Increased Na^+-dependent D-glucose transport and altered lipid composition in renal cortical brush-border membrane vesicles from bile duct-ligated rats

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Abstract Erythrocyte membranes of patients with liver disease are characteristically enriched in cholesterol, a change known to impair several carrier-mediated membrane transport functions. In the present study we have assessed whether experimental liver disease can affect the membrane lipid composition and transport function of kidney epithelial cells. Small (about 5%) but significant ($P < 0.01$) increases were found in the cholesterol-to-phospholipid molar ratio (C/PL) of rat renal cortical brush-border membrane (BBM) vesicles 3, 8, and 15 days after bile duct ligation which correlated closely with increased fluorescence polarization, i.e., decreased membrane fluidity ($r = 0.75$, $P < 0.001$; n = 27). A lipoprotein-mediated pathogenesis was suggested by the close relationship between BBM C/PL and plasma C/PL ($r = 0.69$, $P < 0.001$). The mean high-affinity Na^+-coupled D-glucose uptake by BBM vesicles was higher 1, 3, 8, and 15 days after ligation than in non-operated rats, significantly so at 3 and 8 days (611 ± 37 and 593 ± 22 vs. 507 ± 21 pmol/mg protein per 4 sec; $P < 0.05$), and was positively correlated with BBM C/PL ($r = 0.58$, $P < 0.01$) and fluorescence polarization ($r = 0.41$, $P < 0.05$). Brief incubation of BBM vesicles from normal rats with cholesterol-rich phospholipid liposomes simultaneously increased BBM C/PL and Na^+-dependent D-glucose uptake. Stimulation of BBM Na^+-glucose cotransport in ligated rats was not due to delayed dissipation of the Na^+ gradient or to a more rapid development of membrane potential. High-affinity Na^+-dependent D-glucose uptake kinetics in 3-day bile duct-ligated rats showed a lower K_t, without an alteration in maximum velocity, V_{max}, compared to sham-operated animals (0.298 ± 0.015 vs. 0.382 ± 0.029 mM; $P < 0.05$), whilst the binding dissociation constant, K_d of high-affinity phlorizin binding sites was reduced by ligation (0.453 ± 0.013 vs. 0.560 ± 0.015 μM; $P < 0.001$). We conclude that an early effect of bile duct ligation is to enrich renal cortical brush-border membranes in cholesterol, thereby decreasing membrane fluidity and stimulating Na^+-dependent D-glucose uptake by increasing the affinity of the carrier.—**Imai, Y., J. E. Scoble, N. McIntyre, and J. S. Owen.** Increased Na^+-dependent D-glucose transport and altered lipid composition in renal cortical brush-border membrane vesicles from bile duct-ligated rats. *J. Lipid Res.* 1992. **33:** 473–483.

Supplementary key words LCAT • liver disease • membrane fluidity • membrane lipids

The coupled translocation of Na^+ and glucose across the brush border membrane (BBM) of epithelial cells of the kidney proximal tubule is electrogenic, stereospecific, and sensitive to phlorizin (1–4). Recently, Hediger and coworkers cloned the Na^+-glucose cotransporter in rabbit (5) and human (6) intestine and proposed a structural model based on the predicted amino acid sequence (3–6). Functional similarity has long been recognized between the intestinal brush-border Na^+-glucose carrier and the Na^+-dependent D-glucose cotransporter in renal BBM; on the basis of antibody studies and Northern blot analyses a close structural relationship is also suggested (7). The Na^+-glucose cotransporter appears sensitive to an increase in membrane fluidity inasmuch as addition of n-aliphatic alcohols or benzyl alcohol fluidizes renal BBM and markedly inhibits Na^+-dependent D-glucose transport (8, 9). This finding has been confirmed by Molitoris and Kinne (10) who showed that reversible ischemia alters the lipid composition of rat renal cortical BBM and that the resulting increase in membrane fluidity is associated with suppression of Na^+-coupled D-glucose uptake. However, it is unclear whether the converse applies, namely whether a reduction in the fluidity of renal BBM enhances Na^+-glucose cotransport.

Abbreviations: BBM, brush-border membrane; C/PL, cholesterol-to-phospholipid molar ratio; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; LCAT, lecithin:cholesterol acyltransferase (EC 2.3.1.43); PC/SM, phosphatidylcholine-to-sphingomyelin molar ratio.

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In human liver disease, erythrocyte membranes frequently contain excess cholesterol, apparently by uptake from abnormal plasma lipoproteins (11). The increased cholesterol content of such erythrocytes correlates closely with a reduced membrane fluidity (12) and with impaired carrier-mediated membrane transport of cations (13, 14) and anions (15). Recently, we briefly reported (16) that rats with chronic (3 week) biliary obstruction accumulate cholesterol not only in erythrocyte membranes but also in renal cortical BBM. In the present study, we have assessed whether such cholesterol deposition in vivo is an early event in biliary obstruction and whether it can affect the activity of the Na^+-glucose cotransporter. Renal cortical BBM vesicles were prepared from rats subjected to bile duct ligation for up to 15 days and their ability to cotransport Na^+ and glucose, measured as the initial rate of high-affinity Na^+-coupled D-[^{3}H]glucose uptake, was correlated with changes in membrane lipid composition and fluidity.

MATERIALS AND METHODS

Materials

D-[1-^{3}H]glucose (8.3 Ci/mmol) and [^{3}H]phlorizin (60 Ci/mmol) were purchased from Amersham International Plc (Amersham Place, Buckinghamshire, England) and New England Nuclear (Du Pont (UK) Ltd., NEN Products Division, Stevenage, Hertfordshire, England), respectively. D-Glucose, phlorizin, dipalmitoyl phosphatidylcholine, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and fatty acid-free human albumin were obtained from the Sigma Chemical Co. Ltd. (Poole, Dorset, England). Tetrahydrofuran and 1,6-diphenylhexa-1,3,5-triene were from the Aldrich Chemical Co. Ltd. (Gillingham, Dorset, England).

