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ORIGINAL PAPER

Black pepper and piperine reduce cholesterol uptake and enhance translocation of cholesterol transporter proteins

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Abstract Black pepper (*Piper nigrum* L.) lowers blood lipids in vivo and inhibits cholesterol uptake in vitro, and piperine may mediate these effects. To test this, the present study aimed to compare actions of black pepper extract and piperine on (1) cholesterol uptake and efflux in Caco-2 cells, (2) the membrane/cytosol distribution of cholesterol transport proteins in these cells, and (3) the physicochemical properties of cholesterol micelles. Piperine or black pepper extract (containing the same amount of piperine) dose-dependently reduced cholesterol uptake into Caco-2 cells in a similar manner. Both preparations reduced the membrane levels of NPC1L1 and SR-BI proteins but not their overall cellular expression. Micellar cholesterol solubility of lipid micelles was unaffected except by 1 mg/mL concentration of black pepper extract. These data suggest that piperine is the active compound in black pepper and reduces cholesterol uptake by internalizing the cholesterol transporter proteins.

Keywords Cholesterol · Cholesterol uptake · Black pepper · Piperine · NPC1L1 · SR-BI

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Introduction

Elevated plasma cholesterol levels play a major role in the development of atherosclerosis and cardiovascular disease. Plasma cholesterol levels depend on several parameters, including endogenous cholesterol synthesis, the removal of cholesterol from the circulation, the absorption of dietary cholesterol and the reabsorption of cholesterol from bile [1]. Although many drugs are available to manage hypercholesterolemia, the first line of treatment is by dietary and lifestyle changes. However, lifestyle changes are not easily achieved and sustained by most people. Thus, alternative agents, especially in forms of dietary supplements, continue to be attractive and cost-effective treatments. In recent years, natural components from food and/or herbal medicines have been recommended as cholesterol-lowering agents [2–9].

Circulating cholesterol comes from hepatic cholesterol synthesis and intestinal cholesterol uptake. In the latter process, intestinal cholesterol is solubilized as micelles and uptake occurs via the enterocyte carrier proteins, Niemann-Pick C1-like 1 (NPC1L1) [10–12] and scavenger receptor class B type I (SR-BI) [13]. Cholesterol uptake might therefore be suppressed, reducing micellar cholesterol solubility, increasing micelle size [14–16] or inhibiting carrier protein function.

Black pepper (*Piper nigrum* L.) is commonly used as a spice in various types of food. One of the major active components (5–9 %) in black pepper is an alkaloid amide called piperine [17]. Several beneficial physiological and pharmacological properties of black pepper and piperine have been reported, including preventing oxidative damage, lowering lipid peroxidation and enhancing the bioavailability of some therapeutic drugs [18]. Furthermore, black pepper reduces total cholesterol, free fatty acids, phospholipids and triglycerides in plasma and tissue of rats

on a high-fat diet [19]. Our previous study demonstrated that black pepper extract effectively inhibited cholesterol uptake into differentiated Caco-2 cells [20], which may consequently lower blood cholesterol. Piperine itself can also reduce plasma levels of total cholesterol, LDL and VLDL in high-fat fed rats [21], which suggests that the actions of black pepper extract on cholesterol are via its content of piperine.

The present study therefore aimed to confirm this idea by comparing the actions of piperine and black pepper extract on (1) micellar properties and (2) cholesterol transport in differentiated Caco-2 cells, and (3) the expression of the carrier proteins. We show that most actions of the extract can indeed be replicated by piperine.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM)/F12, other cell culture materials, cholesterol, phosphatidylcholine, sodium taurocholate and piperine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gibco (Carlsbad, CA). [1α,2α(n)-^{3}H]cholesterol was purchased from Perkin-Elmer (Wellesley, MA, USA) and antibodies recognizing NPC1L1 and SR-BI came from Novus Biologicals, USA and Abcam, UK, respectively.

