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Glucose entry into rat mesangial cells is mediated by both Na^+-coupled and facilitative transporters

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Summary Since previous studies from our laboratory have demonstrated that increased glucose consumption by cultured rat mesangial cells is accompanied by an accelerated production of type IV and type VI collagen, we have now examined the manner by which glucose is transported into these cells. A progressive stimulation of glucose uptake by the mesangial cells was observed with increasing concentrations of NaCl so that at 145 mmol/l about twice as much glucose entered the cells as in its absence (substituted by choline chloride). Moreover, since phlorizin inhibited the NaCl-promoted uptake of glucose and this salt was found to increase the accumulation of α-methylglucoside in a manner which could not be duplicated by KCl or mannitol, both Na^+-coupled and facilitative glucose transporters appeared to be present in the cells. K_m values of 1.93 mmol/l and 1.36 mmol/l were determined for the co-transport and facilitated transport pathways, respectively, with their V_{max} being 29.5 and 18.0 nmol · mg protein^{-1} · h^{-1}. Both uptake activities were found to be down-regulated by exposure of the cells to high glucose and furthermore the Na^+-dependent transport could no longer be detected after about 12 passages of the cells. Hybridization of mesangial cell mRNA with cDNA probes revealed transcripts for the Na^+/glucose co-transporter as well as GLUT1 and to a lesser extent GLUT4. The identification of the co-transporter in these non-polarized cells is pertinent to an understanding of the intracellular signals which can lead to the development of the diabetic glomerular lesions; in the hyperglycaemic state this carrier provides an additional route for accelerated glucose entry and furthermore by the attendant increase in Na^+ flux may bring about an alteration in the ionic composition of the cell. [Diabetologia (1995) 38: 291–297]

Key words Na^+-coupled glucose transporter, facilitative glucose transporters, mesangial cells, glomerulopathy, phlorizin.

It has been recognized since the initial description of the diabetic glomerular lesions by Kimmelstiel and his collaborators [1, 2] that the mesangium plays a key role in their development. More recently it has been shown that there is a close correlation between expansion of this region of the glomerulus and the clinical manifestations of diabetic nephropathy [3].

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Abbreviations: FBS, Fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; SGLT, sodium/glucose co-transporter.

Although it has become evident that increased deposits of type IV as well as type VI collagen occur in the diabetic glomerulus [4, 5] and that mesangial cells in culture demonstrate enhanced production of these two proteins in response to elevated glucose levels [6, 7], the mechanism by which glucose and its metabolites regulate the synthesis of the glomerular extracellular matrix remains obscure. Only very limited information is currently available in regard to the manner of entry and subsequent metabolism of glucose by mesangial cells, a subject which is crucial to the understanding of the metabolic hypothesis of diabetic glomerulopathy.

In an attempt to fill this void in knowledge and prompted by our recent observation that mesangial

cells respond to elevated NaCl by increased consumption of glucose [7], we have explored the possibility that glucose entry into these cells may occur by means of Na^+-coupled as well as facilitated transport. On the basis of uptake studies with radiolabelled glucose and non-metabolizable hexoses, as well as the use of transport inhibitors, we have obtained evidence that mesangial cells, quite unexpectedly in view of their apolar and non-epithelial nature, do indeed have representatives of the two distinct recognized families of glucose transporters [8–10] which we have identified by hybridization of their mRNAs with cDNA probes.