Isolation of BBM

Male Wistar rats, weighing 300–400 g and maintained on standard chow, were used in all experiments. Double ligation of the common bile duct, with section between the two ligatures, was carried out while the rats were under diethyl ether anesthesia. Other rats were subjected to sham operations or were non-operated. Renal cortical BBM were prepared by minor modification of the method of Biber et al. (17). While the rats were under ether anesthesia, blood was collected through the abdominal aorta and, after section of the bilateral renal veins, the kidneys were flushed in situ with 50 ml of physiological saline containing 1 mM Tris/HEPES, pH 7.4. Thin cortical sections from both kidneys were homogenized with 50 ml of 10 mM Tris/HEPES, pH 7.4, buffer containing 150 mM mannitol, 2.5 mM ethyleneglycol-bis [B-aminoethylether]-N,N′-tetraacetic acid and 0.05 mM phenylmethylsulfonyl fluoride and then precipated with 15 mM $MgCl_2$ for 20 min. After centrifugation of this suspension at 2,500 *g* for 15 min, the supernatant was collected and re-centrifuged at 48,000 *g* for 30 min. The resulting pellet was resuspended in 30 ml of the above buffer and the Mg^{2+} precipitation and centrifugation steps were repeated. The BBM pellet was washed with 10 mM Tris/HEPES, pH 7.4, buffer containing 300 mM mannitol by centrifugation at 48,000 *g* for 30 min and then resuspended in the same buffer to a final concentration of about 3 mg protein/ml. Vesiculation was induced by repeated passage through a 21-gauge needle; one portion was used immediately for transport studies, whilst other aliquots were stored at –70°C for subsequent enzyme and lipid determinations.

Enzyme assays

These were carried out at 37°C on both whole homogenates and isolated BBM. Alkaline phosphatase was assayed using *p*-nitrophenyl phosphate as substrate (18). Leucine aminopeptidase and Na^+, K^+-ATPase were determined as marker enzymes of BBM and basolateral membranes, respectively (19, 20). Protein concentrations were measured with the Folin phenol reagent using bovine serum albumin as standard. Plasma lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) activity was measured by the Stokke-Norum method (21).

Lipid analyses

Lipids were extracted from BBM with methanol–chloroform 2:1 (v/v) and their concentrations were measured as described previously (16, 22). In brief, total phospholipids were estimated as inorganic phosphorus after digestion with H_2SO_4 whilst their fatty acid composition was determined by gas–liquid chromatography after transmethylation (16). Cholesterol was measured with the appropriate commercial cholesterol oxidase reagent (Boehringer Corporation Ltd., Lewes, East Sussex, UK) added either to plasma or to portions of dried lipid extract redissolved in isopropanol. The individual phospholipid classes were separated by two-dimensional thin-layer chromatography and estimated as inorganic phosphorus (22).

Fluorescence polarization measurements

The fluidity of renal BBM was assessed by measuring the steady-state fluorescence polarization of the hydrophobic probe, diphenylhexatriene (23, 24). This fluorophore was stored in tetrahydrofuran at a concentration of 2 mM and diluted 2000-fold by injection into vigorously stirred phosphate-buffered saline im-

mediately before use. The colloidal suspension was sonicated for two 5-min periods and then incubated with an equal volume of BBM vesicles (final concentration 25 μg protein/ml) for 30 min at 37°C to partition the probe into the bilayer core (8, 23). The diphenylhexatriene was excited at 357 nm and the emission was viewed at 430 nm using the Elscint MV-1a microviscosimeter; as described previously (12) this instrument directly records the polarization ratio, P ($P = I_x - I_y)/(I_x + I_y$), where I_x and I_y are the intensities of the polarized light emitted in parallel and perpendicular, respectively, to the excitation polarizer). Measurements were made in triplicate at 25°C and, in some cases, at 37°C; each sample was routinely corrected for light scattering, using membrane suspensions without probe, although this was minimal at the low protein concentration used. Because the lifetime of diphenylhexatriene is known to be independent of changes in the lipid composition of renal BBM (10, 25, 26) including cholesterol enrichment (26), increases in the value of P indicate a decrease in membrane fluidity (23, 24).

Transport studies

All transport studies were done in at least quadruplicate at 25°C using a rapid filtration technique. The initial velocity of D-glucose uptake was measured after 4 sec incubation using a low concentration (0.1 mM) to selectively examine the high-affinity carrier (10, 27). Twenty μl of BBM vesicles was placed next to 40 μl of incubation medium containing 0.15 mM D-[^{3}H]glucose (1 μCi) in 50 mM mannitol, 150 mM NaCl, and 10 mM Tris/HEPES, pH 7.4. Uptake was initiated by vortexing the mixture and was stopped at 4 sec, using a metronome to count out the seconds, by adding 1 ml of ice-cold stop solution (100 mM mannitol, 300 mM NaCl, 0.2 mM phlorizin, 10 mM Tris/HEPES, pH 7.4). The mixture was rapidly filtered through a prewetted 0.45-μm WCN filter (Whatman) and washed once with 4.5 ml of ice-cold stop solution. The filters were dissolved in 3 ml of Filtron-X (National Diagnostics, Aylesbury, Bucks, UK) and their radioactivity was counted to determine total D-[^{3}H]glucose uptake. Na^+-independent D-glucose uptake was measured with KCl replacing the NaCl and in the presence of 0.5 mM phlorizin (10, 27) and was subtracted from the total uptake to give the Na^+-dependent D-glucose uptake.

The protonophore FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was used to short-circuit diffusion potential differences (27). FCCP in ethanol (final concentrations of 100 μM and 0.25% (v/v), respectively, with omission of FCCP in controls) was dissolved in the incubation medium by brief sonication and then mixed with the BBM vesicles; initial D-glucose uptakes were measured as described above. Dissipation of the sodium gradient was assessed directly by measuring uptake of 1 mM $^{22}Na^+$ (2 μCi in 300 mM mannitol and 10 mM Tris/HEPES, pH 7.4) over 15 sec (10).

