Polymyxin B Enhances Low Density Lipoprotein Catabolism in Hepatic and Extrahepatic Cells

Wei Liao and Claes-Henrik Florin

We investigated the effects of polymyxin B (PMB), an antibiotic that binds to endotoxins, on the uptake and degradation of low density lipoproteins (LDLs) in HepG2 cells, a highly differentiated human hepatoma cell line. The results showed that PMB very effectively enhanced the binding, internalization, and degradation of LDL in HepG2 cells. The PMB-mediated enhancement of LDL uptake was not dependent on the LDL receptor-mediated pathway, as blockage of the LDL receptor by use of a monoclonal anti-LDL receptor antibody had no effect on the PMB-mediated cellular processing of LDL and PMB-mediated enhancement of LDL uptake did not cause an increase in cholesterol esterification. In addition, chloroquine and colchicine, which inhibit lysosomal degradation and cellular endocytosis, respectively, diminished PMB-enhanced degradation of LDL, indicating that PMB mediates uptake through a pathway similar to the LDL receptor-mediated pathway. The PMB-mediated uptake of LDL was sensitive to treatment with phospholipase C and pronase and was dependent on the presence of Ca²⁺. PMB caused similar changes in human skin fibroblasts, bovine smooth muscle cells, and bovine endothelial cells, which suggests that PMB-enhanced LDL uptake is a general cellular phenomenon. Our results thus indicate that PMB increases cellular catabolism of LDL through an endocytotic pathway not involving the LDL receptors. (Arteriosclerosis and Thrombosis 1992;12:503-511)

KEY WORDS • polymyxin B • low density lipoproteins • HepG2 cells • fibroblasts • smooth muscle cells • endothelial cells • endocytosis

Polymyxin B (PMB) is a cyclic polypeptide antibiotic produced by various strains of Bacillus polymyxa, an aerobic spore-forming rod found in soil.¹ In addition to its antimicrobial activity, PMB has potent antiendotoxin properties.² Its clinical use, however, is limited by its neurotoxicity and nephrotoxicity. When we studied the interactions between endotoxins, lipoproteins, and cells, we accidentally discovered that PMB very effectively increased the uptake and degradation of low density lipoproteins (LDLs) in hepatic and extrahepatic cells. LDL, the main cholesterol-carrying lipoprotein in plasma, is associated with atherosclerosis when present in high plasma concentrations.³ Enhanced catabolism of LDL by the liver decreases plasma LDL concentrations. Thus, to study the regulation of plasma concentrations of LDL, one must focus on liver catabolism. In this article, we explore the effects of PMB on the cellular uptake and degradation of LDL in HepG2 cells, a highly differentiated human hepatoma cell line. The study shows that PMB effectively increases LDL binding, internalization, and degradation by an endocytotic non-LDL receptor-mediated pathway.

From the Department of Internal Medicine, Lund University, Malmö General Hospital, Malmö, Sweden.

Supported by grants from the Medical Faculty, University of Lund, the Påhlsson Foundation, the Research Foundation of the Hospital of Malmö, the Heart-Lung Foundation, and the Ermhold Lundström Foundation.

Address for correspondence: Claes-Henrik Florén, MD, PhD, Department of Internal Medicine, Lund University, Malmö General Hospital, S-214 01 Malmö, Sweden.

Received August 20, 1991; revision accepted December 9, 1991.

Methods

Materials

The PMB sulfate (7,610 units PMB base/mg) used in this study was obtained from Sigma Chemical Co., St. Louis, Mo. Before addition to the cells, PMB was added to the tissue culture medium and gently swirled until dissolved. Chloroquine, colchicine, pronase, and phospholipase C were also obtained from Sigma; trypsin was from Cooper Biomedical; a monoclonal anti-LDL receptor antibody (clone C7) was from Amersham, Buckinghamshire, UK; and Spectra/Por standard cellulose dialysis tubing was from Spectrum, Houston, Tex.

