High Doses of Vitamin C Reverse *Escherichia coli* Endotoxin–Induced Hyporeactivity to Acetylcholine in the Human Forearm

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Background—Acute inflammation causes endothelial vasodilator dysfunction that may be mediated by oxidative stress.

Methods and Results—In this randomized, double-blind, crossover study, forearm blood flow responses to acetylcholine (ACh) (endothelium-dependent dilator) and glyceryl-trinitrate (GTN) (endothelium-independent dilator) were assessed before and after induction of acute systemic inflammation by low doses of *Escherichia coli* endotoxin (LPS) (20 IU/kg IV) in 8 healthy volunteers. The acute effect of intra-arterial vitamin C (24 mg/min) or placebo was studied 4 hours after LPS, respectively. Vitamin C alone was administered in control experiments. LPS administration caused systemic vasodilation, increased white blood count, elevated body temperature, and reduced vitamin C plasma concentrations. LPS decreased the responses of forearm blood flow to ACh by 30% ($P<0.05$) but not to GTN. Vitamin C completely restored the response to ACh, which was comparable with that observed under baseline conditions. Vitamin C had no effect on basal blood flow or ACh- or GTN-induced vasodilation in control subjects.

Conclusions—Our data demonstrate that impaired endothelial vasodilation caused by *E coli* endotoxemia can be counteracted by high doses of antioxidants in vivo. Oxidative stress may play an important role in the pathogenesis of endothelial dysfunction during inflammation. **(*Circulation*. 2002;106:1460-1464.)**

Key Words: inflammation ■ endothelial function ■ oxidative stress ■ vitamin C

The vascular endothelium plays an important role in regulating vascular tone, leukocyte adhesion, platelet activation, and vascular remodelling through the production of mediators, including NO, prostacyclin, and endothelin.[1,2] Experimental human studies have demonstrated endothelial dysfunction during acute inflammation in the venous[3] and arterial vascular beds.[4,5] Endothelial dysfunction might provide a mechanism to link the association between acute systemic inflammation and the transiently elevated risk of cardiovascular events.[6] How inflammation leads to endothelial dysfunction remains unclear, but oxidative stress secondary to increased production of reactive oxygen species (ROS) has been implicated in animal and in vitro models.[7,8] ROS, including superoxide and hydroxyl radicals, may inactivate endothelial mediators (including NO) or may directly damage the endothelium.[9] Clinical studies also suggest a link between oxidative stress, inflammation, and vascular dysfunction.[10–12] Serum concentrations of antioxidants are low in patients with cardiovascular disease,[10] and high local concentrations of antioxidants such as vitamin C have been shown to restore endothelial function in patients with cardiovascular risk factors, including diabetes, hypertension, hypercholesterolemia, and smoking.[13]

The purpose of the present study was to test the hypothesis that systemic concentrations of the antioxidant vitamin C are decreased during acute inflammation secondary to *Escherichia coli* endotoxin (LPS) and that endotoxin-associated endothelial dysfunction can be restored by high doses of vitamin C. The administration of low doses of LPS is an established model for generation of an acute systemic inflammatory response in humans,[5,14,15] and forearm vascular function was studied using strain-gauge plethysmography.

Methods

The study was approved by the ethics committee of the University of Vienna and conforms with the principles outlined in the Declaration of Helsinki, including current revisions and the European Guidelines on Good Clinical Practice.

Study Population

Eight healthy male subjects (age 26±3 years) from whom informed consent was obtained before enrolment were included in this double-blind, randomized, crossover study. All subjects were given a complete health examination (including physical examination, ECG, and laboratory screening) within 14 days before the first study day. All subjects were nonsmokers and had no history or signs of arterial hypertension, hypercholesterolemia, or other cardiovascular

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risk factors. Subjects were asked to avoid nonsteroidal anti-inflammatory drugs from 2 weeks before screening until the study was complete. After an overnight fast, studies were conducted in a quiet room with an ambient temperature of 22°C with complete resuscitation facilities.

Forearm Blood Flow Measurements

Forearm blood flow was measured in both arms, as described previously.[5,16] Briefly, strain gauges, placed on the forearms, were connected to plethysmographs (EC-6, D.E. Hokanson) to measure changes in forearm volume in response to inflation of venous congesting cuffs. Drug effects were expressed as the ratio of blood flow in the intervention to the control arm,[5,17] where baseline ratio was defined as 100%. Wrist cuffs were inflated to suprasystolic pressures during each measurement to exclude circulation to the hands. Flow measurements were recorded for 9 seconds at 30-second intervals during drug infusions.

