Brefeldin A (BFA) Inhibits Basolateral Membrane (BLM) Delivery and Dimerization of Transcobalamin II Receptor in Human Intestinal Epithelial Caco-2 Cells

BFA EFFECTS ON BLM CHOLESTEROL CONTENT*

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Brefeldin A (BFA) treatment of Caco-2 cells (5 μg/ml for 12 h) reduced by 90% the cholesterol, but not the phospholipid (PL), levels of the basolateral membrane (BLM), thus altering its PL/cholesterol molar ratio from 2.6 to 22.0, and decreasing its steady state fluorescent anisotropy (r<sub>s</sub>) from 0.27 to 0.15. BFA treatment for 12 h also resulted in complete loss of transcobalamin II receptor (TC II-R) activity/protein levels in the BLM and the disappearance of trans-Golgi network (TGN) morphology as revealed by confocal immunofluorescence microscopy using antibody to TGN 38. However, BFA treatment had no effect on either total cellular cholesterol, TC II-R activity, or PL levels. When cells treated with BFA for 12 h were exposed to BFA-free medium for 0–24 h, all of the effects were reversed, including reappearance of normal TGN morphology. TC II-R delivered to the BLM during this period was progressively sialylated and changed its physical state from a monomer (8 h) to a dimer (12 h), coinciding with increased delivery (11–55 pmol) of cholesterol to the BLM and an increase in the BLM r<sub>s</sub> from 0.15 to 0.21. These results indicate that cholesterol, but not PL, delivery to the BLM of Caco-2 cells is BFA-sensitive, and cholesterol, by influencing the higher order of the BLM, is essential for TC II-R dimerization.

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‡‡‡ The abbreviations used are: Cbl, cobalamin (vitamin B<sub>12</sub>); DMEM, Dulbecco’s modified Eagle’s medium; TC II-R, transcobalamin II receptor; TC II, transcobalamin II; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; PM, plasma membrane; PL, phospholipid; PC, phosphatidylcholine; r<sub>s</sub>, steady state fluorescence anisotropy; TGN, trans-Golgi network; TBS, Tris-buffered saline; BLM, basolateral membrane; BFA, brefeldin A.

Circulatory cobalamin (Cbl<sup>1</sup>; vitamin B<sub>12</sub>) bound to plasma transporter, transcobalamin II (TC II), is taken up by all tissues/cells by receptor-mediated endocytosis via plasma membrane (PM) transcobalamin II receptor (TC II-R) (1). TC II/TC II-R-mediated delivery of Cbl is the only physiological uptake system that provides Cbl to all cells to be utilized as Cbl coenzyme. TC II-R, a glycoprotein with a molecular mass of 62 kDa (2) is expressed in all tissue PMs as a noncovalent functional homodimer with a molecular mass of 124 kDa (2). TC II-R homodimers are resistant to treatment with sodium dodecylsulfate (2, 3) and, thus, can be separated on SDS-PAGE and detected by immunoblotting (3). Studies using this technique (4) have revealed that at steady state, TC II-R dimer levels are 8–10-fold higher than that of the monomer in all total tissue membranes tested and that TC II-R dimers are present in the PM and in some PM-derived vesicles, while TC II-R monomers are the only species present in the microsomes (4).

Earlier in vitro studies (3) using isolated tissue PMs and microsomes have revealed that TC II-R dimerization is supported in the plasma but not in the microsomal membranes due to their higher cholesterol content. Additional studies using symmetrical phosphatidylcholine (PC) vesicles have shown that a minimum of 10 mol % of cholesterol is essential to support TC II-R dimerization above the transition temperatures of these PC vesicles (3). Although the importance of membrane cholesterol levels and cholesterol-phospholipid interactions in the dimerization of TC II-R in tissue-derived PMs and in PC vesicles is established (3), it is not known how the dimerization of TC II-R is regulated at a cellular level and whether cellular PM cholesterol levels are important for the dimerization of TC II-R.

