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Unusual Sterolic Mixture, and 24-Isopropylcholesterol, From the sponge *Ciocalypta* sp. Reduce Cholesterol Uptake and Basolateral Secretion in Caco-2 Cells

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ABSTRACT

An unusual sterolic mixture (82.3% of 24-isopropylated sterols) and its major component, 24-isopropylcholesterol, isolated from a marine sponge, *Ciocalypta* sp. (Halichondriidae), reduce cholesterol uptake, basolateral secretion and ACAT-2 mRNA expression and increase the expression of ABCA1 mRNA in Caco-2 cells. The decreases of cholesterol uptake and secretion induced by 24-isopropylcholesterol alone were more than that of both the sterolic mixture and β-sitosterol. These data add a new sterol, 24-isopropylcholesterol, to sterols that may reduce intestinal cholesterol absorption. J. Cell. Biochem. 106: 659–665, 2009.

KEY WORDS: STEROLS; CHOLESTEROL; 24-ISOPROPYLCHOLESTEROL; INTESTINAL CHOLESTEROL ABSORPTION; CACO-2 CELLS; MARINE SPONGE; HALICHONDRIIDAE; ABCA1; ACAT-2

The abnormal metabolism and elevation of plasma cholesterol are well-documented risk factors for the development of atherosclerosis. Evidence from clinical trials indicates that reducing plasma cholesterol by dietary and/or pharmacological means leads to reduction in the incidence of death from cardiovascular disease [Scandinavian Simvastatin Survival Study Group, 1994]. Cholesterol absorption is defined as the transport of cholesterol from intestinal lumen to its secretion to the plasma [Wilson and Rudel, 1994; Dawson and Rudel, 1999]. There are several steps in this process, among them is the uptake for cholesterol by enterocytes. Several candidate proteins that can facilitate cholesterol uptake by enterocytes have been identified. Among them Niemann-pick C1 like 1 (NPC1L1) might play a key role in this process [Altmann et al., 2004]. Indeed, NPC1L1 gene deletion reduces cholesterol absorption in mice [Altmann et al., 2004; Davis et al., 2004]. Furthermore, ezetimibe, a cholesterol absorption inhibitor, was shown to be inefficient in the absence of NPC1L1. Other proteins, mainly the family of ATP Binding Cassette (the half transporters ABCG5 and ABCG8 and the transporter ABCA1), play an important role in the regulation of cholesterol absorbed by the intestine [Dean et al., 2001]. The two half transporters normally cooperate to restrict the absorption of sterols. Mutations of these two genes result in the excess uptake of sterol including plant sterols such as β-sitosterol [Berge et al., 2000]. ABCA1 is involved in cholesterol efflux, the first step in reverse cholesterol transport and to elevation in plasma HDL-cholesterol level [Rust et al., 1999]. The cholesterol that enters the enterocyte is transferred to endoplasmic reticulum, where it can be esterified by acyl-CoA cholesterol acyltransferase-2 (ACAT-2). Cholesterol esterification involves the covalent attachment of fatty acids to 3-position hydroxyl group of cholesterol to form cholesteryl esters. Employing a number of specific ACAT-2 inhibitors, several studies have shown a reduction in intestinal cholesterol absorption

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[Clark and Tercyak, 1984; Kataoka et al., 1996; Miyazaki et al., 2005]. Furthermore, ACAT-2 knock out mice are resistant to diet-induced hypercholesterolemia due to defective cholesterol esterification by intestinal cells [Buhman et al., 2000].

Phytosterols are naturally occurring sterols in the plant equivalent of mammalian cholesterol. Despite structurally differing from cholesterol by only a methyl (campesterol) or an ethyl (β-sitosterol) group attached to the C-24 position of the side-chain, these major plant sterols are absorbed to a much lesser extent than cholesterol [Lees et al., 1977; Mattson et al., 1982] and reduce cholesterol absorption [Ling and Jones, 1995; Jones, 1999; Ostlund et al., 2002]. These remarkable properties have focused much attention on phytosterols and consequently research activity on such sterols with branched side-chain has increased in order to investigate some unusual structures [Djerassi and Silva, 1991]. Since the discovery of 24-isopropylcholesterol and 24-isopropyl-22-dehydrocholesterol from an Australian *Pseudaxinyssa* sponge [Hofheinz and Oesterhelt, 1979], several marine sponges from the family Halichondriidae [Erpenbeck et al., 2002] were shown to contain these sterols at relatively high levels, including the genera *Topsentia* [Fusetani et al., 1994; Kerr et al., 1997; Yang et al., 2003], *Epipolasis* [Calderón et al., 2004], and *Ciocalypta* [Djerassi and Silva, 1991].

