Taurine and Vitamin C Modify Monocyte and Endothelial Dysfunction in Young Smokers

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Background—Endothelial dysfunction initiated by monocyte-endothelial interactions has previously been observed in many vasculopathies, including chronic cigarette smoking. Taurine, a semiessential amino acid, and vitamin C, a naturally occurring antioxidant, have previously been shown to have endothelial protective effects when exposed to proinflammatory insults. Therefore, we hypothesized that taurine and vitamin C would restore endothelial function in young smokers by modifying monocyte-endothelial interactions.

Methods and Results—Endothelial-dependent vasodilatation was assessed in vivo using duplex ultrasonography, and monocyte-endothelial interactions were assessed in vitro using endothelial cell culture (human umbilical vein endothelial cells [HUVECs]) with monocyte-conditioned medium (MCM). Endothelial-dependent vasodilatation was significantly impaired in young smokers compared with nonsmokers. Pretreatment of young smokers for 5 days with 2 g/d vitamin C and, more significantly, with 1.5 g/d taurine attenuated this response. MCM taken from smokers impaired the release of nitric oxide and increased the levels of endothelin-1 release from HUVECs. When HUVECs were cultured with MCM from smokers who had been treated with taurine, the levels of nitric oxide and endothelin-1 returned toward control levels. This was attributed to an upregulation in endothelial nitric oxide synthase expression.

Conclusions—These observations suggest that taurine supplementation has a beneficial impact on macrovascular endothelial function, and an investigation of its effect on altered endothelial function in dyslipidemic states is warranted. **(*Circulation*. 2003;107:410-415.)**

Key Words: amino acids ■ endothelium ■ atherosclerosis ■ nitric oxide synthase ■ smoking

Endothelial dysfunction is well established as one of the primary events in the pathogenesis of atherosclerosis.[1] Monocyte-endothelial interactions have been implicated in the initiation of endothelial dysfunction seen in many vasculopathies, including dyslipidemic states, diabetes mellitus, and chronic cigarette smoking.[2] These dysfunctions have been partially attributed to the impaired activity of endothelial-derived nitric oxide (NO) and overproduction of the vasospastic cytokine endothelin-1 (ET-1).[1,3]

NO activity is critical in the many potentially proatherogenic processes, namely monocyte adhesion, platelet aggregation, vascular smooth muscle proliferation, and oxidative modification of low density lipoprotein (LDL).[3,4] It is also the regulator of vasomotor tone, impairment of which is one of the earliest measurable features of the atheromatous process, predating structural change and the increase in serum lipid levels.[5–7]

ET-1, an endothelial-derived peptide, is a potent vasoconstrictor and growth promoter that has a short plasma half-life but a prolonged onset and duration of action.[8] It is unclear whether ET-1 plays a role in basal physiological function, but the presence of NO decreases its release.[9] Although the physiological significance of ET-1 is well recognized in proven atherosclerotic disease, hypertension, hyperlipidemia, and congestive heart failure, the potential role it may play in mediating endothelial dysfunction is unknown.[10–12]

Impaired flow-mediated dilatation (FMD), a NO mediated response, has been demonstrated by ultrasonic insonation in the brachial arteries of young, otherwise-healthy cigarette smokers and in patients with dyslipidemia and with proven atherosclerosis.[5–7,13] In FMD of the brachial artery, endothelial NO synthase (eNOS) is activated by the mechanical forces of increased shear and stretch, with the resultant release of NO, which induces relaxation of smooth muscle cells in the vessel wall.[14] A normal response to FMD depends on an intact functioning vascular endothelium. This technique has been shown to be reproducible and correlates well with invasive testing of coronary endothelial function.[15,16]

Taurine, a semiessential amino acid, has been shown to be protective of endothelial structure and function after exposure to inflammatory cells, their mediators, and other chemicals.[17,18] This molecule, which is present in high amounts in inflammatory cells, seems to be uniquely capable of modify-

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ing both target and receptor cell homeostasis through antioxidant, calcium flux, and osmoregulatory pathways.[19,20]

We hypothesize that taurine supplementation in doses comparable to those achievable with the daily intake of 100 g of fish would restore to normal the FMD response in otherwise-healthy, young, chronic cigarette smokers.[21] We also hypothesize that this effect is mediated by the restoration of the normal expression of eNOS, the enzyme responsible for the production of NO from L-arginine within the endothelial cell.

