THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 29, Issue of July 18, pp. 18020–18025, 1997

Printed in U.S.A.

Fumonisin B_1-induced Sphingolipid Depletion Inhibits Vitamin Uptake via the Glycosylphosphatidylinositol-anchored Folate Receptor*

(Received for publication, October 31, 1996, and in revised form, May 13, 1997)

Victoria L. Stevens‡§¶ and Jianhua Tang‡

From the ‡Department of Radiation Oncology, Division of Cancer Biology and the §Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30335

The folate receptor, like many glycosylphosphatidylinositol-anchored proteins, is found associated with membrane domains that are insoluble in Triton X-100 at low temperature and that are enriched in cholesterol and sphingolipids. Depletion of cellular cholesterol has been shown to inhibit vitamin uptake by this receptor (Chang, W.-J., Rothberg, K. G., Kamen, B. A., and Anderson, R. G. W. (1993) *J. Cell Biol.* 118, 63–69), suggesting that these domains regulate this process. In this study, the importance of sphingolipids for folate receptor function was investigated in Caco-2 cells using fumonisin B_1, a mycotoxin that inhibits the biosynthesis of these lipids. The folate receptor-mediated transport of 5-methyltetrahydrofolate was almost completely blocked in cells in which sphingolipids had been reduced by ~40%. This inhibition was dependent on the concentration and duration of the treatment with the mycotoxin and was mediated by the sphingolipid decrease. Neither receptor-mediated nor facilitative transport was inhibited by fumonisin B_1 treatment, indicating that the effect of sphingolipid depletion was specific for folate receptor-mediated vitamin uptake. A concurrent loss in the total amount of folate binding capacity in the cells was seen as sphingolipids were depleted, suggesting a causal relationship between folate receptor number and vitamin uptake. These findings suggest that dietary exposure to fumonisin B_1 could adversely affect folate uptake and potentially compromise cellular processes dependent on this vitamin. Furthermore, because folate deficiency causes neural tube defects, some birth defects unexplained by other known risk factors may be caused by exposure to fumonisin B_1.

The folate vitamins play an essential role as cofactors in many biochemical reactions involving one-carbon metabolism. These include the biosynthesis of purines and thymidine, the regeneration of methionine from homocysteine, and histidine metabolism. Cellular processes dependent upon folate can be compromised if dietary levels of this vitamin are insufficient or if its transport into cells is affected. Two different systems are used for folate uptake into cells. The first uses a high capacity, low affinity transmembrane transporter known as the reduced folate carrier. The second involves a glycosylphosphatidylinositol (GPI)[1]-anchored protein referred to as the folate receptor (1, 2). This high affinity receptor is responsible for the transport of folate into cells of the placenta, kidney, breast, and other tissues with elevated requirements for this vitamin.

The mechanism by which the GPI-anchored folate receptor transports vitamin into the cytosol has received considerable attention in recent years. The immunochemical localization of several GPI-anchored proteins, including the folate receptor, to uncoated membrane invaginations called caveolae (3) led to the suggestion that the uptake of folate is mediated by these structures by a process termed potocytosis (4). In this and other studies, caveolae were equated with membrane domains that could be isolated based on their insolubility in Triton X-100 at 4 °C (5, 6) and that are enriched in cholesterol and sphingolipids (7). More recent evidence has suggested that the Triton X-100-insoluble domains may include caveolae, but are primarily other membrane regions in which the GPI-anchored proteins (including the folate receptor) reside (8). Characterization of the protein components of caveolae isolated using new, detergent-free purification schemes has supported the conclusion that GPI-anchored proteins are not enriched in these structures (9, 10). Collectively, this evidence suggests that the folate receptor is not in caveolae, and therefore, potocytosis may not be the mechanism by which vitamin transport occurs.

Recent evidence suggests that uptake mediated by the folate receptor involves endocytosis (11, 12). However, the association of the folate receptor with Triton X-100-insoluble domains does appear to be important to its function. Depletion of cellular cholesterol through inhibition of its biosynthesis inhibited receptor-mediated folate uptake (13). Interpreting these results in the context of potocytosis, Rothberg *et al.* (14) suggested that this occurred because the clustering of the folate receptor in caveolae was disrupted. In terms of the effect on the endocytosis of the folate receptor, cholesterol depletion has been found to accelerate the rate at which this protein was recycled to the cell surface.[2] How this results in an inhibition of folate uptake is unclear.

The importance of sphingolipids, the other lipids enriched in Triton X-100-insoluble domains, for folate receptor function has not yet been investigated, although several studies have probed the importance of these lipids for other GPI-anchored proteins. Inhibition of sphingolipid biosynthesis influenced both the localization of GPI-anchored proteins to these Triton X-100-insoluble domains (15) and the transport of newly synthesized GPI-anchored proteins to the Golgi in yeast (16) and to the appropriate membrane surface in polarized epithelial cells (17). Therefore, the localization, transport, and targeting of these lipids and GPI-anchored proteins appear to be linked (18).

* This work was supported by Grant 10195 from the March of Dimes Birth Defects Foundation (to V. L. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Radiation Oncology, Loughlin Radiation Oncology Center, 145 Edgewood Ave., S. E., Atlanta, GA 30335. Tel.: 404-616-6945; Fax: 404-616-5689.

[1] The abbreviation used is: GPI, glycosylphosphatidylinositol.

[2] S. Mayor and F. R. Maxfield, submitted for publication.

