The Effects of Pluronic® Block Copolymers and Cremophor® EL on Intestinal Lipoprotein Processing and the Potential Link with P-Glycoprotein in Caco-2 Cells

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Purpose. This investigation was performed to study the effects of Pluronic® block copolymers and Cremophor® EL on intestinal lipoprotein processing and to investigate a potential link between lipoprotein processing and P-glycoprotein.

Methods. Caco-2 cells were used to monitor changes in lipoprotein production and secretion following exposure to excipients. Effects on P-glycoprotein were monitored using cyclosporin A as a model substrate.

Results. A range of surfactants commonly used as pharmaceutical excipients in lipid-based oral drug delivery systems, including Pluronic® block copolymers L81, P85, and F68 and Cremophor® EL, inhibited intestinal lipoprotein secretion. The effects were concentration dependent and reversible. The mechanism of inhibition appears to be related to the assembly and secretion of lipoproteins rather than to initial intracellular triglyceride synthesis. A strong correlation was found between excipient-mediated inhibition of lipoprotein secretion and inhibition of P-glycoprotein efflux, implying a link between the two biochemical processes.

Conclusions. The ability of such bioactive excipients to simultaneously manipulate different cellular processes must be considered in selecting excipients for oral drug delivery systems. Such information is particularly relevant when the drug is lipophilic, a candidate for P-glycoprotein efflux, and where intestinal lymphatic targeting via chylomicron stimulation is desirable.

KEY WORDS: Pluronic® block copolymers; Cremophor® EL; lipoproteins; P-glycoprotein; Caco-2; lipophilic drug delivery.

INTRODUCTION

Lipid-based drug delivery vehicles enhance oral bioavailability of many lipophilic drugs (1). Although the exact mechanisms of absorption enhancement from lipid-based systems remain unclear, it has been suggested that enhanced intestinal lymphatic transport may play a role (1). Oral lipid vehicles usually consist of a blend of oils and excipients, including surfactants, designed to solubilize the drug and simultaneously aid dispersion in the aqueous environment of the intestine. Little is known about the effects such excipients may have on digestive or metabolic functioning of the gastrointestinal tract. Such interactions may potentially significantly affect the *in vivo* performance and influence the mechanism of absorption enhancement of a coadministered drug. This lack of mechanistic information has been identified as a limiting factor in the more widespread acceptance of these potentially useful drug delivery systems and has often resulted in an arbitrary approach to selection of vehicle excipients. However, evidence does exist to indicate the bioactive nature of certain excipients. A wide range of nonionic surfactants, including Cremophor® EL, polysorbate 80, and the block copolymers Pluronic® P85 and L81, have been shown to inhibit intestinal P-glycoprotein (P-gp) efflux (2–4). In addition, Pluronic® L81 has been shown to inhibit intestinal secretion of lipoproteins, including triglyceride-rich chylomicrons, in an *in vivo* rat model (5) and in Caco-2 cells (6). Such studies may have significant implications for oral delivery of lipophilic drugs, as these compounds are likely to be substrates both for P-gp efflux (7) and for transport via the lymphatic system in association with the triglyceride core of chylomicrons (8). Field *et al.* (9) reported that nonsurfactant agents, including verapamil and trifluoperazine, which inhibit P-gp activity, also decreased the secretion of triglyceride-rich lipoproteins (TRL), thus suggesting a link between the two biochemical processes. The possibility that pharmaceutical excipients may also simultaneously modulate these two cellular processes, and the implications that this may have for oral delivery of lipophilic drugs formulated in conjunction with these excipients, was initially discussed by O'Driscoll (10).

The Caco-2 cell model is ideally suited for simultaneous investigation of the effects of excipients on P-gp (11) and on lipoprotein processing. This model is already well established for investigating intestinal drug permeability (12). Caco-2 cells express P-gp on the apical surface and demonstrate good *in vitro* and *in vivo* correlations in terms of both passive and active drug transport (12,13). In addition, Caco-2 cells synthesize and secrete lipoproteins with similar characteristics, in terms of lipid and apolipoprotein composition, to those found *in vivo* (14,15). The Caco-2 cell response to fatty acid stimuli is similar to that reported in animals. Administration of long-chain unsaturated fatty acids, such as linoleic and oleic acids, selectively stimulates secretion of TRL [chylomicrons and very low-density lipoproteins (VLDL)], whereas saturated fatty acids such as palmitic and stearic acids result predominantly in secretion of low-density lipoproteins (LDL) and intermediate-density lipoproteins (IDL) (16). Similar trends of increased chylomicron secretion with long-chain fatty acids have been reported in animals (17).

The aim of this work is to investigate, using the Caco-2 model, the possibility that other nonionic surfactants, commonly used as pharmaceutical excipients, inhibit triglyceride-rich lipoprotein secretion in a fashion similar to that observed for Pluronic® L81. In addition, the potential association between inhibition of P-gp activity and lipid metabolism in the presence of such excipients is explored. This will add to the knowledge available on excipient–drug interactions, particularly in relation to lipid metabolism. Such understanding is critical for facilitating the rational design of oral lipid-based formulations.