For time-course experiments of Na^+-glucose cotransport, 75 μl of BBM vesicles was incubated in 300 μl of 0.15 mM D-[^{3}H]glucose solution containing 150 mM NaCl (or 150 mM KCl and 0.5 mM phlorizin), 50 mM mannitol, 10 mM Tris/HEPES, pH7.4; uptake was stopped at timed intervals by withdrawing 50 μl of the mixture and adding it to 1 ml of ice-cold stop solution. Kinetic studies of Na^+-dependent D-glucose uptake were carried out using glucose concentrations between 0.05 and 1.0 mM, since this restricted range permits selective examination of the high-affinity carrier in BBM vesicles prepared from whole cortex (10, 27).

Initial uptake studies were also carried out in BBM vesicles enriched in cholesterol by preincubation with cholesterol-phospholipid liposomes. Dipalmitoyl phosphatidylcholine liposomes in 300 mM mannitol, 10 mM Tris/HEPES, pH 7.4, buffer, containing increasing amounts of cholesterol to give cholesterol-to-phospholipid molar ratios (C/PL) in the range 0.9–3.6, were prepared by ultrasonication as described previously (12). Defatted albumin (10 mg/ml) was added and the liposomes (10 μmol phospholipid) were incubated for 2 h at 25°C with BBM vesicles from normal rats (5 μmol phospholipid; about 7 mg protein/ml). One portion of the liposome-vesicle suspension was used to measure the initial velocity of D-[^{3}H]glucose uptake; preliminary experiments, in which the various liposome preparations were mixed with BBM vesicles immediately prior to uptake determinations, established that the presence of the liposomes did not affect the measurements. The remainder of the mixture was diluted at least 60-fold with 300 mM mannitol, 10 mM Tris/HEPES, pH 7.4, and centrifuged at 48,000 *g* for 30 min at 4°C. The sedimented BBM vesicles were washed once under the same conditions and their cholesterol and phospholipid contents were measured.

Phlorizin binding

Binding studies with phlorizin, a competitive inhibitor of D-glucose binding by the carrier which is not itself translocated (3, 7), were carried out in quadruplicate at 25°C. Preliminary experiments established that equilibrium binding of phlorizin was achieved by 5 min as reported by others (10, 28). Twenty μl of BBM vesicles was incubated for 5 min with 40 μl of [^{3}H]phlorizin (final concentration 0.05–2.5 μM) in 10 mM Tris/HEPES, pH 7.4, buffer containing 150 mM NaCl and 50 mM mannitol. Incubations were terminated by the addition of 1 ml of ice-cold stop solu-

tion (150 mM NaCl, 50 mM mannitol, 10 mM Tris/HEPES, pH 7.4); subsequent rapid filtration, washing and radioactivity counting were as described above. Nonspecific binding was determined in the presence of at least a 100-fold excess of unlabeled phlorizin and in the absence of Na^+; specific phlorizin binding was defined as the difference between total and nonspecific binding.

Statistics

Regression lines were calculated by the method of least squares and all results are expressed as means ± SEM; statistical differences were determined by Student's two-tailed unpaired *t* test.

RESULTS

Effects of the duration of bile-duct ligation

Renal cortical BBM were prepared from individual non-operated rats or rats 1, 3, 8, and 15 days after bile duct ligation and, in each preparation, high-affinity Na^+-glucose cotransport activity, membrane fluidity, and lipid composition were measured. An increase in mean steady-state fluorescence polarization of diphenylhexatriene (i.e., a decrease in fluidity) was apparent in BBM 1 day after ligation (**Fig. 1**); it rose further at 3 days so that is was significantly higher than in non-operated animals ($P < 0.05$) but thereafter essentially remained at the same elevated level. Similarly, the BBM C/PL was significantly increased 3 days after bile duct ligation, albeit by only 4% compared to non-operated rats (0.658 ± 0.006 vs. 0.631 ± 0.004, $P < 0.01$), and was still elevated at 15 days (0.666 ± 0.006, $P < 0.01$) (Fig. 1) and correlated closely with the fluorescence polarization ($r = 0.75$, $P < 0.001$; n = 27). Renal BBM C/PL was also correlated directly with the C/PL of total plasma lipoproteins ($r = 0.68$, $P < 0.001$; **Fig. 2**) and inversely with plasma LCAT activity ($r = -0.59$, $P < 0.01$).

An increase in the proportion of phosphatidylcholine in biological membranes, most commonly expressed as a rise in the phosphatidylcholine-to-sphingomyelin molar ratio (PC/SM), tends to have a fluidizing effect (29, 30). The mean BBM PC/SM was unchanged 1 and 3 days after ligation, but rose subsequently and at 15 days was increased by 16% ($P < 0.05$; Fig. 1) when the phosphatidylcholine content was significantly higher both as a fractional (21.2 ± 0.7 vs. $19.0 \pm 0.4\%$ of the total phospholipids, $P < 0.05$) and absolute (164 ± 15 vs. 135 ± 4 nmol/mg protein, $P < 0.01$) amount. By contrast, both the sphingomyelin content (273 ± 6 vs. 263 ± 8 nmol/mg protein, $P > 0.05$) and total phospholipid concentration (779 ± 16 vs. 718 ± 24 nmol/mg protein, $P > 0.05$) were unchanged 15 days after ligation. Neither the BBM PC/SM nor the BBM fatty acid composition, as assessed by the ratio of saturated-to-monounsaturated plus polyunsaturated fatty acids, correlated with fluorescence polarization ($r = 0.04$ and 0.11, respectively).

