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New Insights In Intestinal Sar1B GTPase Regulation and Role in Cholesterol Homeostasis

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ABSTRACT

Sar1B GTPase is a key component of Coat protein complex II (COPII)-coated vesicles that bud from the endoplasmic reticulum to export newly synthesized proteins. The aims of this study were to determine whether Sar1B responds to lipid regulation and to evaluate its role in cholesterol (CHOL) homeostasis. The influence of lipids on Sar1B protein expression was analyzed in Caco-2/15 cells by Western blot. Our results showed that the presence of CHOL (200 μM) and oleic acid (0.5 mM), bound to albumin, increases Sar1B protein expression. Similarly, supplementation of the medium with micelles composed of taurocholate with monooleylglycerol or oleic acid also stimulated Sar1B expression, but the addition of CHOL (200 μM) to micelle content did not modify its regulation. On the other hand, overexpression of Sar1B impacted on CHOL transport and metabolism in view of the reduced cellular CHOL content along with elevated secretion when incubated with oleic acid-containing micelles for 24 h, thereby disclosing induced CHOL transport. This was accompanied with higher secretion of free- and esterified-CHOL within chylomicrons, which was not the case when oleic acid was replaced with monooleylglycerol or when albumin-bound CHOL was given alone. The aforementioned cellular CHOL depletion was accompanied with a low phosphorylated/non phosphorylated HMG-CoA reductase ratio, indicating elevated enzymatic activity. Combination of Sar1B overexpression with micelle incubation led to reduction in intestinal CHOL transporters (NPC1L1, SR-BI) and metabolic regulators (PCSK9 and LDLR). The present work showed that Sar1B is regulated in a time- and concentration-dependent manner by dietary lipids, suggesting an adaptation to alimentary lipid flux. Our data also suggest that Sar1B overexpression contributes to regulation of CHOL transport and metabolism by facilitating rapid uptake and transport of CHOL. J. Cell. Biochem. 116: 2270–2282, 2015.

KEY WORDS: Sar1B; CHOLESTEROL; FATTY ACIDS; INTESTINE; LIPOPROTEINS

Coat protein complex II (COPII)-coated vesicles bud from the endoplasmic reticulum (ER) to export newly synthesized proteins to the Golgi complex. The COPII coat consists of the small GTPase Sar1 (secretion associated, Ras related GTPase 1, isoforms Sar1A, and Sar1B) as well as the Sec23/24 and the Sec13/31 complexes that sequentially bind to the ER membrane [Barlowe et al., 1994]. Assembly of the COPII coat is initiated by the activation of Sar1 through conversion of GDP to GTP under the catalytic control of Sec12, an integral membrane protein in the ER, and the guanine-nucleotide-exchange factor for Sar1 [Barlowe and Schekman, 1993; Fryer et al., 2014].

Mutations in the *SAR1B* gene (*SARA2*, HGNC: 10535; MIM *607690), encoding Sar1B GTPase protein, have been associated with chylomicron (CM) retention disease, an inherited disorder that affects the absorption of dietary fats, cholesterol (CHOL), and fat-soluble vitamins [Roy et al., 1987; Levy et al., 1994; Levy, 2014]. Patients display impaired intracellular CM trafficking, thereby resulting in defective lipid delivery to the blood circulation [Peretti et al., 2009]. These clinical findings, combined with our work using Caco-2/15 cells overexpressing Sar1B [Levy et al., 2011], support a central role for Sar1B in CM secretion. As a matter of fact, in a previous report, we have exhibited that Sar1B can promote intestinal lipid transport in a process involving the COPII network in the Caco-2/15 cell model [Levy et al., 2011].

Numerous proteins involved in intestinal lipid transport and CM assembly are modulated by dietary fats and components of lipid digestion [Darimont et al., 1999; Williams et al., 2004; Jackson et al., 2009; Yao et al., 2011]. Specifically, in vivo and in vitro studies have

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suggested that lipid-modifying activities including phospholipase D [Pathre et al., 2003] and lysophospholipid acyltransferase [Brown et al., 2008] are required for COPII vesicle formation. Moreover, synthesis of phosphatidylinositol 4,5-phosphate [Blumental-Perry et al., 2006] and fatty acids (FA) [Shindiapina and Barlowe, 2010] are required for normal transitional ER site structure and function. Altogether, these observations suggest that the secretory pathway, including Sar1B-mediated COPII recruitment, responds to lipid modulation. However, no investigation has yet specifically focused on the direct modulation of Sar1B by effectors and, more specifically, by components of lipid digestion.

Growing evidence has demonstrated the role of the small intestine in regulating plasma CHOL levels [Cohen et al., 2006]. Hence, it is of great importance to understand the mechanisms of actions involved in intestinal CHOL biosynthesis and transport. Among the various proteins involved in these processes, Niemann-Pick C1-like 1 (NPC1L1), the major molecular target of the CHOL lowering agent ezetimibe, plays an important role in intestinal CHOL uptake [Field et al., 2007]. The scavenger receptor class B type I (SR-BI) also contributes to CHOL intestinal absorption at the brush-border membrane [Peretti et al., 2007; Sane et al., 2006]. Importantly, HMG-CoA reductase, a regulatory sterol enzyme catalyzing the rate-limiting step of CHOL synthesis, is crucial in regulating intracellular CHOL levels [Field et al., 2001]. Besides, the low-density lipoprotein receptor (LDLR) and the protein convertase subtilisin/kexin type 9 (PCSK9), a secreted protein inducing LDLR degradation, have also been identified as additional critical regulators of intestinal and circulating CHOL levels [Engelking et al., 2012; Levy et al., 2013]. Genetic studies revealed multiple "gain-of-function" mutations in the *PCSK9* gene that resulted in reduced cellular LDLR levels and increased levels of plasma LDL-CHOL [Cohen et al., 2005; Cohen et al., 2006; Kotowski et al., 2006]. In parallel, PCSK9 mutations with "loss-of-functions" were associated with increased levels of cellular LDLR [Cohen et al., 2006; Kotowski et al., 2006; Zhao et al., 2006].

Although Sar1B appears to play an important role in CM assembly and, thereby, might be affected by CHOL transport and homeostasis, no studies have yet investigated its modulation and its role in CHOL homeostasis. In the current study, using the Caco-2/15 cell line, we first explored Sar1B regulation by dietary lipids. Then, we employed genetic manipulation of Caco-2/15 cells to overexpress human recombinant Sar1B in order to investigate its direct role in modulating intestinal CHOL transport and metabolism.

METHODS

CELL CULTURE

The human epithelial colorectal adenocarcinoma Caco-2/15 cell line, a stable clone of the parent Caco-2 cells (American Type Culture Collection, Rockville, MD), was obtained from Dr. JF Beaulieu (Department of Cellular Biology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Quebec, Canada) [Beaulieu and Quaroni, 1991; Vachon and Beaulieu, 1992; Vachon et al., 1996]. Caco-2/15 cells were grown at 37°C with 5% CO_2 in Eagle's Minimum Essential Medium (EMEM, Wisent Inc., Saint-Bruno, Quebec, Canada) containing 1% penicillin-streptomycin and 1% MEM nonessential amino acids (GIBCO-BRL, Grand Island, NY) and supplemented with 10% decomplemented fetal bovine serum (FBS) (Wisent Inc.) as described previously [Levy et al., 2013]. Caco-2/15 cells (passages 20–25) were maintained in T-75-cm^2 flasks (Corning Glass Works, Corning, NY). Cultures were split (1:5) when they reached 70–90% confluence, by use of 0.05% trypsin-0.5 mM EDTA (GIBCO-BRL). For individual experiments, cells were plated at a density of 1×10^6 cells/well on 9.5 cm^2 polystyrene wells (Costar, Corning Inc., Tewksbury, MA) in EMEM (as described above) supplemented with 5% FBS. Cells were cultured for 14 days post-confluence, at which the Caco-2/15 cells are highly differentiated into mature enterocytes and appropriate for lipid metabolism [Sane et al., 2006; Levy et al., 2013]. The medium was refreshed every second day. Prior to treatments, cells were grown overnight in serum-free EMEM which has been used as carrier for CHOL, oleic acid (OA), ergosterol, eicosapentanoic acid (EPA) or 25-hydroxy-CHOLpreparations.

