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Regulation by metformin of the hexose transport system in vascular endothelial and smooth muscle cells

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1 The effect of the biguanide metformin on hexose transport activity was studied in bovine cultured aortic endothelial (BEC) and smooth muscle cells (BSMC).

2 Metformin elevated the rate of hexose transport determined with 2-deoxyglucose (2DG) in a dose- and time-dependent manner in both cell types. Similar ED_{50} values (0.8–1.0 mM) were determined for the effect of metformin on 2DG uptake in both BEC and BSMC following 24 h exposure to increasing concentrations of metformin, with maximal stimulation at 2 mM.

3 In BEC, metformin increased the hexose transport rate 2–3 fold at all glucose concentrations tested (3.3–22.2 mM). In BSMC incubated with 22.2 mM glucose, metformin elevated the hexose transport ~2 fold. The drug was also effective at lower glucose levels, but did not exceed the maximal transport rate observed in glucose-deprived cells.

4 Similar results were obtained when the effect of metformin on hexose transport activity was assessed with the non-metabolizable hexose analogue, 3-O-methylglucose, suggesting that the drug affects primarily the rate of hexose transport rather than its subsequent phosphorylation.

5 The metformin-induced increase in hexose transport in BSMC treated for 24 h with the drug correlated with increased abundance of GLUT1 protein in the plasma membrane, as determined by Western blot analysis.

6 These data indicate that in addition to its known effects on hexose metabolism in insulin responsive tissues, metformin also affects the hexose transport system in vascular cells. This may contribute to its blood glucose lowering capacity in patients with Type 2, non-insulin-dependent diabetes mellitus.

Keywords: Metformin; glucose transporter; vascular endothelium; vascular smooth muscle; diabetes mellitus; non-insulin dependent; hexose transport

Introduction

The biguanide, metformin, is an antihyperglycaemic drug used in the treatment of patients with Type 2, non-insulin-dependent diabetes mellitus (NIDDM). Unlike the hypoglycaemic sulphonylurea agents (Henquin, 1992), metformin does not modify insulin secretion (Jackson *et al.*, 1987; Baily, 1988; Wu *et al.*, 1990), but acts primarily to increase peripheral glucose utilization (Baily, 1992). Many of the studies on metformin action centred on tissues directly involved in glucose homeostasis, such as skeletal muscle, adipose tissue and liver (Meyer *et al.*, 1967; Wollen & Baily, 1988; Pedersen *et al.*, 1989; Matthaei *et al.*, 1991; Klip *et al.*, 1992; Hundal *et al.*, 1992; Sarabia *et al.*, 1992; Fischer *et al.*, 1995). In recent studies metformin was shown to inhibit the proliferation of human arterial smooth muscle cells and fibroblasts (Koschinsky *et al.*, 1988); in endothelial cells from human umbilical vein, the effect of the drug was variable depending on its concentration (Petty & Pearson, 1992). However, the effect of metformin on the hexose transport system in vascular cells has not been investigated. This is of particular interest in light of the possible involvement of both hyperglycaemia and increased intracellular glucose metabolism in the vascular complications of diabetes (Russel, 1993; Brownlee, 1994).

In a previous study we demonstrated differential adaptive responses of bovine aortic smooth muscle (BSMC) and endothelial cells (BEC) to ambient glucose levels (Kaiser *et al.*, 1993). In BSMC the rate of hexose transport decreased gradually with exposure to increasing glucose concentrations in the culture medium. This modulation of the transport activity was reflected by parallel changes in the maximal velocity (V_{max}) of the transport with no significant changes in the K_m values. In contrast, BEC were termed 'glucose-blind' since they maintained unaltered hexose transport rates in the face of changing ambient glucose levels (Kaiser *et al.*, 1993).

In the present investigation we studied the effect of metformin on the interaction between glucose exposure and glucose transport in cultured vascular endothelial and smooth muscle cells. A drug-induced modulation of the transport system response to ambient glucose may not only counteract hyperglycaemia, but could be of particular importance in light of previous reports that increased glucose flux may augment intracellular glycosylation and alter vascular cell function (Brownlee, 1994; Giardino *et al.*, 1994).

Methods

Cell culture and treatment

Vascular endothelial and smooth muscle cells were prepared from segments of the descending bovine aorta obtained from a local slaughterhouse. Bovine aortic endothelial cells were harvested by a modification of the method described by Gospodarowicz *et al.* (1979).

