A natural protective mechanism against hyperglycaemia in vascular endothelial and smooth-muscle cells: role of glucose and 12-hydroxyeicosatetraenoic acid

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Bovine aortic endothelial and smooth-muscle cells down-regulate the rate of glucose transport in the face of hyperglycaemia, thus providing protection against deleterious effects of increased intracellular glucose levels. When exposed to high glucose concentrations these cells reduced the mRNA and protein content of their typical glucose transporter, GLUT-1, as well as its plasma-membrane abundance. Inhibition of the lipoxygenase (LO) pathway, and particularly 12-LO, reversed this glucose-induced down-regulatory process and restored the rate of hexose transport to the level seen in vascular cells exposed to normal glucose levels. This reversal was accompanied by increased levels of GLUT-1 mRNA and protein, as well as of its plasma-membrane content. Exposure of the vascular cells to elevated glucose concentrations increased by 2–3-fold the levels of cell-associated and secreted 12-hydroxyeicosatetraenoic acid (12-

HETE), the product of 12-LO. Inhibition of 15- and 5-LO, cyclooxygenases 1 and 2, and eicosanoid-producing cytochrome P450 did not modify the hexose-transport system in vascular cells. These results suggest a role for HETEs in the autoregulation of hexose transport in vascular cells. 8-Iso prostaglandin $F_{2\alpha}$, a non-enzymic oxidation product of arachidonic acid, had no effect on the hexose-transport system in vascular cells exposed to hyperglycaemic conditions. Taken together, these findings show that hyperglycaemia increases the production rate of 12-HETE, which in turn mediates the down-regulation of GLUT-1 expression and the glucose-transport system in vascular endothelial and smooth-muscle cells.

Key words: glucose transport, GLUT-1, lipoxygenase, vascular endothelial cells, vascular smooth-muscle cells.

INTRODUCTION

Diabetes-related complications have been linked to an enhanced production of arachidonic acid metabolites [1]. Antonipillai et al. [2] showed higher urinary secretion rate of 12-hydroxyeicosatetraenoic acid (12-HETE), the product of the 12-lipoxygenase (12-LO), in Type 2 diabetes patients with normal renal function and in those with micro- or macro-albuminuria relative to non-diabetic hypertensive micro-albuminuria patients. Similarly, the over-all production of 12-HETE was high in streptozotocin-treated rats [3]. It has also been shown that vascular smooth-muscle cells (VSMC) proliferate faster under hyperglycaemic conditions, while inhibition of LO suppresses this enhanced growth [4]. Wang and Powell [5] found increased levels of HETEs in aortae of atherosclerotic rabbits. Since HETEs are mitogenic, pro-inflammatory, vasoconstrictive and affect cell–matrix interactions, their increased production may play a role in the aetiology of vascular disease [6–8].

Three major mammalian LO enzymes oxygenate carbons 5, 12 or 15 in arachidonic acid, and are thus termed 5-, 12- and 15-LO, generating 5-, 12- and 15-HETE, respectively. Two isoforms of 12-LO were identified in mammalian tissues: the platelet type and the leucocyte type [9,10]. Kim et al. [11] showed that human and porcine VSMC express the leucocyte-type 12-LO, but not the platelet type. No 15-LO mRNA has been detected in human

or porcine VSMC or in human vascular endothelial cells (VEC) [11]. The expression of 12-LO mRNA and the content of 12-HETE are low in porcine aortic VSMC grown with 5.5 mM glucose; both are augmented under hyperglycaemic conditions [12]. Similarly, increased amounts of HETEs are produced in VEC under hyperglycaemic conditions [13].

Previously we reported that VSMC down-regulate the rate of hexose transport under hyperglycaemic conditions, thus reducing the intracellular level of glucose and its metabolites [14]. Recently, we have reported that the esculetin, a general inhibitor of 5-, 12- and 15-LO, reverses the down-regulation of hexose transport in vascular cells under hyperglycaemic conditions [15]. The present study was designed to investigate the hypothesis that specific products of the LO pathway mediate the autoregulatory effect of glucose and glucose transport in VSMC and VEC.

EXPERIMENTAL

Materials and cell cultures

Dulbecco's modified Eagle's medium (DMEM), newborn calf serum, fetal calf serum, bovine fibronectin, glutamine and antibiotics were from Biological Industries (Kibbutz Beth-Haemek, Israel). [U - ^{14}C]Sucrose (500 mCi/mmol) and [α - ^{32}P]dCTP (3000 Ci/mmol) were from Amersham Bioscience (Little Chalfont, Bucks, U.K.). American Radiolabelled Chemicals (St.

Abbreviations used: BW-755C, 3-amino-1-[(*m*-trifluoromethyl)phenyl]2-pyrrazolinand; CDC, cinnamyl-3,4-dihydroxy- α -cyanocinnamate; COX, cyclooxygenase; DMEM, Dulbecco's modified Eagle's medium; dGlc, 2-deoxy-D-glucose; ETYA, 5,8,11,14-eicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; LO, lipoxygenase; MK-866, 3-3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2 dimethylpropanoic acid; NDGA, nordihydroguaiaretic acid; 17-ODYA, 17-octadecynoic acid; VEC, vascular endothelial cells; VSMC, vascular smooth-muscle cells; RT-PCR, reverse transcriptase PCR.