MATERIALS

Cell culture materials were obtained from Gibco Life Science, UK. Essentially fatty acid–free BSA and oleic acid

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(>99% pure) were obtained from Sigma, Cremophor® EL and Pluronic® block copolymers from BASF Corp., [1-^{14}C]oleic acid (2.07 GBq/mmol), [9, 10 (*n*)-^{3}H]oleic acid (370 GBq/mmol), and [mebmt-ß-^{3}H]cyclosporin A (333 GBq/mmol) from Amersham Pharmacia Biotech, UK, the monoclonal antibody, 1D1, from the University of Ottowa Heart Institute, Canada, the antiapolipoprotein B, human from sheep, from Boehringer Mannheim, and alkaline phosphatase-conjugated rabbit affinity purified antibody to sheep IgG (whole molecule) from Cappel. All solvents were of HPLC grade.

METHODS

Cell Culture

Caco-2 cells were obtained from the European Collection of Animal Cell Cultures (ECACC) and were maintained at 37°C with 5% CO_2 and 90% relative humidity. Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, and 0.1 mg/ml gentamycin in 75-cm^2 flasks. Cells were subcultured when 70–90% confluent using trypsin-EDTA, reseeded at a 1:4 ratio, and used at passage 65–85. The cells were seeded at a density of 6.3×10^4 cells/cm^2 onto Transwell filter inserts with 0.4-μm pores (Costar, UK) and cultured for 19–22 days to allow differentiation. Transepithelial electrical resistance (TEER) was measured using an EVOM voltohmmeter with chopstick electrodes (World Precision Instruments).

Lipid Vehicles

"Fed" simulated vehicle comprised 0.5 mM oleic acid bound to 0.125 mM bovine serum albumin (BSA) and was prepared as previously described (18,19). Vehicles were prepared from a 50 mM oleic acid stock solution in ethanol. Aliquots of oleic acid solution were dried under nitrogen and labeled with trace [^{14}C]oleic acid (7.4 KBq/ml), which is used as a marker for lipid synthesis in cell culture work (18,20). The sodium salt was prepared by neutralization with sodium hydroxide (100 mM) in ethanol solution. After drying, the fatty acid salt was reconstituted in hot water (>80°C). BSA in serum-free culture medium (DMEM supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, and 0.1 mg/ml gentamycin) was added to 50% of final volume. After 2 h at 37°C, the resulting clear solution was diluted to full volume with serum-free culture medium or with solutions of excipients in serum-free culture medium to the final working concentration. A "fasted-state" vehicle was prepared for monitoring baseline lipid metabolism, containing 0.125 mM BSA labeled with trace [^{14}C]oleic acid, as outlined above.

Vehicle Administration

Monolayers were rinsed three times and then incubated in serum-free culture medium for 4 h. Test vehicles were added to the apical side of monolayers (1.5 ml), and serum-free culture medium was added to the basolateral well (3 ml). Cells were incubated at 37°C, 5% CO_2 for 20 h to allow sufficient accumulation of secreted lipoproteins for accurate quantification and ultracentrifugation analysis (15).

The ability of cells to recover from the inhibitory effects of Pluronic® L81 was investigated by using a two-step dual-labeling technique. A time–secretion profile of triglyceride was used as a measure of TRL secretion rate. In the initial phase, Pluronic® L81 was added to [^{14}C]oleic acid–labeled "fed-state" vehicle and incubated with Caco-2 monolayers. Basolateral samples were taken at indicated time points over 24 h and assayed for [^{14}C]-labeled triglyceride. Resulting levels were compared to those achieved with a control "fed-state" vehicle. After this preliminary exposure, the test vehicle was removed, and monolayers were washed three times in serum-free culture medium. A recovery "fed-state" vehicle, this time labeled with [^{3}H]oleic acid, was then applied to monolayers to monitor triglyceride synthesis after excipient exposure. Basolateral samples were again taken at intervals over the following 24 h, and results compared to those obtained for monolayers previously exposed to control "fed-state." Because two different labels were used in the recovery phase, it was possible to discriminate between the secretion of newly synthesized triglyceride and the secretion of triglyceride synthesized during the initial excipient exposure phase. In addition, as a measure of background, unstimulated, triglyceride secretion throughout the study, a control "fasted-state" vehicle comprising 0.125 mM BSA was administered in the presence of trace amounts of each label.

Measurement of Cell Viability

Toxicity of excipients was determined by an MTT assay for intracellular dehydrogenase activity (21). MTT results are expressed as an IC_{50} value; this is the concentration of excipient producing a 50% inhibition of intracellular dehydrogenase activity (relative to cells incubated in serum-containing media), indicating a 50% decrease in cell viability. In addition, TEER values were used as a measure of monolayer integrity. The viability of cells following 24 h of exposure to "fasted-" and "fed-state" vehicles (in the absence of serum) was also confirmed using the MTT assay.

