JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2005, 53, 4623–4627 4623

Naturally Derived Micelles for Rapid in Vitro Screening of Potential Cholesterol-Lowering Bioactives

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A high plasma cholesterol level, especially low-density lipoprotein cholesterol, indicates increased risk of cardiovascular diseases. Plasma cholesterol levels are influenced by diet and cholesterol biosynthesis, uptake, and secretion. Cholesterol uptake involves solubilization into complex phospholipid spherical bodies termed micelles that facilitate the transport of lipids through the gut brush border membrane into enterocytes. In vitro assays reported to date to determine potential cholesterol-lowering effects of various compounds require artificial micelle preparations that are elaborate and time-consuming to prepare. The aims of this study were to compare the efficacy of artificially prepared micelles with naturally derived micelles from pig's bile and to test their ability to assess potential inhibitors of cholesterol uptake. The suitability of pig's bile-derived micelles was tested both at the level of the micelle and at cellular uptake using cultured Caco-2 cells. Known cholesterol uptake inhibitors at the micelle (green tea catechins) and at the Caco-2 cell (β-lactoglobulin-derived peptide, IIAEK) were used as reference inhibitory compounds. It was concluded that pig's bile was a rapid, reproducible, convenient, and cost-effective source of micelles for cholesterol micelle solubility and cellular uptake assay systems and is suitable for screening purposes focused on identifying potential cholesterol-lowering agents.

KEYWORDS: Micelle; pig's bile; Caco-2 cells; catechins; IIAEK; cholesterol

INTRODUCTION

An elevated plasma cholesterol concentration is widely regarded as a primary risk factor for atherosclerosis and coronary heart disease (*1*). Recent scientific data have suggested that drinking green tea (*2*) or consumption of plant sterols (*3*) can reduce plasma cholesterol levels and may lower the risk of heart disease. Functional foods have become an emerging trend in the food industry and have been a driving force in world food markets (*4*). As the functional food/nutraceutical market is growing rapidly, there is continued interest to discover novel bioactives with potential health benefits. Accordingly, in vitro based assays for rapid screening of candidate compounds are an indispensable tool in the identification of lead compounds. To date, a variety of food constituents have functionality that can modify blood lipid profiles and promote cardiovascular health. Functional food ingredients now available in the market include plant sterols, green tea, omega-3 fatty acids, grape seed polyphenols, and some herbs (*5*, *6*).

Plasma cholesterol levels are determined by the balance between diet, cholesterol biosynthesis, cholesterol excretion in bile acids from the liver, and cholesterol absorption and reuptake by the small intestine. Dietary lipids are first emulsified in the jejunal lumen by bile salt and lecithin and then encapsulated into micelles. Micelles are water-soluble polymolecular aggregates that can cross the unstirred water layer and therefore facilitate lipid uptake into mucosal cells through the brush border membrane (*7*). Inhibition of cholesterol solubilization into micelles and cellular cholesterol absorption are of growing interest as target sites of interventions for cholesterol reduction. Accordingly, assays have been developed to evaluate natural products aimed at lowering plasma cholesterol levels.

Artificial micelles have been used as a model system for in vitro cholesterol solubilization (*8*, *9*). As an adjunct, monolayer enterocyte-like Caco-2 cells represent a convenient and reproducible model system to study intestinal lipid metabolism and have been used to evaluate cholesterol absorption using the artificially prepared micelles (*8*). However, the preparation of micelles is a time-consuming and relatively expensive process. Preferably, high-throughput screening processes for the evaluation of candidate food constituents as potential cholesterol-lowering agents should be quick, simple, reproducible and cost-effective. In this paper, we describe the use of pig's bile as a convenient, rapid, and reliable source of natural micelles for cholesterol solubilization and cholesterol absorption into the

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10.1021/jf050447x CCC: $30.25 © 2005 American Chemical Society
Published on Web 05/10/2005

Caco-2 cell system. Pig's bile was chosen because of its availability and because the diet, physiology, and bile of pigs closely model those of humans (*10*). Catechin gallates (*9*, *11*, *12*) and bovine milk peptides from tryptic digestion of β-lactoglobulin (*13*) have been shown to inhibit cholesterol solubilization and uptake into micelles and Caco-2 cells, respectively, and were used as reference inhibitory compounds.