Materials and methods

Isolation and culture of cells. Highly purified glomeruli were prepared from the kidneys of Sprague-Dawley rats (approximately 150 g, Taconic Farms, Germantown, N.Y., USA) by a differential sieving procedure [11] and after treatment with collagenase [6] were separated from released cells and plated on 100-mm dishes for growth at 37 °C in an atmosphere of 95 % air and 5 % CO_2 in a medium containing 5 mmol/l glucose and consisting of a 1 : 1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 (both from Sigma, St. Louis, Mo, USA) to which was added 10 % fetal bovine serum (FBS) (ICN, Costa Mesa, Calif., USA), 10 % NuSerum (Collaborative Research, Bedford, Mass., USA) as well as penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin (0.25 µg/ml). The initial glomerular outgrowths contained both mesangial cells, identified as previously described [6], and endothelial cells, which were visualized by their uptake of rhodamine-labelled acetylated low density lipoprotein [6], but no epithelial cells were present. After the third passage, only the mesangial cells remained. The LLC-PK_1 porcine renal epithelial cell line as well as HepG2 and WI-38 cells were obtained from ATCC (Rockville, Md., USA) and grown in the same medium as the mesangial cells; myocytes were prepared from rat hearts [12].

Hexose uptake determinations. In preparation for assay the mesangial cells (after five to nine passages unless otherwise specified) which had reached over 90 % confluency ($\sim 3 \times 10^5$ cells) in 23-mm wells (Falcon, Fisher Scientific Co., Pittsburgh, PA, USA) were incubated in the DMEM-Ham's F-12 medium containing 5 mmol/l glucose, 5 % FBS and 5 % NuSerum for 3 days. Subsequently the medium was changed to one containing 1 % FBS – 1 % NuSerum and variable concentrations of NaCl and choline chloride for a 3-h preincubation. After washing the cells twice with 20 mmol/l Tris/HEPES buffer, pH 7.4, containing 1 mmol/l $CaCl_2$, 5 mmol/l KCl1 and 2.5 mmol/l $MgSO_4$ and the appropriate concentrations of NaCl and choline chloride the radiolabelled hexoses were added in 400 µl of this medium for incubations at 37 °C. The sugars assayed for uptake were [2-^{3}H]-D-glucose (5.0 µCi/ml), 2-[^{3}H(G)]deoxy-D-glucose (1.0 µCi/ml) and methyl-α-D-[^{14}C(U)]glucoside (0.5 µCi/ml) all purchased from DuPont-NEN (Boston, MA, USA) and adjusted to a 0.1 mmol/l concentration with unlabelled hexoses obtained from Sigma. Standard incubations were terminated at 30 min by rapid aspiration of the media followed by three washes with ice-cold phosphate buffered saline. Solubilization of the cells was then achieved at room temperature with 400 µl of 0.5 mol/l NaOH and aliquots were taken for the measurement of radioactivity by scintillation counting in a Beckman LS7500 instrument (Fullerton, CA, USA) following neutralization with acetic acid; protein determinations were performed by the Bradford procedure [13] using bovine serum albumin as a standard. In some experiments phlorizin and cytochalasin B were included during the assay while in others the cells were preincubated for specified times with 20 mmol/l glucose. For the determination of K_m values the glucose uptake assays were carried out at various glucose concentrations in the presence of 145 mmol/l NaCl or choline chloride. All incubations were carried out in quadruplicate and the sugar uptake values were calculated per mg of cell protein.

Preparation and radiolabelling of cDNAs. Clones of *Escherichia coli* containing cDNA for the human facilitative glucose transporters GLUT1, GLUT2, GLUT3 and GLUT4 were obtained from ATCC (catalogue numbers 59630, 61612, 61614, and 61616, respectively) while rabbit SGLT1 cDNA inserted in the EcoRI site of pBSK- was a generous gift of Dr. D.B. Rhoads (Massachusetts General Hospital, Boston, Mass., USA); the latter was used to transform XL1-Blue MRF' Epicurian *E. coli* cells (Stratagene, La Jolla, Calif., USA). After growing the cells in Luria broth (GLUT1, 2, 3 and 4) or *terrific broth* (SGLT1) [14], the plasmids were adsorbed on tip-500 column (Qiagen, Chatsworth, Calif., USA) and after elution treated with restriction enzymes (BamH1 for GLUT1; SalI for GLUT2, GLUT3, and GLUT4; and EcoRI for SGLT, all from GIBCO-BRL, Gaithersburg, Md., USA) for 60 min at 37 °C. After electrophoresis on agarose gels cDNAs of the appropriate size (GLUT1 and GLUT2 = 2.4 kb; GLUT3 = 2.6 kb; GLUT4 = 2.0 kb; and SGLT = 2.2 kb) were recovered from the gel by treatment with GELase (Epicenter Technologies, Madison, Wis., USA) and precipitation with 75 % ethanol – 0.3 mmol/l ammonium acetate and stored in Tris/HCl, pH 7.4, 1 mol/l EDTA at – 20 °C; actin cDNA from chicken was purchased from Oncor, Inc. (Gaithersburg, Md., USA). The probes were radiolabelled with [α-^{32}P]dCTP (DuPont-NEN) with the use of the Multiprime DNA Labelling System (Amersham, Arlington Heights, Ill., USA) and purified after addition of 100 µg of salmon sperm DNA (Sigma) by high performance liquid chromatography as previously described [7].

