

## Cigarette smoking cessation increases plasma levels of several antioxidant micronutrients and improves resistance towards oxidative challenge

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Cigarette smoking is associated epidemiologically with increased risk of cardiovascular diseases, but the pathophysiological mechanisms are still not fully understood. There is evidence that smoking is related to increased free radical production and antioxidant depletion, but the effects of smoking cessation on plasma concentrations of antioxidants and susceptibility to oxidative stress are largely unknown. Plasma levels of vitamins A, C, E, uric acid, total thiols, carotenoids (including lutein, zeaxanthin,  $\beta$ -cryptoxanthin, lycopene,  $\alpha$ - and  $\beta$ -carotene) and malondialdehyde (MDA, a biomarker of lipid peroxidation) were measured in fifteen healthy, normolipidaemic subjects (seven males, eight females, 35.2 (SD 2.3) years) before and 4 weeks after smoking cessation. To determine plasma resistance towards oxidative challenge, plasma was incubated for up to 5 h with the peroxy radical-generator 2,2'-azobis(2-amidinopropane) (AAPH); MDA and ascorbate levels were measured at various time points. The concentrations of all plasma antioxidants were lower before smoking cessation than afterwards; MDA levels were higher before than after termination of smoking. Upon AAPH exposure, the consumption of plasma ascorbate and the production of MDA occurred at a significantly faster rate before smoking cessation as compared with afterwards. Cigarette smoking cessation is followed by a marked increase in plasma antioxidant concentrations and substantially improves plasma resistance towards oxidative challenge. Given the importance of cigarette smoking as a risk factor for cardiovascular diseases and the pathophysiological role played by oxidative stress in these illnesses, quitting smoking represents an irreplaceable preventive strategy against tobacco-induced oxidative stress and vascular damage.

### Smoking: Antioxidants: Micronutrients: Oxidative stress

Cigarette smoking is associated epidemiologically with a high risk for various types of chronic illnesses including vascular disease (McNamara & FitzGerald, 2001). The underlying mechanisms of smoke-related severe damage to tissues and organs are still not completely understood; O- and N-derived free radicals are thought to play a major pathophysiological role (Pryor & Stone, 1993).

Significantly lower plasma carotenoid and vitamin C levels have been shown in Scottish male smokers as compared with non-smokers (Ross *et al.* 1995). Male smokers aged 18–26 years showed significantly lower serum  $\beta$ -carotene levels and significantly higher serum lipid hydroperoxide levels as compared with age-matched non-smokers (al Senaidy *et al.* 1997).

It is known that smoking cessation leads to a significant increase of plasma vitamin C (Lykkesfeldt *et al.* 1996) and of glutathione concentrations (Lane *et al.* 1996), but its effects on plasma concentrations of several antioxidants and susceptibility to oxidative stress are unknown.

The aims of the present study were, therefore, to evaluate the effects of smoking cessation on the plasma

concentrations of a broad spectrum of water-soluble and lipophilic compounds with antioxidant properties and of malondialdehyde (MDA), a marker of free radical-induced lipid peroxidation, as well as on the susceptibility of plasma to oxidative stress.

### Subjects and methods

The investigation conforms to the principles outlined in the Declaration of Helsinki. Fifteen healthy volunteers (seven males, eight females, 35.2 (SD 2.3) years) smoking  $\geq$  seven cigarettes/d for at least 6 years each provided 20 ml blood after an overnight fast. The blood was placed into two 10 ml tubes containing heparin at 08.00 hours. The subjects gave informed consent. They were allowed to smoke on the day of the blood drawing; they did not smoke on the next day or afterwards. After 04 weeks, two other blood samples were obtained from the same subjects at 08.00 hours after an overnight fast. Blood was immediately centrifuged and plasma (8 ml) separated for the assays. Subjects did not change dietary

**Abbreviations:** AAPH, 2,2'-azobis(2-amidinopropane); MDA, malondialdehyde.

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or exercise habits during the time preceding the second blood drawing, as assessed by a standardized interview (performed by an independent researcher blinded to the measurements), which included the administration of a food-frequency questionnaire (Winkler & Döring, 1998). Before smoking cessation, volunteers were asked to recall their average intake of food items in the following six frequency categories: almost daily; several times per week; about once weekly; several times per month; once monthly or less; never. On the day of the first blood withdrawal, subjects were counselled to follow the same dietary pattern for the next 4 weeks. The food-frequency questionnaire was re-administered weekly. There were no missing data for the complete questionnaires or for single items. Exclusion criteria were constituted by BMI <18.5 or >30 kg/m<sup>2</sup>, malnutrition, vitamin consumption, diabetes, hypertension, any organ and/or psychiatric illness and abnormalities of lipid profile as assessed by total cholesterol  $\geq$ 6.2 mmol/l, LDL-cholesterol  $\geq$ 4.1 mmol/l, and triacylglycerol  $\geq$ 3.4 mmol/l.