MATERIALS AND METHODS

Bile and Chemicals. Approximately 1 L of bile from the liver of pigs was collected from a local abattoir and placed on ice. Sodium taurocholate, cholesterol, oleic acid, phosphatidylcholine, monoolein, Dulbecco's modified Eagle's medium (DMEM), erythromycin, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), sodium dodecyl sulfate (SDS), gallic acid (GA), catechin, catechin gallate (CG), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), solvents, and fine chemicals were purchased from Sigma (Sydney, NSW, Australia). Cholesterol standard was purchased from Roche Diagnostics Corp. (Indianapolis, IN). The milk-derived pentapeptide, Ile-Ile-Ala-Glu-Lys (IIAEK), was custom produced by AusPep (Melbourne, Australia). Medium-199/Earle's and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). [1,2-^{3}H]Cholesterol (37 MBq/mL) was purchased from Perkin-Elmer Life Science (Boston, MA). Ultima Gold was purchased from Packard Bioscience (Meriden, CT).

Preparation of Artificial Micelles. Micelles were prepared according to the method of Raederstorff (*9*) with some minor modifications. Lipids, 0.5 mM cholesterol, 1 mM oleic acid, and 2.4 mM phosphatidylcholine, were dissolved in methanol and dried before adding to a 15 mM phosphate-buffered saline (PBS) containing 6.6 mM taurocholate salt, at pH 7.4. The suspension was sonicated twice for 1 min using Branson sonifier cell distruptor B-15 (Danbury, CT) using standard microtip $^1/_8$ in. on 50% output (75 W). The micelle solution was incubated overnight at 37 °C. EGCG (and other inhibitors as indicated) or equivalent PBS as control was added to the mixed micelle solution and incubated for a further 1 h at 37 °C. The solution was then centrifuged at 1000*g* for 10 min and finally filtered through a 0.22 μm Millex-GP (Millipore, Bedford, MA). The concentration of cholesterol was determined on a COBAS MIRA analyzer (Roche Diagnostics). The particle size of micelles was measured using a Coulter counter (Beckman Coulter, Fullerton, CA) using latex monospheres as reference (14 μm).

In Vitro Cholesterol Uptake into Caco-2 Cells. Colon adenocarcinoma (Caco-2) cells were purchased from American Tissue Culture Collection (ATCC). Monolayers of cells were grown in 24-well plastic plates and maintained in DMEM supplemented with 10% (v/v) FBS, 20 mM HEPES, 4 mM L-glutamine, and 50 mg/L-erythromycin and incubated in 37 °C in a humidified atmosphere of 5% CO_2 in air with fresh media added every 2 days. Monolayers become confluent 3−4 days after seeding at 0.25 × 10^6 cells/mL. The cells were dispersed with PBS containing 0.25% (w/v) trypsin. The number of cell passages ranged from 16 to 20. Experiments used cultures of 12−14 days after plating and were performed in medium-199 containing 1 mM HEPES.

Cholesterol uptake into micelle preparations was performed with slight modifications of a method described by Nagaoka (*8*). The ^{3}H-labeled cholesterol artificial micellar solution was prepared as follows: 14.8 kBq of [1,2-^{3}H]cholesterol, 0.1 mM cholesterol, 1 mM monoolein, 5 mM phosphatidylcholine in chloroform, and 5 mM taurocholate salt in ethanol were dried under nitrogen and added with an equivalent volume of medium-199. The suspension was sonicated using a Branson sonifier cell distruptor B-15 using a standard microtip $^1/_8$ in. at 30 W for 3 min. The micelle solution was then incubated overnight at 37 °C.

After 13 days, Caco-2 cells were rinsed twice with PBS. A ^{3}H-labeled cholesterol containing micellar solution with IIAEK or equivalent PBS as control was added to cells and incubated at 37 °C for 45 min in the CO_2 incubator. After incubation, Caco-2 cells were rinsed twice with PBS and then lysed in 0.1% (w/v) SDS solution. Ultima Gold scintillant was added, and the radioactivity of the cellular debris was determined using liquid scintillation (Wallac 1410, Pharmacia).

Preparation of Pig's Bile. Fresh pig's bile recovered on ice was centrifuged at 2000*g* at 4 °C for 10 min to remove debris and was aliquotted into small tubes for storage at −80 °C. Cholesterol concentration in fresh and frozen pig's bile was determined by COBAS MIRA analyzer (Roche Diagnostics), and bile was adjusted to the desired assay cholesterol concentration. For the cholesterol micelle solubility assay, bile with cholesterol equivalent to 0.5 mM was added to 15 mM PBS. Inhibitors or equivalent PBS as control was added to the mixed micelle solution and incubated for 1 h at 37 °C. The solution was then centrifuged at 1000*g* for 10 min and filtered through a 0.22 μm Millex-GP. The concentration of cholesterol was determined on a COBAS MIRA analyzer.