Isolation of mRNA. Total RNA was extracted from confluent plates of mesangial cells (after eight passages) and LLC-PK_1 cells by the guanidinium-thiocyanate-phenol-chloroform procedure [15] using RNAzol (Biotecx Laboratories, Houston, Tex., USA). Isolation of poly(A^+)RNA was accomplished using oligo(dT)-cellulose in spin columns (Biotecx) following the manufacturer's instructions and using 500 mg of the beads for 0.5 to 1 mg of total RNA determined from its adsorption at 260 nm.

Hybridization of mRNA. Samples of purified mRNA were, after denaturation, electrophoresed on 1 % agarose gels containing 2.2 mol/l formaldehyde followed by capillary blotting onto nylon membranes (Nytran, Schleicher and Schuell, Keene, N.H., USA) [14]. Prehybridization and hybridization with ^{32}P-labelled cDNA probes were then carried out as previously described [7]. After washing with 2.0 × SCC (0.3 mol/l NaCl, 30 mmol/l sodium citrate, pH 7.4) containing 0.1 % SDS at 30 °C (three times) followed by an additional high stringency wash with 0.1 × SCC – 0.1 % SDS at 55 °C, radioautography was carried out at – 80 °C using X-Omatic AR film (Eastman Kodak, Rochester, N.Y., USA). Dot blot analyses were performed on aliquots of total RNA in a man-

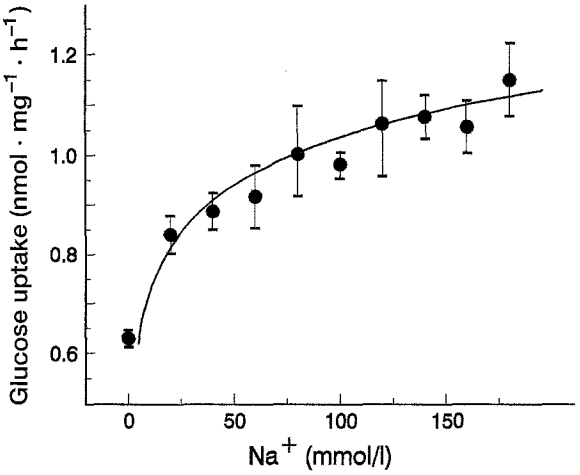

Fig. 1. Effect of Na^+ on glucose uptake by rat mesangial cells. The cells were incubated with [2-^{3}H]-D-glucose (0.1 mmol/l, 5 μCi/ml) at increasing NaCl concentrations under the conditions described in Methods and the uptake of glucose was determined by scintillation counting. To maintain constant osmolarity choline chloride was substituted for NaCl. Determinations were carried out in quadruplicate and expressed as nmoles per mg of protein per h (mean ± SEM). Approximately 100 μg of cell protein was present in each well. The r^2 value for a logarithmic regression curve of the data was 0.91 ($p < 0.001$). From the curve it can be noted that glucose uptake in the absence of Na^+ was 0.63 nmol · mg^{-1} · h^{-1} while half-maximal stimulation occurred at a 34 mmol/l concentration of this ion

