Vitamin C prevents cigarette smoke-induced leukocyte aggregation and adhesion to endothelium in vivo

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ABSTRACT A common feature of cigarette-smoke (CS)-associated diseases such as atherosclerosis and pulmonary emphysema is the activation, aggregation, and adhesion of leukocytes to micro- and macrovascular endothelium. A previous study, using a skinfold chamber model for intravital fluorescence microscopy in awake hamsters, has shown that exposure of hamsters to the smoke generated by one research cigarette elicits the adhesion of fluorescently labeled leukocytes to the endothelium of arterioles and small venules. By the combined use of intravital microscopy and scanning electron microscopy, we now demonstrate in the same animal model that (i) CS-induced leukocyte adhesion is not confined to the microcirculation, but that leukocytes also adhere singly and in clusters to the aortic endothelium; (ii) CS induces the formation in the bloodstream of aggregates between leukocytes and platelets; and (iii) CS-induced leukocyte adhesion to micro- and macrovascular endothelium and leukocyte–platelet aggregate formation are almost entirely prevented by dietary or intravenous pretreatment with the water-soluble antioxidant vitamin C (venules, 21.4 ± 11.0 vs. 149.6 ± 38.7 leukocytes per mm², P < 0.01; arterioles, 8.5 ± 4.2 vs. 54.3 ± 21.6 leukocytes per mm², P < 0.01; aortas, 0.8 ± 0.4 vs. 12.4 ± 5.6 leukocytes per mm², P < 0.01; means ± SD of n = 7 animals, 15 min after CS exposure). No inhibitory effect was observed by pretreatment of the animals with the lipid-soluble antioxidants vitamin E or probucol. The protective effects of vitamin C on CS-induced leukocyte adhesion and aggregation were seen at vitamin C plasma levels (55.6 ± 22.2 μM, n = 7) that can easily be reached in humans by dietary means or supplementation, suggesting that vitamin C effectively contributes to protection from CS-associated cardiovascular and pulmonary diseases in humans.

Cigarette smoke (CS) has been identified as an independent major risk factor for the development of pulmonary and cardiovascular diseases, such as chronic obstructive pulmonary disease, emphysema, atherosclerosis, and coronary artery disease (1). Although the spectrum of adverse effects of CS involves multiple tissue and organ systems, a feature common to the pathomechanism of most CS-associated diseases is the activation and adhesion of circulating leukocytes to micro- and/or macrovascular endothelium (2–4), followed by acute and/or chronic leukocyte-mediated tissue damage (5, 6). In agreement with earlier reports on the sequestration and activation of leukocytes in the pulmonary microcirculation in rabbits (7), hamsters (8, 9), and humans (10), intravital microscopy in a skinfold chamber model in hamsters revealed that the exposure of hamsters to the smoke of one research cigarette elicits the rolling and adhesion of fluorescently labeled leukocytes to the endothelium of small venules and arterioles in striated muscle (11). Leukocyte adhesion was significantly reduced by pretreatment of the animals with superoxide dismutase, implying a key mediator role for superoxide radicals in CS-induced leukocyte/endothelium interaction (11). Indeed, studies by Pryor and coworkers (12, 13) have shown that CS (both gas-phase and particulate matter) introduces into the organism an abundance of reactive oxygen species (ROS), including superoxide radicals.

The action of superoxide radicals and other ROS can be counteracted not only by superoxide dismutase, but also by the water-soluble antioxidant vitamin C (14–16), as well as by the lipid-soluble vitamin E and probucol, both of which act as chain-breaking antioxidants and interfere with the process of lipid peroxidation (17, 18). Progesterone and antioxidant vitamins C and E have been demonstrated to slow the progression of atherosclerosis in laboratory animals (19, 20) and to reduce cardiovascular mortality in large epidemiological surveys (21–24). Among other biological effects, these antioxidant agents reduce leukocyte–endothelium interaction in response to various pathophysiological stimuli in vitro (25, 26). It was thus the aim of the present study to investigate whether vitamins C and E, as well as the Food and Drug Administration-approved antioxidant drug probucol, can counteract CS-induced leukocyte activation/adhesion in the hamster.

