Vitamin C Inhibits Endothelial Cell Apoptosis in Congestive Heart Failure

Lothar Rössig, Jörg Hoffmann, Bénédicte Hugel, Ziad Mallat, Astrid Haase, Jean-Marie Freyssinet, Alain Tedgui, Alexandra Aicher, Andreas M. Zeiher and Stefanie Dimmeler

Circulation 2001;104;2182-2187
DOI: 10.1161/hc4301.098284

Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2001 American Heart Association. All rights reserved. Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/cgi/content/full/104/18/2182

Subscriptions: Information about subscribing to Circulation is online at http://circ.ahajournals.org/subscriptions/

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail: journalpermissions@lww.com

Reprints: Information about reprints can be found online at http://www.lww.com/reprints
Vitamin C Inhibits Endothelial Cell Apoptosis in Congestive Heart Failure

Lothar Rössig, MD; Jörg Hoffmann, BSc; Bénédicte Hugel, PhD; Ziad Mallat, MD, PhD; Astrid Haase; Jean-Marie Freyssinet, PhD; Alain Tedgui, PhD; Alexandra Aicher, MD; Andreas M. Zeiher, MD; Stefanie Dimmeler, PhD

Background—Proinflammatory cytokines like tumor necrosis factor-α and oxidative stress induce apoptotic cell death in endothelial cells (ECs). Systemic inflammation and increased oxidative stress in congestive heart failure (CHF) coincide with enhanced EC apoptosis and the development of endothelial dysfunction. Therefore, we investigated the effects of antioxidative vitamin C therapy on EC apoptosis in CHF patients.

Methods and Results—Vitamin C dose dependently suppressed the induction of EC apoptosis by tumor necrosis factor-α and angiotensin II in vitro as assessed by DNA fragmentation, DAPI nuclear staining, and MTT viability assay. The antiapoptotic effect of vitamin C was associated with reduced cytochrome C release from mitochondria and the inhibition of caspase-9 activity. To assess EC protection by vitamin C in CHF patients, we prospectively randomized CHF patients in a double-blind trial to vitamin C treatment versus placebo. Vitamin C administration to CHF patients markedly reduced plasma levels of circulating apoptotic microparticles to 32\% of baseline levels, whereas placebo had no effect (87\% \( P < 0.005 \)). In addition, vitamin C administration suppressed the proapoptotic activity on EC of the serum of CHF patients (\( P < 0.001 \)).

Conclusions—Administration of vitamin C to CHF patients suppresses EC apoptosis in vivo, which might contribute to the established functional benefit of vitamin C supplementation on endothelial function. (Circulation. 2001;104:2182-2187.)

Key Words: endothelium ■ apoptosis ■ inflammation ■ heart failure ■ antioxidants

Exercise intolerance in congestive heart failure (CHF) results from vasotone dysregulation caused by impaired endothelial function.1–3 The formation of reactive oxygen species (ROS) is enhanced during the progression of CHF4,5 and was hypothesized to contribute to endothelial dysfunction.6 In accordance with this model, antioxidative treatment improves endothelial function in CHF patients.7 We and others have previously shown that serum of CHF patients induces apoptosis in cultured endothelial cells (ECs).8,9 Importantly, the levels of apoptotic membrane microparticles are elevated in the circulation of CHF patients.9 Increasing evidence suggests that oxidative stress regulates apoptosis of ECs. Thus, prooxidant stimuli like H2O2 induce EC apoptosis.10,11 Moreover, cytokine messengers, tumor necrosis factor-α (TNF-α), and the vasoconstrictor peptide angiotensin II (Ang II) trigger apoptosis of EC and stimulate the generation of ROS.12 Thus, enhanced oxidative stress may contribute to endothelial dysfunction in heart failure by the induction of EC apoptosis.13,14 Therefore, this study was designed to investigate the influence of the antioxidant vitamin C15 on TNF-α–, Ang II–, and CHF serum–induced apoptosis signaling in ECs in vitro. In addition, we performed a prospectively randomized, placebo-controlled, double-blind clinical trial to investigate the functional effects of vitamin C therapy on EC apoptosis in patients with CHF.