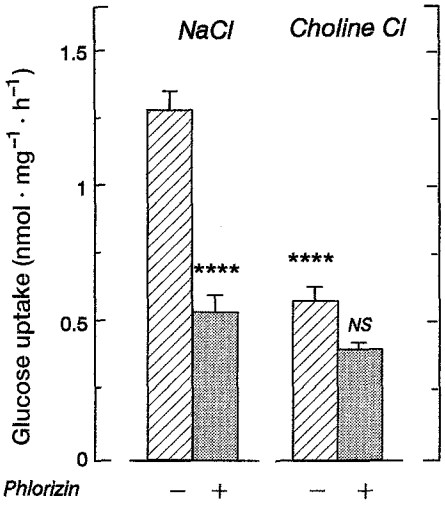

Fig. 2. Effect of phlorizin on glucose uptake by mesangial cells in the presence and absence of Na^+ ions. The cells were incubated with [2-^{3}H]-D-glucose (0.1 mmol/l, 5 μCi/ml) in the presence of 145 mmol/l NaCl or 145 mmol/l choline chloride with (+) or without (−) phlorizin (0.2 mmol/l) as described in Methods. Determinations of glucose uptake are expressed as nmoles per mg cell protein (mean ± SEM). **** $p < 0.001$, compared to NaCl with no inhibitor; NS, not significant

ner previously reported [7]. Quantitation of the components on the autoradiographs was achieved with a Model 300 A laser densitometer (Molecular Dynamics, Sunnyvale, Calif., USA).

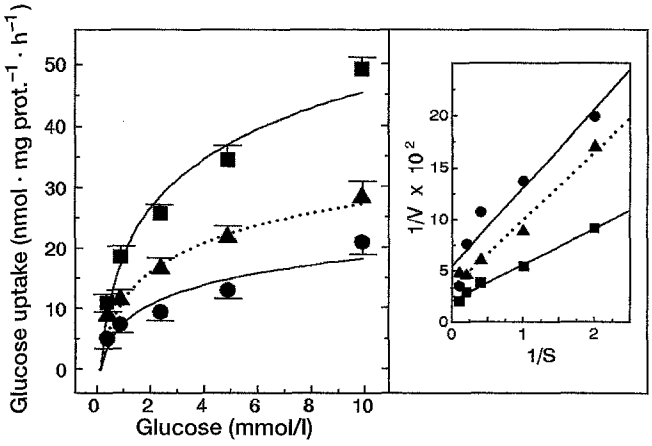

Fig. 3. Glucose uptake by mesangial cells in the presence and absence of Na^+ ions. The cells were incubated with [2-^{3}H]-D-glucose (5 μCi/ml) at glucose concentrations ranging from 0.5 mmol/l to 10 mmol/l and the uptake of this sugar in buffer containing 145 mmol/l NaCl (■) or 145 mmol/l choline chloride (●) was determined as described in Methods and expressed as nmoles per mg cell protein (mean ±SEM); the Na^+-dependent transport (▲, dotted line) was calculated as the difference between the uptake in the presence of NaCl and choline chloride. Inset shows the Lineweaver-Burk plot of the data

Statistical analyses

The Student's *t*-test (two-tailed) was used for statistical determinations.

Results

Demonstration of a Na^+-dependent glucose uptake by rat mesangial cells. A progressive stimulation of glucose uptake by mesangial cells was noted with increasing concentrations of NaCl so that at physiological levels of this salt almost twice as much glucose entered the cells as in the presence of choline chloride (Fig. 1). These observations, which suggested that a Na^+-coupled glucose transporter is responsible for about half of the glucose uptake by mesangial cells, were confirmed by the action of phlorizin. When the cells were incubated with this selective inhibitor [16] the NaCl-stimulated glucose uptake was no longer evident and glucose transport was reduced to the level observed in the presence choline chloride (Fig. 2). Glucose uptake in assays containing choline chloride, which is believed to represent facilitated transport, was only slightly reduced by this inhibitor (Fig. 2), as anticipated from previous studies with this agent at the concentration which we employed [17, 18].

