Intestinal fatty acid binding protein regulates mitochondrion β-oxidation and cholesterol uptake

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Abstract The role of intestinal fatty acid binding protein (I-FABP) in lipid metabolism remains elusive. To address this issue, normal human intestinal epithelial cells (HIEC-6) were transfected with cDNA to overexpress I-FABP and compared with cells treated with empty pQCXIP vector. I-FABP overexpression stimulated mitochondrial [U-^{14}C]oleate oxidation to CO_2 and acid-soluble metabolites via mechanisms including the upregulation of protein expression and the activity of carnitine palmitoyltransferase 1, a critical enzyme controlling the entry of fatty acid (FA) into mitochondria, and increased activity of 3-hydroxyacyl-CoA dehydrogenase, a mitochondrial β-oxidation enzyme. On the other hand, the gene and protein expression of the key enzymes FA synthase and acetyl-coenzyme A carboxylase 2 was decreased, suggesting diminished lipogenesis. Furthermore, I-FABP overexpression caused a decline in [^{14}C]free cholesterol (CHOL) incorporation. Accordingly, a significant lessening was observed in the gene expression of Niemann Pick C1-Like 1, a mediator of CHOL uptake, along with an increase in the transcripts and protein content of ABCA1 and ABCG5/ABCG8, acting as CHOL efflux pumps. Furthermore, I-FABP overexpression resulted in increased levels of mRNA, protein mass, and activity of HMG-CoA reductase, the rate-limiting step in CHOL synthesis. Scrutiny of the nuclear receptors revealed augmented peroxisome proliferator-activated receptor α,γ and reduced liver X receptor-α in HIEC-6 overexpressing I-FABP. Finally, I-FABP overexpression did not influence acyl-coenzyme A oxidase 1, which catalyzes the first rate-limiting step in peroxisomal FA β-oxidation. Overall, our data suggest that I-FABP may influence mitochondrial FA oxidation and CHOL transport by regulating gene expression and interaction with nuclear receptors.—Montoudis, A., E. Seidman, F. Boudreau, J-F. Beaulieu, D. Menard, M. Elchebly, G. Mailhot, A-T. Sane, M. Lambert, E. Delvin, and E. Levy. **Intestinal fatty acid binding protein regulates mitochondrion β-oxidation and cholesterol uptake. *J. Lipid Res.* 2008. 49: 961–972.**

Supplementary key words I-FABP • HIEC-6 • cholesterol metabolism

Fatty acid binding proteins (FABPs) display a high affinity for long-chain fatty acids (LCFA) and appear to function in the metabolism and intracellular transport of lipids (1). Two distinct FABPs are expressed in the small intestine: the liver (L)-FABP encoded by the *FABP1* gene and the intestinal (I)-FABP encoded by the *FABP2* gene (2). The function of these proteins is still under investigation. The presence of two structurally distinct, independently regulated FABPs in the intestine has led to the speculation that these proteins assume unique roles in intestinal fatty acid (FA) metabolism (2, 3).

Because targeted gene disruption studies may shed light on the physiological importance of these proteins, Vassileva et al. (4) invalidated mouse *FABP2* and studied the impact on intestinal lipid transport. The results of their experiments suggest that I-FABP knockout does not produce a detrimental effect on dietary fat absorption but may cause weight gain and hyperinsulinemia. Additionally, our observations using normal human intestinal epithelial cells (HIEC-6) overexpressing sizeable amounts of I-FABP demonstrated a negligible influence of this transporter on lipid synthesis, apolipoprotein biogenesis, and lipoprotein exocytosis (5). Conversely, examination of the human intestinal cell line Caco-2 overexpressing human I-FABP showed minimal effects on FA incorporation (6).

Because no in vivo or in vitro function can be definitively put forward for I-FABP, a puzzling and interesting question pertaining to its specific role in the enterocyte persists. Findings from *I-Fabp*$^{-/-}$ mice (4) and the Ala54Thr human mutation (7–9) suggest that I-FABP functions physiologically as a lipid-sensing component of energy homeostasis and not as a direct part of dietary FA absorption. Vassileva et al. (4) proposed that "I-FABP likely feeds information about dietary lipid status into mechanisms that

Manuscript received 14 August 2007 and in revised form 11 January 2008.

Published, JLR Papers in Press, January 30, 2008.
DOI 10.1194/jlr.M700363-JLR200

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universally control energy utilization, energy storage, and eventually body weight." Therefore, we wonder whether I-FABP *i*) is involved in the movement of FA to specific subcellular compartments other than the endoplasmic reticulum; *ii*) influences β-oxidation in mitochondria; *iii*) intervenes in the metabolism of other lipid classes, such as cholesterol (CHOL); and *iv*) is associated with the alteration of transcription factor expression in the nucleus. These questions were addressed in HIEC-6 with forced expression of I-FABP.

MATERIALS AND METHODS

Cell culture

HIEC-6 was generated with the use of the dissociating enzyme thermolysin as described previously (10). Cells were expanded and then kept frozen (in Dulbecco's modified Eagle's medium containing DMSO and fetal calf serum) at passage two or three in liquid nitrogen. For the following studies, cell aliquots were cultured at 37°C in high-glucose DMEM containing 5% FBS, 10 mM HEPES, 4 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 5 μg/ml epidermal growth factor, and 100 μl/ml insulin, transferrin, and selenium, all from Gibco-BRL. Confluence was reached by day 13. Media were replaced every 2 days. The experiments were carried out at 8 days after confluence.

Virus production and retroviral infections

Wild-type I-FABP-encoding cDNA (kindly provided by Dr. J. H. Veerkamp) was amplified by PCR and hemagglutinin (HA)-tagged using sense primer 5′-GAAGATCTTCATGTATGATGTTCCTGATTATGCTATGGCGTTTGACAGCACTTGG-3′ and antisense primer 5′-CCATCGATGGTCAATCCTTTTTAAAGATCCTTTT-3′ with the *Taq* Plus Precision PCR system (Stratagene, Vancouver, British Columbia, Canada) according to the company's specifications. The amplicon was digested by *Bgl*II and *Cla*I and inserted downstream of the cytomegalovirus promoter in the retroviral vector pLNCX2 (Clontech) and confirmed by sequencing. The insert was subcloned into the *Eco*RI site of pQCXIP (Clontech) after removal from pLNCX2 using *Bgl*II and *Cla*I and Klenow to fill in the overhang. The wild-type I-FABP-HA and empty pQCXIP vectors were used to produce virus in HEK293T cells in cotransfection with helper amphotropic DNA vector (pAmpho) as described previously (11). Selection using 1 μg/ml puromycin (Calbiochem) was applied at 24 h after the end of infection for 10 days to obtain stable populations. All experiments shown were performed within 3 weeks of selection, virus production, and retroviral infection.

β-Oxidation

HIEC-6 was preincubated for 1 h with labeling medium in flasks fitted with central wells, suspended through an air-tight rubber bung. Cells were supplemented with 1 μCi of 100 μM [^{14}C]oleic acid for 16 h while NaOH was added to each central well to trap released [^{14}C]CO_2, and [^{14}C]oleate oxidized to [^{14}C]CO_2 was measured as described by Ferre et al. (12). Briefly, [^{14}C]CO_2 was driven from medium by adding 200 μl of 70% perchloric acid and trapped in the central well saturated with NaOH. Flasks were shaken for a further 45 min at room temperature to increase trapping of [^{14}C]CO_2, after which the center wells were placed in plastic vials, dark-adapted overnight, and assayed for radioactivity in a liquid scintillation counter.

