Daily low-dose folic acid supplementation does not prevent nitroglycerin-induced nitric oxide synthase dysfunction and tolerance: A human in vivo study

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INTRODUCTION: Continuous treatment with nitroglycerin (GTN) causes tolerance and endothelial dysfunction, both of which may involve endothelial nitric oxide synthase (eNOS) dysfunction. eNOS dysfunction may be linked to depletion of tetrahydrobiopterin, and folic acid may be involved in the regeneration of this cofactor. It has been demonstrated that 10 mg/day folic acid supplementation prevents the development of GTN tolerance and GTN-induced endothelial dysfunction. However, the efficacy of daily lower-dose folic acid supplementation for preventing these phenomena has not been investigated.

OBJECTIVE: To determine the effect of 1 mg/day folic acid supplementation on responses to sustained GTN therapy.

METHODS AND RESULTS: On visit 1, 20 healthy male volunteers were randomly assigned to receive either oral folic acid (1 mg/day) or placebo for one week in a double-blind study. All subjects also received continuous transdermal GTN (0.6 mg/h). On visit 2, forearm blood flow was measured using venous occlusion strain-gauge plethysmography in response to incremental intra-arterial infusions of acetylcholine, N-monomethyl-L-arginine and GTN. Subjects in both groups displayed significantly decreased responses to acetylcholine and N-monomethyl-L-arginine infusions compared with a control group that received no treatment. Responses to GTN were also significantly diminished in both groups ($P < 0.05$ for all).

DISCUSSION: The present data demonstrate that daily supplementation with 1 mg folic acid does not prevent the development of GTN-induced eNOS dysfunction or tolerance.

Key Words: Acetylcholine; Blood flow; Endothelial dysfunction; Folic acid; Nitric oxide synthase; Nitroglycerin; Superoxide

Une faible dose quotidienne de suppléments d'acide folique ne prévient pas la dysfonction de la monoxyde d'azote synthase induite par la nitroglycérine : Une étude *in vivo* chez des humains

INTRODUCTION : Un traitement continu à la nitroglycérine (NTG) provoque une tolérance et une dysfonction endothéliale, qui peuvent toutes deux s'associer à une dysfonction de la monoxyde d'azote synthase endothéliale (eNOS). La dysfonction de l'eNOS peut être liée à une déplétion de la tétrahydrobioptérine, et l'acide folique peut participer à la régénération de ce cofacteur. Il est démontré que 10 mg/jour de suppléments d'acide folique préviennent l'apparition d'une tolérance à la NTG et une dysfonction endothéliale induite par la NTG. Cependant, l'efficacité d'une faible dose de suppléments d'acide folique pour prévenir ce phénomène n'a fait l'objet d'aucune exploration.

OBJECTIF : Déterminer l'effet de suppléments quotidiens de 1 mg d'acide folique sur les réponses à une thérapie soutenue à la NTG.

MÉTHODOLOGIE ET RÉSULTATS : À la première visite, 20 volontaires en santé de sexe masculin ont été divisés de manière aléatoire pour recevoir de l'acide folique par voie orale (1 mg/jour) ou un placebo pendant une semaine dans le cadre d'une étude à double insu. Tous les sujets ont également reçu de la NTG transdermique en continu (0,6 mg/heure). À la deuxième visite, on a mesuré le débit sanguin dans leur avant-bras au moyen d'une pléthysmographie par tensiomètre de l'occlusion veineuse en réponse à des perfusions interartérielles incrémentielles d'acétylcholine, de N-monométhyl-L-arginine et de NTG. Les sujets des deux groupes ont présenté une réduction significative de la réaction aux perfusions d'acétylcholine et de N-monométhyl-L-arginine par rapport au groupe témoin non traité. Les réponses à la NTG diminuaient également de manière significative dans les deux groupes ($P < 0,05$ dans tous les cas).

EXPOSÉ : Les présentes données démontrent que des suppléments quotidiens de 1 mg d'acide folique ne préviennent ni l'apparition d'une dysfonction de l'eNOS induite par la NTG ni la tolérance.

