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THE MECHANISM OF DECREASED Na^+-DEPENDENT D-GLUCOSE TRANSPORT IN BRUSH-BORDER MEMBRANE VESICLES FROM RABBIT KIDNEYS WITH EXPERIMENTAL FANCONI SYNDROME

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In our previous paper (Yanase, M. et al. (1983) Biochim. Biophys. Acta 733, 95–101) we reported that the Na^+-dependent D-glucose uptake into brush-border membrane vesicles is decreased in rabbits with experimental Fanconi syndrome (induced by anhydro-4-epitetracycline). In the present paper we investigate the mechanism underlying this decrease. D-Glucose is taken up into the osmotically active space in anhydro-4-epitetracycline-treated brush-border membrane vesicles and exhibits the same distribution volume and the same degree of nonspecific binding and trapping as in control brush-border membrane vesicles. The passive permeability properties of control and anhydro-4-epitetracycline-treated brush-border membrane vesicles are shown to be the same as measured by the time-dependence of L-glucose efflux from brush-border membrane vesicles. D-Glucose flux was measured by the equilibrium exchange procedure at constant external and internal Na^+ concentrations and zero potential. Kinetic analyses of Na^+-dependent D-glucose flux indicate that V_{max} in anhydro-4-epitetracycline-treated brush-border membrane vesicles (79.3 ± 7.6 nmol/min per mg protein) is significantly smaller than in control brush-border membrane vesicles (141.3 ± 9.9 nmol/min per mg protein), while the K_m values in the two cases are not different from each other (22.3 ± 0.9 and 27.4 ± 1.8 mM, respectively). These results suggest that Na^+-dependent D-glucose carriers per se are affected by anhydro-4-epitetracycline, and that this disorder is an important underlying mechanism in the decreased Na^+-dependent D-glucose uptake into anhydro-4-epitetracycline-treated brush-border membrane vesicles.

Introduction

The acquired Fanconi syndrome induced by toxic substances, such as outdated tetracycline and maleic acid, is characterized by a reversible disorder in renal proximal tubule transport affecting glucose, amino acids and other substances [1]. It is of interest that each of the molecular or ionic species whose urine excretion is increased in this condition is coupled to Na^+ influx across the brush-border membrane of the proximal tubule.

There are, however, relatively few studies concerning a direct action of such substances at the level of brush-border membrane transport. In the model Fanconi state produced by maleic acid, there is no alteration in behavior of the Na^+-dependent D-glucose transport system. Moreover, there is no change in the coupling of sugar transport either to the Na^+ chemical or electrical potential gradient across the brush-border membrane [2]. On the other hand, we reported that Na^+-dependent D-glucose uptake into brush-border membrane vesicles is decreased in the

Abbreviation: 10 mM Tris-Hepes, 10 mM 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid buffered with Tris to pH 7.4.

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Fanconi syndrome induced by anhydro-4-epitetracycline [3]. The kinetic approach to this disorder is of considerable interest from the physiological point of view, since this provides a basis for understanding the mechanism of reabsorptive processes.

In the present study we used isolated brush-border membrane vesicles from the kidneys of anhydro-4-epitetracycline-administered rabbits and measured the distribution space of D-glucose, the time dependence of the L-glucose efflux, and some kinetic parameters. These results provide evidence that Na^+-dependent D-glucose carriers per se may be affected by anhydro-4-epitetracycline in vivo.

Materials and Methods

Vesicle preparation and characterization

Brush-border membrane vesicles were prepared from the kidneys of both control and anhydro-4-epitetracycline-treated rabbits by the calcium precipitation method [3]. Relative to the starting tissue homogenate, the activities of trehalase, γ-glutamyltransferase and alkaline phosphatase (a brush-border membrane marker) in the final vesicle fraction were enriched approx. 10-times, while the activities of $(Na^+ + K^+)$-ATPase (an antiluminal membrane marker), glucose-6-phosphatase (a microsomal marker), and succinate dehydrogenase (a mitochondrial marker) were reduced by factors of 6, 1.5 and 4, respectively.