Western blots

To assess the presence of various proteins, cells were homogenized and adequately prepared for Western blotting as described previously (11). Proteins were denatured in sample buffer containing SDS and β-mercaptoethanol, separated by 4–20% gradient SDS-PAGE, and electroblotted onto nitrocellulose membranes. Nonspecific binding sites of the membranes were blocked using defatted milk proteins followed by the addition of primary antibodies directed against the targeted proteins: I-FABP (developed in our laboratory); carnitine palmitoyltransferase 1 (CPT-1) (Calbiochem, San Diego, CA); acetyl-coenzyme A carboxylase 2 (ACC2) (Cell Signaling Technology, Danvers, MA); FAS (Calbiochem); HMG-CoA reductase (Upstate, Bedford, MA); Niemann Pick C1-Like 1 (NPC1L1), scavenger receptor class B type I (SR-BI), and ABCA1 (Novus Biologicals, Littleton, CO); L-FABP, cluster determinant 36 (CD36), and ABCG8 (Santa Cruz Biotechnology, Santa Cruz, CA); sterol-regulatory element binding protein 2 (SREBP-2) (Cayman Chemical, Ann Arbor, MI); and acyl-coenzyme A oxidase 1 (ACOX1) (Abgent, San Diego, CA). The relative amount of primary antibody was detected with species-specific horseradish peroxidase-conjugated secondary antibody. Blots were developed and the mass of proteins was estimated using a Hewlett-Packard Scanjet scanner equipped with a transparency adaptor and software. Data for cellular protein content quantified by Western blotting were expressed as the protein-β-actin ratio.

RT-PCR

Experiments for mRNA quantification as well as for GAPDH (as a control gene) were performed using the GeneAmp PCR System 9700 (Applied Biosystems) as reported previously (11). Approximately 30–40 cycles of amplification were used at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Amplicons were visualized on standard ethidium bromide-stained agarose gels. Under these experimental conditions relative to RT-PCR, 34–36 cycles corresponded to the linear portion of the exponential phase.

Monoacylglycerol acyltransferase and diacylglycerol acyltransferase activity assay

The microsomal activity of monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT) was determined as reported by Coleman (13), whereas that of glycerol-3-phosphate acyltransferase (GPAT) was assayed as described previously (14). Only minor variations in the radioactive precursor and extraction method were made. We used [^{14}C]oleoyl-CoA instead of palmitoyl-CoA, and lipid extracts were chromotographed on a silica gel G plate in heptane-isopropyl ether-acetic acid at 80:20:1 (v/v/v) instead of 60:40:4 (v/v/v).

HMG-CoA reductase activity assay

Enzymatic activity was assayed as described previously (15, 16). The reaction mixture contained 100 mM potassium phosphate (pH 7.4), 200 μg of cellular protein, 20 mM glucose-6-phosphate, 12.5 mM dithiothreitol, 2.5 mM NADP, and 1.2 units of glucose-6-phosphate dehydrogenase. Initiation of the reaction was done by the addition of [^{14}C]HMG-CoA (200 Bq/nmol) for 30 min at 37°C. The [^{14}C]mevalonate formed was converted into lactone by the addition of 10 N HCl, isolated by TLC, and counted using an internal standard to correct for incomplete recovery. For the PCR analysis of HMG-CoA reductase, the nucleotides used were 5′-GTCATTCCAGCCAAGGTTGT-3′ and 5′-GGGACCACTTGCTTCCATTA-3′.

ACAT activity assay

The activity of ACAT was determined by adding 5 nmol of [^{14}C]oleoyl-CoA (specific activity of ~167 Bq/nmol) to the

mixture containing 200 μg of cellular protein to initiate the reaction in a buffer solution (pH 7.5) consisting of CHOL, 0.04 M KH_2PO_4, 50 mM NaF, 0.25 M sucrose, and 1 mM EDTA. After incubation for 10 min at 37°C, the reaction was stopped by adding chloroform-methanol (2:1, v/v) followed by free cholesterol (FC) and cholesteryl ester (CE) as carriers. The FC and CE formed were isolated by TLC and counted. For the PCR analysis of ACAT-2, the nucleotides used were 5′-CAAGGAGGTGAAGGACAAGC-3′ and 5′-ATTGGACATGCTCTCTCCATCC-3′.

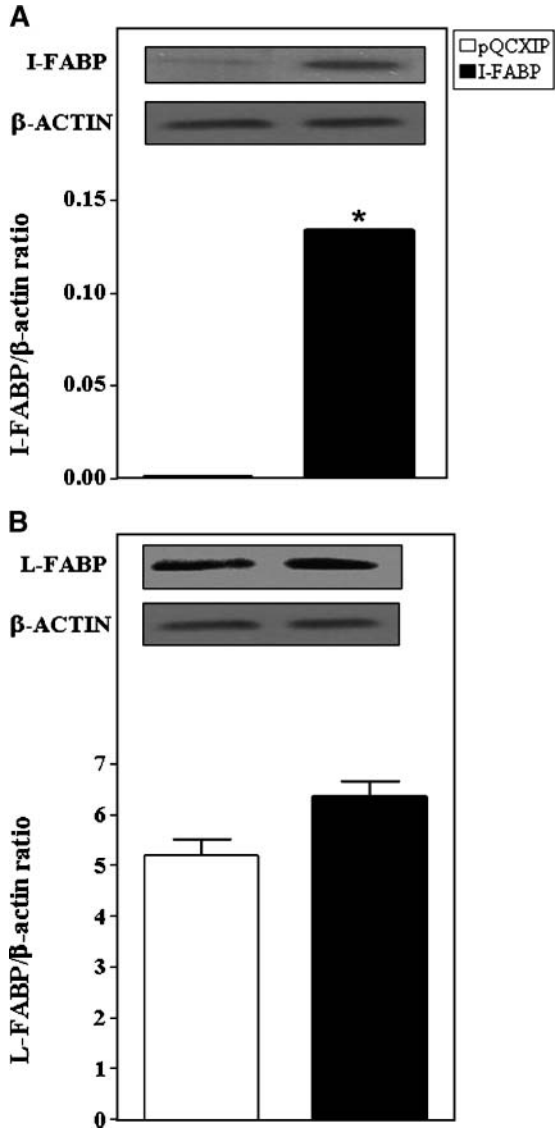

Fig. 1. Characterization of human intestinal epithelial cells (HIECs) overexpressing intestinal fatty acid binding protein (I-FABP). HIECs transfected with empty vector pQCXIP (controls or pQCXIP) or with I-FABP cDNA (I-FABP) were analyzed for I-FABP (A) and liver (L)-FABP (B) expression by Western blot. HIECs were stably transfected with human I-FABP cDNA, and the capacity of our construct to enhance I-FABP expression was examined. I-FABP expression at the protein level was 90-fold higher compared with that in control cells (pQCXIP vector alone), whereas L-FABP content did not differ between the two cell types. Quantitative analysis of immunoblots from three independent experiments is reported. Data are means ± SD. * $P < 0.0001$ versus cells transfected with empty pQCXIP.

CPT-1 and L-3-hydroxyacyl-coenzyme A dehydrogenase activity

A radioisotope assay for the determination of CPT-1 activity was used as described by McGarry and Brown (17). Briefly, the assay was conducted at 37°C and was initiated by the addition of 10 μl of mitochondrial suspension (1:3 dilution) to 90 μl of the following standard reaction medium: 117 mM Tris-HCl (pH 7.4), 0.28 mM reduced glutathione, 4.4 mM ATP, 4.4 mM $MgCl_2$, 16.7 mM KCl, 2.2 mM KCN, 40 mg/l rotenone, 0.5% BSA, 300 μM palmitoyl-CoA, and 5 mM L-carnitine, with 1 μCi of L-[^{3}H]carnitine and a final pH of 7.1. The reaction was stopped after 6 min with the addition of ice-cold 1 N HCl. Palmitoyl-[^{3}H]carnitine was extracted in water-saturated butanol in a process involving three washes with distilled water and subsequent recentrifugation steps to separate the butanol phase, in which the radioactivity was counted. CPT-1 activity was normalized to milligrams of protein.