Black pepper extraction

Black pepper was collected from Chanthaburi province, Thailand. It was identified by Assoc. Prof. Dr. Kornkanok Ingkaninan, Faculty of Pharmaceutical Sciences, Naresuan University. The herbarium specimen was kept at PBM herbarium, Mahidol University, Thailand and the collection number is Fansai0010. Black pepper fruits were washed thoroughly with tap water, dried at 37 °C in an incubator, powdered in a grinder, further dried at 60 °C for 2–3 days and the dried powder was macerated with 95 % ethanol for 3 days. The aqueous extracts were subsequently filtered, dried in a rotavapor at 55–60 °C under pressure and stored at −20 °C. Piperine content was determined using an HPCL system equipped with a SPD-M10AVP photodiode array detector. The mobile phase was a 65:35 mixture of acetonitrile and 25 mM NaH_2PO_4 (aq, pH4.5) and the detection wavelength was 340 nm.

Cell culture preparation

Caco-2 cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in DMEM/F12 containing 10 % FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 37 °C in an incubator in a saturated humidity atmosphere containing 95 % air and 5 % CO_2. Caco-2 cells were cultured on 24-well plates for 2–3 weeks to allow cell differentiation and the culture media was changed every 2–3 days.

Cholesterol micelle preparation

The micelle preparation method was modified from Yamanashi et al. [12]. Briefly, stock [1α,2α(n)-^{3}H]cholesterol (GE Healthcare, UK), cholesterol and phosphatidylcholine were dissolved in chloroform. A solution of sodium taurocholate was prepared in methanol. The lipid and bile salt solutions were mixed and evaporated under a stream of N_2 gas and the lipid film was stored under N_2 at −20 °C until use. The micelle solution was freshly prepared by hydrating the lipid film in serum-free DMEM/F12 so that the final concentrations in the micellar suspension were 1 μM cholesterol, 2 mM sodium taurocholate, 50 μM phosphatidylcholine and 1 μCi/mL [1α,2α(n)-^{3}H]cholesterol.

Cholesterol uptake assay

The uptake of cholesterol micelle was detected by measuring the radioactivity of [^{3}H]cholesterol. Caco-2 cells were seeded on 24-well plates at 50,000 cells/well. Cells were cultured for 14–21 days to allow differentiation. The cells were cultured in serum-free medium overnight before incubating with black pepper extract or piperine for 1 h and [^{3}H]cholesterol micelles for another 3 h. After washing with ice-cold PBS, the cells were disrupted with 0.2 N NaOH/0.1 % Triton-X 100 and the cell lysate used to measure protein concentration standardized to radioactivity.

Cholesterol efflux assay

The efflux assay used the method of Tachibana et al. [22]. To preload with cholesterol, differentiated Caco-2 cells were incubated with serum-free medium overnight before loading with the [^{3}H]cholesterol micelles for 24 h to allow radiolabelled cholesterol uptake by the cells. The excess micellar [^{3}H]cholesterol was removed by washing with PBS 3 times. Efflux was measured by incubating the cells with fresh culture medium containing black pepper extract or piperine for 24 h. The efflux of cholesterol was evaluated by measuring the amount of radioactivity ([^{3}H]cholesterol) in the culture medium and that remaining in the cells after disruption.

Cell membrane preparation

Preparation of membrane proteins was adapted from the method of Yu and coworkers [23]. Briefly, Caco-2 cells

were washed with cold phosphate-buffered saline (PBS) and scraped in cold reagent A consisting of 20 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, and 0.25 M sucrose along with 1 % protease inhibitors. The cells were then ruptured by passing through a 22-gauge needle 30 times and spun down at 1,000*g* for 10 min at 4 °C. The supernatant was transferred to new tubes and spun at 55,000 rpm for 30 min at 4 °C. The cell membrane pellet was resuspended in reagent B [50 mM Tris–HCl (pH 8.0), 2 mM $CaCl_2$, 1 % protease inhibitors and 1 % Triton X-100] and stored at −20 °C until further experiment.