As demonstrated in the previous paper [3], no enzyme activities of renal cortical homogenate and vesicle fraction from anhydro-4-epitetracycline-treated rabbit kidneys were inhibited or activated significantly compared to those from control ones. The sidedness of the vesicle preparations was investigated by measuring the latency of maltase activity upon digestion of the membranes with Triton X-100. No difference in maltase activity could be detected between intact and solubilized vesicles in either preparation (data not shown). Thus both control and anhydro-4-epitetracycline-treated vesicle preparations are oriented right-side-out.

Uptake measurements (rapid filtration technique)

Unless otherwise noted, the procedure for uptake and exchange measurements was as follows. A 20 or 50 μl aliquot of vesicles (1.5–3.0 mg/ml) was placed in a glass test tube and at time zero a 50 or 100 μl aliquot of incubation medium containing radioactively labeled ligands and other constituents as required was added. After an appropriate time the reaction was terminated by the addition of a 10-fold dilution of ice-cold stop solution (see below). After addition of the stop solution the vesicles were applied to a Millipore filter (HAWP 0.45 μm) under light suction. The filter was then washed by a further 4.5 ml of the stop solution. From control experiments in which the time between addition of the stop solution and filtration was prolonged, we have established that no significant loss of D-glucose occurred during the stopping and washing procedure as demonstrated by Turner and Moran [4]. The filter, which retained brush-border membrane vesicles, was dissolved in scintillation fluid and counted along with samples of the incubation medium and appropriate standards.

The detailed compositions of the various media used in each experiment are given in the figure legends. In general 10 mM Tris-Hepes containing 100 mM mannitol and 100 mM KSCN was used as the basis for all media. In this way 100 mM KSCN was present in equilibrium across the vesicle membrane at all times. When appropriate, 12.5 μg valinomycin/mg vesicle protein was added as a stock solution of 25 mg/ml in ethanol. As shown by Turner and Moran [4], 100 mM KSCN equilibrium with this concentration of valinomycin is sufficient to short-circuit transmembrane electrical potential difference. The stop solution was 10 mM Tris-Hepes with 300 mM NaCl, 1 mM phlorizin and sufficient mannitol to compensate for intravesicular osmolarity.

Materials

Anhydro-4-epitetracycline was prepared by the method of McCormick et al. [5] and purified by a high-performance liquid chromatography. D-[^{14}C]Glucose, D-[^{3}H]glucose and L-[^{3}H]glucose were obtained from New England Nuclear Corp. (U.S.A.). Phlorizin was purchased from Sigma Chemical Co. (U.S.A.). Other chemicals were of highest purity available from commercial sources. All solutions were filtered (Sartorius 0.2 μm) before use.

All experiments were carried out in triplicate at

25°C. The errors shown in the figures (provided they are large enough to illustrate) are standard errors. Results of representative experiments are given and evaluated statistically by the independent *t*-test.

Results

Distribution spaces of D-glucose

Fig. 1 shows the results of an experiment in which the distribution spaces of D-glucose in control and anhydro-4-epitetracycline-treated brush-border membrane veiscles were measured as a function of extravesicular osmolarity. The osmolarity of the extravesicular medium was varied by changing its sucrose concentration. Measurements were made after 5 min incubation at 37°C in the presence of sodium, by which time D-glucose uptake is close to its equilibrium value [6]. The plots of uptake vs. inverse osmolarity for both vesicle preparations are linear and superimposable (see Fig. 1), indicating that D-glucose is equilibrating with the same osmolarity active space. The non-zero intercept on the vertical axis represents 'uptake' when the extravesicular osmolarity is extrapolated to infinity. In a similar experiment [6] when the uptake of D- and L-glucose was measured as a function of extravesicular osmolarity, it was found that the value of this intercept was not stereospecific for D-glucose. Thus, this component of 'uptake' is probably due to nonspecific binding and trapping by the membranes and filters.

