Presence of glutamine:fructose-6-phosphate amidotransferase for glucosamine-6-phosphate synthesis in endothelial cells: effects of hyperglycaemia and glutamine

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Abstract

Aims/hypothesis. Recent studies show that glucosamine infusion impairs endothelium-dependent blood flow in normoglycaemic rats. The pathophysiological relevance of this finding, however, depends on whether de novo glucosamine synthesis occurs in endothelial cells. The aim of this study was to test the hypothesis of whether glutamine:fructose-6-phosphate amidotransferase (the first and key regulatory enzyme in hexosamine synthesis) is present for endothelial glucosamine synthesis.

Methods. Bovine venular, bovine aortic, human microvascular, human umbilical vein, and rat coronary microvascular endothelial cells were used to measure glutamine:fructose-6-phosphate amidotransferase activity. To determine glucosamine-6-phosphate synthesis in intact cells, they were incubated for 1 h in Krebs bicarbonate buffer containing 5, 15 or 30 mmol/l [^{14}C]glucose and 0.5, 2 or 4 mmol/l glutamine. The [^{14}C]Glucosamine-6-phosphate and its end products ([^{14}C]UDP-*N*-acetylglucosamine and [^{14}C]UDP-*N*-acetylgalactosamine) were separated by HPLC.

Results. There were high glutamine:fructose-6-phosphate amidotransferase activities in all endothelial cells studied. Exposure of cells to 15 to 30 mmol/l glucose or 2 to 4 mmol/l glutamine increased enzyme activity. Glucosamine-6-phosphate, UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine syntheses increased with increasing extracellular concentrations of glucose from 5 to 30 mmol/l or of glutamine from 0.5 to 4 mmol/l.

Conclusion/interpretation. Our results show the presence of glutamine:fructose-6-phosphate amidotransferase for de novo glucosamine synthesis in endothelial cells and the modulation of this pathway by hyperglycaemia and glutamine. As glucosamine inhibits endothelial nitric oxide synthesis, these findings could have important implications for impaired endothelium-dependent relaxation and vascular dysfunction in diabetes mellitus. [Diabetologia (2001) 44: 196–202]

Keywords Glucosamine-6-phosphate, hyperglycaemia, glutamine, endothelial cells.

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Abbreviations: EC, Endothelial cells; ECGF, endothelial cell growth factor; GFAT, glutamine:fructose-6-phosphate amidotransferase; glucosamine-6-P, glucosamine-6-phosphate; UDP-GlcNAc, UDP-*N*-acetylglucosamine; UDP-GalNAc, UDP-*N*-acetylgalactosamine; UDP, uridine diphosphate; UTP, uridine triphosphate; SNK, Student-Newman-Keuls

Glucosamine-6-phosphate (glucosamine-6-P) is synthesized from L-glutamine and D-fructose-6-P (produced from glucose via glycolysis) in humans and animals [1]. Glucosamine:fructose-6-P amidotransferase (GFAT) catalyses the first and rate-controlling step in the synthesis of UDP-*N*-acetylglucosamine (UDP-GlcNAc) (Fig. 1), a precursor for the formation of all macromolecules containing amino sugars [1]. This enzyme is known to be present in the liver, adipose tissue, smooth muscle, kidney, heart and skeletal muscle [2–6]. An increase in hexosamine biosynthesis has been shown to impair glucose utilization