Nitroglycerin (GTN) has been used in the management of stable and unstable angina, acute myocardial infarction and congestive heart failure for more than a century. The development of tolerance to the beneficial hemodynamic and anti-ischemic effects of GTN usually occurs within 48 h of continuous exposure, greatly limiting the clinical usefulness of this therapy (1-4). The cause of nitrate tolerance is considered to be multifactorial, with several proposed mechanisms (2-9). However, both experimental and clinical observations indicate that the primary cause of nitrate tolerance is an increase in the vascular bioavailability of the reactive oxygen species (ROS) superoxide radical anion and peroxynitrite (10-12).

A major functional consequence of increased vascular ROS bioavailability is endothelial dysfunction, which may be followed by diminished endogenous production and/or increased ROS-mediated scavenging of nitric oxide (NO). Our laboratory at the Mount Sinai Hospital (Toronto, Ontario) previously reported that prolonged exposure to GTN caused endothelial dysfunction in healthy subjects (13) and in the coronary circulation of patients with coronary artery disease (14), and that both in vivo and ex vivo GTN exposure had deleterious effects on endothelial progenitor cells, circulating cells that may participate in endothelial repair, and neoangiogenesis in ischemic and infarcted tissues (15). Based on the role of the endothelial enzyme

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Figure 1 Diagram illustrating the experimental protocol. During visit 1, subjects were randomly assigned to receive either placebo or folic acid (1.0 mg/day orally). All subjects concurrently received 0.6 mg/h nitroglycerin (GTN) for six to seven days. During visit 1, strain-gauge plethysmography was performed to establish baseline forearm blood flow. During visit 2, forearm blood flow was measured in response to incremental infusions of acetylcholine, N-monomethyl-L-arginine and GTN

NO synthase (eNOS) in GTN-induced vascular dysfunction, strategies to overcome nitrate tolerance and nitrate-induced endothelial dysfunction have been designed to modulate the production of NO and ROS. Our laboratory previously demonstrated (13) that supplemental high-dose (10 mg/day) folic acid prevents both endothelial dysfunction and nitrate tolerance in the arterial circulation of healthy volunteers. The mechanism of this effect is not known, but it might be mediated by the antioxidant properties of folic acid (16) along with its role in regenerating tetrahydrobiopterin, a cofactor for the NO synthase (17). To date, the minimum dose of folic acid necessary to prevent GTN tolerance and nitrate-induced endothelial dysfunction has not been determined. We previously reported (13) that 10 mg/day folic acid is effective. However, the efficacy of low-dose folic acid for modifying or preventing nitrate tolerance and nitrate-induced endothelial dysfunction has not been investigated. Thus, we sought to determine the impact of 1 mg/day folic acid on the development of GTN tolerance and GTN-induced endothelial dysfunction.

METHODS

Study population

Twenty healthy male, nonsmoking volunteers (19 to 30 years of age) were enrolled after obtaining written, informed consent. All subjects were instructed to abstain from caffeine on each study day and from any drugs, including supplemental vitamins, for the duration of the study.

Study protocol

The present study was approved by the Mount Sinai Hospital Research Ethics Board in Toronto, Ontario. The protocol is outlined in Figure 1.

Strain-gauge plethysmography: Forearm blood flow (FBF) was determined in both arms by venous occlusion strain-gauge plethysmography (DE Hokanson Inc, USA) as described previously (11). Briefly, circulation to the hand was occluded by inflating wrist cuffs to 200 mmHg during measurement periods. The upper arm cuffs were inflated to 40 mmHg and deflated at 10 s intervals (Hokanson rapid cuff inflator, DE Hokanson Inc). FBF was recorded as the average of five consecutive measurements. An investigator who was blinded to the random assignment of the subjects performed all FBF measurements. During visit 1, only basal FBF was measured. During visit 2, FBF was measured at baseline, and in response to normal saline and vasoactive agents as described below.