Of the 8 ml plasma separated from the blood of volunteers before and after smoking cessation, 1 ml was used for measurements of cotinine concentrations and of antioxidant and MDA concentrations. Plasma cotinine was determined by radioimmunoassay as previously described (Langone *et al.* 1973). Vitamin C and uric acid were detected by HPLC with electrochemical detection as previously described (Frei *et al.* 1989). Carotenoids including lutein, zeaxanthin,  $\beta$ -cryptoxanthin, total lycopene,  $\alpha$ -carotene and  $\beta$ -carotene were measured by HPLC with u.v. and visible detection according to Stahl *et al.* (1993). A second u.v. and visible detector was connected in series and set at 325 nm and at 292 nm for quantification of retinol (vitamin A), and  $\alpha$ -tocopherol and  $\gamma$ -tocopherol, respectively. Total plasma thiol groups were measured spectrophotometrically (Motchnik *et al.* 1994). MDA concentrations were measured by HPLC with fluorescence detection (excitation 513 nm, emission 550 nm) (Suttner *et al.* 1997).

Plasma (7 ml) was incubated for 5 min at 37°C, and 750 mM-2,2'-azobis(2-amidinopropane) (AAPH) (500  $\mu$ l) was added (final concentration, 50 mM). Immediately after the addition of AAPH (time 0), and every 30 min up to 5 h, samples were taken for the determination of vitamin C and MDA concentrations.

All data are presented as mean values and standard deviations. Statistical analysis was performed with the program SPSS 10.0 (SPSS Inc., Chicago, IL). Non-parametric Wilcoxon signed ranking test was used for comparisons between values before and after smoking cessation. A generalized linear model for repeated measures was used for comparisons between AAPH-induced vitamin C consumption and MDA production over time before and after smoking cessation. Significance was accepted if the null hypothesis was rejected at the  $P < 0.05$  level.

## Results and discussion

All subjects studied showed decreased concentrations of plasma cotinine after smoking cessation (1.6 (SD 0.7) v. 0.7 (SD 0.2) ng/ml;  $P < 0.04$ ). Before smoking cessation,

all subjects showed lower plasma antioxidant levels than afterwards. Differences reached statistical significance for vitamin C,  $\alpha$ -tocopherol,  $\gamma$ -tocopherol, vitamin A, lutein, zeaxanthin, lycopene and  $\beta$ -carotene (Table 1). There were no significant differences between values in male and female subjects before and after smoking cessation. In agreement with our previous results (Polidori *et al.* 2001b), as well as with the study by Lykkesfeldt *et al.* (1996), who showed that plasma vitamin C concentrations increased from 45.4 (SD 20.2)  $\mu$ M before smoking cessation to 56.0 (SD 24.2)  $\mu$ M after smoking cessation in 100 subjects, an increase in vitamin C plasma levels from 50.0 (SD 11.8) to 62.9 (SD 13.9)  $\mu$ M was observed in the smoking quitters included in the present study. Eiserich *et al.* (1995) reported that plasma exposure to gas-phase cigarette smoke caused depletion of carotenoids, vitamin C, uric acid and  $\alpha$ -tocopherol, and induction of lipid peroxidation *in vitro*. *In vivo* studies strongly support the relationship between smoking habit and depletion of a number of dietary components with various degrees of antioxidant capacity, including vitamin C,  $\alpha$ -tocopherol and carotenoids (Eiserich *et al.* 1995; Ross *et al.* 1995; al Senaidy *et al.* 1997). It is conceivable that, in a condition of cigarette smoke-related oxidative stress, compounds with stronger antioxidant capacity such as ascorbate, the most potent water-soluble antioxidant in the organism whose levels have been shown to be negatively associated to the severity of both ischaemic (Cherubini *et al.* 2000) and haemorrhagic (Polidori *et al.* 2001a) stroke, are consumed first.

In the present study, plasma levels of lycopene and lutein, among other carotenoids, also markedly increased after smoking cessation (Table 1). Both of these carotenoids possess an effective free radical-scavenging ability, and might have a synergistic antioxidant effect; their plasma levels have been shown to be inversely correlated

**Table 1.** Plasma concentrations ( $\mu$ M) of vitamin C, uric acid, total thiols,  $\alpha$ -tocopherol,  $\gamma$ -tocopherol, vitamin A, lutein, zeaxanthin,  $\beta$ -cryptoxanthin, lycopene,  $\alpha$ -carotene,  $\beta$ -carotene, and malondialdehyde before and after smoking cessation and relative  $P$  values† (Mean values and standard deviations)

