Effect of gamma-oryzanol on the bioaccessibility and synthesis of cholesterol

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Abstract. – Background and Objectives: Gamma-oryzanol (γ-OR) is a unique mixture of triterpene alcohol and sterol ferulates present in rice bran oil. Hypocholesterolemic activity of γ-OR has been reported in various animal and human studies. However, the mechanisms for this hypocholesterolemic activity of γ-OR remain unclear. Therefore, the aim of this in vitro study was to examine the effect of γ-OR on the bioaccessibility and synthesis of cholesterol.

Methods: The effects of γ-OR on the efficiency of incorporation of cholesterol into mixed micelles during digestion and apical uptake of cholesterol by Caco-2 human intestinal cells were determined using the coupled in vitro simulated digestion/Caco-2 human intestinal cell model. The impact of γ-OR on the HMG-CoA reductase activity was also investigated.

Results: Although incorporation of cholesterol into synthetic micelles was significantly inhibited by 15-fold molar excess of γ-OR, efficiency of micellization of cholesterol during simulated digestion of the rice meal was not significantly altered by the presence of as high as 20-fold molar excess of γ-OR. Nevertheless, 20-fold molar excess of γ-OR significantly decreased apical uptake of cholesterol into Caco-2 intestinal cells. In addition, γ-OR inhibited 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity.

Conclusions: These findings suggest that the hypocholesterolemic activity of γ-OR is due in part to impaired apical uptake of cholesterol into enterocytes and perhaps a decrease in HMG-CoA reductase activity.

Key Words:
Oryzanol, Cholesterol, Bioaccessibility, Caco-2 cells, HMG-CoA reductase.

Abbreviations
γ-OR: Gamma-Oryzanol
RBO: Rice Bran Oil
HMG-CoA reductase: 3-hydroxy-3-methylglutaryl-coenzyme A reductase
GDC: Glycodeoxycholate
TDC: Taurodeoxycholate
TC: Taurocholate
DMEM: Dulbecco’s modified Eagle’s medium
PBS: Dulbecco’s Phosphate Buffered Saline
FBS: Fetal Bovine Serum

Introduction
Gamma-oryzanol (γ-OR) is a mixture of 10 distinct ferulic acid (4-hydroxy-3-methoxycinnamic acid) esters of triterpene alcohols and sterols found in rice bran and its oil. 24-methyl-ene-cyclo-artenyl ferulate, cycloartenyl ferulate, campesteryl ferulate, and sitosteryl ferulate are the most abundant components generally accounting for approximately 80% of γ-OR in rice bran oil (RBO)\(^1\). However, the actual concentration of γ-OR in RBO may vary as it is affected by type of rice cultivar and extraction and processing conditions\(^2-4\).

The hypocholesterolemic activity of RBO and γ-OR has been demonstrated in various animal and human studies\(^5-11\). Oral ingestion of γ-OR significantly reduced aortic fatty streak formation and cholesterol absorption in hamsters\(^7\) and sig-
significantly increase the fecal excretion of bile acids and neutral sterols\textsuperscript{12,13}. A daily dose of 300 mg γ-OR lowered blood cholesterol in hyperlipidemic patients\textsuperscript{14,15}. Nevertheless, mechanisms responsible for the hypocholesterolemic activity of γ-OR remain unclear. It has been demonstrated that plant sterols have the capability to displace cholesterol from bile salt micelles, thus decreasing cholesterol absorption\textsuperscript{16}. Other possibilities for the hypocholesterolemic activity of γ-OR include interference with incorporation of cholesterol into mixed micelles during small intestinal digestion, decreased transfer of cholesterol from mixed micelles to enterocytes due to structural similarities of its components and cholesterol, increased fecal excretion of bile acids and neutral sterols, and inhibition of the activity of HMG-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis. Inhibition of HMG-CoA reductase activity is known to decreases hepatic cholesterol content, thus up-regulating expression of hepatic LDL receptors and enhancing uptake of cholesterol-rich LDL to reduce serum cholesterol\textsuperscript{17}.

Here, the effects of γ-OR on the efficiency of cholesterol micellization and apical uptake by Caco-2 human intestinal cells were assessed using the coupled \textit{in vitro} simulated digestion/ Caco-2 human intestinal cell model. The effect of γ-OR on HMG-CoA reductase activity in a partially purified preparation from rat liver was also investigated.

