Modulation of monkey small intestinal brush border membrane D-glucose transport by nonesterified fatty acids

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Brush border membranes isolated from monkey intestinal mucosa was found to contain considerable amount of nonesterified fatty acids. Incubation of brush border membranes with fatty acid free albumin selectively removed the free fatty acids more than 80% without altering the level of phospholipids or cholesterol. The sodium dependent D-glucose transport was stimulated by the albumin treatment. Kinetic study showed that albumin treatment did not alter the apparent affinity ($K_a$) of the transporter for glucose whereas the maximal velocity ($V_{max}$) was increased significantly. The sodium dependent D-glucose transport was inhibited by the exogenously added unsaturated fatty acids. Saturated fatty acids and methyl esters of unsaturated fatty acids showed no inhibition. Based on these results, it may be concluded that free fatty acids inhibit the sodium dependent intestinal D-glucose transport either by directly interacting with the transport protein or by abolishing the sodium gradient.

Enterocytes are highly polarized cells consisting of well defined brush border and basolateral membranes. Brush border membrane is the site where the final stage of digestion occurs and is also the site of absorption of nutrients. It has been shown that transport of glucose, amino acids and inorganic phosphate across the intestinal brush border membrane is by secondary active process. Evidences indicate that alterations in the lipid composition and physical state of the membrane lipids can alter membrane-bound enzyme activities and transport system. Plasma membrane of the intestinal epithelial cells contains considerable amount of nonesterified fatty acids (NEFA) compared to other known biological membranes. NEFA is known to alter a variety of membrane associated cellular functions like membrane-bound enzyme activities, platelet aggregation, energy transduction in chromaffin granules, oxidative phosphorylation, and potassium channels. NEFA may also alter the intestinal transport and play an important role in the pathogenesis of fatty acid-induced diarrhoea. Rhoads et al. showed that synaptosomal Na⁺-dependent uptake of neurotransmitter amino acids was stimulated by fatty acid-free albumin. They concluded that the stimulatory effect of albumin is due to its ability to bind free fatty acids, as proteins which do not bind fatty acids failed to stimulate the uptake. Thus it was suggested that NEFA may be involved in the regulation of synaptosomal uptake of neurotransmitter amino acids. Since intestinal brush border membranes contain considerable amount of NEFA, it will be of interest to study the role of these fatty acids on membrane associated functions such as transport.

Materials and Methods
Authentic fatty acids, lipid standards, Tris, Hepes, bovine serum albumin (BSA), dithiothreitol, fatty acid-free albumin and 1, 6-diphenyl-L-3, 5-hexatriene (DPH) were all obtained from Sigma Chemical Company, USA. [14C]D-Glucose was obtained from Bhabha Atomic Research Centre, Bombay. All other chemicals were used of analytical grade.

Preparation of brush border membrane vesicles—Brush border membrane vesicles (BBMV) were prepared from monkey intestinal mucosa by MgCl₂ precipitation as described. Briefly, overnight fasted monkeys (Macaca radiata) were killed by giving an overdose of pentobarbitone. The intestine was removed and washed with ice-cold 0.9% NaCl containing 2 mM dithiothreitol. The mucosa was scraped using a glass slide and homogenized in 20 volts of ice-cold homogenizing buffer containing 150 mM mannitol, 2.5 mM EGTA, 6 mM Tris buffer adjusted to pH 7.5. The homogenate was allowed to stand at 4°C for 10 min and filtered through a nylon cloth. To the filtered homogenate, 1 M MgCl₂ was added to a final concentration of 10 mM stirred for 1 min and allowed to stand for 15 min. It was then centrifuged at 3000 × g for 15 min. The supernatant collected was centrifuged

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at 42000 × g for 30 min. The pellet obtained was suspended in 1 ml of preloading buffer (300 mM mannitol and 10 mM Hepes, pH 7.5), made into vesicles by using a syringe fitted with a 26 gauge needle, then diluted to 30 ml with preloading buffer and centrifuged again at 42000 × g for 30 min. This process was repeated once again to get purified BBMV. Protein was estimated using BSA as standard19. BBMV protein concentration was adjusted to 10 mg/ml and used immediately or stored in liquid nitrogen until further use. Purity of the BBMV was assessed by the enrichment of the marker enzyme alkaline phosphatase.

