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Enhancement of Naringenin Bioavailability by Complexation with Hydroxypropoyl-\(\beta\)-Cyclodextrin

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Abstract
The abundant flavonoid aglycone, naringenin, which is responsible for the bitter taste in grapefruits, has been shown to possess hypolipidemic and anti-inflammatory effects both in vitro and in vivo. Recently, our group demonstrated that naringenin inhibits hepatitis C virus (HCV) production, while others demonstrated its potential in the treatment of hyperlipidemia and diabetes. However, naringenin suffers from low oral bioavailability critically limiting its clinical potential. In this study, we demonstrate that the solubility of naringenin is enhanced by complexation with \(\beta\)-cyclodextrin, an FDA approved excipient. Hydroxypropoyl-\(\beta\)-cyclodextrin (HP\(\beta\)CD), specifically, increased the solubility of naringenin by over 400-fold, and its transport across a Caco-2 model of the gut epithelium by 11-fold. Complexation of naringenin with HP\(\beta\)CD increased its plasma concentrations when fed to rats, with AUC values increasing by 7.4-fold and C\(_{\text{max}}\) increasing 14.6-fold. Moreover, when the complex was administered just prior to a meal it decreased VLDL levels by 42% and increased the rate of glucose clearance by 64% compared to naringenin alone. These effects correlated with increased expression of the PPAR co-activator, PGC1\(\alpha\) in both liver and skeletal muscle. Histology and blood chemistry analysis indicated this route of administration was not associated with damage to the intestine, kidney, or liver. These results suggest that the complexation of naringenin with HP\(\beta\)CD is a viable option for the oral delivery of naringenin as a therapeutic entity with applications in the treatment of dyslipidemia, diabetes, and HCV infection.

Introduction

In recent years, polyphenols, and flavonoids in particular, have emerged as a class of natural products shown to have anti-oxidant, anti-atherogenic, and normolipidemic effects [1]. One of the most abundant is the citrus flavonoid-glycoside naringin, which is responsible for the bitter taste in grapefruit. Naringin is hydrolyzed to naringenin by gut flora prior to being absorbed [2]. Naringenin has been widely studied, and has been reported to be an antioxidant [3,4], MTP and ACAT inhibitor [5,6], and a regulator of cytochrome P450 (CYP450) enzymes including, CYP1A, CYP3A4, and CYP4A [7,8,9]. The ability of naringenin, and its glucuronide metabolites, to reduce plasma cholesterol levels has been demonstrated in vitro [10,11,12], while its ability to reduce ApoB secretion has been demonstrated extensively in vivo [13,14]. A recent clinical trial in hypercholesterolemic patients demonstrated that a 400 mg/day dose of naringin lowered LDL levels by 17% [3]. Similar cholesterol lowering effects of naringenin were demonstrated in rabbits [11] and rats [12]. More recently, Huff and coworkers have shown that naringenin helps correct many of the lipid disturbances associated with diabetes in transgenic mice lacking the LDL receptor that were fed a western-style diet, including correction of VLDL overproduction, amelioration of hepatic steatosis, and attenuation of dyslipidemia [10], while our group demonstrated that naringenin blocked the assembly of VLDL and infectious hepatitis C virus (HCV) particles in Huh7.5.1 cells and primary human hepatocytes [15].

Importantly, our recent findings demonstrate that naringenin is a dual-PPAR agonist, activating both PPAR\(\alpha\) and PPAR\(\gamma\) through the induction of their co-activator PGC1\(\alpha\) [16]. At the same time, naringenin directly inhibits LXR\(\alpha\), which controls HMG-CoA reductase (HMGCR) expression in the liver [16]. These results suggest that naringenin could potentially replace the actions of fibrates (PPAR\(\alpha\) agonists), thiazolidenediones (PPAR\(\gamma\) agonists), and statins (HMGCR inhibitors) in the treatment of type-2 diabetes or hyperlipidemia [16].