These issues have been addressed in the current study using polarized epithelial Caco-2 cells that express TC II-R predominantly (85%) in the BLM (5, 6) and are known to be sensitive to treatment with BFA (7). BFA, a fungal metabolite, causes Golgi disruption and other morphological changes (8–13) in cells and has been used to study the effect of these changes on the sorting of proteins (14, 15) and lipids (16, 17) in a variety of cells. Our aim in the current study was to test whether BFA affected BLM delivery of major lipids such as cholesterol and PL in Caco-2 cells and, if so, whether it also affected the BLM delivery and dimerization of TC II-R.

The results of the current study show that in Caco-2 cells, delivery of cholesterol, but not PL, to the BLM is BFA-sensitive. As a consequence of decreased cholesterol levels, TC II-R monomers delivered to the BLM during the incubation of BFA-treated cells with BFA-free medium were able to dimerize only after 12 h of incubation, when cholesterol levels of the BLM and its order were restored to nearly normal values.

EXPERIMENTAL PROCEDURES

Materials—[<sup>57</sup>C]Cobalamin (specific activity 15 μCi/μg) was from Johnson and Johnson Clinical Diagnostics (Ontario, Canada),

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carrier-free Na$^{221}$ was from Amersham Pharmacia Biotech, and human serum was obtained from Southeastern Wisconsin Blood Center (Milwaukee, WI). Dulbecco's modified Eagle's medium (DMEM) and trypsin-EDTA were from Life Technologies, Inc.; $^{35}$S(methionine (>1000 Ci/mmol) was from NEW Life Science Products; protein A, BFA, lactoperoxidase, and neuraminidase from Clostridium perfringens were from Sigma; and endo-$\beta$-N-acetylglucosaminidase I from Streptomyces plicatus, peptide-$N$-glycosidase F from Flavobacterium meningosepticum, and O-glycosidase from Diplococcus pneumoniae were from Boehringer Mannheim. Disuccinimidyl suberate sulfovanadocimino biotin and IODO-GEN were from Pierce. 1-(4-Trimethylamino)-phenoxy-6-1,3,5-triene was from Molecular Probes, Inc. (Eugene, OR), and purified $^{35}$Smethionine (10 pmol) on the basolateral side with DMEM containing 125I-TC II-Cbl and the filters were incubated in the presence and absence of TC II-R monomer and dimer forms, respectively, onto nitrocellulose membranes and probed with TC II-R antiserum and $^{125}$I-protein A as described previously (3). Immunoblotting was performed a total of three times for 30 min each as described recently (6, 18).

**Cell Surface Biotinylation—**Biotinylation of BLM of Caco-2 cells recovering from the effects of BFA was carried out by adding disuccinimidyl suberate sulfovanadocimino biotin (0.5 mg/ml) to the basolateral compartments of filter-grown monolayers (12-day growth) and was performed a total of three times for 30 min each as described earlier (6). Anisotropy Measurements—Postconfluent Caco-2 cells grown on culture inserts were incubated with BFA (5 mg/ml) for 12 h. The medium was replaced with fresh medium without BFA, and the cells were transfected into growth medium for 6 h. For BFA effects, the confluence was 1.5 x 10**6** cells/ml. Steady state $r_s$ was determined at 10 times for each sample at each dilution from three sets of cells treated with trimethylammoniumdiphenylhexatriene dissolved in dimethyl sulfoxide (0.1 pmol/filter) was added to the basolateral medium 30 min before the allocated time of 2–24 h. The cells were then harvested at each time interval, washed in TBS, and finally suspended in 3 ml of TBS and used for fluorescent polarization studies in a 3-ml quartz cuvette with constant stirring (250 rpm). Fluorescence anisotropy ($r*$) was determined at room temperature using a model 4800 C spectra fluorometer (SLM-Aminco Inc., Rochester, NY). The excitation and emission wavelengths were 360 and 430 nm, respectively. Correction for light scattering was carried out by successive dilutions of the cell suspension until a plateau value of polarization was obtained. Steady state $r_s$ was determined at least 10 times for each sample at each dilution from three sets of cells treated with trimethylammoniumdiphenylhexatriene from three separate experiments, and the $r_s$ values were calculated according to Van Blitterswijk et al. (20).