In this study, the effects of changes in the cellular levels of sphingolipids on folate receptor-mediated vitamin uptake were investigated in Caco-2 cells. Originally isolated from a human colon adenocarcinoma, Caco-2 cells were chosen for this study because their folate receptor (2) and the association of GPI-anchored proteins with Triton X-100-insoluble domains in these cells (19) have been characterized. Cellular sphingolipids were depleted using fumonisin B_1. A mycotoxin produced by the fungus *Fusarium moniliforme* (20), fumonisin B_1 blocks sphingolipid biosynthesis by inhibiting the reaction catalyzed by sphingosine *N*-acyltransferase (ceramide synthase) (21). The results presented here indicate that sphingolipids play an important role in folate receptor function and that fumonisin B_1 could influence cellular folate status through its effects on these membrane lipids.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum was purchased from Atlanta Biologicals, Inc. RPMI 1640 medium and folate-free RPMI 1640 medium were from Life Technologies, Inc. [3,5,7,9-^{3}H]Folic acid (25–30 Ci/mmol, 99% pure) was obtained from American Radiolabeled Chemicals. 5-[3′,5′,7,9-^{3}H]Methyltetrahydrofolate (30 Ci/mmol, 97.8% pure) was purchased from Moravek Biochemicals, Inc. ^{125}I-Labeled diferric transferrin was from NEN Life Science Products. Lovastatin was a generous gift from Merck. The high performance Silica Gel 60 TLC plates were obtained from Whatman. DEAE-cellulose, charcoal, all lipid standards, and other chemicals were from Sigma. The reagents for the bicinchoninic acid protein assay were purchased from Pierce.

Cell Culture—Caco-2 cells were purchased from the American Type Culture Collection and were routinely grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were plated at 2.5×10^5 cells/25-cm flask or at 8×10^5 cells/125-mm flask for quantitation of folate binding and uptake or for analysis of lipids, respectively. After 2 days of growth, the medium was changed to folate-free RPMI 1640 medium and 10% fetal bovine serum such that the cells were maintained under these conditions for a total of 5 days. Treatments were done for the indicated times during this 5-day period such that they ended at the time of analysis. Lovastatin-treated cells were grown in folate-free RPMI 1640 medium supplemented with lipoprotein-depleted fetal bovine serum prepared as described by Goldstein *et al.* (22). In all cases, the cells were confluent by the end of the experiment, but continued to divide at nearly the same rate as subconfluent cells. Neither the growth rate (as measured by cell counting and [^{3}H]thymidine uptake) nor the viability of the cells (as assessed by trypan blue exclusion) was affected by the treatments (data not shown).

Isolation of Triton X-100-insoluble Domains—Triton X-100-insoluble domains were isolated using the two-step sucrose gradient method described by Arreaza *et al.* (23). Cells were washed twice with phosphate-buffered saline (8 g/liter NaCl, 0.2 g/liter KCl, 1.15 g/liter Na_2HPO_4, and 0.2 g/liter KH_2PO_4) and lysed by the addition of cold TNE buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM EDTA) containing 1% Triton X-100 to the dish. After 20 min of incubation on ice, the cells were scraped from the dish with a rubber policeman and homogenized. The lysate was then adjusted to 40% sucrose with TNE buffer containing 80% sucrose, placed in an ultracentrifuge tube, and overlaid with 5.5 ml of TNE buffer containing 38% sucrose followed by 2 ml of TNE buffer containing 5% sucrose. The samples were ultracentrifuged at 120,000 × *g* at 37 °C for 15–20 h. The Triton X-100-insoluble membranes visible at the interface between the 5 and 38% sucrose layers were harvested with a syringe, diluted ~5-fold with TNE buffer, and pelleted by ultracentrifugation (1 h at 120,000 × *g* at 4 °C).

Quantitation of 5-Methyltetrahydrofolate Uptake—The internalization of the reduced folate derivative into the cytosol was quantitated as described by Smart *et al.* (24). Briefly, cells were incubated with 5 nM 5-[^{3}H]methyltetrahydrofolate (0.5 μCi) in folate-free medium for various amounts of time at 37 °C. The medium was then removed, and the cells were washed four times with cold phosphate-buffered saline before the addition of 1.5 ml of lysis buffer (10 mM Tris-HCl (pH 8.0), 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 μM 5-methyltetrahydrofolate) to each flask. The cells were lysed by placing the flasks at −80 °C for 15 min and thawed on ice. The cells were then collected and centrifuged for 20 min at 100,00 × *g* in an Optima TL ultracentrifuge to separate the membrane (pellet) and cytosolic (supernatant) fractions. The radioactivity in each fraction was quantitated by scintillation counting. Non-specific uptake was measured using 2.5 μM 5-[^{3}H]methyltetrahydrofolate and subtracted from the total radioactivity to give the specific uptake. The results were normalized to protein determined using the bicinchoninic acid assay of Smith *et al.* (25).

Transferrin Uptake Measurements—Internalization of ^{125}I-labeled diferric transferrin was measured on cells plated in 6-well dishes as described (26) with the following minor modifications. Cells were incubated with medium A (RPMI 1640 medium containing 0.2% bovine serum albumin) for 15 min at 37 °C to deplete endogenous transferrin. The cells were then washed once with this medium and incubated in medium A containing 3 μg/ml ^{125}I-transferrin at 37 °C in 5% CO_2 for 2, 4, 6, or 8 min. At the end of the incubation, the cells were placed on ice; the ^{125}I-transferrin-containing medium was removed; and prechilled 0.2 N acetic acid in 0.2 M NaCl was added to each well. After 2 min on ice, this solution was removed, and the cells were washed three times with 150 mM NaCl, 20 mM Hepes (pH 7.4), 1 mM $CaCl_2$, 5 mM KCl, and 1 mM $MgCl_2$. The cells were then solubilized with 0.1 N NaOH in phosphate-buffered saline, and an aliquot was counted in a γ-counter to quantitate the internalized transferrin. Surface transferrin was determined by incubating cells with prechilled medium A containing 3 μg/ml ^{125}I-transferrin on ice for 30 min, followed by four washes with 150 mM NaCl, 20 mM Hepes (pH 7.4), 1 mM $CaCl_2$, 5 mM KCl, and 1 mM $MgCl_2$.