Fig. 1. Pathway of glucosamine synthesis in endothelial cells. Enzymes that catalyse the indicated reactions are: 1, glutamine:fructose-6-P amidotransferase; 2, glucosamine-phosphate *N*-acetyltransferase; 3, phosphoacetylglucosamine mutase; 4, UDP-*N*-acetyl-glucosamine pyrophosphorylase; 5, UDP-*N*-acetylglucosamine 4-epimerase; 6, hexokinase; 7, phosphohexose isomerase

by insulin-sensitive tissues (such as skeletal muscle and adipose tissue) [7–15]. Thus, there has been growing interest in a role for glucosamine in mediating insulin resistance in diabetes mellitus [14, 15]. It was recently reported that intravenous infusion of glucosamine, which enters cells via glucose transporters for conversion into glucosamine-6-P, considerably reduced blood flow in hind limb femoral muscles of normoglycaemic rats [16]. Similarly, increased concentrations of glutamine and glucose inhibit nitric oxide synthesis in endothelial cells and impair endothelium-dependent relaxation [17–20]. These findings suggest that glucosamine or its precursors affect endothelial cell function and raise an important question of whether increased glucosamine synthesis from glutamine and glucose mediates impaired endothelium-dependent relaxation in diabetes.

Glucosamine:fructose-6-P amidotransferase activity or expression has not been reported for endothelial cells. On the basis of immunohistochemical staining, GFAT was thought to be absent from human endothelium [5]. Identifying the presence of GFAT in endothelial cells is therefore necessary to clarify the role for the hexosamine-synthetic pathway in endothelial dysfunction in diabetes. In the light of our recent finding that glutamine metabolism via a hex-

osamine-synthetic pathway is necessary for glutamine inhibition of nitric oxide synthesis in endothelial cells [21], we hypothesized that GFAT is present for glucosamine-6-P synthesis in these cells.

Materials and methods

Chemicals. L-Glutamine, D-glucose, HEPES, bovine serum albumin (BSA, essentially fatty-acid free), NADH, EDTA, dithiothreitol, D-glucosamine-6-P, D-fructose-6-P, D-glucose-6-P, phenylmethylsulphonyl fluoride, aprotinin, chymostatin and pepstatin A were obtained from Sigma Chemicals (St. Louis, Mo., USA). The L-[U-¹⁴C]Glutamine, L-[U-¹⁴C]glutamate, D-[U-¹⁴C]glucose, and [glucose-¹⁴C]UDP-GlcNAc were obtained from American Radiolabeled Chemicals (St. Louis, Mo., USA). The [Galactosamine-1-³H(N)]-UDP-*N*-acetyl-D-galactosamine (UDP-GalNAc) was purchased from New England Nuclear (Boston, Mass., USA). Calcium-free and magnesium-free Dulbecco's phosphate-buffered saline and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco-BRL (Grand Island, N.Y., USA). Fetal bovine serum was obtained from Summit (Greeley, Colo., USA). Endothelial cell growth factor (ECGF) was obtained from Biomedical Technologies (Stoughton, Mass., USA).

Isolation and culture of endothelial cells. Bovine venular and bovine aortic endothelial cells were isolated from coronary venules (15 µm in diameter) and aorta of adult cattle, respectively, as described previously [18, 22]. Human microvascular endothelial cells were isolated from a mesentery segment of an adult human for non-neoplastic bowel obstruction [22]. Rat coronary microvascular endothelial cells were isolated from the hearts of Sprague-Dawley rats (300–325 g), as described previously [23]. The endothelial identity was confirmed by positive staining for factor VIII-related antigen and uptake of modified low-density lipoprotein [22, 23]. Human umbilical vein endothelial cells were obtained from Clonetics (San Diego, Calif., USA). Endothelial cells were cultured in DMEM containing ECGF (100 µg/ml), 10 mmol/l D-glucose, 1 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. After cells neared confluence, ECGF was replaced with 20% fetal bovine serum. Cell lines were passaged by trypsinization in Dulbecco's phosphate-buffered saline containing 0.25% trypsin and 0.02% EDTA. Endothelial cells at passages 9–12 were used for GFAT assay and metabolic studies.