Protein expression analysis

The expression of the carrier proteins NPC1L1 and SR-BI was determined by Western blotting. Differentiated Caco-2 cells were treated with black pepper extract or piperine for 24 h. These cells were harvested in lysis buffer (50 mM Tris–HCl, pH 7.4, containing 0.5 % SDS and protease inhibitors). The cell lysate was separated on a SDS–polyacrylamide gel, transferred to PVDF membrane and blocked with 5 % skimmed milk. The membrane was then incubated with antibody recognizing NPC1L1 or SR-BI at 4 °C overnight. The proteins of interest were detected by immunoglobulin G (IgG) conjugated with horseradish peroxidase and then visualized by chemiluminescence.

Cholesterol micelle size

The cholesterol micelles were prepared as above but using unlabeled cholesterol. The micellar size evaluation was adapted from Kirana et al. [24]. Briefly, the micelle solutions were sonicated and passed through 0.22 μm syringe filters and kept at 37 °C. Black pepper extract or piperine was added to the micelle solution, incubated for 3 h at 37 °C, and micellar size was measured using a particle size analyzer (Model ZetaPALS, Brookhaven, USA).

Micellar cholesterol solubility determination

The micelle solubility determination was adapted from Kirana et al. [24]. The cholesterol micelle preparation consisted of 1 mM cholesterol, 1 mM sodium taurocholate and 0.6 mM phosphatidylcholine, and was prepared as above. Black pepper extract or piperine was added to the mixed micelle solution and incubated for 3 h at 37 °C. The lipid micelles were then filtered through a 0.22 μm membrane to separate precipitated cholesterol from the intramicellar cholesterol. Precipitated cholesterol indicated non-micellar or un-dissolved clumped cholesterol that could not pass through the filter membrane. The amount of cholesterol which remained in the filtrate was taken as the micellar concentration and determined by a kit which measured total cholesterol assay (HUMAN GmbH, Germany).

Statistical analysis

Results were expressed as the mean ± SEM of *n* experiments. The data were analyzed by analysis of variance (ANOVA) with a post-hoc Scheffe's or William's test and Student's *t* test. $p < 0.05$ was considered significant.

Results

Cholesterol uptake

To examine the effect of black pepper extract and piperine on cholesterol uptake in the intestinal lumen, differentiated Caco-2 cells were used. These cells are widely used as an in-vitro model to study intestinal absorption or transport. Although they are originally derived from a human colon adenocarcinoma, they can spontaneously differentiate in culture showing the morphological and functional properties of normal small intestinal cells (enterocytes) [25]. These cells exhibit differentiation-specific markers for small intestinal enterocytes including sucrose-isomaltase, lactase, aminopeptidase and alkaline phosphatases [26]. In the present study, differentiation of Caco-2 cells was evaluated by enzymatic reaction of alkaline phosphatases and α-glucosidase (data not shown).

The uptake of cholesterol was determined by measuring the level of [^{3}H]cholesterol captured by Caco-2 cells. In this study, ezetimibe was used as a positive control since it is known to block the NPC1L1 cholesterol transporter

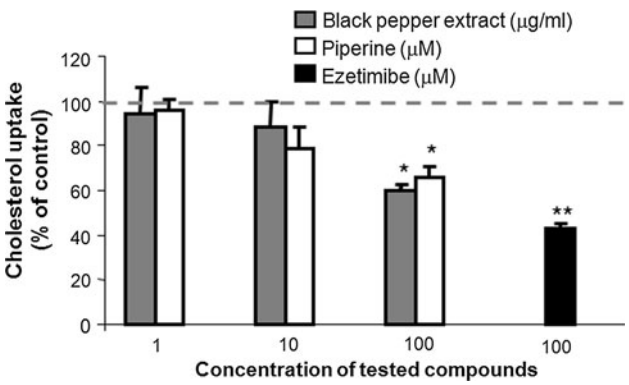

Fig. 1 Black pepper extract and piperine reduce cholesterol uptake in differentiated Caco-2 cells. Cells were incubated with [1α2α(n)-^{3}H] cholesterol micelles in the presence of black pepper extract (1, 10, and 100 μg/mL, containing 1,10 and 100 μM piperine) or piperine (1, 10, and 100 μM) or 100 μM ezetimibe for 3 h. The control was cells incubated with [^{3}H]cholesterol micelles without the test compounds. Values represent mean ± SEM ($n = 3$–4). *$p < 0.05$, **$p < 0.001$ compared to untreated cells (control)