These hypotheses were examined in groups of young cigarette smokers who were given a daily dose of l.5 g of taurine orally for 5 days. After this period, FMD responses in the brachial artery were compared with those seen in healthy normal controls and the same group of smokers given 2 g of vitamin C daily for 5 days. The effect of taurine on the in vitro expression of eNOS and the release of NO and ET-1 from human umbilical vein endothelial cells (HUVECs), when cocultured for 24 to 48 hours with monocyte-conditioned medium (MCM) derived from smokers and nonsmokers, was also examined. The role of the inflammatory cytokines interleukin (IL)-lα and IL-1β, tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF) in the modulation of these responses was also assessed.

Methods

Subjects

Fifteen healthy smokers (20 to 37 years) and 15 control nonsmokers (21 to 35 years) were studied. All were normotensive and nondiabetic and were without a history of hyperlipidemia, a family history of premature vascular disease, or any systemic disease predisposing them to endothelial dysfunction. Smokers had a history of smoking of $\geq$2 pack-years (1 pack-year=20 cigarettes per day for 1 year) and had at least one cigarette in the preceding 12 hours. Control subjects were lifelong nonsmokers.

Treatment Protocol

Smokers were supplemented with 2 g/d vitamin C (Roche Pharmaceuticals) or 1.5 g/d taurine (Twinlab) for 5 days. A 2-week washout period was used between supplementation with vitamin C or taurine. All subjects gave informed consent, and the local ethics committee approved the study protocol.

Assessment of Endothelial-Dependent Dilatation: Study Protocol

All studies were performed in a temperature-controlled room (20°C) with the subject in the resting, supine state. The subject's right arm was immobilized in the extended position to allow constant access to the brachial artery for imaging. Brachial artery diameter and flow velocity were measured from B-mode ultrasound images using a 7.0-MHz linear array transducer ultrasound system and a standard Acuson 128XP/l0. Operating parameters were not changed during each study. Images were recorded on videotape for subsequent offline analysis on the same instrument.[13] FMD was then induced by inflating a pneumatic tourniquet placed around the forearm (distal to the scanned part of the artery) to a pressure of 240 mm Hg for 4.5 minutes, followed by release.[7,13–15]

Assessment of Endothelial-Dependent Dilatation: Data Analysis

Results obtained were expressed as diameter change in millimeters. FMD measurements were taken 45 to 60 seconds after cuff deflation. An average of 4 cardiac cycles was analyzed for each measurement.[15]

Preparation of MCM

Whole blood samples were taken from all groups before and after supplementation with taurine (smokers, n=9; nonsmokers, n=9). Peripheral blood mononuclear cells were isolated under sterile conditions by means of a Ficoll layer gradient technique. The percentage of monocytes was assessed using the Simultest Leucogate CD45/CDl4 antibody and run on a FACScan (Becton Dickinson). The FL_1 filter ($\cong$530 nm) was used to detect FITC-labeled CD45, and the FL_2 filter ($\cong$565 nm) detected phycoerythrin-labeled CD14. A minimum number of 10 000 events was collected and analyzed on the software Lysis 11 using flow cytometry.

Cells were corrected to 2×10^6 monocytes per 5 mL of DMEM per 0.5% fetal calf serum per 0.1% (v/v) penicillin/streptomycin solution and were plated in flat-bottomed microtiter plates (Nunc). DMEM was then concentrated in a time (24 and 48 hours) and concentration (2×10^6 monocytes) dependent manner. Samples were obtained at both 24- and 48-hour time points and stored at −40°C.

Preparation of MCM/HUVEC Coculture

HUVECs were isolated from umbilical veins and cultured as previously described.[22] In all experiments reported, HUVECs were used as individual isolates between passages 3 and 5. Isolated HUVECs were made up to 4×10^5 cells/mL. A 0.5-mL cell suspension of this was added to each well of a 24-well plate that was previously coated with 2% gelatin. These cells were then incubated at 37°C in 5% CO_2 for 12 hours, after which the supernatant was removed from each well and 0.25 mL of DMEM/0.25 mL MCM was added and incubated at 37°C in 5% CO_2 for 12 and 24 hours.