The mean values for the initial rates of Na^+-dependent D-glucose uptake by BBM vesicles from the ligated rats were 7–20% higher than in the non-operated

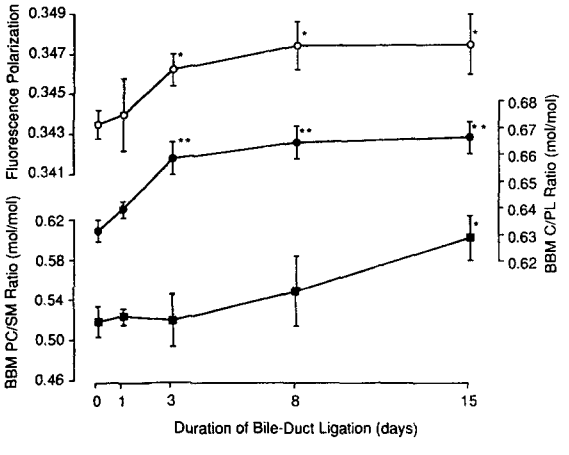

Fig. 1. Effect of the duration of bile duct ligation on the fluorescence polarization and lipid composition of rat renal cortical BBM. Fluorescence polarization (○), C/PL (●), and PC/SM (■) of renal cortical BBM were measured in non-operated rats (n=8) and in rats 1 (n=4), 3 (n=6), 8 (n=4), and 15 (n=5) days after bile duct ligation. Steady-state fluorescence polarization measurements were made at 25°C using diphenylhexatriene as a probe. Individual phospholipids were separated by two-dimensional thin-layer chromatography and measured as inorganic phosphorus. Results are shown as mean ± SEM; significance of differences from non-operated rats is indicated as $^*P < 0.05$ and $^{**}P < 0.01$.

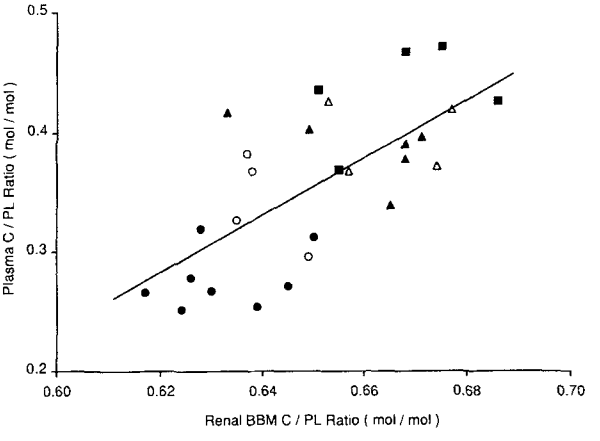

Fig. 2. Correlation between plasma lipoprotein C/PL and renal cortical BBM C/PL in bile duct-ligated rats. BBM were prepared from non-operated rats (●) and rats 1 (○), 3 (▲), 8 (△), and 15 (■) days after bile duct ligation. The correlation coefficient was 0.68 ($P < 0.001$, n=27) by linear regression.

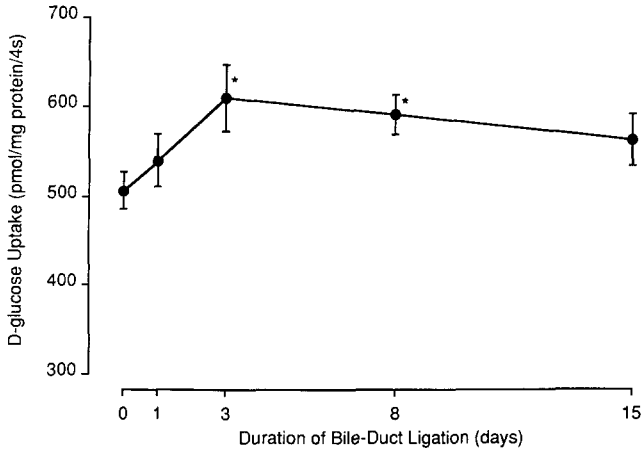

Fig. 3. Effect of the duration of bile duct ligation on high-affinity Na^+-dependent D-glucose uptake by renal cortical BBM vesicles. The initial rate of Na^+-dependent D-glucose uptake over 4 sec was carried out in BBM vesicles from the same rats listed in Fig. 1 by using rapid filtration techniques exactly as described under Materials and Methods. Na^+-independent D-glucose uptake was determined by substituting 150 mM KCl and 0.5 mM phlorizin for the NaCl in the transport buffer and was subtracted from total uptake to give Na^+-dependent D-glucose uptake. Results are expressed as mean ± SEM for non-operated rats (n=8) and for rats 1 (n=4), 3 (n=6), 8 (n=4) and 15 (n=5) days after bile duct ligation; significance of differences from non-operated rats is indicated by $*P < 0.05$.

group (**Fig. 3**). The highest rate occurred 3 days after ligation (611 ± 37 vs. 507 ± 21 pmol/mg protein per 4 sec in the non-operated rats, $P < 0.05$) but thereafter it gradually declined, although it was still significantly higher at 8 days (593 ± 22 pmol/mg protein per 4 sec; $P < 0.05$). There were significant correlations between D-glucose uptake and both the C/PL ($r = 0.58$, $P < 0.01$; **Fig. 4**) and fluorescence polarization ($r = 0.41$, $P < 0.05$) of the BBM. By contrast, the BBM PC/SM was negatively correlated with Na^+-dependent D-glucose uptake ($r = -0.38$, $P < 0.05$). There was no relationship between uptake and the fatty acid composition of the membrane ($r = 0.12$).

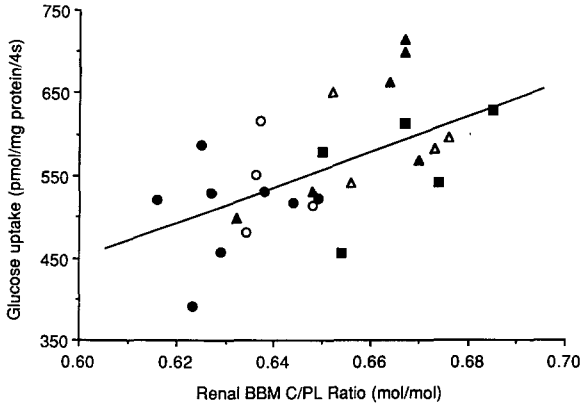

Fig. 4. Relationship between the initial rate of high-affinity Na^+-dependent D-glucose uptake by renal cortical BBM vesicles and their C/PL ratio. Vesicles were prepared from non-operated rats (●) and rats 1 (○), 3 (▲), 8 (△), and 15 (■) days after bile duct ligation. The points are the individual values used to calculate the means shown in Figs. 1 and 3. The correlation coefficient was 0.58 ($P < 0.01$, n=27) by linear regression.

