator used different scanning angles to identify the greatest IMT, defined as the distance between the junction of the lumen and intima and that of the media and adventitia. Three measurements of IMT were obtained from the right and left carotid arteries, respectively, and were averaged to determine the mean IMT for both sides combined. The coefficient of variation was 2.6%.

Flow-mediated vasodilatation
Ultrasound assessment of endothelial dependent and independent FMD of brachial artery was investigated according to the recently reported guidelines.13 Briefly, the study was performed in a temperature-controlled room (22 °C) with the subjects in a resting, supine state between the hours of 8 a.m. and 10 a.m.; brachial artery diameter was imaged using a 7.5-MHz linear array transducer ultrasound system (Siemens) equipped with electronic callipers, vascular software for two-dimensional imaging, colour and spectral Doppler, and internal electrocardiogram; the brachial artery was imaged at a location 3–7 cm above the antecubital crease; to create a flow stimulus in the brachial artery, a sphygmomanometric cuff was placed on the forearm; the cuff was inflated at least 50 mmHg above systolic pressure to occlude artery inflow for 5 min; all vasodilation measurements were made at the end of diastole; FMD was expressed as a change in post-stimulus diameter evaluated as a percentage of the baseline diameter.

NOx/NO2 and 8-OHdG serum levels
Between 8 and 9 a.m. a blood sample was taken from each patient who had fasted at least 12 h. Samples were immediately centrifuged at 3000 rpm for 15 min at 4 °C, and the supernatant was collected and stored at –80 °C until measurement.

NOx serum levels were evaluated through the measurement of metabolic end products (Calbiochem nitritenitrate assay), i.e. nitrite and nitrate (NOx), using enzymatic catalysis coupled with Griess reaction as reported by Verdon et al. Intra- and inter-assay coefficients of variation were 2.2 and 4.0%, respectively.

Serum levels of 8-OHdG were analysed using a commercial competitive enzyme-linked immunosorbent assay for quantitative measurement of the oxidative DNA adduct 8-hydroxy-2-deoxyguanosine, (Bioxytech 8-OHdG ELISA, OXIS Health Products, Portland) following the manufacturer’s protocol. Samples were submitted to filtration to separate interfering substances (cut off molecular weight 10 000). Absorbance was read at 450 nm wavelength and the concentrations of 8-OHdG were determined by comparison with the standard curve. The lower detection limit of 8-OHdG in this assay was 0.15 ng/mL. Intra- and inter-assay coefficients of variation have been determined at multiple points on the standard curve and were 2.1 and 4.5%, respectively.
Statistical analysis

Sample size determination

As reported earlier, for the cross-sectional study, we recruited all the patients (n = 25) attending the ambulatory of our Division between September 2004 and October 2005, who respected the inclusion/exclusion criteria. The number of controls (n = 40) was computed with respect to a two-tailed Student’s t-test for independent groups, considering as (i) clinically relevant difference in FMD to be detected between patients and controls |δ| ≥ 2.5%, (ii) standard deviations homogeneous between groups SD = 2.95%, (iii) type I error probability α = 0.05 and power 1 − β = 0.90.

As regards the interventional cross-over study, we computed the minimum sample size with respect to a two-tailed one-sample Student’s t-test, considering as (i) clinically relevant difference for FMD variation to be detected between PLC and control treatments |δ| ≥ 2.5%, (ii) standard deviation of the paired differences SD = 3.0%, (iii) type I error probability α = 0.05 and power 1 − β = 0.90. This resulted in n = 18, which was increased to n = 21 in order to keep the degrees of freedom for the assessment of treatment effect in the statistical analysis at the required level.

Statistical methods

Categorical variables are reported as counts (percentage) and continuous variables as means ± SD unless otherwise indicated. Independence of categorical variables was tested by χ²-test. Comparisons between PAD patients and controls were carried out by Student’s t-test and were replicated as appropriate with nonparametric tests (Kolmogorov-Smirnov test in case of non-homogeneous variances as verified by Levene’s test).

The cross-over study data were analysed for the assessment of treatment and period effects, by performing a split-plot ANOVA with one between-subject factor (treatment sequence) and two within-subject factors (period 1 vs. 2; pre- vs. post-treatment).15 The full model was considered, allowing for the assessment of all main effects and 5-way interactions. Pairwise comparisons were corrected by the Bonferroni test; results were expressed as means ± SE.