For the cholesterol uptake assay, 14.8 kBq of [1,2-^{3}H]cholesterol and bile with cholesterol equivalent to 0.1 mM were added to medium-199 containing 1 mM HEPES. A ^{3}H-labeled cholesterol containing micellar solution with IIAEK or equivalent PBS as control was added to the cells and then incubated at 37 °C for 45 min in the CO_2 incubator. After incubation, Caco-2 cells were rinsed twice with PBS and then lysed in 0.1% (w/v) SDS solution. Ultima Gold scintillant was added, and the radioactivity of the cellular debris was determined using liquid scintillation (Wallac 1410, Pharmacia).

Statistics. All data are expressed as means ± standard deviation (SD) of at least three replicates. The statistical significance of differences was evaluated by Student's *t* test using InStat Graph Pad 3.0.

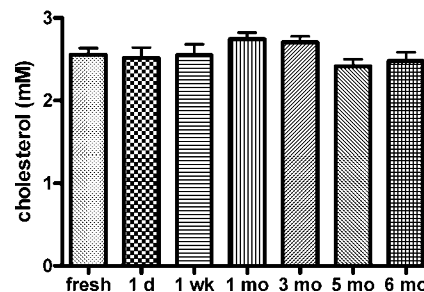

Figure 1. Concentration of cholesterol in a batch of bile, fresh, and the effect of storage at –80 °C for up to 6 months. Values are expressed as means ± SD from at least three determinations.

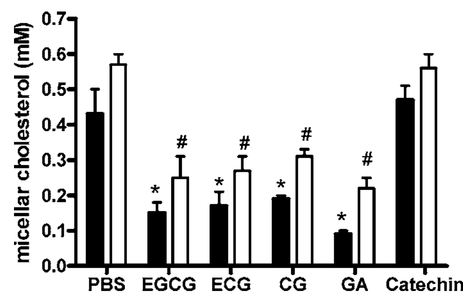

Figure 2. Effects of epigallocatechin gallate (EGCG), epicatechin gallate (ECG), catechin gallate (CG), gallic acid (GA), and catechin (all 1 mM) on micellar solubility of cholesterol in vitro in artificially prepared (solid bars) and pig's bile-derived (open bars) micelles. Values are expressed as means ± SD from at least three determinations. Significant differences between PBS controls and catechins are indicated as follows: ∗, $P < 0.001$ for artificially prepared micelles; #, $P < 0.001$ for bile-derived micelles.

RESULTS

Concentration of Cholesterol in Pig's Bile. The concentration of cholesterol in pig's bile varied between batches (data not shown). However for a given batch, the concentrations of cholesterol in the fresh bile and stored bile were not significantly different, and bile could be stored for at least 6 moths (**Figure 1**).

Micelle Solubility of Cholesterol. EGCG, ECG, CG, and gallic acid significantly reduced the solubility of cholesterol in both artificially prepared and pig's bile-derived micelle preparations, whereas catechin did not show any effect (**Figure 2**). The

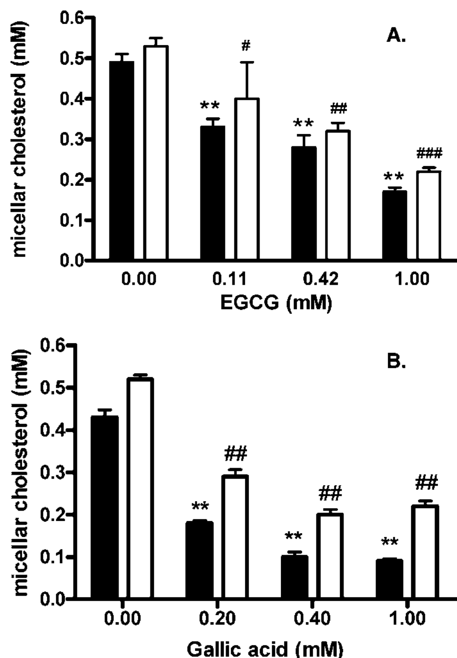

Figure 3. Effects of epigallocatechin gallate (EGCG) (**A**) and gallic acid (GA) (**B**) dose on cholesterol solubility in artificially prepared (solid bars) and pig's bile-derived (open bars) micelle preparations. Values are expressed as means ± SD from at least three determinations. Significant differences between 0 mM and increasing EGCG or GA dose are indicated as follows: ∗∗, $P < 0.001$ for artificially prepared micelles; #, $P < 0.05$, ##, $P < 0.01$, and ###, $P < 0.001$, for bile-derived micelles.

