

# Smoking a Single Cigarette Rapidly Reduces Combined Concentrations of Nitrate and Nitrite and Concentrations of Antioxidants in Plasma

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**Background**—Cigarette smoking is a well-known risk factor for the development of cardiovascular disease, yet the mechanism of action involved is not completely understood. Because cigarette smoke contains superoxide and other reactive oxygen species, it has been hypothesized that some of the adverse effects of smoking may result from oxidative damage to endothelial cells, which results in nitric oxide (NO) shortage. However, little information is available regarding the acute effects of smoking on plasma concentrations of NO and antioxidants. We measured the changes in the combined plasma concentrations of nitrate and nitrite as an index of NO concentration, as well as changes in concentrations of major serum antioxidants (ascorbic acid, cysteine, methionine, and uric acid) in smokers after smoking a single cigarette.

**Methods and Results**—A randomized crossover study of the effects of smoking a single cigarette was performed in 20 smokers. Smoking a sham cigarette induced no significant changes in all assayed parameters. However, smoking a single cigarette significantly decreased nitrate and nitrite plasma concentrations by  $3.5 \pm 1.2$  and  $3.4 \pm 1.1$   $\mu\text{mol/L}$ , compared with plasma concentrations at presmoking and sham smoking, respectively. The concentrations of ascorbic acid and other antioxidants were also significantly lower after smoking a single cigarette. These parameters returned to preexperimental levels 60 minutes after smoking cessation.

**Conclusion**—The present findings indicate that smoking a single cigarette temporarily decreases nitrate, nitrite, and serum antioxidant concentrations in the plasma. These transient changes may partially contribute to coronary vasoconstriction, which is routinely observed after smoking. (*Circulation*. 2002;105:1155-1157.)

**Key Words:** smoking ■ nitric oxide ■ free radicals ■ antioxidant ■ endothelium

A number of studies have shown that chronic smokers have an increased risk for cerebral and coronary artery diseases. However, the precise mechanism by which smoking contributes to the development and clinical manifestations of these diseases is unknown.<sup>1</sup> We previously demonstrated that cigarette smoke contains superoxide and a large number of other reactive oxygen species (ROS).<sup>2,3</sup> It has been hypothesized that the adverse effects of smoking may result from an accumulation of oxidative damage brought about by ROS in endothelial cells.<sup>4</sup> These cells constitutively synthesize nitric oxide (NO) from L-arginine by the endogenous enzyme, NO synthase, in order to regulate vascular tone, local blood flow, and tissue perfusion. Low plasma concentrations of NO,<sup>5</sup> which are a possible sign of endothelial dysfunction, along with low plasma concentrations of ascorbic acid,<sup>6</sup> have been reported in long-term habitual smokers. These conditions could accelerate insufficiency of coronary artery and vasoconstriction in many different tissues.

Little information is currently available on the acute effects of cigarette smoking on plasma NO and antioxidant levels. Some animal studies have indicated that cigarette smoke inhalation paradoxically increases NO concentration in plasma and enhances vascular dilation,<sup>7,8</sup> which is possibly due to exogenous NO contained in the cigarette smoke.<sup>8</sup> Nevertheless, coronary and other vitally important arteries were shown to constrict in human subjects after they smoked just one cigarette.<sup>9,10</sup> These discrepancies might be explained by an NO-independent mechanism, or by limitations inherent to animal experimentation. Thus, the present study sought to clarify the immediate effects of smoking a single cigarette on nitrate and nitrite plasma concentrations, as an index of NO plasma concentration, and on the plasma concentration of the antioxidants (ascorbic acid, cysteine, methionine, and uric acid).

### Methods

After study approval by the Osaka City University Medical School ethics committee, a randomized, crossover study of the effects of

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**Change in Plasma Concentrations (Mean±SEM) of Nitrate and Nitrite, Ascorbic Acid, Cysteine, Methionine, and Uric Acid in Smokers 5 Minutes After Smoking a Single Cigarette or a Sham Cigarette**

Parameter	Cigarette Smoking			Sham Smoking		
	Before	5 min After Smoking	<i>P</i>	Before	5 min After Smoking	<i>P</i>
Nitrate and nitrite, $\mu\text{mol/L}$	27.4±1.2	23.9±1.1	<0.001	27.5±1.3	27.3±1.2	0.613
Ascorbic acid, $\mu\text{mol/L}$	51.8±4.2	39.7±3.9	<0.001	52.3±4.2	51.9±4.4	0.365
Cysteine, $\mu\text{mol/L}$	7.0±0.6	4.7±0.5	<0.001	7.0±0.6	7.1±0.6	0.551
Methionine, $\mu\text{mol/L}$	10.2±0.7	8.1±0.7	<0.001	10.4±0.8	10.4±0.8	0.779
Uric acid, $\mu\text{mol/L}$	195.2±3.4	183.5±3.7	<0.001	196.0±3.6	196.9±3.6	0.387

smoking a single cigarette was performed in 20 healthy Japanese smokers. All subjects were randomly assigned to initially smoke either real or sham cigarettes. A week later, the same subjects smoked the opposite cigarette type (real or sham) depending on their initial random cigarette type assignment. Prior to the experiment, subjects did not eat and drink or smoke for 10 and 6 hours, respectively. The experiment consisted of smoking a single cigarette, either real or sham, in 10 minutes. Before and 5 and 60 minutes after the experiment, 2 mL of whole blood from each subject were drawn into heparinized tubes.