Quantitation of Folic Acid Binding Capacity—The binding of folic acid in solubilized cells and Triton X-100-insoluble fractions was quantitated as described by Antony *et al.* (27). For the total folate binding capacity, cells were solubilized 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 1% octyl glucoside. The Triton X-100-insoluble pellet obtained as described above was solubilized in this octyl glucoside-containing buffer for quantitation of folate receptors in these domains. The different samples were incubated with 5 nM [^{3}H]folate (0.5 μCi) for 20 min at 37 °C to allow ligand binding to the receptor (total volume of 1 ml/tube). The samples were then cooled on ice for 5 min, after which 40 mg of dextran-coated charcoal was added to each tube to absorb unbound radiolabel. After mixing and the addition of 1 ml of 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 1% octyl glucoside, the samples were incubated on ice for 10 min, followed by centrifugation for 30 min at 30,000 × *g* at 4 °C. Aliquots of the supernatant were then counted by scintillation counting. Nonspecific binding was determined in each experiment by measuring binding in the presence of 2.5 μM [^{3}H]folate (500-fold excess).

Lipid Analyses—Lipids were extracted and purified from either whole cells or Triton X-100-insoluble domains using the method of Ariga *et al.* (28). Cells (2.5×10^8) or the appropriate fraction (isolated from 2.5×10^8 cells) was sequentially extracted with chloroform/methanol (2:1, v/v), chloroform/methanol (1:1, v/v), and chloroform/methanol/water (30:60:8, v/v). The extracts were then pooled and applied to a DEAE-cellulose column. The neutral lipids were eluted in chloroform/methanol/water (30:60:8, v/v) and passed through a second DEAE-cellulose column to remove contaminants. The acidic lipids were eluted with chloroform, methanol, and 0.8 M sodium acetate (30:60:8, v/v). The neutral lipids were further fractionated on a silica column from which fatty acids and cholesterol were eluted with chloroform and neutral glycosphingolipids and phospholipids were eluted with chloroform/methanol (80:20, v/v). Glycolipids and gangliosides were purified of any contaminating lipids by base hydrolysis followed by re-chromatography on DEAE-cellulose.

The lipids were identified and quantitated as described by Brown and Rose (7). Identifications were made by comigration on high performance TLC plates with standards; stability (sphingolipids) or lability (glycerolipids) in base; and reactivity with molybdate reagent (phospholipids), orcinol (glycolipids), or resorcinol (gangliosides). Phospholipids, cholesterol, sulfatides, and cerebrosides were visualized by charring with cupric acetate and quantitated by densitometric scanning and comparison to standards. Gangliosides were detected using resorcinol and quantitated by measuring sialic acid. Neutral glycolipids were visualized with orcinol and quantitated by densitometric scanning and comparison to standards. The thin layer chromatography systems used to resolve the various lipids were as follows: chloroform/methanol/ammonium hydroxide (60:35:8, v/v) for phospholipids; chloroform/methanol/acetic acid/formic acid/water (42:18:7.2:2.4:1.2, v/v) for neutral glycolipids; and chloroform, methanol, and 0.25% KCl (50:45:10, v/v) for gangliosides.

RESULTS

Fumonisin B_1-induced Depletion of Cellular Sphingolipids—Cells were treated with either fumonisin B_1 (20 μg/ml (27.7 μM)

TABLE I
Lipid composition of control and fumonisin B_1- and lovastatin-treated cells

Caco-2 cells were grown in folate-free RPMI 1640 medium containing 10% fetal bovine serum alone (control), 20 μg/ml fumonisin B_1 for 2 days, or 25 μM lovastatin for 3 days prior to quantitation of the major lipid species in whole cells and Triton X-100-insoluble domains as described under "Experimental Procedures." These results represent the means ± S.E. obtained from three separate experiments. The numbers shown in parentheses are the percentage decrease in the treated groups relative to the control group.

Lipid	Whole cells			Triton X-100-insoluble fraction		
	Control	Fumonisin B_1	Lovastatin	Control	Fumonisin B_1	Lovastatin
	nmol / 10^8 cells			*nmol / 10^8 cells*		
Cholesterol	1727 ± 88	1829 ± 84 (0)	1365 ± 60 (21)	1388 ± 112	1536 ± 86 (0)	921 ± 76 (34)
TG[a]	475 ± 41	388 ± 15 (18)	508 ± 16 (0)	240 ± 29	243 ± 23 (0)	204 ± 56 (15)
FA	1119 ± 101	1136 ± 23 (0)	1022 ± 172 (9)	806 ± 89	857 ± 163 (0)	777 ± 143 (4)
PC	2762 ± 142	3129 ± 258 (0)	2537 ± 110 (8)	1234 ± 129	1242 ± 213 (0)	1015 ± 86 (18)
PE	1814 ± 50	1943 ± 49 (0)	1777 ± 26 (2)	884 ± 95	1058 ± 97 (0)	664 ± 31 (25)
PI	896 ± 53	934 ± 89 (0)	945 ± 25 (0)	234 ± 50	243 ± 99 (0)	176 ± 24 (25)
PS	898 ± 59	850 ± 82 (5)	1043 ± 25 (0)	266 ± 27	212 ± 36 (20)	280 ± 42 (0)
CL	429 ± 25	424 ± 45 (1)	477 ± 25 (0)	40 ± 13	40 ± 18 (0)	34 ± 5 (15)
SM	1230 ± 38	809 ± 30 (34)	1167 ± 26 (5)	1210 ± 55	762 ± 18 (37)	1081 ± 80 (11)
Ceramides	271 ± 19	117 ± 17 (57)	254 ± 37 (6)	239 ± 26	110 ± 28 (54)	208 ± 35 (13)
Sulfatides	226 ± 28	91 ± 16 (60)	284 ± 31 (0)	172 ± 19	66 ± 9 (62)	199 ± 27 (0)
Glc-Cer	100 ± 10	36 ± 10 (64)	93 ± 8 (7)	87 ± 5	46 ± 23 (47)	74 ± 5 (15)
Gal-Cer	81 ± 8	40 ± 13 (51)	84 ± 11 (0)	71 ± 9	42 ± 14 (41)	61 ± 12 (14)
Lac-Cer	157 ± 25	119 ± 31 (24)	144 ± 22 (8)	147 ± 28	111 ± 36 (24)	119 ± 31 (19)
Gangliosides	45 ± 4	23 ± 7 (49)	52 ± 3 (0)	37 ± 4	21 ± 4 (43)	40 ± 8 (0)