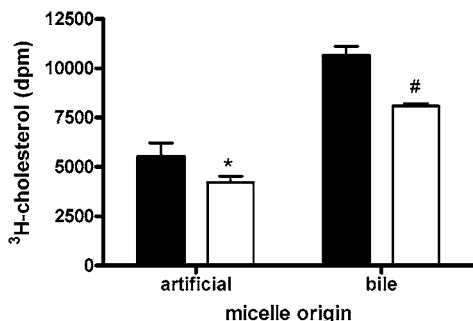

Figure 4. Cholesterol absorption into Caco-2 cells using artificially prepared and pig's bile-derived micelles. Control (solid bar) and effect of a bovine milk β-lactoglobulin-derived peptide, Ile-Ile-Ala-Glu-Lys (IIAEK) (open bars) (at 1.8 mg/mL) are expressed as mean ± SD from three determinations. Significant effect of IIAEK is expressed as follows: ∗, $P < 0.05$ for artificially prepared micelles; #, $P < 0.05$ for pig's bile-derived micelles.

concentration of cholesterol decreased in both systems in a dose-dependent manner by displacement with EGCG or gallic acid (**Figure 3**).

Cholesterol Uptake into Caco-2 Cells. The suppression of cholesterol absorption in Caco-2 cells by IIAEK at 1.8 mg/mL is shown in **Figure 4**. Cholesterol uptake was inhibited by ∼24% by IIAEK when using either artificially prepared or pig's bile-derived micelle preparations.

Distribution of Micelle Particle Size. The distribution of particle size of artificially prepared and pig's bile-derived micelle preparations is shown in **Figure 5**. The particle size of micelles derived from pig's bile approximated those in the artificially prepared micelles. In the artificially prepared micelle, ∼18% of particles had a diameter of 8−9 μm and ∼24% had a diameter of 3−4 μm. In the pig's bile-derived micelles, <8% of the particles had a diameter of 8−9 μm and ∼54% of the particles had a diameter of 3−4 μm.

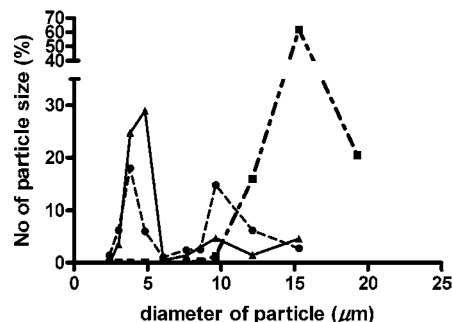

Figure 5. Distribution of particle diameter size of artificially prepared (●) and pig's bile-derived micelle (▲) preparations. The particle size was determined in a Coulter counter using a latex standard of 14 μm (■). The graph is representative of three determinations.

DISCUSSION

Mixed micelle implies a bile salt rich aggregate that is the "traditional" cholesterol carrier in bile and has been studied in model systems to determine cholesterol solubilization (*14*). Native bile fluid has very heterogeneous organic and inorganic compositions, and they are influenced by diet. The major organic components of bile are bile acids, phospholipids, cholesterol, proteins, and bile pigments (*15*). The artificial micelle preparations, however, are typically based on sodium taurocholate and egg lecithins, cholesterol, and oleic acid to reflect the natural mixed micelle (*8*, *9*). The artificial micelle systems contain predominantly uniform particles with measurable structural characteristics. In contrast, native bile rich in mixed micelles also contains nonmicellar particles such as unilamellar phospholipid vesicles and multilamellar structures such as stacked lamellae, multilamellar vesicles, and liquid crystals. The number and type of cholesterol-containing aggregates in bile are still controversial and, not surprisingly, all of the complexes and forms found in mammalian bile have not been reproduced in model solutions (*14*). The artificial micelle, therefore, only partially represents the heterogeneous compositions and structures present in native bile.

The availability of sufficient gut luminal bile-containing micelles is an important factor for cholesterol solubility and for efficient intestinal cholesterol absorption (*16*). The successive and coordinated steps in the process of intestinal cholesterol digestion and absorption are reviewed by Ross (*7*). Transport across the intestinal epithelium may occur by one or more of four different routes (*17*). They are the passive transcellular and paracellular routes, the carrier-mediated route, and transcytosis. Although the mode of entry of lipid digestion products into gut enterocytes remains controversial (*18*), it is generally accepted that it is by passive diffusion down a concentration gradient (*19*). However, it was recently suggested that absorption of cholesterol may also occur via a carrier-mediated binding protein associated with the brush border membranes (*20*). All four routes described above have been shown to occur in Caco-2 monolayers (*17*).

