Effect of systemic vitamin C on free fatty acid-induced lipid peroxidation

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SUMMARY

Objectives: Plasma malondialdehyde (MDA), a reactive product of lipid peroxidation, may be influenced by anti-oxidant therapy. The aim of the present study was to investigate if elevated MDA as induced by increased free fatty acids (FFA) correlates with endothelial function and is affected by high doses of vitamin C.

Methods: The study design was randomised, placebo-controlled, double blind, 2-way cross over. Plasma MDA concentrations and forearm blood flow (FBF) responses to intra-arterial acetylcholine (ACh) and glyceryl trinitrate were assessed during co-administration of vitamin C or placebo in the presence of increased plasma FFA by Intralipid®/heparin infusion in 10 healthy male subjects.

Results: The seven-fold rise in plasma FFA was associated with an increase in plasma MDA concentrations (r = 0.7, p < 0.001) and decreased FBF responses to ACh (r = -0.4, p < 0.01). Co-administration of vitamin C restored the impaired reactivity of FBF to ACh but had no effect on elevated MDA concentrations.

Conclusions: Anti-oxidant vitamin C improves lipid-induced impairment of endothelium-dependent vasodilation, but does not alter MDA formation or breakdown.

Key-words: Malondialdehyde C · Vitamin C · Free fatty acids · Endothelial function.

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RÉSUMÉ

Objectifs : Le malondialdehyde plasmatique (MDA), produit réactif de la peroxydation lipidique, peut être influencé par un traitement anti-oxydant. Le but de cette étude était d’évaluer si un taux élevé de MDA tel qu’induit par l’augmentation des acides gras libres (FFA) est corrélé à la fonction endothéliale et est affecté par de fortes doses de vitamine C.

Méthodes : Il s’agit d’une étude randomisée, contrôlée par placebo, en double insu, avec double crossover. Les concentrations plasmatiques de MDA et les réponses du flux sanguin à l’avant bras (FBF) à l’acétylcholine intra-artérielle (ACh) et au glyceryl trinitrate ont été évaluées au cours d’une co-administration de vitamine C ou de placebo en présence de taux élevés de FFA plasmatiques par perfusion d’Intralipid®/héparine chez 10 sujets sains masculins.

Résultats : L’augmentation d’un facteur 7 des FFA plasmatiques est associée à une augmentation des concentrations plasmatiques de MDA (r = 0.7, p < 0.001) et une diminution des réponses FBF à ACh (r = -0.4, p < 0.01). La co-administration de vitamine C restaure la réactivité de FBF à ACh mais n’a pas d’effet sur les concentrations élevées de MDA.

Conclusions : Le traitement anti-oxydant par vitamine C améliore les altérations de la vasodilatation endothélium-dépendante induites par les lipides, mais n’agit pas sur la formation ou la clairance des MDA.

Mots-clés : Malondialdehyde · Vitamine C · Acides gras libres · Fonction endothéliale.
Free radicals such as reactive oxygen species (ROS) are produced during metabolic processes. These radicals are neutralized by an anti-oxidant defence system comprising enzymes such as superoxide dismutase, glutathione peroxidase, and non-enzymatic anti-oxidants, including vitamins A, E and C [1]. Malondialdehyde (MDA) is a toxic by-product formed from lipid oxidation by free radicals. It has been reported that MDA concentrations are increased in conditions associated with insulin resistance, such as diabetes mellitus, obesity, dyslipidemia, hypertension and chronic heart failure [2, 3, 4, 5, 6]. Increased plasma free fatty acids (FFA) are a typical feature of insulin resistant states [7] and can even induce insulin resistance [8]. FFA have also been found to attenuate endothelium-dependent relaxation in vitro [9] and in vivo [10, 11]. Recently it has been demonstrated that lipid infusions stimulate lipid peroxidation, which is paralleled by impairment of endothelial function [4].

Formation of ROS has been linked to FFA exposure [4, 12]. FFA and hyperglycemia can induce ROS generation in vascular cells by activation of NAD(P)H oxidase in vitro [12]. Experiments in isolated blood vessels demonstrated that impaired endothelium-dependent relaxation in the presence of FFA was reversed by the ROS scavenger superoxide dismutase [9]. Animal experiments have shown that vitamin C prevents oxidative stress by enhancing total antioxidant capacity and reducing lipid peroxidation products [13, 14]. In humans high doses of vitamin C abrogate the detrimental effects of elevated FFA on endothelial function [15]. Formation of ROS is therefore thought to be an important mechanism by which FFA induces endothelial dysfunction.

It was therefore the aim of the study to investigate (a) if plasma MDA concentrations correlate with exogenously administered FFA, (b) if plasma MDA levels are associated with endothelial function as assessed by forearm plethysmography and (c) if lipid peroxidation can be blunted by vitamin C administration in healthy humans.