Regrettably, the clinical relevance of naringenin is limited by its low solubility and minimal bioavailability owing to its largely hydrophobic ring structure. In this study, \(\beta\)-cyclodextrins were examined as potential excipients to enhance the solubility and acceptance of naringenin.
enteral uptake of the flavonoid. Cyclodextrins are a family of cyclic oligosaccharides that create a 3-dimensional toroid structure, providing a cavity that can accommodate small hydrophobic molecules, such as cholesterol or steroids. Cyclodextrins can therefore be used as excipients to improve the solubility of hydrophobic drugs with similar structure [17,18]. Specifically, the bioavailability of rutin, a flavonoid-glycoside similar in structure to naringenin, was significantly enhanced by complexation with 2-hydroxypropyl-β-cyclodextrin (HPβCD) [19]. Here, we demonstrate that HPβCD enhances the solubility of naringenin, increases its transport across a Caco-2 model of human gut epithelium, and elevates its plasma concentrations following oral administration to Sprague-Dawley rats. When the complex is given right before a meal rich in glucose and fat, it decreased VLDL levels by 42% and increased the rate of glucose clearance by 64% compared to naringenin alone. These effects correlated with increased mRNA expression of the PPAR co-activator, PGC1α in both liver and skeletal muscle, strengthening recent evidence of a PPAR-mediated mechanism of action [16]. Combined with HPβCD’s strong safety record, our results suggest that HPβCD-naringenin complexes could be used to efficiently deliver the flavonoid in patients for the treatment of dyslipidemia, arthrosclerosis, and HCV infection.

Results

β-Cyclodextrins increase the solubility of naringenin

Molecules similar to naringenin in structure and size were previously shown to be solubilized by complexation with β-cyclodextrin. To explore if naringenin is similarly solubilized we generated complexes with β-cyclodextrin (βCD), methyl β-cyclodextrin (mβCD), and 2-hydroxypropyl-β-cyclodextrin (HPβCD). UV analysis indicated that complexation with cyclodextrins resulted in a very small shift in naringenin’s absorption spectrum (Figure 1A). Concentrations of naringenin were then extrapolated from the previously obtained standard curve (Figure 1C). As expected, naringenin solubility in water was 36±1 μM, consistent with previously observed results [20]. Upon complexation with cyclodextrins, the amount of solubilized naringenin increased, as summarized in Table 1. The three βCDs solubilized naringenin in decreasing order mβCD > HPβCD > βCD resulting in a significant 526, 437, and 132-fold, enhancement in solubility respectively (p<0.01).

HPβCD enhances the transport of naringenin across a Caco-2 monolayer

While mβCD was the most effective in enhancing the solubility of naringenin, its use is associated with soft tissue and kidney damage due to its detergent-like effect on membranes [21]. On the other hand, HPβCD does not cause hemolysis or irritation due to its low surface tension and is generally regarded as a safe excipient [22]. We therefore examined the ability of HPβCD to enhance the transport of naringenin across a monolayer of Caco-2 cells, an established model for drug transport across the human gut epithelium. Caco-2 cells were grown for 21 days on collagen-coated 1 cm² porous transwell membranes (0.4 μm pores) on which cells formed differentiated monolayers, expressing major tight junction proteins, microvilli, and drug transporters [23]. Transepithelial Electrical Resistance (TEER) and Lucifer yellow transport were used to evaluate epithelial integrity and maturity of the monolayers. The apparent permeability coefficient, P_app, remained between 6 and 7x10⁻⁷ cm/sec through the course of the experiment, demonstrating that the Caco-2 layer was intact. 11 mM naringenin, either alone or in a complex form with 45 mM HPβCD, was added to the top assay chamber. Samples were taken from both the top, apical chamber and the bottom, basal chamber at different time intervals and assayed for concentrations of naringenin (Figure 2A). In the presence of HPβCD, the concentration of naringenin at the basal chamber was increased from 0.04±0.02 μM to 0.51±0.07 μM, representing an 11-fold enhancement of transport across the Caco-2 monolayer. The integrity of the monolayer prior to and following the experiment was similar to control for both treatments.