**Pulse-Chase Labeling of Caco-2 Cells—**Postconfluent cells untreated and treated with BFA (5 mg/ml) for 12 h were first incubated with methionine-free DMEM for 30 min and then pulsed for 1 h with $^{35}$Smethionine (200 Ci/ml) in the presence of BFA. The medium was removed, and the cells were washed with DMEM and then chased for 3 h with methionine (20 mg/ml and BFA). The $^{35}$S-labeled TC II-R isolated at each time interval by immunoprecipitation was further processed for nonreducing SDS-PAGE as described before (18).

**Confocal Immunofluorescence Microscopy—**Postconfluent Caco-2 cells grown on filters were incubated in the presence and absence of BFA (5 mg/ml) for 12 h. In some experiments, the cells were allowed to recover from the effects of BFA by incubation in BFA-free medium for 24 h. Following incubations, the filters were washed with phosphate-buffered saline and fixed in 4% paraformaldehyde followed by sequential incubations with the primary antibody (rabbit polyclonal TGN 38 antibody) for 1 h at 37 °C and Texas red-conjugated secondary antibodies (donkey anti-rabbit) for 30 min at 37 °C in the dark. The samples were then analyzed using a krypton-argon laser coupled with a Bio-Rad MRC 600 confocal head attached to an Optiphot II Nikon microscope with a plan Apo 60 × 1.4 NA objective lens. Other Methods—Reconstitution of pure sialylated and asialo-TG II-R in total BBM lipid extracts was carried out using 2 pmol of total PL and either sialylated or asialo-TG II-R (0.25 mg) as described previously (3). Protein concentration was determined using the Bradford assay with bovine serum albumin as the standard (21). TC II-R assays were performed using partially purified TC II from human serum (22). TC II-[57Co]Cbl complex was prepared for receptor assays by the charcoal adsorption method (23). Basolateral cell surface binding of human TC II-[57Co]Cbl (500 fmol) using filter-grown Caco-2 cells was determined by incubating the ligand at 4 °C for 30 min. After 30 min, the medium was removed, the cells were washed in cold medium, and the amount of TC II-[57Co]Cbl bound to the cell surface was determined by subtracting the amount of ligand from the cell surface in the presence of TC II-R antiserum (5–20 μl) or that bound when the ligand was incubated at 5 °C in the presence of PH5/EDTA buffer. In general, the nonspecific binding was less than 5% of the total ligand bound. Total membranes from scraped Caco-2 cells were subjected to acetone precipitation to remove SDS. The precipitated radioactivity was resolubilized with TBS containing Triton X-100 (1%), and the solubilized radioactivity (20,000 cpm) was incubated with $S_n$ agglutinin-agarose beads. The beads were washed with TBS, and the beads were pelleted and counted. As a control, $^{35}$S-TG II-R immobilized (20,000 cpm) from cells not treated with BFA was digested with sialidase and then allowed to bind to $S_n$ agglutinin-agarose beads, and the radioactivity bound was washed and counted as before. The radioactivity eluted from $S_n$ agglutinin-agarose beads was subjected to nonreducing SDS-PAGE.
BFA Effects on Transcobalamin II Receptor Dimerization