Determination of GFAT activity. We determined GFAT activity as described by other investigators [10] with modifications. Endothelial cells (5×10^6) were suspended in 0.5 ml of 50 mmol/l sodium phosphate buffer (pH 7.5) containing 100 mmol/l KCl, 1 mmol/l EDTA, 2.5 mmol/l dithiothreitol, 0 or 12 mmol/l D-glucose-6-P, a stabilizer of GFAT [2], and protease inhibitors (5 µg/ml phenylmethylsulphonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml chymostatin, 5 µg/ml pepstatin A). Cells were lysed by three cycles of freezing (liquid nitrogen) and thawing (37°C). Cell lysates were centrifuged at 10000 g and 4°C for 1 min and the supernatant was used for enzyme assay. The assay mixture (0.1 ml) consisted of 12 mmol/l L-glutamine, 12 mmol/l D-fructose-6-P, 0 or 12 mmol/l D-glucose-6-P, 1 mmol/l EDTA, 1 mmol/l dithiothreitol and cell extracts (0.1–0.3 mg protein) and was incubated at 37°C for 0, 15, 30 and 45 min. The reaction was terminated by addition of 50 µl of 1.5 mol/l HClO₄, and the acidified solution was neutralized with 25 µl of 2 mol/l K₂CO₃. The neutralized extracts were di-

luted 20 times with HPLC H₂O before use for glucosamine-6-P analysis. Glucosamine-6-P was separated by an HPLC method involving precolumn derivatization with *o*-phthaldialdehyde, as well as Solvent A (0.1 mol/l sodium acetate, 0.5% tetrahydrofuran, 9% methanol, pH 7.2) and Solvent B (methanol) [22]. The gradient used for glucosamine-6-P analysis was as follows: at 0 min, 86% A and 14% B; at 14 min, 86% A and 14% B; at 14.1 min, 0% A and 100% B; at 18 min, 0% A and 100% B; at 18.1 min, 86% A and 14% B and at 24.5 min, 86% A and 14% B. The retention time of glucosamine-6-P was 8.24 min, as identified with an authentic glucosamine-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).

Glucosamine-6-P and UDP-GlcNAc synthesis. To improve the sensitivity for detecting the synthesis of glucosamine-6-P, UDP-GlcNAc and UDP-GalNAc (the product of UDP-GlcNAc's 4-epimeration) in intact endothelial cells, we used a radiochemical technique. Endothelial cells (10×10^6) were incubated for 1 h in 2 ml KHB buffer (pH 7.4) containing 1% BSA, 5 mmol/l D-[U-¹⁴C]glucose (450 dpm/nmol) and 2 mmol/l L-glutamine. Blank media containing all components but no cells were run in parallel. Incubations were terminated by adding 0.2 ml of 1.5 mol/l HClO₄ and the acidified media were neutralized with 0.1 ml of 2 mol/l K₂CO₃. Neutralized extracts were freeze-dried and suspended in 0.3 ml HPLC H₂O for analysis of ¹⁴C-labelled glucosamine-6-P, UDP-GlcNAc and UDP-GalNAc. The [¹⁴C]glucosamine-6-P was separated by HPLC as described above; the fraction containing [¹⁴C]glucosamine-6-P was collected from the HPLC column and radioactivity measured using a liquid scintillation counter. For [¹⁴C]UDP-GlcNAc and [¹⁴C]UDP-GalNAc evaluation, each neutralized sample was desalted using a 1-ml C₁₈ Sep-Pak cartridge (Waters, Milford, Mass., USA), and then applied to a Partisil-10 SAX column (4.6 × 250 mm) (Phenomenex Inc., Torrance, Calif., USA). The [¹⁴C]UDP-GlcNAc and [¹⁴C]UDP-GalNAc were separated by an isocratic, anion-exchange HPLC system using borate buffer, as described [24]. The fractions containing [¹⁴C]UDP-GlcNAc and [¹⁴C]UDP-GalNAc were collected from the HPLC column and radioactivities measured using a liquid scintillation counter. Retention times for UDP-GlcNAc and UDP-GalNAc were 10.8 and 17.4 min, respectively, as identified with authentic [¹⁴C]UDP-GlcNAc and [³H]UDP-GalNAc standards.

