Morphological and functional changes in the enterocyte induced by fructose

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In the presence of 10–50 mM-fructose, enterocytes of organ-cultured pig intestinal-mucosal explants fail to glycosylate correctly their newly synthesized microvillar enzymes, and instead degrade them [Danielsen (1989) *J. Biol. Chem.* **264**, 13726–13729]. In the present work, this degradation was shown to occur extremely rapidly as the microvillar enzyme aminopeptidase N (EC 3.4.11.2) was hardly detectable after a 10 min pulse with [³⁵S]methionine. The abnormal biosynthesis of membrane glycoproteins affected both the morphology and the function of the Golgi complex as well as the microvillar membrane. Thus the stack of Golgi cisternae was condensed and devoid of dilated rims, and the secretion of a non-glycosylated protein, apolipoprotein A-1, was almost completely blocked in the presence of fructose, showing that transport through the secretory pathway is disturbed even for proteins unaffected by the defective glycosylation. The microvilli of the brush-border membrane were markedly shortened (by about 40 %) in the presence of fructose, and incorporation of newly made actin into the microvillar cytoskeleton was similarly decreased. By affecting membrane glycoprotein synthesis, the common dietary sugar fructose thus profoundly perturbs the exocytic membrane traffic in the enterocyte.

INTRODUCTION

The ability of the intestinal absorptive cell (enterocyte) to complete the digestion of dietary carbohydrate and protein relies on its continuous expression of glycosidases and peptidases at the luminal microvillar (brush-border) membrane (Semenza, 1986; Alpers, 1987). Synthesis of more than a dozen microvillar enzymes is thus a major task for the enterocyte; those most abundant, such as aminopeptidase N (EC 3.4.11.2), account for up to 1–2 % of the total protein synthesis and therefore constitute a considerable proportion of the exocytic membrane traffic through the secretory pathway. During biosynthesis, they become heavily glycosylated in the rough endoplasmic reticulum (up to 10 high-mannose carbohydrate chains per polypeptide chain), followed by trimming and attachment of ‘complex’ sugars to *N*-linked carbohydrate, and by *O*-linked glycosylation and tyrosine sulphation during passage through the Golgi complex (Danielsen *et al.*, 1987).

Inhibitors of co-translational glycosylation and carbohydrate trimming (Elbein, 1987) have been shown to decrease the surface expression of newly made microvillar enzymes (Danielsen & Cowell, 1984, 1986; Matter *et al.*, 1989). In the enterocyte, this occurs by a mechanism whereby abnormally glycosylated molecules are recognized by a leupeptin-sensitive ‘quality control’ and rapidly degraded. Recently, the common dietary sugar fructose was observed to suppress the apical expression of microvillar enzymes in enterocytes by inducing an abnormal co-translational high-mannose glycosylation, followed by intracellular degradation (Danielsen, 1989). Among other common dietary mono- and di-saccharides, only sucrose was observed to have a similar effect. Fructose is absorbed by a Na⁺-independent carrier in the intestine and is the only sugar of physiological significance to be transported by this system (Semenza & Corcelli, 1986). The mechanism whereby fructose interferes with *N*-linked glycosylation is not known, but the observation that the fructose effect is rapidly reversible and suppressible by equimolar amounts of mannose (Danielsen, 1989) suggests that it acts by inhibiting

competitively one or more of the steps in the assembly of high-mannose oligosaccharides on dolichol phosphate.

The present work shows how uptake of fructose, by primarily affecting glycosylation of membrane proteins, induces changes in both morphology and function of enterocytes of organ-cultured mucosal explants.

EXPERIMENTAL

Materials

Equipment for performing organ culture, including Trowell’s T-8 medium, plastic dishes with grids and [³⁵S]methionine (sp. radioactivity > 1000 Ci/mmol), was obtained as described previously (Danielsen *et al.*, 1982). An antiserum to apolipoprotein A-1 (apo A-1) was prepared as described by Danielsen (1990).

Pig small intestines were kindly given by the Department of Experimental Pathology, Rigshospitalet, Copenhagen.