Sample Preparation

For intracellular determination, monolayers were washed twice with 3% (w/v) BSA in pH 7.4 phosphate-buffered saline (PBS) to remove adhering fatty acid and then twice with PBS alone. Cells were scraped into ice-cold PBS and suspended by repeated passage through a 22-gauge needle and lysed with Triton X (0.5% w/v). One aliquot was removed for protein determination (Lowry method, using a BioRad assay kit), and the remainder used for lipid analysis.

Basolateral samples were centrifuged at 5000 *g* for 10 min to remove cell debris. ε-Amino-*n*-caproic acid (EACA), aprotinin, and phenyl methyl sulfonyl fluoride (PMSF) were added as protease inhibitors.

Lipid and Apo B Analysis

Lipid analysis was conducted on cell lysate and basolateral samples. Lipids were extracted in chloroform:methanol (2:1) according to the method of Folch *et al.* (22). Extracts were dried under nitrogen and stored at –20°C until further analysis. Dried extracts were reconstituted in chloroform and spotted onto thin-layer chromatography (TLC) plates. Plates were developed in petroleum ether:diethyl ethyl ether:acetic acid (80:20:1) to isolate lipid subclasses (triglyceride and

phospholipid). Olive oil and phosphatidyl choline were used as standards for spot identification. Lipid bands were visualized by exposure to iodine vapor and scraped into scintillation vials. The radiolabel content was determined by a Packard Tri-Carb 2100 liquid scintillation counter using Packard Ultima Gold scintillation fluid.

The apolipoprotein B (apo B) content of basolateral samples was determined using an indirect sandwich ELISA, as previously described (23).

Separation of Lipoproteins

Lipoproteins were isolated from basolateral samples, according to flotation density, by density gradient ultracentrifugation using conditions described previously (6).

P-Glycoprotein Efflux

Cyclosporin A (CsA) was used as a model P-gp substrate for evaluating the effects of Pluronic® L81 and Cremophor® EL on active efflux mechanisms. Efflux activity was measured by comparing the apical–basolateral (influx) and basolateral–apical (efflux) transport of CsA, as described previously (24). The test vehicle contained CsA (0.5 μM), labeled with [^{3}H]CsA (0.2 μCi/ml). For influx experiments, test CsA vehicle, containing various dilutions of excipient, was added to the apical compartment (donor compartment), and blank buffer to the basolateral (acceptor) well. Basolateral samples were taken every 30 min for 2.5 h. For efflux experiments the test CsA vehicle was added to the basolateral well (now the donor), and transport buffer plus excipient to the apical (acceptor) compartment. Samples were again taken every 30 min. The CsA was measured via liquid scintillation counting (as previously described), and apparent permeability coefficients (P_{app}, cm/s) were calculated as follows: $P_{app} = dQ/dt \times 1/AC_0$, where dQ/dt = steady-state flux (mol/s), A = surface area of the membrane (cm^2), and C_0 = initial concentration in donor chamber (mol/cm^3).

Measurement of Surfactant Critical Micelle Concentration

Surfactant critical micelle concentration (CMC) values were determined in "fed-state" media or HBSS at 37°C by measuring surface tension using the Du Noüy ring method with a Lauda TD-1 tensiometer.

Statistical Analysis

Statistical analysis was conducted using Minitab™ statistical software. A one-way ANOVA was used, followed by Dunnett's method of multiple comparisons to determine statistical significance against a control.

RESULTS

Cell Viability and Vehicle Toxicity

The level of dehydrogenase activity, as measured via an MTT assay, following exposure over 24 h to "fed-" or "fasted-state" vehicles was similar to that achieved for cells incubated in serum-containing medium (data not shown), thus confirming cell viability under the experimental conditions used. The IC_{50} as determined from MTT data (Table I) indicates that Pluronic® L81 has the highest toxicity of the investigated block copolymers, with an IC_{50} of 0.02% (w/v). At 0.01% (w/v) Pluronic® L81, dehydrogenase activity was reduced by over 25%. Pluronic® P85 and F68 proved to be less cytotoxic, with IC_{50} values of 0.33% (w/v) and 14.3% (w/v), respectively. Under the experimental conditions described, the cytotoxicity of the tested block copolymers appears to increase with a corresponding decrease in HLB value (increasing lipophilicity). The TEER results (taken after an overnight incubation with test vehicle) followed a similar trend to the MTT data, with a significant decrease (45% decrease, $p < 0.01$) in monolayer resistance observed only for Pluronic® L81 at the highest test concentration (0.012% w/v).

Cremophor® EL demonstrates similar toxicity, as measured by the MTT assay, to Pluronic® P85, with an IC_{50} value of 0.53% (w/v) (Table I). A significant decrease in TEER value (27% decrease, $p < 0.05$) was observed only at 1% (w/v) Cremophor® EL.