HPβCD enhances the bioavailability of naringenin in rats

To test whether cyclodextrin would enhance the oral bioavailability of naringenin, adult Sprague-Dawley rats were fed 20 mg/kg body weight naringenin either alone, or as a 1:16 (wt/wt) HPβCD-naringenin complex, using an oral gavage. Blood samples were collected sequentially for 10 hrs from the carotid artery using the previously placed catheter into tubes containing heparin. Immediately after collection, plasma was separated and stored at −80°C for further analysis. At the conclusion of the experiment, all animals were sacrificed, and liver, kidney, and bowel specimens
Naringenin (mM) Cyclodextrin (mM) Fold increase in solubility K
βCD 4.8±0.3 20±1.0 132 6025
mβCD 190±0.9 50±2.5 526 9975
HPβCD 15.8±1.4 50±2.5 437 8203

were collected for histology. In an additional experiment, animals were placed in metabolic cages and urine was collected and pooled. Total naringenin (flavonoid and glucuronide) was determined by LC-MS as described above.

The complexation of HPβCD with naringenin significantly affected the plasma concentration versus time profile of the flavonoid (Figure 2C). Complexation with HPβCD significantly increased the AUC0-10 of naringenin from 2.0±0.5 hr·μg/ml to 15.0±4.9 hr·μg/ml representing a 7.4-fold increase in bioavailability (p = 0.005, n = 3). Naringenin’s maximal concentration, Cmax, increased from 0.3±0.1 μg/ml to 4.3±1.2 μg/ml representing a 14.6-fold increase (p = 0.002, n = 3). The calculated half-life for naringenin in plasma remained unchanged in both conditions at 2.3 hours, consistent with values previously reported in humans [24,25] and rats [26]. The percentage of free naringenin in plasma was in both cases <3% with the remainder in the glucuronide form. Finally, analysis of urine samples in two animals demonstrated unchanged renal clearance of 4.2±1%.

HPβCD–Naringenin complex reduces VLDL production and enhances glucose clearance following a lipid and glucose rich meal in rats

To assess if a single dose of HPβCD–naringenin could affect rat metabolism we administered naringenin or its complex orally, 30 minutes before the oral administration of a meal high in lipids (1 ml/kg) and glucose (1 g/kg). Glucose levels were measured sequentially for 2 hrs after the meal (Figure S1). Interestingly, rats that were administered the HPβCD–naringenin complex showed a significantly 64% higher (p = 0.05, n = 3) rates of glucose clearance, compared to rats given naringenin alone (Figure 3A). Previous work showed that the maximal level of VLDL in blood is reached 3 to 4 hrs after a meal. Here we show that 3.5 hrs after the meal, plasma levels of ApoB100, the structural protein of VLDL, were significantly 42% lower (p = 0.05, n = 3) then rats given naringenin alone (Figure 3C). Interestingly, triglyceride levels in the same rats increased, but not significantly (p = 0.24, n = 3). This response is similar to that of fructose, that is, naringenin, act through PPARγ, and are thought to occur due to a flux of chylomicrons from the intestine.

Recently, we demonstrated that naringenin is a dual-PPAR agonist, activating both PPARγ and PPARα through the induction of their co-activator PGC1α [16]. To examine if naringenin acts through a similar mechanism in vivo we carried out qRT-PCR analysis on samples of liver and skeletal muscle taken 3.5 hrs after the meal. The expression of PGC1α significantly increased by 230±100% (p = 0.02, n = 3) and 118±60% in skeletal muscle and liver, respectively (Figure 3D).

