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Research paper

Controlling the gastrointestinal fate of nutraceutical and pharmaceutical-enriched lipid nanoparticles: From mixed micelles to chylomicrons

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ABSTRACT

The oral bioavailability of lipophilic bioactive compounds such as many pharmaceuticals and nutraceuticals can be enhanced using triacylglycerol-based lipid nanoparticle delivery systems. These digestible lipid nanoparticles are dissembled in the gastrointestinal tract to form mixed micelles that solubilize and transport the lipophilic bioactives to the intestinal epithelium cells where they are absorbed. In these cells, the lipid digestion products and bioactive agents contained within the mixed micelles are then packaged into biological lipid protein nanoparticles (e.g., chylomicrons) that are secreted into the lymph. In this study, we examined the influence of fatty acid type (i.e., oleic acid, linoleic acid, and linolenic acid) on the properties of mixed micelles, cellular lipid droplets, and lipoprotein nanoparticles, and on the bioavailability of a highly lipophilic nutraceutical: 5-demethylnobiletin (5DN). There were distinct differences in the structural properties of lipoprotein nanoparticles formed depending on fatty acid unsaturation. Oleic acid ($C_{18:1}$) was most effective in enhancing intestinal uptake of 5DN and led to the formation of the largest chylomicrons. Linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) also promoted intestinal uptake of 5DN and formation of chylomicrons, but they were less efficient than oleic acid. The metabolism of 5DN within the intestinal epithelium cells was greatly reduced when 5DN was incorporated into chylomicrons, presumably because they were isolated from metabolic enzymes in the cytoplasm. These results have important implications for the rational design of lipid nanoparticle-based delivery systems for lipophilic nutraceuticals and pharmaceuticals by targeting them to the lymphatic circulation.

1. Introduction

Oral administration of pharmaceuticals and nutraceuticals has been considered to be a more convenient and desirable delivery route where possible, since other routes such as intravenous delivery are often painful, inconvenient, and burdensome (Ng et al., 2011). However, many of these bioactive compounds are so highly lipophilic that they have relatively low and variable oral bioavailability (Gonnet et al., 2010; Porter et al., 2007). Hence their potential beneficial health effects are not fully realized due to their poor absorption and extensive metabolism by the human body. An effective way to enhance the oral bioavailability of lipophilic bioactive compounds is to encapsulate them within delivery systems containing digestible lipids (McClements et al., 2009; Porter et al., 2007; Yeap et al., 2013).

Triacylglycerols (TGs) are the most common form of digestible lipid used in lipid-based delivery systems, with over 95% of them typically being digested within the human gastrointestinal tract (GIT) (Williams et al., 2013). After ingestion, TGs are hydrolyzed by digestive enzymes (lipases) in the stomach and small intestine to form free fatty acids and monoacylglycerols (Williams et al., 2013). These lipid digestion products interact with biological surfactants secreted by the body (e.g., phospholipids, bile salts, and cholesterol) to form various nanostructured assemblies collectively known as "mixed micelles". These mixed micelles have hydrophobic regions capable of incorporating lipophilic bioactive agents, e.g., the non-polar interior of micelles or the non-polar bilayers of liposomes. The mixed micelles can then travel through the intestinal lumen, across the mucus layer, and to the apical side of the epithelial cells, where they release their contents for absorption by various passive and active transport mechanisms.

After absorption by the epithelium cells, the biological fate of lipophilic molecules depends on their molecular characteristics as well as that of any co-absorbed lipid digestion products (Porter et al., 2007;

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Yanez et al., 2011). For example, after intracellular trafficking, medium-chain or short-chain fatty acids may directly enter the portal vein, while long-chain fatty acids are likely incorporated into chylomicrons and then transported to the lymph, thus avoiding the first pass metabolism in the liver (Shen et al., 2001). A chylomicron (CM) is a biological lipid nanoparticle assembled in the epithelium cells that consists of a hydrophobic core containing TGs, cholesterol, and lipophilic bioactives, and a hydrophilic shell consisting of phospholipids and proteins (Bateman et al., 2007).

Previous studies have reported that the formation and properties of chylomicrons depend on the nature of the fatty acids entering the epithelium cells. Monounsaturated fatty acids and polyunsaturated fatty acids were found to produce larger CMs than saturated fatty acids (Bateman et al., 2007). Oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids were also reported to promote greater secretion of CMs than steric ($C_{18:0}$) and palmitic ($C_{16:0}$) acids (van Greevenbroek and de Bruin, 1998). Studies have also shown that the bioavailability of ingested bioactive components depended on the nature of the fatty acids they are ingested with (Holm et al., 2001; van Greevenbroek and de Bruin, 1998). However, there is not always a close correlation between the bioavailability of lipophilic bioactive agents and the nature of the CMs formed. For example, oleic acid was found to stimulate the formation of larger and more numerous CMs than linoleic acid, but the lymphatic transport of a lipophilic bioactive component (halofantrine) was reported to be higher for linoleic acid than oleic acid (Holm et al., 2001; van Greevenbroek and de Bruin, 1998). This means that there is not always a simple correlation between the production of CMs and the lymphatic transport of bioactive components.