[a] TG, triglyceride; FA, fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; SM, sphingomyelin; Cer, ceramide.

for 2 days) or lovastatin (25 μM for 3 days) to decrease the cellular sphingolipids or cholesterol, respectively. The specificity and effectiveness of these treatments were assessed by quantitating the major lipids both from whole Caco-2 cells and from the Triton X-100-insoluble domains. These results are shown in Table I. Consistent with the previous results of Brown and Rose (7), the Triton X-100-insoluble domains were found to be enriched in sphingolipids and cholesterol, containing ~93% of the former and 80% of the latter. Fumonisin B_1 treatment significantly reduced the levels of all the measured sphingolipids in both whole cells and the Triton X-100-insoluble domains. Lovastatin treatment specifically decreased cholesterol among the total lipids, but was found to affect the levels of several other lipids (both glycerolipids and sphingolipids) in the Triton X-100-insoluble domains. Overall, both treatments resulted in an ~20% decrease in the cholesterol/sphingolipid content of the Triton X-100-insoluble domains.

Fumonisin B_1 Inhibition of 5-Methyltetrahydrofolate Uptake—The consequences of these changes in membrane lipid composition on folate receptor function were evaluated by measuring the rate of uptake of 5-methyltetrahydrofolate by fumonisin B_1- and lovastatin-treated cells. As shown in Fig. 1, the rate of uptake of the vitamin was roughly linear in untreated Caco-2 cells grown in folate-free medium with either normal (Fig. 1, *A* and *B*, *closed circles*) or lipoprotein-depleted (Fig. 1*B*, *triangles*) serum. Uptake was inhibited by ~90% in the fumonisin B_1-treated cells (Fig. 1*A*). A similar level of inhibition, which has been reported previously by others (13), was observed in the lovastatin-treated cells (Fig. 1*B*). Uptake of the vitamin was unaffected in cells treated with fumonisin B_1 for only 1 h, indicating that the inhibition was not mediated by the mycotoxin alone. Treatment with either various concentrations of fumonisin B_1 for 2 days (Fig. 2*A*) or 20 μg/ml for various amounts of time (Fig. 2*B*) demonstrated that the inhibition of 5-methyltetrahydrofolate uptake was both concentration- and time-dependent. The sphingolipid levels of these cells also decreased in a concentration- and time-dependent manner (data not shown), establishing that the inhibition of 5-methyltetrahydrofolate uptake in the fumonisin B_1-treated cells was mediated by the changes in the sphingolipid composition.

Coupled with the previous reports (13, 14) of the effects of lowering cellular cholesterol on folate transport, the finding that depletion of cellular sphingolipids by fumonisin B_1 inhibited this process suggests that the cholesterol/sphingolipid-enriched domains are involved in this effect. To determine if fumonisin B_1 specifically inhibits processes dependent on these domains, the effect of this mycotoxin on other types of uptake systems was assessed. Facilitative transport was measured by quantitating 2-deoxyglucose uptake. Treatment with fumonisin B_1 (20 μg/ml for 2 days) had no effect on the rate of uptake of this glucose analog by Caco-2 cells (data not shown). Receptor-mediated endocytosis was assessed by measuring transferrin uptake. Both the rates of internalization (Fig. 3) and externalization (data not shown) of ^{125}I-transferrin were found to be very similar in control and fumonisin B_1-treated cells. The only difference found was in the amount of surface transferrin binding, which was 2.7 times more in the sphingolipid-depleted groups. While the reason for this difference is unclear, it is responsible for the line representing the fumonisin B_1-treated rate of uptake being offset from that of the control cells in Fig. 3. Therefore, transport processes not thought to involve sphingolipid-enriched domains (receptor-mediated endocytosis via clathrin-coated pits and facilitative transport, respectively) were not compromised by changes in the cellular sphingolipid levels.

Lipid Depletion-induced Changes in Cellular Folic Acid Binding Capacity—Previous studies of the effect of depletion of cellular cholesterol on folate uptake suggested that this process was compromised because the clustering of the folate receptor in cholesterol/sphingolipid-rich domains in the plasma membrane was disrupted (13, 14). To determine if the decrease in cellular sphingolipids caused by fumonisin B_1 affected the folate receptor in a similar manner, the amount of this protein localized in the Triton X-100-insoluble domains was determined. This was accomplished by quantitating the high affinity binding of folic acid in either solubilized whole Caco-2 cells or Triton X-100-insoluble domains. Because folic acid is essentially bound irreversibly by the folate receptor (K_d = 0.4 nM (29)), the amount of this ligand bound is an approximate measure of the amount of this protein in the cell or fraction. As shown in Fig. 4, ~80% of the folate binding was localized to the Triton X-100-insoluble domains in untreated cells. Surprisingly, the total amount of folate receptor in the cell, but not its localization, was affected in both the fumonisin B_1- and lovastatin-treated cells. While ~50% of the total folate receptor was lost with either sphingolipid or cholesterol depletion, ~80% of