The activity of L-3-hydroxyacyl-coenzyme A dehydrogenase (L3HOAD) was measured at 25°C by monitoring the disappearance of NADH ($\varepsilon_{340} = 6{,}220\ M^{-1}\ cm^{-1}$) upon the reduction of acetoacetyl-CoA according to Noyes and Bradshaw (18). The assay was performed in citrate/phosphate buffer, pH 7.0, containing 100 μM NADH and 50 μM ethyl-CoA.

Statistical analysis

Values are presented as means ± SD, and the significance of the differences between the means of treatment groups and controls was determined using Student's *t*-test.

RESULTS

To gain insight into I-FABP function in intestinal cells, we transfected HIEC-6 that expressed I-FABP in minute amounts only. The I-FABP protein expression product was electrophoresed by SDS-PAGE and transferred to Western blot membranes. By applying a specific polyclonal antibody, we showed that I-FABP integrated into HIEC-6, because we specifically detected a single protein band of the expected size with a 90-fold increase in its expression compared with normal HIEC-6 transfected with empty pQCXIP (**Fig. 1A**). The overexpression procedure allowed

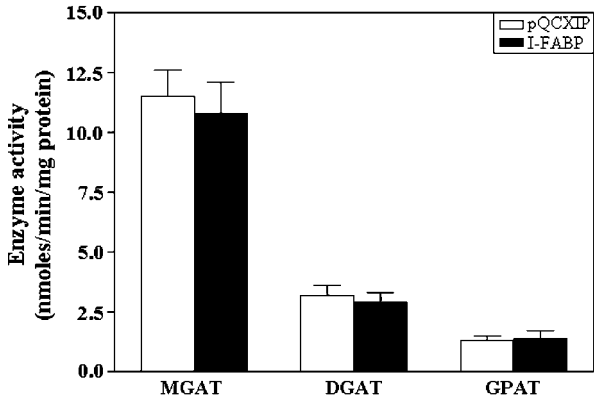

Fig. 2. Monoacylglycerol acyltransferase (MGAT), diacylglycerol acyltransferase (DGAT), and glycerol-3-phosphate acyltransferase (GPAT) activity in HIEC-6 overexpressing I-FABP. Microsomes were prepared from the intestinal epithelial cells and assayed for the three enzymes. Data are means ± SD for four separate experiments.

us to reach in HIEC-6 the I-FABP protein level detectable in human intestinal epithelium as estimated. Importantly, L-FABP protein expression consistently remained unchanged after I-FABP overexpression (Fig. 1B).

We previously showed that I-FABP expression in HIEC-6 had no inducing effects on FA esterification into triglycerides (TGs), CE, and phospholipids (PLs) (5). In the current studies, we determined the status of the enzymes controlling TG esterification via the monoacylglycerol (MG) pathway, which accounts for the large bulk of TG reconstitution in intestinal epithelial cells. As observed in **Fig. 2**, MGAT and DGAT activities were invariable in microsomes isolated from transfected I-FABP-HIEC-6 and cells transfected with empty pQCXIP. We also examined GPAT, which is the first committed step, one that is presumed to be rate-limiting, in glycerol-PL biosynthesis. Again, no differences were noted in the activity of this critical enzyme between cells overexpressing I-FABP and cells treated with empty pQCXIP vector only (Fig. 2). These findings confirm the limited capacity of I-FABP to influence the major glycerolipid synthetic routes.

Because some critical roles pertaining to intracellular FA catabolism have been associated with certain cytosolic FABP families, we then speculated that I-FABP might also be capable of altering the oxidation of LCFAs. To test this hypothesis, we used HIEC-6 to determine whether increasing I-FABP expression would modify their oxidation rates. When HIEC-6 was incubated with [^{14}C]oleate, the overexpression of I-FABP resulted in an increased conversion of [^{14}C]oleate into CO_2 (**Fig. 3A**) and augmented the incorporation of [^{14}C]oleate into acid-soluble metabolites (ASMs) (Fig. 3B), which indicates enhanced total β-oxidation (Fig. 3C). We reasoned that the stimulation

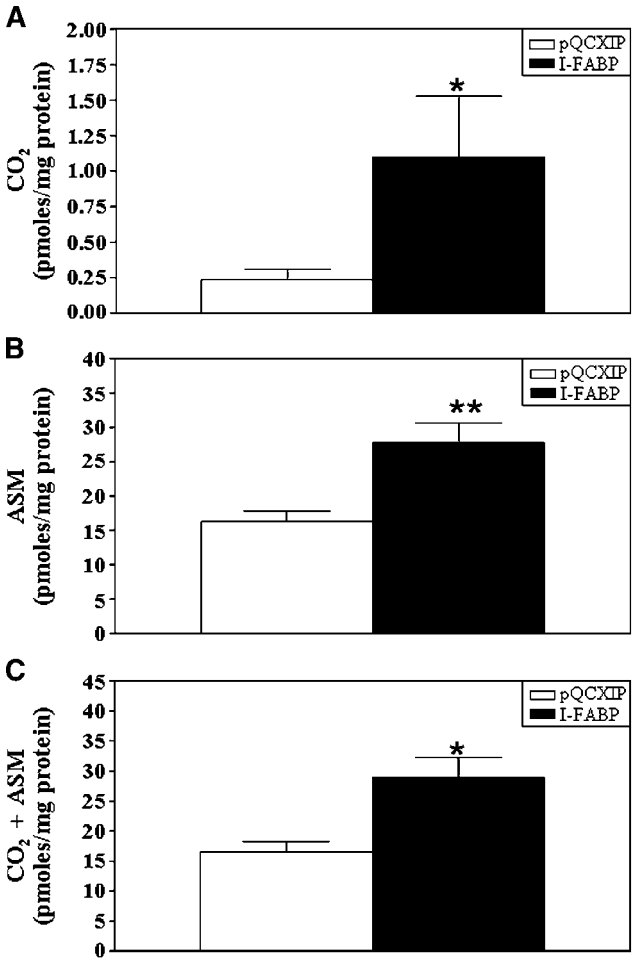

Fig. 3. β-Oxidation of fatty acids in HIEC-6 overexpressing I-FABP. The capacity of intestinal epithelial cells to oxidize [U-^{14}C] oleic acid was measured as described in Materials and Methods. Briefly, cultured HIEC-6 was exposed to [U-^{14}C]oleic acid for 16 h. At the end of the incubation period, [^{14}C]oleic acid oxidized to CO_2 (A), acid-soluble metabolites (ASMs) (B), and CO_2 + ASMs (C) was measured. Data are from six separate experiments and expressed as means ± SD. * $P < 0.01$, ** $P < 0.05$ versus cells transfected with empty pQCXIP.