Our previous research using a Caco-2 cell culture model demonstrated that mixed micelles, consisting of oleic acid and sodium taurocholate, increased the trans-intestinal transport of an encapsulated lipophilic nutraceutical by promoting its incorporation into chylomicrons (Mingfei Yao et al., 2013). The purpose of the current study was to examine the influence of fatty acid type ($C_{18:1}$, $C_{18:2}$, $C_{18:3}$) on the formation and structure of mixed micelles, lipid droplets, and chylomicrons, as well as on the incorporation of a bioactive lipophilic agent into the chylomicrons. This study is particularly important as polyunsaturated fatty acids have been increasingly utilized in the human diet due to their potential beneficial health effects in human (Nelson, 2005) (Abeywardena and Head, 2001; Djousse et al., 2005; Mozaffarian and Wu, 2011; Wijendran and Hayes, 2004). The knowledge gained from this study could be used to rationally design lipid-based delivery systems for pharmaceuticals and nutraceuticals with improved efficacy by oral administration.

2. Materials and methods

2.1. Materials

The following products were purchased from Sigma Chemicals (St. Louis, MO): OsO4, oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$), taurocholic acid (TC), sulfatase from *Heli pomatia*, phosphatungstic acid (PTA) and Optiprep™ density gradient medium. All other chemicals and solvents were of analytical grade and were obtained from Fisher Scientific (Pittsburgh, PA). Apolipoprotein B (Apo B) human Elisa kit was purchased from Abcam (Cambridge, MA). 5-Demethylnobiletin (5-hydroxy-6, 7, 8, 3′, 4′-pentamethoxylflavone, 5DN) was synthesized as we described previously (Zheng et al., 2013).

2.2. Cell culture

Caco-2 cells (passage 55– 65) were cultured in complete Dulbecco's modified essential medium (DMEM) containing high glucose, 10% fetal bovine serum (FBS), 1% antibiotic, and 1% amino acids. Cells were seeded at 3×10^5 cells/mL on transwells (Corning Inc., MA, USA) containing polyester filters (3 μm pore size and 4.7 cm^2 surface area) and grown for 21 days. The transepithelial electrical resistance (TEER) was measured at 37 °C using a Millicell® ERS-2 epithelial voltammeter (World Precision Instruments, Sarasota, FL). Data were expressed as $\Omega \times cm^2$. Before the start of different fatty acid treatments, Caco-2 monolayers were washed and incubated for 4 h with serum-free complete medium as described previously (Mingfei Yao et al., 2013).

2.3. Preparation of mixed micelle systems

Mixed micelles systems containing different types of fatty acids were prepared using a method described previously (Mingfei Yao et al., 2013). Three types of mixed micelles were prepared: oleic acid-sodium taurocholate ($C_{18:1}$-TC); linoleic acid-sodium taurocholate ($C_{18:2}$-TC) and linolenic acid-sodium taurocholate ($C_{18:3}$-TC), with the same fatty acid-to-TC molar ratio of 1.6:0.5. A known amount of fatty acids was added to a TC solution and then homogenized with a sonicator at 4 °C. The solutions were then swirled overnight under a nitrogen atmosphere before being stored at −20 °C prior to use. The particle diameter (Z-average) and charge (ζ-potential) were measured using a combined dynamic light scattering/particle electrophoresis instrument (NanoZS, Malvern Instruments, Malvern, UK). Samples were diluted with DMEM prior to their application to cell culture.

2.4. Quantification of 5DN and its metabolites

Caco-2 cell monolayers were cultured in trans-well for 21 days, then were incubated with cell culture media containing $C_{18:1}$-TC-5DN, $C_{18:2}$-TC-5DN, $C_{18:2}$-TC-5DN or 5DN at the apical side. Aliquots of basolateral medium (100 μL) were collected at 1, 2, 4, 8 and 24 h, and replaced with equal volume of complete basolateral medium each time. The apical medium and the cell monolayers were collected at 24 h. Samples were extracted twice with ethyl acetate to obtain 5DN and its metabolites, i.e., 5, 3′-dihydroxy-6, 7, 8, 4′-tetramethoxylflavone (M1), 5, 4′-dihydroxy-6, 7, 8, 3′-tetramethoxylflavone (M2) and their corresponding conjugates M1′ and M2′. Selected samples were incubated with sulfatase at 37°C to deconjugate M1′ and M2′ before being extracted with ethyl acetate. Extracts were dried under vacuum. The resulting residue was then dissolved with 100 μL of mobile phase for HPLC analysis. The concentrations of 5DN and its metabolites were determined using a HPLC system (CoulArray®, Chelmsford, MA, USA) equipped with a multi-channel electrical conductivity detector (Model 6210, CoulArray®, Chelmsford, MA, USA) as we previously described (Dong et al., 2010; Zheng et al., 2015).

2.5. Isolation characterization of lipoprotein by TEM

Lipoproteins secreted to the basolateral side by Caco-2 cell monolayer were isolated by density gradient ultracentrifugation (Bateman et al., 2007). The media collected from the basolateral side were preserved in saline EDTA mixed with optiprep (60% iodixanol) at the ratio of 4:1 (v/v) with 9% idoixanol-PBS layered on top. The lipoproteins were separated by centrifuging at 28,000 rpm for 3 h in an ultracentrifuge (SW40 rotor, Beckman Coulter, Indianapolis, IN) at 20 °C. The top 1 mL ($d < 1.006$ g cm^{-3}) was collected for further analysis. Lipoproteins were stained by 4% OsO_4 and then 1% PTA before transmission electron microscopy (TEM, Philip, Tecnai 12) analysis.

2.6. Apolipoprotein B analysis

The medium in the basolateral side was collected and centrifuged at 2000 *g* for 10 min to remove any debris. Supernatants were collected and concentrated in Vivaspin tubes and then stored at −20 °C before analysis. Cells in the transwells were rinsed with ice-cold PBS containing 0.5 M EDTA and then scraped into a tube with PBS containing 0.5 M EDTA. The suspension was then centrifuged at 1000 rpm for 10 min at 4 °C and the supernatant was collected. The cells were then