Lipoprotein Secretion

Control

Addition of the "fed-state" control vehicle (no added excipient) to the apical side stimulated Caco-2 cells to secrete

Table I. Characteristics of Pluronic® Block Copolymers [Poly (Ethylene Oxide)-Block-Poly(Propylene Oxide)-Block-Poly (Ethylene Oxide) Copolymers] ($EO_{m/2}$-PO_n-$EO_{m/2}$) and Cremophor® EL (Castor Oil: Ethylene Oxide in Molar Ratio 1:35)

Characteristic	Pluronic® L81	Pluronic® P85	Pluronic® F68	Cremophor® EL
HLB[a]	1–7	12–18	>24	12–14
CMC (% w/v) in:				
"Fed-state" media	0.004	0.030	0.150	0.01
HBSS	0.006	N.D.	N.D.	0.01
Mwt[b]	2500	4500	8800	2515
N_{PO}/N_{EO} (m/n)[c]	43/6.2	40/52	29/153	n/a
IC_{50} (% w/v) (SD)	0.02 (0.006)	0.33 (0.01)	14.30 (0.20)	0.53 (0.11)

Note: CMC values were measured at 37°C using surface tension measurements. IC_{50} was determined using an MTT assay technique. All other values are from published literature.

[a] HLB as per manufacturer (BASF Corp.).

[b] Molecular weight (Mwt) as per manufacturer (BASF Corp.).

[c] Average number of poly (propylene oxide) (PO) and poly (ethylene oxide) (EO) units (27).

lipoproteins of flotation density equivalent to chylomicrons and VLDL as determined by radiolabel incorporation. Under "fed-state" conditions, approximately 5% of administered label was secreted as TRL over 20 h; this is comparable to values reported by van Greevenbroek *et al.* (18) under similar experimental conditions. Addition of "fasted-state" vehicle (labeled with trace [^{14}C]oleic acid) to the apical side produced <0.4% secretion of administered label as TRL (predominantly as VLDL). This promotion of chylomicron secretion only, under fatty acid stimulation, is consistent with *in vivo* data in rats (5) and *in vitro* data using Caco-2 cells (6).

Addition of Pluronic® Block Copolymers to Fed-State

The excipients were added to the "fed-state" vehicle and then applied to cells. Changes in lipoprotein response were expressed relative to the "fed-state" control (taken as 100%). Of the Pluronic® block copolymers investigated, Pluronic® L81, the most lipophilic, proved to be the most potent inhibitor of TRL secretion, producing more than 65% inhibition of chylomicron secretion relative to "fed" state at 0.006% (w/v) (Fig. 1). Impaired secretion of VLDL did not occur at this concentration (0.006% w/v) but was observed only for Pluronic® L81 at a higher concentration of 0.012% (w/v) (Fig. 2); however, this may be related to cytotoxicity as indicated by the MTT results.

Pluronic® P85 also produced inhibition of chylomicron secretion (Fig. 1). However, in contrast to Pluronic® L81 results, the concentration required to induce inhibition was much higher (0.03 vs. 0.006% w/v). As observed with Pluronic® L81, chylomicron secretion was more sensitive to the inhibitory effects of Pluronic® P85 with VLDL secretion affected only at the higher concentration of 0.06% (w/v) (Fig. 2), indicating selective inhibition of chylomicron secretion at the lower concentration of 0.03% (w/v).

The effects of Pluronic® F68, the most hydrophilic of the investigated copolymers as indicated by the HLB, were investigated over the concentration range 0.01 to 8% (w/v).

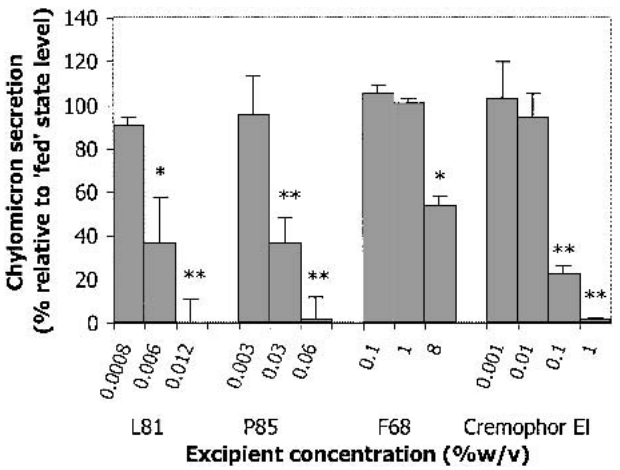

Fig. 1. Effect of increasing concentrations of surfactant excipients on chylomicron secretion. Results are expressed as percentage of administered label secreted as chylomicrons relative to the "fed-state" level. Values are the means of three to five monolayers ± SD. Statistical significance of excipient effects relative to the "fed-state" control is shown: *p < 0.05, **p < 0.01.