Measurements of facilitated and Na^+-dependent glucose uptake were made at varying glucose concentrations in the presence of NaCl or choline chloride (Fig. 3). From these determinations it was evident that the relative contribution of the two transport

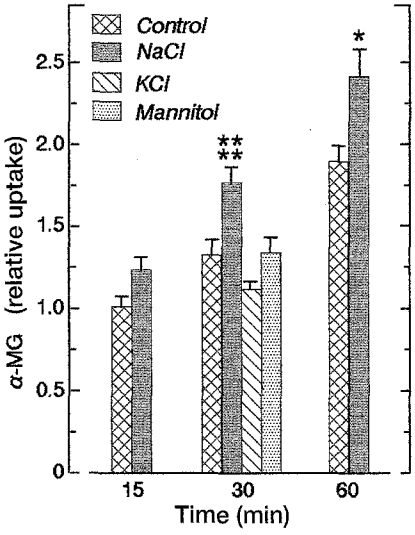

Fig. 4. Time course of uptake of methyl-α-D-glucoside and comparison of the effect of NaCl, KCl and mannitol. Mesangial cells were incubated for varying periods of time with methyl-α-D-[^{14}C]glucoside (α-MG, 0.1 mmol/l, 0.5 μCi/ml) in buffer containing 145 mmol/l NaCl without (Control) or with the further addition of 15 mmol/l each of NaCl, KCl or mannitol, respectively, under the conditions described in Methods. The uptake of the radiolabelled sugar was expressed relative to the 15-min Control incubations (mean ± SEM) in which the values for α-MG was 9.9×10^2 dpm per mg of cell protein per h. * $p < 0.05$; **** $p < 0.001$ compared to the Control at the same time period

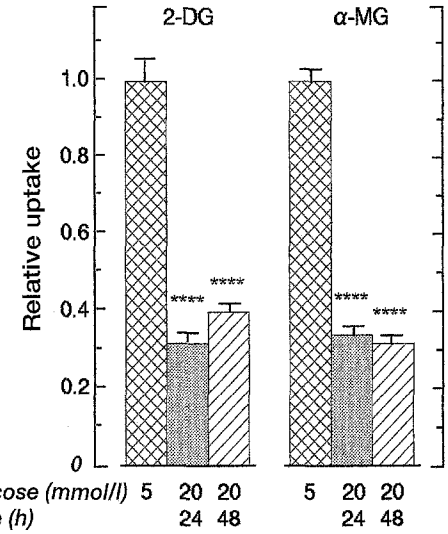

Fig. 5. Effect of medium glucose concentration on non-metabolizable hexose uptake by mesangial cells. Prior to the determinations of 2-DG and α-MG uptake, confluent cells which had been incubated for 3 days in the presence of 5 mmol/l glucose were exposed to medium containing 20 mmol/l glucose for 24 or 48 h or kept under unchanged conditions, and incubated with either [^{14}C]α-MG or 2-[^{3}H(G)]deoxyglucose (2-DG) as described in Methods. The uptake of the radiolabelled sugars was expressed relative to control cells (maintained in 5 mmol/l glucose), for which the values for α-MG and 2-DG were 2.3×10^3 dpm and 1.2×10^4 dpm, respectively. **** $p < 0.001$ in comparison to cells kept in 5 mmol/l glucose

pathways in mesangial cells remained similar at the various glucose levels. The K_m values determined from the Lineweaver Burk plots were 1.93 mmol/l for the Na^+-dependent and 1.36 for facilitated uptake while the V_{max} values were 29.5 and 18.0 nmol · mg^{-1} · h^{-1}, respectively.