METHODS

Animal Model. The study was performed with the dorsal skinfold chamber preparation of Syrian Golden hamsters (body weight, 50–70 g; age, 6–8 weeks) kept on standard rodent diet and water ad libitum. For in vivo fluorescence microscopy a dorsal skinfold chamber and indwelling catheters were implanted in pentobarbital-anesthetized hamsters as described (27). A recovery period of 48–72 hr between the chamber implantation and the experiments was allowed to eliminate the effects of anesthesia and surgical trauma on the microvasculature. All experiments were performed in a blinded fashion.

Treatmen Regimen. Vitamins were administered by supplementation of the homogenized standard laboratory diet (Purina rodent diet, Ralston Purina; basal vitamin E content, 60 mg/kg of diet) with vitamin C (L-ascorbic acid, 10 g/kg of diet; Sigma) or vitamin E [(±)-α-tocopherol acetate, 10,000 units/kg of diet, kindly provided by Omega Pharma, Berlin]. In the same way, probucol (10 g/kg of diet, kindly provided by Marion Merrell Dow, Rüsselsheim, Germany) was added to the homogenized diet. To facilitate absorption of the lipid-soluble vitamin E and probucol, the diet (including the

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Abbreviations: CS, cigarette smoke; ROS, reactive oxygen species.
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vitamin C-supplemented diet and the unsupplemented control diet was supplemented with 2% olive oil (Bertolli Classico, Secaucus, NJ). Vitamin plasma levels were assessed in control animals and in vitamin C- and E-treated animals after 1 week of dietary supplementation from EDTA-anticoagulated blood by HPLC with electrochemical detection (15, 28). Vitamin E analysis assessed the predominant \(\alpha\)-tocopherol form. Probucol levels were assessed by HPLC following extraction with methanol/acetone (19).

**CS Exposure.** Awake hamsters were exposed to the smoke generated by one cigarette (2R1 University of Kentucky research cigarette) as previously described in detail (11). This method of CS exposure resulted in nicotine and carboxyhemoglobin plasma levels comparable to those found in human smokers (11). Neither in control animals nor in animals of any of the treatment groups did CS exposure result in significant changes in mean arterial pressure or heart rate during CS exposure and the 60-min follow-up period as monitored via indwelling catheters in the carotid artery of the animals. This finding is noteworthy, since CS exposure has been reported to increase heart rate and blood pressure in humans, presumably due to activation of the sympathetic nervous system (29).

**Intravital Fluorescence Microscopy.** Quantitative measurements of the microcirculation were evaluated in the striated skin muscle contained within the observation window by means of intravital microscopy in awake hamsters (27). The analyses included the quantification of the leukocyte–endothelium interaction in four to six collecting and post-capillary venules (diameter, 20–60 \(\mu\)m) and in four to six arterioles (diameter, 20–60 \(\mu\)m) per observation chamber. For contrast enhancement, leukocytes were stained in vivo with acridine orange (0.5 mg/kg per min, i.v.; Sigma) and classified according to their interaction with the endothelial lining as adherent, rolling, or free-flowing cells (27). At all time points before and after CS exposure, microvessel diameters were assessed by means of a computer-assisted microcirculation analysis system and centerline red-cell velocities were assessed by dual-slit cross correlation.

**Scanning Electron Microscopy.** Before and 15 min after CS exposure, EDTA-anticoagulated blood was taken through aortic puncture in pentobarbital-anesthetized hamsters \((n = 7\) animals per group). Buffy-coat cells were isolated by density gradient centrifugation and fixed in 2.5% glutaraldehyde/0.05% \(\text{CaCl}_2/0.1\) M sodium cacodylate buffer, pH 7.4, 21°C. Following dehydration in graded ethanol solutions, samples were placed on aluminum stubs, critical-point dried under liquid carbon dioxide (critical point dryer CPD030; Baltec, Vaduz, Liechtenstein), sputter-coated with gold, and examined with a scanning electron microscope (JEOL 35CF) at 15 kV. Likewise, aortas were removed by laparotomy in pentobarbital-anesthetized hamsters before and 15 min after CS exposure \((n = 7\) animals per group). For this purpose, aortas were first flushed retrograde with normal saline and then perfused with the above-described fixation medium. The abdominal part of the aorta and major portions of the thoracic aorta were excised, opened longitudinally along the plane of the celiac and mesenteric arteries, pinned out on cork mounts, dehydrated, and examined by electron microscopy as described above.

