Glutamine metabolism to glucosamine is necessary for glutamine inhibition of endothelial nitric oxide synthesis

Guoyao WU*†[1], Tony E. HAYNES*, Hui LI*, Wene YAN* and Cynthia J. MEININGER†

*Department of Animal Science and Faculty of Nutrition, Texas A&M University, 2471 TAMUS, College Station, TX 77843-2471, U.S.A., and †Cardiovascular Research Institute and Department of Medical Physiology, The Texas A&M University System Health Science Center, College Station, TX 77843-1114, U.S.A.

L-Glutamine is a physiological inhibitor of endothelial NO synthesis. The present study was conducted to test the hypothesis that metabolism of glutamine to glucosamine is necessary for glutamine inhibition of endothelial NO generation. Bovine venular endothelial cells were cultured for 24 h in the presence of 0, 0.1, 0.5 or 2 mM D-glucosamine, or of 0.2 or 2 mM L-glutamine with or without 20 μM 6-diazo-5-oxo-L-norleucine (DON) or with 100 μM azaserine. Both DON and azaserine are inhibitors of L-glutamine:D-fructose-6-phosphate transaminase (isomerizing) (EC 2.6.1.16), the first and rate controlling enzyme in glucosamine synthesis. Glucosamine at 0.1, 0.5 and 2 mM decreased NO production by 34, 45 and 56% respectively compared with controls where glucosamine was lacking. DON (20 μM) and azaserine (100 μM) blocked glucosamine synthesis and prevented the inhibition of NO generation by glutamine. Neither glutamine nor glucosamine had an effect on NO synthase (NOS) activity, arginine transport or cellular tetrahydrobiopterin and Ca^{2+} levels. However, both glutamine and glucosamine inhibited pentose cycle activity and decreased cellular NADPH concentrations; these effects of glutamine were abolished by DON or azaserine. Restoration of cellular NADPH levels by the addition of 1 mM citrate also prevented the inhibiting effect of glutamine or glucosamine on NO synthesis. A further increase in cellular NADPH levels by the addition of 5 mM citrate resulted in greater production of NO. Collectively, our results demonstrate that the metabolism of glutamine to glucosamine is necessary for the inhibition of endothelial NO generation by glutamine. Glucosamine reduces the cellular availability of NADPH (an essential cofactor for NOS) by inhibiting pentose cycle activity, and this may be a metabolic basis for the inhibition of endothelial NO synthesis by glucosamine.

Key words: arginine, endothelial cells, NADPH, pentose cycle.

INTRODUCTION

L-Arginine is the physiological precursor for NO synthesis in endothelial cells [1]. Endothelial NO synthase (NOS), present in both the plasma membrane and the cytosol, requires L-arginine, (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4), NADPH, FAD, FMN, Ca^{2+}, oxygen and calmodulin for its activity [2]. By directly activating soluble guanylate cyclase to generate cGMP from GTP, NO is a potent mediator of various biological responses. Besides serving as a mediator of immune responses, a neurotransmitter and a signalling molecule, NO is an endothelium-derived relaxing factor and plays an important role in regulating vascular tone and permeability [3].

L-Glutamine, the most abundant free amino acid in plasma, is a physiological inhibitor of NO synthesis in endothelial cells [4–7], intact blood vessels [8] and cerebral tissues [9], and therefore has been suggested to have a role in regulating cardiovascular function [6,10,11]. There is evidence to suggest that glutamine inhibits whole-body NO synthesis in rats [12]. Although the inhibition of endothelial NO generation by glutamine has been recognized for 10 years [4,5,8], the mechanism involved remains unknown.

Glutamine is extensively catabolized to glutamate and ammonia in endothelial cells via phosphate-dependent glutaminase [13]. However, neither ammonia nor glutamate has an effect on endothelial NOS activity or NO production [6–9], suggesting that glutamine metabolism via glutaminase is not responsible for the glutamine inhibition of NO synthesis in endothelial cells. An important alternative pathway may be the synthesis of glucosamine, which has recently been reported to markedly reduce blood flow in hind limb femoral muscles [14], implying an inhibition of endothelial NO generation by glucosamine. However, we are not aware of any published report of an effect of glucosamine on endothelial NO production. Furthermore, L-glutamine:D-fructose-6-phosphate transaminase (EC 2.6.1.16; GFAT), the first and rate-controlling enzyme in the synthesis of hexosamine from glutamine and fructose 6-phosphate [15], is, at the present time, thought to be absent from the endothelium [16].

We hypothesized that the metabolism of glutamine to glucosamine is necessary for glutamine inhibition of endothelial NO synthesis. The present study was conducted to test this hypothesis using bovine venular endothelial cells. Our results demonstrate, for the first time, that: (i) glucosamine inhibits endothelial NO synthesis; (ii) endothelial cells are capable of synthesizing glucosamine from glutamine and glucose; (iii) an increase in glucosamine synthesis mediates the inhibiting effect of glutamine on endothelial NO synthesis; (iv) glucosamine, like glutamine, decreases the cellular NADPH concentration by inhibiting pentose cycle activity, and (v) restoration of cellular NADPH levels through an increase in pentose cycle activity completely prevented the inhibiting effect of glutamine or glucosamine on endothelial NO synthesis.

MATERIALS AND METHODS

Chemicals

Calcium- and magnesium-free Dulbecco's PBS and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco

Abbreviations used: BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; DMEM, Dulbecco's modified Eagle's medium; DON, 6-diazo-5-oxo-L-norleucine; GFAT, L-glutamine:D-fructose-6-phosphate transaminase; GlcN-6-P, glucosamine 6-phosphate; KHB, Krebs–Henseleit bicarbonate; NOS, NO synthase; UDP-GlcNAc, UDP-*N*-acetylglucosamine; UDP-GalNAc, UDP-*N*-acetylgalactosamine.

[1] To whom correspondence should be addressed (e-mail g-wu@tamu.edu).