CELL TREATMENTS

CHOL (50, 75, 100, and 200 μM), 25-hydroxy-CHOL (50 μM) and ergosterol (200 μM) (Sigma–Aldrich Canada, Oakville, ON) were dissolved in chloroform, and sodium hydroxide (NaOH, 0.1 M) was added to saponify the sterol. The solution was then dried under a stream of nitrogen, and 1 ml of an albumin solution (10% free FA-BSA in PBS, pH 7.4) was slowly added with continuous stirring. Mixed bile salt micelles, as vehicles of CHOL, were also prepared with 4.8 mM sodium taurocholate and 0.3 mM monooleylglycerol or with 4.8 mM sodium taurocholate and 0.5 mM OA. CHOL-containing micelles were also prepared by adding CHOL dissolved in chloroform to the micellar solution at a concentration of 200 μM. The mixture was evaporated under nitrogen before being mixed with EMEM at their final concentrations. OA and EPA (0.5 mM) (Sigma–Aldrich) were also prepared in the albumin solution as mentioned. After a 24 h-pre-incubation, effectors were mixed to EMEM without FBS before being added to the wells and incubated at 37°C for 4 and 24 h.

ESTABLISHMENT OF SAR1B/GTPASE AND GFP STABLE OVEREXPRESSING CACO-2/15 CELL LINES

The blunt-end PCR fragment encoding *SAR1B* gene was amplified from the Human cDNA clone SC114457 (Origene Technologies, Rockville, MD) with ATGTCCTTCATATTTG as forward primer and CAGATCCTCTTCTGAGATGAGTTTTGTTCATCAATGTTACTGTGC-CAT containing *myc* epitope as reverse primer and TOPO Cloned into pLenti6/V5-D-TOPO (Invitrogen Corp., Carlsbad, CA 92008) to create an expression construct. The pLenti6/V5-D-TOPO expression plasmid of *SAR1B* was then transfected into Caco-2/15 cells with GenJet *In Vitro* Transfection Reagent (SignaGen Laboratories, Ijamsville, MD) according to the supplier's instructions. Generation of stable cell line was achieved using blasticidin selection at a concentration of 1 μg/ml. Control Mock cells were obtained by transfection with pLentiV5/GFPtag (kindly provided by Dr. Jean-François Beaulieu, Université de Sherbrooke) harboring the same features as the pLenti6/V5-D-TOPO system and selected for stability with blasticidin. Cell viability of Mock and Sar1B transfected cells was assessed by MTT assay and showed no difference with non-transduced cells. Cell differentiation was determined by villin protein expression and was not affected by transfection.

MEASUREMENT OF CHOLESTEROL UPTAKE AND TRANSPORT

Caco-2/15 cells were incubated with micelles containing [^{14}C]-CHOL (0.12 μCi; 4.44 kBq; specific activity of 53.0 mCi/mmol, 2.0 GBq/mmol, Perkin Elmer, Boston, MA), non-labeled CHOL (200 μM) and taurocholate (5 mM), with OA (0.5 mM) or monooleylglycerol (0.3 mM) or with [^{14}C]-CHOL 200 uM (0.12 μCi, 0.37 MBq) bound to albumin, as well as ergosterol (200 μM). At the end of the incubation periods (4 and 24 h), cells were washed and scraped with a rubber policeman in a PBS solution containing antiproteases [phenylmethylsulfonyl fluoride (1 mM), pepstatin (1 mM) and Trasylol (1 mM)]. Cellular and basolateral free [^{14}C]-CHOL was assessed by TLC using the solvent mixture of hexane, ether, and acetic acid (80:20:3, vol/vol/vol). The area corresponding to free CHOL was scratched off the TLC plates and the silica powder was placed in a scintillation vial with Ready Safe counting fluid (Beckman, Fullerton, CA). Radioactivity was then measured by scintillation counting (LS 5000 TD, Beckman). Cell protein was quantified using the Bradford method and results were expressed as DPM per mg of cell protein.

MEASUREMENT OF CHYLOMICRON SECRETION

CM secretion (from Mock and Sar1B+) was assayed as described previously [Marcil et al., 2010]. Briefly, cells were incubated for 24 h with micelles containing [^{14}C]-CHOL (0.12 μCi; 4.44 kBq; specific activity of 53.0 mCi/mmol, 2.0 GBq/mmol, Perkin Elmer, Boston, MA), CHOL (200 μM), taurocholate (5 mM) and OA (0.5 mM). At the end of a 24 h-incubation period, the basolateral medium, supplemented with antiproteases as mentioned above, was mixed with plasma as a lipid carrier [4:1 (vol/vol)] to efficiently isolate de novo synthesized lipoproteins. CM were then separated following an ultracentrifugation at 25,000*g* for 20 min using a TL-100 ultracentrifuge (Beckman Instruments, Montreal, Canada) as described previously [Sane et al., 2006; Levy et al., 2013]. Cellular free and esterified [^{14}C]-CHOL was assessed by TLC using the solvent mixture of hexane, ether, and acetic acid (80:20:3, vol/vol/vol). The area corresponding to CHOL was scratched off the TLC plates and the silica powder was placed in a scintillation vial with Ready Safe counting fluid (Beckman, Fullerton, CA). Radioactivity was then measured by scintillation counting (LS 5000 TD, Beckman). Cell protein was quantified using the Bradford method and results were expressed as DPM per mg of cell protein.

WESTERN BLOTS

Sar1B, NPC1L1, SR-BI, HMG-CoA reductase, phospho-HMG-CoA reductase, LDLR, and PCSK9 cellular protein content was determined by immunoblotting 30 μg of total proteins from lysates. Caco-2/15 cells were homogenized and adequately prepared for Western blotting as described previously [Sane et al., 2006; Levy et al., 2013]. The Bradford assay was used to estimate protein concentration. Proteins were denatured at 95°C for 5 min in sample buffer containing SDS and DTT (Thermo Scientific, Rockford, IL), separated on a 15% sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) and electroblotted onto Hybond-C extra nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ) in 25 mM Tris and 192 mM glycine. Nonspecific binding sites of the membranes were blocked with tris-buffered saline [20 mM Tris-HCl (pH 7.5) plus 137 mM NaCl] containing 0.1% Tween 20 and 5% non-fat dry milk for 60 min at room temperature. The blots were then incubated overnight at 4°C in blocking solution with the primary antibodies directed against the targeted proteins. The relative amount of primary antibody was detected with species-specific horseradish peroxidase-conjugated secondary antibody. Ectopic, as well as endogenous Sar1B were detected using Sar1B/A antibody (kindly provided by Dr Randy Schekman, University of California, Berkeley), which reacts with both Sar1A and Sar1B, with an upward shift for the ectopic Sar1B due to the myc tag. The following dilutions of antibodies were used: anti-Sar1B, 1:20,000; anti-NPC1L1 (Novus Biologicals), 1:3,000; anti-SRB1 (Novus Biologicals), 1:10,000; anti-HMG-CoA reductase and anti-phospho-HMG-CoA reductase (Upstate, Millipore), 1:10,000; anti-LDLR (Santa Cruz), 1:1,000; anti-PCSK9 (kindly provided by Dr Nabil Seida, University of Montreal), 1:2,000; and anti-β-actin (Sigma–Aldrich), 1:250,000.