Materials and Methods

\textbf{Chemicals}

γ-oryzanol was purchased from Spectrum Lab Products, Inc. (Gardenia, CA, USA) and characterized by reverse-phase HPLC (Waters 2695 separations module, Milford, MA, USA) coupled with a photodiode array UV detector (Waters 2996 module, Milford, MA, USA), according to the method of Xu and Godber\textsuperscript{1}. Components of γ-OR standard were identified by reported retention time and spectra at wavelength 325 nm. \textsuperscript{14}C-cholesterol (specific activity 53 mCi/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). L-\textalpha-phosphatidylcholine from egg 99\% and \textit{lyso}-phosphatidylcholine were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Glycodeoxycholate (GDC) and taurodeoxycholate (TDC) were purchased from EMD Chemicals, Inc. (San Diego, CA, USA). Taurocholate (TC) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Supplements and antibiotic for cell culture were purchased from Gibco\textsuperscript{8}, Invitrogen Corporation (Chicago, IL, USA). Other chemicals and reagents were purchased from Sigma-Aldrich and Fisher Scientific (Pittsburgh, PA, USA), unless stated otherwise.

\textbf{Preparation of Synthetic Micelles}

Synthetic micelles with physiologically relevant composition were prepared as described elsewhere\textsuperscript{18}. Briefly, appropriate volumes of solvents containing the following compounds were transferred to a large vial to provide the following final concentrations: 500 µmol/L monolein; 100 µmol/L phosphatidylcholine; 300 µmol/L \textit{lyso}-phosphatidylcholine; 10 µmol/L \textalpha-tocopherol; 0.01 µCi of \textsuperscript{14}C-cholesterol; 100 µmol/L cholesterol with 0.01 µCi of \textsuperscript{13}C-cholesterol; and, either 0, 500, or 1500 µmol/L γ-OR. Concentrations of γ-OR were estimated by consideration of relative abundance and molecular weights of the 10 identified ferulic acid esters. After solvent evaporation under a stream of N\textsubscript{2}, Dulbecco’s minimal essential medium containing 1% non-essential amino acids, 1% L-glutamine, bile salts (0.8 mmol/L GDC, 0.46 mmol/L TDC, 0.75 mmol/L TC) and 0.6 mmol/L sodium oleate were added. The mixture was sonicated (ultrasonic bath, room temperature, 30 min), centrifuged (10,000 × g, 4°C, 15 min) to remove insoluble materials, and passed through cellulose acetate filter (0.22 µm pores). \textsuperscript{14}C-cholesterol in aliquots of supernatant (referred to as micelle fraction) and the initial mixture was measured by liquid scintillation spectroscopy (Beckman Coulter, Inc., Fullerton, CA, USA) to assess the effect of γ-OR on cholesterol incorporation in synthetic micelles.

\textbf{Preparation of Cooked Rice Meal}

Jasmine white rice (purchased in a local market) was cooked with 2 volumes of distilled water in a rice cooker. Steamed rice was dried in an oven at 105°C for 5 h. Then, dried rice was blended and sieved (20-mesh) to yield a fine rice powder. For \textit{in vitro} digestion, the cooked rice meal was prepared by mixing fine rice powder with distilled water (1:3, w/v). The slurry was mixed well with peanut oil (50 µL) containing \textsuperscript{14}C-cholesterol and either 0(control), 10 or 20-fold molar excess γ-OR using a disposable plastic spatula.
**In vitro Digestion**

Rice meal (2.0 g) was subjected to three phase (oral, gastric and small intestinal) simulated digestion as described elsewhere. Briefly, rice meal was incubated at 37°C for 10 min with 7 mL synthetic saliva (24 mmol/L KCl, 20 mmol/L NaH2PO4, 8 mmol/L Na2SO4, 10.2 mmol/L NaCl, 40.3 mmol/L NaHCO3, 0.2 mmol/L uric acid, 0.05 mg/mL mucin, and 10.5 mg/mL α-amylase). Then, pH was decreased to 2.5 with 1 mol/L HCl and salt solution (120 mmol/L NaCl, 6 mmol/L CaCl2, and 5 mmol/L KCl) with pepsin (final concentration, 2 mg/mL) was added to initiate the gastric phase. After 1 h at 37°C with shaking (85 rpm), pH was increased to 6.0 with 1 mol/L sodium bicarbonate before addition of pancreatin and pancreatic lipase (final concentrations of 0.4 and 0.2 mg/mL, respectively) and bile salts (0.8 mmol/L GDC, 0.45 mmol/L TDC, 0.75 mmol/L TC). Bile salts were substituted for bile extract to eliminate introduction of additional cholesterol. The final volume was increased to 50 mL with salts solution and pH was increased to 6.5. Sealed tubes were incubated with shaking at 37°C to simulate the small intestinal phase of digestion. Final concentration of cholesterol in the reaction mix was 1.3 µmol/L with 1 µC 14C-cholesterol and either 0, 13, or 26 µmol/L γ-OR. After 2 h, tubes were centrifuged (150,000 g, 30 min, 5°C) to separate aqueous fraction from undigested material in chyme. 14C-cholesterol content in chyme and the filtered supernatant (aqueous fraction) was measured by liquid scintillation spectrometry to assess the effect of γ-OR on cholesterol incorporation in micelles during small intestinal digestion.