Permeability of brush-border membrane vesicles

In Fig. 2 we investigated the passive permeability properties of control and anhydro-4-epitetra-

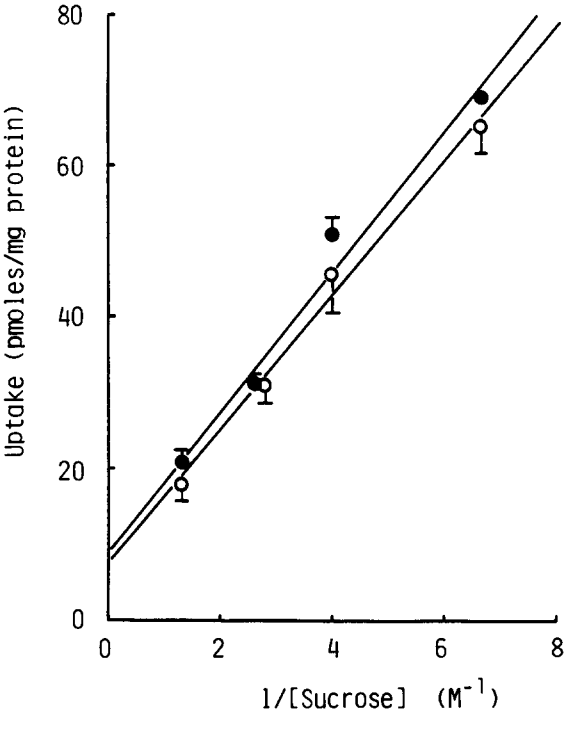

Fig. 1. Effect of extravesicular osmolarity on the equilibrium uptake of D-glucose. Vesicles (○, control; ●, anhydro-4-epitetracycline-treated brush-border membrane vesicles) were prepared in 10 mM Tris-Hepes containing 100 mM NaCl, 100 mM mannitol and 100 mM KSCN plus valinomycin. The incubation medium was the same buffer containing 0.1 mM D-[^{3}H]glucose and 150–750 mM sucrose (final concentration). Uptake was measured after 5 min of incubation.

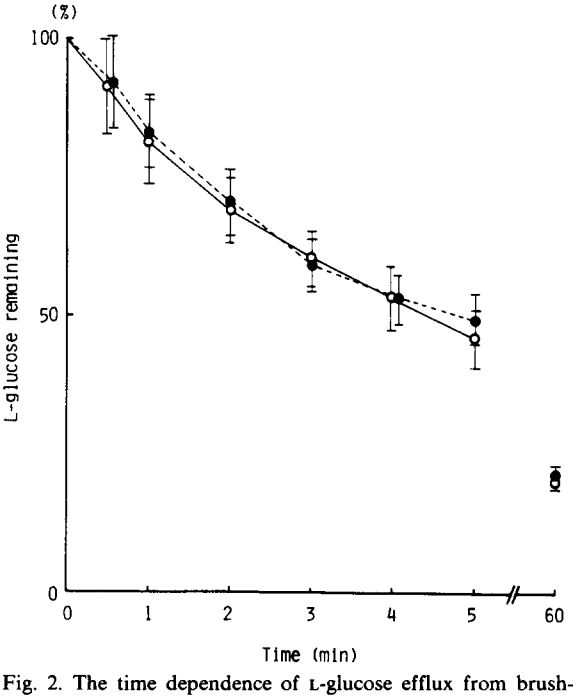

Fig. 2. The time dependence of L-glucose efflux from brush-border membrane vesicles. Vesicles (○, control; ●, anhydro-4-epitetracycline-treated brush-border membrane vesicles) were preloaded with 2.4 μM L-[^{3}H]glucose in 10 mM Tris-Hepes containing 100 mM mannitol, and then diluted (1:6) into the same medium without L-glucose. L-Glucose retained in these vesicles was measured as a function of time. Volumes of control and anhydro-4-epitetracycline-treated brush-border membrane vesicles at time zero (equilibrium volume) were not significantly different from each other (1.56 ± 0.12 and 1.73 ± 0.04 μl/mg protein, respectively).

cycline-treated brush border membrane vesicles as evidenced by the time dependence of L-glucose efflux from brush-border membrane vesicles in the absence of sodium. It is clear from Fig. 2 that the permeability of anhydro-4-epitetracycline-treated brush-border membrane vesicles as reflected in the rate of L-glucose efflux is the same as that of control, the estimated $t_{1/2}$ values being 185 ± 6 s and 185 ± 20 s, respectively. In addition, the uptake of L-glucose at time zero, which provides a measure of the intravesicular space, is not significantly different between control and anhydro-4-epitetracycline-treated brush-border membrane vesicles (1.56 ± 0.12 and 1.73 ± 0.04 μl/mg protein, respectively). Thus, the data in Fig. 2 indicate that there is no significant change in vesicle membrane permeability properties with anhydro-4-epitetracycline administration in vivo.