Cholesterol enrichment of BBM vesicles

To confirm this apparent stimulatory effect of membrane cholesterol enrichment in vivo on Na^+-glucose cotransport, we increased the cholesterol content of renal cortical BBM vesicles from normal rats by preincubation with cholesterol-rich phospholipid dispersions. This treatment can increase the plasma membrane cholesterol content of intact cells (31) and of intestinal BBM (32). In preliminary experiments, time and temperature were varied and a 2-h preincubation at 25°C was found to be sufficient to increase the C/PL of the BBM vesicles even with only a small excess of liposomes. Such cholesterol enrichment of BBM vesicles appeared specific, presumably mediated by exchange-equilibration (31), rather than contamination with the liposomes themselves. This was indirectly confirmed by the negligible increase in total phospholipid concentration of cholesterol-loaded BBM (for example, 697 nmol/mg protein for the BBM with a C/PL of 0.79 compared to 694 nmol/mg protein for nonincubated BBM) and by their unchanged phospholipid composition (the percentage of total phospholipid as phosphatidylcholine, 18.7% was largely unaffected by cholesterol enrichment, 18.3%). Direct support was obtained by using liposomes prepared with traces of [^{14}C]cholesteryl oleate, a nonexchangeable marker; at least 94% of the cholesterol accumulating in the BBM was selectively transferred. Moreover, the cholesterol appeared to be inserted into the membrane bilayer as increasing the C/PL of the BBM from 0.651 to 0.731 and 0.824 also increased their fluorescence polarization (from 0.344 to 0.349 and 0.352, respectively). Importantly, as shown in **Fig. 5**, an essentially linear increase in Na^+-dependent D-glucose uptake was observed (from 510 pmol/mg protein per 4 sec to 609 pmol/mg protein per 4 sec) when the C/PL of the BBM vesicles was increased from 0.647 to 0.774 in a step-wise manner.

Three-day bile duct ligations versus sham operations

A more detailed comparison of renal cortical BBM vesicles from 3-day ligated rats and 3-day sham-operated animals was carried out. This duration of ligation is not only associated with significant increases in BBM C/PL and Na^+-dependent D-glucose uptake, but also with an unchanged BBM phospholipid composition (Figs. 1 and 3). It may be possible, therefore, to regard studies at this time interval as specific effects of membrane cholesterol enrichment in vivo on the

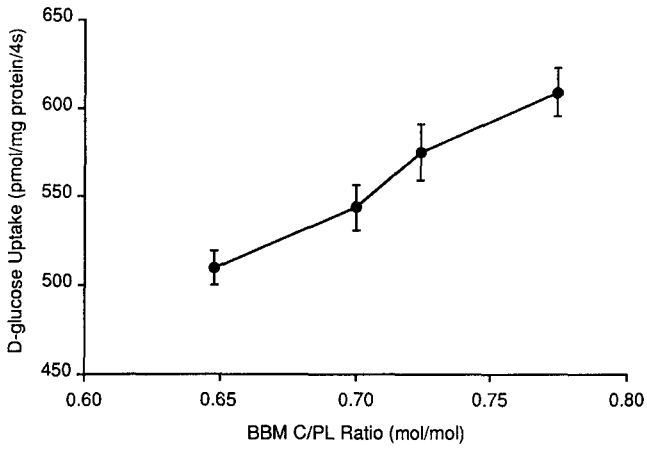

Fig. 5. Na^+-dependent D-glucose uptake by rat renal cortical BBM vesicles as a function of increasing membrane C/PL. The cholesterol content of renal cortical BBM vesicles prepared from normal rats was increased by preincubation at 25°C for 2 h with phospholipid liposomes containing various amounts of cholesterol. Uptake of D-[^{3}H]glucose over 4 sec was carried out in the presence of phospholipid liposomes as a single experiment by the same method indicated in Fig. 1; the bars indicate the SE of quadruplicate measurements. The C/PL of nonincubated BBM vesicles was 0.626 and of the four liposome preparations 0.9, 1.8, 2.7, and 3.6. Two other independent experiments gave similar results.

functioning of the renal cortical BBM Na^+-glucose cotransporter.

Purification of the BBM fraction from sham-operated rats appeared comparable to those reported by other workers (8, 10), as judged by a 12-fold enrichment of the marker enzyme, leucine aminopeptidase and by minimal enrichment of the basolateral membrane enzyme, Na^+, K^+-ATPase (**Table 1**). Moreover, bile duct ligation neither affected the specific activity or enrichment of leucine aminopeptidase nor those of Na^+, K^+-ATPase, suggesting that renal cortical BBM vesicles from ligated rats can be directly compared with the control BBM in studies on glucose uptake, phlorizin binding, and lipid composition. However, the specific activities of alkaline phosphatase were significantly increased in 3-day ligated rats in both the BBM fraction (Table 1) and homogenate (148 ± 12 vs. 110 ± 7 nmol/mg protein per min, $P < 0.05$). Enrichment of this enzyme was also higher in the ligated rats (Table 1). Because these increases were inconsistent with an unchanged leucine aminopeptidase activity, aliquots of renal BBM from non-operated animals and from rats 1, 3, 8, and 15 days after bile duct ligation were also assayed for alkaline phosphatase activity. A significant correlation was found between BBM C/PL and the specific activity of BBM alkaline phosphatase ($r = 0.44$, $P < 0.05$).