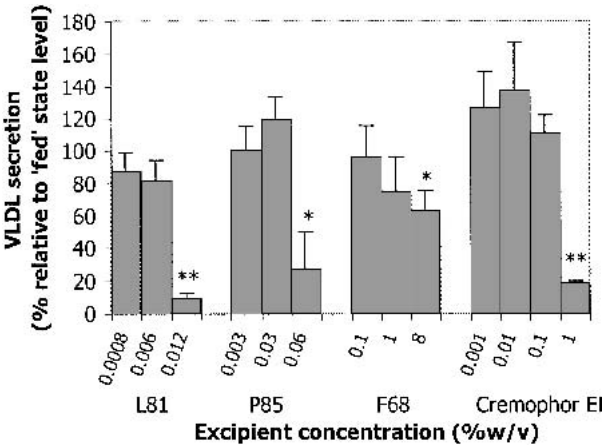

Fig. 2. Effect of increasing concentrations of surfactant excipients on VLDL secretion. Results are expressed as percentage of administered label secreted as VLDL relative to the "fed-state" level. Values represent the means of three to five monolayers ± SD. Statistical significance of excipient effects relative to the "fed-state" control is shown: *p < 0.05, **p < 0.01.

Inhibition of chylomicron and VLDL secretion was detected only at the higher concentration of 8% (w/v) (Figs. 1 and 2).

In the case of Pluronic® L81, in addition to monitoring secretion of chylomicrons (via ultracentrifugation), the total secreted basolateral content of triglyceride (isolated via TLC and quantified via liquid scintillation counting) and apo B (as quantified via ELISA) were determined. Apo B is essential for chylomicron assembly. It is a nonexchangeable protein component and recently has been shown to incorporate into TRL in a fixed 1:1 ratio (25). Consequently, both total triglyceride and apo B can be used as alternative markers of chylomicron secretion, with apo B quantifying the total number of secreted lipoproteins and the triglyceride:apo B ratio indicating the degree of lipid loading of secreted lipoproteins. The level of apo B secretion (Fig. 3a) in the presence of increasing concentrations of Pluronic® L81 followed a similar trend to that of chylomicron secretion (Fig. 1) with a concentration-dependent reduction. However, observed inhibition of apo B secretion was not as pronounced and was significant only at the higher concentration of 0.012% (w/v), as compared to 0.006% (w/v) for chylomicrons. The secreted triglyceride:apo B ratio decreased (Fig. 3b) in a similar manner to that previously observed for chylomicrons (Fig. 1), with significant inhibition at 0.006% (w/v). These results indicate that Pluronic® L81 caused a reduction in not only the number but also the lipid-loading capacity of secreted lipoproteins.

Over the concentration range studied, Pluronic® L81 did not alter the total amount of triglyceride synthesized intracellularly. However, the proportion of intracellular triglyceride secreted into the basolateral compartment did decrease with an increase in excipient concentration (Fig. 4). This indicates that the inhibitory effects of Pluronic® L81 are on the final assembly or secretion steps of TRL metabolism rather than on the intracellular synthesis of triglyceride.

In a separate and single experiment, Pluronic® L81 0.006% (w/v) was applied to the basolateral side of the monolayers only, and "fed-state" vehicle was added to the apical surface of cells. After a 20-h incubation, basolateral contents were analyzed for lipoprotein content. Chylomicron secretion

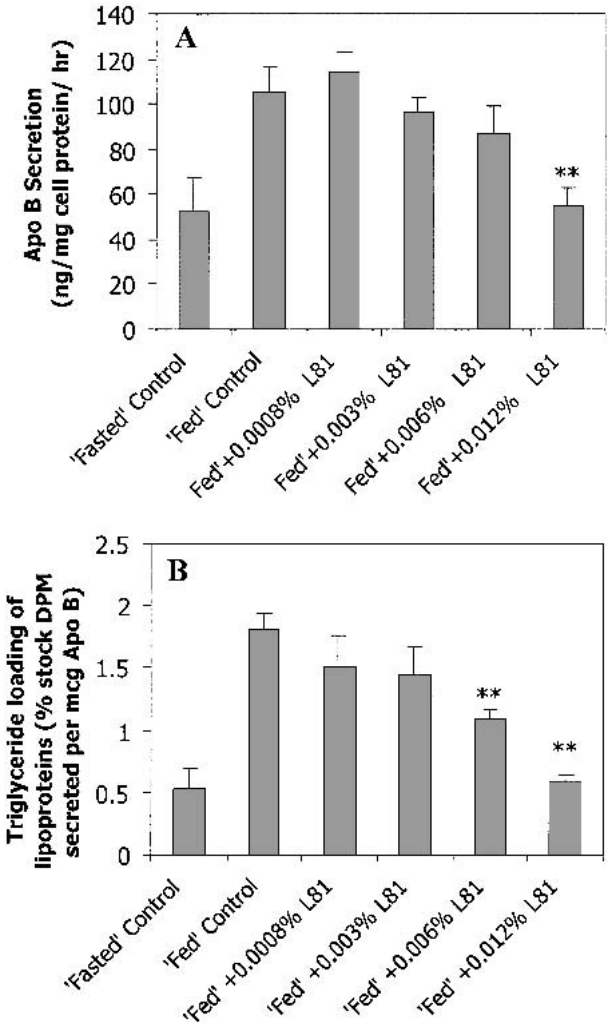

Fig. 3. Effect of increasing concentrations of Pluronic® L81 on apo B secretion (A) and on the triglyceride loading of secreted lipoproteins (B). Values represent the means of three to five monolayers ± SD. Statistical significance of Pluronic® L81 effects relative to the "fed-state" control is shown: **$p < 0.01$.

was found to be decreased by >90% ($p < 0.01$) relative to "fed-state." This shows that effects of Pluronic® L81 are not confined to the apical membrane and may indicate an intracellular target for inhibition.