Plasma was isolated by centrifugation (5 minutes at 750g at 4°C), then deproteinized by the addition of an equal volume of methanol. The samples were then applied to an HPLC system (ENO-20, EICOM) for determining nitrate and nitrite concentrations. The nitrate and nitrite were separated using a reverse-phase column (NO-PAK), after which nitrate was reduced to nitrite in a reduction column packed with copperized cadmium (NO-RED) at 35°C. These nitrites were then mixed with the Griess reagent in a reaction coil, and the change in absorbance was monitored at 540 nm. The flow rate of the mobile phase, which consisted of 10% methanol containing 0.15 mol/L  $\text{BaCl}_2\text{-NH}_4\text{Cl}$  and 0.5 g/L EDTA-4Na, was 0.33 mL/min. The Griess reagent was delivered at a rate of 0.1 mL/min.

The remainder of the plasma sample was then used for ascorbic acid, cysteine, methionine, and uric acid measurements. First, the plasma was deproteinized by the addition of an equal volume of 1 N  $\text{HClO}_4$  containing 0.05% EDTA, and then applied to an HPLC system (Model 5600, ESA Inc), which was equipped with an 8-channel electrochemical detector (CoulArray) and an MCM column (ODS 250×4.6 mm ID, MC Medical). The detectors were set at 100, 200, 300, 400, 500, 600, 700, and 800 mV. Ascorbic acid,

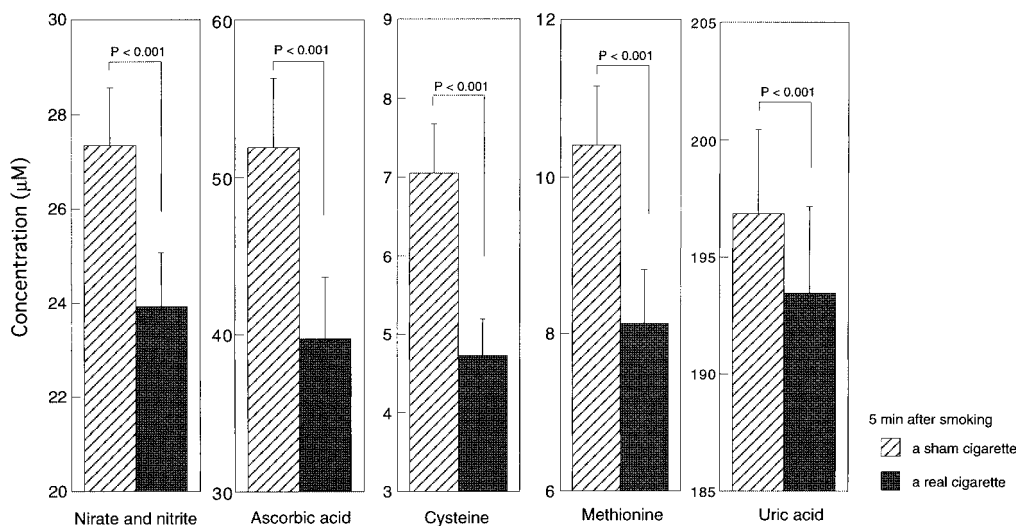
cysteine, methionine, and uric acid were separated using a computer-controlled water-acetonitrile gradient. The two mobile phases were (A) 0.1 mol/L potassium dihydrogenphosphate, 0.15 mmol/L sodium 1-octanesulfonate, and 0.01% v/v KCG reagent in water, and (B) 0.1 mol/L potassium dihydrogenphosphate, 0.15 mmol/L sodium 1-octanesulfonate, and 0.01% v/v KCG reagent in water-acetonitrile (70:30, v/v).

All results are expressed as mean±standard deviation. Statistical evaluation was performed using the Wilcoxon matched-pairs signed-rank test using Statcel with Excel 2001 Macintosh Edition (OMS Co, Japan). Differences were considered significant if  $P<0.05$ .

## Results

Subject age, height, and weight were  $27\pm 4$  years,  $168\pm 8$  cm, and  $62\pm 7$  kg, respectively. Their smoking history was  $7\pm 3$  pack-year (number of cigarettes in packs smoked daily times number of years).