Effect of hyperglycaemia and glutamine on glucosamine-6-P and UDP-GlcNAc synthesis. Rat coronary microvascular and bovine venular endothelial cells were used to evaluate the effect of increased concentrations of glucose and glutamine on glucosamine-6-P and UDP-GlcNAc synthesis. Cell incubations were carried out as described above, with the exception that the incubation media contained 5, 15 or 30 mmol/l D-[U-¹⁴C]glucose (450 dpm/nmol) and 0.5, 2 or 4 mmol/l L-glutamine. These cells were used for this study because they were readily available in large amounts in our laboratories. The concentrations of glucose and glutamine were chosen on the basis of their plasma concentrations in diabetic patients or animals with poor metabolic control [25]. The [¹⁴C]glucosamine-6-P, [¹⁴C]UDP-GlcNAc and [¹⁴C]UDP-GalNAc were evaluated by HPLC methods and liquid scintillation counter, as described above. Our preliminary studies showed that the production of [¹⁴C]glucosamine-6-P, [¹⁴C]UDP-GlcNAc and [¹⁴C]UDP-GalNAc by endothelial cells was linear during a 1-h incubation period in the presence of 5 to 30 mmol/l D-[U-¹⁴C]glucose and 0.5 to 4 mmol/l L-glutamine.

Effect of hyperglycaemia and glutamine on GFAT activity. Because our preliminary study indicated that rat coronary microvascular endothelial cells and bovine venular endothelial cells responded similarly to increased concentrations of glucose and glutamine with glucosamine-6-P synthesis from glutamine and glucose, rat coronary microvascular endothelial cells were chosen to study the effect of hyperglycaemia and glutamine on GFAT activity. Freshly isolated rat coronary endothelial cells were cultured as described above, except that culture media contained 5, 15 or 30 mmol/l glucose in the presence of 0.5, 2 or 4 mmol/l L-glutamine. Conditioned media were replaced daily with fresh media. After a 7-day culture period, cells were obtained for determining GFAT activity, as described above.

GFAT activity in freshly isolated endothelial cells and tissues. Coronary microvascular endothelial cells were isolated from Sprague-Dawley rats (300–325 g) as described previously [23]. Cells were used immediately for measuring GFAT activity as described above. Epididymal fat, gastrocnemius muscle and heart were also obtained from the rats for measuring tissue GFAT activities. Tissues (0.5 g) were homogenized in a glass homogenizer with 2 ml of 50 mmol/l sodium phosphate buffer (pH 7.5) containing 100 mmol/l KCl, 1 mmol/l EDTA, 2.5 mmol/l dithiothreitol, 0 or 12 mmol/l D-glucose-6-P, and protease inhibitors (5 µg/ml phenylmethylsulphonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml chymostatin, 5 µg/ml pepstatin A). Homogenates were centrifuged at 10000 g and 4°C for 1 min and the supernatant was used for GFAT assay in the presence or absence of 12 mmol/l D-glucose-6-P, as described above. The GFAT assay conditions used allow for comparison of tissue GFAT activities between this study and previous studies involving either the presence or absence of 12 mmol/l D-glucose-6-P [11, 26].

Statistical analyses. Results were statistically analysed by two-way analysis of variance with the Student-Newman-Keuls (SNK) multiple range test or by paired *t*-test [27]. We considered *p* values of less than 0.05 to indicate statistical significance.

Results

Activities of GFAT in cultured endothelial cells. Bovine venular, bovine aortic, human microvascular, human umbilical vein and rat coronary microvascular endothelial cells contained high activities of GFAT (Fig. 2). As reported for other cell types, including hepatocytes, brain and muscle [2], we found that the inclusion of 12 mmol/l glucose-6-P in the cell homogenization medium and enzyme assay mixture increased (*p* < 0.01) GFAT activity by about 53 to 56% in all endothelial cells studied (Fig. 2). The activities of GFAT were similar among the bovine, rat and human endothelial cells studied (Fig. 2).