The goal of the present study was to determine whether a sterolic mixture and its major component 24-isopropylcholesterol isolated from a marine sponge, *Ciocalypta* sp., affect the cholesterol uptake and secretion by differentiated Caco-2 cells, an enterocyte cell model.

MATERIALS AND METHODS

The human transformed colonic epithelial Caco-2 cells were purchased from American Type Culture Collection (ATCC). Cell culture media, reagents, and fetal bovine serum (FBS) (certified grade) were from Sigma, L'Isle d'Abeau Chesnes, Saint-Quentin Fallavier, France. ThinCert™ (0.4 μm pore size, inserts of 24.5 mm diameter), tissue culture inserts for multiwell plates and flasks for cell culture were obtained from Greiner bio-one, Les Ulis, Courtaboeuf, France. 1,2-^{3}H(N)]-cholesterol was obtained from PerkinElmer, Boston, MA. Taurocholic acid sodium salt, oleic acid, 2-oleoylglycerol, β-sitosterol, and other common laboratory reagents were from Sigma. RNeasy® Mini Kit was purchased from Qiagen, Courtaboeuf, France. SuperScript™ II Rnase H-Reverse Transcriptase Kit, Platinum Quantitative RT-PCR ThermoScript Kit were obtained from Invitrogen, Cergy-Pontoise, France. Primers for ABCA1, ABCG5, ABCG8, ACAT-2, and NPC1L1 were purchased from Sigma.

SPONGE COLLECTION, ISOLATION, AND IDENTIFICATION OF STEROLS

The sponge *Ciocalypta* sp. (Class Demospongiae; Order Halichondrida; Family Halichondriidae) was collected off Dakar, Senegal [Djerassi and Silva, 1991]. Voucher specimen has been deposited at the Centre d'Océanologie de Marseille. Free sterols were isolated during lipid class separation from fractions eluted with dichloromethane. Total lipids were obtained at 5.1% of the biomass (dried wt), and total free sterols accounted for 11.8% of total lipids. Sterol mixtures were analyzed by gas chromatography–mass spectrometry (EI–MS, 70 eV) in free form or as acetates. A 30 m × 0.32 mm fused silica capillary column coated with HP-1 (0.25 μm phase thickness) was used (carrier gas helium). Column temperature was programmed for free sterols and steryl acetates from 200 to 300°C at 3°C min^{-1}. The GC–MS analysis of the steryl acetates allowed to show readily that all the sterols were Δ5-sterols. Indeed, the Δ5-steryl acetates undergo an easy loss of acetic acid. They do not yield the molecular ion but instead a prominent $[M-60]^{+}$ ion [Goad and Akihisha, 1997], whereas most other steryl acetates yield a molecular ion. Mass spectra of sterol acetates displayed the $[M-AcOH]^{+}$ and $[M-AcOH-iPr]^{+}$ ions as expected for 3β-hydroxy-Δ5 sterol structures, at *m*/*z* 410 and 368 for the acetylated sterol A, and at *m*/*z* 408 and 365 for the acetylated sterol B [Barnathan et al., 1992; Goad and Akihisha, 1997; Barnathan et al., 2003]. Other diagnostic fragment ions were observed for the acetylated sterol A at *m*/*z* 395, 302, 287, 255, and 213, and for the acetylated sterol B at *m*/*z* 309, 282, 255, and 213. The GC–MS profile of the free sterol in mixture M is shown in Figure 1. Mass spectra of the unusual sterols A, B, and C in the free form are depicted on Figure 2 with the characterization of the main fragmentation patterns.