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Louis, MO, U.S.A.) supplied 2-[1,2-³H(N)]deoxy-D-glucose (60 Ci/mmol). Antipain, aprotinin, BSA, caffeic acid, cinnamyl-3,4-dihydroxy- α -cyanocinnamate (CDC), 2-deoxy-D-glucose (dGlc), ebselen[2-phenyl-1,2-benzoselenenazol-3(2H)-one], esculetin (6,7-dihydroxycoumarin), indomethacin, octadecyl-functionalized silica, nordihydroguaiaretic acid (NDGA), 17-octadecynoic acid (17-ODYA), Tri ReagentTM and streptavidin-agarose beads were purchased from Sigma (St. Louis, MO, U.S.A.). D-Glucose was from Merck (Darmstadt, Germany). Sulphosuccinimidyl 6-(biotinamido)biotin (NHS-LC-biotin) was from Pierce (Rockford, IL, U.S.A.). Leupetin and α -macroglobulin were obtained from Boehringer Mannheim (Mannheim, Germany). Baicalein (5,6,7-trihydroxyflavonate) was from Aldrich (Milwaukee, MI, U.S.A.). 5,8,11,14-Eicosatetraynoic acid (ETYA), 5-, 12- and 15-HETE, 8-iso prostaglandin F_{2_{is}}, a mixture of 5-, 8-, 11-, 12- and 15-HETE for HPLC calibration and nimesulide were purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.). 3-3-[1-(4-Chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2 dimethylpropanoic acid (MK-866), 3-amino-1-[(*m*-trifluoromethyl)phenyl]2-pyrazolinand (BW-755C) and SC-41661 were donated kindly by the Merck Frosst Centre for Therapeutic Research (Kirkland, Quebec, Canada), Burroughs Wellcome Co. (Research Triangle Park, NC, U.S.A.) and Searle Chemicals (Augusta, GA, U.S.A.), respectively. All enzyme, buffers and reagents for reverse transcriptase PCR (RT-PCR) were purchased from Promega (Madison, WI, U.S.A.). All other chemicals, reagents and solvents were reagent-, molecular biological- or HPLC-grade.

Primary cultures of bovine aortic endothelial and smooth-muscle cells were prepared and characterized as described previously [14].

Hexose-uptake assay

The [³H]dGlc-uptake assay was performed as described in [14]. At the end of the uptake assay, the cells were solubilized with 1 ml of 1 mg/ml SDS solution (10 min at 37 °C) and taken for liquid-scintillation counting. Extracellular space-associated tritium counts were assessed by parallel incubations with [¹⁴C]sucrose and were less than 1% of total [³H]dGlc. Cytochalasin B-non-inhibitable dGlc uptake (non-carrier-mediated) was less than 2% of total dGlc uptake. The uptake of dGlc was linear up to 15 min. Carrier-mediated dGlc uptake was calculated on the basis of cell number, determined by counting the cells in a haemocytometer following their detachment by trypsinization.

The various HETEs and enzyme inhibitors were added to the cell cultures from stocks in DMSO or ethanol by 1000-fold dilution. DMSO reduced the rate of hexose transport by less than 5% without altering the glucose-induced autoregulation of the transport. Ethanol had no effect on the rate of hexose transport.

Cell-surface biotinylation and GLUT-1 Western-blot analysis

Surface biotinylation of the vascular cells was performed as described previously [16]. Western-blot analysis of total and cell-surface GLUT-1 was performed as described previously [17], using a rabbit antiserum prepared against the human erythrocyte transporter (courtesy of Dr H.-G. Joost, Rheinisch-Westfälische Technische Hochschule, Aachen, Germany).

RNA isolation and cDNA synthesis

Total RNA was extracted from (1–2) × 10⁶ cells using Tri ReagentTM according to the manufacturer's protocol. A mixture

(20 μ l) of 0.5 μ g of total RNA, 0.5 μ g of oligo(dT)₁₅ and 10 pmol of GLUT-1 antisense primer [18] was heated to 70 °C for 3 min and chilled quickly on ice. The cDNA was synthesized following the addition of a reverse transcriptase buffer [10 mM Tris/HCl (pH 9.0)/50 mM KCl/5 mM MgCl₂/0.1% (v/v) Triton X-100/1 unit/ μ l RNasin] containing 1.0 mM of each dNTP and 15 units of avian myeloblastosis virus reverse transcriptase. The reaction was carried out at 42 °C for 1 h and terminated by heating to 65 °C for 10 min. The mixtures were stored at –20 °C until use.

Competitive PCR for quantification of GLUT-1 mRNA

cDNA equivalent to 25 ng of RNA from each sample was mixed in 25 μ l (total volume) of PCR buffer [10 mM Tris/HCl (pH 9.0)/50 mM KCl/1.5 mM MgCl₂/0.1% (v/v) Triton X-100] containing 0.2 mM of each dNTP, 0.2 μ Ci of [α -³²P]dCTP, 12 pmol of each up- and down-stream bovine GLUT-1 primer sequence [18], 0.25 ng of pGEM-4Z-HepG2 plasmid (containing a 1.8 kb insert of GLUT-1; courtesy of Dr G. I. Bell, Howard Hughes Medical Institute, University of Chicago, Chicago, IL, U.S.A.) and 2.5 units of *Taq* DNA polymerase. The PCR reaction was carried out in a PTC-100TM Programmable Thermal Controller (MJ Research, Waltham, MA, U.S.A.) for 35 cycles with denaturation at 94 °C for 45 s, annealing at 59 °C for 45 s and extension at 72 °C for 45 s. The products of the reaction were separated electrophoretically on a 6% polyacrylamide gel. The gel was dried and taken for autoradiography and phosphor-imaging (Bio-Imaging Analyser, Bas 1000; Fujix, Kanagawa, Japan). The pGEM-4Z-HepG2 plasmid and the cDNA give PCR products of 550 and 327 bp, respectively. The nucleotide sequence of each product was identical with the predicted sequences. Sequence analysis was performed at the DNA Analysis Unit of the Hebrew University, Jerusalem, Israel.

The relative efficiency of cDNA synthesis of each sample was assayed in parallel with β -actin PCR (using the primer sequences 5'-GTACCACTGGCATCGTGTGGACT-3' and 3'-ATCCAC-ACGGAGTACTTGCGCTCA-5'). The PCR reaction was carried out as described above with 50 pmol of each β -actin primer for 18 cycles. It should be noted that the glucose-6-phosphate dehydrogenase PCR product could not be used as an internal control in this study since the mRNA content of this enzyme was increased 2–3-fold in VEC and VSMC following treatment with esculetin (S. Sasson and H. Totary, unpublished work).