**Materials and methods**

**Study population**

The study protocol was approved by the Ethics Committee of the Vienna University School of Medicine. The investigation conforms with the principles outlined in the Declaration of Helsinki including current revisions and was conducted according to the Good Clinical Practice guidelines of the European Union. The nature of the study was explained and all subjects gave written consent to participate. Subjects did not take any prescribed medications, or “over-the-counter” drugs containing non-steroidal anti-inflammatory drugs from two weeks prior to screening until the study was complete. All subjects underwent a complete health examination (including physical examination, ECG and laboratory screening) prior to the first study day and were studied after overnight fasting between 8 and 9 AM in a quiet room with an ambient temperature of 22°C.

**Study protocol**

10 healthy male subjects were included in this double blind, randomised, cross over study. Subject characteristics are shown in Table I. Two plastic cannulas were inserted into antecubital veins for monitoring of plasma concentrations of outcome parameters and for administration of Intralipid® and heparin, respectively. An intravenous infusion of a lipid emulsion (Intralipid® 20%, 1.5 ml/min; Pharmacia & Upjohn, Vienna, Austria) plus heparin (bolus: 200 IU; constant infusion rate: 0.2 IU/kg/min; Baxter AG, Vienna, Austria) was administered to achieve systemic plasma FFA levels typical for severely insulin-resistant subjects [8, 9]. Heparin was added to enhance breakdown of triglycerides to FFA in plasma [8]. A very fine needle (27G-needle Sterican, B. Braun, Melsungen, Germany) was inserted into the brachial artery of the non-dominant arm.

After a 20-minute resting period baseline forearm blood flow measurements were recorded and the systemic infusion of the Intralipid®/heparin emulsion started. Vitamin C (Ascorbic acid, 24 mg/min, Mayerhofer GmbH, Linz, Austria) or Placebo (0.9% NaCl) was administered intra-arterially at an infusion rate of 1.5 ml/min [16] into the brachial artery 110 minutes later on alternate study days in random order. Forearm blood flow measurements were repeated during infusion of Intralipid®/heparin and after start of co-infusion with vitamin C or placebo. At baseline and at timed intervals throughout the study blood samples for determination of FFA, MDA, glucose, insulin, and vitamin C concentrations were drawn. Tetrahydrolipstatin was present in vials for FFA measurement to avoid in vitro lipolysis, which could have resulted in artificially high FFA concentrations [17]. After sampling blood was immediately centrifuged, frozen and stored until batch analysis.

**Table I**

Clinical and metabolic characteristics of study subjects.

| Age (years) | 25 (20-29) |
| Body mass index (kg/m²) | 23.6 (20.7-28.1) |
| Mean arterial pressure (mmHg) | 86 (72-84) |
| Total cholesterol (mg/dl) | 124 (96-160) |
| LDL cholesterol (mg/dl) | 77 (38-113) |
| HDL cholesterol (mg/dl) | 38 (26-47) |
| Triglycerides (mg/dl) | 117 (43-243) |
| Glucose (mg/dl) | 89 (74-93) |
| Insulin (U/ml) | 19 (3-22) |

n = 10, data are expressed as means (range).
Measurements

Blood pressure and pulse rate

Systolic, diastolic and mean blood pressures (SBP, DBP, MAP) were measured on the upper arm by an automated oscillometric device (HP-CMS patient monitor, Hewlett Packard, Palo Alto, CA, USA). Pulse rate was automatically recorded from a finger pulse-oxymetric device (HP-CMS patient monitor). This system has been previously evaluated [18].

Forearm blood flow measurements

Forearm blood flow was measured as described previously [19, 20]. Briefly, strain gauges, placed on the forearms, were connected to plethysmographs (EC-6, D.E. Hokanson Bellevue, WA, USA) and traces analysed using the NIVP3 software (Version 5.25, Hokanson). To assess endothelium-dependent vasodilation of the human forearm resistance vasculature increasing doses of acetylcholine (ACh; 25, 50 and 100 nmol/min; Clinalfa, Läufelfingen, Switzerland) were infused into the brachial artery. Endothelium-independent vasodilation was assessed by intra-arterial infusion of glyceryl trinitrate (GTN; 4, 8 and 16 mmol/min; G. Pohl Boscamp GmbH, Hohenlockstedt, Germany). Both vasodilators were infused for 3 minutes per dose level, with a 15 minutes washout period between drugs [19]. Bilateral plethysmography was used, expressing the responses to ACh and GTN as the ratio of blood flow in the intervention arm versus the control arm [19, 20]; baseline ratio was defined as 100%. Cuffs were placed around both upper arms and inflated to 45 mmHg by a rapid cuff inflator (AG 101, Hokanson) during the measurements to occlude venous outflow. Wrist cuffs were inflated to suprasystolic pressures during each measurement to exclude circulation of the hands. Flow measurements were recorded for 9 seconds at 30-second intervals during drug infusion.