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Briefly, the intimal aspect of the aorta was exposed and washed extensively with PBS, then scraped gently with a sterile stainless steel spatula. The scraped cells were plated on fibronectin-coated tissue culture dishes in DMEM containing 10% FCS, 50 u ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (Kaiser *et al.*, 1993). Areas of pure endothelial cells were enclosed with cylinder rings, trypsinized, and replated on fibronectin-coated dishes. BEC cultures derived from several aortae were passaged weekly (1:3 split ratio) and confluent, highly organized cultures between the 4th and the 18th passages were used for experiments. Bovine aortic smooth muscle cells were prepared as described by Gimborne & Cortan (1975): after removal of the endothelium as above, pieces (0.5–1.0 mm^3) of medial section of the aorta were removed by dissection and placed in culture dishes containing DMEM with 10% NBCS and antibiotics. Following migration of smooth muscle cells from the explants they were harvested with a trypsin-EDTA solution and cultured at a split ratio of 1:4. Cultured BSMC originating from several aortae were used for experiments between the 4th and the 15th passages. All cultures were maintained at 37°C in a humidified 5% CO_2 incubator. BEC and BSMC were identified as described previously (Kaiser *et al.*, 1993).

Measurement of 2DG and AIB uptake

Confluent cultures in 35 mm dishes were used for the uptake studies. The regular culture medium (5.5 mM glucose) was changed to glucose-free culture medium supplemented with the indicated glucose concentrations without or with metformin, as described below. For the uptake studies, the cultured cells were rinsed five times with PBS at room temperature, and incubated with 1 ml of PBS containing 0.5–1.0 μCi labelled 2DG and the indicated concentration of unlabelled 2DG. After 5 min incubation at room temperature the incubation was terminated by rinsing the cells five times with ice-cold PBS. The cells were then digested with 1 ml of 1 M NaOH for 60 min at 50°C and neutralized with concentrated HCl. Alternatively, the cells were solubilized with 1 ml 0.1% sodium dodecyl sulphate (SDS) solution. Aliquots were taken for radioactive determination by liquid scintillation counting in 10 ml of 40% Lumax in toluene (Lumac, LSC, Gronigen, The Netherlands). Extracellular space was assessed in parallel incubations with [^{14}C]-sucrose as described by Sasson & Cerasi (1986). The uptake of AIB was determined similarly with 0.1 mM AIB and 1.0 μCi labelled AIB. The uptake data were calculated on the basis of cell number. The uptake of 2DG was linear up to 10 min at all glucose concentrations tested in the absence or the presence of metformin (not shown).

Measurement of 3OMG uptake

The cultures were rinsed as described above and incubated at room temperature for 30 s (BSMC), or 1 min (BEC) with 1 ml PBS containing 0.1 mM 3OMG, 2.0 μCi tritiated 3OMG, and 1.0 μCi [^{14}C]-sucrose. The uptake was terminated by the addition of 40 μM CB for 30 s on ice, followed by five washes with ice-cold PBS. After the washes, the cells were processed for liquid scintillation as described above. Results shown are corrected for counts in the extracellular space and cell number. The uptake of 3OMG was linear up to 2 min for both BEC and BSMC (not shown).

Membrane preparation and Western blot anaylsis

Cells were grown on 100-mm dishes under the various experimental conditions described. Crude membrane preparations were prepared as described previously (Kaiser *et al.*, 1993).

Enriched plasma membrane fractions of BSMC were obtained by subcellular fractionation using the method of Greco-Perotto *et al.* (1992), orginally developed for cultured L8 myocytes. The enrichment in plasma membranes estimated by measuring the changes in the specific activity of 5'-nucleotidase was 7.7±0.9 and 7.7±1.1 fold ($n=3$) relative to the initial cell homogenate from control and metformin-treated BSMC, respectively. Enriched microsomal membrane fractions of BSMC could not be recovered by this method, nor were we successful in applying the procedure to BEC.

Protein concentrations in the various membrane preparations were determined by the Coomassie brilliant blue assay (Bradford, 1976). For Western blot analysis, the membrane preparations were diluted in Laemmli buffer, and aliquots (2–5 μg and 0.8–1.2 μg of crude membrane protein and plasma membrane protein, respectively) separated on 10% SDS/polyacrylamide gels. The resolved proteins were transferred to nitrocellulose membranes, and analyzed by Western blot as described (Kaiser *et al.*, 1993) with specific rabbit antiserum raised against a synthetic peptide corresponding to the C-terminus of mouse GLUT1 (Weiland *et al.*, 1990) at dilutions of 1:100 to 1:250. Immunoreactive species were detected by ECL according to the manufacturer's instructions.

Glucose consumption

The rate of glucose consumption in confluent cells was calculated from the change in the concentration of glucose in the medium under the conditions specified below. Glucose was determined by the glucose oxidase method with a glucose analyzer (Beckman Industries, Fullerton, CA, U.S.A.).

