

Coadministration of Atorvastatin Prevents Nitroglycerin-Induced Endothelial Dysfunction and Nitrate Tolerance in Healthy Humans

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- Objectives** We aimed to assess whether concurrent administration of atorvastatin would modify the development of tolerance and endothelial dysfunction associated with sustained nitroglycerin (GTN) therapy in humans.
- Background** Animal studies have demonstrated that administration of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors can protect against GTN-induced endothelial dysfunction and tolerance, likely through an antioxidant mechanism.
- Methods** Thirty-six healthy male volunteers were randomized to receive continuous transdermal GTN (0.6 mg/h) and placebo, atorvastatin (80 mg/day) alone, or continuous transdermal GTN (0.6 mg/h) with concurrent atorvastatin (80 mg/day), all for 7 days. On the second visit, forearm blood flow was measured with venous-occlusion strain gauge plethysmography in response to incremental infusions of acetylcholine (7.5, 15, and 30 $\mu\text{g}/\text{min}$). Acetylcholine infusions were coinfused first with saline, and repeated during the coinfusion of vitamin C (24 mg/min). Blood pressure responses to sublingual GTN (400 μg) were assessed on both visits.
- Results** Acetylcholine responses in the GTN plus placebo group were significantly attenuated versus those in the GTN plus atorvastatin and atorvastatin groups ($p < 0.01$). Coinfusion of vitamin C completely restored acetylcholine responses in the GTN plus placebo group ($p < 0.01$ vs. saline coinfusion), but caused no change in either the atorvastatin or the GTN plus atorvastatin groups. Blood pressure responses to sublingual GTN did not significantly change between visits in subjects receiving GTN plus atorvastatin and atorvastatin alone, but were significantly blunted in the GTN plus placebo group ($p < 0.05$).
- Conclusions** The present findings demonstrate, for the first time in humans, that atorvastatin prevents both GTN-induced endothelial dysfunction and nitrate tolerance, likely by counteracting the GTN-induced increase in oxidative stress. (J Am Coll Cardiol 2011;57:93–8) © 2011 by the American College of Cardiology Foundation

Nitroglycerin (GTN) and other organic nitrates are widely used in the management of cardiovascular disease. The vascular effects of GTN are mediated by the release of nitric oxide (NO) or an NO-related species by denitrication of the nitrate ester (1). However, the clinical utility of chronic GTN therapy is limited by the rapid loss of the hemodynamic and anti-ischemic effects, a phenomenon termed *tolerance* (2). Both experimental and clinical observations

indicate that an important cause of nitrate tolerance is an increase in the vascular bioavailability of reactive oxygen species (ROS) (3). Multiple sources of ROS have been described in response to nitrate therapy, including nicotinamide adenine dinucleotide phosphate oxidases, mitochondria, and uncoupled NO synthase (NOS) (1). The increased bioavailability of ROS also results in the development of endothelial dysfunction, a consequence of sustained nitrate therapy that is now well documented (4–6). These observations have led to a number of new concepts concerning the etiology of tolerance, the role of nitrate biotransformation as a trigger of increased free radical production, as well as the exploration of multiple therapeutic strategies to prevent the nitrate-induced increase in ROS (7,8). Recent observations in animal models have demonstrated that concurrent therapy with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors can modify the develop-

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**Abbreviations
and Acronyms**

ACh	= acetylcholine chloride
ANOVA	= analysis of variance
FBF	= forearm blood flow
GTN	= nitroglycerin
HMG-CoA	= 3-hydroxy-3-methylglutaryl coenzyme A
NO	= nitric oxide
NOS	= nitric oxide synthase
ROS	= reactive oxygen species
SBP	= systolic blood pressure

ment of tolerance and endothelial dysfunction associated with chronic GTN therapy by directly counteracting the GTN-induced increase in ROS (9–11). Whether HMG-CoA reductase inhibitor administration can preserve GTN responsiveness and prevent the development of GTN-induced endothelial dysfunction in humans, and whether a similar antioxidant mechanism is involved, has yet to be established.