**RESULTS**

**BFA Inhibits BLM but Not the Total Cellular TC II-R Activity or Protein Levels in Filter-grown Caco-2 Cells**—Incubation of Caco-2 cells for 12 h with BFA (0.5–10 μg/ml) revealed inhibition of BLM but not total cellular ligand binding by 50 and 100% at BFA concentrations of 0.5 and 5 μg/ml, respectively (Fig. 1). When incubated with 5 μg/ml BFA for less than 12 h, ligand binding to BLM was inhibited by 50, 75, and 90% at BFA concentrations of 0.5 and 5 μg/ml, respectively. Total lipid extract was prepared from total cellular homogenate (2 mg of protein) isolated from Caco-2 cells that were incubated for 12 h at 37 °C. In some filters, ligand binding to the BLM (●) was determined by the addition of TC II-[¹³⁵I]Cbl (500 fmol). The filters were incubated for 1 h at 4 °C. The cells were then scraped, and the radioactivity was counted. Specific binding was determined by subtracting total radioactivity bound from the radioactivity bound to BLM in the presence of TC II-R antiserum (25 μl). Total cellular TC II-R activity (□) was determined as described earlier (2) using Triton X-100 extracts of the cell homogenates. Other details are provided under "Experimental Procedures." Each data point represents mean ± S.D. of triplicate assays from three separate filter experiments.

**Immunoblotting of total cell membranes** (Fig. 2) from untreated cells revealed that TC II-R was a dimer with a molecular mass of 124 kDa (Fig. 2, *left panel*), and TC II-R monomer with a molecular mass of 62 kDa could not be detected (Fig. 2, *right panel*). When cells were incubated with 0.5 μg/ml BFA for 12 h, ligand binding to BLM was inhibited by 50, 75, and 90% at BFA concentrations of 0.5 and 5 μg/ml, respectively. Thus, in all subsequent experiments cells were incubated with 5 μg/ml BFA for 12 h.

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probing with 125I-streptavidin as described before (6). The bands shown divided membranes from three separate filters.munoprecipitated with antiserum to TC II-R, and subjected to nonreducing SDS-PAGE (7.5%) and immunoblotting as before.

membranes (25 μg of ligand (86 kDa/dimer) to a receptor of molecular mass of 124 kDa. Similar sized cross-linked product was also obtained by cross-linking the BLM from untreated cells (lane 5), and the ligand cross-linked to BLMs of untreated cells are shown in lanes 1 and 5, respectively.


to nonreducing SDS-PAGE (7.5%) and immunoblotting as before. The bands were detected by autoradiography. The labeled ligand alone in the left panel was incubated with BFA for 12 h and then in BFA-free medium for the indicated time intervals. At each indicated time, the BLMs of some filters were biotinylated with disuccinimidyl suberate sulfo-NHS-biotin as described under “Experimental Procedures,” while others were used for binding to the ligand. Ligand binding to BLM (left panel) was carried out using TC II-[57Co]Cbl (500 fmol) as described before (6). The biotinylated cells were scraped, extracted with Triton X-100(1%), immunoprecipitated with antiserum to TC II-R, and subjected to nonreducing SDS-PAGE (7.5%), and the bands (right panel) were detected by probing with 125I-streptavidin as described before (6). The bands shown in the right panel were quantified from gels (left panel) using biotinylated membranes from three separate filters.

II-R delivered to BLM as a monomer is unable to dimerize for up to 8 h of reversal time.

The size of monomeric form of TC II-R retained in the cells following treatment with BFA was 56 kDa, while the size of BLM TC II-R from cells recovering from BFA (Fig. 4, right panel) increased from 56 to 62 kDa. These observations suggested that BFA treatment may inhibit terminal sialylation of the TC II-R oligosaccharides and that progressive recovery of sialylation may occur during the recovery period. In order to directly test these possibilities, the following experiments were carried out.

BFA Inhibits Sialylation of Oligosaccharides of TC II-R—The molecular mass of [35S]methionine-labeled immunoprecipitated TC II-R monomer was 62 kDa in BFA-untreated cells (Fig. 6, lanes 1 and 7) and was 56 kDa in treated cells (lanes 4 and 10). Cells following treatment with sialidase, the 62-kDa TC II-R from untreated cells was converted to 56 kDa (lane 8), while similar treatment of the 56-kDa form of TC II-R obtained from BFA-treated cells resulted in the shift equivalent to 55 kDa (lane 11). When the labeled receptor from both untreated (lane 9) and BFA-treated (lane 12) cells were further digested with O-glycanase, a shift equivalent to 9–10 kDa was noted. This observation clearly indicated that BFA treatment did not inhibit core O-glycosylation of TC II-R but only the terminal sialylation of these sugars. In addition, BFA treatment also had no effect on the maturation of a single N-linked oligosaccharide of TC II-R, since the labeled receptors from both the untreated (lane 1) and treated (lane 4) cells were resistant to endo-β-N-acetylgalactosaminidase (lanes 2 and 5) but not to peptide-N-glycosidase F (lanes 3 and 6) treatments.