Addition of Cremophor® EL to Fed-State

Addition of Cremophor® EL to the "fed-state" vehicle at 0.01% (w/v) did not modify TRL secretion relative to the "fed-state" control. However, at concentrations above this (0.1 and 1% w/v), a dose-dependent inhibition of chylomicron secretion was observed, with >70% inhibition of chylomicron secretion relative to "fed-state" at a concentration of 0.1% (w/v) (Fig. 1). No inhibition of VLDL secretion was observed up to 0.1% (w/v) (Fig. 2); however, at the higher concentration of 1% (w/v), approximately 80% inhibition ($p < 0.01$) was observed.

Recovery of Lipoprotein Secretion

In order to assess if the inhibitory effects of Pluronic® L81 (0.006% w/v) on chylomicron secretion (Fig. 1) were reversible, a recovery experiment was performed. In this experiment, triglyceride output (isolated via TLC and quantified via liquid scintillation counting) was used as an indicator of chylomicron secretion, as the large number of samples and relatively low secretion levels at given intervals made ultracentrifugal separation impractical. The secretion profile for "fed-state" control vehicle showed a 2.5-h lag period, with maximum rate of triglyceride secretion being reached at approximately 10 h. In the presence of Pluronic® L81, a reduced rate of secretion was observed, with significant differences ($p < 0.05$) occurring from 5 h onward (Fig. 5a).

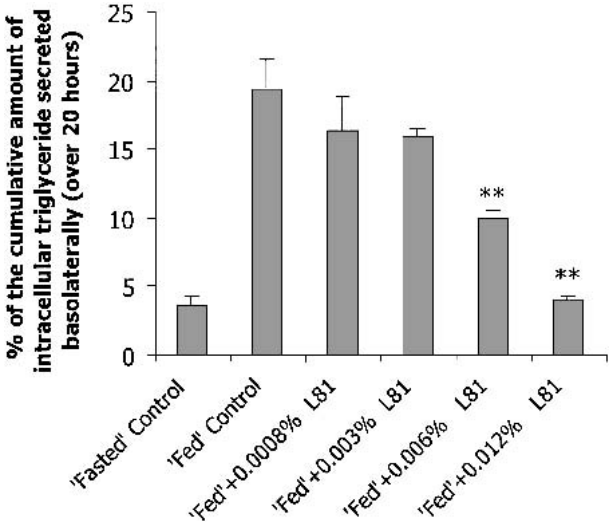

Fig. 4. Effect of increasing concentrations of Pluronic® L81 on the triglyceride secretion efficiency. Values represent the means of three to five monolayers ± SD. Statistical significance of Pluronic® L81 effects relative to the "fed-state" control is shown: **$p < 0.01$.

Following the washout step, cells were administered a "fed-state" recovery vehicle. Cells previously treated with Pluronic® L81 recovered to give secretion profiles similar to "fed-state" control (not previously treated with Pluronic® L81) (Fig. 5b). This observation of acute and reversible inhibition of Pluronic® L81 is consistent with *in vivo* findings in the rat (5). The plateau reached by cells given a recovery "fed-state" vehicle (i.e., cells previously exposed to "fed-state" plus or minus Pluronic® L81) tended to be slightly, but not significantly, higher than that initially reached by "fed-state" exposure (Fig. 5b). Prior exposure to fatty acid may explain this observation.

A control "fasted-state" BSA vehicle (labeled with trace amounts only of [^{14}C]oleic acid or [^{3}H]oleic acid) was administered concomitantly in both phases (Fig. 5) of the experiment to monitor the background secretion level. In each case, cells demonstrated minimal triglyceride secretion.