Organ culture of intestinal mucosal explants

Small-intestinal mucosal explants of about 100 mg (for biochemical determinations) or 10 mg wet wt. (for electron microscopy) were excised and cultured for periods up to 10 h as described previously (Danielsen *et al.*, 1982). After culture, the explants, and, in experiments with apo A-1, also the media, were collected and quickly frozen at –20 °C. Explants used for electron microscopy were fixed immediately after culture.

Subcellular fractionation

Fractionation of mucosal explants by the bivalent-cation technique (Schmitz *et al.*, 1973; Booth & Kenny, 1974) into a Mg²⁺-precipitated membrane fraction (containing intracellular and basolateral membranes), a microvillar fraction and a soluble fraction was carried out as follows. Mucosal explants were thawed and homogenized in 1 ml of 12 mM-Tris/HCl/0.3 M-mannitol, pH 7.1+1.6 ml of distilled water in a 5 ml Potter/Elvehjem homogenizer by 10–15 strokes of the piston (manually operated). The homogenate was centrifuged at 500 g

Abbreviation used: apo A-1, apolipoprotein A-1.

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for 10 min, and the resulting supernatant adjusted to 10 mM in MgCl_2 . After 15 min, the preparation was centrifuged at 2000 g for 10 min to obtain a pellet (Mg^{2+} -precipitated membrane fraction) and a supernatant. The latter was centrifuged at 48 000 g for 1 h, yielding a pellet (microvillar membrane fraction) and a supernatant (soluble fraction).

Immunoisolation of microvillar enzymes and apo A-1

Aminopeptidase N and sucrase-isomaltase from mucosal explants were immunopurified by line-immunoelectrophoresis against a rabbit antiserum raised against the enzymes from the pig (Danielsen & Cowell, 1983). Apo A-1 was immunoprecipitated from mucosal explants, homogenized in 50 mM-Tris/HCl (pH 7.5)/150 mM-NaCl and extracted with 1% (w/v) Triton X-100. Extracts cleared by centrifugation at 20 000 g for 3 min were incubated with anti-apo A-1 antiserum at 4 °C for 20 h before the immunoprecipitates were sedimented by centrifugation as above. Apo A-1 was similarly immunoprecipitated from media which before addition of antiserum had been extracted with 1% Triton X-100 and cleared by centrifugation as described above.

Electrophoresis

SDS/PAGE in 10%-acrylamide gels under reducing conditions was performed as described by Laemmli (1970), and fluorography as described by Bonner & Laskey (1974). Gel tracks were scanned in a LKB Ultrosan XL densitometer (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Electron microscopy

For ultrastructural analysis, mucosal explants were fixed by immersion in 2.5% (v/v) glutaraldehyde in 0.1 M-sodium phosphate, pH 7.2, for 2 h at 4 °C and post-fixed for 1 h at 4 °C in 1% osmium tetroxide (made up in the above buffer). Before dehydration in ethanol and embedding in Epon, some of the explants were block-stained in aq. 1% uranyl acetate for 1 h at 20 °C.

Ultrathin sections were cut on an LKB 8800 III (Pharmacia LKB Biotechnology) or a Reichert-Jung (Wien, Austria) ultratome, with a diamond knife (Jumdi, Stockholm, Sweden) and collected on Formvar and carbon-coated slot grids or on naked 400-mesh nickel grids (rinsed successively in 20% acetic acid, 96% ethanol and distilled water). The sections were stained in lead citrate (Reynolds, 1963) for 1 min (block-stained explants) or in aq. 1% uranyl acetate (10 min) and lead citrate (1 min) before examination in a Philips 201c electron microscope (Eindhoven, The Netherlands), operated at 60 kV. Data were obtained from explants from three animals, and nine micrographs of the brush border and Golgi areas from mid-villar enterocytes were taken at random from each of six explants. Only micrographs of microvilli sectioned in parallel to their longitudinal axis were used, and a total of 125 microvilli from fructose-exposed and control explants were measured with a planimeter (Numonics Corp., Lansdale, PA, U.S.A.) The micrographs of the Golgi areas from fructose-exposed and control explants were randomized, and, according to the criteria of (1) condensed cisternae and (2) dilated rims, more than 90% of the micrographs were assigned the correct culture conditions (presence or absence of fructose).