Generation of Acute Systemic Inflammation

E coli endotoxin (LPS; 20 IU/kg body weight; National Reference Endotoxin, *E coli*, U.S.P. United States Pharmacopeia Convention Inc) was administered intravenously as a bolus to induce acute inflammation. Injection of LPS to humans has been established as a model for acute systemic inflammation[5,14,15] and causes endothelial dysfunction that is most marked 4 hours after LPS bolus administration. The flu-like symptoms are mild and transient, and subjects can be discharged after ≈8 to 10 hours in good health without sequelae.

Study Design

LPS was administered to each subject on 2 study days, and vitamin C or vehicle was infused 230 minutes after administration of LPS in a double-blind randomized crossover design. A sample size calculation based on the established standard deviation of repeated measurements under baseline conditions estimated that a study in 8 volunteers had an 80% power to detect a 14% difference in forearm blood flow ratio at an α of 0.05. In a previous trial,[5] vasodilation to acetylcholine (ACh) was attenuated by ≈34% after LPS administration. Our sample size was based on the assumption that an effect of vitamin C resulting in 50% reduction of this impairment could be detected.

To study the direct effects of vitamin C on endothelium-dependent and -independent vasodilation, control experiments with vitamin C alone, without prior LPS administration, were performed on a third open-label trial day. The schedule for the third study day was identical otherwise.

Experimental Protocol

A plastic cannula was inserted into antecubital veins for monitoring of white blood count (WBC) and plasma concentrations of vitamin C and for administration of LPS, respectively. A fine-bore needle (27G needle Sterican, B. Braun) was inserted into the brachial artery of the nondominant arm for the administration of vasodilators. After a 20-minute resting period, baseline forearm blood flow was measured in response to the endothelium-dependent dilator ACh (25, 50, and 100 nmol/min; each dose for 3 minutes; Clinalfa). After a 15-minute washout period to allow restoration of control blood flow, forearm blood flow was measured in response to the endothelium-independent dilator glyceryl-trinitrate (GTN) (4, 8, and 16 nmol/min; each dose for 3 minutes; G. Pohl Boskamp GmbH).

After an additional 15 minutes, LPS was administered as described above. Two hundred thirty minutes after LPS administration, subjects received a continuous infusion of either saline or vitamin C intra-arterially (24 mg/min; Mayerhofer GmbH),[18] and the responses to ACh and GTN coadministered with vitamin C or saline were assessed after 10 minutes, as above. In the open-label limb of the study, the response to ACh and GTN was determined before and 4 hours later, during coinfusion with vitamin C.

TABLE 1. Clinical and Laboratory Parameters at Baseline and 4 Hours After Induction of Systemic Inflammation by *E coli* Endotoxin (20 IU/kg LPS), With Local Coinfusion of Vitamin C or Placebo

	Baseline	Endotoxemia
Temperature, °C		
Placebo	36.3±0.1	38.2±0.1*
Vitamin C	36.4±0.1	37.7±0.2*
Pulse rate, bpm		
Placebo	65±4	81±3*
Vitamin C	63±4	80±3*
Systolic blood pressure, mm Hg		
Placebo	126±4	115±4*
Vitamin C	125±3	120±5
Diastolic blood pressure, mm Hg		
Placebo	63±3	52±5*
Vitamin C	65±2	57±4
White blood count, 10^9/L		
Placebo	4.9±0.5	10.0±1.2*
Vitamin C	5.1±0.3	9.3±1.0*
Vitamin C concentration, μmol/L		
Placebo	57.8±3.0	36.2±2.8*
Vitamin C	53.6±9.3	376.3±12.0*†

Values are mean±SEM (n=8).

*P<0.05 for significant changes vs baseline (paired t test); †P<0.05 for significant differences between study days (unpaired t test).

Monitoring

Blood for analysis of WBC and vitamin C levels was drawn at baseline and 4 hours after LPS administration. Tympanic temperature (Thermoscan pro, Braun AG) was measured at frequent intervals, and ECG, pulse rate, and blood pressure were recorded with a standard device (Hewlett Packard CMS patient monitor).

Laboratory Tests

Analyses for WBC were carried out according to standard procedures by the Department of Clinical Chemistry, Allgemeines Krankenhaus, Wien. Plasma vitamin C blood concentration was measured by the ascorbate oxidase method on a standard clinical chemistry analyzer (Hitachi 911, Roche).

Statistical Analysis

All data were tested for normal distribution by Kolomogorov-Smirnov test and log transformed if not normally distributed. Systemic hemodynamics and laboratory parameters were expressed as absolute values or percent changes from baseline and compared using Student's paired or unpaired t test, as appropriate. Forearm blood flow measurements were measured at mL/min per 100 mL forearm volume and expressed as percent change from baseline ratio. The effects of ACh and GTN at baseline and after LPS administration were assessed by ANOVA for repeated measurements using the Statistica software package (Release 4.5, StatSoft Inc). $P \leq 0.05$ was considered significant. Values are expressed as mean±SEM unless indicated otherwise.