Relationship between Chylomicron Secretion and Active Efflux Activity

To investigate a possible link between inhibition of P-gp and triglyceride-rich lipoprotein secretion, permeability studies were performed using CsA, a known substrate for P-gp, in the presence of Pluronic® L81 and Cremophor® EL. With increasing concentrations of Pluronic® L81, P-gp–mediated efflux (basolateral to apical movement) of CsA was found to decrease in parallel with inhibition of chylomicron secretion

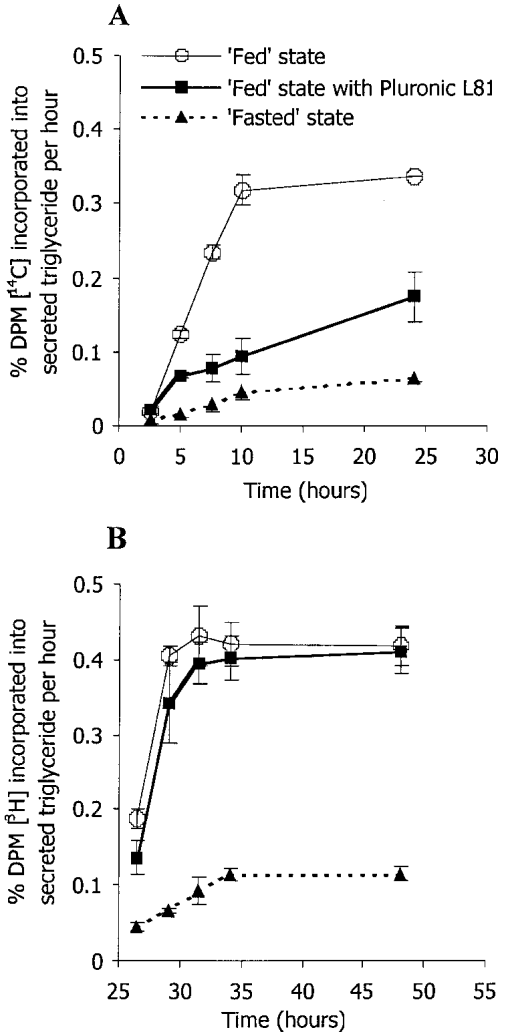

Fig. 5. Effect of coadministered Pluronic® L81 (0.006% w/v) on the rate of triglyceride secretion (expressed as the percentage of [^{14}C]oleic acid incorporated into triglyceride per hour) over a 24-h period in "fed" stimulated Caco-2 monolayers (A) and on the rate of triglyceride secretion (expressed as the amount of [^{3}H]oleic acid incorporated into triglyceride per hour) following washout step at 24 h and administration of recovery [^{3}H]oleic acid–labeled "fed-state" vehicle (B). Values represent the means of four monolayers ± SD.

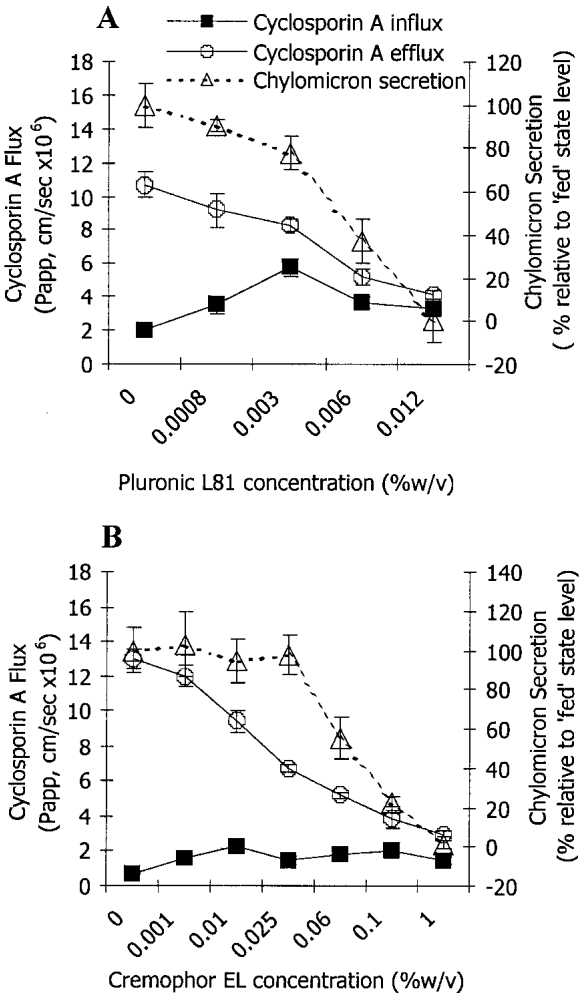

Fig. 6. Comparison of the inhibitory effects of Pluronic® L81 (A) and Cremophor® EL (B) on cyclosporin A flux (apical–basolateral transport and basolateral–apical transport) and chylomicron secretion. Values represent the means of three or four monolayers ± SD.

(Fig. 6a). Below the CMC, at 0.003% (w/v), the inhibition of efflux was significant ($p < 0.05$). A decrease in chylomicron secretion was also observed; however, this was not significant because of higher variability. Inhibited efflux activity by Pluronic® L81 at submicellar concentrations has been previously reported and attributed to surfactant "unimers" (4). At the CMC (0.006% w/v) and above, Pluronic® L81 produced significant inhibition of both chylomicron secretion and efflux activity ($p < 0.05$).