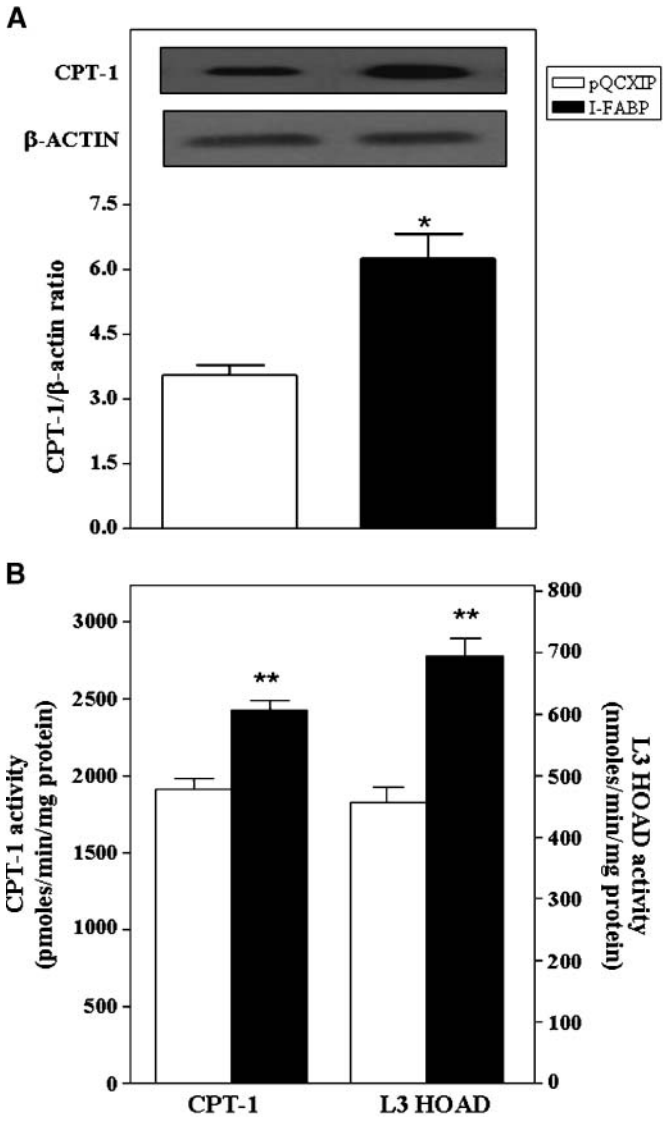

Fig. 4. Effects of I-FABP overexpression on carnitine palmitoyltransferase 1 (CPT-1) and L-3-hydroxyacyl-coenzyme A dehydrogenase (L3-HOAD) in HIEC-6. Mitochondria were isolated and assayed for CPT-1 protein expression (A) and for CPT-1 and L3-HOAD enzymatic activity (B). Data are means ± SD for three separate experiments. * $P < 0.05$, ** $P < 0.03$ versus cells transfected with empty pQCXIP.

of FA consumption might be attributable to mitochondrial β-oxidation; therefore, we assessed the mechanisms by first examining the protein expression and activity of CPT-1 that catalyze the formation of acyl-carnitine from long-chain acyl-CoA and carnitine, a principal regulatory step allowing their subsequent mitochondrial import. Consistent with the enhanced oxidation of [^{14}C]oleate to CO_2 and ASMs, increased levels of CPT-1 protein expression (**Fig. 4A**) and activity (Fig. 4B) were detected in HIEC-6 overexpressing I-FABP. We also measured the activity of L3HOAD, an important enzyme that actively participates in FA catabolism in mitochondria. As illustrated in Fig. 4B, the activity of the L3HOAD was increased in HIEC-6 overexpressing I-FABP. Overall, our findings suggest that I-FABP plays an important role in the induction of β-oxidation.

We also quantified the abundance of FAS, a key enzyme in the lipogenesis pathway, as well as that of ACC2, a critical enzyme anchored to the mitochondrial surface, which produces malonyl-CoA, a negative modulator of CPT-1 that is the rate-limiting enzyme in the fatty acyl-CoA transport system for FA β-oxidation (19). In fact, ACC2 plays a critical role for the regulation of mitochondrial FA oxidation. **Figure 5** illustrates the downregulation of FAS gene and protein expression (indicating that I-FABP overproduction reduces the biosynthesis of FAs in these conditions) as well as of ACC2 gene and protein expression (indicating that more FAs penetrate mitochondria and undergo β-oxidation).

The next series of experiments was aimed at examining whether I-FABP overexpression influenced the intracellular handling of CHOL. Uptake of CHOL was then determined in control and genetically modified intestinal cells. I-FABP overexpression caused a lessened ability of HIEC-6 to capture micellar [^{14}C]FC (**Fig. 6**). Albeit decreased, the intracellular conversion of FC into its esterified form, CE, was not changed significantly. We then explored the impact of I-FABP expression on the regulatory sterol enzymes: HMG-CoA reductase, the rate-limiting step in CHOL synthesis, and ACAT, an integral protein present in the rough endoplasmic reticulum, which catalyzes the formation of CE from FC and fatty acyl-CoA. Overexpression of I-FABP in HIEC-6 led to an increase in mRNA transcripts (**Fig. 7A**), protein expression (Fig. 7B), and activity (Fig. 7C) of HMG-CoA reductase. On the other hand, the mRNA quantity of ACAT-2 (**Fig. 8A**) and activity of ACAT (Fig. 8B) were not changed.

These findings prompted us to focus on the main proteins that control CHOL homeostasis, including carriers that have been reported to mediate intestinal CHOL transport (11, 20). I-FABP overexpression in HIEC-6 resulted in

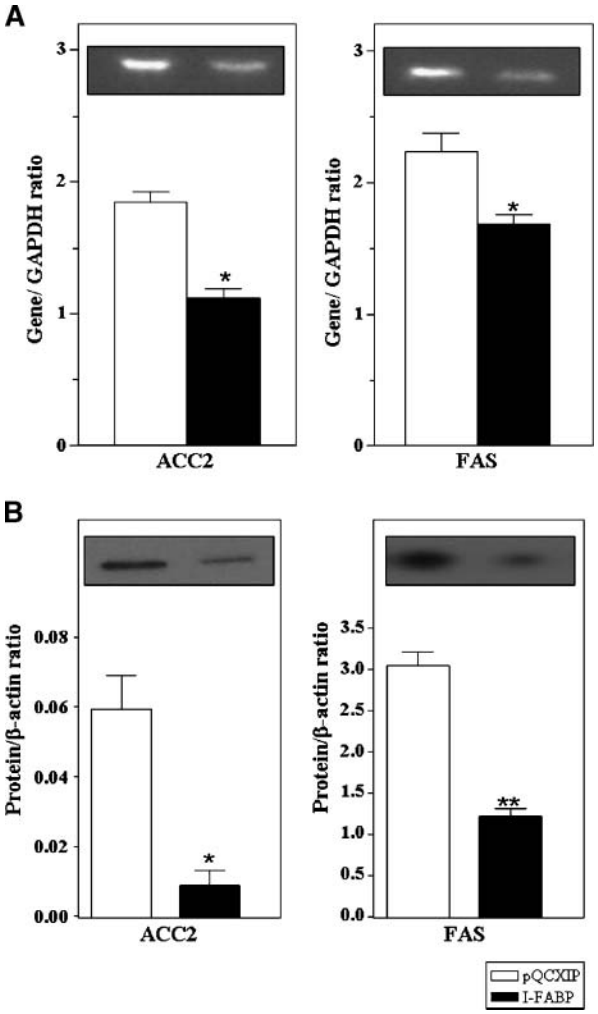

Fig. 5. Gene and protein expression of acetyl carboxylase 2 (ACC2) and FAS in HIEC-6 overexpressing I-FABP. Intestinal epithelial cells were incubated with fresh medium, allowed to grow, and tested for transcript levels (A) and protein mass (B) of ACC2 and FAS by RT-PCR and Western blotting, respectively. Data are means ± SD for three separate experiments. * $P < 0.05$, ** $P < 0.001$ versus cells transfected with empty pQCXIP.