Cell Cultures

The established hepatoblastoma cell line HepG2 was obtained from the American Tissue Type Culture Collection, Rockville, Md. The cells were grown at 37°C in 80-cm² flasks (NUCLON, Roskilde, Denmark) in 25 ml RPMI-1640 with glutamine (Life Technologies Ltd., Paisley, Scotland) containing 10% fetal calf serum (FCS, heat inactivated at 56°C for 30 minutes) from Flow Laboratories, Irvine, Scotland. The cells were grown in humidified air with 5% CO₂ and subcultured approximately once every week at a split ratio of 1 to 6.

Human skin fibroblasts (FBs) were taken from skin punch biopsies of the medial aspect of the left upper arm of healthy adults. Cell culture was established⁴ and maintained in minimum essential medium with 25 mM N₂-hydroxyethylpiperazine-N₂'-ethanesulfonic acid supplemented with 10% FCS, 4 mM glutamine, 1% nonessential amino acids, 75 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of air.
Bovine smooth muscle cells (SMCs) were grown from explanted segments of the thoracic aorta according to Stavenow and Tejler. The cells were grown under the same conditions as used in the FB cultures described above.

Bovine endothelial cells (ECs) from the thoracic aorta were established and maintained in Dulbecco's modified Eagle's medium/Ham's F12 medium (1:5, vol/vol) containing 10% FCS and 0.1 mg/ml gentamicin in gelatin-coated flasks at 37°C in a humidified 5% CO2 atmosphere.

The cells, i.e., human FBs, bovine SMCs, and bovine ECs, from passages two to three were used in the present experiments.

To initiate the experiments, cells were trypsinized with 0.05% trypsin/0.02% EDTA (Life Technologies Ltd.), plated into 35-mm plastic petri dishes (NUNCLO), and grown in 2 ml medium containing 10% FCS. The culture medium was routinely changed every 2-3 days. The experiments started when the cells became subconfluent, i.e., when they covered approximately two thirds of the bottom of the dish. The medium was changed to 1 ml medium containing 0.5% human serum albumin (HSA) without FCS for an LDL binding, uptake, and degradation assay.

**Lipoprotein Isolation and Labeling**

To prepare LDLs, blood was drawn from normocholesterolemic subjects into EDTA-containing Vacutainer tubes (100×16 mm, Becton Dickinson, Rutherford, N.J.). Immediately, thimerosal (sodium ethylmercurithiosalicylate; Sigma) was added to a final concentration of 0.05% trypsin/0.02% EDTA (Life Technologies Ltd.), plated into 35-mm plastic petri dishes (NUNCLO), and grown in 2 ml medium containing 10% FCS. The culture medium was routinely changed every 2-3 days. The experiments started when the cells became subconfluent, i.e., when they covered approximately two thirds of the bottom of the dish. The medium was changed to 1 ml medium containing 0.5% human serum albumin (HSA) without FCS for an LDL binding, uptake, and degradation assay.

**Low Density Lipoprotein Binding Assay**

The binding of LDL to HepG2 cells was performed at 4°C and 37°C. At 4°C before the binding experiment began, the cells were washed twice with 1 ml phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA). One milliliter ice-cold RPMI-1640 containing 0.5% HSA (fraction V; Sigma) was then added to each dish. The cells were then incubated at 4°C for 24 hours at 37°C in humidified air with 0.5% CO2. After incubation, 125I-LDL was then added to the dishes. The cells were then incubated for 4 hours at 37°C, usually for 4 or 24 hours, with the indicated exceptions. After incubation, the cells were washed with 1 ml cold PBS three times and scraped into 1 ml cold NaOH for a subsequent measurement of bound LDL, referred to as the uptake by the cells (the sum of bound and internalized 125I-LDL), and for cell protein determination. Degradation of 125I-LDL was measured as the trichloroacetic acid-soluble noniodine 125I radioactivity in the medium. The results are expressed as nanograms LDL protein taken up or degraded per milligram cell protein.