(Grand Island, NY, U.S.A.). Fetal-bovine serum was obtained from Summit (Greeley, CO, U.S.A.). L-[U-^{14}C]Arginine, D-[1-^{14}C]glucose, D-[6-^{14}C]glucose, D-[U-^{14}C]glucose, and UDP-*N*-acetyl[^{14}C]glucosamine (UDP-[*glucose*-^{14}C]GlcNAc) were obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). UDP-[*galactosamine*-1-^{3}H(n)]*N*-acetyl-D-galactosamine (UDP-GalNAc) was purchased from New England Nuclear (Boston, MA, U.S.A.). Sodium citrate, D-glucosamine, L-glutamine, D-glucose, Hepes, BSA (essentially fatty-acid free), NADPH, EDTA, dithiothreitol, D-glucosamine 6-phosphate (GlcN-6-P), D-fructose 6-phosphate, D-glucose 6-phosphate, *o*-phthaldialdehyde, 2,3-diaminonaphthalene, 6-diazo-5-oxo-L-norleucine (DON), azaserine, PMSF, aprotinin, chymostatin and pepstatin A were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). Glucose-6-phosphate dehydrogenase, phosphoglucose isomerase and NADP were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.).

Isolation and culture of bovine venular endothelial cells

Bovine venular endothelial cells were isolated from coronary venules (15 μm in diameter) using a bead perfusion system, as described previously [6,10]. Endothelial cells were cultured in DMEM supplemented with 2 mM L-glutamine, 20 mM D-glucose, 0.4 mM L-arginine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 20 % (v/v) fetal-bovine serum. Cell lines were passaged after trypsin treatment in Dulbecco's PBS containing 0.25 % (w/v) trypsin and 0.02 % (w/v) EDTA [17] and were used at passages 9–15.

Effect of glucosamine on NO synthesis by bovine venular endothelial cells

The release of nitrite and nitrate (two major stable end products of NO oxidation) by cultured endothelial cells was measured as an indicator of NO synthesis. Endothelial cells (4×10^6) were rinsed with Dulbecco's PBS and then cultured at 37 °C for 24 h in 6 ml of DMEM containing 0, 0.1, 0.5 or 2 mM D-glucosamine. The concentrations of D-glucosamine used in cell cultures were based on plasma concentrations (up to 2 mM) in animals infused with glucosamine [18]. The culture medium was supplemented with 0.2 mM L-glutamine, 0.4 mM L-arginine, 10 mM D-glucose, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Extracellular D-glucose (10 mM) was included in the medium in order to generate sufficient cellular NADPH, and a low concentration of L-glutamine (0.2 mM) was used to minimize endogenous synthesis of glucosamine in endothelial cells. In all experiments, culture medium without cells was used as a control. At the end of the 24 h culture period, nitrite concentrations were determined in the conditioned media by its reaction with 2,3-diaminonaphthalene to form 2,3-naphthotriazole [19], except that the nitrite derivative was separated by HPLC using 15 mM sodium phosphate buffer (pH 7.5) containing 50 % methanol [20]. Nitrate was converted into nitrite by nitrate reductase and the solution was used for nitrite analysis by HPLC [20].

Effect of DON and azaserine on NO synthesis in the presence of elevated medium glutamine concentrations

Endothelial cells (4×10^6) were cultured at 37 °C for 24 h in 6 ml of DMEM as described above, except that the medium contained 20 mM D-glucose, 0.2 mM or 2 mM L-glutamine, and 0 or 20 μM DON or 100 μM azaserine, but no glucosamine. A high concentration of glucose (20 mM) was used to mimic hyperglycaemia in diabetic patients and our previous experimental conditions, in which glutamine markedly inhibited NO synthesis by bovine venular endothelial cells [6]. Culture medium without cells was used as a blank. At the end of the 24 h culture period, the conditioned media were used for nitrite and nitrate analysis [20].

Effect of citrate on NO synthesis in endothelial cells cultured with high glutamine concentrations in the medium

Endothelial cells (4×10^6) were cultured at 37 °C for 24 h in 6 ml of DMEM containing 20 mM D-glucose and 0.2 mM or 2 mM L-glutamine, or 2 mM L-glutamine plus 1 mM or 5 mM sodium citrate. Culture medium without cells was used as a blank. At the end of the 24 h culture period, the conditioned media were used for nitrite and nitrate analysis [20].

Effect of citrate on NO synthesis in endothelial cells cultured with high glucosamine concentrations in the medium

Endothelial cells (4×10^6) were cultured at 37 °C for 24 h in 6 ml of DMEM containing 10 mM D-glucose, 0.2 mM L-glutamine, and 0 or 2 mM D-glucosamine, or 2 mM D-glucosamine plus 1 mM or 5 mM sodium citrate. Culture medium without cells was used as a blank. At the end of the 24 h culture period, the conditioned media were used for nitrite and nitrate analysis [20].

NOS activity in bovine venular endothelial cells

NOS activity was measured in endothelial cell extracts as described previously [6]. Briefly, endothelial cells were lysed by three cycles of freezing in liquid nitrogen and thawing in a water bath at 37 °C. The whole-cell extracts were used for NOS assay. The assay medium (0.2 ml) contained 0.3 mM BH_4, 3 mM dithiothreitol, 1 μg/ml calmodulin, 1 mM $CaCl_2$, 1 mM NADPH, 0.2 mM FAD, 0.1 mM FMN, 0.1 mM L-[U-^{14}C]arginine (1500 d.p.m./nmol), cell extracts (0.2 mg of protein) and 10 mM L-valine (an inhibitor of arginase). At the end of a 30 min incubation period, L-[^{14}C]citrulline was separated from L-[^{14}C]arginine using Dowex 50W-X8 resin (Na^+ form).

Arginine transport by bovine venular endothelial cells

L-Arginine transport by endothelial cells was measured at 37 °C using L-[U-^{14}C]arginine, as described previously [6]. Briefly, the oxygenated [O_2/CO_2, 19:1 (v/v)] Krebs–Henseleit bicarbonate (KHB) medium (final volume 0.2 ml) contained 10 mM glucose, 0.4 mM L-[U-^{14}C]arginine (0.05 μCi/ml) and 0, 0.1, 0.5 or 2 mM D-glucosamine. L-Arginine transport was initiated by the addition of 2×10^6 cells and terminated after 2 min by the addition of 0.2 ml of ice-cold 10 mM L-arginine containing 0.05 μCi of D-[2-^{3}H]mannitol as an extracellular marker. [^{3}H]Arginine was separated from the medium using 0.7 ml of bromododecane/dodecane (20:1, v/v) overlaid on 0.2 ml of 1.5 M $HClO_4$, centrifuged at 10000 ***g*** in a microcentrifuge for 1 min, and the radioactivity in the bottom layer was measured by liquid scintillation spectroscopy.