Bivariate analysis was performed with Pearson’s linear regression test.

A value of P < 0.05 was considered statistically significant. All analyses were carried out with SPSS-13.0 software (SPSS Inc.).

Results

Cross-sectional study

Clinical characteristics of PAD patients and controls are reported in Table 1. As expected, PAD patients had higher prevalence of risk factors for cardiovascular disease, including hypertension and diabetes, compared with controls. Dyslipidaemia was not different between patients and controls likely because many PAD patients were treated with statins.

Compared with controls, patients with PAD had a lower FMD (10.34 ± 2.14 vs. 6.69 ± 2.95%; P < 0.001); also, PAD patients had enhanced oxidative stress, as documented by elevated serum levels of 8-OHdG (4.24 ± 3.11 vs. 2.4 ± 1.2 ng/mL; P < 0.001), and reduced NOx serum levels (11.28 ± 6.02 vs. 17.02 ± 6.11 μM; P < 0.001).

Simple linear regression analysis showed that NOx was correlated with FMD (r = 0.45; P = 0.02) and 8-OHdG (r = −0.672; P < 0.001).

Interventional study

After placebo infusion, no change of brachial basal diameter was observed (from 4.58 ± 0.11 to 4.59 ± 0.11 mm; P = n.s.); conversely after PLC infusion, a significant increase of brachial basal diameter was observed (from 4.6 ± 0.10 to 4.89 ± 0.11 mm; P < 0.001).

Because drugs that produce increases in baseline diameter lead to smaller estimates of FMD,16 we calculated FMD as an absolute change in diameter (expressed as percentage variation) in order to minimize this problem.

From the ANOVA performed on cross-over study data, we found neither significant effect of period nor significant interactions between period and treatment and/or pre–post infusion (0.47 < P < 0.96 for all effects). On the contrary, we observed significant effects of treatment [F(1,19) = 7.69, P = 0.012], pre-post infusion [F(1,19) = 14.14, P = 0.0013], and two-way interaction treatment × pre-post infusion [F(1,19) = 9.98, P = 0.005]. The latter indicates that the variation pre–post infusion was different between the two treatments. In particular, the pairwise comparisons showed that FMD significantly increased in the PLC group (before vs. after infusion: 6.6 ± 0.6 vs. 11.1 ± 1.2%; P = 0.004), while no significant change was observed in the placebo group (before vs. after infusion: 6.8 ± 0.6 vs. 7.4 ± 0.7%; P = 0.645) (Figure 1).

Finally, we analysed NOx and 8-OHdG serum levels in 10 out of the 21 patients enrolled for the interventional study. Clinical characteristics of this subgroup were similar to that of the entire population participating in the interventional study (not shown). After placebo infusion, no changes of NOx (from 14.8 ± 1.1 to 13.9 ± 1.2 μM; P = 0.566) and 8-OHdG (from 3.52 ± 0.33 to 3.45 ± 0.35 ng/mL; P = 0.347) serum levels were observed. Conversely, in patients after PLC infusion an increase of NOx (from 14.5 ± 1.4 to 17.1 ± 1.2 μM; P = 0.012) and a

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risk of subsequent vascular accidents. The present study
of oxidative stress in PAD patients. Previous studies demonstrated a significant increase
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different markers of oxidative stress, such as serum iso-
detected in the atherosclerotic plaque. Also, oxidative
depends upon endothelial release of NO, and several
patients has not been fully clarified. FMD is prevalently
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The mechanism accounting for reduced FMD in PAD
patients has not been fully clarified. FMD is prevalently
dependent upon endothelial release of NO, and several
studies demonstrated a significant direct correlation
between reduced FMD and circulating levels of nitroso
compounds that reflect systemic NO bioactivity. However, the
mechanism accounting for reduced NO bioactivity was
neither clarified nor was it investigated to determine if oxida-
tive stress could play some role in reducing NO bioactivity.
Therefore, we first investigated the behaviour of FMD in PAD
population, then we tried to analyse the role of NO bioactiv-
ty and its interplay with oxidative stress.