Further confirmation that BFA treatment had indeed inhibited terminal sialylation of TC II-R was obtained by testing the ability of [35S]-TC II-R obtained from Caco-2 cells labeled in the
BFA-treated and -untreated cells were pulsed for 1 h with $^{35}$S]-methionine. Labeled TC II-R immunoprecipitate was allowed to bind to \textit{S. nigra} agglutinin-agarose. The values reported are an average of binding using two separate labeling experiments and expressed as percentage of the radioactivity bound. The radioactivity used for binding to \textit{S. nigra} agglutinin-agarose varied (5,000–20,000 cpm) from sample to sample, particularly when the cells were incubated in BFA-free medium. Other details are provided under “Experimental Procedures.”

### Table I

| Treatments | Incubation time | $^{35}$S-TC II-R bound % |
|------------|-----------------|--------------------------|
| Without BFA | 12 h            | 100                      |
| Without BFA, with sialidase | 12 h            | 5                       |
| With BFA   | 12 h            | 6                       |
| With BFA-free medium | 0 h             | 5                       |
|            | 4 h             | 7                       |
|            | 8 h             | 12                      |
|            | 12 h            | 90                      |
|            | 24 h            | 100                     |

Lipid Bilayer Prepared Using BLM Total Lipids from BFA-treated Cells Does Not Support in Vitro the Dimerization of both Native and Asialo-TC II-R—

Postconfluent Caco-2 cells grown on tissue culture flasks were incubated with BFA (5 \(\mu\)g/ml) for 12 h at 37°C. The cells were then harvested for either lipid extraction to measure total PL and cholesterol or for isolating BLM. The values reported represent mean ± S.D. of triplicate assays for each lipid measurement and for lipid extracts from either cells or isolated BLM from three separate flasks. The values reported are expressed as pmol of each lipid in one flask of cells (30 \(\times\) 106 cells) and in the BLM as pmol/total BLM protein/flask of cells. The values in the parenthesis represent the percentage of total cellular lipid present in the BLM. Other details are provided under “Experimental Procedures.”

### Table II

**Effect of BFA treatment on the total cellular and BLM cholesterol and phospholipid levels**

| Treatment | Cholesterol (pmol) | Phospholipid (pmol) |
|-----------|--------------------|---------------------|
|           | Cellular BLM       | BLM                 |
| Without BFA | 526 ± 25          | 95 ± 5 (18)         |
|           | 3300 ± 110         | 260 ± 15 (8)        |
| With BFA  | 495 ± 15           | 11 ± 1 (2)          |
|           | 3270 ± 95          | 245 ± 10 (7.4)      |

### Table III

**Cholesterol and phospholipid levels in the BLM of BFA-treated Caco-2 cells incubated with BFA-free medium**

Caco-2 cells treated with BFA (5 \(\mu\)g/ml) for 12 h were washed in BFA-free medium and incubated further for the indicated times in BFA-free medium. The harvested cells were used for BLM isolation, total lipid extraction, and measuring cholesterol and phospholipid levels. The values represent mean ± S.D. of triplicate assays from BLM isolated from three separate flasks of cells and are expressed as pmol of lipid present in the BLM protein isolated from each flask. The BLM protein/flask was 208 ± 15 \(\mu\)g.