Methods

The Mount Sinai Research Ethics Board approved this investigator-initiated, non-industry-funded study, and all subjects gave written informed consent before beginning the study. Studies were conducted in a quiet, temperature- and humidity-controlled environment.

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Study population. Thirty-six normal healthy, male, non-smoking volunteers (18 to 27 years of age) were enrolled in a randomized, parallel trial. All subjects were required to fast and abstain from caffeine for 14 hours before the study. Exclusion criteria included any active disease, the use of medications (including supplemental vitamins), as well as risk factors for cardiovascular disease such as hypertension, smoking, a family history of premature cardiovascular disease, and hypercholesterolemia as defined by Canadian guidelines (12).

Study visit 1. After admission into the study, standing blood pressure and heart rate were measured using an automatic, calibrated sphygmomanometer (GE Healthcare, Mississauga, Ontario, Canada). Baseline forearm blood flow (FBF) then was measured by forearm venous-occlusion strain gauge plethysmography, as described previously (4,6). After these measurements were complete, subjects were administered 400 μ g GTN as a sublingual spray, and beat-to-beat blood pressure was monitored from the middle finger of the dominant hand using a finometer system (Finapres Medical Systems, Amsterdam, the Netherlands) for the next 4 min. Thirty minutes later, subjects were randomized to receive transdermal GTN 0.6 mg/h and atorvastatin (80 mg/day), transdermal GTN 0.6 mg/h and matching placebo, or atorvastatin alone. Repeat standing blood pressure and heart rate measurements were obtained 3 h later. At the conclusion of visit 1, subjects were given a 6-day supply of either atorvastatin or placebo and were instructed to take 1 pill daily at 9 AM until the end of the study. Subjects assigned to GTN treatment were given a supply of transdermal GTN 0.6 mg/h for the following 6 days. Subjects were instructed to wear the patch continuously and to change it every morning at 9 AM. All oral medications (placebo and atorvastatin) were administered in

a double-blind fashion, whereas GTN patches were administered in an investigator-blinded fashion (in this case study, personnel not involved in data acquisition or analysis were responsible for randomization procedures and answering any questions subjects had during the study).

Study visit 2. After 7 days of study medication, subjects returned to the laboratory for the assessment of: 1) resistance artery endothelial function and tolerance; and 2) the role of oxidative stress in GTN- and atorvastatin-induced changes in endothelial responses. Standing blood pressure and heart rate measurements were repeated as described in visit 1. After being completed, the brachial artery of the nondominant arm was cannulated as previously described (4). The FBF then was measured during intra-arterial infusions of normal saline and in response to increasing concentrations of the endothelium-dependent vasodilator acetylcholine chloride (ACh), as described previously (4,6). ACh was first coinfused with normal saline; ACh responses subsequently were repeated with a vitamin C coinfusion (24 mg/min) (6). When FBF measurements were complete and blood flow values had returned to baseline, subjects were administered 400 μ g GTN as a sublingual spray and beat-to-beat blood pressure from the middle finger of the dominant hand was measured as described for visit 1.

Statistical analysis. All results are expressed as mean \pm SD. All FBF values are presented as the ratio of the infused versus the noninfused arm (4,8). This approach normalizes the results obtained over time because of small alterations in sympathetic activation, blood pressure, or both (13) and is considered more repeatable and reliable than absolute values of FBF in the infused arm alone (14). Normality was assessed for all variables in each of the 3 groups using the Shapiro-Wilk test. Several of the variables were not normally distributed; the data were transformed using the natural log, which yielded normal distribution. Within- and between-group differences were evaluated with a repeated-measures analysis of variance (ANOVA). A doubly repeated-measures ANOVA was used to assess the differences in FBF between groups, the effect over time (coinfusions), as well as the group by time interaction. A value of $p < 0.05$ was set as the threshold for significance. SAS software (version 9.2, SAS Institute Inc., Cary, North Carolina) was used for all statistical analyses.

