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Inhibition of cholesterol transport into skin cells in cultures by phytosterol-loaded microemulsion

S. Rozner[a], L. Verkhovski[b], Y. Nissimov[b], A. Aserin[a], R. Vilensky[c], D. Danino[c,d], C.C. Zouboulis[e,f,g], Y. Milner[b,*], N. Garti[a,*]

[a] Casali Institute of Applied Chemistry, The Institute of Chemistry, Givat Ram Campus, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
[b] Myers Skin Biology and Biochemistry Laboratory, Department of Biological Chemistry, Institute of Life Sciences, Givat Ram Campus, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
[c] Department of Biotechnology & Food Engineering, Technion-Israel Institute of Technology, Haifa 32000, Israel
[d] Russell Berrie Nanotechnology Institute, Technion-Israel Institute of Technology, Haifa 32000, Israel
[e] Department of Dermatology, Venereology, Allergology and Immunology, Dessau Medical Center, Dessau, Germany
[f] Department of Venereology, Allergology and Immunology, Dessau Medical Center, Dessau, Germany
[g] Laboratory for Biogerontology, Dermato-Pharmacology and Dermato-Endocrinology, Institute of Clinical Pharmacology and Toxicology, Charité Universitaetsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany

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ABSTRACT

Cholesterol and plant phytosterols are lipophilic compounds solubilized by intestinal micelles in a competitive manner. In this work, we used radioactive cholesterol- and phytosterol-loaded oil-in-water microemulsions to follow their incorporation and mutual competition in HaCaT keratinocytes, SZ95 sebocytes, and skin pieces in cultures. Dynamic light scattering showed homogenous nanostructures of 10.5 ± 1.5 nm diameter and cryo-transmission electron microscopy confirmed the presence of uniform spherical droplets of 7.0 ± 1.0 nm diameter. Up to 320 nmol/ml of cholesterol can be solubilized and transported into cells with minimal toxic effect by 0.5 wt% nanodroplets in a cell medium. Phytosterols inhibit incorporation of cholesterol into cells, in vitro, at molar ratios (phytosterols/cholesterol) of 4 and above. The loaded nanodroplets accumulate in intracellular vesicles (presumably endosomes). No metabolic conversion of cholesterol or phytosterols was found in these cells, in vitro, after 24 h, at 37 °C.

1. Introduction

Phytosterols (PS), a group of plant sterols that are synthesized mainly in seeds, have structures similar to that of cholesterol (CH), but with an additional short carbon chain attached to carbon number 24 (Fig. 1, Ostlund, 2002).

Clinical studies showed that PS or their esters lower CH levels in mammals (Moghadasian et al., 1999; Pritchard et al., 2003), but the mechanism by which PS inhibits CH incorporation in the gut membrane is still not completely clear. However, kinetic effects, mutual inhibition on intracellular receptor(s), and specific competition with CH transporters have been proposed (Trautwein et al., 2003; Ikeda et al., 2002; Kaneko et al., 2003; Field and Mathur, 1983). Specifically, the potential steps at which PS can interfere with CH intake could be: (1) competitive solubilization in micelles, (2) mutual interference in the co-crystallization of CH and PS from the giant micelles, (3) mutual interference effects at the binding site(s)—for instance, in hydrolysis by lipases and esterases (Ikeda et al., 2002), or (4) interference by competing with transporters and/or inhibiting enzymes in the intracellular transportation system active in the intestinal transport of CH from the intestinal lumen (Kaneko et al., 2003; Field and Mathur, 1983).

Both PS and CH are lipophilic phase soluble materials, and as such are encapsulated into vehicles that have hydrophobic cores and hydrophilic surfaces. In this study we used U-type modified microemulsions (Spernath et al., 2003; Garti et al., 2004) as vehicles for transporting CH and PS molecules into skin cells. Microemulsions were formed from blends of nonionic surfactants, oil phase, and co-solvent (polyols). The multi-component system is fully miscible with water/salt solutions and has many unique physical properties of Newtonian fluids, forming transparent monodispersed droplets of about 10 nm diameter (Spernath and Aserin,

* Corresponding authors. Tel.: +972 2 658 6574/5; fax: +972 2 652 0262; Tel.:+972 2 658 5051; fax:+972 2 658 5429.
E-mail addresses: milner@vms.huji.ac.il (Y. Milner), garti@vms.huji.ac.il (N. Garti).

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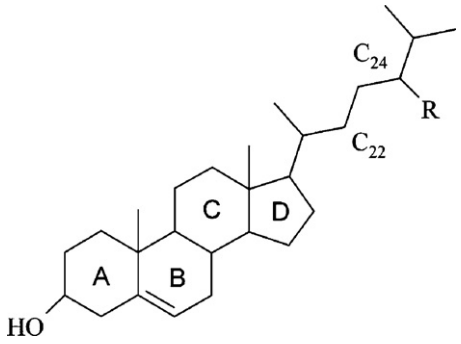

Fig. 1. Molecular structure of cholesterol (CH) and some abundant phytosterol (PS) types. R = H, cholesterol; R = CH_2CH_3, β-sitosterol; R = CH_2CH_3 with an additional double bond at C_{22}, stigmasterol; R = CH_3, campesterol; R = CH_3 with an additional double bond at C_{22}, brassicasterol.

2006). These were shown to increase the permeability of the solutes and to facilitate transdermal transport (Spernath et al., 2003; Garti et al., 2004).