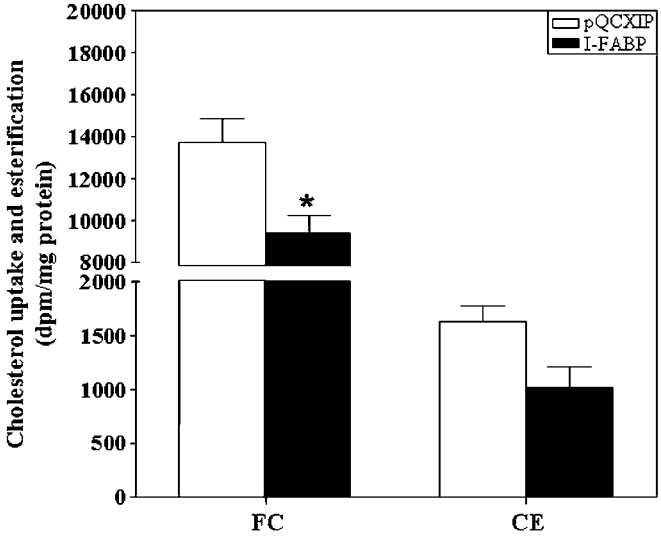

Fig. 6. Influence of I-FABP overexpression on free cholesterol (FC) uptake and cholesteryl ester (CE) in HIEC-6. After incubation with [^{14}C]cholesterol (CHOL), epithelial cells were washed, homogenized, and lipid-extracted before application to TLC. Labeled FC and CE were counted after separation using a liquid scintillation spectrometer. Quenching was corrected using computerized curves generated with external standards. Data are from four separate experiments and expressed as means ± SD. * $P < 0.05$ versus cells transfected with empty pQCXIP.

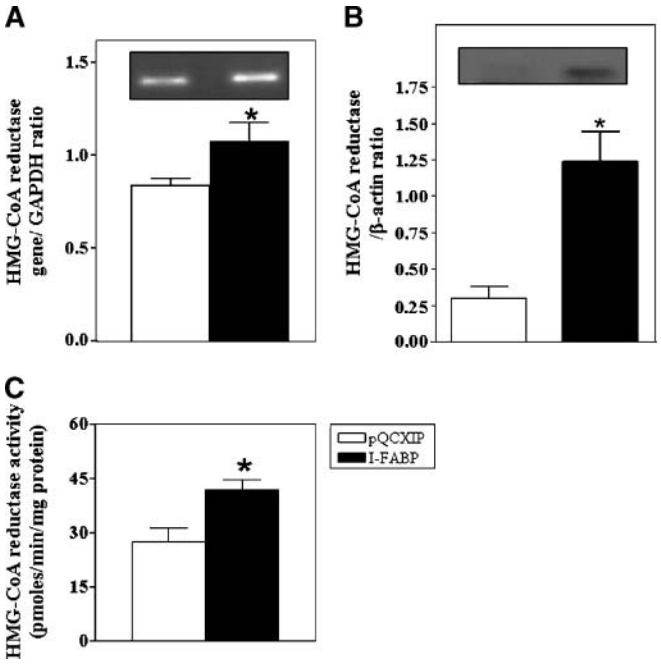

Fig. 7. Impact of I-FABP overexpression on HMG-CoA reductase gene expression, protein mass, and activity. The levels of HMG-CoA reductase mRNA transcripts (A) and protein content (B) were determined by RT-PCR and Western blotting, respectively, as described in Materials and Methods. Cell homogenates were assayed for DL-HMG-CoA reductase activity (C). Values are means ± SD for three independent experiments. * $P < 0.05$ versus cells transfected with empty pQCXIP.

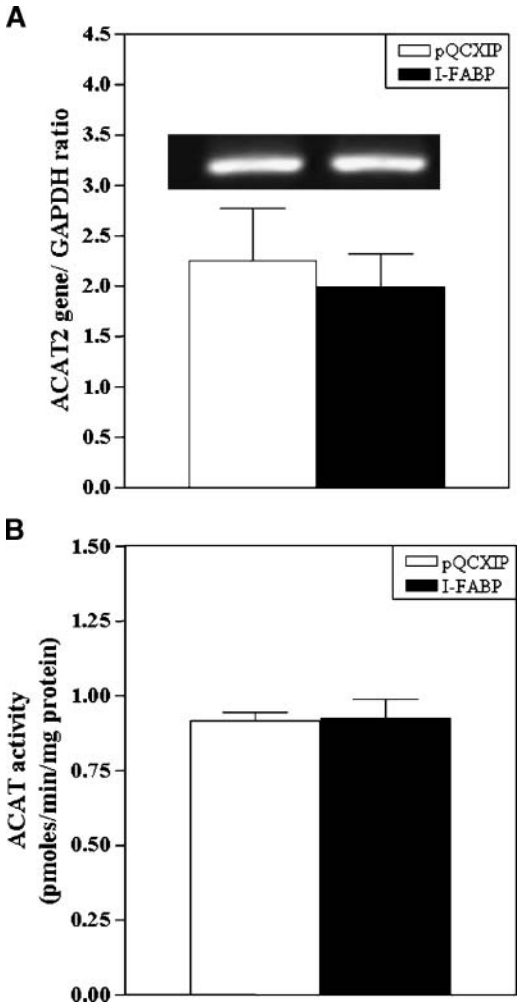

Fig. 8. Impact of I-FABP overexpression on ACAT-2 gene expression and ACAT enzymatic activity. A: The level of ACAT mRNA was determined as described in Materials and Methods. B: Cell homogenates were assayed for ACAT activity. Values are means ± SD for three independent experiments.

diminished mRNA levels of NPC1L1, CD36, and SR-BI (**Fig. 9A**). However, only the protein mass of NPC1L1 followed the same trend of decrease, because the protein expression of CD36 remained unchanged and that of SR-BI revealed an increase (Fig. 9B). Because ABC family members (ABCA1 and ABCG5/ABCG8) act as efflux pumps favoring the CHOL export out of absorptive cells, we evaluated their status. The I-FABP overexpression approach resulted in a significant increase in ABCA1 and ABCG5 gene expression (**Fig. 10A**), whereas ABCG8 mRNA levels remained unchanged. Furthermore, the protein mass of ABCA1 and ABCG8 increased under the influence of I-FABP overexpression (Fig. 10B).

Finally, we turned on the nuclear and transcription factors that affect the transcription of a variety of genes associated with lipid metabolism. We first tested SREBP-2, which regulates CHOL metabolism. SREBP-2 gene and protein expression did nod respond to I-FABP overexpression (**Fig. 11**). We then investigated the mRNA status of peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), and retinoid X receptor (RXR). The data in **Fig. 12** illustrate the induction of PPARα and PPARγ, underline the inactivation of LXRα, and emphasize the invariable condition of PPARβ, LXRβ, RXRα, and RXRβ in HIEC-6 overexpressing I-FABP.

Given the involvement of peroxisomes in FA β-oxidation, it seemed interesting to determine whether I-FABP overexpression might influence ACOX1, which catalyzes the first, rate-limiting step in peroxisomal FA β-oxidation. Neither RT-PCR nor Western blot analysis could detect any differences in mRNA level (**Fig. 13A**) or protein mass (Fig. 13B), respectively, of ACOX1 between control cells with empty vector and experimental epithelial cells overexpressing I-FABP.