**Cholesterol Estimation Assay**

HepG2 cells were grown in 2 ml RPMI-1640 supplemented with 10% FCS until they were subconfluent. Then the medium was changed to 1 ml RPMI-1640 containing 0.5% HSA (pH 7.4) and unlabeled LDL (100 μg protein/ml medium). The cells were incubated for 24 hours at 37°C. After incubation, the media was aspirated and the cells were washed three times with 1 ml cold PBS. The cellular lipids were then extracted with chloroform/methanol (1:2, vol/vol). The chloroform phase was dried under nitrogen, and the lipids were dissolved in 200 μl of chloroform/methanol (1:2, vol/vol). The chloroform phase was then evaporated to dryness. The lipid extract was then placed on a thin-layer chromatography plate (E. Merck, Darmstadt, FRG) and separated with petroleum ether/diethyl ether/acetic acid and the radioactivity detected using a gamma counter (Packard model 2008 liquid scintillation spectrometer). The results are expressed as disintegrations per minute incorporated per milligram cell protein.

**Anti–Low Density Lipoprotein Receptor Antibody**

This antibody blocks LDL receptor-mediated uptake of LDL in cultured human FBs, as described by Beisiegel et al. The dilution and freezing protocol recommended by the manufacturer was followed.
Protein Determination

The protein contents of the cells and LDL were determined with the Lowry method using HSA as the standard.

Statistical Analyses

Experimental results were analyzed for their statistical significance by Student's t test. A probability value less than 0.05 was considered significant.

Results

Polymyxin B Enhances Low Density Lipoprotein Binding, Internalization, and Degradation in HepG2 Cells

Because internalization of ligands by receptor-mediated endocytosis is inhibited at 4°C, binding of LDL to HepG2 cells was examined at this temperature. The binding of LDL to HepG2 cells at 4°C in the presence of 100 μg PMB/ml medium was 4.7 times (p<0.005) higher than that in the absence of PMB (Figure 1). The binding, internalization, and subsequent degradation of LDL in HepG2 cells at 37°C in the presence of 100 μg PMB/ml medium were also much higher than those in the absence of PMB. PMB caused a 712% increase of binding (p<0.001), a 348% increase of internalization (p<0.001), and a 111% increase of degradation (p<0.001) (Figure 2).

PMB was added in increasing amounts (0–1,000 μg/ml medium) to HepG2 cells incubated with a constant amount of 125I-LDL (5.33 μg LDL protein/ml medium). As shown in Figure 3, the uptake of 125I-LDL increased with increasing amounts of PMB in a dose-dependent manner. At a concentration greater than 800 μg PMB/ml medium, the uptake seemed to reach saturation. Incubation of HepG2 cells with 100, 500, and 1,000 μg PMB/ml medium and 125I-LDL for 24 hours resulted in a 107%, 777%, and 1,086% increase, respectively, in uptake. Meanwhile, at first the degradation of 125I-LDL sharply increased with increasing concentrations of PMB. The degradation of LDL peaked with 100 μg PMB/ml medium. Then LDL degradation decreased with increasing amounts of PMB. Incubation of HepG2 cells with PMB and 125I-LDL resulted in an 83% increase of LDL degradation at 100 μg, a 0% increase at 500 μg, and a 35% decrease at 1,000 μg/ml medium. When the concentration of PMB was lower than 50 μg/ml medium, there was no significant change in the uptake and degradation of LDL by HepG2 cells.

The time course for uptake and degradation of LDL in the presence of PMB (100 μg/ml medium) is shown in Figure 4. There was always a much higher uptake of LDL in the presence of PMB than in the absence of PMB at any time point. The uptake of LDL by the cells sharply increased until the fourth hour of incubation and then decreased with time. The degradation of LDL increased in a linear manner during the whole 24-hour period of incubation at this concentration. This suggests that bound LDL was rapidly internalized and subsequently degraded in the cells.