Results

Systemic hemodynamics and laboratory parameters were comparable between the trial days at baseline (Table 1). After LPS, the expected mild and transient flu-like symptoms occurred. LPS increased WBC on both days after 4 hours

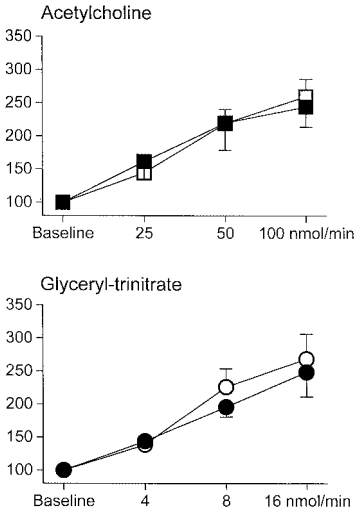

Figure 1. Vasodilation to ACh (top) and GTN (bottom) at baseline before LPS and vitamin C (solid symbols) or placebo (open symbols) administration. FBF ratio (intervention vs control arm) is expressed as mean±SEM. n=8; no significant differences between groups.

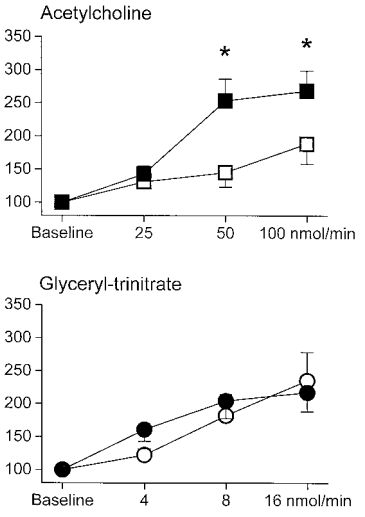

Figure 2. Vasodilation to ACh (top) and GTN (bottom) 4 hours after LPS, with administration of vitamin C (solid symbols) or placebo (open symbols) administration. Forearm blood flow ratio (intervention versus control arm) is expressed as mean±SEM. n=8; *$P<0.05$ vs baseline and between groups (ANOVA).

($P<0.01$), which was paralleled by a significant decrease in systemic blood pressure and an increase in pulse rate. Hemodynamic parameters returned to baseline 8 hours after LPS. Systemic vitamin C levels decreased after LPS by 37% during placebo and increased ≈7.5-fold during local administration of vitamin C ($P<0.05$ for both study days).

Endothelium-Dependent and -Independent Vasodilation After LPS

Baseline forearm blood flow (FBF) was comparable between study days. Four hours after LPS administration, FBF increased from 3.9±0.1 to 5.0±0.2 mL/min per 100 mL ($P<0.05$), and no differences were observed between trial days.

Endothelium-dependent vasodilation to ACh was comparable at baseline on both trial days (Figure 1) but was reduced 4 hours after LPS administration by 30±11% (Figure 2, Table 2). Intraarterial administration of vitamin C had no effect on baseline forearm blood flow during endotoxemia but completely restored the response to ACh (Figure 2).

No differences were observed in response to GTN at baseline or during experimental inflammation on the 2 trial days (Figure 1). Vitamin C or placebo had no additional effect on GTN-induced vasodilation of the forearm (Figure 2, Table 2).

Endothelium-Dependent and -Independent Effects of Vitamin C

Vitamin C alone increased systemic vitamin C levels from 66.1±5.8 to 445.5±36.2 μmol/L ($P<0.001$) in the contralateral arm but had no effect on baseline forearm blood flow or endothelium-dependent or -independent vasodilation (data not shown). No changes in systemic hemodynamics, body temperature, or WBC were observed.

Discussion

This study confirms our previous findings that administration of LPS causes systemic inflammation and profoundly suppresses the response of the resistance vasculature to ACh without altering the response to GTN.[5] These findings are consistent with a direct effect of acute inflammation to impair endothelial function. In addition, acute administration of the antioxidant vitamin C restored the response to ACh, implicating a role of oxidative stress in the endothelial dysfunction caused by inflammation.

Infusion of LPS to healthy humans has been established by other groups and at our institution to evaluate the clinical response to endotoxin.[5,14,15] Similar to previous studies, LPS caused transient flu-like symptoms, paralleled by a rise in body temperature and white blood count and a change in systemic hemodynamics. Selective impairment of endothelium-dependent vasodilation by LPS was first demonstrated in vitro.[19,20] In humans in vivo, this was first confirmed by Bhagat et al[3] in veins. Subsequently, Hingorani et al[4] demonstrated reduced endothelium-dependent vasodilation of resistance and conduit vessels after vaccination with *Salmonella typhi* in healthy volunteers. In the present study, the response to acetylcholine was reduced 4 hours after LPS administration, with preservation of the dilatation caused by the smooth muscle relaxant GTN.