Measurements of cellular arginine and cofactors of NOS

BH_4 in endothelial cells was determined by the HPLC method of Fukushima and Nixon [21], except that 5 mM dithioerythritol (an antioxidant) was included in the BH_4 extraction procedure [22]. NADPH in endothelial cells was determined by the HPLC method of Stocchi et al. [23], except that 1 mM bathophenanthroline disulphonic acid (a bivalent metal chelator) was used to prevent oxidation of NADPH by iron and that NADPH was detected by fluorimetry (excitation 340 nm, emission 460 nm)

to improve sensitivity [22]. Arginine in endothelial cells was determined by an HPLC method involving pre-column derivatization with *o*-phthaldialdehyde [24]. Ca^{2+} levels in endothelial cells were determined with indo-1-acetoxymethyl ester (Molecular Probes, Eugene, OR, U.S.A.), using a stage scanning photometric imaging system (Ultima-Z; Meridian Instruments, Okemos, MI, U.S.A.), exactly as described by Ziche et al. [25]. The fluorescence was monitored at 405 nm and 485 nm, using narrow band-pass filters and dual photomultiplier amplifiers.

Measurement of cellular glucose 6-phosphate and fructose 6-phosphate

Endothelial cells (5×10^6 cells) were lysed in 0.5 ml of 1.5 M $HClO_4$ and the acidified solution was neutralized with 0.25 ml of 2 M K_2CO_3. Cell extracts (0.5 ml) were used for determination of glucose 6-phosphate and fructose 6-phosphate as described by Lang and Michal [26], except that the formation of NADPH was measured using a fluorimeter (excitation 340 nm, emission 460 nm) (Model 450; Turner Fluorimeter; Dubuque, IA, U.S.A.) to improve the assay sensitivity. The amounts of glucose 6-phosphate and fructose 6-phosphate in cell extracts were calculated from glucose 6-phosphate and fructose 6-phosphate standard curves (0–10 nmol/ml).

Determination of GFAT activity

GFAT activity was determined as described by Hebert et al. [27] with modifications. Endothelial cells (5×10^6) were suspended in 0.5 ml of 50 mM sodium phosphate buffer (pH 7.5) containing 100 mM KCl, 1 mM EDTA, 2.5 mM dithiothreitol, 12 mM D-glucose 6-phosphate (a stabilizer of GFAT [28]) and protease inhibitors (5 μg/ml PMSF, 5 μg/ml aprotinin, 5 μg/ml chymostatin and 5 μg/ml pepstatin A). Cells were lysed by three cycles of freezing in liquid nitrogen and thawing at 37 °C. Cell lysates were centrifuged at 10000 ***g*** for 1 min at 4 °C and the supernatant was used for enzyme assay. The assay mixture (0.1 ml) consisted of 12 mM L-glutamine, 12 mM D-fructose 6-phosphate, 12 mM D-glucose 6-phosphate, 1 mM EDTA, 1 mM dithiothreitol and cell extracts (0.1–0.3 mg of protein), and was incubated at 37 °C for 0, 15, 30 or 45 min. The reaction was terminated by the addition of 50 μl of 1.5 M $HClO_4$, and the acidified solution was neutralized with 25 μl of 2 M K_2CO_3. Neutralized extracts were diluted 20 times with water for HPLC (Optima) before GlcN-6-P analysis. GlcN-6-P was separated by an HPLC method which involved precolumn derivatization of GlcN-6-P with *o*-phthaldialdehyde [24]. The mobile phase was as follows: 86 % Solvent A (0.1 M sodium acetate, 0.5 % tetrahydrofuran, 9 % methanol, pH 7.2) and 14 % Solvent B (methanol) from 0 to 14 min; 100 % Solvent B from 14.1 to 18 min; 86 % Solvent A and 14 % Solvent B from 18.1 to 24.5 min. The retention time of GlcN-6-P was 8.24 min, identified with an authentic GlcN-6-P standard. In the GFAT assay, enzyme activity was linear with time (up to 45 min) and cell protein (0.05–0.5 mg).

GlcN-6-P and UDP-GlcNAc synthesis

To improve the sensitivity for detecting the synthesis of GlcN-6-P, UDP-GlcNAc and UDP-GalNAc (the 4-epimerization product of UDP-GlcNAc [15]) in intact endothelial cells, we employed a radiochemical technique. Endothelial cells (1×10^7) were incubated for 1 h in 2 ml of KHB buffer (pH 7.4) containing 1 % BSA, 20 mM D-[U-^{14}C]glucose (450 d.p.m./nmol) and 0.2 mM or 2 mM L-glutamine. Blank media containing all components but no cells were run in parallel. Incubations were terminated by the addition of 0.2 ml of 1.5 M $HClO_4$, and the acidified media were neutralized with 0.1 ml of 2 M K_2CO_3. Neutralized extracts were freeze-dried and suspended in 0.3 ml of purified water (Optima) for determination of ^{14}C-labelled GlcN-6-P, UDP-GlcNAc and UDP-GalNAc. [^{14}C]GlcN-6-P was separated by HPLC, as described above, and the radioactivity in fractions containing [^{14}C]GlcN-6-P was estimated by liquid scintillation spectroscopy. For analysis of [^{14}C]UDP-GlcNAc and [^{14}C]UDP-GalNAc, each neutralized sample was desalted through a 1 ml C_{18} Sep-Pak cartridge (Waters Inc., Milford, MA, U.S.A.) and then applied to a Partisil-10 SAX column (4.6 mm × 250 mm) (Phenomenex Inc., Torrance, CA, U.S.A.). [^{14}C]UDP-GlcNAc and [^{14}C]UDP-GalNAc were separated by an isocratic anion-exchange HPLC system, as described by Weckbecker and Keppler [29]. The fractions containing [^{14}C]UDP-GlcNAc and [^{14}C]UDP-GalNAc were collected and radioactivity was estimated by liquid scintillation spectroscopy. Retention times for UDP-GlcNAc and UDP-GalNAc were 10.8 min and 17.4 min respectively, as identified by authentic [^{14}C]UDP-GlcNAc and [^{3}H]UDP-GalNAc standards.