To elucidate whether PMB was complexed with 125I-LDL before processing of LDL by the cells, the following experiment was done. PMB was mixed with 125I-LDL (the ratio of PMB to 125I-LDL in micrograms per microgram was ~100). Then the mixture was incubated for 30 minutes at 37°C followed by dialysis against 0.9% NaCl containing 1 mM EDTA (pH 7.4) (1,000 ml x2) for 24 hours using a dialysis bag with a molecular weight cutoff of 12,000–14,000 to dialyze...
away free PMB (molecular weight, 1,000). This preparation of the \(^{125}\text{I}-\text{LDL}\)–PMB complex (5.33 \(\mu\text{g} \text{ LDL protein/ml medium}\)) was added to the cells and compared with dialyzed \(^{125}\text{I}-\text{LDL}\) (control) for a 4-hour uptake and degradation assay. As shown in Figure 5, the \(^{125}\text{I}-\text{LDL}\)–PMB complex caused a 236% increase in uptake \((p<0.001)\) and an 80% increase in degradation \((p<0.001)\) of \(^{125}\text{I}-\text{LDL}\) by HepG2 cells. This suggests that PMB first complexes with \(^{125}\text{I}-\text{LDL}\) and then mediates processing of LDL by the cells.

![Figure 3](image3.png)

**Figure 3.** Line plot of dose response of polymyxin B (PMB)–mediated uptake and degradation of low density lipoprotein (LDL) in HepG2 cells. Various amounts of PMB and a fixed amount of \(^{125}\text{I}-\text{LDL}\) (5.33 \(\mu\text{g} \text{ LDL protein/ml medium}\)) were added to the dishes for a 24-hour uptake and degradation assay as described in “Methods.” ••, LDL uptake; △△, LDL degradation. Values are mean of two dishes.

![Figure 4](image4.png)

**Figure 4.** Line plot of time course for polymyxin B (PMB)–mediated uptake and degradation of low density lipoprotein (LDL) in HepG2 cells. \(^{125}\text{I}-\text{LDL}\) (5.33 \(\mu\text{g} \text{ LDL protein/ml medium}\)) with (100 \(\mu\text{g/ml medium}\)) or without PMB was added to the dishes. After that the dishes were incubated for various time intervals to measure uptake and degradation of \(^{125}\text{I}-\text{LDL}\) as described in “Methods.” •• and •• show LDL uptake in the presence and absence of PMB, respectively. △△ and △△ show LDL degradation in the presence and absence of PMB, respectively. Values are mean of two dishes.
Polymyxin B Enhances LDL Catabolism

507

Liao and Florin

FIGURE 5. Bar graph showing effect of 125I-low density lipoprotein (LDL)–polymyxin B complex on LDL uptake and degradation in HepG2 cells. Dialyzed 125I-LDL (5.33 µg LDL protein/ml medium; control) or 125I-LDL–PMB complex (5.33 µg LDL protein/ml medium) was added to the dishes for a 4-hour uptake and degradation assay as described in “Methods.” Open bars show uptake, and hatched bars show degradation of LDL. Values are mean of four dishes.

Polymyxin B–Mediated Uptake and Degradation of Low Density Lipoprotein Is Not Dependent on the Low Density Lipoprotein Receptor–Mediated Pathway

To clarify whether PMB-induced increases in the uptake and degradation of LDL occur by means of stimulating the LDL receptor–mediated pathway in HepG2 cells, the following experiment was carried out. A monoclonal anti-LDL receptor antibody, which blocks the LDL receptor–mediated uptake,14 was added to the cells. The results showed that complete suppression of the LDL receptor–mediated uptake of 125I-LDL by the antibody occurred at an antibody concentration of 4–6 µg/ml medium (data not shown). Both the uptake and degradation of 125I-LDL in the presence of PMB (500 µg/ml medium) were not affected despite the increasing concentration of antibody to as much as 10 µg/ml medium (Figure 6). This suggests that little if any LDL was processed through the LDL receptor–mediated pathway in the presence of PMB.

Polyquin B–Mediated Uptake and Degradation of Low Density Lipoprotein Are Dependent on the Presence of Ca2+ in the Medium

The results shown in Figure 7 indicate that the PMB-mediated uptake and degradation of LDL are dependent on the presence of Ca2+ in the medium. The uptake and degradation of LDL decreased with increasing concentrations of EDTA in the medium. The presence of 4 mM EDTA caused a 19% decrease in the uptake and a 77% decrease in the degradation of 125I-LDL. The presence of 10 mM EDTA caused a 56% decrease in the uptake and a 92% decrease in the degradation of 125I-LDL.