DISCUSSION

In recent years, tremendous progress has been made with respect to the identification, ligand specificity, and molecular switch of FABP species in various tissues. Given the enigmatic presence and roles of the two FABPs present in the intestine, we have concentrated on the potential of I-FABP to exert lipid functions. Using the molecular overexpression approach in HIEC-6, we demonstrated that I-FABP is able to modulate lipid metabolism, possibly by interacting with specific targets. This report specifically indicates that I-FABP overexpression results in the follow-

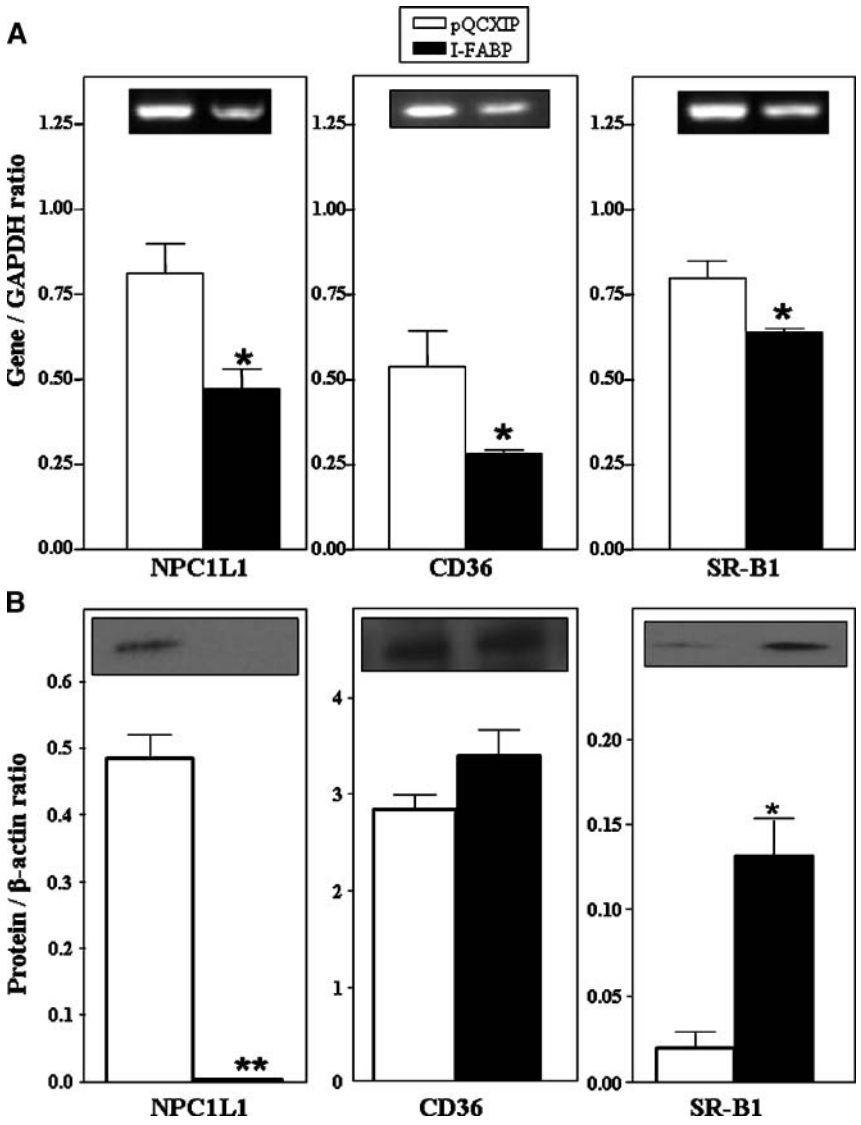

Fig. 9. Gene and protein expression of transporters for CHOL influx, Niemann Pick C1-Like 1 (NPC1L1), cluster determinant 36 (CD36), and scavenger receptor class B type I (SR-BI), in HIEC-6 overexpressing I-FABP. Intestinal epithelial cells were incubated with fresh medium, allowed to grow, and tested for transcript levels by RT-PCR (A) and for protein mass by Western blotting (B) of NPC1L1, CD36, and SR-BI. Data are means ± SD for three separate experiments. * $P < 0.05$, ** $P < 0.001$ versus cells transfected with empty pQCXIP.

ing: *i*) a lack of influence of glycerolipid esterification, as suggested by unchanged activity of enzymes controlling the MG and phosphatidic acid pathways; *ii*) decreased micellar CHOL uptake, which was confirmed by the lessened gene and protein expression of the major transporter NPC1L1 as well as the increased gene and protein expression of ABCA1 along with the augmented protein mass of ABCG8; *iii*) increases in the transcripts, protein level, and activity of HMG-CoA reductase without any alteration in ACAT-2; *iv*) upregulation of mitochondrial FA β-oxidation, documented by the rate of [^{14}C]CO_2 exhalation, the production of ASMs, and the induction of both the pivotal catalytic CPT-1 and L3HOAD enzymes; *v*) downregulation of the gene and protein expression of ACC2 and FAS involved in lipogenesis; *vi*) modulation of the nuclear receptors that mediate the effects of lipidic ligands at the transcriptional level; and *vii*) invariable gene and protein expression of ATOX1, which suggests that FA β-oxidation in peroxisomes is not influenced by I-FABP overexpression.

To address the influence of I-FABP on TG esterification, an essential process for lipoprotein assembly, we determined the activity of the enzymes involved. TG biosynthesis in the intestine essentially arises via the MG pathway, which uses absorbed *sn*-2-MG templates for TG reesterification and predominates over de novo TG synthesis via the glycerol-3-phosphate or phosphatidic pathway (21). In the MG pathway, FA and *sn*-2-MG are used to se-

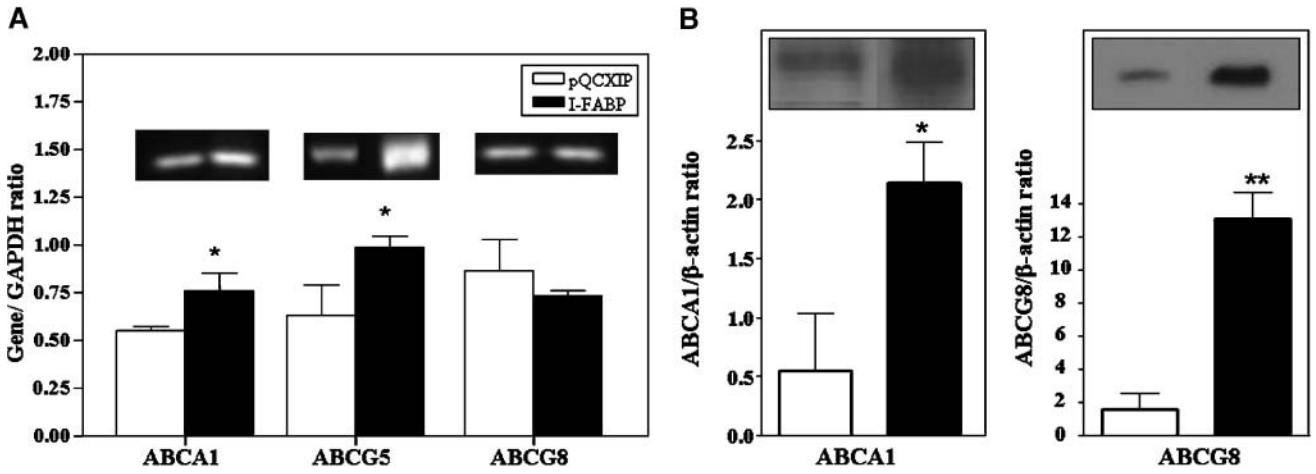

Fig. 10. Gene and protein expression of ABCA1 and ABCG5/ABCG8 in HIEC-6 overexpressing I-FABP. Intestinal epithelial cells were incubated with fresh medium, allowed to grow, and tested for transcript levels by RT-PCR (A) and for protein mass by Western blotting (B) of ABCA1 and ABCG5/ABCG8. Data are means ± SD for three separate experiments. * $P < 0.05$, ** $P < 0.0001$ versus cells transfected with empty pQCXIP.

quentially resynthesize diacylglycerols (DG) and TG by MGAT and DGAT, respectively, allowing for lipids to be transported into the circulation system by chylomicrons. In contrast, glycerol-3-phosphate is formed from glucose metabolism, or glycerol via glycerol kinase, and can result in TG synthesis through a more energy-consuming series of enzyme-catalyzed reactions (22). In this pathway, GPAT catalyzes the initial step by converting glycerol-3-phosphate to lysophosphatidic acid, which is then acylated to form phosphatidic acid, followed by dephosphorylation to DG. The two underlined pathways share the common last step, which consists of the conversion of DG to TG by DGAT. Importantly, the 1,2-DG branch point converts not only to TG but also to PL (23). The present experiments using genetically modified HIEC-6 enabled us to show the inefficient contribution of I-FABP to the MG and phosphatidic pathways, thus confirming the limited capacity of I-FABP to influence lipid esterification and chylomicron assembly, which has been suggested in our recent work (5).