Results

Heart rate and blood pressure responses. Results of heart rate and blood pressure responses are summarized in Table 1. Baseline standing heart rate and systolic blood pressure (SBP) did not differ significantly between the groups on visit 1. Heart rate increased significantly 3 h after the first dose of transdermal GTN in the GTN plus placebo and GTN plus atorvastatin groups, and it remained significantly higher on visit 2 in the GTN plus atorvastatin group. After 6 days, heart rate decreased to baseline values in the GTN plus placebo group. Three hours after the administration of

Table 1 Blood Pressure and Heart Rate Responses to Transdermal GTN

	Visit 1		Visit 2
	Baseline	3 h after GTN	
Systolic blood pressure (mm Hg)			
GTN + placebo	117 ± 10	104 ± 7*	118 ± 6
GTN + atorvastatin	116 ± 10	103 ± 8†	109 ± 7‡
Atorvastatin	117 ± 10	117 ± 10	118 ± 12
Heart rate (beats/min)			
GTN + placebo	79 ± 12	100 ± 15*	81 ± 13
GTN + atorvastatin	78 ± 12	94 ± 12†	85 ± 14‡
Atorvastatin	74 ± 12	77 ± 13	79 ± 11

*p < 0.001 versus baseline and visit 2. †p < 0.01 versus baseline and visit 2. ‡p < 0.05 versus baseline.

GTN = nitroglycerin.

the first transdermal preparation, standing systolic blood pressure was significantly lower in the GTN plus placebo and GTN plus atorvastatin groups. On visit 2, SBP returned to baseline values in the GTN plus placebo group. In contrast, standing SBP in the GTN plus atorvastatin group remained lower when compared with baseline. There were no significant differences in blood pressure or heart rate at any time point in the atorvastatin group. Blood pressure and heart rate did not change significantly in response to any of the intra-arterial drug infusions.

Blood pressure responses to sublingual GTN administration. On visit 1, SBP responses to a single sublingual dose of 400 µg GTN were similar between groups (-11 ± 3 mm Hg, -9 ± 3 mm Hg, and -10 ± 4 mm Hg for the GTN plus placebo, GTN plus atorvastatin, and atorvastatin alone groups, respectively; p = NS). On visit 2, SBP responses remained similar in the GTN plus atorvastatin and atorvastatin alone groups and did not differ significantly from the values obtained on visit 1 (-9 ± 3 mm Hg and -9 ± 4 mm Hg for the GTN plus atorvastatin and atorvastatin groups, respectively; p = NS). In contrast, the reduction in SBP with sublingual GTN was significantly blunted in the GTN plus placebo group (-5 ± 3 mm Hg, p < 0.001 vs. visit 1, p < 0.05 vs. GTN plus atorvastatin and atorvastatin groups) (Fig. 1).

FBF responses. On visit 1, FBF was similar between the 3 groups (data not shown). On visit 2, when coin fused with saline, a dose-dependent increase in FBF in response to each infused concentration of ACh was observed in all groups. However, FBF responses were blunted significantly in the GTN plus placebo group compared with the GTN plus atorvastatin and the atorvastatin alone groups (p < 0.01, for the effect of group) (Table 2) (Fig. 2). When coin fused with vitamin C, ACh responses in the GTN plus placebo group were restored compared with saline confusion (p < 0.01, for the interaction of time and group) and did not differ significantly from those in the GTN plus atorvastatin and atorvastatin groups (p = NS, for the effect of group) (Table 2) (Fig. 3). Vitamin C did not alter ACh-

induced responses in the GTN plus atorvastatin or the atorvastatin alone groups.

Changes in lipid profiles. The changes in lipid profiles are summarized in Table 3. After 7 days of atorvastatin administration, significant decreases were observed in total cholesterol levels and low-density lipoprotein cholesterol in the GTN plus atorvastatin and atorvastatin groups. There were no significant differences in lipid profiles between visits in the GTN plus placebo group, nor were there any significant between-group differences.