Measurement of pentose cycle activity

The activity of the pentose cycle, a major source of NADPH in endothelial cells, was determined using [1-^{14}C]glucose and [6-^{14}C]glucose, as described previously for endothelial cells and other cell types [30,31]. To determine the effect of glucosamine on pentose cycle activity, endothelial cells (2×10^6 cells) were incubated in 25 ml Erlenmeyer flasks containing 2 ml of oxygenated [O_2/CO_2, 19:1 (v/v)] KHB buffer (pH 7.4) supplemented with 20 mM Hepes (pH 7.4), 1 % (w/v) BSA, 0.2 mM L-glutamine, 10 mM D-[1-^{14}C]glucose or D-[6-^{14}C]glucose (250 d.p.m./nmol) and 0, 0.1, 0.5 or 2 mM D-glucosamine at 37 °C for 0.5, 1, 1.5 or 2 h. In some experiments, 1 mM or 5 mM sodium citrate was added to the incubation medium containing 2 mM D-glucosamine. To determine the effect of L-glutamine, DON and azaserine on the pentose cycle activity, endothelial cells were incubated as described above, except that the medium contained 20 mM D-[1-^{14}C]glucose or D-[6-^{14}C]glucose (250 d.p.m./nmol), 0.2 mM or 2 mM L-glutamine and 0 or 20 μM DON or 100 μM azaserine. In some experiments, 1 mM or 5 mM sodium citrate was added to the incubation medium containing 2 mM L-glutamine. Incubations were terminated by the addition of 200 μl of 1.5 M $HClO_4$. $^{14}CO_2$ was collected in NCS-II (Amersham, Arlington Heights, IL, U.S.A.) and measured by liquid scintillation spectroscopy. The difference in $^{14}CO_2$ production by [1-^{14}C]glucose and [6-^{14}C]glucose was used to estimate the flux of glucose into the pentose cycle [30,31].

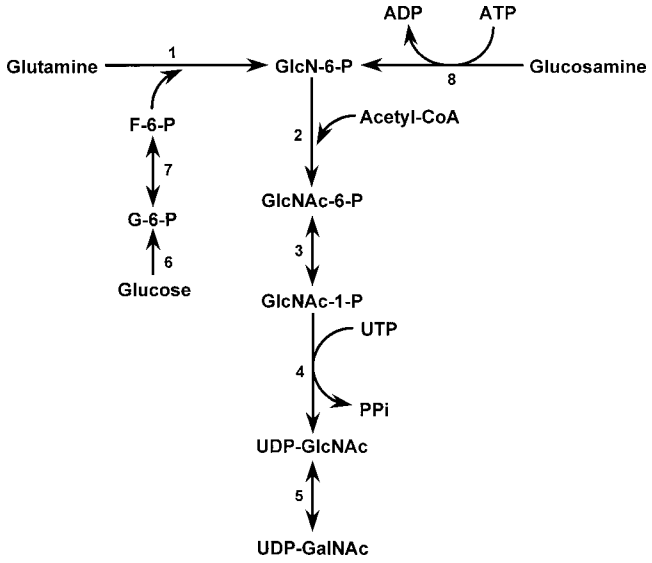
Statistical analyses

Results were statistically analysed by ANOVA with the Student–Newman–Keuls multiple range test, to identify significance among means, by paired Student's *t* test or by correlation analysis [32]. $P < 0.05$ was taken to indicate statistical significance.

RESULTS AND DISCUSSION

Effect of glucosamine on NO synthesis

Glucosamine enters cells via glucose transporters for conversion into GlcN-6-P (Scheme 1) [15]. Glucosamine exerts various important effects, including the inhibition of glucose utilization by insulin-sensitive tissues (e.g. skeletal muscle and adipose tissue) [18,27,33,34] and the regulation of expression of growth factors in smooth muscle cells [35]. Interestingly, recent studies have shown that the intravenous infusion of glucosamine to rats



Scheme 1 Pathway of glucosamine synthesis in endothelial cells

Enzymes that catalyse the indicated reactions are: 1, GFAT; 2, glucosamine-phosphate *N*-acetyltransferase; 3, phosphoacetylglucosamine mutase; 4, UDP-GlcNAc pyrophosphorylase; 5, UDP-GlcNAc 4-epimerase; 6, hexokinase; 7, phosphohexose isomerase; 8, glucosamine kinase. Abbreviations: G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate.

Table 1 Effect of glucosamine on NO synthesis in bovine venular endothelial cells

Bovine venular endothelial cells were cultured at 37 °C for 24 h in DMEM containing 0.2 mM L-glutamine, 10 mM D-glucose and 0, 0.1, 0.5 or 2 mM D-glucosamine. Data are the means ± S.E.M. ($n = 8$) and were analysed by one-way ANOVA. Values with different superscripted letters are significantly different ($P < 0.01$) from each other.

Glucosamine (mM)	Production of nitrite + nitrate (pmol/h per 10^6 cells)
0	238 ± 18^a
0.1	156 ± 14^b
0.5	131 ± 10^c
2.0	105 ± 8^d

markedly reduced blood flow in hind limb femoral muscles [14]. This finding implies, but does not necessarily indicate, an inhibition of endothelial NO synthesis by glucosamine. The results of the present study demonstrate, for the first time, that glucosamine (0.1, 0.5 or 2 mM) inhibited ($P < 0.01$) endothelial NO production in a concentration-dependent manner (Table 1). The inhibition of NO synthesis (34 %) in the presence of 0.1 mM glucosamine was similar to that observed in the presence of 2 mM glutamine (38 %) (Table 2), indicating that glucosamine was a potent inhibitor of endothelial NO synthesis. As NO is the endothelium-derived relaxing factor [3], our novel finding may explain the recent observation of impaired blood flow in glucosamine-infused rats [14].