Effect of Na^+ on uptake of α-methylglucoside by mesangial cells. In order to further evaluate the nature of the carrier systems occurring in mesangial cells, we measured the uptake of the non-metabolizable derivative of glucose, α-methylglucoside, which is known to utilize the Na^+-coupled clucose transporter to effect entrance into the cell [19, 20]. A time-dependent uptake of this sugar was observed and this was enhanced in the presence of an increased concentration of NaCl (Fig. 4). That the stimulatory effect of NaCl on a α-methylglucoside transport can be attributed to the Na^+ ion was evident from the finding that neither KCl nor mannitol increased its uptake (Fig. 4).

Response of transport activities to high glucose and cell generation number. After exposure of mesangial cells to 20 mmol/l glucose, a reduction of approximately 70% in the uptake of α-methylglucose from that occurring in cells maintained in a glucose concentration of 5 mmol/l was noted within a 24-h period (Fig. 5); a similar decline in the uptake of 2-deoxyglucose, a specific indicator of facilitated transport [19], was also observed (Fig. 5). These observations suggested a down-regulation of both Na^+-coupled and facilitative glucose transporters by elevated levels of this sugar.

Since after about 10 passages rat mesangial cells were noted to exhibit a faster growth rate (approximately 3 days instead of 10 days to reach confluence), we evaluated the possibility that this behavioural change could be reflected in their glucose transport activities. Indeed these assays indicated that the substantial Na^+-stimulation of glucose uptake occurring in early passages of cells was no longer evident in later generations, irrespective of whether measured at 1 or 5 mmol/l glucose concentrations (Fig. 6). Furthermore, it was apparent that the generational decrease in Na^+/glucose-co-transporter activity was accompanied by a pronounced elevation in facilitated glucose transport (Fig. 6).

Identification of mRNAs for Na^+-dependent and facilitative glucose transporters in mesangial cells. Northern blot examination of the purified mRNA from the rat mesangial cells demonstrated the presence of a transcript (2.2 kb) for the sodium/glucose co-transporter (SGLT) which migrated to the same position (Fig. 7) as the one present in LLC-PK_1 cells [21]. Moreover, a GLUT1 mRNA (2.8 kb) was evident in the mesangial cells which also co-migrated with the transcript occurring in the kidney epithelial cells

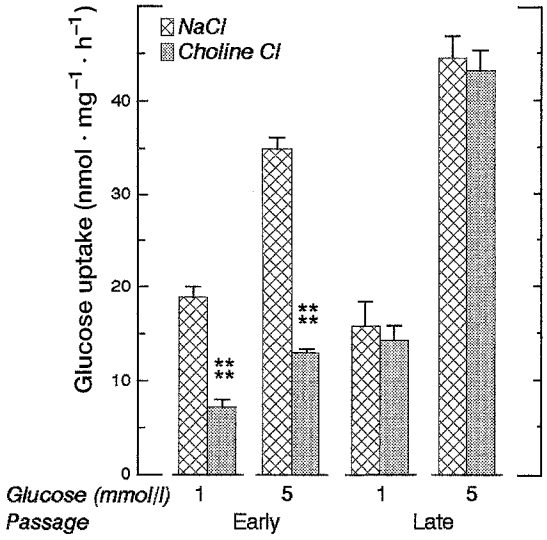

Fig. 6. Effect of passage number of Na^+-dependent glucose uptake by mesangial cells. After 8 (Early) or 14 (Late) passages glucose uptake was measured with [2-^{3}H]-D-glucose (5.0 μCi/ml) at 1 mmol/l and 5 mmol/l glucose concentrations in the presence of 145 mmol/l NaCl or choline chloride as described in Methods. Glucose uptake is expressed as nmoles per mg cell protein per h (mean ± SEM). **** $p < 0.001$ compared to incubation containing NaCl