Kinetics of D-glucose transport

In homogeneous membrane vesicle preparation, the uptake of trace amounts of labeled non-electrolyte under equilibrium exchange conditions is described as a first-order rate process which obeys

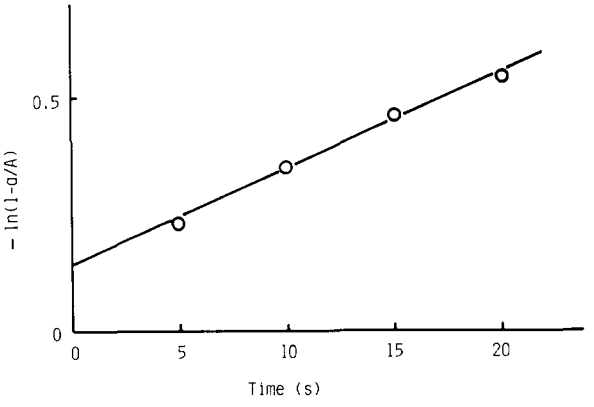

Fig. 3. Kinetics of tracer D-glucose uptake as measured with the equilibrium exchange procedure. Vesicles were preequilibrated with 10 mM Tris-Hepes containing 100 mM NaCl, 0.1 mM D-glucose, 100 mM mannitol and 100 mM KSCN plus valinomycin for at least 1 h before. The incubation medium was the same buffer containing trace amounts of D-[^{3}H]glucose. 50 μl of incubation medium were added to a 50 μl aliquot of vesicles. The unit of ordinate is the fractional uptake with respect to equilibrium.

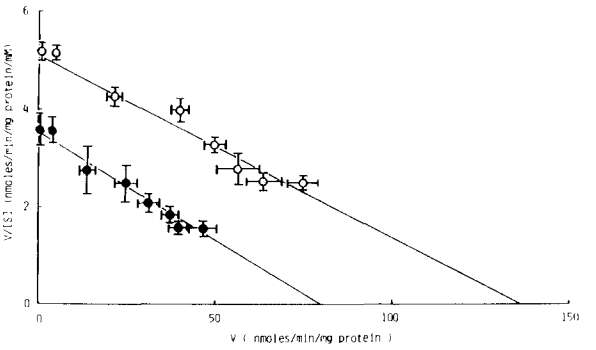

Fig. 4. Eadie-Hofstee plots of Na^+-dependent components of the D-glucose flux. The sodium-dependent component of D-glucose flux was measured as a function of D-glucose concentration. Data were from each single membrane preparation (○, control; ●, anhydro-4-epitetracycline-treated brush-border membrane vesicles). Least-squares fits to these plots yield $K_m = 27.4 \pm 1.8$ and 22.3 ± 0.9 mM, and $V_{max} = 141.3 \pm 9.9$ and 79.3 ± 7.6 nmol/min per mg protein, respectively.

the relationship

$$\ln(1 - a/A) = -\frac{\sigma J_s}{L[S]} t$$

where a = tracer uptake at time t, A = tracer uptake at equilibrium, σ = surface area of vesicles, J_s = flux of S per unit surface area at concentration [S], L = volume of vesicles, and [S] = unlabeled preequilibrated non-electrolyte substrate concentration [7–9].

As shown in Fig. 3 the plot of $-\ln(1 - a/A)$ vs. t is linear, and the slope gives the velocity of Na^+-dependent D-glucose transport. The regression line has the non-zero intercept. An explanation for the non-zero intercept is the heterogeneity of vesicle size, and, thereby, the surface to volume ratio, which is determinant for the rate of isotope equilibration [9,10]. With regard to this point Hopfer [8] has suggested an empirical test for determining if one is working with a vesicle preparation which is heterogeneous with respect to size but homogeneous with respect to membrane type. He proposed the use of $t_{1/2}$ as the velocity. For further discussion see Refs. 7–9. However, in this paper we use the slope of the regression line as the approximation of velocity of D-glucose transport [10].