TABLE 1
Forearm blood flow responses

	Placebo	Folic acid	Control
Saline	1.3±0.2	1.1±0.1	1.0±0.2
ACh 7.5 µg/min	1.8±0.2*	1.7±0.2*	2.0±0.1
ACh 15 µg/min	2.1±0.4*	1.8±0.3*	3.3±0.1
ACh 30 µg/min	2.3±0.4*	2.1±0.5*	3.5±0.1
Saline	1.5±0.2	1.3±0.1	1.4±0.3
GTN 11 nmol/min	1.8±0.2*	1.6±0.1*	2.9±0.1
GTN 22 nmol/min	2.0±0.2*	1.8±0.2*	3.6±0.1
Saline	1.3±0.2	1.1±0.1	1.8±0.2
L-NMMA 1 µmol/min	1.3±0.2*	1.1±0.1*	1.8±0.1
L-NMMA 2 µmol/min	1.2±0.2*	1.0±0.1*	1.3±0.1
L-NMMA 4 µmol/min	1.1±0.2*	0.9±0.1*	1.1±0.1

Data presented as mean ± SEM and expressed as the ratio of the forearm blood flow in the infused to the noninfused arm. *P<0.05 compared with control. ACh Acetylcholine; GTN Nitroglycerin; L-NMMA N-monomethyl-L-arginine

Study day 1: After screening for admission into the study, standing blood pressure and heart rate measurements were taken in triplicate using an automatic, calibrated sphygmomanometer (Critikon Company LLC, USA), and the mean of three measurements was determined. Baseline FBF was measured using forearm venous occlusion strain-gauge plethysmography. Subjects were subsequently given GTN in the form of a 0.6 mg/h transdermal patch (Transderm-Nitro, Novartis, Switzerland), and repeat standing blood pressure and heart rate were measured 3 h later. At the conclusion of visit 1, subjects were randomly assigned in a double-blinded fashion to receive either folic acid (1.0 mg/day orally [folic acid group]) or placebo (placebo group), and instructed to take one tablet daily at 09:00 until the end of the study. All subjects were then given a supply of transdermal GTN 0.6 mg/h for the following seven days. Subjects were instructed to wear the patch continuously and to change it every morning at 09:00.

Study day 2: Subjects returned to the laboratory after seven days of continuous therapy with GTN and either folic acid or placebo. Standing blood pressure and heart rate measurements were taken. Under local anesthesia, a 21-gauge catheter was inserted into the brachial artery of the nondominant arm. A minimum of 15 min later, FBF was measured at baseline and in response to intra-arterial infusions of the endothelium-dependent vasodilator acetylcholine chloride (ACh; 7.5 µg/min, 15 µg/min and 30 µg/min), GTN (11 nmol/min and 22 nmol/min) and the NO synthase inhibitor N-monomethyl-L-arginine (L-NMMA: 1 µmol/min, 2 µmol/min and 4 µmol/min). The infusion rate was kept constant at 0.4 mL/min using a precision pump (Harvard Apparatus, Canada). Each concentration of these vasoactive medications was infused for 6 min, with FBF measurements performed during the last 3 min of the infusion. Between different drug infusions, normal saline was infused until the FBF returned to baseline values, which required no less than 30 min. FBF data are expressed as mL/min/100 mL of forearm in the results section and, in Table 1, as the ratio in the infused versus the noninfused arm to control for systemic variations in blood flow. This latter approach reduces the variability of the data acquired using forearm plethysmography (18). All responses were evaluated as changes from a baseline value (normal saline infusion) measured immediately preceding each infused drug. Intra-arterial blood pressure was recorded after each infusion (Horizon 2000, Mennen Medical Inc, USA) using the average of at least 15 cardiac cycles. The electrocardiogram was also monitored continuously.

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

Control group: Ten additional volunteers were recruited to undergo FBF measurement at baseline and in response to intra-arterial infusion of ACh, GTN and L-NMMA. Subjects in this control group were not treated with transdermal GTN or supplemental folic acid. Their

TABLE 2
Heart rate and blood pressure responses to transdermal nitroglycerin (GTN)

	Visit 1		Visit 2
	Baseline	3 h post-GTN	
Systolic blood pressure, mmHg			
Placebo group	123±4	108±3*	120±3
Folic acid group	118±3	104±3*	118±3
Heart rate, beats/min			
Placebo group	73±4	91±3*	77±4
Folic acid group	70±4	87±5*	76±3

Data presented as mean ± SEM. * $P < 0.05$ compared with baseline and visit 2

responses to ACh, GTN and L-NMMA were compared with those observed in the placebo and folic acid groups.