Glucosamine-6-P and UDP-GlcNAc synthesis in cultured endothelial cells. All endothelial cells studied were capable of synthesizing glucosamine-6-P, UDP-GlcNAc and UDP-GalNAc from glutamine and glucose (Table 1). Because glucosamine-6-P was further

Fig. 2. Glutamine:fructose-6-phosphate amidotransferase (GFAT) activity in bovine, rat and human endothelial cells. Data are means $\pm$ SEM, $n = 8$, and were analysed by paired t -test. Endothelial cells were cultured in the presence of 10 mmol/l D-glucose and 1 mmol/l L-glutamine. Confluent cells were used for homogenization and GFAT assay in the presence of 0 or 12 mmol/l D-glucose-6-phosphate, as described in the text. * $p < 0.01$ vs the 0 mmol/l D-glucose-6-phosphate group, $\square$ Bovine venular EC; $\blacksquare$ Bovine aortic EC; ▨ Human microvascular EC; ▩ Human umbilical vein EC; ▤ Rat coronary microvascular EC

metabolized to UDP-GlcNAc (Fig. 1), the amount of glucosamine-6-P measured at the end of the 1-h incubation period represents its net accumulation in endothelial cells. On the basis of the utilization of glutamine ($250 \text{ nmol} \cdot 10^6 \text{ cells}^{-1} \cdot \text{h}^{-1}$ in the presence of 2 mmol/l glutamine) and glucose ($60 \text{ nmol} \cdot 10^6 \text{ cells}^{-1} \cdot \text{h}^{-1}$; in the presence of 5 mmol/l glucose) by endothelial cells [28, 29], production of total UDP-GlcNAc (based on net accumulation of UDP-GlcNAc plus UDP-GalNAc) accounted for approximately 0.3 and 1.5% of the glutamine and glucose metabolized by endothelial cells, respectively. There was no synthesis of [^{14}C]glucosamine-6-P, [^{14}C]UDP-GlcNAc or [^{14}C]UDP-GalNAc in endothelial cells incubated in the presence of 5, 15 or 30 mmol/l D-[U- ^{14}C]glucose but the absence of L-glutamine in the incubation medium.

Table 1. Glucosamine-6-phosphate, UDP-N-acetylglucosamine and UDP-N-acetyl-galactosamine synthesis in bovine, rat and human endothelial cells

Products	Bovine venular EC	Bovine aortic EC	Human microvascular EC	Human umbilical vein EC	Rat coronary microvascular EC
Glucosamine-6-P	2.84 ± 0.18	2.97 ± 0.21	3.06 ± 0.25	2.88 ± 0.37	3.15 ± 0.23
UDP-GlcNAc	0.58 ± 0.07	0.54 ± 0.08	0.61 ± 0.07	0.56 ± 0.06	0.66 ± 0.08
UDP-GalNAc	0.18 ± 0.03	0.17 ± 0.02	0.18 ± 0.02	0.17 ± 0.03	0.20 ± 0.03

Data are means $\pm$ SEM, $n = 8$; in $\text{nmol} \cdot 10^6 \text{ cells}^{-1} \cdot \text{h}^{-1}$ for UDP-GlcNAc and UDP-GalNAc and in $\text{pmol} \cdot 10^6 \text{ cells}^{-1} \cdot \text{h}^{-1}$ for glucosamine-6-P. Data were analysed by one-way

Table 2. Effects of glucose and glutamine on glucosamine-6-phosphate, UDP-N-acetylglucosamine and UDP-N-acetyl-galactosamine synthesis in rat coronary microvascular endothelial cells