**14C-cholesterol Uptake by Caco-2 Cells**

Stock cultures of Caco-2 cells (HTB-37) were obtained from The American Type Culture Collection (Rockville, MD, USA) and maintained in T75 flasks (vented caps) containing high-glucose DMEM supplemented with 15% heat-inactivated fetal bovine serum (FBS), 4 mmol/L L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin-streptomycin, 0.5 µg/mL fungizone, 44 mmol/L sodium bicarbonate, and 15 mmol/L HEPES, pH 7.0, in a humidified atmosphere of 95% air and 5% CO2, at 37°C. For experiments, cultures of Caco-2 at passages 30-35 were seeded in 6-well plates at 2.6x104 cells/cm2 in above medium and maintained as previously reported. Cultures were used at 11-14 d post-confluence to determine apical uptake of 14C-cholesterol from the filtered aqueous fraction of chyme generated during the small intestinal phase of simulated digestion.

Filtered aqueous fraction of chyme was diluted 1:4 with DMEM containing 4 mmol/L L-glutamine and 1% non-essential amino acids, but without FBS or antibiotics. Monolayers of Caco-2 cells were washed once with DMEM at 37°C before adding medium containing aqueous fraction. After incubation (37°C, 95% air; 5% CO2, 90% humidity) for 4 h, test medium was removed and monolayers were washed once with ice-cold Dulbecco’s phosphate buffered saline (PBS) containing bovine serum albumin (BSA) (2 g/L) to reduce non-specific adsorption of micelles to cell surface. The monolayers were washed two additional times with ice-cold PBS. Monolayers were scraped into a small volume of PBS and cells were collected by centrifugation (400 × g, 4°C, 5 min). The cell pellet was resuspended in a small volume of PBS and homogenized using a probe sonicator (Sonics and Materials, Inc., Newtown, CT, USA). 14C-cholesterol in aliquots of homogenates was determined by liquid scintillation spectroscopy. Protein content of each cell homogenate was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL, USA) with BSA as the standard.

**Determination of the HMG-CoA Reductase Activity**

Four male Wistar rats (180-200 g body weight) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Thailand. Animal facilities and protocol were approved by the Laboratory Animal Care and Use Committee at Faculty of Veterinary Science, Chulalongkorn University, Thailand. Animals were housed 2 rats per cage in a room with natural light cycle from 06:00 to 18:00. Standard chow diet and water were available *ad libitum*. Three days before killing, rats were fed powdered standard chow diet containing 5% cholestyramine. Rats were killed at 24:00 h for maximum HMG-CoA reductase activity.

A crude fraction enriched in HMG-CoA reductase was prepared from rat liver and HMG-CoA reductase activity was determined as described by Kim et al. The reaction mixture (pH 6.8) contained 200 mmol/L KCl, 160 mmol/L potassium phosphate, 4 mmol/L sodium EDTA, and 1 mmol/L dl-dithiothreitol, 0.2 mmol/L...
NADPH, 10 mmol/L HMG-CoA, and either 0, 100, 200, 400 µmol/L γ-OR in chloroform vehicle (1% chloroform in final mixture). HMG-CoA reductase activity was determined by measuring the rate of oxidation of NADPH at 340 nm. Before initiating the reaction by addition of HMG-CoA, absorbance of each reaction mixture was determined. HMG-CoA reductase activity in samples with γ-OR were subtracted from the activity in the mixture lacking γ-OR to calculate the extent percent of inhibition.

Statistical Analysis of Data
Each experiment was repeated to provide a minimum of three independent observations. All data are expressed as mean ± SEM. Statistical analysis was performed using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA). Differences among groups were statistically determined using one-way ANOVA followed by Tukey HSD test. Regression analysis was used to examine linearity between concentration of γ-OR and percent inhibition of HMG-CoA reductase activity. Statistically significant differences were considered at p < 0.05.