Activities of GFAT and the synthesis of GlcN-6-P and UDP-GlcNAc

Our finding extends the role of glucosamine to the inhibition of endothelial NO synthesis, but its physiological or pathological relevance will depend on whether endothelial cells can make glucosamine and whether glucosamine synthesis can be modulated by glutamine. GFAT and synthesis *de novo* of GlcN-6-P from glutamine and glucose have previously been considered to be absent from the endothelium [16]. In contrast with this belief, we found that bovine venular endothelial cells contained high GFAT activity (262 ± 13 nmol/h per mg protein) and that these cells were capable of synthesizing GlcN-6-P, UDP-GlcNAc and UDP-GalNAc from glutamine and glucose (Table 3). Increasing extracellular glutamine concentrations from 0.2 mM to 2 mM dramatically increased ($P < 0.01$) the synthesis of GlcN-6-P, UDP-GlcNAc and UDP-GalNAc in endothelial cells (Table 3), indicating a high capacity for endothelial hexosamine synthesis. Because GlcN-6-P can be further metabolized to UDP-GlcNAc (Scheme 1), the amount of GlcN-6-P measured at the end of the 1 h incubation period represents its net accumulation in endothelial cells. UDP-GlcNAc is the end product of the hexos-

Table 2 Effects of glutamine, DON and azaserine on NO synthesis in bovine venular endothelial cells

Bovine venular endothelial cells were cultured in DMEM containing 0.2 or 2 mM L-glutamine, 20 mM D-glucose, and 0 or 20 μM DON or 100 μM azaserine at 37 °C for 24 h. Data are the means ± S.E.M. ($n = 8$) and were analysed by two-way ANOVA. Values with different superscripted letters are significantly different ($P < 0.01$) from each other. $^*P < 0.01$ compared with the control group (no addition of DON or azaserine).

	Production of nitrite + nitrate (pmol/h per 10^6 cells)		
Glutamine (mM)	Control	DON (20 μM)	Azaserine (100 μM)
0.2	230 ± 16	255 ± 22	248 ± 27
2.0	$144 \pm 11^{b*}$	237 ± 26^a	243 ± 29^a

Table 3 Effect of glutamine on the synthesis of GlcN-6-P, UDP-GlcNAc and UDP-GalNAc in bovine venular endothelial cells

Bovine venular endothelial cells were incubated in 2.0 ml of KHB buffer (pH 7.4) containing 0.2 or 2 mM L-glutamine and 20 mM D-[U-^{14}C]glucose at 37 °C for 1 h. Data are the means ± S.E.M. ($n = 8$) and were analysed by paired Student's t test. $^*P < 0.01$ compared with the 0.2 mM glutamine group.

Glutamine (mM)	GlcN-6-P (pmol/h per 10^6 cells)	UDP-GlcNAc (nmol/h per 10^6 cells)	UDP-GalNAc (nmol/h per 10^6 cells)
0.2	1.50 ± 0.17	0.32 ± 0.05	0.08 ± 0.01
2.0	$20.3 \pm 1.64^*$	$3.46 \pm 0.29^*$	$0.98 \pm 0.10^*$

Table 4 Effect of DON or azaserine on the synthesis of GlcN-6-P plus UDP-GlcNAc plus UDP-GalNAc in bovine venular endothelial cells

Bovine venular endothelial cells were incubated for 1 h in 2.0 ml KHB buffer (pH 7.4) containing 2 mM L-glutamine, 20 mM D-[U-^{14}C]glucose, and 0, 20 or 100 μM DON or 0, 20 or 100 μM azaserine. Data are the means ± S.E.M. ($n = 8$) and were analysed by one-way ANOVA. Values with different superscripted letters within the two groups are significantly different ($P < 0.01$) from each other. ND, not detected.

Inhibitor (μM)	Synthesis of GlcN-6-P + UDP-GlcNAc + UDP-GalNAc (nmol/h per 10^6 cells)
DON	
0	4.37 ± 0.32^a
20	0.36 ± 0.04^b
100	ND
Azaserine	
0	4.24 ± 0.38^a
20	3.10 ± 0.25^b
100	0.39 ± 0.05^c

Table 5 Effect of glucosamine on NOS activity, arginine transport and cellular concentrations of arginine, BH_4 and NADPH in bovine venular endothelial cells

Bovine venular endothelial cells were cultured in DMEM containing 0.2 mM L-glutamine, 10 mM D-glucose and 0, 0.1, 0.5 or 2 mM D-glucosamine at 37 °C for 24 h. Cells were then harvested and NOS activity and cellular concentrations of arginine, BH_4 and NADPH were determined. Data are the means ± S.E.M. ($n = 8$) and were analysed by one-way ANOVA. Values with different superscripted letters are significantly different ($P < 0.01$) from each other.

			Cellular concentrations		
Glucosamine (mM)	NOS activity (pmol/min per mg of protein)	Arginine transport (nmol/min per 10^6 cells)	Arginine (nmol/10^6 cells)	BH_4 (pmol/10^6 cells)	NADPH (pmol/10^6 cells)
0	126 ± 15	0.63 ± 0.08	0.49 ± 0.04	2.58 ± 0.21	149 ± 12[a]
0.1	133 ± 17	0.58 ± 0.06	0.47 ± 0.05	2.62 ± 0.19	102 ± 10[b]
0.5	140 ± 21	0.55 ± 0.07	0.50 ± 0.06	2.47 ± 0.17	85 ± 6.4[c]
2.0	124 ± 14	0.60 ± 0.07	0.46 ± 0.05	2.51 ± 0.22	71 ± 5.5[d]

amine synthetic pathway and is a precursor for the formation of all macromolecules containing amino sugars [15]. On the basis of the utilization of glutamine (250 nmol/h per 10^6 cells in the presence of 2 mM glutamine [13]), production of total UDP-GlcNAc (based on net accumulation of UDP-GlcNAc plus UDP-GalNAc) accounted for 0.3 % of the glutamine metabolized by endothelial cells. There was no synthesis of [^{14}C]GlcN-6-P, [^{14}C]UDP-GlcNAc or [^{14}C]UDP-GalNAC in endothelial cells incubated in the presence of 20 mM D-[U-^{14}C]glucose but in the absence of L-glutamine in the incubation medium, indicating glutamine-dependent synthesis of GlcN-6-P and its derivatives in endothelial cells.