Extraction of HETEs and F₂-isoprostanes

Cell extraction

The extraction procedure followed that described by Powell [19] with some modifications. Briefly, the cultured cells were rinsed three times with PBS at room temperature and collected after rapid treatment with 200 μ l of trypsin/EDTA solution. Following cell detachment, 0.8 ml of methanol containing 0.2 mM NaOH and 0.25 mM propyl gallate was added to the culture plate. NaOH hydrolyses HETE esters and propyl gallate prevents non-specific oxidation [20]. The cells were then collected in an Eppendorf tube followed by three freeze–thaw cycles in liquid N₂. The cell lysates were incubated in the dark under N₂ atmosphere for 40 min at 4 °C, then acidified to pH 3.0 with 1 M HCl before loading on pre-washed (7 ml of methanol and 7 ml of water) octadecyl-functionalized silica columns (2.2 g/column). Traches suction sets (UnoPlast, Hundested, Denmark) were used for columns. The columns were eluted successively and rapidly

under N_2 pressure (10 p.s.i., equivalent to 69 kPa) with 7 ml of 15% ethanol, 7 ml of water, 2 ml of light petroleum (boiling range 35–60 °C) and finally 10 ml of ethyl acetate. The ethyl acetate fraction was collected, evaporated to dryness under an N_2 stream and the dry material dissolved in 500 μ l of ethyl acetate.

Extraction of culture medium

Ethanol and propyl gallate were added to culture medium as aliquots, to final concentrations of 15% (v/v) and 0.25 mM, respectively. The rest of the extraction procedure was identical with that described above for cells. The recovery of HETE standards, added to fresh medium prior to extraction, was 80–85%.

Extraction of F_2 -isoprostanes from culture medium

This procedure was similar to that described above but the final elution step was with methyl formate. The extracted material was dried under N_2 and dissolved in 500 μ l of methyl formate. The recovery of 8-iso prostaglandin F_{2x} was $\approx$ 85%.

HPLC analysis of HETE

Extract aliquots (20 μ l) were analysed by reversed-phase HPLC in an L-6200 Merck-Hitachi chromatography system using a Lichrosphere RP-18 pre-column (5 μ m, 4 mm $\times$ 4 mm) and column (5 μ m, 250 mm $\times$ 4 mm; Merck) connected to an L-4200 UV/Vis detector (235 nm). Elution was at a flow rate of 1.5 ml/min with a three-solvent isocratic and gradient mixture (solvent A, 0.01% acetic acid; solvent B, acetonitrile; solvent C, methanol) as follows: the initial solvent mixture was 33% A/10% B/57% C. A convex (non-linear) gradient over 25 min then followed to 10% B/90% C. Isocratic elution with 10% B/90% C followed for an additional 10 min. The system was then regenerated to the initial solvent ratio with a linear programme over 10 min. With this programme, 15-, 12- and 5-HETE were eluted at 14.7, 15.7 and 16.9 min, respectively, as confirmed with pure 5-, 12- and 15-HETE standards and a HETEs mixture for HPLC calibrations.

HPLC analysis of F_2 -isoprostanes

Measurement of F_2 -isoprostanes was performed as described by Mori et al. [21] using the same instrument, columns and elution programme described above. UV detection was at 205 nm. Extract aliquots (200 μ l) were eluted with a two-solvent gradient and isocratic mixture as follows (solvents A and B were as described above): the initial mixture was 90% A/10% B. A linear gradient over 20 min then followed to 50% A/50% B. At 25 min it reached 100% B. Isocratic elution with 100% B followed for an additional 10 min. The system was then regenerated to the initial solvent ratio with a linear programme over 10 min. With this programme, 8-iso prostaglandin F_{2x} was eluted at 17.5 min, as confirmed with a pure 8-iso prostaglandin F_{2x} standard.

Glucose determination

Glucose concentration in culture-medium samples was determined with Glucometer Elite™ and blood glucose test strips (Bayer, Puteaux, France).

Statistical analysis

Statistical analysis was done using Mann–Whitney test.

RESULTS

Effects of glucose and esculetin on the rate of hexose transport in vascular cells

The effect of LO inhibition by esculetin on the rate of hexose transport was studied in vascular cells. VEC and VSMC cultures were preconditioned at 5.5 and 23.0 mM glucose for 48 h (medium was changed once after 24 h) to induce up- and down-regulation of hexose transport. The LO inhibitor esculetin

Figure 1 Time course of esculetin-dependent stimulation of hexose transport in VEC

Confluent VEC cultures were preincubated with 5.5 mM (□, ■) or 23.0 mM (○, ●) glucose for 48 h. The cells were then washed and received fresh media with the same glucose concentration in the absence (○, □) or the presence (●, ■) of 100 μ M esculetin. [3 H]dGlc-uptake assay was performed at the indicated times. Means $\pm$ S.E.M. are shown; $n = 3$.

Figure 2 Time course of esculetin-dependent stimulation of hexose transport in VSMC

Confluent VSMC cultures were treated and processed as described in the legend to Figure 1. The symbols correspond to those in Figure 1. Means $\pm$ S.E.M. are shown; $n = 3$.

Figure 3 Dose-response curves showing the effect of esculetin on hexose transport in VEC

Confluent VEC cultures were maintained for 48 h at 5.5 or 23.0 mM glucose. Cells pre-exposed to 5.5 mM glucose received fresh medium containing 5.5 mM (□) or 23.0 mM (■) glucose with increasing concentrations of esculetin (0–100 μM). Cells pre-exposed to 23.0 mM glucose received fresh medium containing 23.0 (●) or 5.5 mM (○) glucose, also with increasing concentrations of esculetin. After 36 h of incubation the cells were taken for the standard [³H]dGlc-uptake assay. (A) dGlc uptake (means ± S.E.M.; *n* = 3). (B) The effect of esculetin on dGlc uptake relative to esculetin-free controls that were incubated with the same glucose concentration.

(100 μM) or vehicle was then added and the incubation continued for the indicated times. Pre-exposure to hyperglycaemic conditions in the absence of esculetin reduced the rate of hexose transport in VEC (Figure 1) and VSMC (Figure 2) by 55 and 60%, respectively, in comparison with the normoglycaemic conditions. Esculetin augmented the rate of hexose transport in a time-dependent manner under both glycaemic conditions and in both cell types. This effect of esculetin was obtained 6–10 h after its addition. However, the relative stimulatory effect of esculetin was higher in cells exposed to 23.0 mM glucose than to 5.5 mM glucose (increases of 163 and 118%, respectively, for VEC, and of 191 and 141%, respectively, for VSMC).