Methods

Patients

We studied 34 patients (26 men, 8 women; age, 28 to 76 years) who suffered from CHF symptoms according to NYHA classes II through IV. The clinical characteristics of these patients are shown in the Table. All patients received standard treatment for heart failure, including ACE inhibitors/angiotensin receptor-1 blockers, diuretics, β-blockers, and for many digitalis. Patients with elevated serum creatinine levels (>2.0 mg/dL) and those with concomitant infectious or primary pulmonary disease were excluded. None of the patients had a history of myocardial infarction within 3 months of the onset of treatment. Written, informed consent was obtained from all patients included in this study. The study protocol was approved by the Research Ethics Committee of the Johann Wolfgang Goethe University of Frankfurt.

Received July 6, 2001; revision received August 17, 2001; accepted August 24, 2001.


Correspondence to Stefanie Dimmeler, PhD, Department of Internal Medicine IV, Division of Cardiology, University of Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany. E-mail dimmeler@em.uni-frankfurt.de

© 2001 American Heart Association, Inc.

Circulation is available at http://www.circulationaha.org
were begun, complete medium was changed to endothelial basal plated in 35-mm culture dishes. Twelve hours before the experiments the MTT assay as previously published. 17 The amount of apoptotic assay. Results were expressed as nanomolar phosphatidylserine microparticles in human plasma samples was measured according to the method of Aupeix et al 16 with a solid-phase prothrombinase of 500
supplemented with hydrocortisone (1
77/3 6/9/2 NS
8/9 10/7 NS
27±3 24±2 0.37
ACE I/V, blockers
17 (100) 17 (100) NS
β-Blockers
8 (47) 11 (65) NS
Diuretics
14 (82) 16 (94) NS
Digitals
13 (76) 13 (76) NS

Study Protocol
Patients were randomized to receive vitamin C or placebo according to a sequential administration protocol combining initial intravenous bolus with prolonged oral application of the treatment and placebo substances. Initially, a baseline serum sample was drawn, followed by a 10-minute bolus infusion of 2.5 g vitamin C (Wörgä Pharma) or sodium chloride 0.9%. Fifteen minutes later, a second serum sample was taken. Throughout the next 3 days, patients received 2 g oral vitamin C or placebo tablets (Merck) twice daily, and a third serum sample was obtained in the morning of the fifth day.

At each of the 3 respective time points, venous blood samples were drawn into 10-mL serum tubes without additives, immediately stored on ice for 30 minutes to allow clotting, and centrifuged at 3500 rpm and 4°C for 10 minutes; then, serum was frozen in aliquots of 500 µL at −80°C. For use in cell culture, aliquots were thawed on ice shortly before experiments were performed. To perform shed membrane particle analysis, blood was drawn into plasma monovettes containing 0.138 mol/L trisodium citrate at a final volume ratio (blood:anticoagulant) of 9:1, and sample preparation was performed according to the method of Aupeix et al.16

Cell Culture
Human umbilical venous ECs (HUVECs) were purchased from Cell Systems/Clonetics and were cultured in endothelial basal medium supplemented with hydrocortisone (1 µg/mL), bovine brain extract (3 µg/mL), gentamicin (50 µg/mL), amphotericin B (50 µg/mL), epidermal growth factor (10 µg/mL), and 10% FCS until the third passage. After detachment with trypsin, cells were grown in culture dishes or flasks for ≥12 hours before experiments were performed.