protein and, at 100 μM, it reduced uptake by ~50 % (Fig. 1). Ezetimibe at higher concentrations (>100 μM) showed no further inhibition of cholesterol uptake (data not shown). With piperine (1–100 μM), the uptake of cholesterol into Caco-2 cells was reduced in a dose-dependent manner but less potently than with ezetimibe (Fig. 1). Black pepper extract contained 28.2 % piperine analyzed by HPLC and the amount of extract tested used concentrations (1–100 μg/mL) adjusted to contain the same amounts of piperine. This extract produced a similar range of inhibitions as did the equivalent concentrations of piperine (Fig. 1). This result suggests that the cholesterol uptake inhibitory activity of black pepper extract may result entirely from its piperine content.

Cholesterol efflux

Re-export of cholesterol from the apical surface of the cultured cells also influences the net uptake. This appeared to be slightly reduced (~15 %) by both black pepper extract (100 μg/mL) or piperine (100 μM) (Fig. 2). Clearly, the main action of extract/piperine is on the uptake, rather than promoting cholesterol efflux/re-export, although this might have had a small moderating influence on net uptake.

Micellar cholesterol solubility and lipid micellar size

The physicochemical properties of micelles can also influence cholesterol uptake. The micellar concentrations of cholesterol were unaffected by most of the concentrations of black pepper extract and of piperine (Table 1). Only with the highest concentration of black pepper extract (1,000 μg/mL) was there a small decrease in cholesterol micellar solubility, a concentration higher than that which inhibited transport. Neither black pepper extract nor piperine had any effect on the size of the cholesterol micelles (data not shown).

The expression of proteins involved in intestinal cholesterol absorption

The important cholesterol transporters are NPC1L1 and SR-BI. Black pepper extract and piperine may affect their expression, hence influencing cholesterol uptake, and thus this was determined by immunoblotting of these proteins. We used both 3 h incubations to assess functional protein changes and also 24 h incubations to assess changes in expression, and measured these in whole lysates and membrane fractions. NPC1L1 immunoreactivity in whole cell lysates was unaffected by either black pepper extract or piperine for both time points (Fig. 3). However, the membrane fraction isolated from these cells clearly showed reduced NPC1L1 immunoreactivity with pepper extract. For piperine, this was only apparent at 24 h but could not be detected at the shorter incubation time (Fig. 3b). These observations indirectly imply that black pepper extract and piperine may promote the translocation of NPC1L1 from the cell membrane, causing diminished cholesterol uptake.

With SR-BI, the action of black pepper extract and piperine showed a different pattern of effects. While again the cell lysate levels were unaffected, 3 h incubations showed reduced immunoreactivity in the membrane fractions (Fig. 4), but by 24 h this effect was smaller (for piperine) or absent for the extract. Taking all data together, both black pepper extract and piperine may cause