A molecular ion is observed for each free sterol analyzed. All spectra of these Δ5-sterols contained the $[M-sc-42-H_2O]^{+}$ ion (sc = side-chain) at *m*/*z* 213, and the $[M-sc-H_2O]^{+}$ ion at *m*/*z* 255 [Goad and Akihisha, 1997]. Other major diagnostic ions are showed in Figure 2. There is no major ion at *m*/*z* 296 in the mass spectrum of sterol C excluding a 24(24′)-double bond. Identification of the sterols D, E, and F was obtained from their known spectra and their GC–MS retention times, and by comparison with spectra of authentic compounds available in the Hewlett Packard Spectra Library. In addition, β-sitosterol was also compared with an authentic compound commercially available. All these 24-alkylated sterols are generally mixtures of 24*R* and 24*S* epimers that are not distinguishable in the usual GC conditions. Thus, sterol mixture M was devoid of cholesterol and contained 24-isopropylcholesterol A, 24-isopropyl-22-dehydrocholesterol B, 24-isopropylcholesta-5,24′-dienol (or 24-isopropenylcholesterol) C, β-sitosterol/clionasterol D, poriferasterol/stigmasterol E, and brassicasterol/crinosterol F as shown in Figure 3.

Preparative HPLC separation (Gilson, pump 305, UV detector 115, interchrom C-18, intersphere 5 μm ODS1 250 mm × 4.6 mm column, MeOH) of the sterol mixture M yielded the unusual 24-alkyl sterols A and B identified by ^{1}H-nuclear magnetic resonance (^{1}H-NMR, Brucker Avance-III 300 MHz) by comparison with previous reported data [Hofheinz and Oesterhelt, 1979; Djerassi and Silva, 1991; Calderón et al., 2004]. The singlets at δ 0.68 and 1.00, and the doublet at δ 5.35 (J = 5.4 Hz), correspond to those expected for the C-18 and C-19 angular methyl groups, and for the olefinic proton H-6, respectively, of a Δ5-sterol nucleus. The proton NMR spectrum displays five methyl doublets at δ 0.95 (J = 6.8 Hz) for 21-H, and 0.91 (J = 6.6 Hz), 0.84 (J = 6.5 Hz), 0.82 (J = 6.6 Hz), 0.79 (J = 5.8 Hz) for the two isopropyl groups. ^{1}H-NMR spectrum of

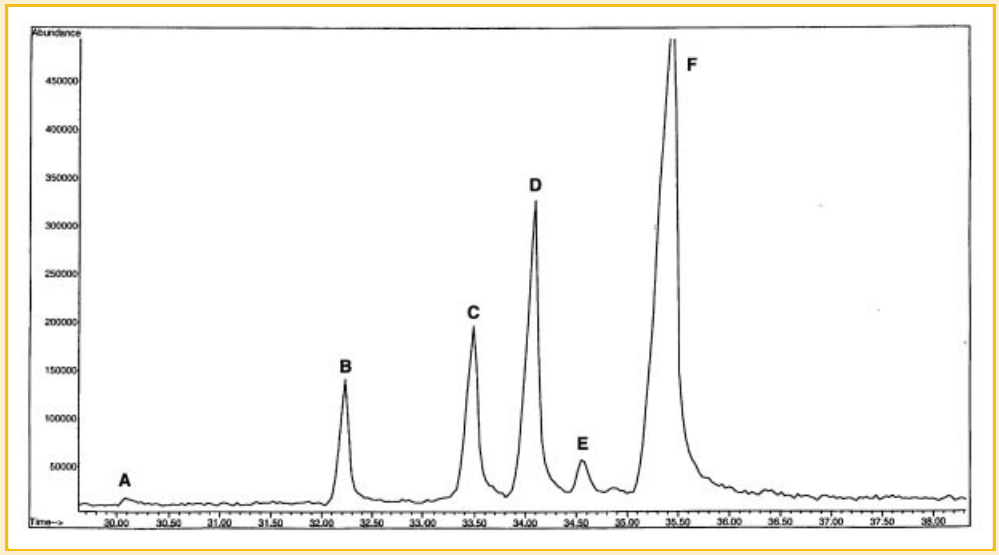

Fig. 1. GC–MS profile of the sterol mixture M.

sterol B also displays five doublets expected for H-21 and for two isopropyl groups. In addition to the olefinic proton H-6 at δ 5.35 (J = 4.9 Hz), two olefinic signals were at δ 5.03 (dd, 17.1, 8.1 Hz, 23-H) and 5.13 (dd, 17.1, 9.2 Hz, 22-H) corresponding to a sterol Δ22 side-chain [Calderón et al., 2004].