Chemicals and materials

2-[1,2-^{3}H]-deoxyglucose (2DG, 40 Ci $mmol^{-1}$), [^{3}H-methyl]-3-O-methylglucose (3OMG, 37 Ci $mmol^{-1}$), and [1-^{14}C]-α-aminoisobutyric acid (AIB, 51 mCi $mmol^{-1}$) were purchased from DuPont-New England Nuclear (Boston, MA, U.S.A.). [U-^{14}C]-sucrose (485 mCi $mmol^{-1}$) and enhanced chemiluminescence kit (ECL) were from the Radiochemical Centre (Amersham, Bucks, UK). D-Glucose was from Merck (Darmstadt, Germany). Cytochalasin B (CB), 2DG and 3OMG were from Sigma (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), penicillin, streptomycin and 0.05% trypsin-0.02% EDTA solution were purchased from Biological Industries (Kibbutz Beth-Haemek, Israel). Glucose-free DMEM was purchased from Gibco (Grand Island, NY, U.S.A.). Newborn calf serum (NBCS) was from Bio Lab (Jerusalem, Israel). Human fibronectin was obtained from the New York Blood Center (New York, NY, U.S.A.). Tissue culture plates were from Nunc (Roskilde, Denmark). All other chemicals were reagent grade. Phosphate buffered saline (PBS) contained (in mM): NaCl 137, Na_2HPO_4 9.6, KCl 2.7, KH_2PO_4 1.5, $MgCl_2$ 0.5, $CaCl_2$ 1.0, pH 7.4.

Data presentation and statistical analysis

Data were computed as mean ± s.e.mean and compared by the two sample t test, because each experiment made use of cells from the same seeding.

Results

Regulation of 2DG uptake by glucose and metformin

Vascular endothelial (BEC) and smooth muscle (BSMC) cells exhibited differential regulation of hexose transport in response to the level of ambient glucose (Figure 1). BEC incubated in media containing glucose concentrations between 3.3 and 22.2 mM maintained a constant rate of hexose uptake at the level of ~0.05 nmol 2DG min^{-1} per 10^6 cells. Metformin augmented the rate of hexose uptake in these cells 3 fold. Glucose consumption in confluent endothelial cells, as determined by the rate of glucose disappearance from the culture medium during the last 24 h of the experiments, was 1–2 μmol 24 h^{-1} per 10^6 cells. Metformin augmented the glucose con-

sumption rate by a further 4–5 μmol 24 h^{-1} per 10^6 cells.

BSMC adapted to medium glucose by reducing the rate of hexose uptake in 24 h incubations in medium containing increasing concentrations of glucose. The uptake of 2DG decreased progressively from 0.63 ± 0.01 nmol min^{-1} per 10^6 cells in cultures exposed to 3.3 mM glucose to 0.42 ± 0.02 and 0.15 ± 0.01 nmol min^{-1} per 10^6 cells in cells incubated with 5.5 and 22.2 mM glucose, respectively. The basal glucose consumption in BSMC initially exposed to DMEM containing 22.2 mM glucose was 9.9 ± 1.2 μmol min^{-1} per 10^6 cells. In cells incubated with DMEM initially containing 3.3 or 5.5 mM glucose, medium glucose decreased to undetectable levels at 24 h. Despite the fact that glucose levels were similarly reduced to an undetectable level, BSMC in the two media exhibited significant variations in the rate of 2DG uptake, suggesting that under the extreme condition of glucose deprivation, the autoregulatory response is dependent on the duration of the exposure to glucose-free medium. Metformin (2 mM) increased the 2DG uptake in BSMC exposed to 22.2 mM glucose by 1.7 fold, and augmented the rate of glucose consumption by 3.3 ± 0.5 μmol min^{-1} per 10^6 cells. The efficiency of metformin to increase hexose uptake showed a progressive decline as medium glucose decreased, the effect disappearing at 3.3 mM glucose. Therefore, in further experiments the effect of the drug was studied in BSMC exposed to 22.2 mM glucose, in which its effect was most pronounced.