Results
γ-OR used in this study contained 10 distinct compounds according to retention time and spectral characteristics reported by Xu and Godber\(^1\). These included Δ\(^7\)-stigmasteryl ferulate (0.3%), stigmasteryl ferulate (0.8%), cycloartenyl ferulate (31.5%), 24-methylenecycloartanyl ferulate (37.6%), Δ\(^7\)-campestenyl ferulate (1.1%), campesteryl ferulate (17.0%), Δ\(^7\)-sitostenyl ferulate (0.5%), sitosteryl ferulate (8.4%), campestanol ferulate (1.9%), and sitostanol ferulate (0.9%) (Figure 1).

Effect of γ-OR on the Bioaccessibility of Cholesterol
Incorporation of \(^{14}\)C-cholesterol into synthetic micelles was inhibited in the presence of 15-fold molar excess of γ-OR (Figure 2) (control: 94.0 ± 2.4%; +1.5 mmol/L γ-OR: 74.9 ± 4.3%; p = 0.002). In contrast, the efficiency of partitioning of cholesterol into the filtered aqueous or bioaccessible fraction during simulated digestion of the rice meal containing peanut oil was not significantly affected by the presence of 10- and 20-fold higher concentrations of γ-OR (Figure 3) (control: 56.3 ± 1.3%; 13 µmol/L γ-OR: 53.3 ± 1.9%; 26 µmol/L γ-OR: 58.6 ± 2.7%). When cultures of Caco-2 cells were incubated with diluted aqueous fraction from digested meals with either 0 or 13 mmol/L γ-OR, uptake of \(^{14}\)C-cholesterol was not significantly (p > 0.05) different (13.9 ± 0.5 vs. 12.9 ± 0.3 ng cholesterol uptake/mg cell protein, respectively) (Figure 4). In contrast, apical uptake of \(^{14}\)C-cholesterol by differentiated cultures of Caco-2 intestinal cells was decreased 21.8 ± 2.1% when incubated with aqueous fraction from the digested rice meal containing 26 µmol/L γ-OR (10.9 ± 0.5 ng total cholesterol/mg cell protein, p = 0.008) (Figure 4).

γ-OR inhibits HMG-CoA Reductase Activity
γ-OR inhibited hepatic HMG-CoA reductase activity in a dose-dependent manner (R = 0.758, p = 0.004) (Figure 5). The extent of inhibition ranged from 27.1 ± 5.2% to 66.3 ± 8.3% in presence of 100 to 400 µmol γ-OR/L, respectively.

Discussion
Rice bran oil and γ-OR have been reported to decrease serum cholesterol in animal and human
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**Figure 2.** 15-fold molar excess of γ-OR inhibits incorporation of cholesterol into synthetic micelles. Synthetic micelles containing 100 µmol/L cholesterol with 0.01 µCi 14C-cholesterol were prepared in the absence (control) or presence of 500, and 1500 µmol/L γ-OR to determine efficiency of incorporation of cholesterol. Data are means ± SEM for the percentage of cholesterol incorporated into synthetic micelles, n = 5. Different letters above the error bars indicates that means differ significantly (p < 0.05).

**Figure 3.** Efficiency of micellarization of cholesterol is not altered during simulated digestion of rice meal containing 14C-cholesterol (1.3 µmol/L) along with as much as 20-fold molar excess of γ-OR. Rice meals containing peanut oil with cholesterol (1.3 µmol/L) and either 0 (control), 10-, or 20-fold molar excess γ-OR were digested. Partitioning of cholesterol in the aqueous fraction of chyme (i.e., micellarization) was determined. Data are means ± SEM for percentage of cholesterol incorporated into mixed micelles, n = 3. Similar letters above error bars indicates that means are not significantly different (p > 0.05).
significantly reduced cholesterol absorption and aortic fatty streak formation in hamsters. Also, serum cholesterol was decreased in hyperlipidemic patients ingesting a daily dose of 300 mg γ-OR. Nevertheless, mechanisms responsible for these effects remain unclear.

For example, consumption of γ-OR (0.5% of total dietary fat) significantly decreased blood and liver cholesterol levels in rats. Oral ingestion of γ-OR (1% γ-OR and 0.1% cholesterol of total fat in a hypercholesterolemic diet) significantly reduced cholesterol absorption and aortic fatty streak formation in hamsters. Also, serum cholesterol was decreased in hyperlipidemic patients ingesting a daily dose of 300 mg γ-OR. Nevertheless, mechanisms responsible for these effects remain unclear.