Metabolism of glutamine to glucosamine is necessary for glutamine inhibition of endothelial NO synthesis

NO production by endothelial cells was 38 % lower ($P < 0.01$) in the presence of 2 mM L-glutamine compared with 0.2 mM L-glutamine (Table 2). On the basis of our findings that glucosamine was an inhibitor of NO synthesis in endothelial cells and that these cells synthesized glucosamine from glutamine and glucose, we hypothesized that glucosamine may mediate the inhibiting effect of glutamine on endothelial NO synthesis. To test this hypothesis, we used 20 μM DON and 100 μM azaserine, potent inhibitors of GFAT [34]. DON (20 μM) or azaserine (100 μM) had no effect on glutamine metabolism via the phosphate-dependent glutaminase pathway in endothelial cells [13], but effectively blocked glucosamine synthesis (Table 4). Furthermore, DON and azaserine prevented the inhibiting action of glutamine on endothelial NO synthesis (Table 2). Taken together, our results suggest that glutamine metabolism to glucosamine is necessary for glutamine inhibition of endothelial NO synthesis.

Effects of glucosamine on NOS, arginine transport and intracellular concentrations of arginine and cofactors

To provide a metabolic basis for the inhibition of endothelial NO generation by glucosamine, we determined NOS activity, arginine uptake and cellular levels of arginine and cofactors for NOS. Glucosamine had no effect on arginine uptake, NOS activity or intracellular concentrations of arginine, BH_4 (Table 5) and Ca^{2+} (Figure 1) in endothelial cells. The fluorescence ratio (405/485 nm), an indicator of relative changes in cellular Ca^{2+} concentration [25], did not differ ($P > 0.05$) between control endothelial cells (0.598 ± 0.014, means ± S.E.M., $n = 6$) and endothelial cells treated with 2 mM glucosamine (0.604 ± 0.014, means ± S.E.M., $n = 6$). The lack of response to glucosamine was not due to alteration in cell viability, as bradykinin (1 μM) or ionomycin (1 μM) could elicit changes in cellular Ca^{2+} concentrations (Figure 1). The peak fluorescence ratio (405 nm : 485 nm) after the addition of bradykinin and ionomycin was 0.897 ± 0.020 and 1.25 ± 0.026 (means ± S.E.M., $n = 6$) respectively. Remarkably, glucosamine at 0.1, 0.5 or 2 mM decreased intracellular NADPH levels in a concentration-dependent manner (Table 5). Similarly, glutamine (2 mM) had no effect on arginine uptake, NOS activity or cellular concentrations of arginine [6] or BH_4 (Table 6) in bovine venular endothelial cells. Glutamine also had no effect on cellular Ca^{2+} concentration in bovine venular endothelial cells (results not shown). However, like glucosamine, glutamine decreased intracellular NADPH concentration, and this effect was abolished by 20 μM DON or 100 μM azaserine (Table 6). These results suggest that glucosamine decreased cellular NADPH availability in endothelial cells, and that an increase in glucosamine synthesis mediates the effect of glutamine on decreasing cellular NADPH levels, which may be a metabolic basis for the inhibition of endothelial NO synthesis by glucosamine.

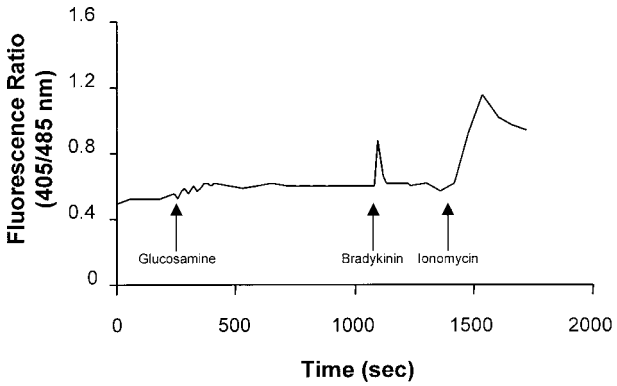


Figure 1 Effect of glucosamine on cellular Ca^{2+} levels in bovine venular endothelial cells

Glucosamine (2 mM) was added to the assay medium, at the time indicated by the arrow. Bradykinin (1 μM) and 1 μM ionomycin were added as positive controls, as indicated. The *y*-axis represents the ratio of the fluorescence of the dye (indo-1-acetoxymethyl ester) bound to Ca^{2+} and the non-bound dye, which gives a reference for changes in intracellular Ca^{2+} concentrations. A representative trace is shown.

Inhibition of pentose cycle activity by glucosamine

As the pentose cycle is the major source of NADPH in endothelial cells [30], we hypothesized that glucosamine (the substrate of GlcN-6-P, a potent competitive inhibitor of glucose-6-phosphate

Table 6 Effects of glutamine, DON and azaserine on cellular concentrations of BH_4 and NADPH in bovine venular endothelial cells

Bovine venular endothelial cells were cultured in DMEM containing 0.2 or 2 mM L-glutamine, 20 mM D-glucose and 0 or 20 μM DON or 100 μM azaserine at 37 °C for 24 h. Cells were then analysed for BH_4 and NADPH content. Data are the means ± S.E.M. ($n = 8$) and were analysed by two-way ANOVA. Values with different superscripted letters are significantly different ($P < 0.01$) from each other. *$P < 0.01$, different from the corresponding value for the 0.2 mM glutamine group.

Additions to culture medium	Cellular BH_4 (pmol/10^6 cells)	Cellular NADPH (pmol/10^6 cells)
0.2 mM Glutamine	2.41 ± 0.27	156 ± 16
0.2 mM Glutamine + 20 μM DON	2.82 ± 0.34	143 ± 13
0.2 mM Glutamine + 100 μM azaserine	2.66 ± 0.29	147 ± 15
2.0 mM Glutamine	2.66 ± 0.30	103 ± 9.2[b*]
2.0 mM Glutamine + 20 μM DON	2.79 ± 0.36	148 ± 14[a]
2.0 mM Glutamine + 100 μM azaserine	2.51 ± 0.32	157 ± 17[a]

Table 7 Effect of glucosamine on $^{14}CO_2$ production from [1-^{14}C]glucose and [6-^{14}C]glucose and the flux of glucose into the pentose cycle in bovine venular endothelial cells

Bovine venular endothelial cells were incubated in KHB (pH 7.4) containing 0.2 mM L-glutamine, 10 mM D-[1-^{14}C]glucose or 10 mM D-[6-^{14}C]glucose and 0, 0.1, 0.5 or 2 mM D-glucosamine at 37 °C for 2 h. Data are the means ± S.E.M. ($n = 8$) and were analysed by one-way ANOVA. Values with different superscripted letters are significantly different ($P < 0.01$) from each other.