Sample size

To calculate the number of subjects required to detect a significant change in FBF due to treatment, previously reported values were used for healthy subjects after no treatment or one week of exposure to 0.6 mg/h GTN. Previous data (11,19) show that GTN therapy reduces ACh, GTN and L-NMMA responses by approximately 50%. It was hypothesized that folic acid would improve FBF responses by 30% and, accordingly, calculated that the present study would require 10 subjects per group for $1-\beta=0.8$ and two-sided $\alpha=0.05$.

Statistical analysis

FBF data are presented in mL/min/100 mL of forearm in the results section. Table 1 presents data as the ratio of FBF in the infused versus the noninfused arm – an approach that reduces variability of the method. Within-group differences were evaluated using one-way ANOVA for repeated measures. Between-group differences were analyzed using two-way ANOVA. For all results, post hoc comparisons between groups were conducted using the Bonferroni correction. $P < 0.05$ was set as the threshold for statistical significance. All results are expressed as mean ± SEM. Statview software (SAS Institute Inc, USA) was used for all statistical analyses.

RESULTS

Blood pressure and heart rate responses

All results are presented in Table 2. Compared with baseline values, standing systolic blood pressure was significantly lower in both groups 3 h after administration of the first GTN patch. On visit 2, after seven days of transdermal GTN exposure, systolic blood pressures returned to near baseline in both groups.

Heart rate was significantly increased compared with baseline after 3 h of transdermal GTN in both groups. Heart rate remained slightly higher than baseline values in both groups on visit 2.

Blood pressure and heart rate were not significantly altered in response to any of the intra-arterial drug infusions.

Baseline FBFs

On visit 1, absolute FBF in the infused arm was similar between the groups (GTN + placebo: 4.2 ± 0.4 mL/min/100 mL; GTN + folic acid: 4.3 ± 0.5 mL/min/100 mL of forearm). On visit 2, baseline absolute FBF remained similar among groups (GTN + placebo: 5.5 ± 0.6 mL/min/100 mL; GTN + folic acid 4.0 ± 0.3 mL/min/100 mL; control: 3.8 ± 0.7 mL/min/100 mL of forearm [Table 1]) and within each group. Baseline FBF values obtained during visit 2 did not significantly differ from those obtained during visit 1.

Responses to intra-arterial infusions

Table 1 and Figure 2 refer to FBF data calculated as the ratio of the infused to the noninfused arm. FBF did not change significantly in the noninfused arm. The dose-dependent increase in FBF in response to incremental infusions of ACh was significantly blunted

Figure 2 Forearm blood flow responses to acetylcholine infusion (responses are expressed as the percentage change in the ratio of the infused versus noninfused arm blood flow from baseline). * $P < 0.05$

in both the placebo and the 1 mg/day folic acid groups compared with the control group. The mean percentage increase in absolute blood flow in response to the highest concentration of ACh in the placebo and folate groups was $66 \pm 24\%$ and $99 \pm 44\%$, respectively. These values were significantly attenuated compared with the $241 \pm 44\%$ increase observed in the nontreated control group ($P < 0.05$). Similarly, compared with the nontreated controls and historical controls from previous studies (11,12), subjects receiving placebo and 1 mg/day folic acid showed a blunted dose-dependent vasoconstrictive effect of L-NMMA (Figure 3). The placebo and folic acid groups displayed nearly identical percentage decreases in FBF in response to the highest concentration of L-NMMA at $21 \pm 3\%$ and $21 \pm 5\%$, respectively. These values are significantly lower than the $42 \pm 4\%$ decrease observed in the nontreated controls ($P < 0.05$), although it needs to be acknowledged that a drift in the baseline FBF value in this group might have contributed to this difference. The response to infusions of GTN was not significantly different between the placebo and folic acid groups (Table 1 and Figure 4). These responses were significantly lower than those in the control group (maximal responses in absolute flow: $57 \pm 11\%$ and $49 \pm 12\%$; control group: $178 \pm 35\%$; $P < 0.05$).