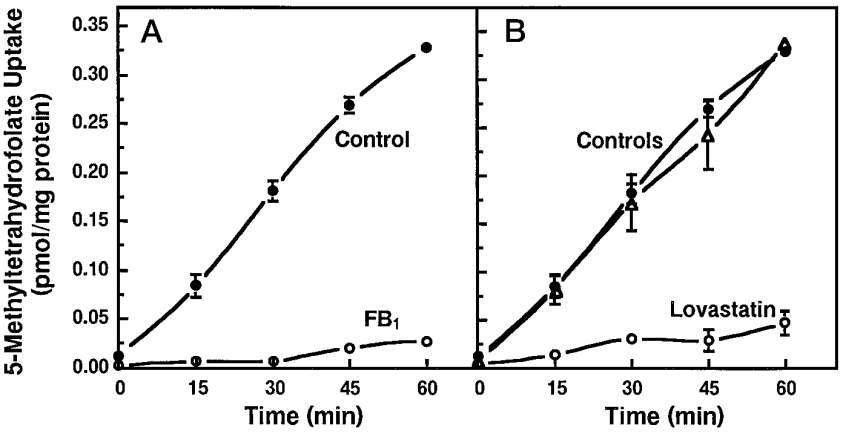

FIG. 1. **5-Methyltetrahydrofolate uptake by lipid-depleted Caco-2 cells.** The transport of 5-methyltetrahydrofolate into the cytosol was measured in control cells grown in folate-free medium supplemented with 10% fetal calf serum (*A* and *B*, *closed circles*) or 10% lipoprotein-depleted fetal calf serum (*B*, *triangles*) or treated with either 20 μg/ml fumonisin B_1 (FB_1) for 2 days (*A*, *open circles*) or 25 μM lovastatin for 3 days (*B*, *open circles*) at the indicated times. The data shown are the means ± S.E. of three experiments, in which each result was determined in triplicate.

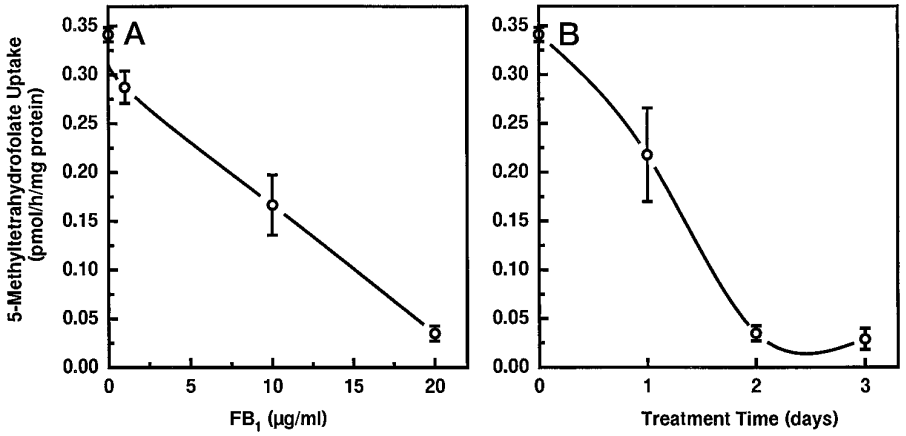

FIG. 2. **Concentration and time dependence of fumonisin B_1 inhibition of vitamin transport.** The rate of 5-methyltetrahydrofolate uptake was determined in Caco-2 cells treated with various concentrations of fumonisin B_1 (FB_1) for 2 days (*A*) or with 20 μg/ml fumonisin B_1 for 0–3 days (*B*). The data shown are the means ± S.E. of three experiments, in which each result was determined in triplicate.

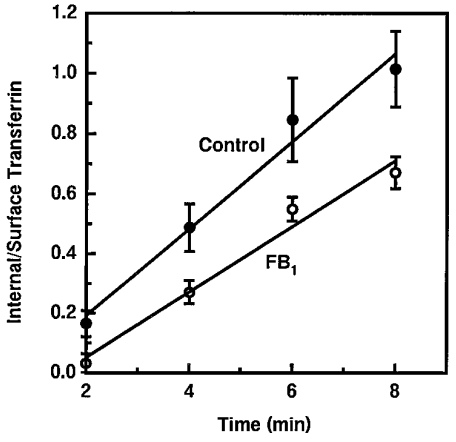

FIG. 3. **Transferrin uptake by fumonisin B_1-treated cells.** The rate of internalization of ^{125}I-transferrin was measured in untreated cells (*Control*; *closed circles*) and in cells treated with 20 μg/ml fumonisin B_1 (FB_1) for 2 days (*open circles*) as described under "Experimental Procedures." The results shown are the means ± S.E. of triplicate determinations.

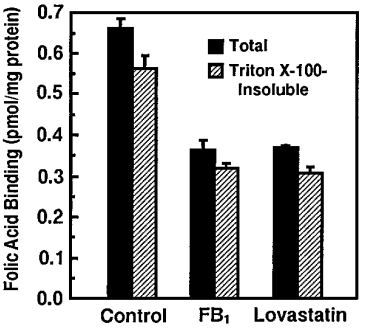

FIG. 4. **Folic acid binding by fumonisin B_1- and lovastatin-treated cells.** Specific binding of [^{3}H]folic acid was quantitated in whole cells (*solid bars*) or in Triton X-100-insoluble domains (*hatched bars*) solubilized in 1% octyl glucoside after growth in medium alone (*Control*), with 20 μg/ml fumonisin B_1 (FB_1) for 2 days, or with 25 μM lovastatin for 3 days. The data shown are the means ± S.E. of three experiments, in which each result was determined in triplicate.

the remaining folate binding was found in the Triton X-100-insoluble domain. As with the inhibition of 5-methyltetrahydrofolate uptake, the decrease in total folate binding was dependent on both the duration of the treatment (Fig. 5*A*) and the concentration of fumonisin B_1 used (Fig. 5*B*). While these results indicate that the decrease in folate binding is mediated by the changes in lipid content in the cell, the possibility that fumonisin B_1 directly affected binding of the vitamin was further ruled out by the finding that the mycotoxin was unable to alter this parameter when added directly to the binding assay. Therefore, changes in the cholesterol or sphingolipid levels appear to decrease the total amount of folate receptor in the cells, but do not change its enrichment in Triton X-100-insoluble domains.