Discussion

The present study demonstrates, for the first time in humans, the ability of the HMG-CoA reductase inhibitor atorvastatin to prevent the development of tolerance and endothelial dysfunction associated with sustained GTN therapy in normal volunteers, an effect mediated, at least in part, by a reduction of oxidative stress in the vasculature.

Continuous treatment with GTN and other organic nitrates is associated with a loss of clinical efficacy and the development of important abnormalities in endothelial function. The mechanism behind these phenomena seems to be multifactorial, including impaired biotransformation of GTN by mitochondrial aldehyde dehydrogenase and possible neurohumoral activation (15). Furthermore, a large body of work points to the generation of ROS during sustained GTN therapy, initially from mitochondrial and eventually cytosolic sources, as a central component to this pathophysiology (16). Although the interactions of ROS are multiple, an important target is endothelium-derived NO, leading to the formation of peroxynitrite. Peroxynitrite can oxidize tetrahydrobiopterin, uncoupling the NOS enzyme and reducing endothelial NO bioavailability. In support of these hypotheses, previous reports have confirmed that therapy with GTN is associated with the development of significant endothelial dysfunction and NOS uncoupling in

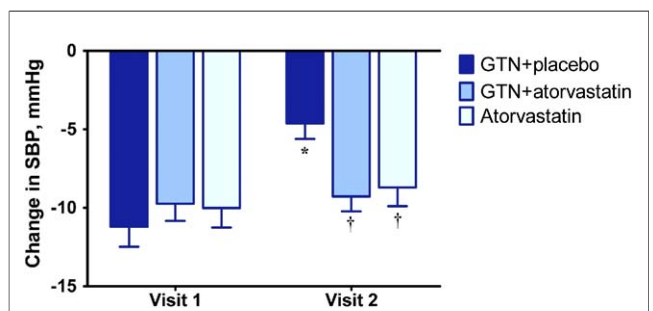


Figure 1 Blood Pressure Responses to Sublingual GTN Administration

Bar graph showing systolic blood pressure (SBP) responses to a single sublingual dose of 400 µg GTN expressed as the change in SBP from baseline. After chronic therapy with GTN, responses were significantly blunted in the GTN plus placebo group but were preserved in the GTN plus atorvastatin group and in the atorvastatin alone group. Data are mean ± SEM. *p < 0.001 for within-group comparison versus visit 1. †p < 0.05 for between-group comparison versus GTN plus placebo group.

Table 2 FBF Responses

	GTN + Placebo	GTN + Atorvastatin	Atorvastatin
Saline/saline	1.1 ± 0.2 (0.1 ± 0.2)*	1.0 ± 0.2 (0.0 ± 0.1)	1.0 ± 0.3 (-0.1 ± 0.3)
Saline/ACh (7.5 μg/min)	1.3 ± 0.5 (0.2 ± 0.4)*	3.5 ± 2.1 (1.1 ± 0.6)	2.9 ± 1.1 (1.0 ± 0.3)
Saline/ACh (15 μg/min)	2.2 ± 1.3 (0.7 ± 0.6)*	4.6 ± 2.5 (1.4 ± 0.6)	4.1 ± 2.0 (1.3 ± 0.4)
Saline/ACh (30 μg/min)	2.7 ± 1.3 (0.9 ± 0.5)*	5.3 ± 2.8 (1.5 ± 0.5)	5.1 ± 2.1 (1.6 ± 0.4)
Vitamin C/saline	1.0 ± 0.1 (0.0 ± 0.1)†	1.0 ± 0.2 (0.0 ± 0.2)	1.0 ± 0.3 (0.0 ± 0.3)
Vitamin C/ACh (7.5 μg/min)	2.9 ± 1.3 (1.0 ± 0.4)†	2.8 ± 1.3 (0.9 ± 0.5)	3.1 ± 0.7 (1.1 ± 0.2)
Vitamin C/ACh (15 μg/min)	4.3 ± 1.8 (1.4 ± 0.4)†	4.2 ± 2.6 (1.2 ± 0.7)	3.2 ± 1.4 (1.1 ± 0.4)
Vitamin C/ACh (30 μg/min)	6.1 ± 2.9 (1.7 ± 0.5)†	4.8 ± 2.7 (1.4 ± 0.5)	4.9 ± 2.5 (1.5 ± 0.5)

Values are expressed as the ratio of the infused to the noninfused arms. Data presented as mean ± SD (mean ln ± SD ln). *p < 0.01, for the effect of group. †p < 0.01, for the interaction of time and group.