| Incubation time | Cholesterol (pmol) | Phospholipid (pmol) | Phospholipid/Cholesterol |
|-----------------|--------------------|---------------------|-------------------------|
| 0 h             | 11.3 ± 1.1         | 248 ± 15            | 22.0                    |
| 4 h             | 13.6 ± 0.9         | 230 ± 13            | 16.9                    |
| 8 h             | 14.5 ± 1.3         | 240 ± 17            | 16.6                    |
| 12 h            | 53.0 ± 3.9         | 210 ± 19            | 4.0                     |
| 24 h            | 90.0 ± 5.2         | 235 ± 18            | 2.6                     |

rose dramatically from about 14 pmol at 8 h of incubation to about 53 pmol at 12 h and 90 pmol at 24 h of incubation, respectively. The PL (Table II) and protein (data not shown) levels of the BLM did not reveal any significant changes during incubation of BFA-treated cells with BFA-free medium (Table III). Steady-state fluorescent anisotropy (which is inversely proportional to fluidity), \(r_e\), of the BLM fell from 0.27 in BFA-untreated cells to 0.15 following 12 h of incubation of filter-grown cells with BFA for 12 h (Table IV). When the cells were exposed to BFA-free medium, the \(r_e\) value rose linearly, and in 24 h of incubation it reached normal values (Table IV).

To confirm that the BFA-induced changes noted in this
Filter-grown cells were incubated in the presence or absence of BFA (5 μg/ml) for 12 h at 37 °C. BFA-treated cells were washed with BFA-free medium and incubated in the absence of BFA for 0–24 h. The cationic probe TMA-DPH was added to the BLM. The $r_\text{s}$ values reported are mean ± S.D. from 10 determinations at each time interval using three separate sets of filters. Other details are provided under “Experimental Procedures.”

| Incubation time | $r_\text{s}$  |
|-----------------|-------------|
| h               |             |
| 12 (no BFA)     | 0.27 ± 0.02 |
| 12 (+ BFA)      | 0.15 ± 0.01 |
| 0 (no BFA)      | 0.15 ± 0.01 |
| 4               | 0.16 ± 0.01 |
| 8               | 0.19 ± 0.02 |
| 12              | 0.21 ± 0.01 |
| 24              | 0.28 ± 0.02 |

The interesting observation that cholesterol but not PL levels of the BLM were depleted in BFA-treated Caco-2 cells raised important questions regarding the mechanism(s) by which this could occur. Since there was no change in the total cellular cholesterol levels following BFA treatment of Caco-2 cells noted in this (Table II) and a previous study (34), it is unlikely that depletion of BLM cholesterol in BFA-treated cells is due to an
effect on the de novo synthesis of cholesterol. BFA treatment of Caco-2 cells for 8–24 h has been previously (34) shown to increase cholesterol ester formation. However, there is no evidence that free cholesterol that is esterified is derived from the PM pool. Thus, the most likely explanation for the depletion of BLM cholesterol is that it is due to BFA-induced morphological disruptions, the delivery of cholesterol to the BLM is inhibited.

The potential involvement of the Golgi as an intermediate in the intracellular sorting of cholesterol to the cellular PM is not fully known. Available evidence suggests that PM cholesterol is derived from both the newly synthesized pool in the ER (35) by biliary excretion and that free cholesterol that is esterified is derived from the lysosomes (37) by a BFA-sensitive, Golgi-dependent pathway (38). It is not known which one (if not both) of the two pathways operates to deliver cholesterol to BLM in Caco-2 cells and is BFA-sensitive. One could speculate that in Caco-2 cells the bulk of the BLM cholesterol is derived from the lysosomes via the Golgi, disruption of which results in increased delivery of cholesterol to the ER, where it is esterified. This speculation is supported by the following observations: (a) lysosomal cholesterol must pass through the PM first on its way to the ER (39) for esterification, (b) lysosomal cholesterol delivery to the PM is BFA-sensitive (38), and (c) BFA treatment increases cholesterol ester formation in Caco-2 cells (34), and BFA depletes BLM cholesterol levels (Table II).

Due to decreased BLM cholesterol and unaltered PL levels in BFA-treated cells, the PL/cholesterol ratio of BLM increased from 2.6 to 22.0, thus causing a dramatic decrease in the mol % BFA depletes BLM cholesterol levels (Table II).

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