Folic Acid Prevents Nitroglycerin-Induced Nitric Oxide Synthase Dysfunction and Nitrate Tolerance

A Human In Vivo Study

Tommaso Gori, MD; Jason M. Burstein, MD; Sofia Ahmed, MD; Steve E.S. Miner, MD; Abdul Al-Hesayen, MD; Susan Kelly, RN; John D. Parker, MD

Background—In healthy humans, continuous treatment with nitroglycerin (GTN) causes nitric oxide synthase dysfunction, probably through the reduced bioavailability of tetrahydrobiopterin. Recent studies proposed that folic acid is involved in the regeneration of tetrahydrobiopterin in different disease states. Therefore, we investigated whether folic acid administration would prevent this phenomenon. We also sought to determine if folic acid supplementation could prevent the development of tolerance to GTN.

Methods and Results—On the first visit, 18 healthy male volunteers (aged 19 to 32 years) were randomized to receive either oral folic acid (10 mg once a day) or placebo for 1 week in a double-blind designed study. All subjects also received continuous transdermal GTN (0.6 mg/h). 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 μg/min), N-monomethyl-L-arginine (1, 2, and 4 μmol/min), and GTN (11 and 22 nmol/min). Folic acid prevented GTN-induced endothelial dysfunction, as assessed by responses to intraarterial acetylcholine and N-monomethyl-L-arginine (P<0.01). Moreover, in the subjects treated with folic acid plus transdermal GTN, responses to intraarterial GTN were significantly greater than those observed after transdermal GTN plus placebo (P<0.05).

Conclusion—Our data demonstrate that supplemental folic acid prevents both nitric oxide synthase dysfunction induced by continuous GTN and nitrate tolerance in the arterial circulation of healthy volunteers. We hypothesize that the reduced bioavailability of tetrahydrobiopterin is involved in the pathogenesis of both phenomena. Our results confirm the view that oxidative stress contributes to nitrate tolerance. (Circulation. 2001;104:1119-1123.)

Key Words: acetylcholine ■ nitroglycerin ■ endothelium ■ nitric oxide synthase ■ blood flow

Nitroglycerin (GTN) has been used for more than a century in the treatment of coronary artery disease and congestive heart failure. Despite widespread clinical application, the use of GTN and other organic nitrates is limited by the development of tolerance during continuous therapy. The exact mechanism of nitrate tolerance remains uncertain, although evidence suggests that specific vascular biochemical responses may play an important role.1–3

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Human studies documented that GTN treatment is associated with impaired endothelium-mediated vasomotor function in both the coronary and peripheral circulation.4,5 Interestingly, elegant animal studies demonstrated that exposure to GTN causes endothelium-dependent increased bioavailability of superoxide anion.6,7 Superoxide anion and other oxygen free radicals can directly impair nitric oxide bioavailability and also reduce the responsiveness of its messaging mechanisms in smooth muscle cells.7 Although membrane-bound oxidases have been considered responsible for the increased superoxide anion production,3 recent experimental data suggested that abnormalities in nitric oxide synthase (NOS) function might also contribute.6,8 The mechanism for the NOS-dependent superoxide production remains uncertain, and a number of hypotheses have been proposed.6,8,9 Including reduced availability of tetrahydrobiopterin, a cofactor for NOS, and/or L-arginine, its substrate.6,8 Supplemental tetrahydrobiopterin has been shown to reverse NOS dysfunction in conditions of increased oxidative stress, such as hypercholesterolemia, chronic smoking and, recently, GTN treatment.8,10,11

Folic acid and its derivatives have been shown to reverse the endothelial dysfunction associated with hypercholesterolemia,12 hyperhomocysteinemia,13 and after fatty meal.14 The mechanism of action of folic acid has not yet been fully elucidated, but it is hypothesized that folic acid might contribute to the restoration of the bioavailability of tetrahydrobiopterin.15

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preceding each infused drug. Intraarterial blood pressure was rec-

tration was infused for 5 minutes, and FBF measurements were

mL/min with a precision pump (Harvard apparatus). Each concen-

chloride (CIBA) was infused at 7.5, 15, and 30

infused drugs. The endothelium-dependent vasodilator acetylcholine

-NMMA; 1, 2, and 4

N

Inc) was infused at 11 and 22 nmol/min. Finally, FBF responses to

vascular reactivity in response to GTN, intraarterial GTN (Sabex

subject to wear the patch continuously and to change it

given a supply of transdermal GTN 0.6 mg/h for the following 6 days

take one pill daily until the end of the study. All subjects were then

treated with transdermal GTN and/or supplemental folic acid. They received a sequential infusion of GTN at

rates of 11 and 22 nmol/min as described above. Their responses to

intraarterial GTN were compared with those observed in the placebo

and folic acid groups.