DISCUSSION

Therapy with organic nitrates has profound effects on vascular and, particularly, endothelial homeostasis. Studies have shown that chronic exposure to GTN or isosorbide mononitrate, even in doses used in clinical practice, is associated with decreased responsiveness to endothelium-dependent vasodilators and vasoconstrictors, and with impaired autonomic control of heart rate (2-4). The mechanisms of these changes are not entirely understood; however, inappropriate production of oxygen free radicals from mitochondria, membrane oxidases and NO synthase seems to play a major role (2-4). Interestingly, it has been shown that in the setting of chronic GTN exposure, eNOS is uncoupled, a condition in which the enzyme reduces molecular oxygen to the free radical superoxide anion, but is unable to use this molecule to oxidize its substrate L-arginine (5). The net result of this enzymatic dysfunction is that the enzyme produces oxidant free radicals instead of its natural product NO. Folic acid may either act directly as a coenzyme for NO synthase, or regenerate its natural coenzyme tetrahydrobiopterin and recouple the enzyme. We previously documented (13) that supplemental folic acid at a dose of 10 mg/day prevents the development of GTN tolerance and GTN-induced

Figure 3 Forearm blood flow responses to *N*-monomethyl-*L*-arginine infusion (responses are expressed as the percentage change in the ratio of the infused versus noninfused arm blood flow from baseline). * $P < 0.05$

endothelial dysfunction in healthy volunteers. The present study was designed to determine whether folic acid supplementation at a lower dose (1 mg/day) was capable of producing similar effects. Our findings show that 1 mg/day (in addition to current dietary sources) does not prevent these adverse effects of sustained GTN therapy. Taken together, the data from these studies suggest a dose-response effect for folic acid in the setting of continuous exposure to transdermal GTN, and reveal that supplementation must exceed the published recommended daily intake if the negative effects of tolerance and endothelial dysfunction are to be attenuated.

Since several western countries mandated the addition of folic acid to breads, cereals, flours, corn meals, pasta, rice and other grain products in the late 1990s, dietary intake has increased significantly (20-23). In addition to this, many patients take folic acid supplements (usually 1 mg/day to 5 mg/day), particularly if they are identified to have elevated levels of homocysteine. Despite its widespread use, our understanding of the effects and safety of long-term folic acid supplementation is incomplete. Although the risk of toxicity from higher dosages of folic acid (in the range of 10 mg/day, as previously used in our nitrate tolerance study [12]) is low (24), there has been concern about the interaction between vitamin B₁₂ and folic acid because higher doses of folic acid may mask early signs of B₁₂ deficiency (25,26). Furthermore, data confirming a clinical benefit associated with folic acid supplementation in cardiovascular diseases are also lacking, and there has been at least one report (27) that folic acid and vitamin B₁₂ supplementation may even increase the risk of cardiovascular events after acute myocardial infarction. Despite some positive results from short-term clinical studies, in which folic acid and its derivatives were shown to reverse the endothelial dysfunction associated with hypercholesterolemia (28), hyperhomocysteinemia (29) and high-fat meals (30), and in the prevention of nitrate-induced endothelial dysfunction and nitrate tolerance (13), recent large clinical trials (27,31-33) showed no identifiable benefit from supplementation with folic acid and B vitamins. Thus, more research is necessary to definitively characterize the clinical efficacy and safety of folic acid supplementation in cardiovascular disease states.

Limitations

Limitations of the present study need to be acknowledged. These include the limited sample size, the parallel design (and the fact that vasomotor responses were not studied in visit 1), as well as changes in the baseline (preinfusion) FBFs on visit 2, which complicates the interpretation of the present findings. While these limitations warrant some caution in

Figure 4 Forearm blood flow responses to nitroglycerin infusion (responses are expressed as the percentage change in the ratio of the infused versus noninfused arm blood flow from baseline). * $P < 0.05$

drawing definitive conclusions, the present data suggest that supplementation with a low dose of folic acid (including that from dietary sources) might have a neutral effect on GTN side effects. While benefit and safety considerations do not encourage folate supplementation in all cardiovascular patients, high-dose (ie, 10 mg/day) folic acid supplementation, along with concurrent monitoring of serum vitamin B₁₂ and appropriate supplementation to avoid folate-related toxicity, could be used in specific situations, such as in the attempt to attenuate GTN tolerance and GTN-induced endothelial dysfunction.

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