The lipid content and fluorescence polarization of renal cortical BBM in 3-day bile duct-ligated and sham-operated rats is shown in **Table 2**. Both cholesterol concentration per mg of protein and C/PL were significantly elevated in BBM vesicles from ligated animals. The phospholipid composition of the BBM from our sham-operated rats was very similar to that in other reports (10, 33); as there are clear differences between the phospholipid pattern of basolateral membranes and BBM (33) this finding is additional evidence for the purity of our BBM fraction. The percentage distribution of phospholipids in BBM of ligated rats was virtually identical to that of sham-operated rats and there was no significant difference in the PC/SM (Table 2). However, fluorescence polarization was significantly higher ($P < 0.05$) in BBM vesicles from ligated rats, presumably reflecting their increased BBM cholesterol content since this sterol is known to primarily affect the order component of membrane fluidity (23, 24). The initial rate of D-glucose uptake by BBM vesicles from 3-day sham-operated rats was unchanged compared to that in non-operated animals (507 ± 28 vs. 490 ± 25 pmol/mg protein per 4 sec $P > 0.05$; Table 2 and Fig. 3), suggesting that any effects of surgical stress were negligible. As expected from the time course experiments (Fig. 3) Na^+-dependent D-glucose uptake was 20% higher ($P < 0.01$) in the 3-day ligated rats (Table 2). In four additional pairs of animals we carried out fluorescence polarization and D-glucose uptake measurements at 37°C; in agreement with the data at 25°C, the values were significantly greater in the 3-day ligated rats compared to the sham-operated animals (0.306 ± 0.001 vs. 0.303 ± 0.001, $P < 0.05$ and 935 ± 30 vs. 719 ± 17 pmol/mg protein per 4 sec, $P < 0.01$, respectively).

The time course of total D-glucose uptake showed typical transient "overshoot" (3, 10, 27) with a peak at

TABLE 1. Enzyme specific activities in renal cortical BBM from 3-day bile duct-ligated and sham-operated rats

Animal	Leucine Aminopeptidase (n = 9)		Alkaline Phosphatase (n = 9)		Na^+, K^+-ATPase (n = 6)	
	Specific Activity	Enrichment	Specific Activity	Enrichment	Specific Activity	Enrichment
Sham-operated	977 ± 78	12.4 ± 45	1265 ± 45	11.7 ± 0.3	218 ± 19	1.6 ± 0.2
3-Day ligated	973 ± 44	12.5 ± 0.5	1979 ± 128[b]	13.5 ± 0.3[a]	209 ± 30	1.6 ± 0.2

All enzyme activities are expressed as the mean nmol/mg protein per min ± SEM for the number of individual preparations given in parentheses. Enrichment refers to the enzyme specific activity in the BBM relative to the initial homogenate. Significance of differences between preparations from bile duct-ligated and sham-operated rats is indicated by [a]$P < 0.01$ and [b]$P < 0.001$.

TABLE 2. Lipid composition, fluorescence polarization and Na^+-dependent D-glucose uptake in renal cortical BBM from 3-day bile duct-ligated and sham-operated rats

Animals (n)	Cholesterol	Phospholipid	C/PL	Phospholipid Composition					PC/SM	Fluorescence Polarization	D-Glucose Uptake
				SM	PC	PE	PS	PI			
	nmol/mg protein		*mol/mol*	*mol%*					*mol/mol*		*pmol/mg protein/ 4 sec*
Sham-operated (6)	447 ± 5	709 ± 7	0.630 ± 0.002	37.1 ± 0.3	19.5 ± 0.4	27.4 ± 0.3	13.9 ± 0.2	2.1 ± 0.1	0.527 ± 0.015	0.339 ± 0.001	490 ± 25
3-Day ligated (6)	504[b] ± 7	769[b] ± 9	0.655[c] ± 0.003	37.1 ± 0.4	19.8 ± 0.2	27.3 ± 0.3	13.7 ± 0.1	2.1 ± 0.1	0.534 ± 0.008	0.342[a] ± 0.001	589[b] ± 18

Values are expressed as means ± SEM; significance of differences from sham-operated rats is indicated by [a]$P < 0.05$, [b]$P < 0.01$, and [c]$P < 0.001$. SM, sphingomyelin; PC, phosphatidylcholine, PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol. C/PL, cholesterol-to-phospholipid molar ratio; n,number.

1 min for BBM vesicles from both ligated rat and sham-operated animals (**Fig. 6**). During the initial 5 min the total uptake was up to 26% higher in the bile duct-ligated rat, whereas the Na^+-independent D-glucose uptakes were virtually the same. The equilibrium uptakes obtained at 2 h were similar in the 3-day ligated and sham-operated rats (317 vs. 339 pmol/mg protein) indicating that bile duct ligation did not change intravesicular volume.

Bile duct ligation was not associated with a slower collapse of the Na^+ gradient, as ^{22}Na uptake in the presence of a 1 mM external Na^+ gradient was unchanged compared to sham-operated animals (471 ± 41 vs. 483 ± 75 pmol/mg protein per 15; n=4, $P > 0.05$). Furthermore, BBM vesicles from ligated rats were not more permeable to the anion Cl^- (from NaCl), since uptake of D-glucose in the presence of FCCP to clamp the voltage was still significantly higher in the ligated rats (681 ± 32 vs. 523 ± 19 nmol/mg protein per 4 sec; n=4, $P < 0.01$). Woolf-Augustinsson-Hofstee plots (10, 27) of the initial 4-sec rates of Na^+-dependent D-glucose uptake by the BBM vesicles showed that K_t was reduced by bile duct ligation (0.298 ± 0.015 compared to 0.382 ± 0.029 mM in the sham-operated rats, $P < 0.05$), while the V_{max} remained constant (2429 ± 156 vs. 2463 ± 198 pmol/mg protein per 4 sec) (**Fig. 7**). Scatchard plots of high-affinity phlorizin binding (data not shown) revealed that bile duct ligation reduced the binding dissociation constant, K_d (0.453 ± 0.013 vs. 0.560 ± 0.015 μM for the sham-operated rats, $P < 0.001$), suggesting an enhanced affinity of the carrier for D-glucose. A simultaneous decrease in the number of binding sites also occurred (262 ± 15 vs. 311 ± 6 pmol/mg protein, $P < 0.05$), implying that carrier turnover (V_{max}/carrier number) was increased by bile duct ligation from 1.98 to 2.32 per sec. However, although others have also estimated K_d, carrier number, and carrier turnover for rat renal BBM from whole cortex (10, 27), the possible heterogeneity of Na^+-glucose cotransport systems in the rat proximal tubule may make such data equivocal. Phlorizin is bound by the low-affinity Na^+-glucose cotransporter in the outer cortex of rabbit kidney (34, 35) as well as the high-affinity carrier (36) under investigation in the present study.