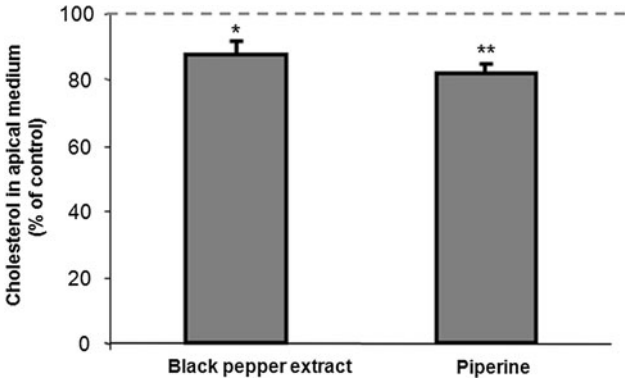

Fig. 2 Effect of black pepper extract and piperine on [^{3}H]cholesterol efflux in differentiated Caco-2 cells. After loading cells with [1α2α(n)-^{3}H]cholesterol micelles, black pepper extract (100 μg/mL) or piperine (100 μM) was added and incubated for 24 h. The apical media was then collected and radioactivity measured. Values represent mean ± SEM ($n = 4$). *$p < 0.05$, **$p < 0.01$ compared to untreated cells (control)

Table 1 Effect of black pepper extract and piperine on cholesterol solubility in micelles

Concentration of black pepper extract (μg/mL) or piperine (μM)	Cholesterol concentration in micelles (mg/dL)	
	Black pepper extract	Piperine
Control	52.4 ± 3.9	51.2 ± 3.3
100	45.5 ± 4.4	49.6 ± 1.8
200	46.8 ± 3.6	48.9 ± 2.9
400	46.7 ± 4.9	48.8 ± 2.5
500	46.2 ± 4.3	49.0 ± 3.5
1,000	39.7 ± 3.9*	49.5 ± 2.2

Micelles were suspended in solution of various concentrations of black pepper or piperine and content of cholesterol in micelles determined. Values represent mean ± SEM ($n = 6$–8)

* $p < 0.05$ compared to control by ANOVA with William's test

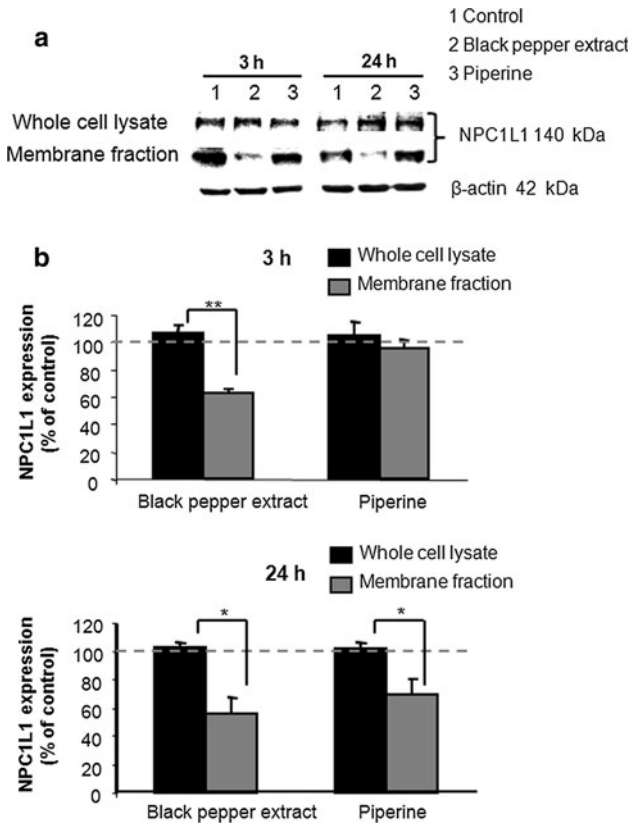

Fig. 3 NPC1L1 protein expression in whole cell lysates and membrane fraction of differentiated Caco-2 cells. Cells were incubated with black pepper extract (100 μg/mL) or piperine (100 μM) for 3 and 24 h. The appropriate fractions were analyzed by immunoblotting (**a**). Protein band densities were normalized to the density of β-actin and then calculated as percentage of control (**b**). The data represent mean ± SEM ($n = 3$–4). *$p < 0.05$, **$p < 0.001$ by Student's t test