Effects of Chloroquine and Colchicine Treatment on the Polymyxin B–Mediated Processing of Low Density Lipoprotein in HepG2 Cells

The roles of microtubules and lysosomes in the PMB-mediated processing of LDL were determined by studying the effects of chloroquine, a general inhibitor

FIGURE 6. Line plot of effects of a monoclonal anti-low density lipoprotein (LDL) receptor antibody on polymyxin B (PMB)–mediated uptake and degradation of LDL in HepG2 cells. Increasing amounts of an anti-LDL receptor antibody were added to the dishes. Then 125I-LDL (5.33 µg LDL protein/ml medium) with PMB (500 µg/ml medium) was added to the dishes for a 24-hour uptake and degradation assay as described in “Methods.” —— shows LDL uptake, and △—△ shows LDL degradation. Values are mean of two dishes.
of lysosomal hydrolysis,16 and colchicine, an inhibitor of microtubular polymerization,17 on the metabolism of 

\[ {^{125}}\text{I-LDL} \] in the presence of PMB (100 \( \mu \)g/ml medium) by HepG2 cells. As shown in Table 1, the addition of chloroquine (50 \( \mu \)M) caused a 54% increase in the uptake and an 85% reduction in the degradation of 

\[ {^{125}}\text{I-LDL} \] (\( p<0.001 \)). At 100 \( \mu \)M, chloroquine had no further effect on the uptake of 

\[ {^{125}}\text{I-LDL} \] but inhibited its degradation to an even higher extent (95%). Colchicine also inhibited the degradation of 

\[ {^{125}}\text{I-LDL} \]. When tested at 1 \( \mu \)M, colchicine caused a 45% increase in the uptake of 

\[ {^{125}}\text{I-LDL} \] and a 49% reduction in its degradation (\( p<0.001 \)). This suggests that the PMB-mediated LDL uptake occurs through an endocytotic pathway, followed by lysosomal hydrolysis.

**TABLE 1. Effects of Chloroquine and Colchicine on Polymyxin B-Mediated Uptake and Degradation of Low Density Lipoprotein in HepG2 Cells**

| Addition (\( \mu \)M) | 
|---------------------|------------------|
|                     | \( {^{125}}\text{I-LDL} \) (ng/mg cell protein) | Uptake | Degradation |
| None | 141±31 (100) |
| Chloroquine (50) | 217±26 (154) | 5.7±0.7 (15) |
| Chloroquine (100) | 208±16 (148) | 1.7±0.2 (4.5) |
| Colchicine (1) | 205±34 (145) | 19.0±0 (31) |
| Colchicine (10) | 177±0.7 (126) | 19.5±0 (52) |

Chloroquine or colchicine was added to the cells for a 3-hour preincubation. Then 

\[ {^{125}}\text{I-low density lipoprotein (LDL)} \] (5.33 \( \mu \)g LDL protein/ml medium) and polymyxin B (100 \( \mu \)g/ml medium) were added to each dish for a 4-hour uptake and degradation assay as described in "Methods." All values are mean±SD of two dishes. Numbers in parentheses are percentages of control values.

**Effects of Pronase, Trypsin, and Phospholipase C on the Polymyxin B-Mediated Processing of Low Density Lipoprotein in HepG2 Cells**

To determine whether the PMB-mediated binding of LDL to HepG2 cells was dependent on binding sites containing protein or phospholipid moieties, the following experiments were done. The cells were pretreated with pronase, trypsin, or phospholipase C for 30 minutes at 37°C. The pretreatment was stopped by washing the cells once with RPMI-1640 containing 10% FCS and twice with PBS. The treated cells were then incubated with PMB (100 \( \mu \)g/ml medium) and 

\[ {^{125}}\text{I-LDL} \] (5.33 \( \mu \)g LDL protein/ml medium) for a 4-hour uptake and degradation assay. The results are shown in Table 2 and indicate that the PMB-mediated processing of LDL was sensitive to phospholipase C, mildly sensitive to pronase, and relatively resistant to trypsin treatment. As shown in Table 2, pretreatment of the cells with 10 \( \mu \)g trypsin/ml medium caused a 3% reduction in the uptake and a 28% reduction in the degradation of 