DENSITOMETRIC QUANTIFICATION AND STATISTICAL ANALYSIS

Films of Amplicons from RT-PCR and Western Blots were scanned with an HP Scanjet 8300 digital scanner equipped with a transparency adapter. Densitometric evaluation was then performed using UN-SCAN-IT gel 6.1 software. Data are expressed as means ± SE of 3 independent experiments in triplicates. Statistical analysis was performed using either student *t*-test or one-way ANOVA with post hoc Bonferroni multiple comparison test, and $P < 0.05$ was considered significant.

RESULTS

In the first set of experiments, we aimed to investigate the regulation of Sar1B by different lipid effectors after short (4 h) and long term (24 h) incubation periods. We first evaluated the effects of CHOL, solubilized in albumin, on Sar1B. Figure 1A shows that CHOL (200 μM) led to a slight increase in Sar1B protein expression after 4 and 24 h-incubations (9.5% and 12%, respectively), but only the latter was significant. However, incubation with 50, 75, and 100 μM of CHOL did not induce a significant change in Sar1B (data not shown). In the same manner, treatment of Caco-2/15 cells with 25-hydroxy-CHOL, a hydroxylated derivative of CHOL, did not affect Sar1B levels (data not shown). Besides, OA significantly stimulated Sar1B protein expression at the two incubation periods by 20% and 10%, respectively (Fig. 1B). Since albumin-bound CHOL induced Sar1B expression, we also treated cells with 200 μM ergosterol, an inefficient modulator of cholesterol metabolism. As expected, Supplementary Figures S1 A and B show no significant difference between cells treated with ergosterol and control cells, demonstrating its lack of effect on Sar1B gene and protein expression. However, these results also support the effectiveness of CHOL in modulating Sar1B for longer incubation time (Supplementary Figure S1B) albeit the transcriptional expression was not modified (Supplementary Figure S2).

During the process of lipid digestion, micelles composed mostly of bile acids; FA and CHOL are formed in the intestinal lumen to solubilize lipids and to allow their absorption. Hence, we designed experiments to study the influence of CHOL and FA incorporated

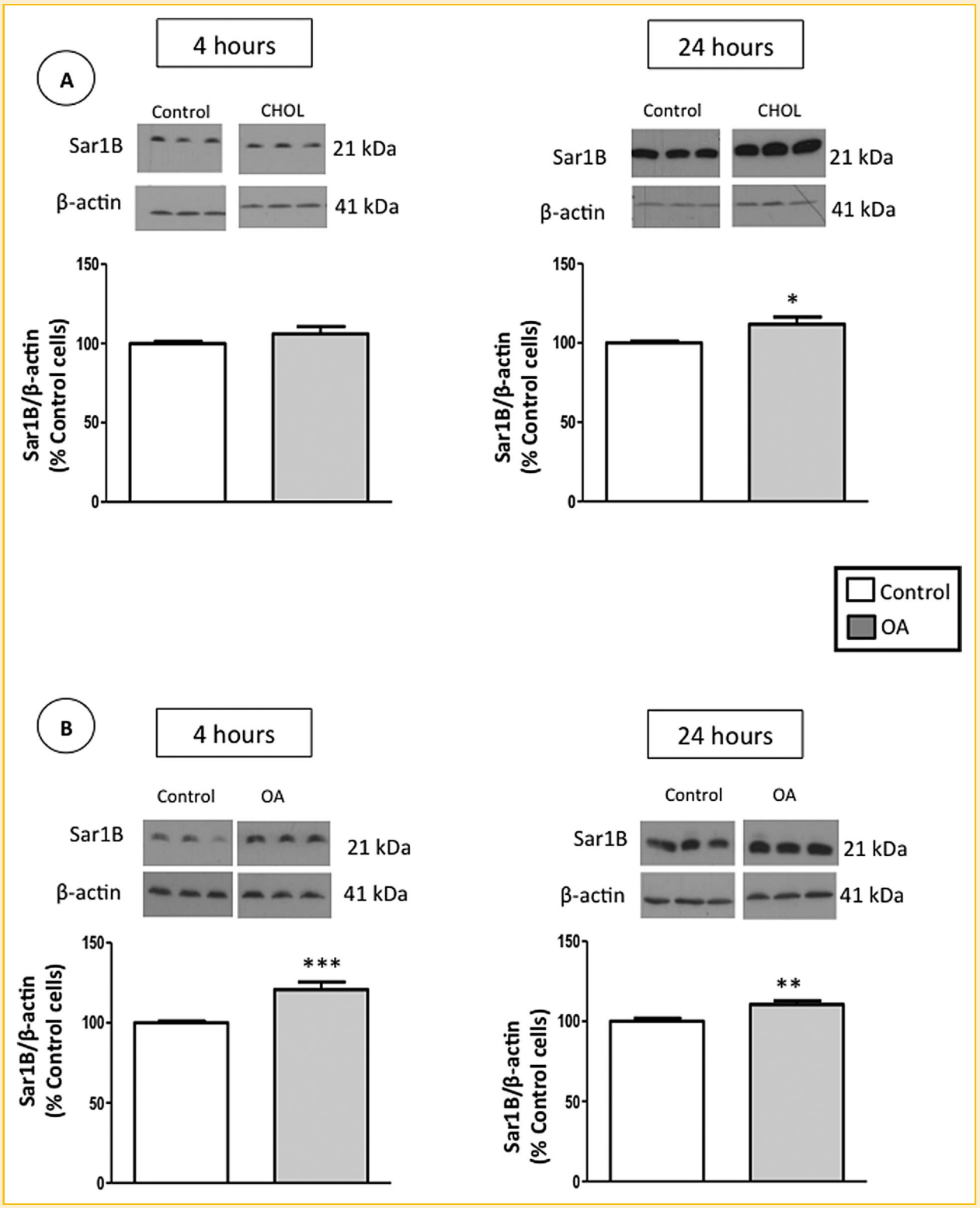

Fig. 1. Effect of cholesterol and oleic acid on Sar1B protein expression in Caco-2/15 cells. Caco-2/15 cells were incubated with albumin (control), 200 μM cholesterol (CHOL) (A) or 500 μM oleic acid (OA) (B) both solubilized in albumin for 4 and 24 h as described in the Material and Methods section. Protein expression of Sar1B was determined by Western blots. Results represent the means ± SEM of 4 independent experiments each in triplicate. Results are illustrated as % of control cells after the data were calculated as densitometric ratios of Sar1B to β-actin. A representative blot is shown, illustrating an experiment in triplicate on the same gel and at the same exposure. $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$ versus controls.

into micelle components on Sar1B expression. In Figure 2, we compared the effects of micelles composed of monoolein or OA, without CHOL. While both types of micelles stimulated Sar1B expression at 4 h, only the micelles containing OA could sustain this stimulation after a 24 h-incubation period. Noteworthy, the addition of taurocholate alone did not affect Sar1B protein expression when compared to untreated cells (data not shown). We then aimed to isolate the specific impact of CHOL. The addition of 200 μM CHOL did not affect the regulation of Sar1B by micelles composed of monooleylglycerol or OA at any incubation time (Supplementary Figure S3).

In the next set of experiments, we intended to study the impact of Sar1B overexpression on CHOL metabolism. To this end, we generated Caco-2/15 cells expressing stable human recombinant *SAR1B*. The rise in gene (385%) and protein (462%) expression of Sar1B in cells transfected with the pLenti6/V5-D-TOPO expression plasmid of *SAR1B* (Sar1B+), compared to Mock cells, was verified by RT-PCR (Fig. 3A) and Western blot (Fig. 3B), respectively. Using this cell model, we assessed the effect of CHOL uptake and transport on Sar1B overexpression, using micelles containing monooleylglycerol instead of OA. Under these conditions, there were no significant differences in the levels of [^{14}C]-CHOL uptake between Mock and Sar1B+ cells at 4 h (Fig. 4). Nevertheless, there was an accumulation of cellular [^{14}C]-CHOL with a longer incubation period (24 h) (Fig. 4A), indicating reduced CHOL secretion. Similarly, measurement of the uptake of [^{14}C]-CHOL bound to albumin did not reveal any differences in cellular CHOL levels between Mock and Sar1B+ cells (Fig. 4B).