Biochemical assays

Plasma concentrations of FFA were measured using enzymatic methods as described previously [21]. MDA was measured by the thiobarbituric acid (TBA) reaction, HPLC separation and fluorescence detection of the adduct, according to modifications of previous methods [22, 23, 24]. 10 ml each of 6 calibrators (0-10 nmol/l, 1,1,3,3-tetraethoxypropane in 40% ethanol), control samples or EDTA-plasma samples were incubated with 1 ml 0.2% TBA in 2 mol/l sodium acetate pH 3.5. After cooling and centrifugation at 15000 g for 5 minutes, 10 ml of the supernatant was injected by an auto-sampler through a guard cartridge (Phenomenex, USA) into a 150 mm 4.6 mm stainless-steel column packed with 5 mm Hypersil ODS2. The mobile phase consisted of a 3:2 mixture of 50 mmol/l KH2PO4 pH 6.8 with methanol. The flow rate was maintained at 1 ml/minute without circulation during the measurements. Fluorescence was detected at 553 nm emission and excitation at 515 nm. The interassay coefficient of variation was 7.8% at 1.7 nmol/l. Vitamin C was analysed by HPLC and UV detection with kits from Immundiagnostik (Germany). Heparinized plasma samples were treated immediately after arrival in the laboratory by addition of a precipitation agent and centrifugation. The stabilized supernatants were stored at -80°C until analysis. Interassay coefficients of variation were 13% and 8.6% at 23.5 and 76.9 nmol/l, respectively. All HPLC equipment was from Merck-Hitachi (Germany) and concentrations were calculated from peak areas by the “Chromleon” software ( Dionex, USA) [15]. Serum insulin was measured by radioimmunoassay with kits from Biochem Immunosystems (Germany), the interassay variations were 7% and 5% at 13.5 and 61.5 mU/ml, respectively. Glucose concentration was determined using the glucose oxidase method (Glucose analyzer II, Beckmann Instruments, Fullerton, CA) [21].

Statistical analysis

All statistical analyses were done using the Statistica® software package (Release 6.1, StatSoft Inc., Tulsa, OK, USA). Data are presented as means ± SE unless indicated otherwise. All data sets were tested for normal distribution and log transformed if appropriate. Differences within groups and between trial days at baseline were compared by Student’s t-test. Between group differences of outcome parameters on trial days were assessed by analysis of variance for repeated measurements (ANOVA). P < 0.05 was considered the level of significance. Correlation between outcome parameters was calculated by linear regression analysis.

Results

All interventions were well tolerated. Systemic hemodynamics and metabolic parameters did not differ between the trial days at baseline (data not shown). Intralipid®/heparin infusion had no effect on blood pressure or pulse rate on study days (p = ns).

Effect of FFA on lipid peroxidation and metabolic parameters

As expected administration of the Intralipid®/heparin emulsion increased plasma FFA concentrations by approximately 7 fold (p < 0.01) on both trial days. MDA plasma concentration increased by 116 % 16% (p < 0.001) and by 80 % 21% (p < 0.001) from baseline, respectively (p = ns. between trial days, Tab II). In an analysis of pooled data, plasma FFA correlated with MDA plasma concentrations (r = 0.7, p < 0.001, Fig 1). Plasma glucose, triglyceride or insulin concentrations were not affected by Intralipid®/heparin infusion (data not shown).
Effect of FFA on forearm blood flow (FBF)

FBF and vasodilation to ACh and GTN was comparable at baseline on study days. Administration of Intralipid®/heparin resulted in a small but significant increase in resting FBF without differences between trial days. Increased plasma FFA reduced the responses of FBF to ACh (p < 0.01, Fig 2) but not to GTN (data not shown). Reactivity to ACh was inversely related to plasma MDA (r = -0.4, p < 0.01, Fig 3) and to plasma FFA concentrations (r = -0.6, p < 0.01).

Effect of vitamin C on lipid peroxidation and forearm blood flow

Systemic vitamin C concentrations increased by 1902 ± 427% after local infusion of vitamin C (p < 0.001), whereas a small non-significant decrease in vitamin C levels was noted during placebo (Tab II). Vitamin C or placebo had no effect on elevated MDA plasma concentrations. In contrast, intra-arterial administration of vitamin C but not placebo completely reversed the impaired responsiveness of the forearm vasculature to ACh (Fig 2). Vitamin C or placebo had no effect on GTN-induced forearm vasodilation (data not shown).