Detection of Cell Death In Vitro and In Vivo
For morphological assessment of apoptotic nuclei, 106 cells were plated in 35-mm culture dishes. Twelve hours before the experiments were begun, complete medium was changed to endothelial basal medium with no FCS and no supplements. Then, 10% human serum or the indicated concentrations of TNF-α or Ang II (both from Sigma) were added. Eighteen hours later, cells underwent nuclear staining and were fluoromicroscopically analyzed as described previously.9 To assess internucleosomal DNA laddering, cells were plated in culture flasks (106 cells) and underwent the same experimental protocol as described above. Isolation and radioactive labeling of DNA and subsequent Southern blot analysis were performed as described in detail elsewhere.17 Cell viability was determined with the MTT assay as previously published.17 The amount of apoptotic microparticles in human plasma samples was measured according to the method of Aupeix et al16 with a solid-phase prothrombinase assay. Results were expressed as nanomolar phosphatidylserine equivalent normalized to a reference curve.

Caspase-9 Activity, Cytochrome C, Thiobarbituric Acid Reactive Substances, and Cytokine Assays
For caspase activity measurements, HUVEC were lysed as stated before.17 Then, proteolytic cleavage of a fluorescent caspase-9 substrate (Calbiochem, Bad Soden/Taunus) in cell lysates was measured at excitation and emission wavelengths of 400 and 505 nm, respectively. Cytochrome C detection in cytoplasmic and nuclear fractions was performed according to the method of Walter et al.18 TNF-α, soluble TNF-α receptor I (sTNFRI), soluble Fas (sFas), and soluble Fas Ligand (sFasLigand) serum concentrations were measured with ELISA kits according to the manufacturer’s instructions (TNF-α, sTNFRI: R&D Systems; sFas, sFasLigand: Coulter Immunotech); and determination of thiobarbituric acid reactive substances (TBARS) was performed with the OXI-TEK TBARS assay kit (ZeptoMetrix).

Statistical Analysis
Data are expressed as mean±SEM or mean±SD as indicated from ≥3 independent experiments. Continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test. Statistical analysis was performed by t test for comparison of the 2 treatment groups (variance analysis by the Levene test) and for multiple groups by 1-way ANOVA analysis, followed by post-hoc analysis adjusted with a least-significant-difference correction. Categorical variables were compared by means of Fisher’s exact test. Statistical significance was assumed if a null hypothesis could be rejected at P<0.05.

Results
Effect of Vitamin C on TNF-α- and Ang II–Induced EC Apoptosis
Morphological analysis of cell nuclei demonstrated that TNF-α elicited an ~3-fold increase in the number of apoptotic ECs (Figure 1A). Coincubation with vitamin C dose dependently inhibited TNF-α–induced EC apoptosis (P<0.001; Figure 1A). Suppression of TNF-α–induced apoptosis by vitamin C was confirmed by analysis of DNA laddering (Figure 1B). TNF-α induced a marked reduction in cell viability as assessed by MTT assay that was prevented in the presence of vitamin C (P<0.005). Likewise, vitamin C completely inhibited apoptosis induction in ECs by Ang II (P<0.05; Figure 1C). The antiapoptotic effect of vitamin C depended on endogenous nitric oxide (NO) generation because coinoculation of the NO synthase inhibitor Nω-nitro-L-arginine monooacetate (L-NMMA, 1 mmol/L) with vitamin C reversed the inhibition of TNF-α–induced apoptosis by vitamin C (Figure 1D), whereas L-NMMA had no effect on the extent of TNF-α–induced apoptosis in the absence of vitamin C (data not shown).

Apoptosis signaling in ECs involves the release of cytochrome C from mitochondria18 and the subsequent activation of caspase-9, which initiates execution of the cell death program.19 Therefore, we investigated whether the antiapoptotic activity of vitamin C interferes with TNF-α–induced cytochrome C release and caspase-9 activation. Coincubation of ECs with vitamin C inhibited the translocation of cytochrome C from mitochondria to the cytosol in response to TNF-α (Figure 1E) and suppressed caspase-9 activation in response to TNF-α almost to baseline (Figure 1F). Taken together, these data indicate that vitamin C interferes with apoptosis signaling in activated ECs via the inhibition of cytochrome C release from mitochondria and the subsequent suppression of caspase-9 activation.
Effects of Vitamin C Therapy on EC Apoptosis in Patients With CHF

Our next aim was to demonstrate the antiapoptotic effect of vitamin C therapy in CHF patients in vivo. Therefore, 34 patients were prospectively randomized in a double-blind fashion to receive either vitamin C or placebo. As the Table illustrates, there were no significant differences between the vitamin C and the placebo groups with respect to age, sex, clinical severity of CHF according to the NYHA classification, CHF origin, left ventricular function, and concomitant therapy.

