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Modulation of Intestinal and Liver Fatty Acid-Binding Proteins in Caco-2 Cells by Lipids, Hormones and Cytokines

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Abstract Intestinal and liver fatty acid binding proteins (I- and L-FABP) are thought to play a role in enterocyte fatty acid (FA) trafficking. Their modulation by cell differentiation and various potential effectors was investigated in the human Caco-2 cell line. With the acquisition of enterocytic features, Caco-2 cells seeded on plastic progressively increased L-FABP quantities, whereas I-FABP was not detectable even very late in the maturation process. On permeable filters that improved differentiation markers (sucrase, alkaline phosphatase, transepithelial resistance), Caco-2 cells furthered their L-FABP content and expressed I-FABP. Western blot analysis showed a significant increase in I- and L-FABP expression following an 8-hour incubation period with butyric acid, oleic acid, and phosphatidylcholine. However, in all cases, I-FABP levels were higher than L-FABP concentrations regardless of the lipid substrates added. Similarly, hydrocortisone and insulin enhanced the cellular content of I- and L-FABP whereas leptin triggered I-FABP expression only after an 8-hour incubation. Finally, tumor necrosis factor-α was more effective in increasing the cytosolic amount of I-FABP levels. In conclusion, our data demonstrate that I-FABP expression is limited to fully differentiated Caco-2 cells and can be more easily regulated than L-FABP by lipids, hormones, and cytokines. J. Cell. Biochem. 81:613–620, 2001. © 2001 Wiley-Liss, Inc.

Key words: fatty acids; phosphatidylcholine; hydrocortisone; insulin; cytokines; fat absorption

INTRODUCTION

Dietary fat represents a major caloric source in most Western countries. A complex series of digestive and absorptive mechanisms is required to efficiently process the 120–150 g of lipids consumed daily [Davidson, 1994; Tso, 1994; Black, 1995]. Yet, the assimilation of digestive products by intestinal absorptive cells is not very well understood. This process comprises several intricate events within enterocytes, which encompass vectorial lipid delivery for assembly with apolipoproteins (apo) and their export as lipoproteins [Levy, 1992; Levy et al., 1995].

In the two last decades, many laboratories have attempted to elucidate a multistep pathway leading to the assembly of chylomicrons, which includes the uptake of lipolytic products, lipid esterification in the endoplasmic reticulum, synthesis and posttranslational modification of different apolipoproteins (apo), and the packaging of lipid and apo components into lipoprotein particles [Levy, 1992; Davidson, 1994; Tso, 1994; Black, 1995; Levy et al., 1995]. Two fatty acid-binding proteins (FABP) were identified for the transport of fatty acids (FA) in the enterocyte, intestinal (I) and liver (L) FABP [Bass and Manning, 1986; Bass, 1988].

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Based on a variety of physico-chemical studies, various functions have been proposed for cytosolic FABP, such as FA transport and compartmentalization, the modulation of enzyme activities involved in lipid metabolism, and the protection of the cellular integrity from the detrimental effects of hydrophobic FA or other noxious substances [Gordon et al., 1983; Lowe et al., 1987; Sweetser et al., 1987; Cistola et al., 1989]. However, the physiological significance of the two FABP forms in the intestinal absorptive cells remains unknown. Moreover, there is insufficient information on the modulation of I- and L-FABP by fatty acids, hormones and cytokines, all known to influence intestinal lipid transport and triglyceride-rich lipoprotein production [Lowe et al., 1987; Loirdighi et al., 1992; Mehran et al., 1995; Levy et al., 1996; Loirdighi et al., 1997; Stan et al., 1999]. Therefore, this study was undertaken to further our understanding of the regulation of the two FABP forms.

MATERIALS AND METHODS

Cell Culture

Caco-2 cells (American Type Culture Collection (ATCC), Rockville, MD) were cultured as previously reported [Mehran et al., 1995, 1997]. Briefly, they were grown at 37°C with 5% CO_2 in a complete medium consisting of MEM (GIBCO BRL, Grand Island, NY) containing 1% penicillin/streptomycin, 1% DMEM nonessential amino acids (GIBCO BRL), and 10% decomplemented FCS (Flow Laboratories, McLean, VA). Caco-2 cells (between passages 30–40) were maintained in 175 cm^2 flasks (Corning Glass Works, Corning, NY) in a 95% air-5% CO_2 atmosphere at 37°C. Cell cultures were split (generally 1:3 to 1:6) when they reached 70–90% confluence, using 0.05% trypsin (0.5 nM) in ethylenediaminetetraacetic acid (EDTA GIBCO BRL). For individual experiments, cells were plated (1×10^6 cells/well) on plastic supports or 24.5 mn polycarbonate Transwell filter inserts with 0.4 μm pores (Costar, Cambridge, MA) in complete medium (as described above), supplemented with 5% FCS. The inserts fit into six-well culture plates, allowing separate access to the apical and basolateral surfaces of the cell monolayers. The cells were used for experiments 18–24 days after plating, as previously described [Mehran et al., 1995, 1997]. The medium was refreshed every second day. Transepithelial resistance, an index of cell confluence and tight junction formation, was evaluated using a Millicel-ERS apparatus (Millipore, Bedford, MA).