**Statistical Analysis.** Although parametric distribution was not uniformly found in all data sets, the data in the figures are given as mean \(\pm\) SD to facilitate interpretation. \(P\) values were calculated by the Mann–Whitney \(U\) test or the Wilcoxon test with Bonferroni correction.

**RESULTS**

As observed in previous studies (11), CS exposure elicited the rolling and adhesion of fluorescently stained leukocytes to the endothelial lining of both arterioles and postcapillary venules (Fig. 1; see also Fig. 4). Likewise, CS exposure elicited in all animals \((n = 7)\) the formation of loose leukocyte aggregates, tumbling down the microvessels and firmly adhering to the microvascular lining (Fig. 1). These leukocyte aggregates were further characterized by scanning electron microscopy.

**FIG. 1.** Intravital microscopic demonstration of leukocyte adhesion and aggregate formation in the microcirculation of hamsters 15 min after CS exposure. Postcapillary venules (Upper) and arteriole (Lower) were visualized with a \(\times 25\) water-immersion objective. White dots represent leukocytes stained by intravenous injection of the fluorescent marker acridine orange. Blood flow was from left to right. Note the formation of leukocyte aggregates tumbling down the endothelial lining. (Bars = 100 \(\mu\)m.)

**FIG. 2.** Scanning electron micrographic demonstration of leukocyte aggregates in hamster bloodstream 15 min after CS exposure. An aggregate of three leukocytes is shown. Note the involvement of activated, dendritic platelets forming broad and thread-like bridges between individual leukocytes. (Bar = 1 \(\mu\)m.)
Leukocyte adhesion to junctions bridges (Middle). Vitamin C and E supplementation resulted in a 3.4- and 4-fold increase in vitamin C and E plasma levels, respectively, over levels measured in control hamsters (Table 1). A comparable increase in vitamin C plasma levels was reached by intravenous injection of vitamin C (5 mg/kg of body weight; Table 1). Supplementation of the homogenized diet with probucol at 10 mg/kg of laboratory diet resulted in a probucol level of 4.7 ± 1.7 μg/ml of plasma (n = 7 animals).

Vitamin C treatment, both by diet and by intravenous injection, significantly attenuated CS-induced leukocyte adhesion to microvascular endothelium (Fig. 4). These changes were not secondary to alterations in local shear-force conditions, since we observed no differences in microhemodynamic parameters (microvessel diameter and red-cell velocity) between animals of the different treatment groups (data not shown). Likewise, we no longer observed the formation of leukocyte–platelet aggregates in vitamin C-treated animals (0 of 7 animals). Finally, a significant inhibition was observed of CS-induced leukocyte adhesion to aortic endothelium, where only 0.8 ± 0.4 (vitamin C diet) and 0.7 ± 0.5 (intravenous vitamin C) leukocytes were counted per square millimeter, respectively (n = 7 animals per group; data in both groups were significantly different from values in control animals at the P < 0.01 level).

No inhibition of CS-induced leukocyte adhesion was observed in animals pretreated with vitamin E or probucol (Fig. 4). Neither did we observe a reduction in leukocyte aggregate formation. Also, leukocyte adhesion to aortic endothelium in vitamin E and probucol-treated animals was not different from that in control animals (vitamin E, 12.7 ± 7.8 cells per mm²; probucol, 14.0 ± 6.4 cells per mm²; n = 7 animals per group). Neither vitamin C- nor vitamin E- nor probucol-treated animals showed a reduction of CS-induced leukocyte rolling along the endothelial lining of venules and arterioles (data not shown).

**DISCUSSION**

The principal observations in this study are that (i) CS induces leukocyte adhesion to the endothelium of small venules and arterioles (Figs. 1 and 4), as well as to aortic endothelium (Fig. 3); (ii) CS elicits the formation of aggregates between leukocytes and activated dendritic platelets, tumbling down the micro- and macrovascular endothelium.