For immuno-electron microscopy the mucosal explants were fixed and processed as described by Hansen *et al.* (1989). Gold particles (5 nm), prepared as described by Slot & Geuze (1985), were conjugated to sheep anti-rabbit IgG as described by Wang & Larsson (1985) with the modification that the IgG-gold complexes were purified by ultracentrifugation. Immunogold labelling with rabbit anti-(aminopeptidase N) IgG was performed as described by Hansen *et al.* (1987, 1989). Data were obtained from explants from two animals, and 10 micrographs of the microvillar membrane from mid-villar enterocytes were taken at random from each of four explants. Only micrographs of microvilli sectioned in parallel to their longitudinal axis were measured (as described above) and the number of gold particles in the membrane was counted.

The significance of the effect of fructose on the height of

Fig. 1. Rapid degradation of newly made aminopeptidase N induced by fructose

Mucosal explants, cultured for 2 h in the absence or presence of 50 mM-fructose, were pulse-labelled for 10 min with 200 μCi of [^{35}S]methionine/ml. The labelled explants were homogenized and solubilized in 25 mM-Tris/HCl/75 mM-NaCl (pH 7.3)/5% (w/v) Triton X-100, and the detergent-extracted homogenates were cleared by centrifugation at 25 000 g for 5 min. Aminopeptidase N and apo A-1 were immunopurified from the homogenates as described in the Experimental section, and the immunoprecipitates (I) and samples of homogenate (H) were subjected to SDS/PAGE. After electrophoresis and fluorography the gel tracks of the homogenates were scanned in a densitometer. Molecular-mass values (kDa) of the high-mannose glycosylated forms of aminopeptidase N (140 kDa), actin (42 kDa) and apo A-1 (25 kDa) are indicated.

microvilli and on the labelling intensity of aminopeptidase N was determined by Student's *t* test.

RESULTS

Fructose induces rapid degradation of newly made microvillar enzymes

Fig. 1 shows the labelling of mucosal explants with a 10 min pulse of [³⁵S]methionine, after a 2 h preincubation in the absence

or presence of 50 mM-fructose. In detergent extracts of whole mucosa, the overall labelling pattern of polypeptides in SDS/PAGE was not greatly affected, indicating that the sugar does not markedly interfere with the overall protein synthesis of the enterocyte. However, a band of 140 kDa, representing the transient high-mannose-glycosylated form of aminopeptidase N, was absent from fructose-exposed explants. (In contrast, synthesis of the non-glycosylated secretory protein apo A-1 was unaffected.) Densitometric scanning of gel tracks of

Fig. 2. Low-magnification electron micrographs of the villar region of mucosal explants cultured for 5 h in the absence (a) or presence (b) of 50 mM-fructose. Enterocytes (EN), goblet cells (GC) and an endocrine cell (EC) are seen. Bars indicate 5 µm.

Fig. 3. Apical region of villar enterocytes from mucosal explants cultured for 5 h in the absence (a) or presence (b) of 50 mM-fructose. Bars indicate 0.5 µm. Abbreviation: MV, microvilli.

Table 1. Effect of fructose on the microvillar membrane

The height of the microvilli of the brush-border membrane and the labelling density of aminopeptidase N were determined in explants cultured for 5 h in the absence or presence of 50 mM-fructose as described in the Experimental section (values in parentheses indicate S.D.).

	Control	+ Fructose
Microvillar height (μm)	1.80 (0.04)	1.14 (0.03)
Labelling density (particles/ μm of membrane)	5.63 (0.45)	4.60 (0.42)

immunopurified aminopeptidase N showed that less than 10% labelling remained associated with the enzyme after the 10 min pulse in fructose-exposed explants compared with controls (results not shown). An accurate determination of the half-life of the newly made microvillar enzyme in the presence of fructose was therefore not feasible, but the pulse-labelling experiment indicates that it must be below 10 min.