In accordance with previous reports, our PAD population
had reduced FMD, which was significantly associated with
PAD, independent from the coexistence of risk factors,
which may potentially affect FMD. To measure NO gener-
ation, we analysed the plasma concentration of nitrate
and nitrite, which are formed in vivo upon NO oxidation. Nitrite, in particular, is one of the major oxidative metab-
olites of NO and greatly contributes to the circulating pool
of bioactive NO. Patients with PAD had significantly lower
NOx serum levels compared with controls suggesting, in
accordance with previous report, that NO generation is
reduced in this clinical setting. The significant association
between NOx and FMD in PAD population is novel and
extends previous data indicating that circulating levels of
NO-derived compounds are predictors of FMD.

Thus, NO is rapidly inactivated by superoxide anion to give
formation of peroxynitrite, an important oxidant species
detected in the atherosclerotic plaque. Also, oxidative stress
may affect NO generation by inhibiting eNOS. Using
different markers of oxidative stress, such as serum iso-
prostanes, and the titre of antibodies against oxidized
LDL, previous studies demonstrated a significant increase
of oxidative stress in PAD patients. The present study
reinforces these data as serum levels of 8-OHdG, a marker
of oxidative stress, were significantly higher in patients
with PAD compared with controls and suggests that it may be
implicated in reducing NO bioactivity. The cross-sectional
study seemed to support this hypothesis as serum levels of
8-OHdG and NOx were inversely correlated, thereby
suggesting that oxidative stress could be implicated in
eliciting arterial dysfunction via reducing NO generation.
To further explore this hypothesis, we performed an
interventional study with LPC, a molecule that possesses
antioxidant property via inhibition of arachidonic acid-
induced NADPH oxidase activation. Owing to the low bioa-
vailability of oral LPC, we preferred the intravenous
route to investigate if LPC had antioxidant property and
was able to influence FMD. The interventional trial showed
that LPC infusion was associated with enhanced arterial
vasodilatation as documented by the significant increase of
arterial diameter compared with placebo. Such vasodilating
effect was associated with a reduction of 8-OHdG and an
increase of NO generation, so reinforcing the hypothesis
that oxidative stress was implicated in arterial dysfunction
of PAD patients. These data are consistent with an
experimental study performed in spontaneously hyper-
tensive rats in which PLC infusion was associated with
amelioration of arterial dysfunction and increased serum
levels of NOx.

The intrinsic mechanism through which PLC inhibited
oxidative stress in vivo can only be a matter of speculation.
We have previously shown that PLC inhibits in vitro platelet
NADPH oxidase activation. This enzyme is also present in
the vascular wall where it has a key role in arterial dysfunc-
tion via the production of superoxide anion. Further study
is, therefore, necessary to investigate if the vasodilating
effect of carnitine is dependent on inhibition of vascular
NADPH oxidase activation.

Our study has potential limitations that should be
acknowledged. Patients with PAD have several risk factors
that may contribute to lower FMD independently from
PAD. Even if small sample size limits definite conclusions,
it is of interest that PAD is significantly associated with low
FMD. Measurement of NOx as markers of NO generation
have been studied in atherosclerotic patients and provided
divergent results. This may depend on the fact that
NOx is influenced by many exogenous and endogenous
factors including dietary nitrate uptake, inhalation of
atmospheric gaseous nitrogen oxides, salivary formation,
and renal function. Even if we cannot exclude that these
factors may have influenced NOx, our data are in accordance
with previous ones showing low NO generation in PAD
patients and are consistent with a relationship between
circulating levels of nitrite and nitrate and FMD. The
mechanism through which oxidative stress inhibits NO gener-
ation and in turn FMD was not investigated. Even if it is
arguable that oxidative stress affects NO synthase and/or
NO degradation, further study is necessary to deeply
explore the underlying mechanism.

In conclusion, this study shows that in PAD patients there
is an imbalance between oxidative stress and NO, which may
be responsible for reduced FMD. This hypothesis is supported
by interventional study with an antioxidant that ameliorated
arterial dysfunction. Owing to the relevance of arterial
dysfunction in predicting poor outcome, our findings
warrant further study to investigate the potential usefulness
of antioxidants that prevent NADPH oxidase activation in
PAD population.
Acknowledgement

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Conflict of interest: none declared.

References