NO Assay

NO production was determined indirectly by measuring the concentration of the stable end product nitrite in the supernatant taken from the endothelial cells treated using the Griess reaction.[23]

ET-1 Assay

The concentration of ET-1 was measured directly in the supernatant taken from endothelial cells using an ELISA kit for ET-1 detection (R&D Systems).

Western Blot for eNOS Expression

HUVECs were cultured as previously described, trypsinized at the second passage, and incubated with MCM from each sample group for 6 hours, and then the endothelial cytosolic proteins were extracted. Western blot analysis for eNOS expression was then performed as previously described.[24]

Cytokine Assays

TNF-α, IL-1α, IL-Iβ, and VEGF were detected using ELISA kits for cytokine detection (R&D Systems). These assays used the quantitative sandwich enzyme immunoassay technique.

Results

Effect of Cigarette Smoking on Endothelial Function In Vivo and Investigating the Effects of Vitamin C and Taurine Supplementation

The effect of FMD on brachial artery diameter in smokers (n=15) and nonsmokers (n=15) was assessed using duplex ultrasonography. FMD was observed in control subjects, but this parameter was significantly impaired in smokers. Actual arterial diameter measurements in nonsmokers before and after stimulus were 3.39±0.43 mm before and 3.7±0.38 mm after FMD (P=0.0007 by Wilcoxon signed rank). For smokers, measurements were 3.33±0.49 mm before and

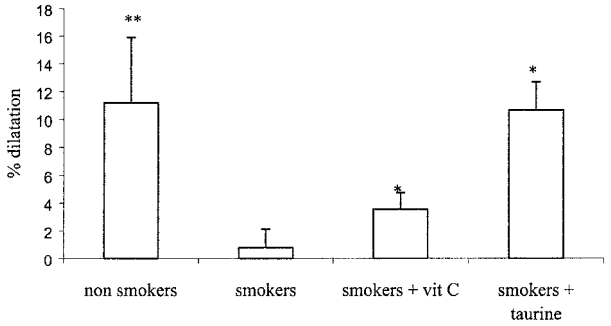

Figure 1. This graph demonstrates the percentage dilatation from baseline before stimulus in response to FMD, therefore testing endothelial-dependent vasodilatation. Smokers had an impaired response to FMD. Supplementation of smokers with both vitamin C (vit C) and taurine partially restored this response, with a more significant effect in the taurine group. *$P<0.05$, **$P<0.01$ vs smokers by Mann-Whitney *U* test.

3.36±0.5 mm after FMD (P=NS by Wilcoxon signed rank; Figure 1).

Oral supplementation of smokers with vitamin C had a significant effect on FMD. Actual arterial diameter measurements in smokers before and after vitamin C supplementation were 3.33±0.49 mm before and 3.45±0.48 mm after supplementation ($P=0.0005$ by Wilcoxon signed rank; Table). However, FMD was not restored to that of normal controls; baseline preflow stimulus values between control nonsmokers and vitamin C–supplemented smokers were not significantly different ($P=0.53$ by Mann-Whitney *U* test), but post-FMD diameters were significantly different between the 2 groups, with the larger increase in diameter in the control group ($P<0.05$ by Mann-Whitney *U* test; Figure 1).

The effect of taurine supplementation on FMD in smokers was also investigated. Oral supplementation with taurine significantly improved FMD in smokers when compared with diameter changes obtained before supplementation. Actual baseline arterial diameter measurements in smokers before and after taurine supplementation were 3.33±0.49 mm before and 3.7±0.47 mm after supplementation ($P=0.0005$ by Wilcoxon signed rank; Table). FMD was restored to that of normal controls; baseline preflow stimulus values between control nonsmokers and smokers on taurine were not significant ($P=0.82$ by Mann-Whitney *U* test), and post-FMD diameters remained nonsignificantly different, which indicates a similar magnitude of response ($P=0.82$ by Mann-Whitney *U* test; Figure 1).