Oral administration of HPβCD-naringenin was not associated with adverse effects

Lastly, we wished to examine if the administration of the HPβCD-naringenin complex was associated with tissue or organ damage. Liver, kidneys and intestine were removed 10 hrs following oral administration of the complex and showed no gross pathological changes (data not shown). Histological characterization by a blind observer demonstrated that the small intestine, kidney, and liver sections showed no evidence of tissue injury or inflammation in both groups. Liver sections showed no evidence of hepatocyte damage or neutrophil infiltration to the portal area, while kidney and intestine sections show no tubular/glomerular damage, edema or epithelial damage, respectively (Figure 4). One intestine section in a single rat showed a localized small infiltrate, which did not appear to be related to the experiment.

Comprehensive metabolic analysis was carried out on serum samples taken from rats, 10 hrs after the treatment with HPβCD-naringenin, naringenin alone, as well as rats treated with saline as a control. The biochemical examination revealed no major changes (Table 2). Glucose and electrolytes levels were within normal values, as were urea and creatinine levels, suggesting kidney function was unchanged. Biochemical liver damage parameters were also within normal, with alkaline phosphatase (ALP) levels being actually lower in treated groups compared to control (p = 0.03), while ALT and AST showing no significant differences (p = 0.44 and p = 0.17, respectively). Total bilirubin (TBIL), albumin (ALB) and total protein (TP) content of the blood was also unchanged. Together with the histological and pathological analysis these results suggest that oral administration of HPβCD-naringenin complex was not associated with any adverse effects.

Discussion

Naringenin is an abundant flavonone-glycoside known to cause the bitter taste in grapefruit (citrus paradisi). The compound is highly soluble and can be found in grapefruit juice at concentrations of up to 0.5 g/L [26]. Following ingestion, naringin is broken down by gut flora to its aglycone form, naringenin [27]. Naringenin has been the focus of multiple studies in recent years, which began to elucidate its clinical potential as an antioxidant with anti-carcinogenic, anti-inflammatory, and hypolipidemic properties [2]. The flavonoid’s ability to reduce the secretion of very-low density lipoprotein (VLDL) from hepatocytes has been demonstrated in tissue culture, and attributed both to signaling events, through the insulin-PI3K and MAPK pathways [6,28]; and most recently, to the modulation of the PPARz, PPARγ and LXRz nuclear receptors [16,29]. Recently, our group demonstrated that this inhibition of VLDL assembly blocked the production of infectious HCV particles from infected hepatocytes [15], while others point to naringenin’s hypolipidemic properties in the treatment of diabetes [10]. As with other drugs, efficacy would depend on the ability to deliver the molecule to patients in a reproducible manner [21].

Cyclodextrins are a family of cyclical oligosaccharides, composed of varying numbers of glucopyranoside rings that form a three-dimensional toroid structure. The inner face of the toroid is significantly less hydrophilic than the surrounding water, providing an energetic advantage to the insertion of hydrophobic molecules into the cavity. β-cyclodextrins, specifically, are composed of seven sugar rings, and have been shown to be nontoxic to humans [21]. These cyclodextrins are widely used by the food and pharmaceutical industries and a generally regarded as safe [22]. The potential of β-cyclodextrins to enhance the solubility and gut absorption of flavonoids was demonstrated by Uekama and coworkers [19]. The group complexed the flavonoid-glycoside, rutin with HPβCD and found a 10-fold increase in solubility. Following the oral administration of the complex in
Naringenin suffers from low solubility in aqueous environments, up to 36 μM in our hands, and is generally dissolved in organic solvents [2]. In the presence of β-cyclodextrins, however, the solubility of naringenin increased by several orders of magnitude, up to 500-fold. Of the three cyclodextrin types tested, solubility increased in the order mβCD > HPβCD > βCD. Despite the superior ability of mβCD to solubilize naringenin, we chose to conduct further experiments with HPβCD, which does not exert a detergent-like effect on biological membranes causing irritation and hemolysis [30] and is used in multiple drug formulations [31].