The dose-response relationships of esculetin's effect on VEC and VSMC are depicted in Figures 3(A) and 4(A), respectively. The cells were pre-exposed to 5.5 or 23.0 mM glucose for 48 h and then received fresh DMEM containing the same or the opposite glucose concentrations and increasing concentrations of esculetin (0–100 μM). VEC and VSMC completed the process

Figure 4 Dose-response curves showing the effect of esculetin on hexose transport in VSMC

Confluent VSMC cultures were treated and processed as described in the legend to Figure 3. The [³H]dGlc-uptake assay was performed 10 h after the addition of esculetin. The symbols correspond to those in Figure 3. Means ± S.E.M. are shown; *n* = 3.

of autoregulation, in the absence of esculetin, within 36 and 10 h, respectively. Esculetin reversed the down-regulatory response in a dose-dependent manner in cells that were pre-exposed to 5.5 mM glucose and then switched to 23.0 mM glucose. Maximal and half-maximal effects of esculetin were observed at 30–40 and 100 μM, respectively, for both types of cell. Conversely, the up-regulatory mechanism (upon transfer of cells from 23.0 to 5.5 mM glucose) continued to function in the presence of the inhibitor. Consequently, the relative stimulatory effect of esculetin was significantly higher in cells that were switched from 5.5 to 23.0 mM glucose than in cells exposed to the opposite medium change (Figures 3B and 4B). Esculetin was toxic to the cells at concentrations greater than 100 μM and reduced cell viability (results not shown).

The slight increase in osmolarity of the 23.0 mM glucose culture medium did not affect the glucose-transport system: VEC and VSMC were exposed to DMEM containing 5.5 mM glucose and 17.5 mM sucrose or L-glucose for 48 h in the absence or presence of 100 μM esculetin during the last 10 h of incubation. The rate of hexose transport in these cells was similar to that measured in the respective control cells that were exposed to 5.5 mM glucose, without or with esculetin (results not shown).

Table 1 Effect of glucose and esculletin on the hexose-transport kinetics in VEC and VSMC

VEC and VSMC cultures were treated as described for Figures 1 and 2. Esculetin (100 μ M) or the vehicle (0.1% v/v, DMSO) was included during the last 10 h of incubation. The cells were then taken for the standard [3 H]dGlc-uptake assay in the presence of increasing dGlc concentrations (0.05–10.0 mM). The uptake data were analysed and the K_m and V_{max} values calculated according to Lineweaver and Burk. K_m is expressed in mM; V_{max} is expressed in nmol of dGlc/ 10^6 cells per min.

Conditions	VEC		VSMC	
	K_m	V_{max}	K_m	V_{max}
5.5 mM Glc	0.73	0.84	0.93	2.43
5.5 mM Glc + esculletin	0.72	1.23	0.85	3.79
23.0 mM Glc	0.69	0.49	0.70	1.60
23.0 mM Glc + esculletin	0.95	1.14	0.53	3.23

Figure 5 Effect of glucose and esculletin on total and plasma-membrane-associated GLUT-1 in VEC

Confluent VEC cultures were maintained for 48 h at 5.5 or 23.0 mM glucose. Esculetin (ESC; 100 μ M) was present during the last 10 h of incubation. The preparation of total cell lysates and the purification of cell-surface biotinylated proteins are described in the Experimental section. (A) A representative GLUT-1 Western blot. (B) Relative intensities of GLUT-1 signals in total lysate (open columns) and in plasma-membrane fractions (PM; hatched columns). The 100% value is assigned to the intensities of lysates and PM fractions of cells incubated at 5.5 mM glucose. Means $\pm$ S.E.M. are shown; $n = 3$.

Kinetic analysis and reversibility of the effect of esculletin

Kinetic analysis of the hexose-transport system (Table 1) shows that hyperglycaemic conditions reduced the V_{max} of hexose transport in VEC and VSMC without affecting the K_m significantly. Esculetin (100 μ M) increased the values of V_{max} in cells maintained at either 5.5 or 23.0 mM glucose to nearly the same maximal value without changing the K_m significantly.

The up-regulatory effect of esculletin was reversible in both cell types: following esculletin washout [eight washes with 5 ml of DMEM containing 23.0 mM glucose and 0.1% (v/v) DMSO] of cell cultures that were pre-exposed to 23.0 mM glucose and esculletin (100 μ M for 10 h), the rate of hexose transport returned to the down-regulated state, as in control cells maintained with 23.0 mM glucose in the absence of esculletin (results not shown).

Figure 6 Effect of glucose and esculletin on total and plasma-membrane-associated GLUT-1 in VSMC

Confluent VSMC cultures were prepared and treated for GLUT-1 Western-blot analysis as described in the legend to Figure 5. (A) A representative GLUT-1 Western blot. (B) Relative intensities of GLUT-1 signals. All details are as for Figure 5. Means $\pm$ S.E.M. are shown; $n = 5$.

The times required for 50% down-regulation of hexose transport following esculletin washout were 16.5 and 11.4 h, for VEC and VSMC, respectively.