Vitamin C therapy in CHF patients by combined intravenous and oral treatment significantly reduced apoptosis induction by patient serum to 76 ± 6% of baseline (P = 0.001 versus baseline), whereas placebo administration had no such effect (95 ± 3%; P = 0.02 versus vitamin C; Figure 2A). After the initial intravenous dose only, vitamin C administration significantly though to a lesser extent decreased the proapoptotic activity of patient serum (82 ± 7%; P = 0.01 versus baseline, P < 0.05 versus placebo). Responses to vitamin C did not differ between patients with CHF caused by ischemic heart disease (74 ± 9%) and those with nonischemic heart disease (78 ± 7%; P = NS). Ex vivo addition of vitamin C to the serum of CHF patients also profoundly reduced serum-induced EC apoptosis induction as assessed by morphological analysis of DAPI-stained nuclei (Figure 2B) or DNA laddering (Figure 2C).

To assess the in vivo relevance of our findings and to exclude that vitamin C remaining in the serum used to stimulate EC apoptosis contributes to the observed effects on ex vivo apoptosis induction by CHF patient serum, we examined whether vitamin C therapy reduced the plasma load
patients confirmed that a significant proportion of the detected residual bodies was of endothelial origin. On vitamin C treatment, CD31-positive microparticles decreased to about the same degree as the total amount of microparticles detected by the prothrombinase assay, whereas they remained unchanged in the placebo group (data not shown). These data indicate that the observed decrease in total apoptotic microparticles can be attributed at least in part to an effect on EC apoptosis. The reduction of apoptotic microparticles was significant only after sequential intravenous and oral treatment with vitamin C, whereas the initial single intravenous bolus dose had no effect different from placebo (Figure 3A). Taken together, these data suggest that vitamin C therapy reduces apoptosis in vivo in patients with CHF.

**Effect of Vitamin C on Markers of Oxidative Stress and TNF-α Serum Concentrations in CHF**

To examine whether the observed protective effects of vitamin C administration were associated with an antioxidative effect of vitamin C, we measured plasma TBARS as a marker of oxidative stress. In accordance with previous reports,5,20,21 CHF patients revealed significantly higher levels of oxidative stress than healthy control subjects (data not shown). Vitamin C treatment significantly reduced TBARS, whereas placebo had no effect (Figure 3B). In addition, TNF-α serum concentrations in CHF patients were determined before and after vitamin C administration. As reported previously, TNF-α levels in CHF patients were significantly elevated by ≈2.5-fold compared with an age- and sex-matched control population (data not shown). However, neither vitamin C nor placebo treatment significantly altered TNF-α serum concentrations (Figure 3C). Likewise, serum levels of other cytotoxic factors capable to induce EC apoptosis in vitro, like sFas, sFasLigand, and sTNFRI, were elevated in CHF patients compared with healthy volunteers but did not change in response to vitamin C or placebo administration (data not shown). Therefore, altered serum levels circulating cytokines do not appear to contribute to the observed protective effect of vitamin C on EC apoptosis in CHF patients.

**Discussion**

Our results demonstrate that vitamin C interferes with apoptosis signaling in ECs stimulated by TNF-α, Ang II activation, and serum of patients with CHF. The antiapoptotic activity of vitamin C is associated with the prevention of mitochondrial cytochrome C release and suppression of subsequent caspase activation in activated ECs. Vitamin C treatment in CHF patients is effective in reducing both the proapoptotic activity of CHF serum and the amount of circulating apoptotic membrane microparticles. Thus, protection of ECs from proapoptotic stimuli may contribute to the observed beneficial effect of vitamin C administration on endothelial function in CHF patients.