Study Population

Eighteen healthy, male, nonsmoking volunteers (19 to 32 years old)

were enrolled. All subjects were requested to abstain from caffeine

on each study day and from any drug, including supplemental vitamins, for the duration of the study.

Study Protocol

This randomized, double-blind, placebo-controlled study was ap-

proved by the University of Toronto Human Subjects Review

Committee, and written, informed consent was obtained from all subjects. The protocol is outlined in Figure 1.

Study Day 1

After screening for admission into the study, standing blood pressure and heart rate measurements were obtained using an automatic, calibrated sphygmomanometer (Critikon Company LLC). The mean of 3 measurements was determined. Baseline forearm blood flow (FBF; Hokanson Inc) was measured with forearm venous occlusion strain gauge plethysmography, as previously described. Subjects were subsequently given the first dose of GTN in the form of a 0.6 mg/h transdermal patch (Transderm Nitro, Ciba Geigy), and repeat standing blood pressure and heart rate measurements were taken 3 hours later.

At the conclusion of visit 1, subjects were randomized in a double-blind fashion to receive either folic acid (10 mg/d PO; folic acid group) or placebo (placebo group), and they were instructed to take one pill daily until the end of the study. All subjects were then given a supply of transdermal GTN 0.6 mg/h for the following 6 days and were instructed to wear the patch continuously and to change it every morning at 9 AM

Study Day 2

Subjects returned to the laboratory after 6 days of continuous therapy with GTN and coadministration of folic acid or placebo. Standing blood pressure and heart rate measurements were taken as described above. After cannulation of the brachial artery as previously de-
scribed,5 FBF was measured at baseline and in response to the infused drugs. The endothelium-dependent vasodilator acetylcysteine

chloride (CIBA) was infused at 7.5, 15, and 30 μg/min. Then, to test vascular reactivity in response to GTN, intraarterial GTN (Sabex Inc) was infused at 11 and 22 nmol/min. Finally, FBF responses to N-monon-methyl-L-arginine (L-NMMA; 1, 2, and 4 μmol/min; Cli-
nalga AG) were measured. The infusion rate was kept constant at 0.4

mL/min with a precision pump (Harvard apparatus). Each concentra-
tion was infused for 5 minutes, and FBF measurements were performed during the last 2 minutes. All responses were evaluated as changes from a baseline value (normal saline infusion) immediately preceding each infused drug. Intraarterial blood pressure was rec-

Values are mean±SE.

*P<0.05 compared with baseline and visit 2; †P<0.05 compared with baseline.

ordered after each infusion (Horizon 2000, Menne Medical Inc) using the average of at least 15 cardiac cycles. ECG was monitored continuously. Between different drug infusions, normal saline was infused until the flow had returned to baseline values.

At the end of the study, the arterial line was removed, all study medications were discontinued, and subjects were discharged from the laboratory.

Control Group

Nine additional volunteers were recruited to undergo FBF measure-

ment at baseline and in response to intraarterial GTN. Subjects in this control group were not treated with transdermal GTN and/or supplement folic acid. They received a sequential infusion of GTN at

rates of 11 and 22 nmol/min as described above. Their responses to intraarterial GTN were compared with those observed in the placebo and folic acid groups.

Statistical Analysis

All FBF values are presented as the ratio of the infused versus the noninfused arm.17 This approach to the measurement of FBF normalizes the results obtained for the normal variability of FBF over time and is considered more repeatable and reliable than the absolute values of FBF in the infused arm alone.17 Within-group differences were evaluated with 1-way ANOVA for repeated measures. Between-group differences were analyzed using 2-way ANOVA. For all results, post hoc comparisons between groups were done with the Bonferroni correction. P<0.05 was set as the threshold for significance. All results are expressed as mean±SE. Statview software, version 5 (SAS Institute Inc), was used for all statistical analyses.

Results

Folic Acid and Homocysteine Levels

In the placebo group on visit 2, the erythrocyte levels of folic acid were all in the normal range (average, 1043±82

nmol/L). At the same time point in the folic acid group, the erythrocyte folic acid concentration in each subject was >1500 nmol/L, which represented the upper detection limit for our local assay. Homocysteine levels were not signifi-
cantly different between groups (placebo group, 7.1 μmol/L; folic acid group, 6.6 μmol/L).