Fructose affects the ultrastructure of enterocytes

As shown in Fig. 2, culture of mucosal explants for 5 h in the presence of fructose did not affect the overall morphology of the villar intestinal epithelium. At low magnification the only struc-

tural alteration observed was a marked decrease in the height of the microvilli of the apical brush-border membrane. The extent of this shortening averaged almost 40% in three series of experiments (Fig. 3, Table 1). In some cases, the decreased microvillar height was accompanied by a decrease in the surface density of microvilli, resulting in a poorly organized brush border.

In immuno-electron microscopy, an antibody to aminopeptidase N labelled the microvillar surface of control explants as well as of fructose-exposed explants (Fig. 4). Quantitative analysis showed that the surface density of the enzyme was decreased by approx. 18% in the presence of fructose, a difference not considered significant by the *t* test ($P > 0.05$).

The height of microvilli is defined by the length of the actin filaments of their cytoskeleton. In previous work (Cowell & Danielsen, 1984), it was shown that the newly synthesized cytoskeletal components actin, villin and the 110 kDa protein are rapidly (< 5 min) incorporated into the microvillar fraction of enterocytes, indicating a rapid assembly of cytoskeletal components in dynamic maintenance of the brush-border structure. Fig. 5 shows the densitometric scanning of the gel tracks of the soluble and microvillar fractions of mucosal explants, pulse-labelled for 10 min after 4 h preincubation in the absence or presence of fructose. The conspicuous peak of actin (42 kDa) is considerably decreased in size in the microvillar fraction of the fructose-exposed explant, compared with the control. In four experiments, the amount of actin in the microvillar fraction was

Fig. 4. Immunolocalization of aminopeptidase N in the microvillar membrane of villar enterocytes from mucosal explants cultured for 5 h in the absence (a) or presence (b) of 50 mM-fructose

Bars indicate 0.2 μm .

Fig. 5. Fructose-induced decreased assembly of newly made actin into microvilli

Mucosal explants were cultured for 2 h in the absence or presence of 50 mM-fructose before labelling for 10 min with 200 μ Ci of [35 S]methionine/ml. After culture, a soluble fraction and a microvillar fraction were prepared and subjected to SDS/PAGE ($\frac{1}{10}$ of the soluble fraction and $\frac{1}{5}$ of the microvillar fraction from the explants was applied to the gel). After electrophoresis and fluorography, the distribution of labelled actin (the most abundant protein in the enterocyte) between the two subcellular fractions was determined by densitometric scanning of the gel tracks. The 42 kDa band of actin is indicated by arrows.

Fig. 6. Golgi complexes of villar enterocytes from mucosal explants cultured for 5 h in the absence (a) or presence (b) of 50 mM-fructose

Bars indicate 0.5 μ m.

$15.0 \pm 3.0\%$ of the amount of actin in the soluble fraction of control explants, whereas this ratio in fructose-exposed explants was $6.8 \pm 1.9\%$, indicating a markedly decreased incorporation of actin into the microvillar cytoskeleton. This finding correlates well with the observed shortening of microvilli and points to an interactive relationship between the exocytic membrane traffic of microvillar membrane glycoproteins to the apical cell surface and the rate whereby components of the microvillar cytoskeleton are assembled.

The only intracellular organelle markedly affected by fructose was the Golgi complex (Fig. 6). Thus 5 h of exposure to fructose resulted in a condensation of the stack of Golgi cisternae, and the dilated rims, normally seen in enterocytes of control explants, were missing.

Fructose decreases membrane traffic through the secretory pathway

The structural alterations of the Golgi complex observed in the presence of fructose (Fig. 6) suggest that this organelle might be functionally affected by the sugar, leading to an impaired protein transport through the enterocyte's secretory pathway. To test

this, the ability of mucosal explants to synthesize and secrete apo A-1, a major secretory protein of the small intestine (Shiau, 1987), was studied. Since apo A-1 is not a glycoprotein and its synthesis in the rough endoplasmic reticulum is therefore not directly affected by fructose, it is an ideal marker for the protein transport through the Golgi complex to the cell surface in the presence of fructose. As shown in Fig. 7(a), during 2 h of continuous labelling a much smaller proportion of newly made apo A-1 was recovered from the medium from fructose-exposed explants; thus densitometric scanning of the gel tracks revealed that only 17% of the total amount of apo A-1 from these explants had been secreted, as compared with 61% from the controls. A pulse-chase experiment (Fig. 7b) showed that in control explants maximal secretion of apo A-1 synthesized in a 10 min pulse is achieved after 60–90 min. A minor proportion of the protein remained associated with the explant even after 150 min, presumably because of entrapment in the intercellular space between neighbouring enterocytes. In the presence of fructose, apo A-1 could be detected in the medium after 60 min of chase, but it failed to accumulate during longer periods of chase. Fructose therefore not merely retards the transport of apo