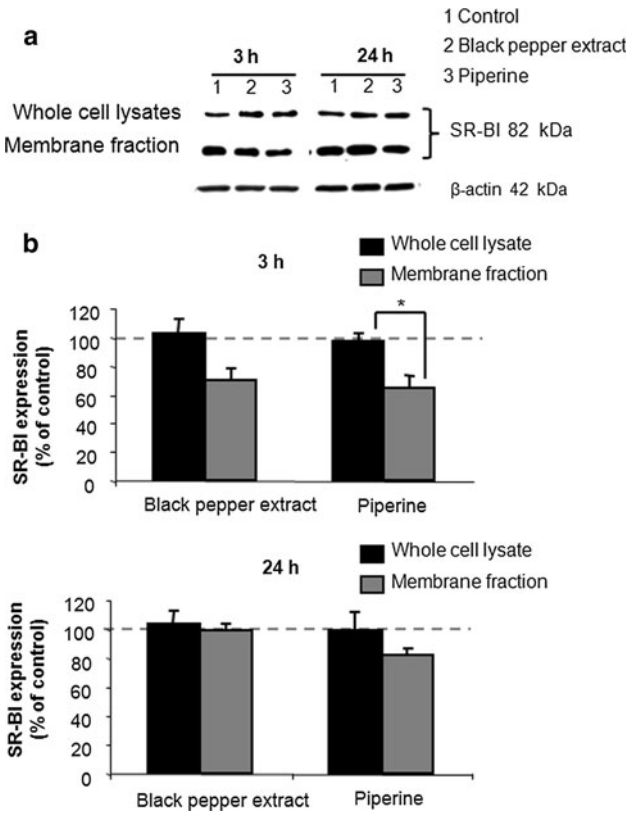

Fig. 4 SR-BI protein expression in whole cell lysates and membrane fraction of differentiated Caco-2 cells. Cells were incubated with black pepper extract (100 μg/mL) and piperine (100 μM) for 3 and 24 h. The appropriate fractions were analyzed by immunoblotting (**a**). Protein band densities were normalized to the density of β-actin and then calculated as percentage of control (**b**). The data represent mean ± SEM ($n = 3$–4). *$p < 0.05$ by Student's t test

internalization of the cholesterol transporter proteins NPC1L1 and SR-BI.

Effect of anti-SR-BI antibody on cholesterol uptake inhibition mediated by black pepper extract or piperine

Antibody binding can also interfere with protein function, so we explored the possibility that SR-BI and NPC1L1 antibodies might reduce cholesterol transport. However, anti-NPC1L1 failed to affect cholesterol uptake into Caco-2 cells (data not shown) and this might reflect a remoteness of the epitope from the cholesterol binding site. For anti-SR-BI antibody, cholesterol uptake was increased in the presence of this antibody (Table 2). These data suggest that anti-SR-BI was interacting with the target protein by cholesterol uptake, which accords with a similar study [27]. The inhibitory effects of black pepper extract, piperine and ezetimibe on cholesterol uptake were reduced in the presence of anti-SR-BI (Table 2), which suggests that this antibody produced a uniform enhancement of cholesterol transport regardless of the presence of piperine, extract and ezetimibe.

Discussion

The hypocholesterolemic effect of black pepper in rats fed a high-fat diet [19] and its ability to reduce the cholesterol uptake in differentiated Caco-2 cells [20] have been previously demonstrated. Piperine, a major active substance of black pepper, is thought to play a critical role in these effects since it was also found to reduce total plasma cholesterol, LDL and VLDL in rats fed a high-fat diet [21]. In the present study, most effects of commercial piperine are correlated to those of black pepper extract containing an equivalent concentration of piperine, suggesting that piperine was the active compound in the inhibition of cholesterol uptake. The mechanism of this effect was not due to the interference of either the size of lipid micelles or the cholesterol efflux pathway, nor changing of solubility

Table 2 Cholesterol uptake into Caco-2 cells in the presence of black pepper extract, piperine, and ezetimibe in the presence and absence of anti-SR-BI