CELL CULTURE

The transformed human intestinal Caco-2 cells were grown in Dulbecco's modified essential medium (DMEM), supplemented with 10% (v/v) FBS, 1% Glutamine, and 0.5% penicillin–streptomycin, (complete media) at 37°C under 5% CO_2. Cells were seeded at a

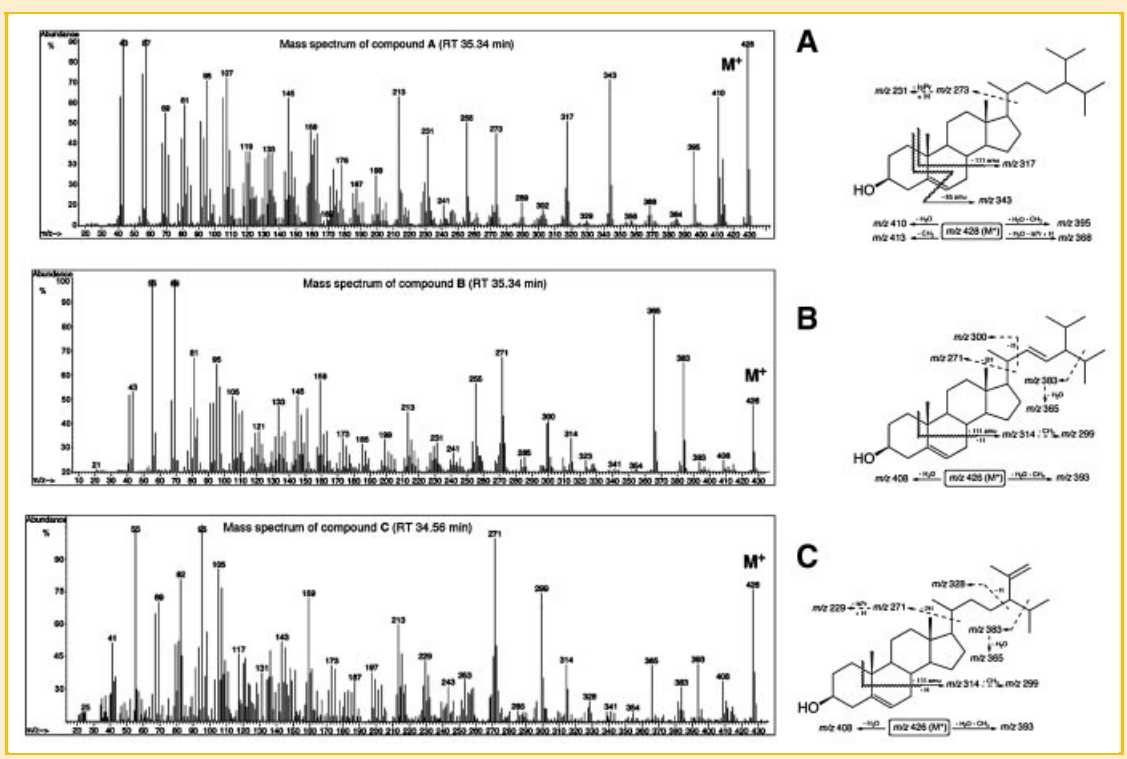

Fig. 2. Mass spectra and typical fragmentations of 24-isopropylcholesterol A, 24-isopropyl-22-dehydrocholesterol B, and 24-isopropylcholesta-5,24′-dienol C.

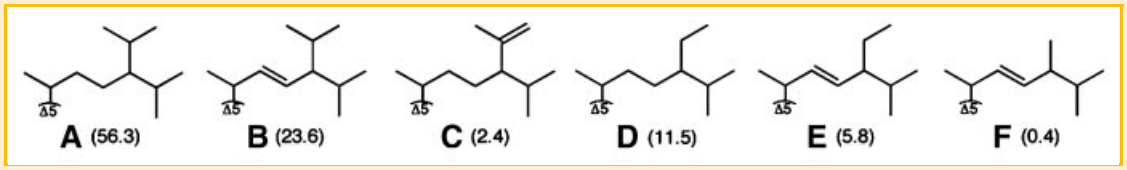

Fig. 3. Sterol composition of mixture M from *Ciocalypta* sp. (% of total sterols in brackets): 24-isopropylcholesterol A, 24-isopropyl-22-dehydrocholesterol B, 24-isopropylcholesta-5,24′-dienol C, β-sitosterol/clionasterol D, poriferasterol/stigmasterol E, and brassicasterol/crinosterol F.

density of 1×10^6 in 75 cm^2 flasks. The media was replaced every 2–3 days. Cells were sub-cultured from flasks at 90% confluency, to inserts (ThinCert™) and plated at a density of 3×10^5 cells/well. Fully differentiated Caco-2 cells were used for all experiments (21 days post-subculturing).