Fig. 7. Decreased secretion of apo A-1 induced by fructose

(a) Mucosal explants were cultured for 2 h in the absence (upper panel) or presence (lower panel) of 50 mM-fructose before continuous labelling for 2 h with 100 μ Ci of [35 S]methionine/ml. After culture, apo A-1 was immunoprecipitated from the explant (Exp) and medium (Med) and subjected to SDS/PAGE. (b) Mucosal explants were cultured for 2 h in the absence (upper panel) or presence (lower panel) of 50 mM-fructose before pulse-labelling for 10 min with 500 μ Ci of [35 S]methionine/ml. The medium was then replaced with chase medium (containing 1 mM-methionine), and at the indicated periods of time (20–150 min) 75 μ l samples of medium (Med) were taken for analysis by SDS/PAGE. After culture, apo A-1 remaining in the explant was immunoprecipitated (2) from a Triton-X-100-solubilized extract of the explant (1). Molecular-mass values (kDa) are indicated.

A-1 through the secretory pathway of the enterocyte, but virtually blocks its release from the cell. This result clearly demonstrates that the transport capacity of the secretory pathway is severely affected in the presence of fructose.

Danielsen (1989) showed that the proteinase inhibitor leupeptin as well as equimolar amounts of mannose are able to suppress partially the effect of fructose. Leupeptin presumably acts by inhibiting the degradatory 'quality control' in a pre-Golgi compartment, whereas mannose probably competes with fructose in the assembly of lipid-linked high-mannose oligosaccharide chains. However, leupeptin either alone or together with mannose failed to restore secretion of apo A-1 from fructose-exposed mucosal explants under conditions otherwise similar to those employed in Fig. 7(a) (Fig. 8). Sucrase-isomaltase was immunopurified from the explants together with apo A-1, and as shown, a blur of 'underglycosylated' enzyme molecules was visible in the presence of leupeptin, whereas only the electrophoretically normal high-mannose form of the microvillar enzyme appeared when mannose was present.

Fig. 8. Effects of leupeptin and mannose

Mucosal explants were cultured for 2 h in the absence (1) or presence of 50 mM-fructose + 50 μ g of leupeptin/ml (2) or 50 mM-fructose + 50 mM-mannose + 50 μ g of leupeptin/ml (3) before continuous labelling for 2 h with 100 μ Ci of [35 S]methionine/ml. After culture, apo A-1 and sucrase-isomaltase were immunopurified from the explants (Exp) and apo A-1 was immunopurified from the media (Med) and subjected to SDS/PAGE. Molecular-mass values of mature (265 kDa) and transient (245 kDa) forms of sucrase-isomaltase and of apo A-1 (25 kDa) are indicated.

However, in neither case was the normal Golgi-associated processing, yielding the mature form of the enzyme, significantly restored (compared with the control). It can therefore be concluded that although the fructose effect is rapidly reversible (Danielsen, 1989), neither leupeptin nor mannose is capable of restoring protein transport along the secretory pathway as long as fructose is present.