FAS catalyzes all of the reaction steps in the conversion of acetyl-CoA and malonyl-CoA to palmitate. Because it is essential for lipogenesis, we have examined its gene and protein expression and found that I-FABP overexpression downregulated it. These findings add further support to the aforementioned observations of the weak capacity of HIEC-6 to synthesize different classes of lipids and to transport them in the form of lipoproteins. Confirmation of this statement was obtained with our observations related to the decline of ACC2 gene and protein expression as a function of I-FABP overexpression. ACC2 is anchored to the mitochondrial surface via a unique N-terminal domain that includes 20 hydrophobic amino acids (19, 24). ACC2 produces malonyl-CoA on the mitochondrial surface, which represents a potent endogenous inhibitor of carnitine palmitoyl. In these conditions, ACC2 indirectly prevents the influx of FAs into the mitochondria and their subsequent β-oxidation (25). In view of the ACC2 reduction, we conclude that HIEC-6 is able to incorporate more FAs for β-oxidation when I-FABP is overexpressed.

Because exogenous FA has not massively entered synthetic pathways in HIEC-6 expressing high levels of I-FABP, we reasoned that they may alternatively be channeled toward mitochondria for degradation via β-oxidation. This hypothesis turned out to be true. Indeed, our data disclosed a significant increase in the conversion of [^{14}C] oleate to CO_2 and ASMs, which are considered a more accurate measure of β-oxidation (26). Additional evidence was obtained by illustrating a substantial enhancement of CPT-1 protein mass and activity. This enzyme, residing in the outer mitochondrial membrane, catalyzes the formation of acyl-carnitine from long-chain acyl CoA and carnitine (17), which constitutes the rate-limiting regulated step of FA oxidation. In fact, *trans*-esterification to acyl-carnitine is required for FA entry into mitochondria and subsequent β-oxidation (17, 27). Concomitantly, we observed an increase in L3HOAD, one of the predominant enzymes in mitochondria, located in the inner mitochondrial membrane, which catalyzes the third step of long-chain FA β-oxidation, converting L3HOAD in the presence of NAD^+ to 3-ketoacyl-CoA, NADH, and H^+. This is the first study that documents high mitochondrial performance in response to the forced expression of I-FABP. Therefore, apart from being an important ligand transporter, I-FABP gains much prominence for its role in regulating FA consumption via important β-oxidation players. Interestingly, overexpression of L-FABP was reported to be associated with induced β-oxidation activity in McA-RH7777 hepatoma cells (28). Conversely, fasted L-FABP-null mice were characterized by lower hepatic FA oxidation than wild-type controls (29, 30). In the present studies, it is important to note that I-FABP accounts for >85% of the two cytosolic FABPs in HIEC-6 and that its overexpression has not resulted in compensatory upregulation of L-FABP.

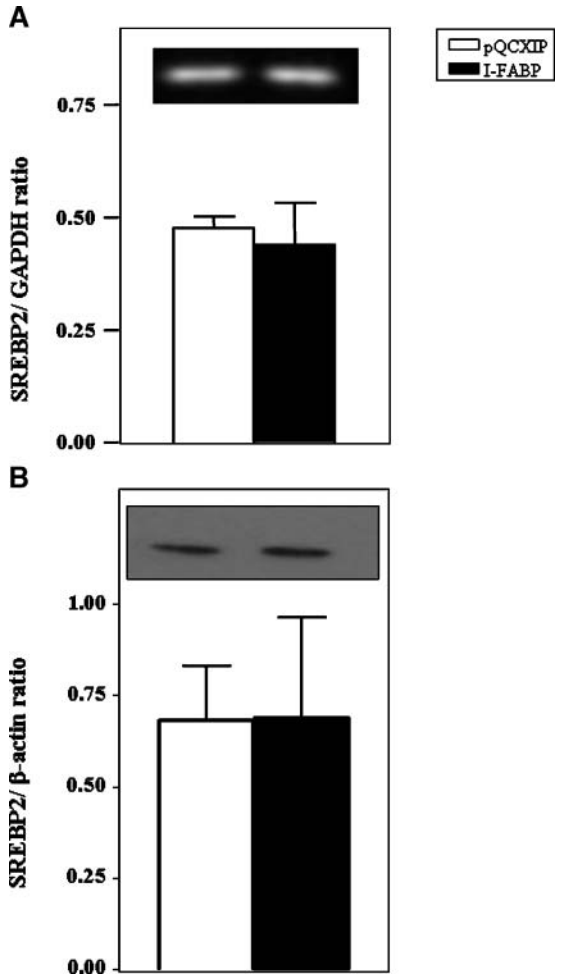

Fig. 11. Impact of I-FABP overexpression on the gene and protein expression of sterol-regulatory element binding protein 2 (SREBP-2). Intestinal epithelial cells were incubated with fresh medium, allowed to grow, and tested for transcript levels by RT-PCR (A) and for protein mass by Western blotting (B) of SREBP-2. Data are means ± SD for three separate experiments.

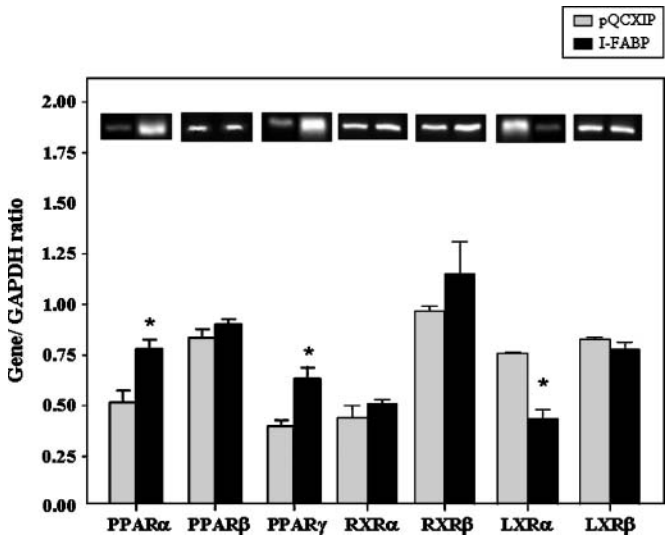

Fig. 12. Impact of I-FABP overexpression on the gene expression of the nuclear receptors peroxisome proliferator-activated receptor (PPAR), retinoid X receptor (RXR), and liver X receptor (LXR). Total mRNA from intestinal epithelial cells was analyzed by RT-PCR. Primers specific for the different gene regions were used to generate the amplicons. Representative autoradiograms of the different amplicons are shown. Values represent means ± SD for three separate experiments. * $P < 0.05$ versus cells transfected with empty pQCXIP.

Peroxisomes are known to accommodate FA β-oxidation, and the first reaction is catalyzed by an ACOX1, which is regarded as the main enzymatic step controlling the flux through the pathway (31). Although similar in mechanism, mitochondrial and peroxisomal β-oxidation fulfill different functions, as concluded from the usually severe but different clinical signs and symptoms associated with inherited defects in either mitochondrial β-oxidation (31, 32). In fact, mitochondrial β-oxidation is coupled to the respiratory chain, leading to the production of ATP. In contrast, in higher vertebrates, peroxisomal β-oxidation is incomplete and cannot fully degrade long-chain fatty acyl-CoAs. Thus, its function is to shorten or convert FAs into a form that can be accepted by mitochondrial enzymes and exported into the mitochondria, where β-oxidation is completed (31, 33, 34). In our studies, the overexpression of I-FABP did not alter ACOX1 gene and protein expression, which suggests that peroxisomes, in contrast to mitochondria, are not regulated by I-FABP. These data accentuate the differences between mitochondria and peroxisomes.