Medium glutamine concentrations (mmol/l)	Medium glucose concentrations (mmol/l)		
	5	15	30
Glucosamine-6-phosphate			
0.5	0.87 ± 0.08^c	4.37 ± 0.39^b	7.83 ± 0.68^a
2	2.61 ± 0.25^{cd}	17.7 ± 1.2^{bd}	30.3 ± 2.6^{ad}
4	3.94 ± 0.33^{ce}	25.6 ± 2.2^{be}	42.7 ± 3.5^{ae}
UDP-N-Acetylglucosamine			
0.5	0.19 ± 0.02^c	0.86 ± 0.07^b	1.51 ± 1.2^a
2	0.56 ± 0.07^{cd}	2.94 ± 0.23^{bd}	5.54 ± 0.53^{ad}
4	0.78 ± 0.09^{ce}	3.91 ± 0.36^{be}	7.38 ± 0.68^{ae}
UDP-N-Acetylgalactosamine			
0.5	0.06 ± 0.01^c	0.27 ± 0.02^b	0.42 ± 0.04^a
2	0.17 ± 0.02^{cd}	0.80 ± 0.06^{bd}	1.33 ± 0.12^{ad}
4	0.24 ± 0.02^{ce}	1.29 ± 0.10^{be}	2.05 ± 0.18^{ae}

Data are means $\pm$ SEM, $n = 8$; in $\text{nmol} \cdot 10^6 \text{ cells}^{-1} \cdot \text{h}^{-1}$ for UDP-GlcNAc and UDP-GalNAc and in $\text{pmol} \cdot 10^6 \text{ cells}^{-1} \cdot \text{h}^{-1}$ for glucosamine-6-P. Data were analysed by two-way ANOVA and the SNK test. Rat coronary microvascular endothelial cells were incubated at 37°C for 1 h in 2.0 ml KHB buffer (pH 7.4) containing 5, 15 or 30 mmol/l D-[U- ^{14}C]glucose and 0.5, 2 or 4 mmol/l L-glutamine

^{a-c}: Means displaying different letters (a, b, c) within a row are different ($p < 0.01$)

^d $p < 0.01$ vs the 0.5 and 4 mmol/l L-glutamine groups

^e $p < 0.05$ vs the 2 mmol/l L-glutamine group

Modulation of glucosamine-6-P and UDP-GlcNAc synthesis by hyperglycaemia and glutamine in cultured endothelial cells. In rat coronary microvascular endothelial cells, glucosamine-6-P, UDP-GlcNAc and UDP-GalNAc synthesis increased ($p < 0.01$) with increasing extracellular glucose concentration from 5 to 30 mmol/l in the presence of 0.5, 2 or 4 mmol/l glutamine (Table 2). Glucosamine-6-P, UDP-GlcNAc and UDP-GalNAc synthesis also increased ($p < 0.01$) with increasing extracellular glutamine concentration from 0.5 to 4 mmol/l in the presence of 5, 15 or 30 mmol/l D-glucose in rat coronary microvascular endothelial cells (Table 2). Similar results on the effect of hyperglycaemia and glutamine on glucosamine-6-P, UDP-GlcNAc and UDP-GalNAc synthesis were obtained for bovine venular endothelial cells (data not shown).

ANOVA and the SNK test. Endothelial cells were incubated at 37°C for 1 h in 2.0 ml KHB buffer (pH 7.4) containing 5 mmol/l D-[U- ^{14}C]glucose and 2 mmol/l glutamine

Fig. 3. Effects of hyperglycaemia and glutamine on glutamine:fructose-6-phosphate amidotransferase (GFAT) activity in rat coronary microvascular endothelial cells. Data are means $\pm$ SEM, $n = 6$, and were analysed by two-way analysis of variance and the SNK test. Coronary microvascular endothelial cells freshly isolated from rat hearts were cultured in the presence of 5, 15 or 30 mmol/l D-glucose and 0.5, 2 or 4 mmol/l L-glutamine. Confluent cells were used for homogenization and GFAT assay in the presence of 12 mmol/l D-glucose-6-phosphate, as described in the text. a-c: Means displaying different letters within each glutamine concentration are different ($p < 0.01$). * $p < 0.01$ vs the 0.5 mmol/l L-glutamine group. $\square$ 5 mmol/l glucose, $\blacksquare$ 15 mmol/l glucose, $\blacksquare$ 30 mmol/l glucose