Figure 4. γ-OR inhibits uptake of 14C-cholesterol delivered in mixed micelles by Caco-2 cells. Aqueous fraction generated during in vitro digestion of rice meal with peanut oil and cholesterol (1.3 µmol/L) and 0, 10 or 20-fold molar excess γ-OR was diluted 1:4 with medium and added to wells containing Caco-2 cells for 4h. Data are means ± SEM, n = 3. The presence of different letters above error bars indicates that mean differs significantly (p < 0.01).

Figure 5. γ-OR inhibits HMG-CoA reductase activity. Data are expressed as means ± SEM, n = 4. Presence of different letters above error bars indicate that means differ significantly (p < 0.05).
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for the hypocholesterolemic activity of γ-OR remains unclear. It has been demonstrated that plant sterols have the capacity to displace cholesterol from bile salt micelles to inhibit cholesterol absorption in small intestine, suggesting that γ-OR-mediated inhibition dietary cholesterol absorption contributes to the improved serum cholesterol profile. This is supported by our observation that 15-fold molar excess of γ-OR inhibited incorporation of cholesterol into synthetic micelles. However, when γ-OR was added to a food matrix, the efficiency of micellarization of cholesterol was not significantly altered by the presence of as much as 20-fold excess of γ-OR. This observation challenges the likelihood that the hypocholesterolemic activity of γ-OR is primarily mediated by impairing the micellarization of cholesterol during small intestinal digestion. We observed that γ-OR significantly decreased apical uptake of cholesterol into Caco-2 intestinal cells. Intestinal absorption of cholesterol requires the participation of numerous intestinal transport proteins including Niemann-Pick C1 Like1 (NPC1L1), scavenger receptor class B type 1 (SR-B1), and ATP-binding cassette (ABC) proteins (ABCG5, ABCG8 and ABCA1). The possible interaction of one or more components in γ-OR with these proteins merits investigation.

γ-OR was reported to be poorly absorbed after administering 50 mg/kg body weight, although metabolites such as ferulic acid, dihydroferulic acid, and their conjugates were detected in urine. Plasma concentrations of γ-OR and ferulic acid as high as 37.6 and 36.6 ng/mL, respectively, were detected after human subjects were administered a single dose of 300 mg γ-OR. Ferulic acid (2.4-2.8% of dose), but not intact γ-OR, was detected in urine. These findings support the possibility that absorbed γ-OR and its metabolites may affect cholesterol metabolism in tissues. It is noteworthy that ferulic acid has been shown to exhibit hypolipidemic activity. Inhibition of HMG-CoA reductase activity, the rate-limiting enzyme of the mevalonate pathway, decreases hepatic cholesterol content and increases hepatic expression of LDL receptors resulting in a lowering of serum cholesterol. It has been suggested that γ-OR reduces plasma lipid levels by activating the hepatic LDL receptor, increasing in HMG-CoA reductase mRNA expression, and increasing fecal excretion of bile acids and neutral sterols in rats. Oral administration of γ-OR (1% γ-OR of total dietary fat) was found to reduce hepatic and intestinal HMG-CoA reductase activities (15 and 17%, respectively) in hamsters, although these observations were not statistically significant. Our data suggest that γ-OR can dose-dependently inhibit hepatic HMG-CoA reductase activity. Inhibition of HMG-CoA reductase activity along with increasing expressions of LDL receptors would be expected to enhance uptake of cholesterol-rich LDL to decrease serum cholesterol. Further studies are needed to assess if the limited extent of absorption of γ-OR and its metabolites is sufficient to attenuate HMG-CoA reductase activity in dose-dependent manner in vivo.

According to the National Cholesterol Education Program – Adult Treatment Panel III (NCEP-ATP III) guideline, dietary ingestion of less than 200 mg cholesterol/day is recommended for hyperlipidemic patients in order to lower total blood cholesterol and LDL cholesterol to reduce the risk of atherosclerosis. Recently, dietary intake from plant food products has been of considerable interest to hypercholesterolemic patients. The development of food products rich in γ-OR may represent a feasible strategy for the prevention and treatment of hypercholesterolemic patients.

In conclusion, the results of this study support the possibility that the hypocholesterolemic activity of γ-OR is due in part to inhibiting uptake cholesterol into absorptive gut epithelial cells and inhibition of HMG-CoA reductase activity.

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References


2) Rogers EJ, Rice SM, Nicolosi RJ, Carpentier DR, McClelland CA, Romanczyk JJJ. Identification and quantitation of gamma-oryzanol components and


20) Thakkar S, Mazha Dixon B, Dixon AG, Failla ML. β-carotene micellization during in vitro digestion and uptake by Caco-2 cells is directly proportional to β-carotene content in different genotypes of cassava. J Nutr 2007; 137: 2229-2233.