Effects of esculletin on total and plasma-membrane content of GLUT-1

Total GLUT-1 content was determined in whole cell lysates of VEC and VSMC. Plasma-membrane content of the transporter was measured using the cell-surface biotinylation procedure. Figures 5(A) and 6(A) depict results of typical experiments in which VEC and VSMC were incubated with 5.5 and 23.0 mM glucose for 48 h, with esculletin present during the last 10 h of incubation. A summary of between three and five experiments (Figures 5B and 6B) shows that hyperglycaemic conditions reduced the total cell GLUT-1 content and the plasma-membrane-associated GLUT-1 by 44 $\pm$ 7 and 52 $\pm$ 7% in VEC, respectively, and by 50 $\pm$ 13 and 43 $\pm$ 10%, respectively, in VSMC. Esculetin had a profound effect on GLUT-1 in cells exposed to 23.0 mM glucose; it increased total GLUT-1 content in VEC and VSMC by 180 $\pm$ 17 and 228 $\pm$ 34%, respectively, in comparison with cells incubated with 23.0 mM glucose alone. Correspondingly, the plasma-membrane GLUT-1 content was increased by 256 $\pm$ 16 and 289 $\pm$ 32%, respectively. At 5.5 mM glucose, esculletin increased total GLUT-1 content by only 122 $\pm$ 5 and 148 $\pm$ 9% in VEC and VSMC, respectively, and plasma-membrane transporter by 146 $\pm$ 7 and 133 $\pm$ 7%, respectively, in comparison with cells incubated with 5.5 mM glucose only. These changes in plasma-membrane content of GLUT-1 correspond well to the respective changes in hexose-transport capacity in vascular cells under similar experimental conditions (Figures 1–4).

Both VSMC and VEC express GLUT-4 [22,23]. Therefore, the effect of hyperglycaemia on GLUT-4 expression was also studied in VSMC and VEC. The total content of GLUT-4 was determined in VEC and VSMC following 48 h exposure to 23.0 mM

Figure 7 Effects of glucose and esculentin on GLUT-1 mRNA in VEC

Confluent VEC were maintained for 48 h at 5.5 or 23.0 mM glucose with or without esculentin (ESC; 100 μ M) during the last 10 h of incubation. RNA extraction, cDNA synthesis, PCR of GLUT-1 cDNA in the presence of the competing pGEM-4Z-HepG2 plasmid and product analysis are described in the Experimental section. (A) A representative PCR product image. (B) Relative intensities of GLUT-1 PCR product versus the competing pGEM-4Z-GT-1 PCR product. The ratio of GLUT-1 to pGEM-4Z-GT-1 at 5.5 mM glucose was taken as 100%. Means $\pm$ S.E.M. are shown; $n = 3$.

glucose and was the similar to levels found at 5.5 mM glucose (results not shown). This finding confirms the report of Cooper et al. [22] on the lack of effect of high glucose level on GLUT-4 expression in A-10 VSMC.

Competitive RT-PCR for GLUT-1 mRNA

The changes in the total GLUT-1 protein content can result from glucose- and esculentin-induced alterations in the expression of GLUT-1 mRNA. Therefore, a competitive PCR analysis of GLUT-1 mRNA was performed. Figures 7(A) and 8(A) show results of a representative experiment on VEC and VSMC that were maintained at 5.5 and 23.0 mM glucose for 48 h in the presence or absence of esculentin (100 μ M) during the last 10 h of incubation. The relative change in the signal of the GLUT-1 product and the competing pGEM-4Z-HepG2 plasmid product was calculated for each experimental condition of three independent experiments and normalized to esculentin-free incubations at 5.5 mM glucose (Figures 7B and 8B). Exposure to 23.0 mM glucose caused a 44 ± 12 and 48 ± 19 % reduction in total GLUT-1 mRNA in VEC and VSMC, respectively. Esculetin treatment of VEC reversed these glucose-induced down-regulatory effects and caused 2.1 ± 0.2 - and 3.8 ± 0.4 -fold increases in total GLUT-1 mRNA levels, relative to esculentin-free incubations

Figure 8 Effects of glucose and esculentin on GLUT-1 mRNA in VSMC

Confluent VEC were maintained for 48 h at 5.5 or 23.0 mM glucose without or with the addition of esculentin (100 μ M) during the last 10 h of incubation. Competitive RT-PCR analysis of GLUT-1 mRNA was performed as described for Figure 7. (A) A representative PCR product image. (B) Relative intensities of GLUT-1 PCR product versus the competing pGEM-4Z-GT-1 PCR product. The ratio of GLUT-1 to pGEM-4Z-GT-1 at 5.5 mM glucose was taken as 100%. Means $\pm$ S.E.M. are shown; $n = 3$.

at 5.5 and 23.0 mM glucose, respectively. In VSMC the corresponding values for esculentin's effect were 2.4 ± 0.5 - and 3.6 ± 0.8 -fold. The RT-PCR product of β -actin served as an internal control.

HPLC analyses of cell-associated and secreted HETEs

Figure 9 shows a representative HPLC profile of media extracts of VSMC cultures incubated for 48 h at 5.5 and 23.0 mM glucose. Among the three major LO metabolites, only 12-HETE secretion was significantly higher in cells exposed to hyperglycaemic conditions. It should be noted that the peak ascribed to 15-HETE is broad and may contain additional metabolites. Table 2 summarizes HPLC analyses of cell-associated and secreted 12- and 15-HETE of vascular cells maintained for 48 h at 5.5 or 23.0 mM glucose. The level of 5-HETE was not analysed due to its relatively small and often indistinguishable peak. Overall, VSMC produced and secreted significantly higher levels of 12- and 15-HETE than VEC. Yet, both cell types produced and secreted significantly higher amounts of 12-HETE at 23.0 than at 5.5 mM glucose. The production and secretion of 15-HETE under hyperglycaemic conditions, although showing a tendency to be increased, was not statistically different from the controls (Table 2).

Figure 9 HPLC analysis of HETEs in VSMC culture media

Media of VSMC cultured for 48 h at 5.5 and 23.0 mM glucose were extracted and taken for a HPLC analysis as described in the Experimental section. Specific retention times for 15-, 12- and 5-HETE were 14.7, 15.7 and 16.9 min, respectively.

Table 3 shows an inverse relationship between the stimulatory effects of the inhibitors esculetin and NDGA on the hexose-transport system and their inhibitory effect on 12- and 15-HETE production in VEC and VSMC. Both inhibitors increased the hexose-transport rate in cells incubated at 23.0 mM glucose by ≈ 2 -fold while decreasing cell-associated and secreted 12- and 15-HETE by 60–80 and $\approx 65\%$, respectively.