CHOLESTEROL UPTAKE AND BASOLATERAL SECRETION

To study the influence of sterolic mixture M or 24-isopropylcholesterol A on cholesterol uptake and secretion by differentiated Caco-2 cells, the complete media containing 10% FBS were replaced with a mixed micellar solution containing 5 mM sodium taurocholate, 400 μM oleic acid, 100 μM 2-oleoylglycerol, 100 μM cholesterol in the presence of 1,2-^{3}H(N)]-cholesterol at the activity of 1 μCi/ml in DMEM [Plat et al., 2005]. In the case when the effect of sterolic mixture M or 24-isopropylcholesterol were examined, they were added on the apical side of the membrane. After 5 or 20 h of incubation, the media on the apical and basolateral sides were withdrawn. Cells were washed and their lipids were extracted by incubation with isopropanol. Radioactivity was then measured in the basolateral media and in isopropanol fraction by scintillation counting on PACKARD Tri-carb 2100TR.

ESTIMATION OF MRNA LEVELS BY REAL-TIME RT-PCR

Total RNA was isolated from Caco-2 cells using RNeasy® Mini Kit (Qiagen). The mRNA (1 μg) was reverse-transcribed using SuperScript™ II RNase H-Reverse Transcriptase Kit (Invitrogen). The cDNA was then used for RT-PCR with Platinum Quantitative RT-PCR ThermoScript Kit (Invitrogen) in the presence of 0.1 μl CYBR Green 1/100. The gene-specific primers were designed to yield a single amplification based on dissociation curves. The sequences were analysed by BLAST (National Center for Biotechnology Information, National Institute of Health) to verify that the primers used were specific for a given gene. Real-time quantitative PCR was performed on Rotorgene 2000 system (Corbett Research, Mortlake, New South Wales, Australia). The thermal cycle parameters were as follows: hold for 5 min at 95°C for one cycle followed by amplification of cDNA for 40 cycles with melting for 15 s at 95°C, annealing at specific temperatures (Table I) for 10 s, extention for 15 s at 72°C, and detecting temperatures (Table I) for 10 s. The values were normalized using 6S rRNA or β-actin as endogenous internal standards. A serial dilution of standard was run for each mRNA and used to calculate the relative levels of mRNA studied (NPC1L1, ABCG5/G8, ABCA1, and ACAT-2).

STATISTICAL ANALYSIS

Data are shown as means ± SD from three independent experiments performed in triplicate. Student's test was used, and P values of <0.05 were considered significant.

RESULTS

To study the effects of sterolic mixture M (including 24-isopropylcholesterol at 56.3%) or 24-isopropylcholesterol A on the cholesterol uptake and secretion in Caco-2 cells, the differentiated cells were incubated with a mixed micelles (see Materials and Methods Section) containing ^{3}H-cholesterol (1VCi/ml) in the presence of the mixture M (containing 24-isopropylcholesterol (25.4 μM), 24-isopropyl-22-dehydrocholesterol (10.7 μM), 24-isopropenylcholesterol (1.1 μM), β-sitosterol/clionasterol (5.4 μM), poriferasterol/ stigmasterol (2.7 μM), and brassicasterol/crinosterol (0.2 μM)) or the sterol A 50 μM.

The presence of mixture M in the apical medium (20 h) induced a reduction of ^{3}H-cholesterol uptake by 55% ($P < 0.01$) (Fig. 4A). Given the activity of the sterolic mixture M on cholesterol uptake and secretion, we decided to investigate the major sterol component A in that way. The presence of 50 μM of sterol A (24-isopropylcholesterol) in apical medium (20 h) also induced a reduction of ^{3}H-cholesterol uptake by 67% ($P < 0.01$) (Fig. 4B). Moreover, the presence of the mixture M and sterol A in the apical medium reduced ^{3}H-cholesterol secretion into basolateral compartment by 40% and 50% respectively ($P < 0.01$) (Fig. 5A,B). A grossly similar effect on cholesterol secretion, has been observed after 5 h incubation with the mixture M (39%) ($P < 0.01$) (data not shown). Significantly lower ^{3}H-cholesterol uptake and secretion (5 h