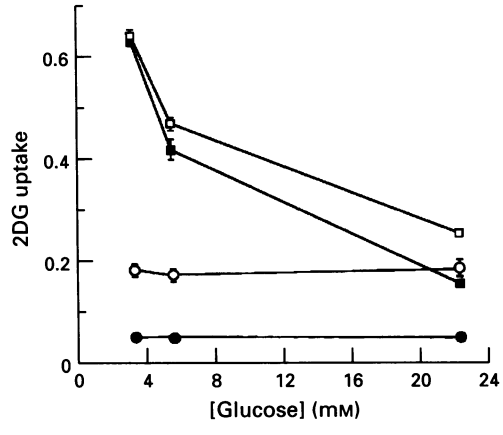

Figure 1 Effect of medium glucose and metformin on 2DG uptake into vascular cells. Confluent cultures of BEC (●, ○) and BSMC (■, □) were preincubated for 24 h in DMEM containing 3.3, 5.5, or 22.2 mM glucose without (closed symbols) and with 2 mM metformin (open symbols). Uptake of 2DG (nmol min^{-1} per 10^6 cells) was performed as described under Methods. Results are mean ± s.e.mean of triplicate plates. When not shown, symbols mask the small error bars. The differences between control and metformin-treated cells were significant ($P < 0.05$) for all glucose concentrations tested in both cell types except for BSMC exposed to 3.3 mM glucose.

Since the 2DG uptake assay measures both free and phosphorylated hexose it cannot differentiate between 2DG transport across the cell membrane and its intracellular phosphorylation by hexokinase. Therefore, we tested the effect of metformin on the transport of the non-phosphorylated hexose analogue, 3OMG. In BEC incubated for 24 h with 5.5 and 22.2 mM glucose, the transport rates of the analogue were 0.031 ± 0.001 and 0.034 ± 0.001 nmol min^{-1} per 10^6 cells, respectively. Metformin (2 mM) increased these rates to 0.063 ± 0.001 and 0.059 ± 0.001 nmol min^{-1} per 10^6 cells, respectively. In BSMC incubated at 22.2 mM glucose, basal transport of 3OMG was 0.076 ± 0.006 $nmol^{-1}$ per 10^6 cells and metformin increased it to 0.110 ± 0.001 nmol min^{-1} per 10^6 cells. These changes in the transport rate of 3OMG were similar to those obtained with 2DG (Figure 1), suggesting that the drug modulates primarily the rate of hexose transport into vascular cells. Cytochalasin B, a potent competitive inhibitor of the glucose transporter, inhibited effectively (at 10–50 μM) in both cell types the uptake of both 2DG and 3OMG in the presence or the absence of metformin (not shown).

The specificity of the action of metformin on membrane transport processes was evaluated by comparing its effect on the uptake of 2DG and the non-metabolizable amino acid analogue, AIB (Table 1). The rate of AIB uptake was not augmented by the drug, nor was it affected by the ambient glucose in both BEC and BSMC.

Dose-response of the effect of metformin on 2DG uptake

Figure 2 depicts the dose-dependent effect of metformin on 2DG uptake in BEC and BSMC following a 24 h incubation with the drug. Metformin augmented the 2DG uptake rate in BEC with a similar dose-dependency whether the cells were incubated at 5.5 or 22.2 mM glucose. Half maximal stimulation was obtained at ~0.8 mM metformin, with a maximal increase of 3 fold above control observed with 2 mM metformin. In BSMC preincubated in 22.2 mM glucose, metformin increased the rate of hexose uptake maximally by 1.6 fold. The half maximal effect of the drug was obtained at 1 mM, with a near maximal effect at 2 mM. Therefore, in the following the action of metformin was usually evaluated at 2 mM.

Time course of metformin effect on 2DG uptake

The time course of the effect of metformin on the hexose transport activity in BEC and BSMC is shown in Figure 3. Cells conditioned for 24 h at 5.5 or 22.2 M glucose-containing media were exposed to media containing the same glucose concentrations in the absence or presence of 2 mM metformin for up to 24 h. The effect of metformin at each of the indicated times was calculated relative to the uptake in control cells in-

Table 1 Effect of metformin on 2DG and AIB uptake in BEC and BSMC

Glucose (mM)	*AIB uptake* (pmol min^{-1} per 10^6 cells)		*2DG uptake* (nmol min^{-1} per 10^6 cells)	
	BEC	*BSMC*	*BEC*	*BSMC*
5.5	9.5 ± 1.0	35.9 ± 1.3	0.085 ± 0.002	0.433 ± 0.011
5.5 + Metformin	8.1 ± 0.6	29.2 ± 3.0	0.197 ± 0.011[a]	0.474 ± 0.013
22.2	8.7 ± 1.1	31.4 ± 2.0	0.085 ± 0.006	0.131 ± 0.005
22.2 + Metformin	6.7 ± 0.6	27.5 ± 2.0	0.165 ± 0.004[a]	0.210 ± 0.011[a]

Confluent BEC and BSMC cultures were incubated with DMEM supplemented with 5.5 or 22.2 mM glucose for 24 h. The cells were then washed and received the same medium without or with 2 mM metformin for additional 24 h. The 2DG and AIB uptake assays were performed as described under Methods. Results are mean ± s.e.mean of 3 plates per treatment.
[a]Significantly different from metformin-free control ($P < 0.005$, by Student's t test).