Relationship between Folate Receptor Function and Number—These results indicate that both the fumonisin B_1 and lovastatin treatments induce a loss or down-regulation of the folate receptor. The relationship between the change in folate receptor number and the inhibition of 5-methyltetrahydrofolate uptake was investigated by comparing these two parameters (Fig. 6). A linear relationship (r = 0.967) between the amount of folate receptor in the cell and the ability to transport 5-methyltetrahydrofolate into the cytoplasm was found. Therefore, it seems more likely that the inhibition of vitamin uptake is caused by the loss of the folate receptor than by a change in the membrane localization of the protein.

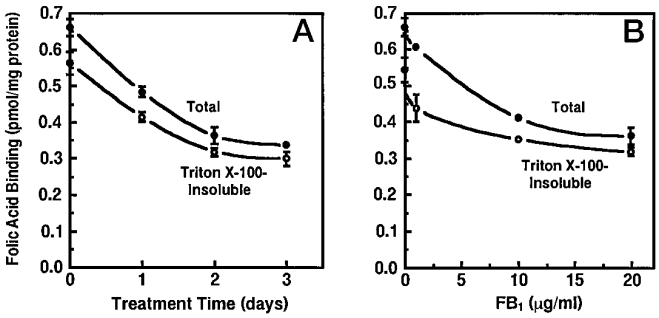

FIG. 5. **Time and concentration dependence of the fumonisin B_1-induced reduction in folic acid binding capacity.** The [^{3}H]folic acid binding activity was quantitated in octyl glucoside-solubilized cells (*Total*; *closed circles*) and in Triton X-100-insoluble domains (*open circles*) after treatment with 20 μg/ml fumonisin B_1 for 0–3 days (*A*) or the indicated concentrations of fumonisin B_1 (*FB_1*) for 2 days (*B*). The data shown are the means ± S.E. of triplicate determinations.

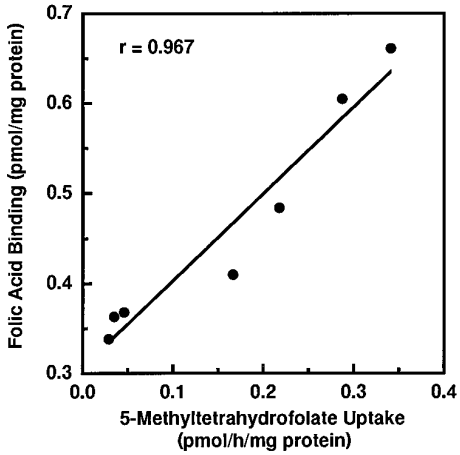

FIG. 6. **Correlation of 5-methyltetrahydrofolate uptake ability and folic acid binding capacity in Caco-2 cells.** The values of these two parameters measured in whole cells for each of the treatments shown in Figs. 1, 2, 4, and 5 (control and fumonisin B_1- and lovastatin-treated) were compared and resulted in a correlation coefficient (*r*) of 0.967.

DISCUSSION

Treatment of Caco-2 cells with fumonisin B_1 resulted in almost complete inhibition of uptake of 5-methyltetrahydrofolate by the folate receptor. Consistent with it being mediated by the depletion of cellular sphingolipids, this inhibition was dependent on the concentration of mycotoxin used and the duration of the treatment. Fumonisin B_1 did not perturb either 2-deoxyglucose or transferrin uptake, indicating that the effect of sphingolipid depletion was specific for folate receptor-mediated transport. The inhibition caused by lowering the cellular sphingolipid levels was very similar to that previously observed when cellular cholesterol was depleted by inhibition of hydroxymethylglutaryl-CoA reductase (13). Therefore, it seems likely that decreases in the cellular levels of these two lipids perturb folate receptor function by the same mechanism and involve the Triton X-100-insoluble domains.

Quantitation of the amount of folic acid binding activity in the Triton X-100-insoluble domains in fumonisin B_1- and lovastatin-treated cells revealed that the lipid depletion did not cause redistribution of the folate receptor. Approximately 80% of the cellular folic acid binding capacity was localized to these cholesterol/sphingolipid-enriched domains in control and lipid-depleted cells. While localization was unaffected, the total amount of folic acid binding capacity was decreased by ~50% in fumonisin B_1- and lovastatin-treated cells. That folate receptor function and number decrease in parallel as sphingolipids are depleted from Caco-2 cells suggests that there is a causal relationship between these two parameters. Growth of cells in medium containing low concentrations of folate, which presumably increases the need to take up this vitamin, has been found to induce folate receptor function (29). Therefore, inhibition of folate uptake, or compromised folate receptor function, could lead to a drop in the amount of this protein in the cell. Alternatively, a decrease in the level of folate receptor in the cell could result in inhibition of vitamin uptake. The data presented here do not address the question of whether the decrease in folate receptor number caused inhibition of vitamin uptake or vice versa. Both parameters appear to decrease with roughly similar rates (Figs. 2*B* and 5*A*). Curiously, conditions that resulted in nearly complete inhibition of 5-methyltetrahydrofolate uptake (20 μg/ml fumonisin B_1 for 2 or 3 days) led to the loss of only half of the folate receptors in the cell. If vitamin uptake is compromised because of the decrease in the number of receptors, then why did the remaining folate receptors not support an intermediate level of folate transport into the cytosol? Perhaps the remaining folate receptors are not functional, or the altered lipid composition has affected some other critical component of the folate uptake pathway (*e.g.* folate polyglutamation).