TABLE I. Primers' Sequences

Gene	Primers' sequences
ABCA1	Forward: CTTTTGCTGTATGGGTGGTC; reverse: TACAGGTCTGGGCCTGATGAA
ACAT-2	Forward: GGACGTTGAGTTCCACCAGT; reverse: ACCTTTGCCCTGACCTCTGG
S6	Forward: CCAAGCTTATTCAGCGTCTTGTTACTCC; reverse: CCCTCGAGTCCTTCATTCTCTTGGC
β-actin	Forward: TGCTATCCAGGCTGTGCTATCC; reverse: GCCAGGTCCAGACGCAGG

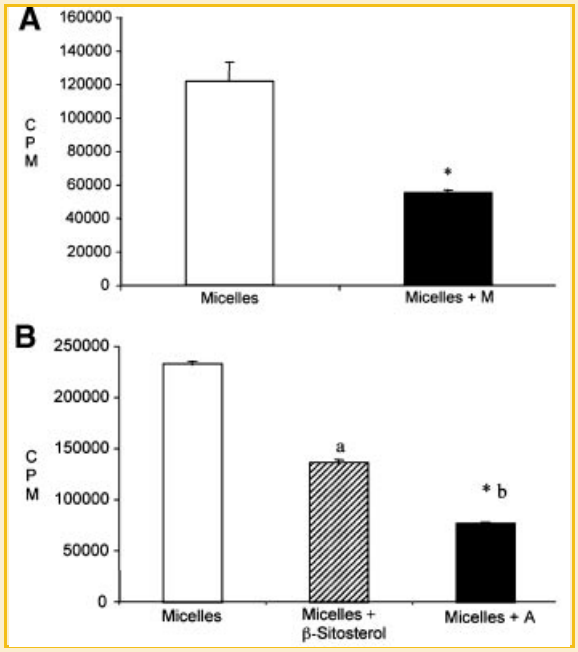

Fig. 4. A: Effect of 20 h incubation of the sterolic mixture M on ^{3}H-cholesterol uptake ($^{*}P < 0.01$). B: Effect of 24-isopropylcholesterol A on cholesterol uptake compared with β-sitosterol. *, Micelles + A versus Micelles, $P < 0.01$, a: Micelles + β-Sitosterol versus Micelles, $P < 0.05$, b: Micelles + A versus Micelles + β-Sitosterol, $P < 0.05$.

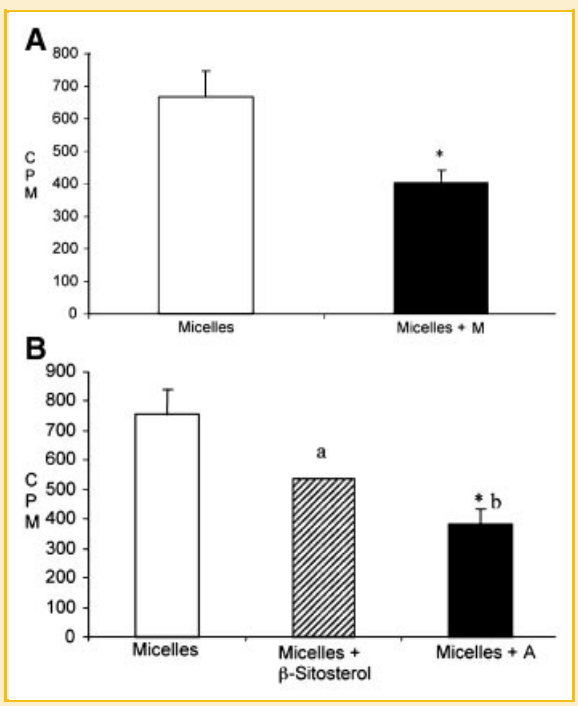

Fig. 5. A: Effect of 20 h incubation of the sterolic mixture M on ^{3}H-cholesterol secretion ($^{*}P < 0.01$). B: Effect of 24-isopropylcholesterol A on cholesterol secretion compared with β-sitosterol. *, Micelles + A versus Micelles, $P < 0.01$, a: Micelles + β-Sitosterol versus Micelles, $P < 0.05$, b: Micelles + A versus Micelles + β-Sitosterol, $P < 0.05$.

incubation) with the sterol A (100 μM) compared to micelles alone (53% and 52%, $P < 0.01$) and to sterol A (50 μM) (28% and 27%, $P < 0.02$) (data not shown). The presence of the well established inhibitor of cholesterol absorption, β-sitosterol (50 μM) also reduced cholesterol uptake (42%, $P < 0.05$) and secretion (29%, $P < 0.05$) but to a lower extent than 24-isopropylcholesterol (67% and 50%) (Figs. 4B and 5B).