Effects of various LOs, cyclo-oxygenase (COX) and cytochrome P450 inhibitors on the rate of hexose transport in vascular cells

To characterize further the role of arachidonic acid metabolites in the hexose-transport system we tested a variety of inhibitors of LO, COX and eicosanoid-producing cytochrome P450 enzymes. All inhibitors were tested under similar experimental conditions as described above for esculetin. Dose-response and time-course experiments were performed for each inhibitor. Table 4 shows the results obtained under optimal conditions and the concentration for each inhibitor, which was added to VEC and VSMC pre-exposed for 48 h to 23.0 mM glucose. The non-selective LO inhibitors esculetin, NDGA and ETYA and a dual inhibitor of COX and LO, BW-755C, augmented the rate of hexose transport in VEC and VSMC. Another non-selective LO inhibitor, CDC, had no effects on hexose transport, but it did not change the levels of cell-associated and secreted 12- and 15-HETE in VEC and VSMC (HPLC analyses; results not shown). Ebselen, a 15-LO inhibitor [24], and the specific 5-LO inhibitors MK-866, SC-41661 [25,26] and caffeic acid [27] failed to modulate the hexose-transport system in vascular cells. These inhibitors

Table 2 Effect of glucose on 12- and 15-HETE production in VSMC and VEC

VSMC and VEC cultures were maintained for 48 h at the indicated glucose concentrations (with a medium change at 24 h). At the end of the incubation period the cells and culture media were collected, extracted and taken for HPLC analysis of HETEs as described in the Experimental section. Control (%) shows the average of the percentage increase in HETE levels in cell and medium extracts prepared from cell cultures and media that were incubated with 23.0 mM relative to 5.5 mM glucose. The data are given as ng of 12- or 15-HETE/ 10^6 cells (means $\pm$ S.E.M.).

Cell type	Extract	12-HETE			15-HETE		
		5.5 mM Glc	23.0 mM Glc	Control (%)	5.5 mM Glc	23.0 mM Glc	Control (%)
VSMC	Cells	64.8 $\pm$ 20.3 (<i>n</i> = 7)	202.1 $\pm$ 55.7 (<i>n</i> = 8)	311.9 $\pm$ 41.7* (<i>n</i> = 7)	113.4 $\pm$ 25.2 (<i>n</i> = 8)	176.1 $\pm$ 61.5 (<i>n</i> = 7)	155.3 $\pm$ 41.4 (<i>n</i> = 7)
	Medium	14.1 $\pm$ 3.0 (<i>n</i> = 9)	40.9 $\pm$ 13.7 (<i>n</i> = 8)	290.1 $\pm$ 39.7* (<i>n</i> = 8)	59.3 $\pm$ 26.8 (<i>n</i> = 9)	111.1 $\pm$ 52.2 (<i>n</i> = 8)	187.5 $\pm$ 65.2 (<i>n</i> = 7)
VEC	Cells	5.1 $\pm$ 2.3 (<i>n</i> = 3)	11.3 $\pm$ 1.4 (<i>n</i> = 3)	221.6 $\pm$ 46.8* (<i>n</i> = 3)	33.4 $\pm$ 21.1 (<i>n</i> = 3)	67.6 $\pm$ 35.8 (<i>n</i> = 3)	202.4 $\pm$ 82.4 (<i>n</i> = 3)
	Medium	4.3 $\pm$ 0.4 (<i>n</i> = 3)	15.0 $\pm$ 1.4 (<i>n</i> = 3)	348.8 $\pm$ 13.2* (<i>n</i> = 3)	11.4 $\pm$ 2.0 (<i>n</i> = 3)	34.2 $\pm$ 3.4 (<i>n</i> = 3)	300.0 $\pm$ 20.2* (<i>n</i> = 3)

* *P* < 0.05, significantly different than the respective control (5.5 mM Glc); Mann–Whitney test.

Table 3 Effect of LO inhibitors on dGlc uptake and cell-associated and secreted 12- and 15-HETE in vascular cells

VSMC and VEC cultures were maintained for 48 h at 23.0 mM glucose (with a medium change at 24 h). During the last 10 h of incubation the cells were exposed to esculetin (100 μ M), NDGA (50 μ M) or the vehicle (0.1%, v/v, DMSO). At the end of the incubation period the cells and culture media were collected, extracted and the various HETE metabolites were determined by HPLC, as described in the Experimental section. Similar cell cultures were used for the standard [3 H]dGlc-uptake assay. 100% dGlc uptake and HETE levels were the corresponding values for cells incubated at 23.0 mM glucose without inhibitors. Means $\pm$ S.E.M. are shown.

Cell type	Treatment	[3 H]dGlc uptake (% of control)	HETE level (% of control)			
			12-HETE		15-HETE	
			Cell-associated	Medium	Cell-associated	Medium
VSMC	Esculetin (<i>n</i> = 4)	208.6 $\pm$ 13.4	13.9 $\pm$ 3.6	23.0 $\pm$ 13.5	19.6 $\pm$ 10.5	37.7 $\pm$ 11.0
	NDGA (<i>n</i> = 3)	186.3 $\pm$ 18.3	40.6 $\pm$ 5.9	26.3 $\pm$ 7.7	36.2 $\pm$ 26.1	35.1 $\pm$ 2.8
VEC	Esculetin (<i>n</i> = 3)	226.7 $\pm$ 47.0	14.3 $\pm$ 4.8	19.0 $\pm$ 5.5	29.2 $\pm$ 7.7	46.4 $\pm$ 9.1
	NDGA (<i>n</i> = 3)	197.1 $\pm$ 12.7	56.2 $\pm$ 7.0	29.8 $\pm$ 12.6	44.6 $\pm$ 15.6	56.2 $\pm$ 11.1

Table 4 Effects of various LO, COX and cytochrome P450 inhibitors on the rate of dGlc uptake in VEC and VSMC

VEC and VSMC were exposed to 23.0 mM glucose for 48 h. At the end of the incubation the various inhibitors or their solvents were added to cultured cells for 8–12 h. The cells were then taken for the standard [^3H]dGlc-uptake assay. Data are given as the percentage change in [^3H]dGlc-uptake compared with the control cells that received vehicle only (means $\pm$ S.E.M., $n = 3-6$).