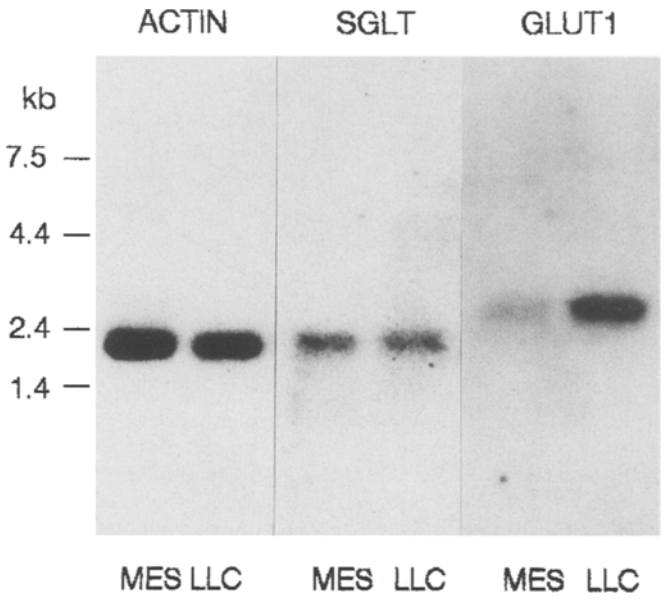

Fig. 7. Northern blot analysis demonstrating the presence of mRNAs for Na^+-dependent and facilitative glucose transporters in rat mesangial cells. After electrophoretic separation of purified mRNA (3 μg) from mesangial (MES) and LLC-PK_1 (LLC) cells, hybridizations were carried out sequentially with ^{32}P-labelled cDNA probes for SGLT, GLUT1 and actin as described in Methods and the components were visualized by autoradiography. The kilobase values were determined with an RNA ladder (GIBCO BRL) stained with ethidium bromide

(Fig. 7). Densitometric analyses indicated that in comparison to GLUT1 mRNA the SGLT message was greater in mesangial than in LLC-PK_1 cells. While the ratio of actin-normalized levels of SGLT in mesangial to LLC-PK_1 cells was 0.67, this value for GLUT1 was only 0.22. Upon hybridization of a Northern blot with GLUT4 cDNA only a very weak band (2.6 kb) was revealed although this probe reacted well with the mRNA from cardiac myocytes [12]. The mesangial mRNA did not react with GLUT2 and GLUT3 probes by Northern blot analyses nor by dot blot assay; however, the mRNA from HepG2 cells [22, 23] and WI-38 [8] cells gave strong reactions with GLUT2 and GLUT3 cDNA, respectively as anticipated.

A comparison of the transcripts from early and late-passage cells indicated that in the latter the mRNA level for GLUT1 was increased by 30% while that for SGLT was not detectable (data not shown), which would be consistent with the glucose uptake studies (Fig. 6).

Discussion

The present investigation indicates that the uptake of glucose by rat mesangial cells is mediated by both Na^+-coupled and facilitative transporters and that indeed this function is about equally shared between these two classes of carriers. The identification of a Na^+/glucose co-transporter (SGLT) in the highly purified mesangial cells was unexpected as it is generally believed that this membrane component is only present in specialized epithelia such as those from intestine and renal tubules [9, 21, 24–26] where it participates in transcellular passage of glucose; recent reports, however, have demonstrated that *Xenopus* oocytes have endogenous co-transporter activity [27, 28]. Our studies have shown that the mesangial cell Na^+-coupled glucose transporter has close similarities to the carrier from epithelial cells in regard to its specificity of response to the Na^+ ion, capacity to promote the uptake of α-methyl-glucoside and inhibition by phlorizin [9, 19, 21, 24–26]. The size of the co-transporter transcript in the rat mesangial cells (2.2 kb) is similar not only to that of LLC-PK1 cells [21] but also to that reported for rat kidney cortex SGLT1 [29, 30]. Furthermore its K_m value is comparable to that observed in oocytes after injection of SGLT1 cRNA together with the cRNA of membrane-associated protein (RS1) from porcine kidney [31]. The magnitude of this K_m indicates that uptake of glucose by the co-transporter would be affected by the glucose level in the fluid surrounding the cells which is believed to be approximately 50% of that present in blood [32].

On the basis of our studies with cDNA probes it would appear that GLUT1 is the major representative of the family of facilitative glucose transporters present in mesangial cells although the message for the previously identified [33] GLUT4 carrier is also present.