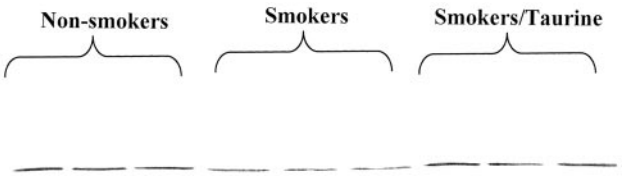

Figure 2. Photomicrograph of a Western blot depicting eNOS expression on HUVECs after coincubation with MCM from nonsmokers (first 3 lanes), smokers (middle 3 lanes), and smokers treated with taurine (last 3 lanes).

Effect of MCM From Smokers, Nonsmokers, and Smokers Treated With Taurine on eNOS Expression

The effect of MCM from smokers and nonsmokers on eNOS production using medium conditioned with monocytes for 24 and 48 hours and coincubated with HUVECs for 24 and 48 hours demonstrate that monocytes isolated from smokers had a downregulatory effect on eNOS production compared with those from nonsmokers. MCM of taurine-treated smokers upregulated eNOS expression (Figure 2).

Effects of MCM on Endothelial NO Production in Smokers, Nonsmokers, and Smokers Supplemented With Taurine

Medium conditioned with monocytes for 24 hours and coincubated with HUVECs for 24 hours demonstrated that monocytes isolated from smokers had a downregulatory effect on endothelial NO production compared with that from nonsmokers; values were 0.002±0.03 nmol/L nitrite for smokers and 0.114±0.02 nmol/L nitrite for nonsmokers ($P=0.0004$ by students 2-sample *t* test). Monocytes isolated from smokers treated with taurine upregulated endothelial NO production compared with pretreatment; values were 0.187±0.022 nmol/L nitrite after supplementation with taurine versus 0.002±0.003 nmol/L nitrite before supplementation ($P<0.0001$ by student's paired *t* test; Figure 3). This relationship remained constant throughout these in vitro experiments at the various time points.

Effects of MCM on Endothelial ET-1 Production in Smokers, Nonsmokers, and Smokers Supplemented With Taurine

The effect of MCM from smokers and nonsmokers on endothelial ET-1 production with medium conditioned by monocytes for 24 hours and coincubated with HUVECs for 24 hours showed that monocytes isolated from smokers upregulated endothelial ET-1 production compared with nonsmokers; values were 68.54±8.1 pg/mL for smokers versus

Actual Brachial Artery Diameter Measurements at Baseline Conditions and After FMD in Smokers, Nonsmokers, and Smokers Supplemented With Vitamin C and Taurine

	Nonsmokers	Smokers	Smokers Given Vitamin C	Smokers Given Taurine
Baseline, mm	3.39±0.43	3.33±0.49	3.33±0.49	3.33±0.49
After FMD, mm	3.70±0.38	3.36±0.5	3.45±0.48	3.7±0.47
P	<0.0007	NS	<0.0005	<0.0005

Values are mean±SD.

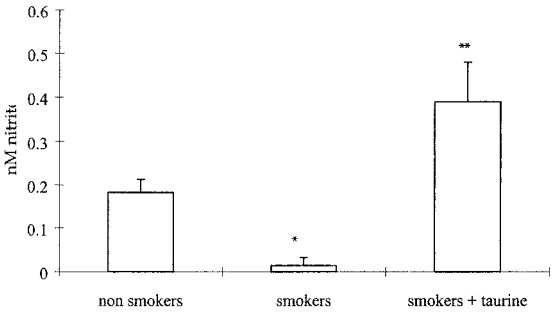

Figure 3. Graph demonstrates the effects of MCM from smokers, nonsmokers, and smokers treated with taurine that was coincubated with HUVECs for 24 hours on NO release, as assessed by nitrite production. A significant reduction in NO release is seen in endothelial cells treated with MCM from smokers. This effect is significantly attenuated by pretreatment with taurine. *$P<0.05$, smokers vs nonsmokers; **$P<0.0001$ for smokers vs smokers on taurine by student's paired *t* test.