We next examined the ability of HPβCD to enhance the delivery of naringenin across the intestinal mucosa. We used the well-characterized Caco-2 transwell model of the human gut epithelium [23]. In this experiment, a monolayer of Caco-2 gut epithelial cells was grown on a transwell membrane, and the ability of naringenin to cross this barrier was measured over time. When complexed to HPβCD, naringenin reached a concentration 11-fold higher than in the absence of the excipient. Interestingly, the rate of transport of naringenin across the membrane was not different between the groups, set as 5±1 μM/min (p>0.10). The integrity of the monolayer was verified both at the beginning and end of the experiment suggesting that neither HPβCD nor naringenin damaged the monolayer at the concentrations and time-scales examined.

We next examined the ability of HPβCD to enhance the bioavailability of naringenin in a rat model. Two groups of male Sprague-Dawley rats were fed 20 mg/kg body weight naringenin. One group was fed naringenin alone, while the other was fed a HPβCD-naringenin complex. Our results indicate a substantial improvement in the delivery of naringenin complexed with HPβCD, with AUC0-10 of naringenin increasing 7.4-fold and maximal concentration, Cmax, increasing 14.6-fold over naringenin alone. This increase in bioavailability represents an increase in the absorption rate from Ka = 26.9 hr−1 to Ka = 63.7 hr−1, a 2400-fold increase. Several effects could explain this increased rate of transport, including enhancement of dissolution kinetics, increase in solubility, decrease in degradation, change in the properties of the intestinal membrane, and shuttling and enhancement of drug concentration at the intestinal wall [31]. However, it is unlikely that complexation with HPβCD changes the plasma pharmacokinetics of naringenin, as cyclodextrins are poorly transported across the intestinal wall [31]. The calculated half-life of naringenin in plasma under both conditions was 2.3 hrs, consistent with values previously reported in humans [24,25] and rats [26]. The ratio of free naringenin to its glucuronide form were also unchanged by the complex and remained <3% in both cases.

Using this information we devised a study in which naringenin or the HPβCD-naringenin complex is given orally to rats 30 min prior to a controlled meal rich in glucose and fat. This 30 min period was judged sufficient to allow the flavonoid to induce PPARα in liver and skeletal muscle through our recently described induction of the PPAR co-activator PGC1α [16]. We show that
Figure 3. HPβCD-naringenin complex ameliorates the effects of a fat and glucose-rich meal. (a) Male Sprague-Dawley rats were fasted overnight, and then fed 20 mg/kg body weight naringenin either alone or as a HPβCD-naringenin complex. 30 min later, the rats were administered a meal composed of a suspension of 1 ml/kg olive oil and 1 g/kg glucose. Glucose clearance was measured as the rate of return to normal from maximal concentration (about 65 min). (b) Plasma cholesterol, HDL, triglycerides, and ApoB100 (VLDL) were measured 3.5 hrs after the meal. Cholesterol and HDL levels did not change. Triglyceride levels in rats fed the HPβCD-naringenin complex were elevated, but not significantly (p = 0.24, n = 3). In contrast, plasma levels of ApoB100, the structural protein of VLDL, were significantly 42% lower (p = 0.05, n = 3) than rats given naringenin alone. (c) mRNA abundance of PGC1α increased by 230 ± 100% and 118 ± 60% in skeletal muscle and liver, respectively. Tissue samples were collected 3.5 hrs after the meal.

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Figure 4. Male Sprague-Dawley rats were fed 20 mg/kg body weight naringenin either alone, or as a HPβCD-naringenin complex. Animals were sacrificed 10 hrs post-treatment and tissue samples were collected and preserved. Representative images of H&E histological preparations from bowel, kidney, and liver are presented. Tissues were evaluated by a blinded pathologist and were judged to be normal with no signs of inflammation or necrosis.