This study clearly demonstrates the importance of sphingolipids for folate receptor-mediated vitamin uptake. However, the mechanism by which this process is affected is not clear. The finding that depletion of cellular cholesterol also inhibits folate receptor function (13) suggests that this effect is mediated by changes in the membrane domains enriched in cholesterol and sphingolipids to which the GPI-anchored folate receptor has been localized. Alternatively, folate receptor number and function could be altered in response to changes in the level of one or more intermediates in the synthesis of these lipids that have a signaling role in the cell. Recent evidence has implicated several sphingolipids, including sphingosine, sphingosine 1-phosphate, ceramide, and sphingomyelin, in signal transduction (reviewed in Refs. 30–33). Fumonisin B_1 inhibition of sphingolipid biosynthesis should result in elevation of cellular long-chain bases and decreased levels of ceramide (34). In preliminary experiments, supplementation of fumonisin B_1-treated cells (20 μg/ml for 2 days) with 1 μM C_6-ceramide for 1 additional day (in the presence of fumonisin B_1) has been found to reverse the effects on the folate receptor.[3] Because long-chain bases should still be elevated, this result suggests that increases in the levels of sphinganine and/or sphingosine are not responsible for the effect on the folate receptor. However, whether it is the loss of mature sphingolipids or ceramide, which has been suggested to play a role in the regulation of endocytosis (35), that mediates the effects of fumonisin B_1 cannot be determined from these experiments. Unraveling which of these is the critical factor will provide important information regarding the regulation of vitamin uptake mediated by the GPI-anchored folate receptor and will be addressed in future studies.

While fumonisin B_1 has proven to be a useful reagent for probing the role of sphingolipids in various cellular processes (34), the fact that it is a naturally occurring compound raises the possibility that these same events could be adversely affected by dietary exposure to this mycotoxin. *F. moniliforme*, the fungus that produces fumonisin B_1, is a common contaminant of corn. Exposure to fumonisin B_1 causes a variety of animal diseases and has been linked to an increased incidence of esophageal cancer in humans in areas of southern Africa and China (36–39). Investigation into the consequences of fetal

[3] E. R. Smith and V. L. Stevens, unpublished results.

exposure to this mycotoxin using either mice or hamsters has shown that it causes developmental toxicity (40, 41). The mouse fetuses that survived to birth had gross skeletal and visceral abnormalities (42). Inhibition of folate uptake through fumonisin B_1-induced depletion of sphingolipids could lead to an intracellular deficiency in this vitamin. Since folate deficiency during the first trimester of pregnancy is associated with an increased risk of neural tube defects in the developing fetus (43–45), it is possible that some instances of high rates of occurrence of these birth defects unexplained by known causes might be linked to dietary exposure to fumonisin B_1. For instance, high rates of neural tube defects have been observed in Cameron County, TX from 1990 to 1991 (46, 47) and in Harris County, TX from 1989 to 1991 (48, 49). The prevalence of these birth defects was high among Hispanics (48), for whom corn and corn products are expected to represent a sizable portion of their diet. The occurrence of a high number of clusters of the fatal equine disease caused by fumonisin B_1 in Texas in 1989 established that the corn crop was contaminated with this mycotoxin during this period. Coupled with the present finding that fumonisin B_1-induced depletion of cellular sphingolipids blocked folate uptake, this evidence suggests that there should be further investigation into the possibility that this mycotoxin may contribute to some birth defects not accounted for by other known risk factors.

Acknowledgments—We thank Dr. Alfred Merrill for the gift of fumonisin B_1 and for helping to bring this problem to our attention and Drs. David Lambeth and Satyajit Mayor for helpful discussions regarding this research.