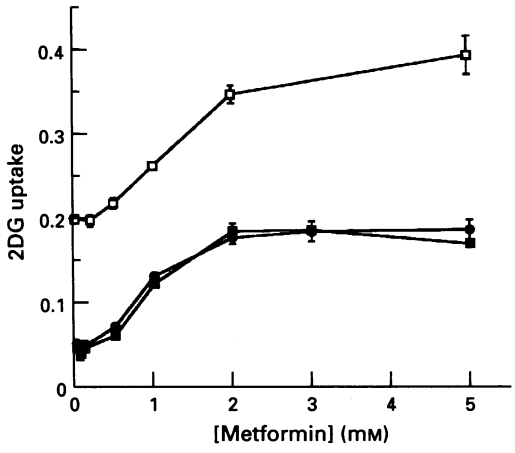

Figure 2 Dose-response of the effect of metformin on 2DG uptake. BEC (closed symbols) and BSMC (open symbols) were preincubated with DMEM containing 5.5 mM glucose (● BEC) or 22.2 (■, BEC and □ BSMC) for 24 h, followed by an additional 24 h incubation in media containing the same level of glucose with increasing concentrations of metformin (0–5 mM). The uptake of 2DG (nmol min^{-1} per 10^6 cells) was determined at the end of the incubations as described under Methods. Results are mean ± s.e.mean of triplicate plates from a representative experiment. When not shown, symbols mask the small error bars.

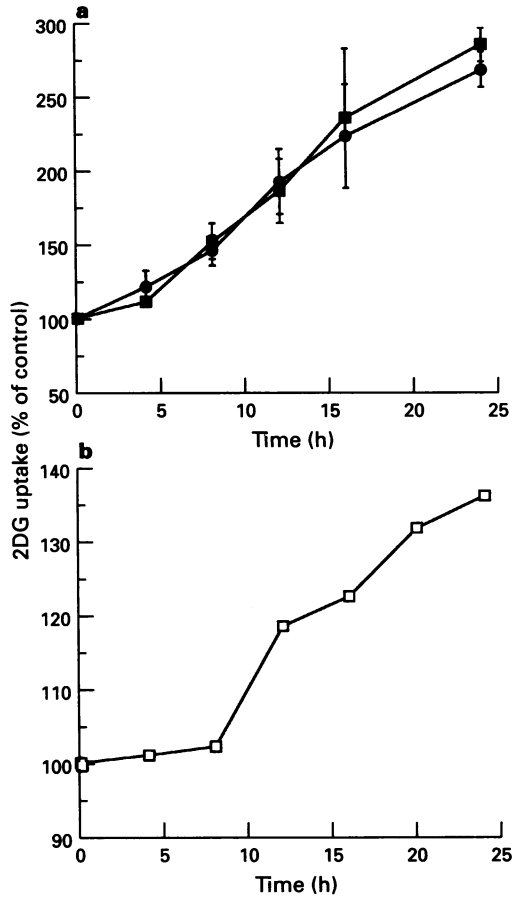

Figure 3 Time course of the effect of metformin on 2DG uptake. Confluent cultures of BEC (a) and BSMC (b) were preincubated for 24 h in DMEM containing either 5.5 (●, BEC) or 22.2 mM glucose (■, BEC and □, BSMC), followed by different times of incubation in media containing the same glucose level as in the preincubation with and without 2 mM metformin. The effect of metformin on 2DG uptake (nmol min^{-1} per 10^6 cells) was calculated relative to control plates incubated for the same time period without the drug. Results for BEC (a) are shown as mean ± s.e.mean of 3 individual experiments, each run on triplicate plates, and for BSMC (b) as an average of 2 experiments each on triplicate plates. The 100% values assigned to 2DG uptake of control cultures at 0 time of incubation were 0.046 ± 0.004 and 0.052 ± 0.007 nmol min^{-1} per 10^6 cells ($n=3$) in BEC preincubated with 5.5 and 22.2 mM glucose, respectively, and 0.46 nmol min^{-1} per 10^6 cells ($n=2$) in BSMC preincubated with 22.2 mM glucose. The uptake of 2DG was determined as described under Methods.

cubated with metformin-free medium. Following a lag period of >4 h, a significant increase ($P < 0.01$, relative to control cells incubated for the same time period without metformin) was observed at 8 h of incubation in BEC; the uptake continued to increase almost linearly throughout the 24 h of incubation (Figure 3a). A lag period of 8 h was observed in BSMC, followed by a gradual increase which reached the level of ~40% above control by 24 h of exposure to metformin (Figure 3b).