Fatty acid-free albumin treatment of BBMV—BBMV were treated with fatty acid-free albumin (protein, 1:15 ratio) and incubated at 4°C for 30 min with frequent shaking. It was centrifuged at 42000 × g for 30 min and the pellet obtained was washed twice with preloading buffer and vesiculated using a 26 gauge needle.

Transport studies—Glucose uptake by BBMV was measured at room temperature by rapid filtration technique18. Briefly, 50 μl of BBMV corresponding to 100 μg protein was incubated with 150 μl of uptake buffer containing 150 mM NaSCN, 50 mM D-glucose and 0.5 μCi [14C]D-glucose, 10 mM Hepes (pH 7.5) at varying time intervals. At the end of incubation, the mixture was diluted with 2 ml of ice-cold stop buffer (150 mM NaCl, 10 mM Hepes, pH 7.5) and filtered rapidly through 0.45 μm Millipore filter under constant vacuum. The filter was washed thrice with 5 ml of stop buffer and transferred to counting vials. The radioactivity retained in the filter was counted by liquid scintillation spectrometry. To study the effect of individual fatty acids on glucose uptake, both native and fatty acid-free albumin-treated BBMV were separately incubated with various fatty acids in ethanol to the final concentration of 0.5 mM. Total volume of incubation medium was 0.5 ml with BBMV corresponding to 1 mg protein. Control experiments had an equal concentration of ethanol alone. These fatty acid-d incubated BBMV were also used for transport studies as described above.

Lipid analysis—Lipids from BBMV were extracted by Bligh & Dyer method20 and neutral lipids were separated by TLC using silica gel plates in a solvent system consisting of hexane-diethyl ether-acetic acid (80:20:1, v/v). Separated lipids were visualized by exposure to iodine and individual spots were scraped, eluted with chloroform-methanol (2:1, v/v) and dried using nitrogen. Phospholipids were quantitated by estimating free phosphate after acid digestion21 and cholesterol was estimated as described22. Nonesterified fatty acids were separated and quantitated using gas chromatography after methylation.

This was done using Pye Unicam PU 4550 gas chromatograph equipped with flame ionization detector and Spectrophysics PU 4811 integrator. Fatty acids were separated on EGSS-X column and heptadecanonic acid was used as internal standard.

Enzymes—BBMV enzymes sucrose, alkaline phosphatase, ATPase, leucine aminopeptidase and gamma glutamyl transpeptidase were measured beforehand and after fatty acid-free albumin treatment.

Results

Alkaline phosphatase measurement showed a 15-fold enrichment of the marker enzyme in the final preparation of BBMV as compared to the homogenate, an observation in agreement with earlier reports. Lipid analysis of the monkey intestinal BBMV showed that it had considerable amount of nonesterified fatty acids (NEFA) as part of the membrane lipids (Table 1). Fatty acid-free albumin treatment removed more than 80% of the NEFA from the membrane without significant alteration in the phospholipid or cholesterol content of the membrane. As shown in Table 1, albumin had no specificity towards any single fatty acid and had affected all fatty acids equally. Activities of BBMV associated enzymes, when studied in both native and albumin-treated BBMV, showed no significant alteration after removal of NEFA from the membrane (data not shown).

Time-dependent study of D-glucose uptake across monkey intestinal BBMV in the presence of

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Native membrane</th>
<th>Albumin treated membrane</th>
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<tr>
<td>Phospholipids (total)</td>
<td>711 ± 112</td>
<td>671 ± 84</td>
</tr>
<tr>
<td>Cholesterol (total)</td>
<td>495 ± 25</td>
<td>466 ± 61</td>
</tr>
<tr>
<td>Total free fatty acids</td>
<td>310 ± 31</td>
<td>46 ± 4</td>
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</table>

*Each value represents mean ± SD of three separate experiments. ND = Not detected.
Fig. 1—Effect of fatty acid-free albumin treatment on the time course of \(\Delta\)-glucose uptake by monkey intestinal BBMV, ([\(\bigcirc\)–\(\bigcirc\)], native membrane in the presence of inward directed \(K^+\) gradient; (\(\bigotimes\)–\(\bigotimes\)), native membrane in presence of inward-directed \(Na^+\) gradient; (\(\bigtriangledown\)–\(\bigtriangledown\)), albumin-treated membrane in presence of inward-directed \(Na^+\) gradient; (\(\bigtriangleup\)–\(\bigtriangleup\)), albumin-treated membrane in presence of inward-directed \(K^+\) gradient. Each value represents mean ± SD of three separate estimations. The experimental details are given in the text).