	$^{14}CO_2$ production (nmol/2 h per 10^6 cells)		
Glucosamine (mM)	From [1-^{14}C]glucose	From [6-^{14}C]glucose	Flux of glucose into the pentose cycle
0	7.74 ± 0.62[a]	3.51 ± 0.21	4.23 ± 0.32[a]
0.1	6.44 ± 0.50[b]	3.60 ± 0.16	2.84 ± 0.21[b]
0.5	5.65 ± 0.46[c]	3.47 ± 0.24	2.18 ± 0.16[c]
2.0	4.80 ± 0.35[d]	3.01 ± 0.28	1.79 ± 0.12[d]

Table 8 Effect of DON and azaserine on $^{14}CO_2$ production from [1-^{14}C]glucose and [6-^{14}C]glucose and the flux of glucose into the pentose cycle in bovine venular endothelial cells

Bovine venular endothelial cells were incubated in KHB containing 20 mM D-[1-^{14}C]glucose or 20 mM [6-^{14}C]glucose, 0.2 or 2 mM L-glutamine and 0 or 20 μM DON or 100 μM azaserine at 37 °C for 2 h. Data are the means ± S.E.M. ($n = 8$) and were analysed by two-way ANOVA. Values with different superscripted letters within each group are significantly different ($P < 0.01$) from each other. *$P < 0.01$, compared with the corresponding value for the 0.2 mM glutamine group.

	$^{14}CO_2$ production (nmol/2 h per 10^6 cells)		
Additions to culture medium	From [1-^{14}C]glucose	From [6-^{14}C]glucose	Flux of glucose into the pentose cycle
0.2 mM Glutamine	14.0 ± 0.98	6.90 ± 0.52	7.11 ± 0.46
0.2 mM Glutamine + 20 μM DON	13.7 ± 1.12	6.82 ± 0.70	6.88 ± 0.75
0.2 mM Glutamine + 100 μM azaserine	13.4 ± 1.03	6.39 ± 0.64	7.02 ± 0.71
2.0 mM Glutamine	10.6 ± 0.71[b*]	6.02 ± 0.51	4.57 ± 0.24[c*]
2.0 mM Glutamine + 20 μM DON	13.3 ± 0.84[a]	6.10 ± 0.64	7.20 ± 0.69[b]
2.0 mM Glutamine + 100 μM azaserine	13.6 ± 0.91[a]	6.51 ± 0.60	7.10 ± 0.75[a]

dehydrogenase [36]) inhibited this pathway, thereby reducing cellular NADPH concentrations. To test this hypothesis, we used [1-^{14}C]glucose and [6-^{14}C]glucose to determine pentose cycle activity [30,31]. As previously reported for bovine venular endothelial cells [30], the production of $^{14}CO_2$ from 10 mM or 20 mM [1-^{14}C]glucose or [6-^{14}C]glucose in bovine venular endothelial cells increased with increasing incubation time from 0.5 to 2 h (results not shown). Table 7 summarizes the data on the production of $^{14}CO_2$ from [1-^{14}C]glucose and [6-^{14}C]glucose, as well as the flux of glucose into the pentose cycle after incubation for 2 h. The addition of glucosamine (0.1, 0.5 or 2 mM) to the incubation media decreased the production of $^{14}CO_2$ from [1-^{14}C]glucose by endothelial cells in a concentration-dependent manner ($P < 0.01$), but had no effect on $^{14}CO_2$ production from [6-^{14}C]glucose compared with 0 mM glucosamine ($P > 0.05$). As a result, glucosamine at 0.1, 0.5 or 2 mM inhibited endothelial pentose cycle activity by 33, 48 and 58 % respectively compared with that in the absence of glucosamine. Similarly, glutamine (2 mM) markedly decreased the production of $^{14}CO_2$ from [1-^{14}C]glucose ($P < 0.01$) and inhibited pentose cycle activity by 36 % in endothelial cells ($P < 0.01$), compared with 0.2 mM glutamine (Table 8). Furthermore, the inhibiting effect of glutamine on the pentose cycle activity was prevented by 20 μM DON or 100 μM azaserine, which is consistent with the observation that DON or azaserine prevented the decrease in cellular NADPH concentration in endothelial cells incubated with 2 mM glutamine (Table 6). These data indicate that glucosamine inhibits NADPH synthesis via its effect on the pentose cycle, and that an increase in glucosamine synthesis mediates the inhibiting effect of glutamine on this metabolic cycle.

Effect of increasing cellular concentrations of glucose 6-phosphate and NADPH on NO production

Because glucose 6-phosphate cannot enter mammalian cells, we used citrate (a potent inhibitor of phosphofructokinase-I [37]) to

Table 9 Effect of citrate on the flux of glucose into the pentose cycle, cellular concentrations of glucose 6-phosphate, fructose 6-phosphate and NADPH, and NO synthesis in bovine venular endothelial cells incubated with increasing glutamine concentrations

Bovine venular endothelial cells were cultured in DMEM containing 20 mM glucose and 0.2 mM or 2 mM L-glutamine, or 2 mM glutamine plus 1 mM or 5 mM citrate. After the 24 h culture period, cells were harvested for determination of glucose 6-phosphate, fructose 6-phosphate and NADPH, and the medium was used for nitrite/nitrate analysis. For determining the flux of glucose into the pentose cycle, cells were incubated at 37 °C for 2 h in KHB containing 20 mM D-[1-^{14}C]glucose or 20 mM [6-^{14}C]glucose and 0.2 mM or 2 mM glutamine, or 2 mM glutamine plus 1 mM or 5 mM citrate. Data are means ± S.E.M. ($n = 8$) and were analysed by one-way ANOVA. Values with different superscripted letters within each column are significantly different ($P < 0.01$) from each other.