32.9±3.9 pg/mL for nonsmokers ($P=0.004$ by student's 2-sample *t* test). When MCM cultured for 24 hours from these same smokers after treatment with taurine was coincubated with HUVECs for 24 hours, ET-1 production was downregulated compared with pretreatment; values were 31.3±6.5 pg/mL after treatment versus 68.5±8.14 pg/mL before treatment ($P=0.01$ by student's paired *t* test; Figure 4). Again, this relationship remained constant throughout these in vitro experiments at the various time points.

Cytokine Content of MCM of Smokers, Nonsmokers, and Smokers Supplemented With Taurine

The MCM of smokers did not contain a significantly greater amount of TNF-α than that of nonsmokers (83±41 pg/mL supernatant versus 62±9 pg/mL supernatant). Also, supplementation of smokers with taurine did not have an effect on TNF-α levels in MCM (83.4±41 pg/mL supernatant before treatment versus 82.5±40 pg/mL supernatant after supplementation).

The MCM of smokers did not contain a significantly different amount of IL-1α than that of nonsmokers (4.2±l.9 pg/mL versus 5.7±l.3 pg/mL). However, supplementing smokers with taurine significantly reduced IL-1α levels in MCM (4.2±l0.8 pg/mL supernatant before supplementation versus 1.7±l0.2 pg/mL supernatant after supplementation; $P<0.05$ by student's paired *t* test).

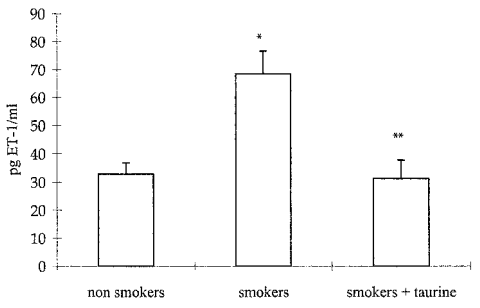

Figure 4. Graph demonstrates the effects of MCM from smokers, nonsmokers, and smokers treated with taurine that was coincubated with HUVECs for 24 hours on ET-1 release, as assessed by ELISA. A significant increase in ET-1 release is seen in endothelial cells treated with MCM from smokers. This effect is significantly attenuated by pretreatment with taurine. *$P<0.05$, nonsmokers vs smokers; **$P<0.01$ for smokers vs smokers on taurine by student's paired *t* test.

In a similar fashion, the MCM of smokers did not contain a significantly different amount of IL-lβ than that of nonsmokers (12.9±15 pg/mL supernatant versus 20.3±4 pg/mL supernatant). Likewise, supplementation of smokers with taurine did not have an effect on IL-lβ levels in MCM (12.9±15 pg/mL supernatant before versus 28±27 pg/mL supernatant after supplementation).

The MCM of smokers did contain a significantly greater amount of VEGF than that of nonsmokers (192±250 pg/mL supernatant in smokers versus 19±95 pg/mL supernatant in nonsmokers; $P<0.05$ by student's 2-sample *t* test). After supplementation of smokers with taurine, no effect on VEGF levels in MCM was seen (192±250 pg/mL supernatant before treatment versus 145±152 pg/mL supernatant after treatment).

Discussion

Flow-mediated endothelial-dependent dilatation of conduit arteries is impaired in young smokers and in subjects with known risk factors for atherogenesis.[7,13] Enhanced monocyte-endothelial interactions are implicated in this disease process.[2] Research in this area has focused principally on monocyte adherence to endothelium and subsequent transendothelial migration as important early steps in the genesis of the atheromatous plaque. However, endothelial dysfunction, such as loss of FMD, antedates plaque formation.[2,13] If the monocyte is involved at these earlier stages, it may well be through the release of soluble mediators, which in turn alter basal endothelial NO bioavailability. Decreased release of NO from endothelial cells might result from deficiencies of substrate or cofactors, increased breakdown of NO, or reduced expression of eNOS. Current perspectives on decreased NO availability in the pathogenesis of atherosclerosis point to a reduction in NO production rather than increased inactivation.[25] Barua et al[26] recently described an association between reduced endothelium-dependent vasodilatation and decreased NO production and eNOS activity in smokers.