\[ {^{125}}\text{I-LDL} \] (\( p=0.05 \)). On the other hand, pretreatment of the cells with 15 \( \mu \)g pronase/ml medium caused a 29% reduction in the uptake (\( p<0.05 \)) and a 44% reduction in the degradation (\( p<0.05 \)) of 

\[ {^{125}}\text{I-LDL} \]. In contrast, pretreatment of the cells with 15 \( \mu \)g phospholipase C/ml medium caused a 33% reduction in the uptake (\( p<0.05 \)) and a 92% reduction in the degradation of 

\[ {^{125}}\text{I-LDL} \] (\( p<0.005 \)).

**Figure 7. Line plot of effects of EDTA on polymyxin B (PMB)-mediated uptake and degradation of low density lipoprotein (LDL) in HepG2 cells. Various amounts of EDTA and a fixed amount of PMB (100 \( \mu \)g/ml medium) with 

\[ {^{125}}\text{I-LDL} \] (5.33 \( \mu \)g LDL protein/ml medium) were added to the dishes for a 24-hour uptake and degradation assay as described in "Methods." •• shows LDL uptake, and •• shows LDL degradation. Values are mean of two dishes.**

**Polymyxin B-Mediated Processing of Low Density Lipoprotein Also Occurs in Extrahepatic Cells**

We also determined whether the PMB-mediated processing of LDL occurred in extrahepatic cells. The results shown in Table 3 indicate that the PMB-medi-
TABLE 3. Polymyxin B-Enhanced Uptake and Degradation of Low Density Lipoprotein in HepG2 Cells and Extrabiliary Cells

<table>
<thead>
<tr>
<th>Cells/PBM (µg/ml)</th>
<th>Uptake</th>
<th>Degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>44±0.1 (100)</td>
<td>32±4.9 (73)</td>
</tr>
<tr>
<td>100</td>
<td>181±14.8 (411)</td>
<td>32±4.9 (73)</td>
</tr>
<tr>
<td>500</td>
<td>1462±12.7 (850)</td>
<td>32±4.9 (73)</td>
</tr>
<tr>
<td>Human skin fibroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>89±6.6 (100)</td>
<td>32±4.9 (73)</td>
</tr>
<tr>
<td>100</td>
<td>474±43 (533)</td>
<td>32±4.9 (73)</td>
</tr>
<tr>
<td>500</td>
<td>464±38.6 (521)</td>
<td>32±4.9 (73)</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>72±14.6 (100)</td>
<td>23±1.0 (100)</td>
</tr>
<tr>
<td>100</td>
<td>474±43 (533)</td>
<td>32±4.9 (73)</td>
</tr>
<tr>
<td>500</td>
<td>222±27.9 (965)</td>
<td>32±4.9 (73)</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>12±1.9 (100)</td>
<td>32±4.9 (73)</td>
</tr>
<tr>
<td>100</td>
<td>262±15 (217)</td>
<td>32±4.9 (73)</td>
</tr>
<tr>
<td>500</td>
<td>247±32.9 (2058)</td>
<td>32±4.9 (73)</td>
</tr>
</tbody>
</table>

Polymyxin B (PMB) and 125I-low density lipoprotein (LDL) (5.33 µg LDL protein/ml medium) were added to the dishes for a 4-hour uptake and degradation assay as described in "Methods." Values are mean±SD of two to four dishes. Numbers in parentheses are percentages of control values.

TABLE 4. Effect of Pretreatment of HepG2 Cells With Polymyxin B on Subsequent Low Density Lipoprotein Uptake and Degradation in HepG2 Cells

<table>
<thead>
<tr>
<th>Preincubation with PMB (µg/ml)</th>
<th>Incubation with PMB (µg/ml)</th>
<th>125I-LDL (ng/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uptake</td>
<td>Degradation</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>82±3.5 (100)</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>682±4.9 (83)</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>27±0 (33)</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>496±9.9 (100)</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>489±43.8 (98)</td>
</tr>
<tr>
<td>500</td>
<td>100</td>
<td>276±2.1 (55)</td>
</tr>
</tbody>
</table>