Additions to culture medium	Flux of glucose into pentose cycle (nmol/2 h per 10^6 cells)	Cellular concentrations (pmol/10^6 cells)			Production of nitrite + nitrate (pmol/h per 10^6 cells)
		Glucose 6-phosphate	Fructose 6-phosphate	NADPH	
0.2 mM Glutamine	6.88 ± 0.37^b	117 ± 5.8^c	73.6 ± 4.3^c	151 ± 12^b	225 ± 17^b
2 mM Glutamine	4.45 ± 0.20^c	112 ± 5.5^c	70.4 ± 4.1^c	106 ± 8.9^c	132 ± 10^c
2 mM Glutamine + 1 mM citrate	6.73 ± 0.34^b	158 ± 8.4^b	86.9 ± 6.7^b	153 ± 14^b	214 ± 15^b
2 mM Glutamine + 5 mM citrate	8.84 ± 0.42^a	268 ± 13.5^a	151 ± 8.2^a	249 ± 18^a	363 ± 29^a

Table 10 Effect of citrate on the flux of glucose into the pentose cycle, cellular concentrations of glucose 6-phosphate, fructose 6-phosphate and NADPH, and NO synthesis in bovine venular endothelial cells incubated with increasing glucosamine concentrations

Bovine venular endothelial cells were cultured in DMEM containing 0.2 mM L-glutamine, 10 mM D-glucose and 0 or 2 mM D-glucosamine, or 2 mM glucosamine plus 1 mM or 5 mM citrate at 37 °C for 24 h. The cells were then harvested for determination of glucose 6-phosphate, fructose 6-phosphate and NADPH concentrations, and the medium was used for nitrite/nitrate analysis. For determination of the flux of glucose into the pentose cycle, cells were incubated in KHB containing 10 mM D-[1-^{14}C]glucose or 10 mM [6-^{14}C]glucose, and 0 or 2 mM glucosamine or 2 mM glucosamine + 1 mM or 5 mM citrate at 37 °C for 2 h. Data are the means ± S.E.M. ($n = 8$) and were analysed by one-way ANOVA. Values with different superscripted letters within each column are significantly different ($P < 0.01$) from each other.

Additions to culture medium	Flux of glucose into pentose cycle (nmol/2 h per 10^6 cells)	Cellular concentrations (pmol/10^6 cells)			Production of nitrite + nitrate (pmol/h per 10^6 cells)
		Glucose 6-phosphate	Fructose 6-phosphate	NADPH	
0 mM Glucosamine	4.16 ± 0.26^b	97.3 ± 4.7^c	61.3 ± 4.1^c	142 ± 13^b	221 ± 15^b
2 mM Glucosamine	1.86 ± 0.20^c	88.5 ± 5.1^c	57.2 ± 4.4^c	75 ± 6.6^c	102 ± 10^c
2 mM Glucosamine + 1 mM citrate	4.09 ± 0.32^b	121 ± 6.8^b	77.3 ± 5.0^b	135 ± 11^b	207 ± 17^b
2 mM Glucosamine + 5 mM citrate	5.48 ± 0.39^a	206 ± 9.7^a	131 ± 9.0^a	224 ± 16^a	330 ± 24^a

inhibit the metabolism of fructose 6-phosphate and consequently of glucose 6-phosphate via the glycolytic pathway, thereby increasing the availability of glucose 6-phosphate for the pentose cycle. The addition of 1 mM and 5 mM citrate to the culture medium did indeed increase the flux of glucose into the pentose cycle, in a concentration-dependent manner, in endothelial cells incubated with 2 mM glutamine or 2 mM glucosamine ($P < 0.01$) (Table 9 and Table 10). Consistent with this finding, addition of 1 mM and 5 mM citrate increased ($P < 0.01$) cellular glucose 6-phosphate, fructose 6-phosphate and NADPH levels in a concentration-dependent manner. It should be borne in mind that cellular concentrations of metabolites depend on rates of their synthesis and utilization via multiple pathways. For example, glucosamine inhibits glucose 6-phosphate degradation via the pentose cycle [36], but stimulates the conversion of glucose 6-phosphate into glycogen [27,38]. This may explain the lack of an effect of 2 mM glucosamine on glucose 6-phosphate and fructose 6-phosphate concentrations in endothelial cells compared with the absence of glucosamine (Table 10). It is noteworthy that the restoration of cellular NADPH concentrations with addition of 1 mM citrate completely prevented the inhibiting effect of glutamine or glucosamine on NO synthesis in endothelial cells. Strikingly, a further increase in cellular NADPH concentration with the addition of 5 mM citrate resulted in greater production of NO compared with addition of 1 mM citrate ($P < 0.01$) (Tables 9 and 10). There was a positive correlation between cellular NADPH concentrations and NO synthesis in bovine venular endothelial cells ($r = 0.972$, $P < 0.01$). These findings further suggest that a decrease in cellular NADPH availability is a metabolic basis for inhibition of endothelial NO synthesis by glutamine or glucosamine.

Possible physiological and pathophysiological significance

Like NO, glucosamine may exhibit either beneficial or detrimental effects, depending on physiological or pathophysiological conditions. For example, as an inhibitor of endothelial NO synthesis, glutamine exerted a cardioprotective effect during ischaemia/reperfusion injury [39], which is thought to involve excess production of free radicals such as NO and superoxide anion (O_2^-) [40]. As the production of both NO and O_2^- is NADPH dependent [41], our results raise the question of whether glucosamine, like glutamine, may be beneficial in alleviating or preventing endothelial oxidative damage. On the other hand, recent studies have shown that glucosamine mediates insulin resistance in diabetes [18,27,33,34] and may also have important implications to diabetes-associated cardiovascular complications. The activity of GFAT is enhanced in diabetic animals and humans [38,42]. In addition, plasma concentrations of glucose and glutamine [43], as well as tissue concentrations of fructose 6-phosphate [38], are increased in diabetic subjects with poor metabolic control. Increases in both GFAT activity and its substrate concentrations result in enhanced glucosamine synthesis in diabetes [38], which may explain decreased endothelial NO synthesis and impaired endothelium-dependent relaxation in diabetes [44,45].

In conclusion, the results of this study demonstrate, for the first time, that glutamine metabolism to glucosamine is necessary

for glutamine inhibition of endothelial NO synthesis. Glucosamine formation from glutamine and glucose decreases the cellular availability of NADPH (an essential cofactor for NOS) by inhibiting pentose cycle activity, and this may be a metabolic basis for the inhibition of endothelial NO synthesis by glucosamine. Our findings may have important implications for diabetes-associated cardiovascular complications.

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