The mixture M and the sterol A (50 μM) had no effect on NPC1L1 and ABCG5 gene expression (data not shown). However, ABCA1 gene expression increased with both the mixture M and the sterol A (4.8- and 5.8-fold) respectively (Fig. 6A,B). The treatment of cells with sterol A (50 μM) decreased ACAT-2 mRNA level (54%, $P < 0.01$) as shown in Figure 6C.

DISCUSSION

Effect of plant sterols on the intestinal absorption of cholesterol has been demonstrated 50 years ago. Many researches show that plant sterols reduce the absorption of cholesterol both in vitro and in vivo

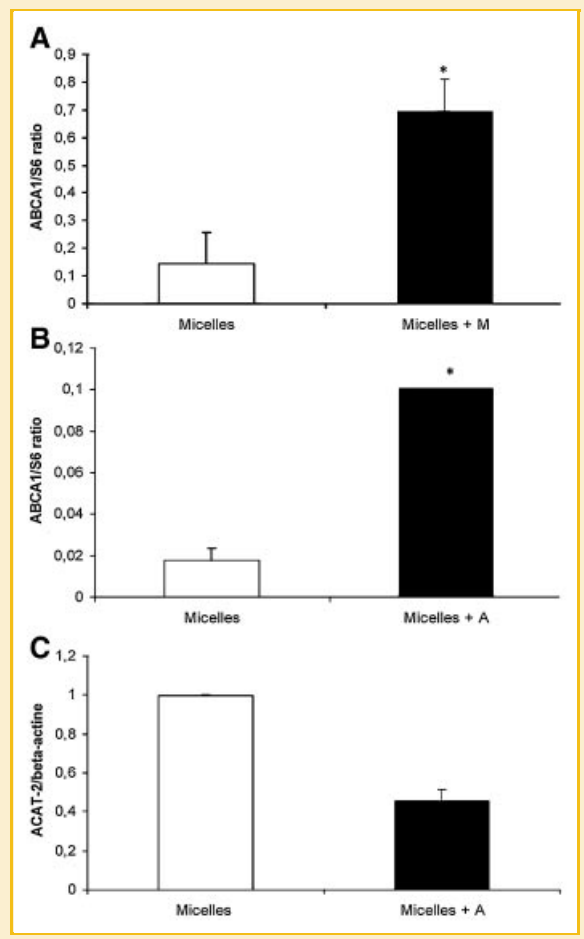

Fig. 6. A: Effect of 20 h incubation of the sterolic mixture M on the expression of ABCA1 gene ($^{*}P < 0.01$). B: Effect of 24-isopropylcholesterol A on the expression of ABCA1 gene ($^{*}P < 0.01$). C: Effect of 24-isopropyl-cholesterol A on the expression of ACAT-2 gene ($^{*}P < 0.01$).

[Peterson, 1951; Lees et al., 1977; Ling and Jones, 1995; Grundy, 2001; Ostlund et al., 2002; Ho and Pal, 2005]. Although the mechanism of the effect has not been determined yet, foods enriched with plant stanol or sterol esters have gained a prominent position in decreasing cardiovascular risk by dietary means [Plat et al., 2000; Grundy, 2001].

In this study, we report for the first time that the apical cholesterol uptake into Caco-2 cells and cholesterol secretion into their basolateral side are both decreased in the presence of the sterolic mixture M (mainly containing 24-isopropylcholesterol, 56.3%, 24-isopropyl-22-dehydrocholesterol, 23.6%, and β-sitosterol, 11.5%) and of 24-isopropylcholesterol (sterol A). The decreases induced by 24-isopropylcholesterol alone are more than the sterolic mixture and the known phytosterol, β-sitosterol.

The precise mechanisms of these inhibitions have not been fully elucidated yet because of the variety of the target genes.

NPC1L1 was recently identified as a major component of the cholesterol uptake pathway by the enterocyte, evidenced by the fact that mice lacking NPC1L1 have dramatically reduced intestinal cholesterol absorption and are resistant to diet-induced hypercholesterolemia [Altmann et al., 2004; Davis et al., 2004]. Although NPC1L1 expression is postulated to be influenced by plant sterol, in our study, 24-isopropylcholesterol shows no influence on the expression of NPC1L1. The same result was found in a study on hamster [Field et al., 2004].