Table 2—Kinetic parameters of \(\Delta\)-glucose transport by monkey BBMV with and without albumin treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(V_{max}) (pmole/mg protein/20 sec)</th>
<th>(K_m) ((\mu)M)</th>
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<tr>
<td>Native</td>
<td>133 ± 10</td>
<td>78 ± 9.9</td>
</tr>
<tr>
<td>Albumin-treated</td>
<td>182 ± 8</td>
<td>74 ± 1.1</td>
</tr>
</tbody>
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NaSCN gradient ([\(Na^+\)] = 0; [\(Na^+\)]_o = 150 mM) is shown in Fig. 1. In presence of \(Na^+\) gradient, \(\Delta\)-glucose uptake showed an overshoot phenomenon with a peak at 20 sec. Treatment of BBMV with fatty acid-free albumin increased the uptake. In the presence of \(K^+\) gradient, the uptake values were same in the native and albumin-treated membranes. This clearly indicated that NEFA removal from BBMV increased the active transport without altering the passive diffusion. In order to find out the nature of stimulation of uptake, the effect of albumin treatment on kinetic parameters of \(\Delta\)-glucose uptake was studied. This showed that the maximum velocity \((V_{max})\) of the albumin-treated BBMV was significantly higher than that of the untreated membrane vesicles (Table 2, Fig. 2). However, albumin treatment did not alter the apparent affinity \((K_m)\) of the transporter for \(\Delta\)-glucose.

To study the specificity of fatty acid inhibition of glucose transport by BBMV, albumin-treated membranes were incubated with various individual fatty acids and these BBMV were tested for the uptake of \(\Delta\)-glucose. As shown in Table 3, unsaturated fatty acids were more inhibitory than the saturated ones.

Table 3—Effect of various long chain fatty acids on \(\Delta\)-glucose uptake

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Native membrane</th>
<th>Albumin treated membrane</th>
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<tbody>
<tr>
<td>Control</td>
<td>125 ± 3 (100)</td>
<td>174 ± 13 (100)</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>120 ± 5 (96)</td>
<td>158 ± 15 (97)</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>126 ± 7 (100)</td>
<td>162 ± 9 (93)</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>50 ± 3 (40)</td>
<td>67 ± 8 (39)</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>39 ± 2 (31)</td>
<td>49 ± 3 (28)</td>
</tr>
<tr>
<td>Elaidic acid</td>
<td>63 ± 5 (50)</td>
<td>83 ± 4 (48)</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>30 ± 2 (24)</td>
<td>39 ± 3 (22)</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>22 ± 4 (18)</td>
<td>25 ± 6 (14)</td>
</tr>
<tr>
<td>Oleic acid methyl ester</td>
<td>121 ± 6 (97)</td>
<td>158 ± 20 (91)</td>
</tr>
<tr>
<td>Linoleic acid methyl ester</td>
<td>123 ± 9 (98)</td>
<td>156 ± 10 (90)</td>
</tr>
</tbody>
</table>

*Each value represents mean ± SD of three separate experiments. Values in parentheses represent percentage of control.

Among the unsaturated fatty acids studied, the extent of inhibition increased with increase in the number of double bonds. Thus linolenic acid was more inhibitory than linoleic acid which was more inhibitory than oleic acid. The configuration of the double bond also determines the inhibitory effect. Elaidic acid, a trans monounsaturated fatty acid, was less inhibitory than the cis isomer oleic acid. Apart from this, the presence of carboxyl group was crucial for the inhibitory effect since methyl ester of oleic and linoleic acid failed to inhibit the \(\Delta\)-glucose uptake compared to that of free acid. The equilibrium (60 min uptake) remained same.
in control albumin-treated and unsaturated fatty acid supplemented membranes suggesting that fatty acid removal and supplementation did not alter the intravesicular volume (control = 18.2 ± 1.0 pmole/mg protein; albumin-treated = 20.6 ± 1.4 pmole/mg protein; unsaturated fatty acid (oleic acid) — supplemented = 21.3 ± 1.9 pmole/mg protein).