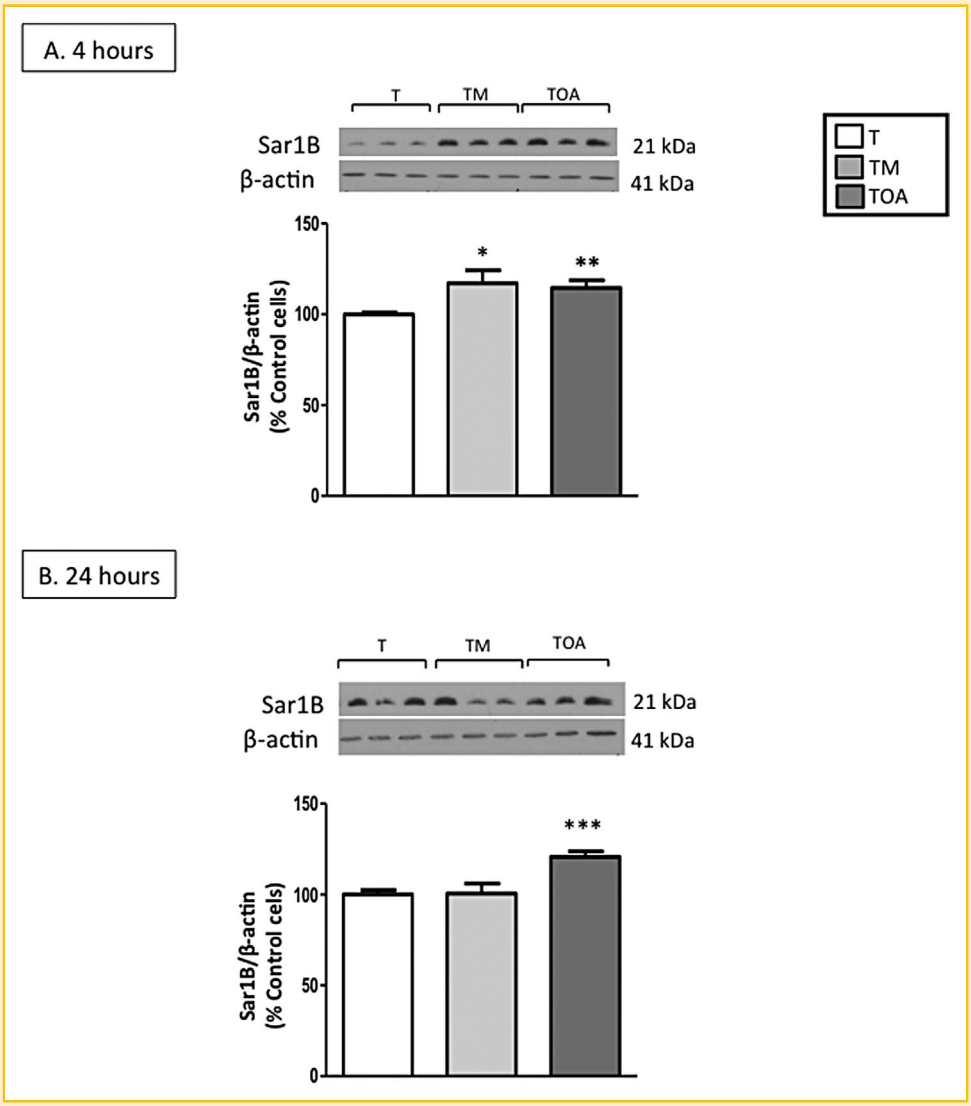

Fig. 2. Effect of micelles components on Sar1B protein expression in Caco-2/15 cells. Caco-2/15 cells were incubated with taurocholate only (T) or micelles consisting of taurocholate and monoolein (TM) or taurocholate and oleic acid (TOA) for 4 (A) and 24 h (B) as described in the Material and Methods section. Protein expression of Sar1B was determined by Western blots. Results represent the means ± SEM of 2 (TM) and 3 (T and TOA) independent experiments each in triplicate. Results are illustrated as % of control cells (T) after the data were calculated as densitometric ratios of Sar1B to β-actin. A representative blot is shown. *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$ versus T.

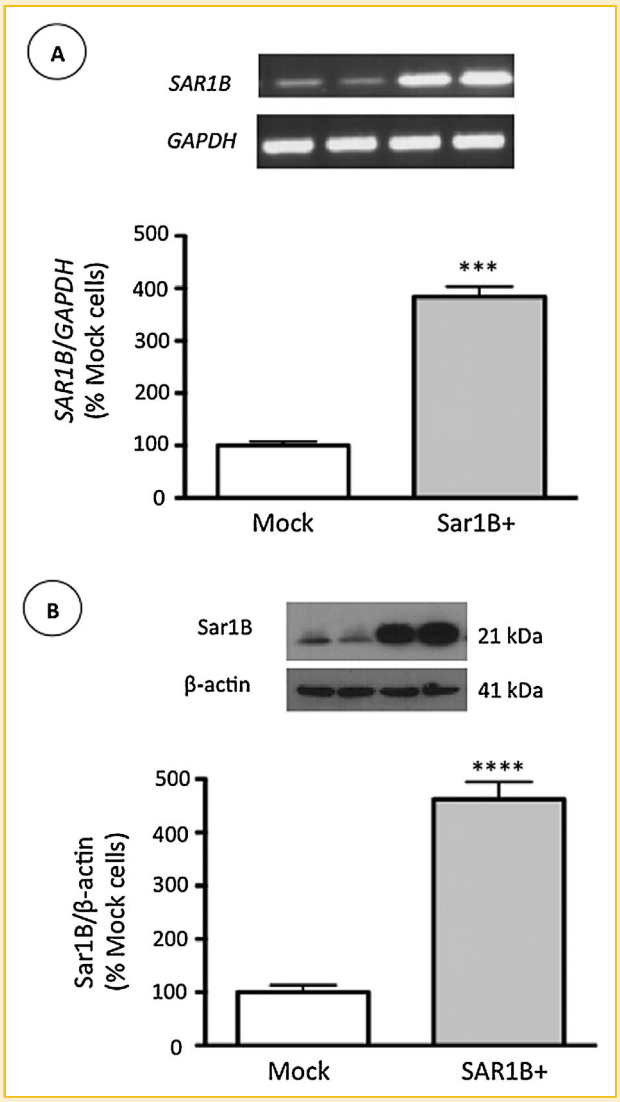

Fig. 3. Establishment of the Sar1B overexpressing stable cell line in Caco-2/15 cells. After transfection with GFP and Sar1B constructs, transduced Caco-2/15 cells were selected with blasticidin for stable clonality and allowed to differentiate for 14 days before being tested for Sar1B gene (A) and protein (B) expression by RT-PCR and Western blot, respectively. Results represent the means ± SEM of 3 to 6 experiments. Results were calculated as densitometric ratios of Sar1B to GAPDH (A) or β-actin (B) and are illustrated as % of Mock cells. A representative blot is shown, illustrating an experiment in triplicate on the same gel and at the same exposure. ***$P < 0.0002$; ****$P < 0.0001$ versus Mock cells.