Effect of glucose and metformin on GLUT1 protein

To test whether the increase in the rate of hexose transport induced by metformin was the result of an increase in the steady state amount of the glucose transporters and/or changes in their subcellular distribution, we measured the cellular level of GLUT1, the only transporter isoform identified in bovine cultured vascular cells (Kaiser *et al.*, 1993), in crude membranes and enriched plasma membrane fractions by Western blot analysis. A band corresponding to a 45,000-Mr protein was detected in both BSMC and BEC. Metformin at maximal effective doses of 2 or 5 mM did not affect the total cellular content of GLUT1 in either type of cell incubated for 24 h with 22.2 mM glucose (Figure 4a). However, the abundance of GLUT1 protein in the plasma membrane fraction of BSMC exposed to 22.2 mM glucose was increased 1.9 ± 0.3 fold by 5 mM metformin; the uptake of 2DG increased 2.1 ± 0.2 fold (mean ± s.e.mean of 3 individual experiments) under similar experimental conditions. Exposure to low glucose level (3.3 mM) also resulted in increased abundance of GLUT1 protein in the plasma membrane-enriched fraction relative to cells exposed to 22.2 mM glucose; the observed increase of ~3 fold ($n=2$ individual experiments) in plasma membrane GLUT1 transporter protein (Figure 4b) was matched by a 3.2 ± 0.3 fold increase (mean ± s.e.mean of 7 individual experiments, and Figure 1) in the rate of 2DG uptake observed under similar experimental conditions. Control experiments indicated that metformin did not affect protein synthesis in the vascular cells as determined by [^{3}H]-leucine incorporation into cellular proteins following 24 h exposure to the drug (not shown).

Kinetic analysis of metformin effect on 2DG uptake

To determine whether increased transporter abundance in the plasma membrane in the presence of metformin can account for the observed increase of hexose transport activity (Figure 2), we further analyzed the kinetics of 2DG uptake over increasing concentrations of the analogue. Figure 5a and b shows a 1.5 fold increase in the maximal capacity (V_{max}) of BSMC to take up 2DG. Similarly, BEC (Figure 6a and b) exhibited a ~2 fold increase in the maximal capacity to take up the hexose analogue into the cells. The K_m of the system was not affected by metformin in either type of cell. It should be noted that the V_{max} values calculated in the above experiments could be underestimated due to inhibition of hexokinase by accumulated 2DG-6-phosphate in the cells (Gliemann & Rees, 1983). Nevertheless, the observed relative changes in the V_{max} of the uptake, clearly indicate that metformin affected the capacity of the hexose transport system in both types of cells.

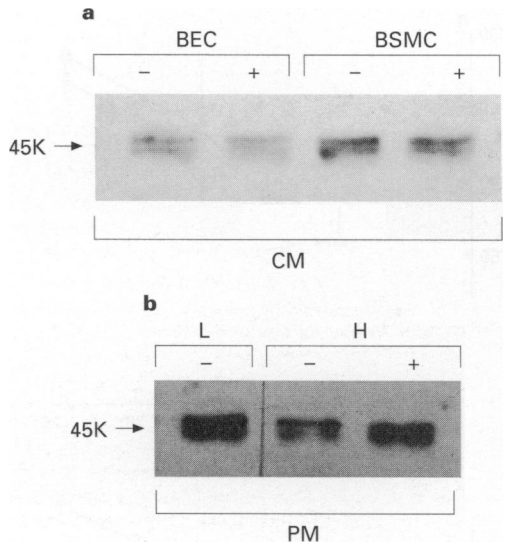

Figure 4 Immunoblot analysis of GLUT1 transporter in BEC and BSMC exposed to metformin. Crude membranes (CM) and enriched plasma membrane preparations (PM) were prepared from confluent vascular cells pre-exposed for 24 h to 22.2 mM glucose followed by a futher 24 h exposure to the same concentration of glucose alone (−) or together with 5 mM metformin (+). BSMC were also studied after pre-exposure to 3.3 mM glucose for 24 h followed by a further 24 h exposure to the same concentration of glucose. Five μg of crude membrane protein and 0.8 μg of plasma membrane protein were resolved on 10% SDS-polyacrylamide gels and processed as described in Methods. The figure is a representative experiment of $n=2$ and $n=3$ experiments performed for BEC and BSMC, respectively. Western blot analysis of crude membranes (CM) from BEC and BSMC exposed to 22.2 mM glucose with (+) and without (−) metformin is shown in (a). Immunoblot analysis of the plasma membrane-enriched fractions (PM) from BSMC exposed to either 3.3 (L), or 22.2 (H) mM glucose with (+) and without (−) metformin is shown in (b).