REFERENCES

1. Luhrs, C. A., and Slomiany, B. L. (1989) *J. Biol. Chem.* **264,** 21446–21449
2. Lacey, S. W., Sanders, J. M., Rothberg, K. G., Anderson, R. G. W., and Kamen, B. A. (1989) *J. Clin. Invest.* **84,** 715–720
3. Rothberg, K. G., Ying, Y.-S., Kolhouse, J. F., Kamen, B. A., and Anderson, R. G. W. (1990) *J. Cell Biol.* **110,** 637–649
4. Anderson, R. G. W., Kamen, B. A., Rothberg, K. G., and Lacey, S. W. (1992) *Science* **255,** 410–411
5. Yu, J., Fischman, D. A., and Steck, T. L. (1973) *J. Supramol. Struct.* **3,** 233–248
6. Davies, A. A., Wigglesworth, N. M., Allan, D., Owens, R. J., and Crumpton, M. J. (1984) *Biochem. J.* **219,** 301–308
7. Brown, D. A., and Rose, J. K. (1992) *Cell* **68,** 533–544
8. Mayor, S., and Maxfield, F. R. (1995) *Mol. Biol. Cell* **6,** 929–944
9. Parton, R. G., and Simons, K. (1995) *Science* **269,** 1398–1399
10. Schnitzer, J. E., McIntosh, D. P., Dvorak, A. M., Liu, J., and Oh, P. (1995) *Science* **269,** 1435–1439
11. Birn, H., Selhub, J., and Christensen, E. I. (1993) *Am. J. Physiol.* **264,** C302–C310
12. Rijnboutt, S., Jansen, G., Posthuma, G., Hynes, J. B., Schornagel, J. H., and Strous, G. J. (1996) *J. Cell Biol.* **132,** 35–47
13. Chang, W.-J., Rothberg, K. G., Kamen, B. A., and Anderson, R. G. W. (1993) *J. Cell Biol.* **118,** 63–69
14. Rothberg, K. G., Ying, Y.-S., Kamen, B. A., and Anderson, R. G. W. (1990) *J. Cell Biol.* **111,** 2931–2938
15. Hanada, K., Nishijima, M., Akamatsu, Y., and Pagano, R. E. (1995) *J. Biol. Chem.* **270,** 6254–6260
16. Horvath, A., Sutterlin, C., Manning-Krieg, U., Roa Movva, N., and Riezman, H. (1994) *EMBO J.* **13,** 3687–3695
17. Mays, R. W., Siemers, K. A., Fritz, B. A., Lowe, A. W., van Meer, G., and Nelson, W. J. (1995) *J. Cell Biol.* **130,** 1105–1115
18. Futerman, A. H. (1995) *Trends Cell Biol.* **5,** 377–380
19. Garcia, M., Mirre, C., Quaroni, A., Reggio, H., and Le Bivic, A. (1993) *J. Cell Sci.* **104,** 1281–1290
20. Bezuidenhout, C. S., Gelderblom, W. C. A., Gorstallman, C. P., Horak, R. M., Marasas, W. F. O., Spiteller, G., and Vleggaar, R. (1988) *J. Chem. Soc. Chem. Commun.* 743–745
21. Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T., and Merrill, A. H., Jr. (1991) *J. Biol. Chem.* **266,** 14486–14490
22. Goldstein, J. L., Basu, S. K., and Brown, M. S. (1983) *Methods Enzymol.* **98,** 241–260
23. Arreaza, G., Melkonian, K. A., LaFevre-Bernt, M., and Brown, D. A. (1994) *J. Biol. Chem.* **269,** 19123–19127
24. Smart, E. J., Foster, D. C., Ying, Y.-S., Kamen, B. A., and Anderson, R. G. W. (1994) *J. Cell Biol.* **124,** 307–313
25. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85
26. McGraw, T. E., and Maxfield, F. R. (1990) *Cell Regul.* **1,** 369–377
27. Antony, A. C., Utley, C., Van Horne, K. C., and Kolhouse, J. F. (1981) *J. Biol. Chem.* **256,** 9684–9692
28. Ariga, T., Macala, L. J., Saito, M., Margolis, R. K., Greene, L. A., Margolis, R. U., and Yu, R. K. (1988) *Biochemistry* **27,** 52–58
29. Kamen, B. A., and Capdevila, A. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5983–5987
30. Hannun, Y. A. (1994) *J. Biol. Chem.* **269,** 3125–3128
31. Divecha, N., and Irvine, R. F. (1995) *Cell* **80,** 269–278
32. Kolesnick, R., and Golde, D. W. (1994) *Cell* **77,** 325–328
33. Spiegel, S., and Milstien, S. (1995) *J. Membr. Biol.* **146,** 225–237
34. Merrill, A. H., Jr., Wang, E., Schroeder, J. J., Smith, E. R., Yoo, H.-S., and Riley, R. T. (1995) in *Molecular Approaches to Food Safety: Issues Involving Toxic Microorganisms* (Eklund, M., Richard, J. L., and Mise, K., eds) pp. 429–443, Alaken, Inc., Fort Collins, CO
35. Chen, C.-S., Rosenwald, A. G., and Pagano, R. E. (1995) *J. Biol. Chem.* **270,** 13291–13297
36. Marasas, W. F. O., Kellerman, T. S., Gelderblom, W. C. A., Coetzer, J. A. W., Thiel, P. G., and van der Lugt, J. J. (1988) *Onderstepoort J. Vet. Res.* **55,** 197–203
37. Sydenham, E. W., Gelderblom, W. C. A., Thiel, P. G., and Marasas, W. F. O. (1990) *J. Agric. Food Chem.* **38,** 285–290
38. Marasas, W. F. O., Kriek, N. P. J., Fincham, J. E., and van Rensburg, S. J. (1984) *Int. J. Cancer* **34,** 383–387
39. Yang, C. S. (1980) *Cancer Res.* **40,** 2633–2644
40. Floss, J. L., Casteel, S. W., Johnson, G. C., Rottinghaus, G. E., and Krause, G. F. (1994) *Mycopathologia* **128,** 33–38
41. Gross, S. M., Reddy, R. V., Rottinghaus, G. E., Johnson, G., and Reddy, C. S. (1994) *Mycopathologia* **128,** 111–118
42. Gross, S. M., Reddy, C. S., Reddy, R. V., Johnson, G., and Rottinghaus, G. E. (1994) *FASEB J.* **8,** 2357 (abstr.)
43. Hibbard, B. M. (1993) *Br. J. Obstet. Gynaecol.* **100,** 307–309
44. Butterworth, C. E., Jr. (1993) *J. Am. Coll. Nutr.* **12,** 438–441
45. Dansky, L. V., Rosenblatt, D. S., and Andermann, E. (1992) *Neurology* **42,** 32–42
46. Albrecht, L. J. (1994) *Tex. Med.* **90,** 16–18
47. Texas Department of Health (1992) *An Investigation of a Cluster of Neural Tube Defects in Cameron County, Texas*, pp. 4–35, Texas Department of Health, Austin, TX
48. Canfield, M. A., Annegers, J. F., Brender, J. D., Cooper, S. P., and Greenberg, F. (1996) *Am. J. Epidemiol.* **143,** 1–11
49. Canfield, M. A., Annegers, J. F., Brender, J. D., Cooper, S. P., and Greenberg, F. (1996) *Am. J. Epidemiol.* **143,** 12–24