DISCUSSION

Our earlier work (Danielsen, 1989), describing the suppressive effect of fructose on the expression of intestinal microvillar enzymes in a mucosal explant system, was extended in the present paper to an investigation of the morphological and functional consequences of an impaired biosynthesis and transport of the enterocyte's apical membrane glycoproteins. The virtually instant degradation of aminopeptidase N clearly localizes this event to a pre-Golgi compartment, since under normal conditions the microvillar enzyme only reaches the Golgi complex 20–30 min after synthesis (Danielsen, 1982). Newly made microvillar enzymes normally constitute a major proportion of the total amount of proteins transported through the secretory pathway, and other types of membrane glycoproteins might well be similarly degraded in the presence of fructose. Morphological changes of the Golgi complex resembling those induced by fructose are caused by culture at low temperature (20 °C), which in addition inhibits the post-translational molecular processing and transport of newly synthesized microvillar enzymes (Danielsen *et al.*, 1989). Low temperature inhibits the vesicular transport between the endoplasmic reticulum and the Golgi complex, and the decreased secretion and intracellular accumulation of apo A-1 in response to fructose indicates that this sugar likewise interferes with the membrane traffic between these two organelles. This transport block may well be a consequence of the induced defective glycosylation/degradation of membrane glycoproteins, since there will be much less newly synthesized proteins available for the transport vesicles operating between the endoplasmic reticulum and the Golgi complex. However, it is also possible that fructose affects other functions than the co-translational glycosylation 'machinery' which in turn cause the observed structural and functional alterations of the Golgi complex. Interestingly, normalizing the defective

glycosylation with mannose/leupeptin was observed not to restore the membrane traffic, as judged from the failure to secrete apo A-I and to process sucrase-isomaltase from transient to mature form. Recently obtained data show that the seemingly normal high-mannose-glycosylated forms of sucrase-isomaltase, as well as aminopeptidase N, seen in the presence of fructose and leupeptin, are retained and eventually degraded in a pre-Golgi compartment (with a half-time of about 40 min) and that fructose, by inducing a defective glycosylation, retards and probably perturbs the polypeptide folding of the nascent microvillar enzyme molecules (E. M. Danielsen, unpublished work).

The capacity of a cell to form microvilli at its surface depends on its ability to express the highly specialized cytoskeletal components, responsible for the microvillar architecture (Friederich *et al.*, 1989). Since the height of microvilli is physically limited by the available amount of apical surface membrane, a major determinant controlling the size of microvilli is probably the balance between the rates of exocytosis and endocytosis. With a functionally impaired Golgi complex, this balance is bound to be affected by fructose, and the microvillar shortening and decreased incorporation of actin are therefore most likely explained as the secondary consequences of a disturbed intracellular membrane traffic. In fact, a similar microvillar shortening has been observed in enterocytes of cultured mucosal explants that have been exposed to cycloheximide, an agent that effectively arrests protein biosynthesis and intracellular transport (G. H. Hansen, unpublished work). However, we cannot exclude the possibility that fructose directly affects the size and shape of the brush-border membrane.

In summary, the present work shows how a common dietary sugar, fructose, by inducing a defective glycosylation of microvillar enzymes and probably other types of membrane glycoproteins, virtually deprives the enterocyte of its secretory pathway. A close relationship between the nature of the carbohydrate source of energy and cellular differentiation (expression of microvillar enzymes) is known for the human colon cancer cell line HT-29, which, in the absence of glucose, undergoes an enterocyte-like differentiation after reaching confluency (Pinto *et al.*, 1982). In this case, differentiation was reported to correlate with an ability to maintain low intracellular pools of UDP-*N*-acetylhexosamines, important intermediates in protein glycosylation, during growth (Wice *et al.*, 1985). Furthermore, when HT-29 cells are maintained in the undifferentiated state (characterized by absence of a microvillar membrane with glycosidases and peptidases), sucrase-isomaltase is synthesized in large amounts, but fails to undergo the normal Golgi-associated carbohydrate processing and instead becomes degraded intracellularly with a half-time of 6 h (Trugnan *et al.*, 1987). Thus HT-29 cells and enterocytes obviously share some properties, linking the stable expression of plasma-membrane glycoproteins with the carbohydrate source of energy. This relationship may reflect a basic mechanism underlying the differentiation of polarized epithelial cells, and, if so, it highlights

the importance of the co-translational glycosylation of membrane proteins as a regulator of this process.

Professor Hans Sjöström, Professor Ove Norén and Dr. Gillian M. Danielsen are thanked for valuable discussions of the manuscript, and Ms. Lis Frandsen is thanked for expert technical assistance with part of the electron-microscopy work. E.M.D. is the recipient of a Hallas-Møller stipend from the Novo Foundation. The project was part of a program under the Biomembrane Research Center, Aarhus University.

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