It is known that CH is also synthesized and transported into skin cells by the same, drug inhibitable transporters that function in the small intestine (Jiang et al., 2006; Jiang et al., 2005; Ponec et al., 1992; Smythe et al., 1998). An in vitro study conducted on keratinocytes showed that PS delivered into cells by micelles or liposomes or in solvents, can replace 30–50% of the CH in the keratinocyte membranes and affects the membrane composition and fluidity (Mora et al., 1999). In another study, it was found that β-sitosterol can be used as an anti-aging agent while stigmasterol cannot, and that PS can regulate some enzyme activities in keratinocytes (Mora-Ranjeva et al., 2006). In light of the importance of CH in the preservation of trans-epidermal water loss (Ishi and Mikami, 1996; Schurer and Elias, 1991; Zettersten et al., 1997; Feingold et al., 1990; De Paepe et al., 2002) and in steroid synthesis (Thiboutot et al., 2003; Hoffmann, 2001), the role of PS in the skin in general and in its appendages, in particular, should also be investigated. Sebaceous glands, for example, are target organs for androgenic steroids and produce sebum, which in humans is uniquely composed of about 12% squalene, an intermediate in the CH biosynthesis pathway (Zouboulis, 1996).

Using the model systems of skin in organ and keratinocyte and sebocyte cultures in vitro, we show in this study that in microemulsion, PS and CH compete for binding to skin cells. Furthermore, through encapsulation of water-insoluble components such as nutraceuticals, CH and PS microemulsions increase their solubilization and may also increase their incorporation efficiency, a good indicator of a potential clinical application for microemulsions (Spernath and Aserin, 2006).

2. Materials and methods

4-[^{14}C]-cholesterol (4-[^{14}C]-CH), with a specific radioactivity of 56 mCi/mmol, was purchased from Amersham (Buckinghamshire, UK); and 4-[^{14}C]-β-sitosterol with specific radioactivity of 53 mCi/mmol, was purchased from ARC (St. Louis, MO, USA); Tween 60, triacetin (99% purity), CH (98% purity), stigmasterol (~95% purity), and β-sitosterol (from soybean, ≥97% purity) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tocopherol (96% purity) and PS (40–58% β-sitosterol, 20–30% campesterol, 14–22% stigmasterol, and 0–6% brassicasterol) were purchased from ADM Nutraceuticals (Decatur, IL, USA). TLC plates (10 cm × 10 cm and 20 cm × 20 cm DC-plastic sheets of Kieselgel 60 containing F254) were purchased from Merck (Whitehouse Station, NJ, USA). Sebomed media for sebocyte cultures were from Biochrom AG (Berlin, Germany). Dulbecco's modified Eagle's medium (DMEM) keratinocyte growth medium and phosphate-buffered saline (PBS) were purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Trypsin was purchased from USB (Staufen, Germany). Other analytical grade laboratory reagents used in this research were purchased from various other companies.

2.1. Microemulsion preparation

Concentrated microemulsion was prepared by mixing 30 wt% oil phase (comprising triacetin, tocopherol, and ethanol in a 1:1:2 weight ratio) with 70 wt% surfactant (Tween 60). Non-radioactive materials (i.e., PS and CH) were solubilized into the concentrated microemulsion at 90 °C for 2–5 min in sealed Viton-corked tubes (to prevent ethanol evaporation) to form a transparent, solution-like system, termed the "concentrate" (100 wt%). In the case of radioactive materials (i.e., 4-[^{14}C]-CH or 4-[^{14}C]-β-sitosterol), the organic solvent content was dried under a N_2 stream before solubilization into the concentrated microemulsion solution by stirring. After the sterol-loaded microemulsions were vortexed and cooled to room temperature, the concentrated microemulsion solutions were diluted 10–20 times with buffer A (10 mM HEPES, 150 mM NaCl, pH 7.24) or by PBS.

For direct use on the cells, this microemulsion system was diluted by MDEM cell medium to a 0.05–0.5 wt% microemulsion. Identically loaded microemulsions containing different active species (i.e., 4-[^{14}C]-CH vs. PS or vs. non-labeled CH) were combined in variable volume ratios, creating different molar ratios, with 4-[^{14}C]-CH in the microemulsions, as above. Every experiment also included a control sample consisting of an empty microemulsion suspension that was added to the 4-[^{14}C]-CH-loaded microemulsion used in the experiments.

2.2. Cryogenic-transmission electron microscopy (cryo-TEM)

Samples were equilibrated at 25 °C in Controlled Environment Vitrification System (CEVS) (Bellare et al., 1988), or in the Vitrobot (FEI) for 20 min and the chamber were saturated with water and respective solvents to avoid evaporation of volatile components during specimen preparation. Vitrified specimens were prepared on a 400 mesh copper grid coated with a perforated formvar film (Ted Pella). A small drop (5–8 μl) was applied to the grid and blotted with filter paper to form a thin liquid film of solution. The grid was plunged into liquid ethane at its freezing point (−196 °C). The procedure was performed manually in the CEVS and automatically in the Vitrobot.

The vitrified specimens were stored in liquid nitrogen. Some samples were examined in a Philips CM120 transmission electron microscope, operated at 120 kV, using an Oxford 3500 cryo-holder maintained below −178 °C. Images were recorded on a Gatan 791 MultiScan cooled charge-coupled device (CCD) camera. Other samples were studied using a Philips Tecnai 12 G2 TEM, at 120 kV with a Gatan cryo-holder maintained below −173 °C, and images were recorded on an Ultrascan 1000 2 k × 2 k CCD camera. In both microscopes images were recorded with the digital micrograph software package at low dose conditions to minimize electron beam radiation damage (Danino et al., 2001). Brightness and contrast enhancement were done using the Adobe Photoshop 7.0 ME package.

2.3. Dynamic light scattering (DLS)

The hydrodynamic radius (R_H) of non-loaded controls and droplets loaded with CH, PS, and a 1:1 CH/PS mole ratio was determined. The microemulsion droplets contained 5 wt% oil and