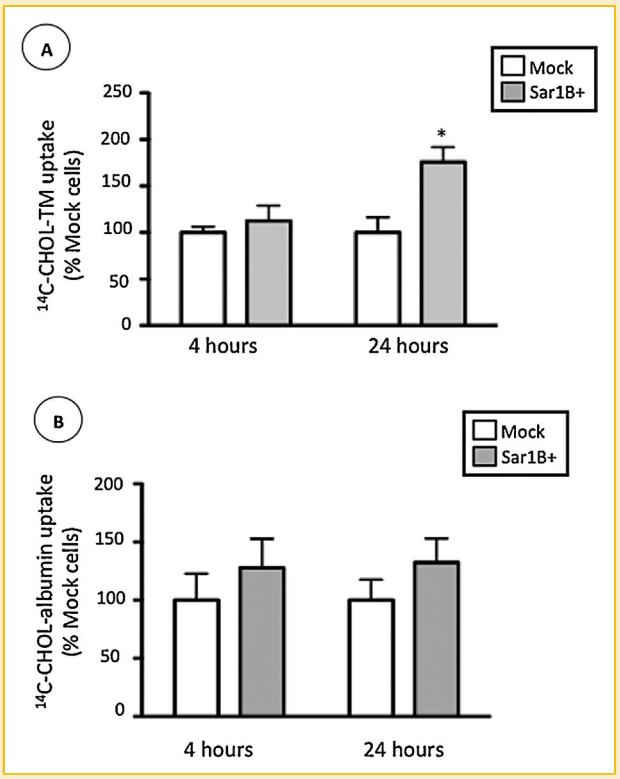

Fig. 4. Influence of Sar1B overexpression on cholesterol transport using micelles containing monoolein and cholesterol, or cholesterol bound to albumin. Mock and Sar1B+ Caco-2/15 cells were incubated with micelles containing taurocholate, [^{14}C]-cholesterol and monooleylglycerol (A) or with [^{14}C]-cholesterol bound to albumin (B) for 4 and 24 h. [^{14}C]-cholesterol from cell homogenates was extracted and measured as described in Materials and Methods. Results represent the means ± SEM of 3 independent experiments each in triplicate. Results were calculated in DPM/mg protein and illustrated as % of Mock cells. *$P < 0.05$ versus Mock cells.

Employing micelles containing OA, our findings showed that Sar1B+ cells had higher levels (195%) of cellular [^{14}C]-CHOL after a 4 h-incubation compared to Mock cells, thereby suggesting an increase in CHOL uptake (Fig. 5A). However, the cellular content in [^{14}C]-CHOL was lower (49%) in Sar1B+ cells, compared to Mock cells, after a 24 h-incubation with OA micelles (Fig. 5A). Concomitantly, [^{14}C]-CHOL secretion was enhanced by 73% in Sar1B+ cells after the 24 h-incubation, while no CHOL secretion was noted after 4 h (data not shown). To confirm that Sar1B+ cells displayed an enhancement in CHOL transport and secretion, we measured CM secretion after a 24 h-incubation with [^{14}C]-CHOL-containing micelles. Results show that Sar1B+ cells secreted more CM than Mock cells at 24 h and that the CM content in free and esterified CHOL was also higher in Sar1B+ cells (Fig. 5B). Given the cellular depletion observed in Sar1B+ cells after a 24 h-incubation with micelles containing OA and CHOL, we evaluated the expression of the HMG-CoA reductase, the enzyme regulating de novo CHOL synthesis. Our experiments revealed that, compared to Mock cells, Sar1B+ Caco-2/15 cells had a declined protein expression of the phosphorylated form of HMG-CoA reductase and an increase in HMG-CoA reductase (Fig. 6), resulting in a reduced phosphorylated/non phosphorylated ratio, which is equivalent of elevated enzymatic activity.

Subsequently, we aimed to study the effect of micelle incubation, combined to Sar1B overexpression, on the intestinal CHOL transporters NPC1L1 (Fig. 7A) and SR-BI (Fig. 7B). Our findings revealed that Sar1B overexpression alone led to a reduction in NPC1L1 and SR-BI protein content with taurocholate (28% and 26% reduction, respectively). However, adding CHOL and OA to the micelle content

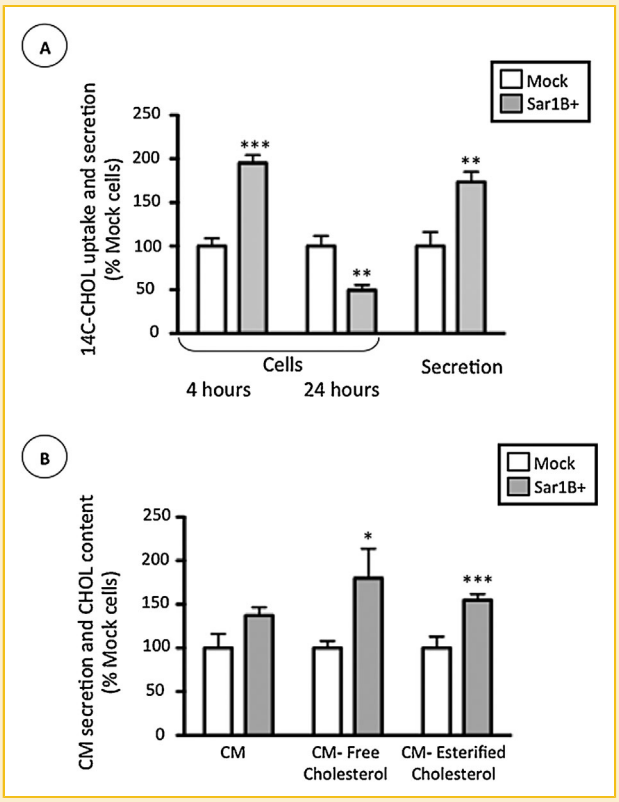

Fig. 5. Influence of Sar1B overexpression on cholesterol uptake and transport using micelles containing oleic acid and cholesterol. (A) Mock and Sar1B+ Caco-2/15 cells were incubated with micelles containing taurocholate, [^{14}C]-cholesterol and oleic acid for 4 and 24 h. [^{14}C]-cholesterol from cell homogenates and basolateral media was extracted and free CHOL measured as described in Materials and Methods. Results represent the means ± SEM of 3 independent experiments each in triplicate. (B) Caco-2/15 cells were incubated with micelles containing [^{14}C]-cholesterol, taurocholate and oleic acid for 24 h. Chylomicrons (CM) were isolated from the basolateral medium by ultracentrifugation and radioactivity was measured as described in Materials and Methods in order to determine chylomicron secretion as well as their content in free (FC) and esterified cholesterol (EC). Results represent the means ± SEM of 2 independent experiments each in triplicate. Results were calculated in DPM/mg protein and illustrated as % of Mock cells. *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$ versus Mock cells.

induced a larger decrease in Sar1B+ cells (80%). This was also the case for LDLR (Fig. 8A) with a similar trend for PCSK9 (Fig. 8B), the key proteins regulating CHOL metabolism.

DISCUSSION

Despite the importance of dietary fat in regulating peripheral lipid levels and metabolic diseases, much is still unknown about the physiology of intestinal lipid transport and lipoprotein assembly. Recently, our laboratory has demonstrated that Sar1B, a protein involved in the process of COPII vesicle assembly, is required for CM formation [Charcosset et al., 2008; Peretti et al., 2009; Levy et al., 2011]. However, limited information is available about the regulation of Sar1B and its impact on CHOL homeostasis in the gut. In the present paper, we used Caco-2/15 cells, an efficient and largely used enterocyte cell model, to show for the first time that Sar1B protein expression can be modulated by dietary lipid components, including CHOL and FA (Figs. 1 and 2). The second goal of our studies was also to highlight whether Sar1B overexpression leads to alterations in CHOL metabolism (Figs. 3–6) and transport (Figs. 7 and 8) in the enterocyte. Indeed, we were able to evidence that Sar1B overexpression along with the presence of a high CHOL load modified key CHOL protein transporters and regulatory enzymes.