Discussion

This study was designed to investigate the association between exogenously increased FFA concentrations, lipid peroxidation as assessed by quantification of MDA plasma levels, and endothelial function. We demonstrated that FFA increases plasma MDA concentrations, which is paralleled by impairment of endothelium-dependent vasodilation of resistance arteries. High doses of vitamin C completely reversed the effect of FFA on forearm blood flow, but did not affect elevated plasma MDA concentrations.

The increase in plasma FFA concentrations was accompanied by an increase of plasma MDA levels, which is in accordance with previous experiments in vivo [4] and vitro where polyunsaturated fatty acids but not other fatty acids served as MDA precursors [25]. MDA is formed during oxidation of...
polyunsaturated fatty acids containing three or more double bonds [26, 27] and react with primary amine groups of biological molecules. In vivo MDA acts as a free aldehyde or as an adduct to other components, forming mutagenic and carcinogenic substances, mediates pro-atherogenic processes, or even provoke cell death [28, 29, 30]. Plasma MDA concentration is generally used as an index of in vivo lipid peroxidation [26, 27, 31]. In conditions associated with high oxidative stress such as neurodegenerative diseases, diabetes or cancer, high plasma MDA concentrations have been reported [30, 32, 33]. It has been shown that urine excretion of MDA adducts is an unreliable indicator of peroxidative stress in vivo because it is influenced by numerous factors such as physical activity and environmental temperature, as well as by wide variations in the intake of peroxides in the diet [34].

Anti-oxidant treatment with a-tocopherol decreased MDA concentrations in patients with amyotrophic lateral sclerosis and chronic supplementation of vitamin C abolished elevated MDA levels in smoke-exposed rats after 3 months [35, 36] suggesting a slow decomposition of MDA. About 30% of MDA predominantly binds to proteins [34, 37] and is therefore prevented from fast renal elimination in rats. In our experiment unbound MDA plasma concentrations (f-MDA) were measured, which should reflect acute anti-oxidative burden. One could speculate that the anti-oxidative defence mechanisms were
overloaded by the supraphysiologic FFA concentrations exceeding the potential salutary effect of vitamin. Nevertheless chronic treatment with vitamin C alters the dynamic equilibrium between production and elimination and thereby decrease MDA plasma levels. [35, 36]. In our setting high systemic doses of vitamin C did not affect elevated MDA. In agreement with a previous report [38] we postulate that acute pharmacological anti-oxidant vitamin therapy is not capable to decrease the enhanced production of f-MDA.

Plasma FFA elevation substantially impaired endothelium-dependent vasodilation consistent with previous experiments [4, 9, 10, 11]. In contrast, GTN-mediated vasodilation was not changed by FFA. The detrimental influence of FFA on endothelium-dependent vasodilation in our acute experimental setting is most likely caused by increased formation of ROS leading to inactivation of vasoactive nitric oxide. This would explain that endothelial dysfunction is acutely restored by anti-oxidant vitamin C rather than direct or indirect inhibition of the NO synthesis [39, 40, 41] or alterations of cyclooxygenase activity [11] by FFA. This is also compatible with the close correlation between plasma MDA concentrations and endothelium-dependent vasodilation in our pooled analysis. However, the analysis of correlation between outcome parameters is limited by the fact that data pooling may overestimate the degree of correlation. Thus, these data have to be interpreted with caution.

Reduction of oxidative stress by vitamin C administration augmented endothelial function without reducing MDA plasma levels: Since infusion of vitamin C increased its plasma concentration to almost 20 times the physiologic level, it is obvious that effective plasma vitamin C concentrations were achieved. Previous studies have demonstrated that MDA concentrations did not significantly change during oral glucose loading alone or by vitamins C and D substitution [42]. Further, experimental hyperinsulinemia did not affect plasma MDA [4]. This confirms that MDA may be used as a marker of lipid oxidation of non-esterified fatty acids but does not reflect total oxidative stress and is not influenced by secondary changes of glucose or insulin following a mixed meal containing polyunsaturated fatty acids. In our cohort, low average total cholesterol and triglyceride plasma levels were observed. This may be due to the fact that samples were not immediately analysed but frozen and quantified later. Further, our study comprised well trained young males under fasting conditions. Effects of Intralipid®/heparin on MDA concentrations may therefore differ in subjects with higher plasma lipid concentrations.

In conclusion these experiments demonstrate that increased plasma FFA concentrations are paralleled by enhanced formation of MDA. Impaired endothelium-dependent vasodilation as induced by FFA is restored by anti-oxidant vitamin C, which does not change elevated plasma MDA concentrations.

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References