Inhibitor	Concentration (μM)	[^3H]dGlc uptake (% of control)	
		VEC	VSMC
Esculetin	100	213 $\pm$ 16*	246 $\pm$ 12*
NDGA	10	212 $\pm$ 4*	164 $\pm$ 8*
BW-755C	100	224 $\pm$ 5*	202 $\pm$ 7*
ETYA	500	169 $\pm$ 3*	175 $\pm$ 4*
Baicalein	100	197 $\pm$ 1*	166 $\pm$ 5*
CDC	10	121 $\pm$ 7	119 $\pm$ 6
Ebselen	50	109 $\pm$ 4	91 $\pm$ 8
Caffeic acid	100	98 $\pm$ 6	109 $\pm$ 5
MK-866	50	110 $\pm$ 7	108 $\pm$ 4
SC-41661	50	109 $\pm$ 18	114 $\pm$ 9
Indomethacin	50	107 $\pm$ 6	118 $\pm$ 11
Nimesulide	100	103 $\pm$ 5	92 $\pm$ 4
17-ODYA	2.5	93 $\pm$ 9	87 $\pm$ 13

* $P < 0.05$, significantly different from the respective control; Mann–Whitney test.

were also ineffective when the cells were switched from 5.5 to 23.0 mM glucose or vice versa (results not shown). However, the 12-LO inhibitor baicalein [28] successfully reversed the glucose-induced down-regulation of hexose transport.

Indomethacin and nimesulide, inhibitors of COX1 and the inducible COX2, respectively [29], did not modify the hexose-transport system in vascular cells exposed to 23.0 mM glucose or in cells that were switched from high glucose to normal glucose (results not shown). Both 19- and 20-HETE are formed by the cytochrome P450 system through ω and $\omega-1$ oxidation of arachidonic acid [30]. Inhibition of this reaction with 17-ODYA [31] had no effect on the hexose-transport system or the autoregulatory process in both cell types.

Direct effects of 12 and 15-HETE in VEC and VSMC

Glucose-induced down-regulation of hexose transport is associated with increased production of HETEs. Thus it was reasonable to ask whether exogenous 12- or 15-HETE mimic the effect of high glucose. A single addition of 1.5 μM 12- or 15-(S)HETE for 8 h or five repeated additions (0.3 μM every 1–2 h) to cells maintained at 5.5 mM glucose reduced marginally the rate of hexose transport in vascular cells (results not shown). Similarly, additions of both HETEs to cells exposed to 23.0 mM glucose together with or 8 h following the addition of 100 μM esculetin produced no significant effects (results not shown). The reason for this became clear following HPLC analysis of extracts prepared from culture media supplemented with 12-HETE, which indicated a rapid oxidation of this eicosanoid with a half-life of 10–12 min. Therefore, it is assumed that this rapid and non-enzymic oxidation precludes the accumulation of 12-HETE to biologically active levels in cells.

8-Iso prostaglandin $\text{F}_{2\alpha}$ is not involved in glucose-induced down-regulation of hexose transport

Non-enzymic oxidation of arachidonic acid produces $\text{F}_{2\alpha}$ -isoprostanes in vascular cells exposed to hyperglycaemic conditions

[32]. Among these molecules, 8-iso prostaglandin $\text{F}_{2\alpha}$ was shown to be biologically active [33]. HPLC analysis of 8-iso prostaglandin $\text{F}_{2\alpha}$ revealed $\approx$ 2-fold higher levels of this molecule in VEC and VSMC that were maintained at 23.0 mM glucose for 48 h than in cells kept at 5.5 mM glucose (results not shown). Unlike HETEs, 8-iso prostaglandin $\text{F}_{2\alpha}$ is more stable in culture medium; its calculated half-life, as determined by HPLC analyses, is 5–7 h. Nevertheless, a direct addition of this molecule to VEC and VSMC cultures (0.03–3 μM for 8–10 h, single or repeated additions) had no measurable effect on the hexose-transport mechanism (results not shown).

DISCUSSION

Hyperglycaemia is a major determinant in the pathological changes leading to vascular complications in diabetes. These complications have also been linked to enhanced production of eicosanoids, particularly 12- and/or 15-HETE [2,5]. Hyperglycaemia has also been shown to increase the expression and activity of 12-LO in vascular cells [12,13,34]. These cells, like many other cell types [35], operate a regulatory mechanism that reduces the deleterious effects of increased intracellular glucose levels by limiting the rate of glucose entry into the cell in the face of hyperglycaemia. This mechanism protects the cells against excessive production of intracellular glucose-derived reactive oxygen species and against exaggerated intracellular glycation of macromolecules.

In contrast to the reports on the adverse role of 12- and 15-HETE in the initiation and progression of vascular cell dysfunction, this study assigns 12-HETE a protective role by mediating the substrate regulation of the glucose-transport mechanism. This conclusion is based on the following observations. Hyperglycaemia reduces the V_{max} of hexose transport, the total amount of GLUT-1 protein and its plasma-membrane localization, as well as GLUT-1 mRNA content in VEC and VSMC. Hyperglycaemia also increases the production rate of 12-HETE in these cells. Inhibition of 12-LO activity prevents the autoregulatory effects of high glucose levels on the hexose-transport system by increasing the levels of GLUT-1 mRNA and protein and its plasma-membrane content and the rate of glucose transport to similar levels found in VEC and VSMC under normal glucose conditions. LO inhibition augmented the hexose-transport system in cells under hyperglycaemic conditions, while under normoglycaemic conditions such inhibition produced moderate stimulatory effects.

We have previously characterized the process of substrate autoregulation of the hexose-transport system in vascular cells and termed VEC as ‘glucose blind’ because they failed to operate this mechanism when exposed to hyperglycaemic conditions for up to 24 h [14]. Here we show that a longer exposure period (36 h) of VEC to hyperglycaemic conditions does induce a similar down-regulatory mechanism. It is suggested that this slower response is related to the low basal rate of glucose transport in VEC, which delays the accumulation of intracellular intermediates necessary for increasing 12-LO activity and operating the down-regulatory machinery. Indeed, the V_{max} of the hexose-transport system in VEC is just one third of that measured for VSMC (Table 1).