Blood Pressure and Heart Rate Responses

All results are presented in Table 1. Standing heart rate and systolic blood pressure did not differ significantly between groups on visit 1. Compared with baseline values, standing systolic blood pressure was significantly lower in both groups.
at 3 hours after the administration of the first patch. On visit 2, after 6 days of transdermal GTN treatment, systolic blood pressure returned to baseline values in the placebo group. In the folic acid group, systolic blood pressure was persistently lower compared with baseline.

Heart rate increased significantly 3 hours after the first dose of transdermal GTN in both groups, and it remained significantly higher after 6 days in the folic acid group. After the development of nitrate tolerance, heart rate decreased to baseline values in the placebo group.

Blood pressure and heart rate did not change significantly in response to any of the intraarterial drug infusions.

Baseline Blood Flows
On visit 1, FBF was similar between the 2 groups (data not shown). On visit 2, baseline FBF remained similar between groups (Table 2), and within each group, it did not significantly differ from the values obtained on visit 1.

Responses to Acetylcholine Infusions
The dose-dependent increase in FBF in response to each infused concentration of acetylcholine was significantly blunted in the placebo group compared with the folic acid group. In the placebo group, the mean percent increase in response to the highest infused concentration of acetylcholine was 123% of the baseline FBF, whereas in the folic acid group, it was 583% (P<0.01; Table 2 and Figure 2).

Responses to L-NMMA Infusions
In the placebo group, the infusion of the low concentration of L-NMMA caused a small vasodilatation (FBF increase from baseline, 5%) but, as expected, vasoconstriction was observed in the folic acid group (FBF decrease from baseline, 13%; Table 2 and Figure 3). At the higher infused concentration of L-NMMA, the placebo group displayed blunted vasoconstriction (15%) compared with those treated with folic acid (36%; Table 2 and Figure 3). The 2-way ANOVA revealed a highly significant difference between the responses to L-NMMA in the placebo versus the folic acid groups (P<0.01; Table 2 and Figure 3).

Responses to GTN Infusions
Intraarterial infusions of GTN caused a dose-dependent increase in FBF in both groups. In the placebo group, the increase in FBF to the maximal GTN infusion rate (22 nmol/min) was 93%, but in the folic acid group, the increase was significantly greater (183%; P<0.05 by 2-way ANOVA). The response in the folic acid group was not significantly different from that observed in the control group composed of subjects who did not receive any treatment (maximum vasodilatation, 146%; Table 2 and Figure 4).

Discussion
Several animal studies proposed that sustained treatment with GTN causes impaired vascular responses to endothelium-dependent vasodilators.18–20 Two reports in humans, both from our laboratory, confirmed that therapy with GTN is

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<td>GTN 22 nmol/min</td>
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Values are mean±SE and are expressed as the ratio of the infused to noninfused arm. Ach indicates acetylcholine.

*P<0.01 and †P<0.05 compared with placebo group.
associated with the development of significant endothelial dysfunction in both the coronary and forearm circulation in humans.4,5

The cause of the NOS dysfunction associated with GTN therapy remains controversial. It is now accepted that therapy with GTN is associated with increased endothelial bioavailability of superoxide anion. The source of this superoxide anion was originally reported to be membrane-bound NADH and NADPH oxidases.2 More recently, NOS dysfunction has also been implicated as a cause of increased free radical production, and it seems that both these enzyme systems contribute to increased superoxide bioavailability during GTN therapy.6,9 Some have suggested that the NOS-dependent increased bioavailability of superoxide is mediated by protein kinase C phosphorylation of the enzyme, whereas others have proposed that it is caused by local, intracellular depletion of L-arginine.6,21 Interestingly, the activation of protein kinase C and the production of asymmetric dimethylarginine, an arginine analogue that competes with L-arginine for NOS, are increased in conditions of oxidative stress.22,23 These processes might cause NOS dysfunction and further oxidative stress.

In a recent publication, Gruhn et al8 demonstrated that GTN treatment in tolerance-inducing doses causes NOS dysfunction and that tetrahydrobiopterin can reverse this process. It is now recognized that tetrahydrobiopterin is an important cofactor for the activity of NOS. In its reduced form, tetrahydrobiopterin serves to prevent "uncoupling" of NOS, a condition where the enzyme produces increased amounts of superoxide anion and reduced amounts of nitric oxide.24,25 Human experimental evidence has documented that supplemental tetrahydrobiopterin administration can reverse the NOS dysfunction associated with hypercholesterolemia and smoking.10,11 In these conditions, superoxide anion production is increased, reacting rapidly with nitric oxide to form peroxynitrite anion.26 Interestingly, Laursen et al27 recently demonstrated that peroxynitrite-mediated oxidation of tetrahydrobiopterin might cause uncoupling of NOS. An increased production of peroxynitrite has also been demonstrated to occur in nitrate tolerance.28 These findings suggest that under conditions associated with NOS dysfunction, tetrahydrobiopterin availability is reduced because of an increased rate of oxidation to dihydrobiopterin. A number of studies have now demonstrated that folic acid and folate derivatives can reverse and/or prevent the development of abnormal responses to endothelium-dependent vasodilators and vasoconstrictors.12–14 The mechanism of this effect remains uncertain; however, it has been suggested that folic acid is implicated in the regeneration of the reduced form of tetrahydrobiopterin and/or facilitates the functional interaction between tetrahydrobiopterin and NOS.15