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animals which received the complex showed significantly 64% higher (p = 0.05, n = 3) rates of glucose clearance, compared to rats given naringenin alone. Correspondingly, skeletal muscle expression of PGC1α measured by qRT-PCR significantly increased by 230±100% (p = 0.02, n = 3). In addition, 3.5 hrs after the meal, plasma levels of ApoB100, the structural protein of VLDL, were significantly 42% lower (p = 0.03, n = 3) in rats given the complex than rats given naringenin alone. Not surprisingly, the expression of PGC1α in the liver was also increased by 118±60%. Interestingly, triglyceride levels in the complex-fed rats increased, but not significantly (p = 0.24, n = 3). This response is similar to that of fibrates that, like naringenin, act through PPARα, and is thought to occur due to a flux of chylomicrons from the intestine being ‘ignored’ by the liver.

Previous studies demonstrated the low bioavailability of naringenin. Niopas and coworkers orally administered 135 mg naringenin to six healthy volunteers. Plasma concentrations peaked after 3.5 hrs, and bioavailability was estimated to be 5.8% [24]. Erlund and coworkers found similarly low bioavailability when the source of naringenin was grapefruit juice. The researchers also noted the high variability in bioavailability, which is ‘ignored’ by the liver.

For the transport studies, Caco-2 cells were seeded on Transwell (0.4-μm pore size, 1-cm² growth area; Corning Costar Co.) at a cell density of 1x10⁵ cells/filter. Cell growth and maintenance were performed as previously described [32]. The cell monolayer was fed fresh growth medium every 2 days and used on day 21 for the transport experiments. HBSS supplement with 20 mM glucose and 10 mM HEPES (pH 7.35) was used as the transport medium. To determine the amount of drug crossing the polarized Caco-2 cell monolayer from the donor to the receiver (i.e., apical to basolateral), the Caco-2 cells were rinsed twice with pre-warmed transport buffer.
medium and incubated by pre-warmed transport medium 0.2 ml for apical chamber and 0.5 ml for basolateral chamber at 37°C for 30 min. A 60 mg/ml (1% DMSO in HBSS) stock solution of test compounds, either naringenin or HPβCD-naringenin, was added and samples from both apical and basolateral were taken (30 µl) at different time points: 30, 60, 120, 130, 180, 240, and 300 min. The integrity of the culture was confirmed by transepithelial electrical resistance (TEER) and by detecting fluorescently labeled cells using 60 µM of Lucifer Yellow as a standard. The concentrations of naringenin or HPβCD-naringenin were determined as described and plotted as a concentration on the basolateral side vs. time. Concentrations were corrected by the dilution factor as fresh buffer was added after sampling.

Animal experiments
Adult male Sprague-Dawley rats were purchased from Charles Rivers Laboratories (Wilmington, MA). Upon arrival, each rat was isolated for 3–5 days towards adaptation to the new environment. Animals were housed under 12h cycle of day/night with free access to drinking water and fed ad libitum unless otherwise noted. To measure the pharmacokinetic profile of naringenin, rats weighing between 280 and 300 g were anaesthetized using intraperitoneal injections of ketamine and xylazine at 110 and 0.4 mg/kg, respectively. The left carotid artery was cannulated using a 0.76-mm diameter ×60-cm length heparanized catheter. The catheter was tunneled subcutaneously from the opening made in the anterior face of the neck to the dorsal site of the neck and permanently anchored in the skin. The catheter was secured by the use of a rat jacket. Animals were placed in their cages during the term of the study. Animals were orally administered with 20 mg/kg body weight HPβCD using a rat oral gavage. Precisely 30 min after the oral administration of naringenin, the rats were administered a high fat (1 ml/kg) high glucose (1 g/kg) meal at time zero. 30 minutes prior to the meal, the rats were administered a high fat (1 ml/kg) high glucose (1 g/kg) meal at time zero. 30 minutes prior to the meal, the rats were administered 200 mg/kg body weight HPβCD using a rat oral gavage. 200 mg/kg body weight HPβCD using a rat oral gavage (18G 1 1/2