Ellis et al22 have recently shown that the antioxidant vitamin C both improved endothelial function and reduced oxidative stress in CHF patients. However, because they found no correlation between the improvement in endothelial function and the reduction in systemic oxidative stress, the
authors speculated that additional effects of vitamin C could account for the observed functional benefit. Recent data established a role for EC apoptosis in the pathophysiological sequelae of CHF by demonstrating elevated plasma concentrations of apoptotic membrane microparticles in CHF patients. Moreover, increased EC apoptosis was shown to correlate with a depressed endothelial vasodilator response to acetylcholine. Thus, suppression of EC apoptosis provides a potential site of interaction by which vitamin C could interfere with the development of endothelial dysfunction in CHF. Indeed, the placebo-controlled data presented here demonstrate that vitamin C therapy markedly decreases the plasma load of apoptotic microparticles in patients with CHF (Figure 3). Because the maintenance of the integrity of the endothelium can be assumed to be prerequisite for its preserved function, we hypothesize that the prevention of enhanced EC apoptosis may be a mechanism by which vitamin C protects the vascular wall in CHF.

The profound decrease in circulating apoptotic membrane microparticles on vitamin C therapy of CHF patients may be secondary to a reduction in proapoptotic stimuli in the serum of CHF patients and/or to blockade of intracellular apoptosis signaling pathway by vitamin C, leading to reduced sensitivity of ECs to proapoptotic stimuli. In accordance with previous findings showing inhibition of lipopolysaccharide- and high glucose–induced EC apoptosis by vitamin C in vitro, our observation that vitamin C application inhibits EC apoptosis when applied ex vivo in cell culture supports an intracellular mode of action rather than the dependency on a systemic effect in cell culture. Moreover, because proapoptotic cytokine serum levels did not change on vitamin C administration, a mechanism that involves the regulation of cytokine serum levels by vitamin C appears highly unlikely. In contrast, vitamin C treatment significantly decreases plasma levels of TBARS as a marker of increased oxidative stress in CHF patients. In addition, we demonstrate that vitamin C interferes with intracellular apoptosis signaling at the level of mitochondrial cytochrome C release and upstream of caspase-9 activation. This effect resembles the intracellular mechanism by which carvedilol, the β-blocking agent with antioxidant properties, inhibits apoptosis in activated ECs. Mitochondria release cytochrome C in response to ROS that are either derived from systemic oxidative stress or produced after TNF-α or Ang II stimulation. Thus, antioxidants like vitamin C or carvedilol might interfere with ROS-mediated cytochrome C liberation by directly neutralizing, ie, reducing, ROS and/or by increasing endogenous NO bioavailability. NO interferes with proapoptotic signaling via multiple pathways, leading to stabilization of the mitochondrial membrane. Indeed, the present study demonstrates that the antiapoptotic effect of vitamin C ex vivo in activated ECs depends to a large degree on the endogenous bioavailability of NO. We therefore propose that vitamin C might suppress EC apoptosis by increasing bioactive intracellular NO, which prevents cytochrome C release and blocks apoptosis signaling.

In conclusion, the inhibition of EC apoptosis by vitamin C treatment of CHF patients in vivo and of ECs in vitro provides thorough evidence for a mechanism of clinical relevance by which vitamin C can interfere with disturbances in endothelial function. These findings extend the established involvement of EC death mechanisms in the pathogenesis and progression of atherosclerotic vascular disease to CHF independent of its cause.

Acknowledgments

The Deutsche Forschungsgemeinschaft supported this work (SFB 553 project C2). Dr Rössig additionally received financial support from a young investigator’s grant from the University of Frankfurt. Jörg Hoffmann was supported by a grant from Boehringer Ingelheim Fonds. We are grateful to Susanne Ficus, Christine Göbel, and Iris Henkel for expert assistance.

References