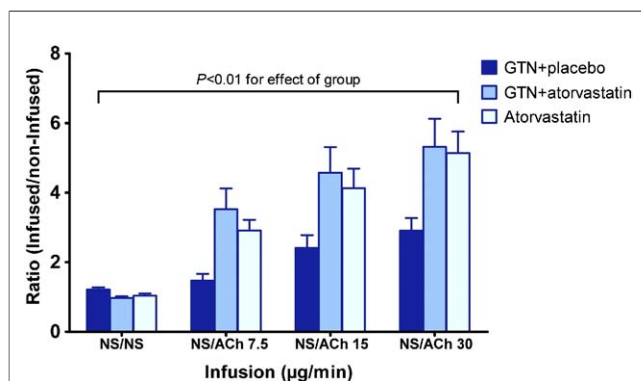
ACh = acetylcholine chloride; FBF = forearm blood flow; ln = natural log; other abbreviations as in Table 1.

both the coronary and forearm circulation in humans (4,5), an effect that can be prevented by the coadministration of antioxidants (6).

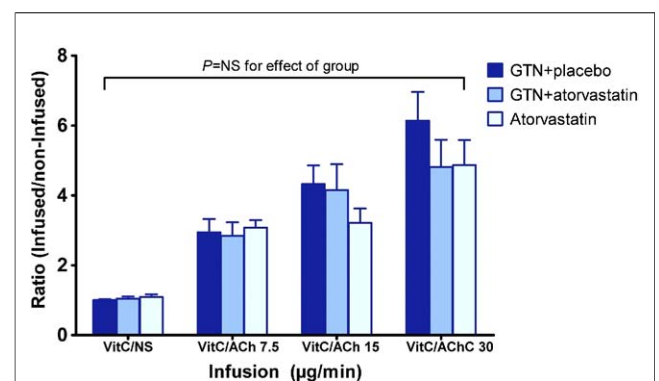
The HMG-CoA reductase inhibitors have become standard therapy in patients with hypercholesterolemia and coronary artery disease and are effective in the primary and secondary prevention of cardiovascular events (17–19). It is now clear that the benefits associated with the HMG-CoA reductase inhibitors extend beyond cholesterol reduction and that these drugs possess cholesterol-independent, or pleiotropic, effects (20). Such effects include antioxidant, anti-inflammatory, antithrombotic, as well as vascular-protective properties, possibly mediated by an increase in NO bioavailability. The HMG-CoA reductase inhibitors have been hypothesized to increase NO bioavailability through multiple mechanisms. These include a direct increase in NOS enzymatic activity, through phosphatidylinositol-3-kinase/protein kinase Akt-mediated phosphorylation; increased production of its essential cofactor tetrahydrobiopterin; and an

increase in NOS messenger ribonucleic acid half-life (20,21). Collectively, these pleiotropic effects may act both to improve and preserve vascular function in response to a number of risk factors or exposures that are associated with the development of endothelial dysfunction and atherosclerosis. Because treatment with organic nitrates, particularly GTN, is associated with the development of endothelial dysfunction, it has been hypothesized that HMG-CoA reductase inhibitors could modify the vascular responses to sustained nitrate therapy. This hypothesis was tested in 3 separate reports where atorvastatin, pravastatin, and rosuvastatin administration were shown to prevent both endothelial dysfunction and nitrate tolerance in the arterial circulation of normocholesterolemic rats (9–11), at least in part, by preventing GTN-associated oxidative stress.