Under our experimental conditions, CHOL solubilized in albumin slightly induced the expression of Sar1B, which is in line with other studies showing that CHOL can regulate COPII assembly [Levy et al., 2011]. If albumin-CHOL (200 μM) induced Sar1B expression, overexpression of Sar1B did not produce additional increase in albumin-CHOL uptake. One could speculate that Sar1B overexpression did not result in a raised albumin-CHOL uptake since a maximal level has already been reached with the substrate concentration. As a matter of fact, the modulatory effect of CHOL on COPII vesicle transport has been demonstrated by its actions on sterol regulatory element-binding proteins (SREBPs). The latter are synthesized in the ER and their activation depends on a polytopic membrane protein called Scap. When CHOL accumulates in ER membranes, the sterol binds to SCAP, alters its conformation and causes the Scap–SREBP complex to bind to Insig-1 or Insig-2, which are closely related polytopic membrane proteins [Goldstein et al., 2006]. When the SCAP–SREBP complex binds to Insig, the complex can no longer be incorporated into COPII-coated vesicles [Sun et al., 2005]. SREBP is then retained in the ER and cannot be cleaved in the Golgi, a necessary step for the release of the transcriptionally active fragment [Goldstein et al., 2006]. Hence, via this mechanism, CHOL inhibits SREBP transcriptional activity. Our results show that CHOL enhanced Sar1B protein expression, thereby suggesting that COPII vesicle is more active in response to sterols. Therefore, it seems important to systematically investigate the regulatory role of CHOL in CM assembly and secretion in relation with COPII components and in association with SREBPs and other valuable transcription factors.

We have recently demonstrated that Sar1B overxpression in Caco-2/15 cell induces a major increase in CM assembly and secretion via the modulation of apolipoprotein (apo) B and lipid moieties [Levy et al., 2011]. Our data showing the effect of CHOL on Sar1B expression are in line with these previous observations and suggest that components influencing CM assembly may concomitantly act on Sar1B-mediated COPII formation. As a matter of fact, CHOL is an important regulator of TG-rich lipoprotein secretion [Nozaki et al., 1990]. Accordingly, the inhibition of intestinal and hepatic CHOL synthesis and/or esterification was found to be associated with the down-regulation of apo B synthesis and secretion [Carr et al., 1995; Burnett et al., 1999; Zhang et al., 1999]. Similarly, ezetimibe, a CHOL absorption inhibitor that acts by blocking the sterol-induced internalization of the key transporter NPC1L1 protein in enterocytes [Ge et al., 2008], was also able to decrease triglyceride (TG) and apo B levels in plasma [Stein et al., 2004; McKenney et al., 2006; Naples et al., 2012].

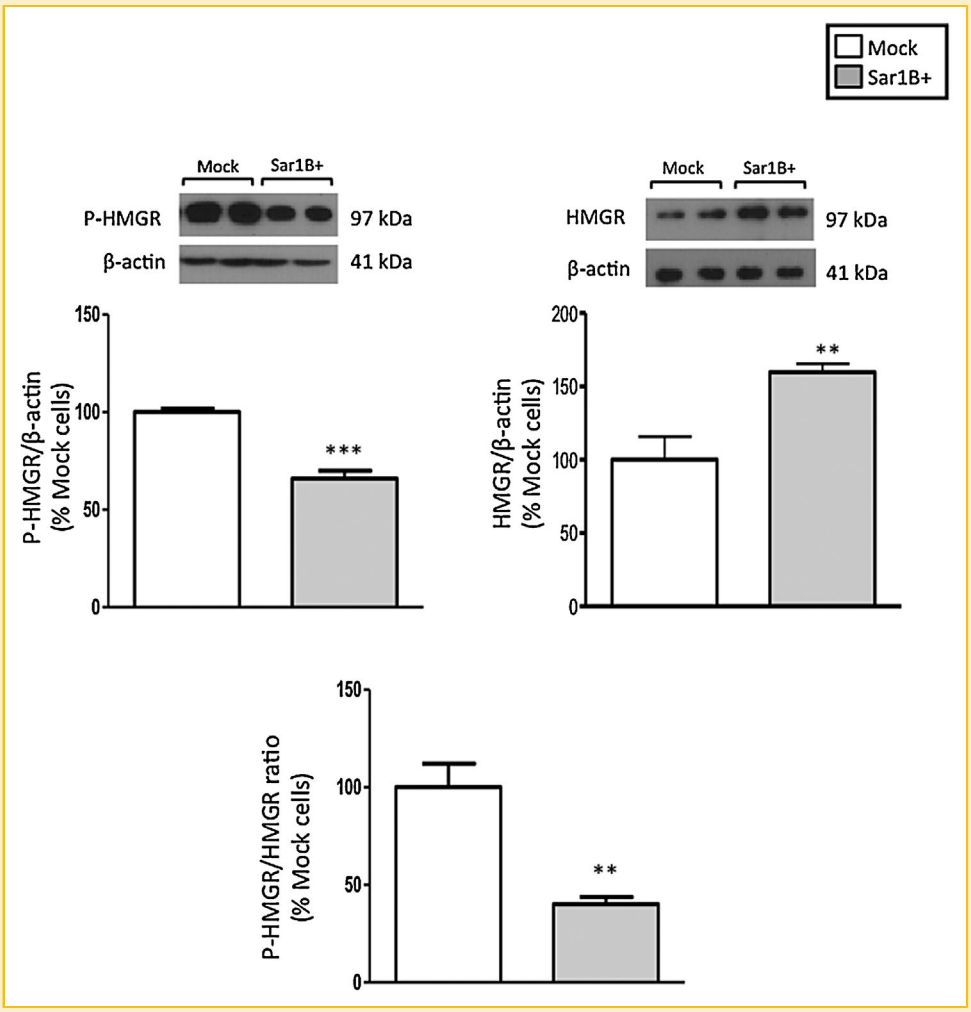

Fig. 6. Influence of Sar1B overexpression on HMG-CoA reductase protein mass. Mock and Sar1B+ Caco-2/15 cells were incubated with micelles containing taurocholate, cholesterol and oleic acid for 24 h as described in the Material and Methods section. Protein expression of phosphorylayed-HMG-CoA reductase (P-HMGR) and HMG-CoA reductase (HMGR) was determined by Western blots and the ratio between phosphorylated/non-phosphorylated HMG-CoA reductase was calculated. Results represent the means ± SEM of two independent experiments each in triplicate. Results were calculated as densitometric ratios of protein to β-actin and are illustrated as % of Mock cells. A representative blot is shown illustrating an experiment in triplicate on the same gel and at the same exposure. $^{**}P < 0.01$; $^{***}P < 0.0001$ versus Mock cells.

A quite recent report [Fryer et al., 2014] established comparison between Sar1A and Sar1B in the McArdle-RH7777 rat hepatoma cell line. Whereas Sar1B promoted hepatic apo B100- and apo B-48 lipoprotein secretion, Sar1A showed antagonizing activity. The Sar1B lipoprotein secretion-promoting activity was accompanied with changes in the expression of *APOB* and *MTTP (*microsomal triglyceride transfer protein), but not with the genes involved in CHOL synthesis. In line with these observations, our results evidenced the substantial contribution of Sar1B to intestinal lipid transport and CM secretion (Fig. 5). In contrast, our experiments could document a reduced phosphorylated HMG-CoA reductase/HMG-CoA reductase ratio (indicating a higher CHOL synthesis) along with a decreased protein level of LDLR and PCSK9 in Caco-2/15 cells. These discrepancies may be due to intrinsic metabolic differences between the two cell models.

Our results also show that OA raised Sar1B protein expression (Figs. 1 and 2). This is also in concordance with the premise that Sar1B plays a role in CM assembly and secretion [Levy et al., 2011]. In fact, FAs act at different levels in the process of lipoprotein production in the enterocyte. When they enter the small intestine, dietary lipids, mainly in the form of TG, are hydrolyzed by pancreatic lipase to form free FAs and monooleylglycerol, which can be transported to the apical membrane of enterocytes. Within these epithelial cells, FAs are reesterified and assembled along with CHOL, phospholipids and apos into CM particles. More specifically, the lipidation of apo B by esterified lipids is crucial for CM formation since it preserves apo B from proteasome degradation [Wetterau et al., 1997]. FAs also up-regulate MTTP, the master factor for apo B lipidation [Yao et al., 2011]. Therefore, FAs as precursors of esterified lipids trigger Sar1B while being the major modulators of CM synthesis [Luchoomun and Hussain, 1999].