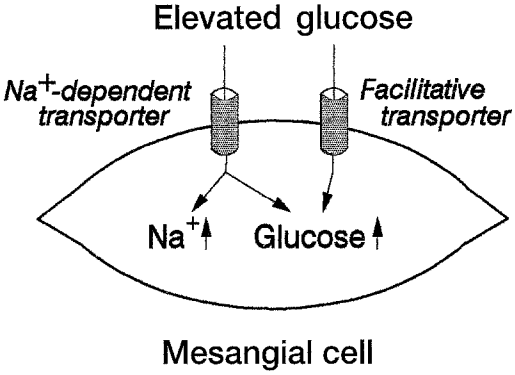

Fig. 8. Schematic representation of the proposed effect of elevated glucose on mesangial cells as brought about by the presence of both facilitative and Na^+-dependent transporters. In addition to the increased uptake of glucose mediated by the action of both transporters, the enhanced passage of glucose across the cell membrane via the co-transporter may lead to an elevated intracellular level of Na^+. The disturbed ionic composition of the mesangial cells brought about by the accelerated Na^+ flux may in addition to the increased intracellular glucose metabolites provide signals promoting the overproduction of glomerular extracellular matrix collagens observed in the hyperglycaemic state

The reported inability to detect the SGLT1 immunohistochemically in the glomerulus [34] is not unexpected since the co-transporter in the non-polarized mesangial cells, in contrast to the tubular epithelial cells, would not be concentrated in a specific region of the cell surface. Furthermore, mesangial cells are embedded in a matrix which could impede the accessibility of antibodies and indeed this latter possibility is strengthened by the finding that even GLUT1 could not be visualized immunochemically in the glomerulus [34].

Our observation that Na^+-dependent glucose transport is no longer evident in late generation mesangial cells would explain the previously reported failure to detect this function in rat mesangial cells which were examined after 15 to 46 passages [35]. Indeed the increase in facilitated glucose uptake which we noted in such multiple passaged cells as well as their accelerated rate of growth could be a consequence of dedifferentiation.

The decrease which we noted in both the Na^+-dependent and facilitated transport of mesangial cells in response to their exposure to high glucose is similar to that observed in the LLC-PK_1 kidney epithelial cell line [21]; glucose-induced down-regulation of hexose transporters has been reported to occur in a variety of cells [25, 36, 37] as well as in renal brush border membrane vesicles from diabetic rats [38]. However, we have noticed that in cultured mesangial cells, despite down-regulation of the transporters in the presence of elevated glucose, the net glucose consumption remains substantially increased and is accompanied by a highly significant enhancement in type IV and VI collagen production [7].

The identification of two genetically distinct transport systems in mesangial cells is clearly pertinent to an understanding of the pathogenesis of the diabetic glomerular lesions in which these cells are believed to play a major role [39]. Since our previous studies [6, 7] and those of others [40, 41] have indicated that high glucose initiates a series of events in mesangial cells which lead to the accelerated production of matrix components, the occurrence of two separate mechanisms of glucose entry provides expanded possibilities for regulation and channelling of metabolites. While it is generally appreciated that Na^+ can be the driving force in promoting the uptake of glucose by cells which have a coupled transporter, and indeed we have previously observed that in mesangial cells this ion stimulates glucose consumption as well as collagen synthesis [7], the converse of this mechanism, namely the enhancement of Na^+ flux by glucose, is rarely considered even though it has been experimentally demonstrated to occur in renal brush border membrane vesicles [42]. We would therefore like to propose that in the diabetic state the co-transport system may bring about an accelerated entry of Na^+ into the cell (Fig. 8) and in fact increased levels of this ion have been observed in hyperglycaemic kidneys [43]. To what extent the altered ionic composition of mesangial cells brought about by the enhanced glucose-stimulated Na^+ entry contributes to the accelerated production of collagens is currently not known, although swelling such as has been observed in SGLT1 transfected Sf9 cells [44], may be indicative of such a role.

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