Effects of hyperglycaemia and glutamine on GFAT activity in cultured endothelial cells. Increasing glucose concentrations in culture medium from 5 to 30 mmol/l in the presence of 0.5 or 2 mmol/l glutamine increased endothelial GFAT activity in a concentration-dependent manner. The greatest effect was observed when extracellular glucose concentrations increased from 5 to 15 mmol/l. Similarly, increasing glutamine concentrations in culture medium from 0.5 to 2 mmol/l in the presence of 5, 15 or 30 mmol/l glucose increased ($p < 0.01$) GFAT activity in endothelial cells. The GFAT activity did not increase further ($p > 0.05$) when extracellular glutamine concentrations increased from 2 to 4 mmol/l (Fig. 3).

GFAT activity in freshly isolated endothelial cells and insulin-sensitive tissues. Glutamine: fructose-6-phosphate aminotransferase activity was present in freshly isolated endothelial cells, which represents about 4.6% of GFAT activity in cultured endothelial cells (Fig. 2). It is noteworthy that GFAT activity in freshly isolated endothelial cells was 43% greater ($p < 0.01$) than that in epididymal fat and 470% greater than that in gastrocnemius muscle and heart (Table 3).

Table 3. Glutamine:fructose-6-phosphate amidotransferase (GFAT) activities in freshly isolated coronary microvascular endothelial cells and tissues of rats

Rat cells or tissues	Glucose-6-phosphate concentrations in GFAT assay medium (mmol/l)	
	0	12
Freshly isolated endothelial cells	90.2 $\pm$ 7.4 ^a	197 $\pm$ 15 ^{da}
Epididymal fat	66.4 $\pm$ 5.2 ^b	138 $\pm$ 11 ^{db}
Gastrocnemius muscle	17.9 $\pm$ 1.4 ^c	36.5 $\pm$ 3.0 ^{dc}
Heart	16.8 $\pm$ 1.2 ^c	34.4 $\pm$ 2.7 ^{dc}

Data are means $\pm$ SEM, $n = 6$; in pmol $\cdot$ mg protein⁻¹ $\cdot$ min⁻¹. Data were analysed by two-way analysis of variance and the SNK test. Freshly isolated rat coronary microvascular endothelial cells and rat tissues were used for GFAT assay in the presence of 0 or 12 mmol/l D-glucose-6-phosphate, as described in the text.

a-c means sharing different letters (a, b, c) within a column are different ($p < 0.01$).

^d $p < 0.01$ vs the 0 mmol/l D-glucose-6-phosphate group.

Discussion

The GFAT enzyme catalyses the first and rate-controlling step in hexosamine biosynthesis [1]. This enzyme has been shown to be present in liver, adipose tissue, heart and skeletal muscle [2–6] but was thought to be absent from the human endothelium [5]. Our results show the presence of GFAT activity in freshly isolated rat coronary microvascular endothelial cells, which was 43 to 470% greater than that in the insulin-sensitive tissues (adipose tissue, skeletal muscle and heart) examined. It is possible that the vascular component of skeletal muscle and heart provides a major portion of the GFAT activity measured in the tissue homogenates. Our values of tissue GFAT activities are similar to those reported by other investigators for rat epididymal fat, gastrocnemius muscle and heart [11,26]. For example, GFAT activities, measured in the absence of added glucose-6-P, averaged 62, 15 and 16 (pmol $\cdot$ mg protein⁻¹ $\cdot$ min⁻¹), respectively, in rat epididymal fat, gastrocnemius muscle and heart have been reported [26]. Greater GFAT activities in these tissues (e.g. 32–38 pmol $\cdot$ mg protein⁻¹ $\cdot$ min for rat hindlimb muscles composing primarily of gastrocnemius muscle) were obtained when 12 mmol/l glucose-6-P (a stabilizer of GFAT) was included in the enzyme assay solution [11]. These data on tissue GFAT activities indicate that the GFAT assay method used in our laboratory gave results similar to those reported from other laboratories.