One of the most striking phenotypes emerging from our experiments was that I-FABP overexpression in HIEC-6 led to reduced CHOL uptake. In evaluating the mechanisms underlying this effect, both types of CHOL transporters active in influx and efflux were involved. Indeed, decreased gene expression of SR-BI, NPC1L1, and CD36, as well as enhanced transcript levels of ABCA1 and ABCG5, were recorded. Western blot analysis confirmed the downregulation of NPC1L1 (the key mediator of CHOL absorption) and the upregulation of ABCA1 and ABCG8, which facilitate the efflux of enterocyte CHOL (35–39). In fact, NPC1L1 represents the most important mechanism by which CHOL moves from the intestinal lumen into the absorptive enterocytes lining the proximal small intestine (11, 40, 41). Other candidate proteins, like SR-BI and CD36 (42, 43), have been identified, but their precise function in intestinal CHOL needs further investigation. According to our experiments, I-FABP seems to inhibit CHOL influx and to stimulate CHOL export out of absorptive cells.

Our results showed an increase in the nuclear factors PPARα and PPARγ, which accompanied the stimulation of FA oxidation in HIEC-6 overexpressing I-FABP. This is in agreement with previous studies demonstrating that the mouse adipocyte FABP gene, named *AP2*, has a specific enhancer able to bind a mPPARγ2/RXRα heterodimer (44). PPARs were further shown to be good candidates for the transcription of L-FABP, and an interaction was shown between the PPARs and unique

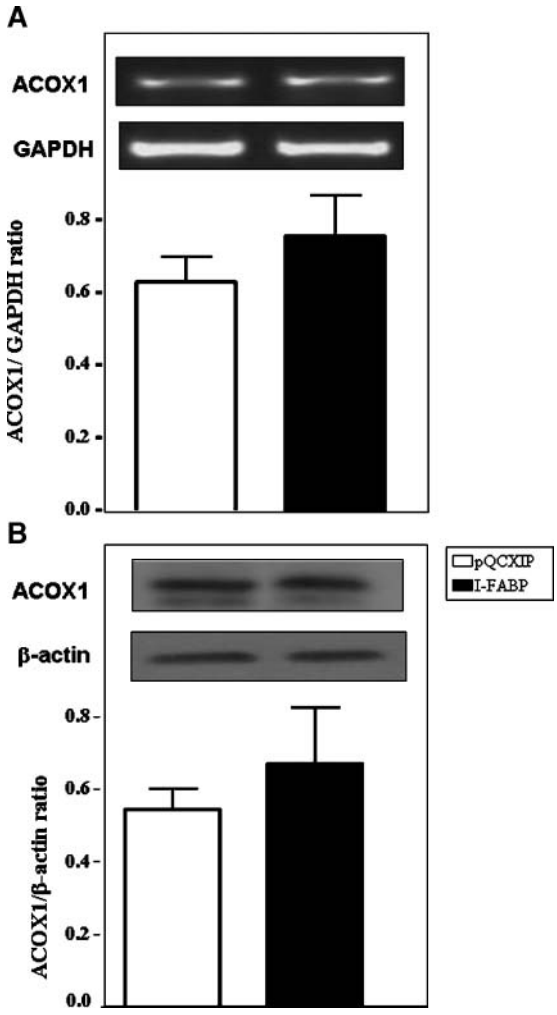

Fig. 13. Gene and protein expression of acyl-coenzyme A oxidase 1 (ACOX1) in HIEC-6 overexpressing I-FABP. Intestinal epithelial cells were incubated with fresh medium, allowed to grow, and tested for transcript levels by RT-PCR (A) and for protein mass by Western blotting (B) of ACOX1. Data are means ± SD for three separate experiments.

sequence PPAR response elements (PPREs) located in the proximal part of the L-FABP promoter (45). Because I-FABP is believed to play a role in the cellular binding and trafficking of FAs in enterocytes and seems to be an important agent for the coordinated regulation of FA-responsive genes (probably through the modulation of the intracellular concentration of long-chain FAs), it is reasonable to suggest that I-FABP represents a cytosolic-nuclear shuttle protein implicated in the ligand activation of PPAR and, therefore, in the transcription of PPAR target genes in the intestine.

As mentioned above, PPARs heterodimerize in the nucleus with another nuclear receptor, known as RXR, and the mouse PPAR/RXR heterodimer interacts in target genes with PPREs. Consensus PPREs are direct repeat AGGTCA separated by one nucleotide DR1 (with a 5′ extension of AACT) for increased specificity (46). A wide variety of natural or synthetic compounds were identified as PPAR ligands. Among the synthetic ligands, the lipid-lowering drugs (fibrates) and insulin sensitizers (thiazolidinediones) are PPARα and PPARγ agonists, respectively, which underscores the important role of PPARs as therapeutic targets (47–49). Given that *i*) PPARα is expressed at high levels in organs that carry out significant FA catabolism and transport, such as the brown adipose tissue, liver, heart, kidney, and intestine (50), and *ii*) PPARγ is involved in glucose and lipid metabolism through an improvement of insulin sensitivity and thus represents a molecular link between lipid and carbohydrate metabolism (51, 52), we assessed their gene expression and found it augmented in HIEC-6 overexpressing I-FABP. Together, our results indicate that the overexpression of I-FABP was linked to PPARα induction, which could stimulate CPT-1 activity and the β-oxidation of FAs. Conversely, targeted I-FABP gene disruption might impede I-FABP functioning, thereby preventing FA consumption and enhancing their availability in the blood circulation, which may boost insulin resistance, as is the case in I-FABP-null mice (1, 4). In the present work, I-FABP overexpression did not enhance RXRαs, the partner of PPARαs, and additional studies are needed to clarify whether RXRα is present in sufficient amounts for PPAR/RXRα heterodimer action or whether the ligand for RXRα, 9-*cis* retinoic acid, is required for maximal dimerization and interplay.

PPARs and LXRs control the transcription of a number of specific genes involved in the central pathways in CHOL metabolism, transport, and elimination (53, 54), and the most common synthetic PPAR agonists are fibrates (PPARα) and thiazolidinediones (PPARγ) that include compounds with varying selectivity. Both compounds are used as first-line drugs in the treatment of hypertriglyceridemia, hypercholesterolemia, and type 2 diabetes (55). In our studies, I-FABP overexpression resulted in the decreased expression of intestinal CHOL transporters and in the increased expression/activity of HMG-CoA reductase. LXRα and PPARα are crucial regulators of CHOL homeostasis, belonging to the nuclear receptor superfamily (56, 57). LXR agonists effectively block intestinal CHOL absorption in mice (38) through the induction of ABC sterol transporter genes, including ABCA1, ABCG5, and ABCG8, which are responsible for CHOL efflux (58, 59). Furthermore, the gene for NPC1L1, a protein critical for intestinal CHOL absorption, is under the control of LXR, because LXR activators downregulate NPC1L1 mRNA levels in human enterocytes (60).

In conclusion, this study demonstrates for the first time the potential role of I-FABP in FA and CHOL regulation. If our findings strengthen the notion that I-FABP is not specifically required for the assimilation of alimentary TG or for their intracellular reesterification to serve in chylomicron packaging, they emphasize its important implications regarding intestinal CHOL transport and FA delivery to mitochondria for β-oxidation. A deficiency in I-FABP may in turn enhance CHOL absorption and prevent FA oxidation, which may increase FA and CHOL availability in the blood circulation, thereby affecting cardiovascular health.

This study was supported by the Canadian Institutes of Health Research (Grant MOP 49433) and the Canadian Institutes of Health Research Team Grant Program (Grant CTP 82942). The authors thank Mrs. Schohraya Spahis for her technical assistance.

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