Findings from the current study suggest that atorvastatin prevents the development of nitrate tolerance in humans. On visit 1, standing blood pressure values were significantly lower than baseline values after 3 h in those who received

**Figure 2** FBF Responses to ACh Coinfused With Saline

Bar graph showing responses to incremental intra-arterial infusions of acetylcholine chloride (ACh) (7.5, 15, and 30 μg/min) coinfused with normal saline in the 3 groups. The FBF is expressed as the ratio of infused to noninfused arm. The forearm blood flow (FBF) responses were significantly blunted in the nitroglycerin (GTN) plus placebo group (p < 0.01, for the effect of group). Data are mean ± SEM. Statistical analysis was performed after natural log transformation. NS = normal saline.

**Figure 3** FBF Responses to ACh Coinfused With Vitamin C

Bar graph showing responses to incremental intra-arterial infusions of ACh (7.5, 15, and 30 μg/min) coinfused with vitamin C (VitC) in the 3 groups. The FBF is expressed as the ratio of infused to noninfused arm. The ACh responses were normalized in the GTN plus placebo group when coinfused with vitamin C (p = NS, for the effect of group). The ACh responses were significantly different when coinfused with vitamin C than during normal saline coinfusion (p < 0.01, for the interaction of time and group). Data are mean ± SEM. Statistical analysis was performed after natural log transformation. Abbreviations as in Figure 2.

Table 3 Analysis of Lipid Parameters

	Visit 1				Visit 2			
	Total Cholesterol	HDL	LDL	Triglycerides	Total Cholesterol	HDL	LDL	Triglycerides
GTN + placebo	4.0 ± 0.7	1.5 ± 0.4	2.1 ± 0.6	0.85 ± 0.41	4.0 ± 0.7	1.5 ± 0.4	2.0 ± 0.6	0.83 ± 0.39
GTN + atorvastatin	3.6 ± 0.7	1.4 ± 0.3	1.9 ± 0.6	0.86 ± 0.52	2.4 ± 0.4*	1.3 ± 0.2	0.8 ± 0.4*	0.58 ± 0.24
Atorvastatin	3.8 ± 0.7	1.5 ± 0.3	1.9 ± 0.5	1.01 ± 0.84	3.0 ± 1.2†	1.4 ± 0.3	1.3 ± 0.9†	0.76 ± 0.54

Data are in millimoles per liter. *p < 0.0001 versus corresponding value on visit 1. †p < 0.05 versus corresponding value on visit 1.
 HDL = high-density lipoprotein; LDL = low-density lipoprotein; other abbreviations as in Table 1.

GTN. In contrast to the GTN plus placebo group, where blood pressure values returned to baseline after 7 days of continuous therapy, those in the GTN plus atorvastatin group had values that remained significantly lower than baseline, indicating a sustained vasodilatory effect of GTN. In addition, blood pressure responses to sublingual GTN were blunted significantly in the GTN plus placebo group after 7 days of transdermal GTN therapy, an effect that was prevented by the coadministration of atorvastatin. These results are in agreement with the previously mentioned animal studies documenting a prevention of GTN tolerance through coadministration of an HMG-CoA reductase inhibitor (9–11).