The medium was changed to 1 ml RPMI-1640 containing 0.5% human serum albumin (HSA) (pH 7.4) and polymyxin B (PMB) for a 24-hour pretreatment. Then the dishes were washed once with RPMI-1640 containing 10% fetal calf serum and twice with phosphate-buffered saline. After that, RPMI-1640 (1 ml/dish) containing 0.5% human serum albumin, 125I-low density lipoprotein (LDL) (5.33 µg LDL protein/ml medium), and polymyxin B (100 µg/ml medium) were added to each dish for a 4-hour uptake and degradation assay as described in "Methods." Values are mean±SD of two dishes. Numbers in parentheses are percentages of control values.

...were then washed once with RPMI-1640 containing 0.5% HSA and with or without PMB. After that, 125I-LDL (5.33 µg LDL protein/ml medium) with or without PMB was added to the dishes for a 4-hour uptake and degradation assay. Pretreatment of the cells with PMB inhibited subsequent LDL uptake and degradation in the absence of PMB and also inhibited PMB-mediated uptake and degradation of LDL in HepG2 cells (Table 4). Pretreatment of the cells with 500 µg PMB/ml medium for 24 hours caused a 67% reduction in the uptake (p<0.005) and 95% reduction in the degradation (p<0.005) in the presence of PMB. However, a much shorter pretreatment (1 hour) of the cells with 500 µg PMB/ml medium did not cause an inhibition of subsequent processes of 125I-LDL in both the presence and absence of PMB (data not shown). The data thus suggest that prior treatment of cells with PMB for a longer time may lead to toxic effects on the cells.

Polymyxin B-Enhanced Uptake of Low Density Lipoprotein Does Not Induce Cholesterol Esterification

One of the consequences of LDL receptor-mediated cellular LDL catabolism is the activation of acyl-coenzyme A:cholesterol O-acyltransferase (ACAT), an enzyme that esterifies cholesterol. An LDL-induced increase in cellular cholesterol esterification occurs merely after uptake of LDL via the LDL receptor. The following experiment was undertaken to study whether PMB by itself affected cholesterol esterification and whether uptake of PMB-LDL increased cholesterol esterification. The results show that LDL (100 µg LDL protein/ml medium) caused a 266% increase in cellular cholesterol esterification (p<0.005) (Figure 8). The addition of 100 µg PMB/ml medium resulted in no significant change in basal cholesterol esterification (p>0.05) and also no significant change in stimulated...
binding, internalization, and subsequent degradation of LDL by HepG2 cells. This cell line is a highly differentiated of PMB is used, it dramatically increases the experiments demonstrate that when a suitable concenterations, once inside the cell, can act as an ACAT inhibitor. These data thus show that PMB-mediated uptake of LDL does not lead to increased cholesterol esterification and does not occur through the LDL receptor pathway.

Discussion

Polymyxins, which are polycationic antibiotics, are relatively simple basic peptides with molecular weights of about 1,000. PMB itself is a mixture of PMB₁ and PMB₂. PMB has surface-active amphipathic properties. Thus, it contains both lipophilic and lipophobic groups within the molecule. PMB has many biologic effects. It interacts strongly with phospholipids and penetrates into and disrupts the structure of cell membranes. Once bacteria sensitive to PMB come into contact with it, the permeability of the bacterial membranes changes immediately. PMB inactivates endotoxins by binding to the lipid A portion of endotoxins (the lipopolysaccharide of the outer membrane of Gram-negative bacteria) and blocks many of the biologic activities of endotoxins.

To our knowledge, this study is the first to present the effects of PMB on cellular LDL processing. The present experiments demonstrate that when a suitable concentration of PMB is used, it dramatically increases the binding, internalization, and subsequent degradation of LDL by HepG2 cells. This cell line is a highly differen-
tiated human hepatoma cell line. Because about two thirds of the LDL is catabolized in the liver in the hepatocytes, it is relevant to study hepatocytic uptake of LDL. However, PMB caused similar changes in human FBs, bovine SMCs, and bovine ECs, showing that the effects of PMB on LDL uptake are not limited to liver cells.