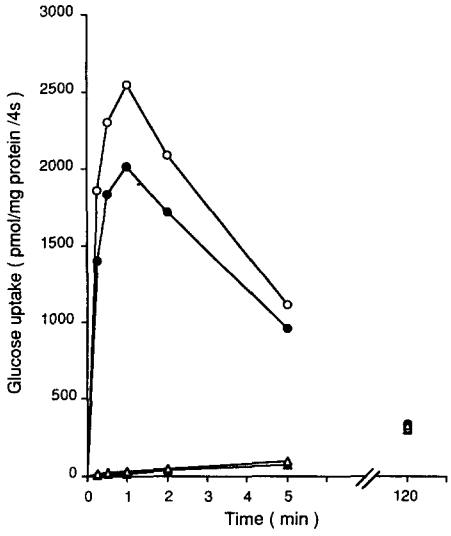

Fig. 6. Effect of bile duct ligation on the time course of D-glucose uptake by rat renal cortical BBM vesicles. Seventy-five μl of a BBM vesicle suspension was mixed with 300 μl of transport buffer as described under Methods. Fifty μl of the incubation mixture was collected at the time intervals shown and immediately added to 1 ml of ice-cold stop solution. The measurements were carried out in quadruplicate and the results from one representative experiment are presented. Total and Na^+-independent D-glucose uptakes are shown for BBM vesicles (C/PL = 0.658) from a 3-day ligated rat (○;△, respectively) and for vesicles (C/PL = 0.611) from a sham-operated animal (●; ▲).

DISCUSSION

Proteins in cell-surface membranes serve as receptors or carry out enzymatic or transport processes, but their activities can be influenced by the lipid con-

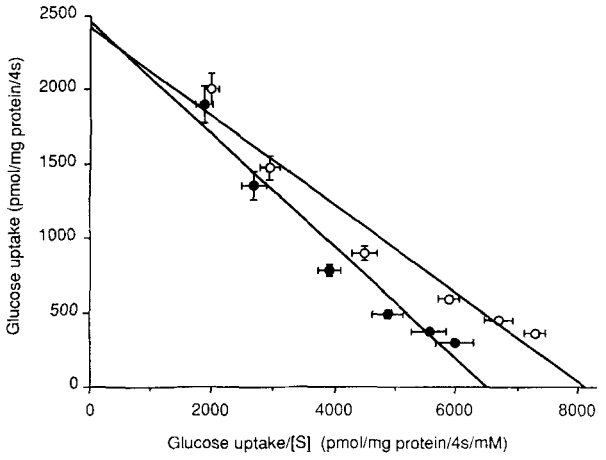

Fig. 7. Woolf-Augustinsson-Hofstee plots of the initial rate of Na^+-dependent D-glucose uptake by renal cortical BBM vesicles as a function of D-glucose concentration in 3-day ligated rats (○) and sham-operated rats (●). Uptake studies of D-[^{3}H]glucose over 4 sec were carried out for five pairs of animals as indicated in Fig. 2 using the low glucose concentration range (0.05–1.0 mM) described by others for high-affinity Na^+-glucose cotransport (12, 25). The experimental points from each animal were fitted by linear regression and individual values for V_{max} and K_t were obtained before calculating statistical differences. The points shown in the figure are means ± SEM (n = 5). The mean values of the BBM C/PL of the ligated and sham-operated rats used were 0.659 and 0.633, respectively.

stituents of the membrane, most commonly through a fluidity change (12, 23, 37). Although increases in fluidity of the lipid bilayer matrix are reported to impair high-affinity Na^+-glucose cotransport by renal BBM (8–10), our results provide the first evidence that the converse applies, namely that reductions in membrane fluidity, even if relatively small, can enhance renal Na^+-glucose cotransport. This finding also constitutes the first report that cholesterol enrichment of membranes in vivo can enhance a carrier-mediated transport process.

The cholesterol content of cell-surface membranes, including renal BBM (23, 33), is the major determinant of their fluidity. It was not surprising, therefore, that a close relationship was found between BBM C/PL and fluorescence polarization ($r = 0.75$), nor that both were correlated with the enhanced Na^+-dependent D-glucose uptake. However, no inverse relationship existed between the BBM PC/SM and fluorescence polarization ($r = 0.04$), even though increases in PC/SM, albeit much larger than in the present study, are considered to fluidize membranes (30), including renal BBM (10, 29, 33). Nevertheless, the increased BBM PC/SM was not without importance; it inversely correlated with the initial rate of Na^+-dependent D-glucose uptake, suggesting that it might counteract, at least in part, the cholesterol-induced stimulation of Na^+-glucose cotransport. Multiregression analysis supported this opposing influence; subtracting the effect of BBM PC/SM from the correlation between BBM C/PL and Na^+-dependent D-glucose uptake ($r = 0.58$, $P < 0.01$; Fig. 4) resulted in a closer relationship without a change of slope ($r = 0.64$, $P < 0.001$). Similarly, it can be argued that the gradual decline in Na^+-glucose cotransport after 3 days of ligation, despite the virtually constant elevation of BBM C/PL and fluorescence polarization, largely reflects the 6% and 16% increases of BBM PC/SM on days 8 and 15, respectively. These proposals imply that alterations in the phospholipid composition of renal BBM can regulate the activity of the Na^+-glucose transporter in more subtle ways than by changing bulk membrane fluidity. Such a contention is not new; certain membrane protein-mediated activities are influenced more by lipid composition per se than by the fluidity of the lipid bilayer (reviewed in 38).