Our results are in agreement with prior reports in animal models that atorvastatin prevents the development of endothelial dysfunction during continuous GTN therapy in humans. We found FBF responses to ACh in the GTN plus atorvastatin group to be significantly greater than those in the GTN plus placebo group after 7 days of treatment. In contrast, we observed a significant increase in FBF responses when ACh was coinjected with vitamin C in the GTN plus placebo group, whereas there was no change in the GTN plus atorvastatin group. Taken together, these results suggest that an important component of atorvastatin's ability to preserve endothelial responses is via a direct or indirect antioxidant mechanism. As mentioned above, the hypothesized sources of ROS in the setting of GTN-induced endothelial dysfunction are multiple; thus, it is possible that atorvastatin may exert its antioxidant effects on multiple targets. Previous studies have suggested that HMG-CoA reductase inhibitors may counteract the GTN-induced increase in ROS by directly decreasing nicotinamide adenine dinucleotide phosphate oxidase activity, by preventing NOS uncoupling and preserving NOS-mediated NO production, or both (9–11). Our group has previously demonstrated that the abnormal responses to ACh during chronic GTN therapy are at least partly related to abnormalities in NOS activity (4,8). This point was emphasized by the fact that these responses can be normalized by pharmacologically preventing NOS uncoupling (8). Similarly, chronic nitrate therapy has been demonstrated to cause an up-regulation in endothelial NOS expression, but a decreased NO bioavailability resulting from uncoupling of the enzyme (22). Our observations suggest that HMG-CoA reductase inhibitors, in this case atorvastatin, have the capacity to restore NOS function via a mechanism that

modifies the increased free radical response that occurs during sustained nitrate exposure (20). This, in turn, seems to prevent both the development of endothelial dysfunction and nitrate tolerance.

In the current study, the FBF responses to ACh in the GTN plus atorvastatin group were the same as those observed in the atorvastatin alone group. This raises the possibility that the effect of atorvastatin may depend on an independent potentiation of FBF responses, rather than a true prevention of nitrate-induced endothelial dysfunction. Two considerations suggest that this is not the case. First, the ACh-induced FBF responses in the atorvastatin alone group were very similar to those previously observed in our laboratory in healthy volunteers receiving no therapy (4), and previous reports consistently showed that HMG-CoA reductase inhibitors have a marginal (if any) impact on FBF responses to ACh in healthy volunteers (23,24). Second, the coadministration of vitamin C markedly improved the FBF responses in the GTN plus placebo group, but did not change the responses in the GTN plus atorvastatin group, which suggests that atorvastatin and vitamin C act via similar mechanisms (i.e., by preventing GTN-induced oxidative stress).

Study limitations. We observed significant reductions in both total cholesterol and low-density lipoprotein levels in subjects who received GTN plus atorvastatin or atorvastatin alone, and thus we cannot discount that the lipid-lowering effect of atorvastatin may have contributed to the preservation of vascular function with chronic GTN. However, the above considerations suggest that our observations do not depend on simple potentiation of FBF responses induced by atorvastatin. We believe that these observations allow us to exclude the lipid-lowering effect of HMG-CoA reductase inhibitor administration as a potential explanation for our observations. The current study used a treatment period of 7 days of continuous therapy, and the impact of longer periods of concurrent therapy, particularly in clinical practice, warrants further investigations. Importantly, endothelial dysfunction in patients with cardiovascular disease also is brought about by risk factors for cardiovascular disease such as age, smoking, hypertension, hyperlipidemia, and diabetes. For this mechanistic study, we elected to recruit normal volunteers to avoid the confounding effects of concurrent drug therapy that also may act to improve endothelial function independently. One intriguing hypothesis raised by these findings is that almost all previous studies describing

the phenomenon of nitrate tolerance and nitrate-induced endothelial dysfunction involved patients not receiving HMG-CoA reductase inhibitors. It is possible that the entire area of nitrate tolerance will require re-exploration in light of the potential effects of cotreatments such as vitamin supplements, HMG-CoA reductase inhibitors, angiotensin-converting enzyme inhibitors, and so forth, which are now common treatment strategies in cardiovascular disease. Further studies will be needed in patients with overt atherosclerotic disease receiving treatment regimens currently being used in clinical practice.

Conclusions

We demonstrate the ability of atorvastatin to prevent the development of tolerance and endothelial dysfunction associated with chronic GTN therapy in healthy volunteers. Furthermore, our data suggest that an antioxidant effect of atorvastatin is at least partly responsible in the prevention of these GTN-induced effects. We believe that our observations emphasize the need for more clinical investigations concerning the impact HMG-CoA reductase inhibition on the efficacy and the vascular impact of organic nitrates.

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