Discussion
The present work shows that monkey intestinal brush border membranes contain significant amount of nonesterified fatty acids. Fatty acid-free albumin specifically removes the NEFA resulting in stimulation of transport activity. Albumin treatment increased the $V_{max}$ of the glucose transporter without altering the $K_m$, suggesting that the fatty acid inhibition is non-competitive. The presence of high levels of NEFA in biological membranes is unusual. It is not known whether the fatty acids are inherently present in the BBMV or artificially generated during isolation. Intestinal brush border membranes are rich in phospholipases and it has been shown that it has a calcium-independent phospholipase22-25. This phospholipase also shows lyso phospholipase activity24. In addition, these membranes contain esterases capable of hydrolysing triglycerides25. These lipolytic enzymes are likely to generate free fatty acids during membrane isolation.

Sodium-dependent D-glucose transport was inhibited by exogenously added fatty acids, the extent of inhibition depending on the structure of the fatty acids. Unsaturated fatty acids were more inhibitory than the saturated ones. A similar inhibitory effect by unsaturated fatty acids on Na⁺H⁺ exchanger has been reported23. Earlier it was shown that unsaturated fatty acids act as potent inhibitors of brush border membrane Mg²⁺ ATPase possibly by influencing the surface pH26. The failure of saturated fatty acids to inhibit BBM Mg²⁺ ATPase may be due to its poor solubility in the incubation medium. Methyl esters of unsaturated fatty acids failed to inhibit transport suggesting that the free carboxyl group is necessary for the inhibitory effect. The configuration of the double bond also determined the inhibitory effect. cis Unsaturated fatty acid was more inhibitory than the trans fatty acid and it is known that cis unsaturated fatty acids cause perturbation of membrane structure27.

The mechanism by which unsaturated fatty acids inhibit D-glucose uptake is not known. Our results clearly show that integrity of the vesicles was not altered since equilibrium uptake values were same for the native and fatty acid supplemented membranes. Evidence show that BBMV fluidity may influence several important membrane functions20-23. One possibility by which unsaturated fatty acids inhibit transport may be through increasing membrane fluidity. But there are contradictory reports in literature on the relation between membrane fluidity and Na⁺ dependant D-glucose transport. Studies using in vivo dietary manipulation have demonstrated a direct correlation between membrane fluidity and Na⁺-dependent D-glucose transport in rat small intestinal brush border membranes29. In vivo experiments using known fluidizer like benzyl alcohol have shown an inverse relationship between fluidity and D-glucose transport30. Based on these results it may be concluded that unsaturated fatty acids inhibit Na⁺-dependent D-glucose transport by a mechanism other than altering the membrane fluidity. It has been shown that intestinal brush border membranes possess various lipid domains25. Klauser et al.31 using DPH and amilinaphyl sulphonate as fluorescent probes showed that membranes of mouse lymphocytes and baby hamster kidney cells contain lipid domain of different fluidities. Saturated and trans-unsaturated fatty acids were taken up by the less fluid domains without affecting the DPH polarization. Cis-unsaturated fatty acids, on the other hand, were taken up by the more fluid domain where they caused disruption of acyl chain packing and lowering of DPH fluorescence polarization. It is also possible that unsaturated fatty acids may alter the sodium permeability. Intestinal perfusion with long chain fatty acids showed that fatty acids alter water and electrolyte absorption32-34. On the basis of these studies, two possible mechanisms by which fatty acids could alter the Na⁺ dependant D-glucose transport can be postulated; (i) It is likely that the glucose transport protein resides in more fluid domain of the brush border membrane and unsaturated fatty acids were taken up by such domain. Presence of free carboxyl groups near the vicinity of the transporter could alter the conformation or modify the charged groups of the transporter leading to decreased transport. Removal of fatty acids stimulates the transport. (ii) Unsaturated fatty acids might increase the sodium permeability and, since sodium gradient is the driving force for D-glucose transport, collapse of sodium gradient by unsaturated fatty acids might inhibit the transport.

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References