LC-MS detection of naringenin
LC-MS analysis was performed on an Agilent Technologies series 1100 LC-MSD system (Santa Clara, CA), which included an Agilent 1100 quaternary pump, autosampler, column oven, online vacuum degasser, and single quadrupole mass spectrometer equipped with electrospray ion source (ESI). Mass spectrometry conditions: Electrospray ionization (ESI), positive, selected ion monitoring scan (SIM); SIM: naringenin m/z 273.1. LC conditions: Eclipse XDB-C18 column (4.6×130mm, 5.0 µm). The mobile phase was composed of methanol-water with 0.1% formic acid (65:35,v/v). The isocratic flow rate was set at 0.8 ml/min and injection volume was only 10 µl. To each 100 µl of rat serum sample, 100 µl of 0.1N sodium acetate (pH = 5.0) and 100 µl of β-glucuronidase enzyme (5000 units/ml, type HP-2 from Helix Pomatia) were added and vortexed for 5 seconds. This process hydrolyzes the conjugated form of naringenin to determine total naringenin in plasma. After addition of 20 µl IS buffer solution (5 µg/ml), the sample was then incubated at 37°C water bath for 18 h.

The sample was extracted with 0.9 ml of ethyl acetate after 18 h incubation, and centrifuged at 13000 rpm for 10 min. The supernatant was collected and evaporated to dryness under nitrogen at room temperature. The residue was reconstituted with 100 µl of mobile phase and filtered through a micro nylon n filter (0.45 µm). 10 µl of the filtrate was forwarded to LC-MS analysis. A calibration curve was established and QC samples conducted (data not shown). Data acquisition was performed using ChemStation software (Agilent). Linear regression (weighted by 1/x) between serum concentration and peak area ratio of naringenin to IS was constructed using SPSS11.0 statistical software. The concentrations of naringenin in samples were calculated by interpolation of the linear equation.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)
Liver and skeletal muscle tissues were ground in liquid nitrogen, homogenized and RNA was purified using BioRad Aurum Total RNA Fatty and Fibrous Tissue Kit (Hercules, CA). Total RNA was quantified on NanoDrop Technologies, ND-1000 spectrophotometer (Wilmington, DE) and mRNA transcript abundance was measured on a BioRad CFX96 real-time PCR Detection System using Bio-Rad SsoFast™ EvaGreen® supermix (Hercules, CA), according to the manufacturers’ instructions. PCR primers used were, forward GACCCCAAGTAGCAAAATGA and reverse GGCCCTGCAGTCTCAGAGAT, while β-Actin (ACTB) primers were, forward AGCCATGTAGCTAGCCAACAG and reverse TCTCCGGATGTCACCACAC (Integrated DNA Technologies, Coralville, IA). Expression was normalized to β-Actin using ΔΔCt.

Metabolism
Liver metabolic and lipid plasma levels were analyzed using Piccolo Blood Analyzer (Abaxis, Union City, CA) and confirmed by A.M.I. veterinary department (Herzilia, Israel). Levels of rat apolipoprotein B100 (APOB100) were measured using ELISA (Uscn Life Sciences, Wuhan, China).

Liver Histology
Histological sections of each organ were taken 10 hours after treatment. Formalin-fixed, paraffin-embedded liver, intestine, and kidney samples were sectioned at 4 µm and stained with hematoxylin & eosin (H&E). Histological characterization was performed by a blinded observer using standard assessment of damage.

Statistics
Data are expressed as the mean ± standard deviation. Statistical significance was determined by a one-tailed Student’s t-test. A P-value of 0.05 was used for statistical significance.

Supporting Information
Figure S1 Glucose plasma concentrations over time in rats administered a high fat (1 ml/kg) high glucose (1 g/kg) meal at time zero. 30 minutes prior to the meal, the rats were administered...
either naringenin alone or HPβCD-naringenin complex. Curves represent average ± standard deviation of 3 rats in each group. (PDF)

Author Contributions
Conceived and designed the experiments: YN CWL-P. Performed the experiments: MS MC AS-G HY HW OB-R, YN. Analyzed the data: MS MC HW YN. Contributed reagents/materials/analysis tools: HW OB-R. Wrote the paper: YN, JG. Contributed research space: MLY.

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