Fig. 4 shows the Eadie-Hofstee plots of experi-

ments where the Na^+-dependent components of D-glucose flux were measured as a function of D-glucose concentration. Sodium-dependent component of D-glucose flux was calculated from the total flux by subtracting the Na^+-independent flux measured with choline replacing sodium. Least-squares analyses of these plots yield $K_m = 27.4 \pm 1.8$ vs. 22.3 ± 0.9 mM, and $V_{max} = 141.3 \pm 9.9$ vs. 79.3 ± 7.6 nmol/min per mg protein (control vs. anhydro-4-epitetracycline-treated brush-border membrane vesicles, respectively).

Discussion

In this paper we present the results of a series of experiments which examine the properties of the Na^+-dependent D-glucose transporter in anhydro-4-epitetracycline-treated brush-border membrane vesicles.

As shown in the previous paper [3], the purity of isolated membrane vesicles, as judged by enzyme activity, is not different between control and anhydro-4-epitetracycline-administered rabbits. Both vesicle preparations are oriented right-side-out, evidence by the latency of maltase activity upon digestion of the membranes with Triton X-100. We find that D-glucose is taken up into osmotically active space in anhydro-4-epitetracycline-treated brush-border membrane vesicles, and exhibits the same distribution volume and the same degree of nonspecific binding and trapping as in control brush-border membrane vesicles (Fig. 1). Moreover, we demonstrate that there is no difference in the passive permeability properties between control and anhydro-4-epitetracycline-treated brush-border membrane vesicles, measured by the time dependence of the L-glucose efflux from brush-border membrane vesicles (Fig. 2). Thus, we cannot find any alteration in the integrity or the membrane property of anhydro-4-epitetracycline-treated brush-border membrane vesicles. In other words, the decreased D-glucose transport in anhydro-4-epitetracycline-treated brush-border membrane vesicles is not due to any change of vesicle size, of tracer binding or trapping by the membranes, or of leakage of uptaken glucose.

We have demonstrated [3] that the exposure of intact brush-border membrane vesicles from control rabbit kidneys to anhydro-4-epitetracycline at the concentrations of 1 and 20 mM (up to 1 h incubation) has no effect on the glucose uptake in a voltage-clamped condition. The results indicate that anhydro-4-epitetracycline does not dissipate the driving force for influx of D-glucose. There appears to be no evidence supporting an ionophore-type of effect of anhydro-4-epitetracycline in vitro.

Kinetic analyses of Na^+-dependent components of D-glucose flux in control and anhydro-4-epitetracycline-treated brush-border membrane vesicles indicate transport sites obeying Michaelis-Menten kinetics with $K_m = 27.4 \pm 1.8$ and 22.3 ± 0.9 mM, and $V_{max} = 141.3 \pm 9.9$ and 79.3 ± 7.6 nmol/min per mg protein, respectively, measured under equilibrium exchange conditions at zero potential (Fig. 4). These apparent K_m and V_{max} values are different from those previously reported by other authors. Two possible reasons are considered [7]. (1) Transport, as measured by the equilibrium exchange procedure, does not necessarily involve translocation of the unloaded form of the carrier across the membrane. Therefore, K_m and V_{max} may be different for net flux conditions. (2) The measured effects of Na^+ in the vesicle system do not take into account the influence of a membrane potential. In particular, it may be possible that the permeability of the Na^+-loaded carrier, if charged, is dependent on the electrical field within the membrane. More recently, Kessler and Semenza [11] reported that a roughly 10-fold difference in the apparent K_m values for D-glucose transport from net uptake conditions and from tracer equilibrium exchange conditions is found, although the membrane potential is clamped near zero.

We, however, demonstrate that V_{max} in anhydro-4-epitetracycline-treated brush-border membrane vesicles is significantly smaller than that in control vesicles ($P < 0.01$), while the apparent K_m values are not different from each other. One simple explanation for these results is a decrease in the number of D-glucose transporters which are affected by anhydro-4-epitetracycline or its metabolites in vivo.

There appears to be a difference in the underlying mechanisms of the model Fanconi states induced by anhydro-4-epitetracycline and by maleic acid. The molecular defect in the former must be

at brush-border membrane, involving a decrease in the number of Na^+-dependent D-glucose transporters, while that in the latter [12,13] must be either at some intracellular site, for example, mitochondria, interfering with cell metabolism and decreasing the production of ATP, or at antiluminal membrane, involving the inhibition of some component of the Na^+ pump (($Na^+ + K^+$)-ATPase), thereby affecting existing transmembrane (luminal) electrochemical Na^+ gradients.

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