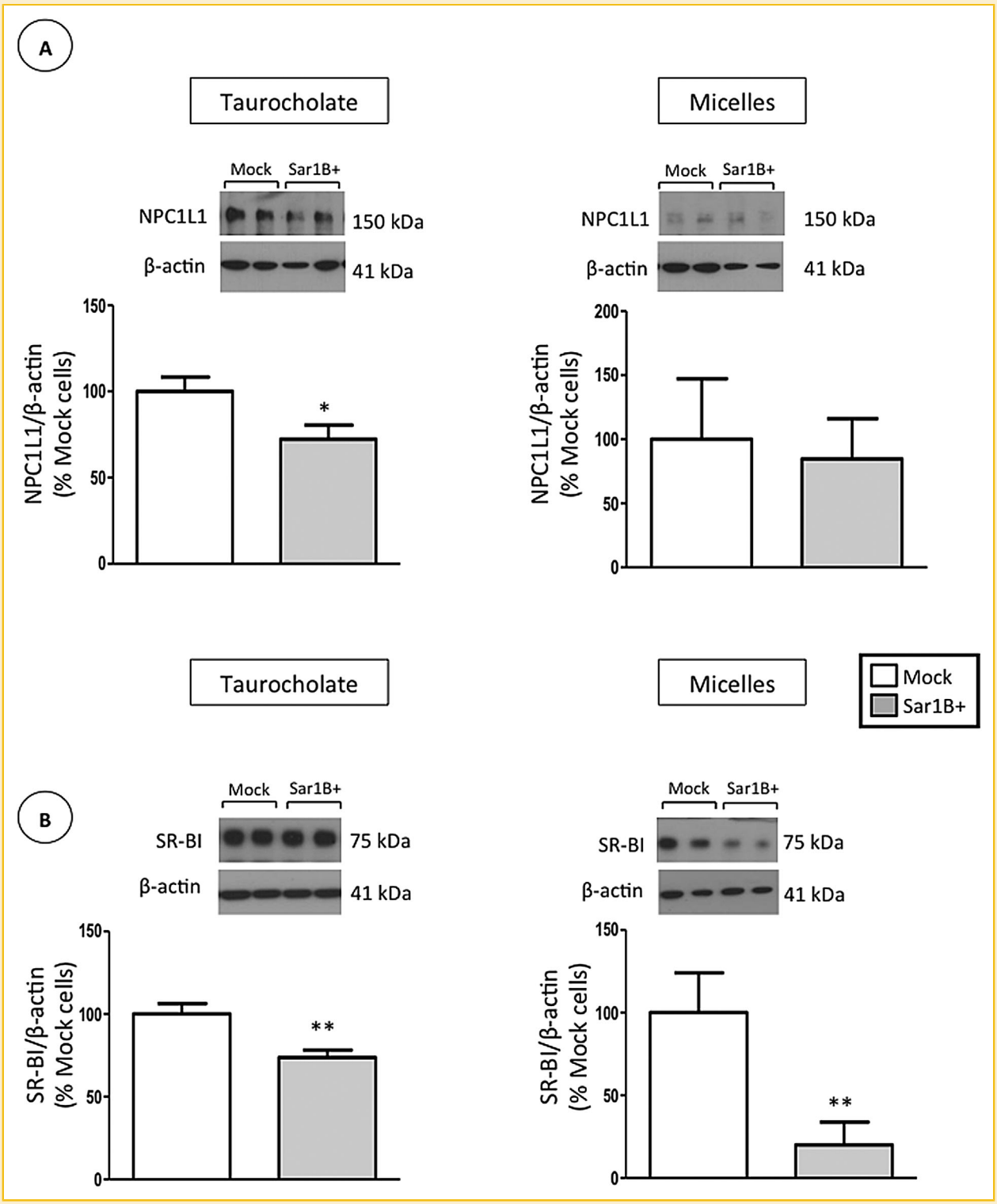

Fig. 7. Influence of Sar1B overexpression on cholesterol transporters content. Mock and Sar1B+ Caco-2/15 cells were incubated with taurocholate alone (control) or with micelles containing taurocholate, cholesterol and oleic acid for 24 h as described in the Material and Methods section. Protein expression of NPC1L1 (A) and SR-BI (B) was determined by Western blots. Results represent the means ± SEM of 3 independent experiments each in triplicate. Results were calculated as densitometric ratios of protein to β-actin and are illustrated as % of Mock control cells. A representative blot is shown illustrating an experiment in triplicate on the same gel and at the same exposure. *$P < 0.05$; **$P < 0.001$ versus Mock cells.