Of note, we found that GFAT activities were particularly high in all cultured bovine, rat and human endothelial cells studied. For example, GFAT activities in cultured endothelial cells were about 10-times to 100-times those reported for freshly isolated endothelial cells and insulin-sensitive tissues and about 4 times those reported for incubated rat epididymal

adipocytes [2–5]. The much greater GFAT activity in cultured endothelial cells compared with freshly isolated endothelial cells suggests that endothelial GFAT expression could be largely influenced by the state of cell differentiation and up-regulated under in vitro culture conditions. The factors responsible for the regulation of endothelial GFAT expression have not been identified. Nevertheless, our finding that all cultured endothelial cells studied contained high GFAT activity is striking and raises an important question about the presence and regulation of the endothelial glucosamine-6-P synthetic pathway.

Consistent with the presence of endothelial GFAT activity, results of this study indicate glucosamine-6-P synthesis from glutamine and glucose in all endothelial cells studied. Furthermore, we found that rates of the endothelial hexosamine synthesis increased with increasing extracellular concentrations of glucose from 5 to 30 mmol/l and of glutamine from 0.5 to 4 mmol/l. In addition, increased concentrations of glucose (15 and 30 mmol/l) or glutamine (2 and 4 mmol/l) increased endothelial GFAT activity. Thus, hyperglycaemia and glutamine regulate endothelial glucosamine-6-P synthesis via at least two mechanisms: enhancing GFAT activity and increasing substrate concentrations for the enzyme. These findings could have important implications for patients with diabetes mellitus in poor metabolic control, who are associated with increased plasma concentrations of both glucose and glutamine [25]. Of note GFAT activity has been reported to be enhanced in skeletal muscle of patients with Type II (non-insulin-dependent) diabetes mellitus [30] and hexosamine synthesis has been shown to be increased in skeletal muscle and liver of diabetic rats [11]. Considering a recent report on two GFAT isozymes in mammalian tissues [31], it would be of great interest to determine which GFAT isozyme is present in endothelial cells and how its activity is regulated by hyperglycaemia and glutamine.

As in smooth muscle cells, skeletal muscle and adipocytes [2–5], glucosamine-6-P, UDP-GlcNAc and UDP-GalNAc are quantitatively minor products of endothelial glutamine and glucose metabolism, accounting for about 0.3% and 1.5% of the glutamine and glucose used by endothelial cells, respectively. This does not, however, necessarily negate an important role for the hexosamine biosynthetic pathway in cell physiology or function. Enhanced hexosamine biosynthesis, for example, has been reported to exert a variety of biological functions, including mediating insulin resistance in diabetes mellitus [7–15]. A recent report showed that intravenous infusion of glucosamine considerably reduced endothelium-dependent blood flow in hind limb femoral muscles of normoglycaemic rats [16]. This result implies impaired endothelial nitric oxide generation in glucosamine-infused subjects. We have recently provided direct

evidence for the inhibition by glucosamine of nitric oxide synthesis in endothelial cells [21]. It is noteworthy that increasing extracellular concentrations of both glutamine and glucose results in decreased nitric oxide production by endothelial cells [18–20, 32, 33]. Because of the important role for nitric oxide in regulating vascular tone and blood flow [34], we suggest that the glucosamine-biosynthetic pathway mediates the inhibition of endothelial nitric oxide synthesis by hyperglycaemia and glutamine.

In summary, our study shows the presence of GFAT activity for glucosamine-6-P and UDP-GlcNAc synthesis in endothelial cells and the modulation of this pathway by hyperglycaemia and glutamine. This knowledge might pave a new path for elucidating the mechanism responsible for cardiovascular dysfunction in diabetes mellitus.

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