To confirm that excess membrane cholesterol is a specific stimulant of high-affinity Na^+-glucose cotransport in kidney epithelial cells, we incubated normal BBM with cholesterol-rich phospholipid dispersions. Good evidence was obtained that some cholesterol selectively partitioned into the membrane and, as no change occurred in the phospholipid profile, these BBM vesicles resemble those from 3-day ligated rats. In both these examples of cholesterol-rich BBM vesicles, Na^+-dependent D-glucose uptake was elevated but, intriguingly, a 5% rise in BBM C/PL induced by the liposomes (Fig. 5) caused two- to threefold less stimulation of Na^+-glucose uptake than the corresponding C/PL increase induced by bile duct ligation (Fig. 4). This diminished effect of cholesterol enrichment in vitro is unlikely to be a consequence of either the 2-h preincubation period or the presence of liposomes and albumin in the assay buffer; D-glucose uptake by BBM vesicles treated with control liposomes of C/PL 0.9 was similar to nonincubated vesicles. Rather, we suspect that the accumulated cholesterol distributed differently in the membrane: because of insufficient time for 'flip-flop' equilibration to occur (39), the outer leaflet of the BBM may contain more of the excess cholesterol when it is acquired in vitro than in vivo. The possibility that each of the BBM bilayer leaflets has quantitatively different effects on Na^+-glucose cotransport activity merits further investigation; it might explain why enrichment of renal BBM in vitro with cholesteryl hemisuccinate (a hydrophilic cholesteryl ester which would not readily flip-flop into the inner membrane leaflet) is reported not to affect Na^+-glucose cotransport (26); it might also be relevant to the inhibitory action of increased PC/SM in the present study since both these phospholipids are asymmetrically distributed in the renal cortical BBM (40).

As an alternative to a membrane lipid effect, enhanced Na^+-glucose cotransport activity could simply be explained by a greater degree of purification of the BBM vesicles from ligated rats. We have rejected this explanation for two reasons. First, because BBM from sham-operated rats appeared no more contaminated with basolateral or other membranes than those from 3-day ligated animals: their phospholipid compositions were comparable as were the low Na^+/K^+-ATPase activities, whilst the specific activity of the BBM marker enzyme, leucine aminopeptidase, and the carrier numbers per mg of membrane protein (as estimated by the number of phlorizin binding sites) were not increased by bile duct ligation. Second, kinetic studies of the cotransporter were inconsistent with simple BBM enrichment: bile duct ligation neither increased V_{max} nor left the characteristics of the carrier unaltered (a reduced K_t was found). But if the BBM from ligated and sham-operated animals were purified to similar extents, why was the specific activity of the other BBM marker enzyme studied, alkaline phosphatase, increased by 50%? Because BBM alkaline phosphatase activity was found to increase directly with BBM C/PL, one possible explanation is that the enzyme, which is anchored to the membrane by covalent attachment to glycosylphosphatidylinositol (41), is particularly sensitive to small increases in membrane cholesterol. It may also be affected by reductions in C/PL since ischemia caused a decrease, albeit not significant, in its specific activity (10). On the other hand, the response of alkaline phosphatase in intestinal BBM is the exact opposite: an increase or decrease in membrane C/PL decreases or increases, respectively, alkaline phosphatase activity (38). Presumably, structural differences between the two enzymes account for their different responses; the alkaline phosphatases of intestine and kidney are products of different genes and can readily be distinguished from each other by immunological and biochemical techniques (42).

The stimulatory effect of 3-day bile duct ligation on Na^+-dependent D-glucose uptake was not due to an augmentation of the driving forces; a slower collapse of the Na^+ gradient was not detectable nor was there evidence of greater anion diffusion potential since voltage clamping did not normalize D-glucose uptake. Rather, there appeared to be a direct effect of cholesterol enrichment, and associated reduced membrane fluidity, on the carrier itself and stimulation was due to a decrease in K_t and not to an increase in V_{max}. This conclusion is consistent with an increased affinity (reduced K_d) of Na^+-dependent phlorizin binding sites 3 days after bile duct ligation. In intestinal BBM, binding of Na^+, the obligatory first substrate of the cotransporter, induces a rapid conformational change in the carrier that increases its affinity for either glucose or its competitive inhibitor, phlorizin (43, 44). Conceivably, decreases in BBM fluidity induced by bile duct ligation may impose constraints or conformational changes on the carrier such that Na^+ binding is facilitated and/or glucose and phlorizin binding sites are made more accessible. By contrast, increasing BBM fluidity appears to evoke a qualitatively different response from the Na^+-glucose transporter; both ischemia (10) or the addition of 20 mM benzyl alcohol (8, 9) halved the V_{max} of the carrier without altering its binding affinity for glucose of phlorizin.

The changes in renal BBM lipid composition associated with bile duct ligation, like those of the erythrocyte membrane (16), appear to be induced by abnormal plasma lipoproteins secondary to LCAT deficiency. Thus, not only was BBM C/PL inversely correlated with plasma LCAT activity ($r = -0.59$, $P < 0.01$) and directly with plasma lipoprotein C/PL ($r = 0.68$, $P < 0.001$; Fig. 2), but the content of phosphatidylcholine in the BBM was also raised 15 days after ligation. Presumably, excess cholesterol and phosphatidylcholine are first deposited in renal basolateral membranes from abnormal lipoprotein particles in the peritubular fluid, followed by a similar alteration in the BBM lipid profile by exchange and equilibration processes (11, 16) during membrane turnover and recycling (45). The more rapid accumulation of cholesterol by BBM compared to phosphatidylcholine is consistent with this scenario as phospholipid exchange is relatively slow (46).

In summary, an early effect of bile duct ligation in rats is to enrich renal cortical BBM in cholesterol, apparently by cellular uptake from abnormal plasma lipoproteins; the resulting decrease in membrane fluidity enhances Na^+-dependent D-glucose cotransport by increasing the affinity and possibly turnover of the carrier. However, when the ligation period is prolonged, the BBM also accumulates phosphatidylcholine and this appears to partially counteract the cholesterol-induced stimulation of Na^+-glucose cotransport by a mechanism not involving a change in membrane fluidity. Whether these lipid changes constitute a renal membrane defect that contributes to sodium and water retention by the kidney in biliary-obstructed rats (47, 48) is unknown. However, this possibility merits further investigation; it seems probable that similar lipoprotein-induced membrane lipid changes will also occur in kidney epithelial cells of jaundiced patients and thus potentially have pathophysiologic significance for the renal dysfunction that is a frequent complication of chronic human liver disease (49, 50).

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