We hypothesized that cigarette smoking results in altered FMD, this is associated with a monocyte-mediated reduction in the endothelial expression of eNOS, and these effects are abrogated by a 5-day course of supplementation with taurine in a dose of 1.5 g daily. In the present studies, we confirmed the observations of others that there is loss of FMD in otherwise-healthy young cigarette smokers.[7] We report that 5 days of supplementation with vitamin C in a daily dose of 2 g significantly improves but does not restore to normal the dilatory response of the brachial artery to increased flow. This observation is in keeping with the report that an intra-arterial infusion of vitamin C markedly improves forearm blood flow in response to acetylcholine.[27]

Taurine supplementation resulted in the restoration of a normal FMD response. The loss of a normal FMD response has previously been reported in the brachial arteries of both active and passive smokers using the ultrasonic evaluation

used in the present study.[7,28] Endothelial dysfunction has also been demonstrated in the coronary circulation of smokers with angiographically normal coronary arteries, thus supporting the suggestion that abnormalities of the endothelium may be one of the earliest manifestations of vasculopathies.[2,5,6]

In these present studies, MCM harvested from cigarette smokers and cocultured with HUVECs resulted in decreased release of NO, increased release of ET-1, and decreased expression of eNOS. Taurine supplementation for 5 days was associated with normalization of NO and ET release and, critically, of eNOS protein expression. In vitro decreased availability of NO in smokers has been reported by others. However, the emphasis has been on decreased activity because of lack of substrate or cofactor or an increased deactivation of NO itself by oxidants such as superoxide.[3,4] Others, however, have demonstrated that the inhibitory effect of activated monocytes on eNOS expression correlates with NO release from cultured aortic endothelial cells and is not mediated by reactive oxygen species.[29] This suggests that other soluble mediators, such as cytokines like IL-1α, IL-1β, and TNF-α, may be responsible for the observed effects. We found that the release of these mediators by smoker's monocytes were not significantly different from normal. Furthermore, although there was a significant increase from smoker's monocytes in the release of VEGF, a cytokine with a suggested reciprocal relationship with NO, this was not altered by taurine supplementation; thus, VEGF was not responsible for the downregulation of eNOS seen in this study.

The effects seen in this clinical study are supported by previous observations of the cytoprotective ability of this amino acid under many in vitro and in vivo experimental and clinical studies of endothelial injury.[17–19] Uniquely, taurine seems to have the ability to maintain or restore both target and effector cellular homeostasis in a hostile environment. The protective effects of taurine on cell function are critically dependent on intracellular levels of the amino acid, which in turn are influenced by the concentration of taurine in the extracellular environment and by the inhibitory influences of pro-inflammatory mediators and cytokines. Although osmoregulation and intracellular antioxidant activities contribute to taurine's involuntary effects on inflammatory cell function, it is probable that control of calcium fluxes is its dominant regulatory function.[19,20]

Taurine seems to be remarkably free of toxicity and has been used clinically in similar or greater daily doses in patients with diabetes mellitus and congestive cardiac failure.[30] Cigarette smoking interacts adversely with the cardiovascular system at many different sites, including conduit arteries, the microvasculature, and the heart. In addition to its effect on monocyte-endothelial interactions, it may act directly or indirectly through its myriad of components (at least 4000).[31,32] It is therefore unlikely that a single agent will block or reverse the effects of cigarette smoking.

It is interesting to note that a worldwide epidemiological study on dietary prevention and cardiovascular disease, the Cardiovascular Diseases and Alimentary Comparison (CARDIAC) study, an average of 100 g of fish per day was sufficient to keep coronary artery disease mortality as low as that in the Japanese race.[33] The dose of taurine used in the present study is equivalent to that found in 100 g of fresh fish. These studies suggest that taurine supplementation has a beneficial impact on macrovascular endothelial function and that this is mediated, at least in part, by restoration of eNOS protein expression. The soluble mediator or mediators responsible for the downregulation of eNOS expression were not identified. Given the recent observations of decreased expression of this enzyme in atheromatous blood vessels, an evaluation of taurine's effect on altered endothelial function in dyslipidemic states is warranted.

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