**Table 1. Plasma levels of the vitamins C and E in control animals and after dietary or intravenous vitamin administration**

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<thead>
<tr>
<th>Vitamin</th>
<th>μM</th>
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<tr>
<td>Control laboratory diet</td>
<td>16.4 ± 5.7</td>
<td>9.4 ± 4.9</td>
<td></td>
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<tr>
<td>Dietary supplementation</td>
<td>55.6 ± 22.2**</td>
<td>38.1 ± 15.2**</td>
<td></td>
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<tr>
<td>Intravenous administration</td>
<td>62.7 ± 23.0**</td>
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Vitamin plasma levels were assessed in EDTA-anticoagulated blood by HPLC with electrochemical detection (15, 30). Vitamin C was also injected intravenously as a bolus in a dose of 5 mg/kg of body weight, 5 min prior to determination of plasma levels. Data are means ± SD in n = 7 animals per group. **, P < 0.01 versus respective values in animals on control laboratory diet (Mann-Whitney U test).
incorporated merely needs from vitamin (12, 13).

sponse E and probucol C, but vitamin vitamin 1
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Leukocyte isolated, cigarette CS-induced adhesion LC
adhesion of CS, and microvascular involvement (41). PAF and microvascular aggregata in platelets (42).

Vitamin C intercepts aqueous-phase ROS before they can attack lipoprotein lipids and thus prevents the abstraction of hydrogen atoms from polyunsaturated fatty acid chains and the initiation of lipid peroxidation (16, 28). Recent in vitro evidence suggests that certain lipid peroxidation products can activate leukocytes and induce leukocyte adhesion to endothelial cells via the receptor for platelet-activating factor (PAF) and have thus been termed PAF-like lipids (PAF-L; ref. 41). PAF and PAF-L have been identified in increased amounts in the plasma of smokers (42). As for P-selectin, the involvement of PAF/PAF-L, too, could explain not only the adhesion of leukocytes to endothelial cells but also the formation of leukocyte–platelet aggregates in CS-exposed hamsters, as well as the inhibition of both phenomena by vitamin C.

CS exposure has been shown by immunogold electron microscopy to induce the upregulation of β3-integrin adhesion molecules on leukocytes sequestered in the upper layers of rabbit lungs (31), to reduce leukocyte deformability (32); and to increase in rats and humans the production by endothelial cells of proaggregatory thromboxane while at the same time inhibiting the generation of antiaggregatory prostacyclin (30). Whether these effects of CS are ROS-dependent and could thus be affected by vitamin C remains to be shown.

The fact that, under the conditions of our experiments, lipid-soluble antioxidants did not prevent leukocyte aggregation and adhesion does not rule out that these antioxidants may confer inhibitory effects on other steps of CS-associated
tissue damage. However, the absence of protection against CS-induced biological effects by lipid-soluble antioxidants in this and previous studies (15, 16, 28, 35, 36, 44) strongly emphasizes the importance of aqueous-phase ROS in CS-induced pathology. Corroborative evidence can be derived from epidemiological surveys which consistently demonstrate a significant consumption of vitamin C, but not of vitamin E, in the plasma of smokers (21, 22, 45) and from the results of a large epidemiological study in which the cardio-protective effects of vitamin C were no longer apparent when the data were corrected for cigarette smoking, indirectly suggesting that smokers may profit most from vitamin C ingestion (21).

Based on epidemiological data, the threshold levels for effective protection from cardiovascular diseases by vitamins C and E have been estimated at 40–50 μM and 27.5–30 μM, respectively (24). These threshold levels were surpassed by all the supplemented hamsters in our study, indicating that the vitamin levels in the animals translate well to data in human subjects, with low baseline vitamin levels in control hamsters corresponding to a predicted high risk of cardiovascular disease and high post-supplementation levels corresponding to a predictive low risk of cardiovascular diseases (21–24).

In conclusion, the results of this study suggest that simple dietary supplementation with vitamin C may exert a powerful protection from CS-associated disease processes that involve ROS and leukocyte-inflicted tissue damage. It is our belief that enough experimental and epidemiological evidence has accumulated to warrant testing of dietary vitamin C in prospective clinical trials aimed at the prevention or retraction of CS-associated diseases such as atherosclerosis and its complications, as well as pulmonary emphysema and chronic obstructive pulmonary disease.

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