	Cholesterol uptake (% of control)				
	Without anti-SR-BI	*p* value*	With anti-SR-BI	*p* value*	*p* value**
Control	100 ± 0.00	–	116.69 ± 8.87	–	0.089
Black pepper extract 100 μg/mL	67.17 ± 3.49	<0.001	86.45 ± 3.78	0.012	0.004
Piperine 100 μM	66.41 ± 1.52	<0.001	80.77 ± 4.90	0.003	0.019
Ezetimibe 100 μM	73.76 ± 3.09	<0.001	84.56 ± 3.62	0.008	0.046

Values represent mean ± SEM ($n = 6$)

* Compared to control

** Compared between with and without anti-SR-BI by Student's *t* test

of lipid micelles, but translocation of cholesterol transporter NPC1L1 and SR-BI proteins to the cytosol.

NPC1L1 is the target of ezetimibe [10, 11] and here it (at a supramaximal concentration) inhibited cholesterol uptake into Caco-2 cells by ~50 %, implicating uptake via other non-NPC1L1 pathway(s). However, the antibody recognizing NPC1L1 failed to identify a functional role for this cholesterol transporter in the present study, but the use of antibodies in such protocols often fails. NPC1L1 contains 13 transmembrane domains with an extracellular N-terminal and cytoplasmic C-terminus [28]. Cholesterol binds to the N-terminal region whereas ezetimibe binds to a different site of NPC1L1 [29]. The anti-NPC1L1 antibody used here might not bind to an amino-acid sequence directly involving cholesterol transport.

SR-BI is one of the 'scavenger' receptors on the apical membrane of intestinal cells but is not specific for cholesterol transport. However, it has been proposed to contribute to intestinal cholesterol absorption [30] and also binds ezetimibe [29, 31]. Although SR-BI is a functional cholesterol transporter, it appears not to be essential for absorption [32, 33]. The suggestion of some enhancement of cholesterol uptake with anti-SR-BI antibody was also made previously [27] and possibly this arose from reduced efflux, because SR-BI also facilitates cholesterol efflux to micelles [27]. However, piperine, extract or ezetimibe were similar, irrespective of whether anti-SR-BI IgG was present. Thus, the role of SR-BI in cholesterol uptake was somewhat complicated.

NPC1L1 is found both in enterocyte brush border membranes and the intracellular compartment [34]. Cholesterol transport involves translocation of the NPC1L1/cholesterol complex to the cytoplasm and ezetimibe inhibits this internalization [23, 35]. Basolateral-to-apical transcytosis of SR-BI has also been observed [36]. The present study provides evidence that NPC1L1 and SR-BI are influenced by black pepper extract and piperine. With black pepper extract or piperine, the presence of NPC1L1 and SR-BI in the plasma membrane of differentiated Caco-2 cells is reduced, while their overall expression appears to be unaffected. But the discrepancy between actions of black pepper extract and piperine with incubation time suggests that other constituents of the extract were active. These observations imply that piperine reduces cholesterol absorption by promoting the internalization or preventing the recycling of NPC1L1 and SR-BI proteins back to the plasma membrane. Thus, these compounds possibly promote the internalization of these proteins which then may limit the accessibility of cholesterol to the transporters.

Lipid micellar size and cholesterol micellar solubility also influence intestinal cholesterol absorption [37]. Many food ingredients including plant sterols, dietary polyphenols and vegetable proteins have been shown to reduce cholesterol absorption by decreasing cholesterol micellar solubility and/or increasing lipid micellar size [14, 16, 38, 39]. In the present study, the size of cholesterol micelles was apparently not affected by black pepper extract and piperine. Likewise, compounds binding to bile acids or displacing cholesterol from lipid micelles also reduce cholesterol absorption [14, 40]. At very high extract concentration (1,000 μg/mL), there was reduced micellar cholesterol. This might to be due to actions of other amides such as piperidine, piperamide and pipericide [41] and also plant sterols such as sitosterol [41] which competes with cholesterol in lipid micelles [42, 43]. Further interactions may also involve the sodium taurocholate used to make the micelles.

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