In the present study, we confirmed the findings of others (*11*, *12*) that cholesterol solubilized in mixed micelles was reduced by the addition of catechin gallate and epicatechin gallate esters but not free catechin using both artificially produced and pig's bile-derived micelles. It has been reported that gallate esters of catechin have a hydrophobic domain and show a higher affinity for hydrophobic lipid bilayers than to free catechins (*21*). Ikeda et al. (*12*) suggested, therefore, that the gallate esters of catechins can be hydrophobically bound to cholesterol. EGCG, a major polyphenolic component of green tea, has also been shown to

reduce plasma cholesterol levels in rats (*9*), and it was further demonstrated that EGCG inhibited cholesterol solubility in an artificial micelle preparation. In the present study, we found that not only EGCG but gallic acid itself was effective in displacing cholesterol in both delivery systems and in a similar dose-dependent manner. Our finding suggests that the gallic acid moiety is the key determinant in cholesterol solubilizing activity. Our finding lends support to an earlier report (*22*) that consumption of green tea increased fecal bile acid and cholesterol excretion and decreased plasma cholesterol levels. The effects of green tea observed by Yang (*22*) were not influenced by the key lipid metabolizing enzymes (HMG-CoA reductase, fatty acid synthase, and cholesterol 7α-hydroxylase).

A series of peptides derived from β-lactoglobulin including IIAEK have been shown to reduce plasma cholesterol in an animal study (*13*). The hypocholesterolemic effect of IIAEK in suppressing cholesterol absorption into Caco-2 cells in vitro using artificial micelles reported earlier by Nagaoka et al. (*8*) was confirmed in the study we report here. We observed a comparable inhibitory effect (24%) on cholesterol uptake by Caco-2 cells by IIAEK using both natural and artificial micelle preparations. In this study, we found that IIAEK was without any effect on cholesterol micellar solubility in either micelle preparation (results not shown). These observations validate the use of pig's bile as an alternative source of artificial micelles.

In this study, Caco-2 cells absorbed relatively greater amounts of [^{3}H]cholesterol when pig's bile-derived micelles were used as the "solubilizer" compared to artificial micelles. Native bile has more complex organic and inorganic compounds than prepared micelle. The complexity of especially major compounds in natural bile influences the physicochemical properties and therefore gives greater solubilization capacity and biological surfactant effect to accelerate cholesterol uptake by the cells (*7*, *23*). In addition, we observed that artificial and pig's bile micelles have comparable ranges of particle sizes, but the proportions were different (**Figure 4**). The proportion of small particles (3−4 μm) in the bile was ∼54%, whereas in the artificial micelle solution it was ∼24%. It has been reported that transepithelial uptake of compounds into Caco-2 monolayers was influenced by particle size (*17*). Transcellular and paracellar routes are passive and relatively rapid methods of transport and influenced by the size and shape of the solutes, with the permeability of the membrane decreasing with increasing particle size (*24*). Therefore, it is likely that the greater uptake of cholesterol in natural bile by Caco-2 cells was also due to the presence of a greater proportion of small particles in this preparation.

The present investigation addressed two early steps important in determining an individual's plasma cholesterol level. They were the solubilization of cholesterol into micelles, followed by micelle uptake into enterocytes using in vitro based techniques. These methods offer a rapid and convenient means to discover natural bioactives with potential for cholesterol-lowering activity. Combining the use of various catechin gallates and IIAEK as inhibitors of micellar solubility of cholesterol and cellular cholesterol absorption assays, respectively, we have demonstrated that pig's bile and artificial micelle preparations produced comparable results in a reproducible manner. Nevertheless, the greater cholesterol uptake by Caco-2 cells using pig's bile may be a better reflection of the natural physiological situation. In addition, the preparation time for artificial micelle is ∼24 h, compared to natural micelle, with which this lengthy process is not required. Natural micelle is an easy and relatively cost-effective source compared to the artificial preparation. Finally, these assays could be employed to identify potential bioactives to lower cholesterol and are complementary to those described earlier for the low-density lipoprotein (LDL) oxidation and LDL receptor assays, which have also been reported to be modulated by several dietary constituents (*25*).

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Received for review February 27, 2005. Revised manuscript received April 8, 2005. Accepted April 12, 2005. This study was supported by the Preventative Health Flagship of CSIRO Australia.

JF050447X