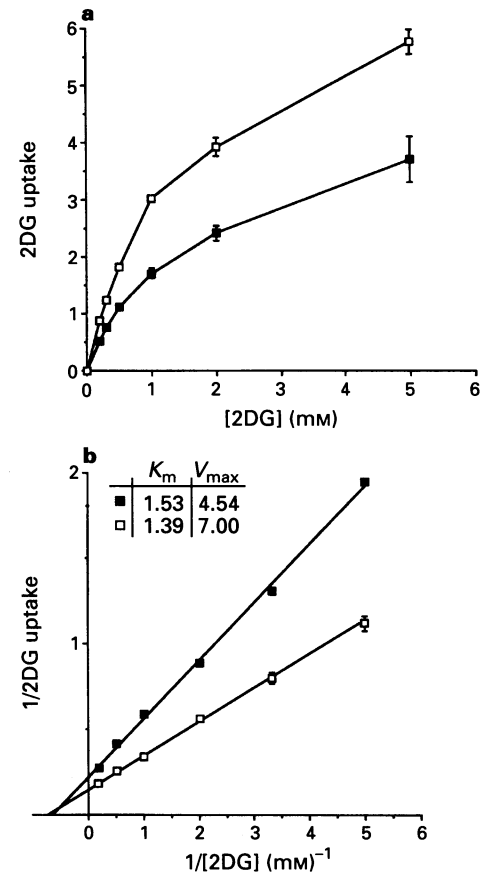

Figure 5 Dose-response and kinetic analysis of the uptake of 2DG into BSMC. Cultures were preincubated in 22.2 mM glucose for 24 h followed by additional 24 h in the same medium without (■) or with (□) 2 mM metformin. The uptake of tritiated 2DG (nmol min^{-1} per 10^6 cells) was determined in triplicate plates in the presence of varying concentrations of unlabelled 2DG. (b) Lineweaver-Burk analysis of the data in (a). Calculated K_m and V_{max} values are shown in the inset. Results are depicted as mean ± s.e.mean of triplicate plates from a representative experiment. When not shown, symbols mask the small error bars.

Discussion

In the present study metformin was found to stimulate the rate of 2DG uptake into both BEC and BSMC in a dose- and time-dependent manner (Figures 2 and 3). The observation that metformin was equally effective in augmenting the uptake of 2DG and 3OMG into vascular cells suggests that it stimulates primarily hexose transport across the vascular cell membrane. The action of metformin on hexose transport was selective, with no stimulatory effect on the transport of the neutral, non-metabolizable amino acid analogue, AIB (Table 1).

The time course of metformin action on hexose transport which showed a considerable delay (4 and 8 h in BEC and BSMC, respectively, Figure 3), is unlikely to result from low permeability of the drug into an intracellular site of action, since the maximal uptake of the drug was reached by 60 min following either *in vitro* or *in vivo* administration (Wilcock *et al.*, 1991; Khan *et al.*, 1992).

The concentration required for maximal effect following 24 h exposure to metformin was 2 mM in both types of vascular cells (Figure 2), with ED_{50} of 0.8–1.0 mM. These levels, although similar to those obtained in the L6 skeletal muscle cell line (Hundal *et al.*, 1992; Klip *et al.*, 1992), heart muscle (Fischer *et al.*, 1995) and rat adipocytes (Matthaei *et al.*, 1991), are higher than the suggested therapeutic concentrations in diabetic patients (~10 μM) (Marchetti *et al.*, 1990; Caille *et al.*, 1993). The reason for this discrepancy is not known, but may be related to the general tendency of cultured cells and isolated organ preparations to have lower sensitivities to a number of pharmacologically active agents and hormones. Indeed, Galuska *et al.* (1994) have shown recently that therapeutic concentrations of metformin failed to increase basal or insulin-stimulated 3OMG transport in isolated skeletal muscle strips obtained from patients with NIDDM and healthy subjects.