PMB-mediated uptake of LDL by HepG2 cells was shown to occur in a dose- and time-dependent manner. PMB also greatly enhanced the degradation of LDL by HepG2 cells when PMB concentrations were 50–200 μg/ml medium but decreased LDL degradation by the cells at higher PMB concentrations, presumably reflecting the inhibitory effects of PMB on lysosomal enzymes. Our study thus indicates that cell surface–bound LDL is rapidly internalized and subsequently degraded by HepG2 cells in the presence of PMB.

PMB-mediated uptake and degradation of LDL did not seem to be dependent on the LDL receptor, as the PMB-stimulated uptake and degradation of LDL were not affected by high concentrations of an anti-LDL receptor antibody. Thus, this suggests that little if any LDL was processed through the LDL receptor–mediated pathway in the presence of PMB. However, there is a slight possibility that PMB alters LDL, so that the PMB–LDL complex can still bind to the LDL receptor but that the binding is not blocked by the anti-LDL receptor antibody. Another characteristic of PMB-mediated LDL uptake was that it did not cause increased cholesterol esterification, which occurs when LDL is taken up through the LDL receptor. However, higher concentrations of PMB led to a decreased cholesterol esterification and a decreased degradation of ¹³⁵I-LDL, which may suggest that PMB in high concentrations, once inside the cell, could be a general inhibitor of ACAT and lysosomal enzymes.

Like the LDL receptor–mediated processing of LDL, the PMB-mediated uptake of LDL was also inhibited by the addition of EDTA. This suggests that the PMB-mediated uptake of LDL is dependent on the presence of divalent cations, presumably Ca²⁺. In the presence of Colchicine also inhibited the PMB-mediated catabolism of LDL, which suggests that transport through an endocytotic pathway is involved. Degradation of PMB–LDL presumably occurs in lysosomes, as chloroquine inhibited the PMB-mediated degradation of LDL. The demonstration that the PMB-mediated processing of LDL is sensitive to phospholipase C and mildly sensitive to pronase indicates that the binding sites are phospholipid–protein complexes.

A 24-hour pretreatment of HepG2 cells with PMB led to a decreased subsequent processing of LDL by the cells in both the presence and absence of PMB. This may be explained by a nonspecific cytotoxic effect of PMB that is not complexed with albumin. The nature of the binding sites for the PMB-mediated processing of LDL is uncertain. Because the structure of PMB contains lipophilic groups, it is reasonable to speculate that PMB complexes with LDL and that the PMB–LDL complex then enters into the cells by an endocytotic pathway after binding to the cell membrane. The fact that dialyzed ¹³⁵I-LDL–PMB retained the effect supports this speculation. Recently, Bysani et al. demonstrated that PMB specifically binds to rat alveolar macrophages and monocytes. It is not clear whether these binding
sites are the same as those in this study, which mediate the PMB-enhanced uptake of LDL, but the former seem to occur only in the macrophage system and are also very sensitive to trypsin.

From the present study, our data show that the PMB-mediated uptake of LDL has some features similar to the LDL receptor-mediated pathway, i.e., Ca\(^{2+}\) dependency, the involvement of the endocytotic pathway in the internalization process, and the occurrence of degradation in lysosomes. The PMB-mediated LDL uptake is, however, more effective and does not cause an increased cholesterol esterification in the cells. Future studies will reveal more details of the intracellular trafficking of the PMB-LDL complex and the type of mechanism by which PMB-LDL binds to the cell surface. Experiments already carried out (W. Liao and C.-H. Florén, unpublished data) show that PMB cationizes LDL. Presumably, then, the positively charged LDL interacts with negative charges on the cellular plasma membrane, which causes an enhanced uptake and degradation of LDL by the cells.

Acknowledgments

We gratefully acknowledge Mrs. Asta Lundquist for excellent technical assistance. We also thank Mrs. Kerstin Kautsson for preparation of fibroblasts and Dr. C.B. Xu for preparation of smooth muscle cells and endothelial cells.

References

Polymyxin B enhances low density lipoprotein catabolism in hepatic and extrahepatic cells.

W Liao and C H Florén

doi: 10.1161/01.ATV.12.4.503

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/12/4/503

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/