In contrast, Cremophor® EL did not significantly inhibit either chylomicron secretion or efflux activity at its CMC (0.01% w/v). At a higher concentration of 0.025% (w/v), significant inhibition of efflux ($p < 0.05$) was observed without a corresponding decrease in chylomicron secretion (Fig. 6b). However, when the concentration was increased further (>0.025% w/v), Cremophor® EL induced inhibition of both P-gp efflux and chylomicron secretion to a similar extent. CsA influx (apical-to-basolateral movement) initially increased with increasing concentration of both surfactants because of reduced drug efflux. However, at concentrations approaching the CMC, the influx of CsA decreased again. This effect was particularly evident with Pluronic® L81 (Fig. 6a) and may be caused by a reduction in free CsA concentration following micelle formation and solubilization. Similar decreases in the influx of a model peptide in the presence of Cremophor® EL, at and above 1% (w/v), have been reported (3).

DISCUSSION

The potential benefits of lipid-based vehicles for enhancing oral absorption of lipophilic drugs, including the possible promotion of lymphatic uptake via chylomicron association, have recently been reviewed (1). The majority of such research has been conducted in whole-animal models (1). The results presented here demonstrate that the Caco-2 model is capable of distinguishing between the effects of different pharmaceutical excipients on lipoprotein production and secretion. The response of the cells to Pluronic® L81 clearly shows similarities with *in vivo* rat data, especially in relation

to the preferential inhibition of chylomicron vs. VLDL secretion (5) and in the reversible nature of inhibition on withdrawal of the excipient (5). Such findings imply a role for the Caco-2 cell model in the initial screening of pharmaceutical vehicle effects on lipid metabolism.

The results demonstrate that the inhibitory effects previously observed for Pluronic® L81 on TRL secretion also apply to other members of the Pluronic® block copolymer family as well as to Cremophor® EL, an ethoxylated caster oil derivative, thus suggesting that this feature may be associated with a larger range of nonionic surfactants. Chylomicron secretion was seen to be more sensitive to inhibition than VLDL secretion. This is consistent with the theory of alternative paths of assembly for these two TRL classes (26).

Of particular note in the current study is the observed apparent HLB dependence of the response to Pluronic® block copolymers. The more lipophilic derivatives (L81 and P85, lower HLB values) proved to be more potent inhibitors of lipoprotein secretion in Caco-2 cells than Pluronic® F68 (HLB > 24). Such HLB dependence has also been observed in relation to P-gp efflux activity as monitored using a rhodamine-123 probe in multiple-drug-resistant (MDR) cancer cells (27). Pluronic® block copolymers have been shown to deplete intracellular levels of ATP (28). P-gp is a member of the ATP-binding cassette (ABC) transporter protein superfamily and hence is dependent on ATP for its efflux activity. Thus, in addition to possible membrane fluidization effects, energy depletion has been implicated as an important contributor to the P-gp inhibitory activity observed for this group (29). Indeed, the greater P-gp inhibitory potential of the more hydrophobic derivatives has been largely attributed to their ability to enter cells and to interact with intracellular organelles. Recent findings in bovine brain microvessel endothelium cells (BBMEC), using a range of fluorescently labeled block copolymers, reveal that ATP-depleting activity of Pluronic® block copolymers can be correlated to their ability to gain access to intracellular sites. More hydrophobic derivatives (HLB < 19) are effectively transported within cells, whereas hydrophilic derivatives (HLB 20–29) fail to enter (29). It is possible that such a mechanism may be relevant to our findings, especially if energy-dependent processes are involved in the final assembly or secretory steps of the TRL pathway.

The observation of a lipoprotein inhibitory effect following basolateral (vs. apical) application of Pluronic® L81 in our work indicates that the effects are not confined to the apical membrane and further supports the concept of an intracellular site of activity.

A significant cross-sensitivity of P-gp efflux and chylomicron secretion to the inhibitory effects of Pluronic® L81 and Cremophor® EL was observed. This suggests that an association exists between the two biochemical processes at the intestinal level, possibly through involvement of ABC-related proteins or other energy-dependent processes in lipid transport. This concept is supported by the work of Field *et al.* (9), which demonstrated >90% inhibition of basolateral triglyceride secretion with 50 μM verapamil, a recognised nonsurfactant P-gp inhibitor. In addition, recent findings indicate the direct involvement of ABC-family proteins in human lipid metabolism. Berge *et al.* (30) reported that mutations in the genes encoding two members of this family (ABCG5 and ABCG8) may be responsible for the excessive intestinal absorption and impaired biliary output of sterols observed in the autosomal recessive condition sitosterolemia.

In summary, the results presented here show that P-gp efflux activity and chylomicron secretion are sensitive to manipulation by a similar range of pharmaceutical surfactants. Such excipients are frequently used in the design of lipid-based oral delivery systems for lipophilic drugs. Although inhibition of P-gp may result in an increase in absorption, a simultaneous inhibition of chylomicron secretion may result in altered drug distribution by reducing the potential for chylomicron-mediated lymphatic transport. Thus, simultaneous manipulation of cellular processes may dramatically affect oral drug absorption. Consequently, the bioactive nature of excipients should be given serious consideration in formulating oral drug delivery systems.

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