Glucose transport activity may be regulated at the transcriptional level, or at the level of the transporter protein (for review see, Klip *et al.*, 1994), e.g., by redistribution of transporters between intracellular membranes and the plasma membrane, by changes of transporter turnover (synthesis and/or degradation) and by changes of intrinsic activity of the transporter. The mode of regulation appears to be tissue specific and dependent on the type of transporter and the nature of the regulatory agent (Kahn & Flier, 1990; Klip *et al.*, 1994). The autoregulatory effect of glucose on the hexose transport system of BSMC under normoglycaemic or hyperglycaemic conditions (5.5 or 22.2 mM glucose) did not involve a change in either the steady state level of GLUT1 mRNA, or total transporter protein (Kaiser *et al.*, 1993). Likewise, the total cellular transporter protein was not affected by 24 h exposure to metformin in downregulated BSMC exposed to 22.2 mM glucose. Given the significant increase (1.5–2.0 fold) in the rate of hexose transport induced by metformin under these experimental conditions, the drug could act by either changing the intrinsic activity of the existing transporters, or affecting their subcellular distribution, i.e., increasing the concentration of active transporters at the plasma membrane, as was also observed in metformin-treated L6 myotubes and in rat adipocytes (Matthaei *et al.*, 1991; Hundal *et al.*, 1992). The ob-

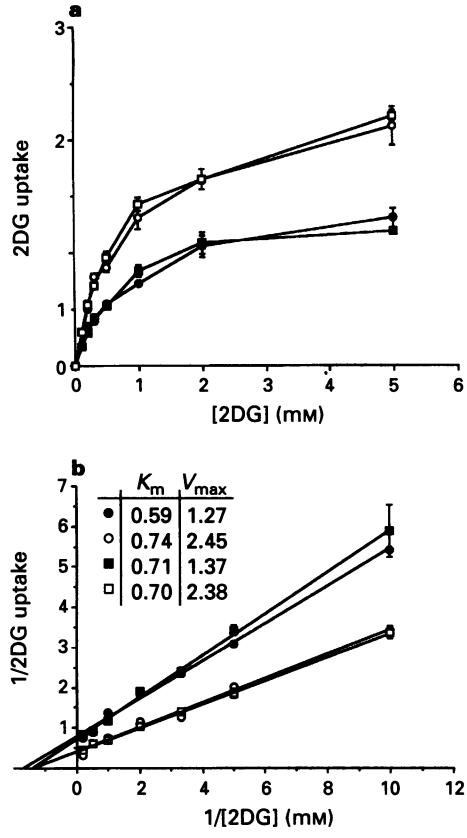

Figure 6 Dose-response and kinetic analysis of the effect of metformin on the uptake of 2DG into BEC. Cultures were preincubated for 24 h in DMEM containing 5.5 or 22.2 mM glucose. At the end of the preincubation the cells were changed to DMEM containing the same glucose concentrations without (●, 5.5 mM glucose; ■, 22.2 mM glucose) or with 2 mM metformin (○, 5.5 mM glucose; □, 22.2 mM glucose). Experimental protocol and presentation of the data are as described in legend to Figure 5. The calculated kinetic parameters are shown in the inset.

servation that transport activity, maximal transport capacity and plasma membrane GLUT1 levels in BSMC exposed to 22.2 mM glucose were similarly augmented by metformin, suggests that the drug modulates transport activity by increasing transporter abundance at the plasma membrane, rather than affecting its intrinsic activity. Moreover, no change in the total cellular content of GLUT1 protein following exposure to stimulatory concentrations of metformin was observed in BEC. However, since plasma membrane fractions prepared from BEC were of a low purity (as judged by 5' nucleotidase activity), the mechanism of metformin action in these cells could not be investigated.

BSMC, operate an effective autoregulatory mechanism of glucose transport (Kaiser *et al.*, 1993) protecting the cells from the possible adverse effect of an increase in glucose flux (Brownlee, 1994; Giardino *et al.*, 1994). BEC, on the other hand, oppose the autoregulatory effect of glucose observed in BSMC and many other types of cells (Klip *et al.*, 1994). Yet the inherent lower activity of the hexose transport system in BEC was proposed to protect the cells against increase in intracellular glucose and alleviate the need for an additional control (Kaiser *et al.*, 1993). In downregulated BSMC, metformin augmented the rate of hexose transport and plasma membrane content of GLUT1, but these effects never exceeded those observed in upregulated cells (Figures 1 and 4b), whereas, in BEC, the drug was equally effective in augmenting hexose transport at all glucose concentrations tested. This indicates that a cellular mechanism for modulating hexose transport, although unresponsive to changes in extracellular glucose, is present in endothelial cells, and can be activated by metformin.

Irrespective of the precise mechanism involved, the observation that metformin stimulated hexose transport in vascular cells may be of therapeutic significance. On the other hand, this may be a positive effect which contributes to the blood glucose lowering capacity of the drug. On the other hand, the ability of metformin to augment glucose transport into vascular cells could be associated with increased intracellular glycosylation and hence contribute to the adverse effects associated with chronic hyperglycaemia, i.e., diabetic angiopathy. The beneficial and possible deleterious effects of metformin in vascular cells deserve further studies.

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