The hypothesis that arachidonic acid metabolites are involved in the autoregulation of hexose transport in vascular cells was tested with a variety of LO, COX and cytochrome P450 inhibitors (Table 4). The lack of effect of indomethacin and nimesulide attests to the negligible role of the constitutive and inducible COX in the regulation of the hexose-transport system in vascular cells. Maffei et al. [36] reported that nimesulide and its metabolites are

also antioxidants. However, neither its COX inhibitory activity nor its antioxidant potential was of any significance in attenuating the process of down-regulation of hexose transport under hyperglycaemic conditions. No specific effect of cytochrome P450 eicosanoid-producing enzymes was detected since the rate of hexose transport of the cells remained unaltered in the presence of 17-ODYA, an inhibitor of this pathway.

General LO inhibitors (esculetin, NDGA and ETYA) and the dual LO/COX inhibitor, BW-755C [37], interfered with the process of glucose-induced down-regulation of hexose transport. The similar biological activity of these divergent and structurally and functionally unrelated LO inhibitors [38–40] supports the hypothesis that the LO pathway is intimately involved in the regulation of the hexose-transport machinery in vascular cells.

Since ebselen (a 15-LO inhibitor), SC-41661, MK-866 and caffeic acid (5-LO inhibitors) did not modify the hexose-transport system in both types of cells it is concluded that 5- and 15-LO and their respective products 5- and 15-HETE are not involved in the regulation of glucose transport.

Baicalein, a reported 12-LO inhibitor [28], reversed glucose-induced down-regulation of hexose transport similarly to the non-selective LO inhibitors. Therefore, among all tested pathways of arachidonic acid metabolism only 12-LO and its product 12-HETE seem to be involved in the complex mechanism of down-regulation of hexose transport in VEC and VSMC. This conclusion is supported further by the finding that hyperglycaemia increases the production and secretion of 12-HETE in these cells.

8-Iso prostaglandin $F_{2\alpha}$, which is generated non-enzymically from arachidonic acid in VEC and VSMC under hyperglycaemic conditions [33,41], did not contribute to the process of glucose-induced down-regulation of hexose transport. Thus this biologically active arachidonic acid product does not participate in the regulation of hexose transport in vascular cells.

The role of 15-LO in bovine aortic vascular cells in regulating hexose transport is questionable: 15-LO mRNA has not been detected in human or porcine VSMC and human VEC [11,42]; however, it has been suggested that bovine VEC contain 15-LO and synthesize 15-HETE [43]. Indeed, the present HPLC analysis shows that VEC and VSMC produce a 2–4-fold excess of 15-HETE over 12-HETE, at both 5.5 and 23.0 mM glucose (Table 2). Revtyak et al. [44] made a similar observation in bovine coronary endothelial cells. Nonetheless, the role of 15-HETE in the regulation of hexose transport seems insignificant because its inhibition by ebselen was inconsequential (Table 4) and since, except in one case (Table 2), exposure of cells to 23.0 mM glucose did not affect significantly its production by the cells. Moreover, there is evidence that 15-HETE may also be a product of the COX pathway [45]. Since both COX and LO pathways function in vascular cells, a reciprocal relationship may exist: decreased production of 15-HETE through the LO pathway may be compensated for by increased production via the COX pathway and vice versa.

Attempts to reverse the glucose-induced down-regulation of hexose transport with 12- and 15-HETE added directly to cell cultures were not successful due to the instability and the very short half-life of these compounds. This strategy has been successful in experimental systems that required short-term exposure periods (5–15 min) to these compounds [46]. Yet, the slow onset of the effect of hyperglycaemia-induced down-regulation of hexose transport and its reversal by 12-LO inhibition in vascular cells seems not to be related to such rapid interactions but more likely to long-term interactions, perhaps at the nuclear level, by regulating, directly or indirectly, either the transcription of GLUT-1 gene and/or the stability of GLUT-1 mRNA. The

mechanism underlying this phenomenon is not entirely clear but may involve protein synthesis *de novo*. Some previous studies provide a basis for the suggestion that 12-HETE may control such nuclear interactions. Herbertsson et al. [47] have identified a specific and high-affinity nuclear receptor for 12(*S*)-HETE, resembling the steroid/thyroid receptors. Moreover, other arachidonic acid metabolites [i.e. 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 , leukotriene B_4 and 8(*S*)-HETE] were shown to be ligands for specific members of the nuclear peroxisome-proliferator-activated receptor (PPAR) family [48]. It remains to be investigated whether 12-HETE shares similar properties.

20-HETE regulates K^+ channel activity and vascular tone in arterioles [49]. The lack of effect of 17-ODYA, a selective inhibitor of 20-HETE synthesis, indicates that such interactions are not linked to the regulation of glucose transport in vascular cells. Tang and Vanderhoek [50] have shown that various mono(*S*)hydroxy fatty acids, including HETEs, are ligands for cytosolic actin and suggested that some of the effects of HETEs may be mediated by these interactions. Thus it is possible that, in addition to possible nuclear effects, 12-HETE also regulates GLUT-1 compartmentalization and translocation by controlling actin–GLUT-1 interactions.

In summary, the present study shows that the natural protective mechanism against hyperglycaemia in vascular cells is dependent on glucose-induced increase of 12-HETE production. The latter operates the down-regulatory response by reducing GLUT-1 expression and abundance in the plasma membrane, thus limiting excessive glucose entry and its damaging effects in vascular cells.

Preliminary results from this study were presented at a conference on Eicosanoids and other bioactive lipids in cancer, inflammation and related diseases, held at La Jolla, CA, U.S.A. in September 1997, and published in the conference proceedings [15]. We thank Dr Israel Ringel for his assistance in performing the HPLC analyses, and Ms Ana Davarashvili and Ms Edna Oliver for their excellent technical help. S.S. and R.R. are members of The David R. Bloom Center for Pharmacy at the Hebrew University of Jerusalem. This work was supported by grants from The Juvenile Diabetes Foundation International (no. 1-1998-40), The Yedidut Foundation Mexico, the Chief Scientist of The Israel Ministry of Health and The David R. Bloom Center for Pharmacy at the Hebrew University of Jerusalem.

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