No significant changes in the plasma concentrations of nitrate, nitrite, and tested antioxidants were noted in the subjects that smoked the sham cigarettes. However, after smoking a single cigarette, the nitrate and nitrite plasma concentrations markedly decreased compared with either the presmoking value ( $23.9\pm 1.1$  versus  $27.4\pm 1.2$   $\mu\text{mol/L}$ ,  $P<0.001$ ) (Table) or sham smoking value ( $23.9\pm 1.1$  versus  $27.3\pm 1.2$   $\mu\text{mol/L}$ ,  $P<0.001$ ) (Figure). The concentration of ascorbic acid, cysteine, methionine, and uric acid also significantly decreased compared with either presmoking



Plasma concentrations (mean±standard deviation) of nitrate and nitrite, ascorbic acid, cysteine, methionine, and uric acid in smokers 5 minutes after smoking a single cigarette compared with those who smoked a sham cigarette. The bar above each shaded rectangle indicates the standard deviation. The  $P<0.001$  range is noted for each cigarette and sham cigarette chemical pair.

values or sham smoking values (Table and Figure). After 60 minutes, the concentrations of nitrate and nitrite, ascorbic acid, cysteine, methionine, and uric acid returned to the presmoking level (26.9  $\mu\text{mol/L} \pm 1.2$ , 50.4  $\mu\text{mol/L} \pm 4.3$ , 6.8  $\mu\text{mol/L} \pm 0.6$ , 9.9  $\mu\text{mol/L} \pm 0.7$ , and 193.3  $\mu\text{mol/L} \pm 3.4$ , respectively).

### Discussion

We measured the plasma concentration of nitrate and nitrite, which are metabolic end products of NO, in smokers after smoking a single cigarette. Although measurements of NO itself cannot be performed, previous studies indicate that these end products are reliable indicators of the extent of NO formation in vivo.<sup>11</sup> However, it is also possible that food or water intake, daily activity, or the time of blood sampling affect the nitrate and nitrite plasma concentration.<sup>5</sup> Both food and water contain substantial amounts of nitrate, thus it is especially important to control these factors before attempting to determine endogenous NO formation. Therefore, we limited and standardized food and water intake by our subjects prior to the study and collected blood samples at the same time each day while the subjects were at rest.

Cigarette smoke is known to contain NO, and thus it is reasonable to assume that an elevation of NO and vasodilation are transiently induced by cigarette smoking. This hypothesis has been supported by several animal studies.<sup>7,8</sup> As shown by the results of human NO inhalation therapy, the usual effect of inhaled NO is temporary and limited to pulmonary circulation and airways because only a large amount of NO can induce a prolonged systemic effect.<sup>12</sup> The same may be true of cigarette smoke. In animal studies, the cigarette smoke exposure level may have been higher than that received by most smokers, and this may explain the elevation in NO plasma concentration. Recently, it was reported that inhalation of cigarette smoke transiently increased NO concentrations in the human lower respiratory tract.<sup>13</sup> Therefore, it would seem that NO is inhaled along with cigarette smoke, even in humans. Although such NO may have localized effects on the respiratory system, the results of the present study indicate that the net transient effect of cigarette smoking is actually to decrease NO in the circulation.

It has been reported that the nitrate half-life in plasma is relatively long (approximately 230 minutes) and the distribution volume is greater than the plasma volume (approximately 21% of body weight).<sup>11</sup> Thus, the simple measurement of nitrate and nitrite plasma concentration may underestimate the actual changes in the total amount of NO in the body. Notwithstanding the pharmacokinetics of NO, the amount and duration of the nitrate and nitrite plasma decrease are still not large, indicating that the effect of smoking a single cigarette is most likely transient. Plasma nitrate and nitrite are mainly derived from coronary circulation, except for the larger amount of exogenous nitrate that is obtained from food absorption.<sup>11</sup> Thus, although such a small reduction may not induce significant systemic physiological changes, it might partially contribute to increased susceptibility of coronary vasoconstriction, which is frequently seen after cigarette smoking in habitual smokers.

Epidemiological studies have shown that smokers have significantly lower plasma concentrations of ascorbic acid than nonsmokers,<sup>6</sup> which could be due to either impaired ascorbic

absorption or increased turnover. Interestingly, a decrease in dietary ascorbic acid intake has been reported among smokers.<sup>6</sup> However, there is no known report stating that smoking directly lowers plasma ascorbic acid levels. The present study demonstrates that smoking a single cigarette rapidly reduces the plasma ascorbic acid concentration, indicating the possibility that smoking indeed accelerates ascorbic acid turnover. After smoking, a similar reduction in the concentration of other plasma antioxidant compounds, such as cysteine, methionine, and uric acid, indicates that oxidative stress increases every time a cigarette is smoked. This is in agreement with a previous study that reported high levels of 8-hydroxydeoxy-guanosine and malondialdehyde in the plasma of smokers.<sup>14</sup>

In vitro studies reported that cigarette smoke transiently impairs NO release from endothelial cells, which is considered an oxidant-mediated reaction attenuated by ascorbic acid. Our results indicate that these reactions could also happen in vivo. Typically, habitual smokers who have a long smoking history and chronic functional impairment of the endothelium,<sup>1</sup> also have lower plasma antioxidant activity<sup>6</sup> as compared with younger subjects with a limited smoking history. Thus, the decrease in plasma nitrate and nitrite and other antioxidants after smoking one cigarette might be more dramatic in such populations.

In conclusion, smoking a single cigarette decreases plasma concentrations of nitrate and nitrite, ascorbic acid, cysteine, methionine, and uric acid.

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