In the present study, the responses of FBF to acetylcholine and L-NMMA in the folate group were similar to those observed in a previously reported cohort receiving no GTN.3 Our results demonstrate that supplemental folic acid prevents both the development of NOS dysfunction and GTN tolerance during continuous GTN therapy. Our findings are consistent with the view that therapy with GTN might reduce the functional availability of tetrahydrobiopterin. As mentioned before, there is now considerable evidence to suggest that therapy with GTN is associated with increased production of superoxide anion and peroxynitrite, a situation that would be expected to decrease tetrahydrobiopterin bioavailability. Thus, the results of the current study support the view that NOS dysfunction induced by therapy with GTN may be caused by changes in tetrahydrobiopterin metabolism.

Different potential mechanisms have been proposed to explain the beneficial effects of supplemental folic acid on abnormal endothelial function. Folic acid has been reported to have direct antioxidant effects and to reduce superoxide anion production from xanthine oxidase in vitro.29 Another investigation suggested that the reported inhibitory effect of folic acid on xanthine oxidase was an artifact of the in vitro analytical approach.30 It could also be suggested that folic acid might mediate its beneficial effect on endothelial function through modification of homocysteine concentrations. This effect would be relevant to the present investigation if therapy with GTN increased homocysteine levels. Of note, in a separate experimental cohort (n=10), we found that continuous GTN therapy (0.6 mg/h for 5 days) caused no change in plasma homocysteine levels (data not shown).

In the present study, no significant difference was observed at the end of the treatment period between the 2 groups. In addition, the fact that the positive effect of folic acid can be demonstrated in vitro and during local vascular infusions lends further support to the conclusion that changes in homocysteine concentrations are not involved.15,29 Importantly, there is now clear in vitro evidence confirming that the effect of folic acid on NOS dysfunction is mediated by tetrahydrobiopterin and is independent of both the production of superoxide anion by xanthine oxidase and the direct antioxidant effect of folic acid itself.15 Whether folic acid at the doses used can also influence the activity of other endothelial enzymatic sources of superoxide anion such as vascular NAD(P)H oxidases in the setting of GTN therapy is not yet known. Our study did not include a group not treated with GTN to study the independent effect of folic acid, but previous investigations confirm that folic acid alone does not change the responses to endothelium-dependent mediators.12

We also investigated the effect of folic acid supplementation on the development of nitrate tolerance. The intraarterial infusion of 2 different concentrations of GTN yielded significantly higher responses in the folic acid group. These responses were similar to those obtained in a control group that did not receive transdermal GTN treatment. Thus, our findings suggest that folic acid prevents the development of nitrate tolerance. This effect of folic acid cannot be related to an unspecified potentiation of NO activity, because previous studies demonstrated that folic acid supplementation does not change the effect of sodium nitroprusside.12,29 Also, in our previous study, GTN treatment was not associated with a decreased effect of the NO-donor sodium nitroprusside.5

Previous studies provided evidence that treatment with the antioxidant vitamin C can also prevent nitrate tolerance.31 This effect might be mediated by superoxide anion and peroxynitrite scavenging, leading to preserved activity of NO-dependent signaling mechanisms.7 In addition, a study showed that the beneficial effect of vitamin C is, at least in
part, independent of its antioxidant properties.\textsuperscript{32} This fact and the demonstration that vitamin C stimulates NOS activity through an interaction with tetrahydrobiopterin\textsuperscript{33,34} seem to suggest a mechanism that is similar to that proposed for folic acid.

In summary, our findings suggest that the abnormalities in NOS function observed after treatment with GTN might play a causal role in the development of GTN tolerance. We think that this is compatible with the view that an intact endothelial function is necessary for GTN effect. The beneficial effect of tetrahydrobiopterin in nitrate tolerance has been clearly demonstrated in a recent animal study,\textsuperscript{8} but safety and cost considerations render tetrahydrobiopterin supplementation unfeasible. Nitrate tolerance continues to be a clinical issue. We think that the effectiveness, low cost, and safety of folic acid supplementation confer direct clinical relevance to our findings.

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