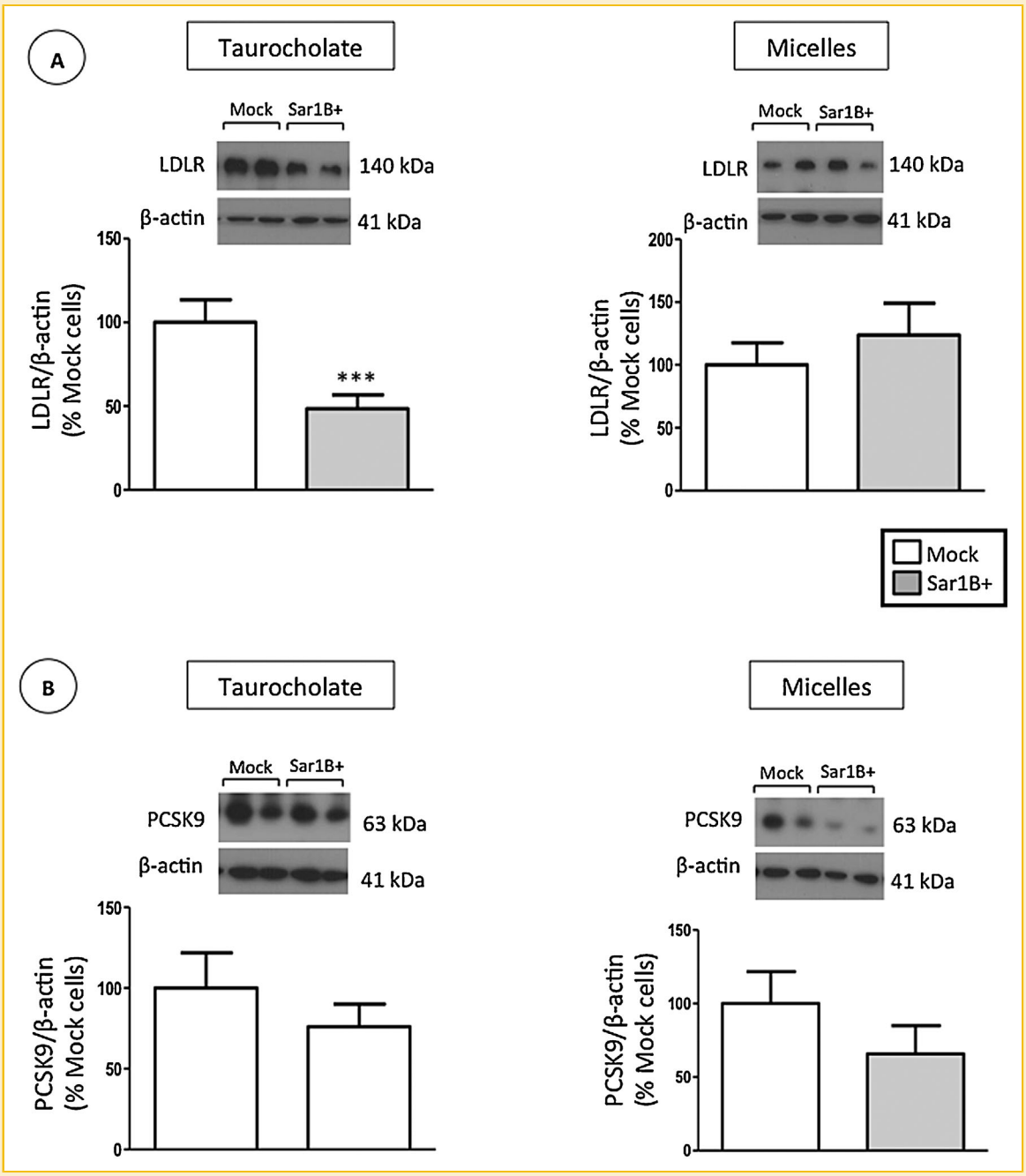

Fig. 8. Influence of Sar1B overexpression on LDLR and PCSK9 mass. Mock and Sar1B+ Caco-2/15 cells were incubated with taurocholate alone (control) or with micelles containing taurocholate, cholesterol and oleic acid for 24 h as described in the Material and Methods section. Protein expression of (A) LDLR and (B) PCSK9 was determined by Western blots. Results represent the means ± SEM of three independent experiments each in triplicate. Results were calculated as densitometric ratios of protein to β-actin and are illustrated as % of Mock control cells. A representative blot is shown, illustrating an experiment in triplicate on the same gel and at the same exposure. ***$P < 0.001$ versus Mock cells.

During the intestinal digestion phase, lipids are emulsified and solubilized with biliary acids into micelles [Tsuzuki, 2007]. In order to investigate if lipid vehicles influence the regulation of Sar1B expression, we decided to use micelles as transporters of CHOL and FA. With this set of experiments, we demonstrated that monooleylglycerol and OA, rather than CHOL, are the preferable micellar components for the stimulation of Sar1B protein expression. Since albumin-bound CHOL induced Sar1B expression, we also treated cells with ergosterol, an irrelevant component for CHOL metabolism, which resulted in no effect on Sar1B protein levels after a 24 h-incubation. These findings confirmed the effectiveness of CHOL in modulating Sar1B protein expression at least for longer incubation

times, but without affecting its transcriptional expression in response to CHOL or OA treatment. Noteworthy, recent data have shown that Sar1B modulation requires its post-translational phosphorylation within the COPII complex, thereby allowing mobilization of FA binding protein 1 for pre-CM transport vesicles [Siddiqi and Mansbach, 2012].

To further explore the role of Sar1B in intestinal CHOL metabolism, we transfected Caco-2/15 cells and overexpressed Sar1B. When cells were incubated 24 h with micelles composed of CHOL and OA, cellular CHOL uptake dropped while secretion was stimulated, which may explain the enhanced output of CM containing free and esterified CHOL. Since other investigators reported that fat feeding increased CM size [Hayashi et al., 1990; Martins et al., 1996], we believe that Caco-2/15 cells induction of Sar1B may form larger CM particles with higher CHOL content in the presence of CHOL influx. This was not observed when cells were incubated with micelles composed of monooleylglycerol instead of OA or with CHOL bound to albumin, indicating that OA itself contributes to the stimulation of CHOL transport when Sar1B is overexpressed, which is in line with our results showing that OA in micelles stimulated Sar1B expression. It is important to emphasize that huge efforts have been consecrated during the last decades to highlight the impact of FAs on CHOL metabolism. In particular, substitution of saturated FAs by monounsaturated FAs or the rise in OA cellular intake resulted in a decrease in total CHOL and LDL-CHOL [Chung et al., 2004; Haban et al., 2004; Kralova, I et al., 2008]. In addition, various FAs were able to modulate the protein expression of several transporters involved in CHOL intestinal absorption in Caco-2 cells [Alvaro et al., 2010].

Concomitantly with enhanced CHOL uptake and transport, cells overexpressing Sar1B had a lower phospho-HMG-CoA reducatse/HMG-CoA reductase ratio, indicating increased CHOL synthesis activity. We propose that the decreased cellular CHOL content in Sar1B+ cells leads to elevated cellular HMG-CoA reductase activity. Accordingly, it has been demonstrated that Caco-2 cells incubated with micellar CHOL have reduced levels of HMG-CoA reductase [Field et al., 2001] and that CHOL-enriched diet caused a strong reduction of intestinal HMG-CoA reductase activity in mice [Nguyen et al., 2001].

In a previous study from our laboratory, the knocked-down of CHOL transporter NPC1L1 in Caco-2/15 cells led to increased HMG-CoA reductase activity [Sane et al., 2006]. Likewise, Alrefai et al. documented that inhibiting HMG-CoA reductase activity with mevilonin led to an elevation of NPC1L1 expression [Alrefai et al., 2007]. In the present study, both cell exposure to micelles and Sar1B overexpression led to a decrease in NPC1L1 and SR-BI expression. In the same line, our previous studies showed that the treatment of Caco-2 cells with high CHOL doses suppressed SR-BI [Peretti et al., 2007] and also that NPC1L1 ablation affected SR-BI expression, suggesting cooperation between the two CHOL transporters [Sane et al., 2006].

Liver fatty acid-binding protein (L-FABP) plays a role in the influx of long-chain FAs into hepatocytes and enterocytes. Recently, Mansbach's group showed that L-FABP in cooperation with other proteins can generate cargo-carrying pre-CM transport vesicles [Neeli et al., 2007; Siddiqi et al., 2010]. Since our previous studies clearly demonstrated that the incubation of Caco-2 cells with FAs up-regulated L-FABP and induced lipoprotein assembly and secretion [Dube et al., 2001; Levy et al., 2009], and given the findings of the present work that emphasized the induction of Sar1B in a time- and concentration-dependent manner by OA, it is tempting to suggest a potential interaction or cooperation between L-FABP and Sar1B. In line with this suggestion, L-FABP is present in native ER [Bass et al., 1985] and readily binds to ER membranes during incubation with cytosol and ATP [Neeli et al., 2007].

In the current work, we have also assessed the role of Sar1B in the modulation of PCSK9 and LDLR expression. Forcing Sar1B resulted in the concomitant down-regulation of PCSK9 and LDLR. These results could appear as a paradox but this phenomenon was previously described by our group: we could demonstrate that PCSK9 and LDLR are either stimulated or down-regulated simultaneously via the SREBP-2 transcription factor [Leblond et al., 2009]. Also, Engelking et al. recently showed that, in mice and in contrast to liver, the increased jejunal PCSK9 expression did not define the increase in LDLR protein in response to ezetimibe and that IDOL, an E3 ubiquitin ligase that promotes LDLR degradation, trumped PCSK9 in the regulation of jejunal LDLR [Engelking et al., 2012]. Hence, other proteins other than PCSK9 could explain the regulation of Sar1B overexpression and micelles on LDLR expression.

In conclusion, the present work showed that Sar1B is regulated in a time- and concentration-dependent manner by OA and CHOL. Furthermore, our work using Caco-2/15 cells overexpressing Sar1B sheds light on the role of Sar1B in CHOL homeostasis by introducing new aspects in intestinal lipid metabolism, namely its implication in CHOL transport and homeostasis. The influence of Sar1B overexpression on CHOL transporters, such as NPC1L1, seems to be linked to CHOL loading since no difference was seen between Mock and Sar1B+ cells at the untreated basal condition (data not shown). Interestingly, we recently reported susceptibility to obesity and insulin sensitivity in Sar1B transgenic mice fed a high-fat diet [Levy et al., 2014]. To date loss-of-function mutations have been described to be the cause of CM retention disease in human but no CHOL homeostasis impairment was reported in case of overexpression or gain of function. This work supports and extends previous observations documenting that Sar1B is a key player in CM formation. Moreover, we believe that additional studies, employing in vivo and in vitro systems, are needed to explore in details the regulation of Sar1B and other COPII vesicle components during the intestinal lipid absorption process in order to elucidate the inherent molecular mechanisms.

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