Compound	Before smoking cessation		After smoking cessation		$P$ value
	Mean	SD	Mean	SD	
Vitamin C	50.0	11.8	62.9*	13.9	0.0007
Uric acid	303	26	306	50	0.394
Total thiols	336	62	339	65	0.108
$\alpha$ -Tocopherol	23.5	3.5	26.2*	3.2	0.0010
$\gamma$ -Tocopherol	2.0	0.4	2.2*	0.4	0.0007
Vitamin A	2.4	0.6	2.8*	0.6	0.0007
Lutein	0.34	0.1	0.45*	0.1	0.0010
Zeaxanthin	0.09	0.03	0.10*	0.02	0.018
$\beta$ -Cryptoxanthin	0.10	0.02	0.11	0.02	0.077
Lycopene	0.51	0.1	0.72*	0.1	0.0007
$\alpha$ -Carotene	0.02	0.01	0.03	0.01	0.388
$\beta$ -Carotene	0.18	0.06	0.21*	0.07	0.0010
Malondialdehyde	0.81	0.12	0.58*	0.15	0.0007

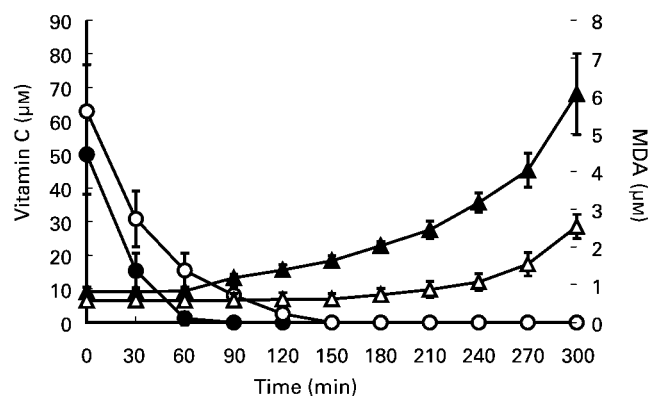
\* Mean value was significantly different from the before smoking cessation value ( $P < 0.05$ ; non-parametric Wilcoxon signed ranking test).

† For details of procedures, see p. 148.

with the severity of ischaemic stroke (Polidori *et al.* 2002a) and congestive heart failure (Polidori *et al.* 2002b). Simultaneously, a significant decrease in plasma MDA concentrations in smoking quitters was observed; interestingly, plasma levels of this biomarker of free radical-induced lipid peroxidation are directly correlated with the severity of ischaemic stroke (Polidori *et al.* 2002a) and congestive heart failure (Polidori *et al.* 2002b).

Exposure of plasma to AAPH, which produces peroxy radicals at a known constant rate, caused more rapid vitamin C consumption ( $P < 0.001$ ) and MDA production ( $P < 0.001$ ) in smokers as compared with quitters (Fig. 1). Most importantly, plasma vitamin C was completely consumed after 70 (SD 15) min before smoking cessation and 140 (SD 15) min after smoking cessation ( $P < 0.001$ ). One possible reason for the lower rate of ascorbate oxidation following smoking cessation might be the influence of biomolecules with antioxidant activity other than vitamin C (for instance, bilirubin) on the reaction of ascorbate with AAPH-derived peroxy radicals. MDA plasma levels started to increase after vitamin C was completely consumed both before and after smoking cessation. Our findings are highly consistent with previous studies (Frei, 1991) showing that only when ascorbate, but no other antioxidants, is completely consumed after plasma AAPH exposure does lipid peroxidation start (Frei, 1991). In later experiments, it was shown that plasma devoid of ascorbate, but no other antioxidants, and exposed to AAPH is extremely vulnerable to lipid peroxidation, and that this protection exerted by ascorbate against lipid peroxidative damage is highly effective even in the presence of bleomycin-detectable Fe (Berger *et al.* 1997).

The results shown in the present paper demonstrate that smoking cessation is accompanied by the elevation of several critical components of the antioxidant defence system of the organism. The finding that this elevation occurs in as little time as 4 weeks is in agreement with the recent observation that smoking a single cigarette rapidly lowers nitrate, nitrite and plasma levels of ascorbate, urate and cysteine (Tsuchiya *et al.* 2002).



**Fig. 1.** Time course of plasma vitamin C (●, ○) and malondialdehyde (MDA; ▲, △) concentrations after 2,2'-azobis(2-amidinopropane) addition. (●, ▲), Before smoking cessation; (○, △), after smoking cessation. Mean values are shown for plasma from seven male and eight female volunteers, with standard deviations being represented by vertical bars.

In our study, quitters also showed lower plasma lipid peroxidation than before stopping smoking, as well as a decrease in plasma susceptibility to vitamin C depletion and MDA formation after exposing plasma to a flux of aqueous peroxy radicals generated at a known constant rate. Lipid peroxidation and antioxidant depletion in smokers may be significant contributors to biomolecular endothelial vascular damage. Although antioxidant supplementation might be helpful in preventing or attenuating some cigarette smoke-related adverse effects (Reilly *et al.* 1996), quitting smoking remains a secure means by which smokers can prevent tobacco-induced oxidative stress and cardiovascular diseases.

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