Another target gene of phytosterol is the cholesterol half transporters ABCG5/G8 [Repa et al., 2002; Plat et al., 2005]. These two transporters play an important role in the regulation of intestinal cholesterol absorption. Indeed, it has been described that the expression of ABCG5/G8 is induced by LXR (liver X receptor) activators [Repa et al., 2002; Plat et al., 2005] and that treatment of intestinal cells with phytosterol increases the expression of LXR target gene [Plat et al., 2005] suggesting that phytosterol and their metabolites act as LXR ligands and influence cholesterol metabolism. In our study, we could not show any influence of the sterolic mixture, and 24-isopropylcholesterol, on mRNA of ABCG5/G8 since the gene is expressed at low level in Caco-2 cells [Plat and Mensink, 2002]. ABCA1 is another target gene of phytosterols since these latter and their derivatives activate LXR and that they induce the sterol-exporting transporter ABCA1. Indeed, it has been reported that that mixed micelles enriched with plant sterols (sitostanol and sitosterol) increased ABCA1 expression in Caco-2 cells (by 244% and 273% respectively) and LXR activation [Plat et al., 2005]. In another study, it has been suggested that metabolites of phytosterols (oxyphytosterols) might be responsible for the effects on gene expression [Plat and Mensink, 2002].

In our study, we show that a sterolic mixture, and the very unusual 24-isopropylcholesterol, induce the expression of ABCA1 mRNA in Caco-2 cells. The role of ABCA1 in cholesterol absorption remains to be clarified since this transporter is dominantly expressed on the basolateral surface of intestinal cells [Ohama et al., 2002]. Moreover, ABCA1 is involved in cholestrol efflux, the first step in reverse cholesterol transport and in the elevation in plasma HDL-cholesterol level [Rust et al., 1999]. The significant effect of the sterolic mixture and 24-isopropylcholesterol on ABCA1 gene expression may suggest that this sterol could play a role in raising HDL-cholesterol level. The cholesterol that enters the enterocyte is transferred to endoplasmic reticulum, where it can be esterified by ACAT-2. The role of ACAT-2 in intestinal cholesterol absorption has been demonstrated in many in vitro and in vivo studies [Clark and Tercyak, 1984; Kataoka et al., 1996; Miyazaki et al., 2005].

The expression and activity of the enzyme are regulated by intracellular cholesterol content. Indeed, incubation of cells with free cholesterol has been shown to potentiate ACAT-2 mRNA expression and ACAT activity. Moreover, removal of cholesterol by incubation of cells with HDL (an acceptor of cholesterol) or deprivation of cholesterol by incubation of cells with LPDS (lipoprotein deficient serum) decreased the ACAT mRNA expression [Pramfalk et al., 2007]. In another study [Rudel et al., 2002] it was proposed that cholesterol regulates the enzyme stability when cholesterol esterification is preferred (i.e., increased intracellular availability). In our study we show that 24-isoprpylcholesterol led to a decrease in ACAT mRNA expression. Thus, we can speculate whether the mechanisms of regulations cited above are also operative in Caco-2 cells. Thus, there would be less cholesterol available which would lead to lower ACAT mRNA, lower cholesterol esterification and then lower secretion. Detailed studies of the effects of this sterol on ACAT activity are therefore needed to confirm or refute our hypothesis.

The sterol 24-isoprpoylcholesterol decreased ACAT-2 mRNA and increased ABCA1-expressions. Our finding is supported by the observation that the loss of ACAT-2 from enterocytes leads to an increase in the level of expression of ABCA1 mRNA [Repa et al., 2004]. Given the localization of ABCA1 on basolateral surface one might speculate that in this case, unesterified cholesterol would be packaged into nascent HDL that enters basolateral compartment.

In conclusion, our study shows for the first time that a sterolic mixture from a sponge *Ciocalypta* sp., and its major component, 24-isopropylcholesterol, decrease the uptake and secretion of cholesterol and ACAT-2 mRNA expression in Caco-2 cells. Moreover, these sterols upregulate ABCA1 gene expression. Thus, these data add a new sterol, 24-isopropylcholesterol to sterols that may reduce cholesterol absorption. Further analyses are required to elucidate the mechanism of action by which 24-isopropylcholesterol can influence cholesterol absorption and ABCA1 gene expression.

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