The mechanisms underlying inflammation-induced endothelial dysfunction are not fully characterized in humans. In acute and chronic inflammation, oxidative stress occurs when the production of ROS (including superoxide anions [O_2^-]) exceeds the capacity of the endogenous antioxidant defense systems.[21] The result of such imbalance results in ROS-mediated damage. The role of O_2^- in the inflammatory response was suggested in the 1970s by McCord and Fridovich.[22] Important proinflammatory roles for O_2^- include endothelial cell damage and increased microvascular permeability,[23] lipid peroxidation and oxidation, DNA single-strand damage,[24] and formation of peroxynitrate.[8,25] Presently, there is no method available to assess directly O_2^- formation in vivo in humans. However, vitamin C is a scavenger of O_2^-

TABLE 2. Effect of Increasing Intra-Arterial Doses of ACh and GTN on Percent Change of FBF Ratio at Baseline and 4 Hours After Induction of Systemic Inflammation by *E coli* Endotoxin (20 IU/kg LPS), With Local Coinfusion of Vitamin C or Placebo

	Baseline			Endotoxemia		
ACh, nmol/min	25	50	100	25	50	100
Vitamin C	162±28	220±41	244±30	143±11	254±33	269±31
Placebo	145±12	219±21	260±26	131±9	146±22*	189±30*
GTN, nmol/min	4	8	16	4	8	16
Vitamin C	144±9	196±15	248±37	161±18	204±26	217±29
Placebo	139±13	226±28	268±38	122±10	182±32	235±43

Values are mean±SEM (n=8).
*P<0.05 vs baseline and between groups, ANOVA.

and has been used to assess the bioactivity of endogenous O_2^- in biological systems.[26,27] In clinical studies, vitamin C increases endothelium-dependent dilatation in patient groups at increased oxidative stress. In the present study, vitamin C prevented endothelial dysfunction caused by LPS administration without altering the responsiveness of the vascular smooth muscle and had no effect on vascular reactivity in subjects not exposed to LPS. However, vitamin C concentrations seen in the present experiments cannot be achieved by oral supplementation. Even large oral doses of 2.5 g vitamin C increased circulating vitamin C concentrations from 30 to 60 μmol/L in unsupplemented individuals to a maximum of only 120 μmol/L,[28] which is much lower than in this study. The limited gastrointestinal absorption prevents much higher vitamin C concentrations after oral intake, and increasing plasma concentrations are paralleled by an elevated renal clearance of vitamin C as a result of saturable tubular reabsorption.[29]

These data suggest a role for oxidative stress in inflammation-induced endothelial dysfunction. However, the effect of endotoxin in a different pathophysiological milieu, such as in atherosclerosis, which in itself impairs endothelial function, cannot be determined from the present results. We observed that plasma concentrations of vitamin C were reduced during inflammation, consistent with consumption of vitamin C during inflammation. Clinical studies indicate that vitamin C concentrations are reduced in patients with unstable angina[30] or at risk of myocardial infarction[31] and in patients with peripheral artery disease.[10] In agreement with these studies, a reduction of circulating vitamin C concentrations was a consistent finding in our experiments. It might therefore be possible to use vitamin C as a marker reflecting oxidative stress during inflammation.

Our data indicate that an altered antioxidant balance can be restored by exogenous vitamin C in vivo. Although it can be speculated that increased levels of ROS contribute to endothelial dysfunction after LPS, the study is limited to functional findings from healthy subjects and does not explain the mechanisms of formation or source of ROS during acute inflammation. It is also possible that the NO release to ACh is normal after LPS, but NO metabolism increased during experimental inflammation. In animals, impaired endothelium-dependent relaxation after LPS treatment was associated with increased generation of O_2^- by xanthine oxidase and NAD(P)H oxidase and an increase in peroxynitrate and nitrotyrosine formation.[32] Whether this is also the case in humans remains to be established. Moreover, vitamin C has other effects to alter endothelial function, including increasing the production of intracellular tetrahydrobiopterin, a cofactor for NO synthesis.[33] Whether these mechanisms are involved in the salutary effect of vitamin C on endothelial function remains to be determined.

In summary, we have demonstrated that impaired endothelial vasodilation caused by *E coli* endotoxin in healthy humans can be counteracted by an antioxidant strategy using vitamin C in vivo. Given the central vasoprotective role of the endothelium, our data suggest that inflammation or infection may play an important role in the pathogenesis of endothelial dysfunction through increased oxidative stress.

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