Anti-L-FABP and Anti-I-FABP Antibody Production

The fusion proteins GST-FABP-L and I were isolated from E. coli cells transformed with the pGEX-L-FABP and I prokaryotic expression constructs, and purified on glutathione sepharose 4B column according to the manufacturer's protocol. The proteins were used to raise anti-L-FABP and I antibodies in rabbits according to the standard immunization protocol approved by the institutional Animal Protection Committee, which included the following steps: priming with 200 μg purified recombinant protein in 0.25 ml PBS in 1:1 emulsion with 0.2 ml complete Freund adjuvant, and subcutaneously injecting (4×0.1 ml) at four places at the back of the animal. Two weeks later the immune response was boosted with 200 μg purified recombinant protein in 0.25 ml PBS in 1:1 emulsion by a similar injection procedure. Serum samples were verified for the presence of anti-L-FABP and anti-L-FABP antibodies at days 0, 14 and 28, by Western blotting and hybridization. The final collection of anti-serum was performed at Day 30. Various studies revealed the specificity, affinity, and binding capacity of the antibodies, and confirmation was obtained by using Dr. Veerkamp's antibodies.

Western Blot Analysis

Cells were harvested after 2 or 8 h incubation with either oleic acid (0.5 mM), butyric acid (4 mM), phosphatidylcholine (800 μM), hydrocortisone (50 ng/ml), insulin (3 mM), leptin (50 nM), interleukin-6 (IL-6) (10 ng/ml) or TNF-α (100 ng/ml). Cells were lysed and rinsed in phosphate-buffered saline (PBS). Lysis buffer consists of TBS, 5 mM EDTA, 0.2% SDS, 1% Triton, 0.5% sodium desoxycholate and a cocktail of protease inhibitors (PMSF, BHT and pepstatin). The buffer was added to the cells and placed on ice for 10 min. All lysates were sonificated for 10 s and then centrifuged for 10 min at 4°C. The supernatant was collected and stored at −80°C. The protein concentration was measured spectrophotometrically using protein standard (Biorad). Cytosolic proteins (30–80μg) were fractionated by SDS-PAGE using 15% polyacrylamide gels and transfered on to a nitrocellulose membrane (Amersham) [Mehran

et al., 1995; Levy et al., 1996; Stan et al., 1999]. After blocking with 5% nonfat milk, the membrane was probed with either anti-human I-FABP antiserum (1:500) or anti-human L-FABP antiserum (1:1 000). Antigen–antibody complexes were revealed with enhanced chemiluminescence detection (Boehringer-Mannheim, Germany).

Morphology

Cells were plated and grown on plastic supports or filters as described above. Monolayers were fixed in situ with 1% gluteraldehyde buffered with 0.1M sodium cacodylate for at least 1 h at 4°C. Cells were then postfixed for 1h in 1% osmium tetroxide at 4°C. Subsequently, cells were dehydrated in graded ethanol and embedded in Epon according to standard techniques [Bendayan, 1984; Levy et al., 1990]. Ultrathin sections were cut, mounted on nickel grids, stained, and examined with a Phillips 410 electron microscope.

Statistical Analysis

Results are reported as mean ± SEM of at least triplicate samples representative of not less than three separate experiments. Statistical analyses were performed using Student's *t*-test.

RESULTS

Cell Differentiation and FABP

Caco-2 cells plated on plastic support (Fig. 1A) progressively differentiated into villous-like cells in the postconfluence of growth (Fig. 1B). Along with time in culture they formed a polarized columnar monolayer of mature-appearing enterocytes (Fig. 1B). The presence of microvilli and junctional complexes became more evident as the duration of culture was extended. At 21 days of culture, they formed a polarized monolayer with a brush border consisting of numerous microvilli, functionally competent tight junctions, and complex lateral membrane interdigitations (Fig. 1B). Concomitant with the acquisition of ultrastructural enterocytic characteristics, Caco-2 cells appeared to increase the expression of L-FABP visualized on Western blot (Fig. 2). However, I-FABP was not detectable even very late in the differentiation process of Caco-2 cells seeded on plastic supports. Then Caco-2 cells were grown on permeable polycarbonate filters known to hasten and increase cell maturation (morphological results not shown), which was confirmed by the combined measurements of sucrase and alkaline phosphatase activity as well as transepithelial resistance (Table I). Under these conditions, Caco-2 cells augmented their L-FABP content and expressed I-FABP. The calculated ratio of L-FABP-to-I-FABP was equivalent to 50, which indicates the differential regulation of these small cytosolic proteins by the differentiation process.

Regulation of I- and L-FABP by Lipids

The cellular content of I- and L-FABP was evaluated following the apical addition of butyric acid, oleic acid and phosphatidylcholine. The presence of all these lipid substrates induced an increase in the amount of I- and L-FABP at the two incubation periods, especially at 8 h, compared with the control cultures without lipid supplementation (Fig. 3). However, Western blot analysis at 8 h showed that butyric acid, oleic acid, and phosphatidylcholine triggered more I-FABP (61, 106, and 72%, respectively) than L-FABP (22, 40, and 20%, respectively).

Hormones Modulation of I- and L-FABP

In order to examine the hormonally mediated effect on I- and L-FABP, hydrocortisone, insulin, and leptine were added to the culture medium. Hydrocortisone and insulin were found to increase the protein cellular levels of both types of FABP (Fig. 4). However, they enhanced I-FABP (78 and 49%, respectively) more than L-FABP (36 and 7%, respectively) at the 2 h incubation period. Moreover, no marked changes in L-FABP cytosolic content occurred with leptin supplementation at 2 and 8 h, while I-FABP levels rose by 86 and 43%, compared with controls, at the 2- and 8-hour incubation periods.

Cytokines Regulation of I- and L-FABP

The influence of IL-6 and TNF-α on I- and L-FABP was evaluated in cultured Caco-2 cells. At the 2- and 8-hour incubation periods, IL-6 increased L-FABP by 12 and 22%, and I-FABP to a similar extent (28 and 25%) (Fig. 5). Treatment with TNF-α did not modify the cellular levels of L-FABP, while it enhanced those of I-FABP at 2 and 8 h by 37 and 40%, respectively.

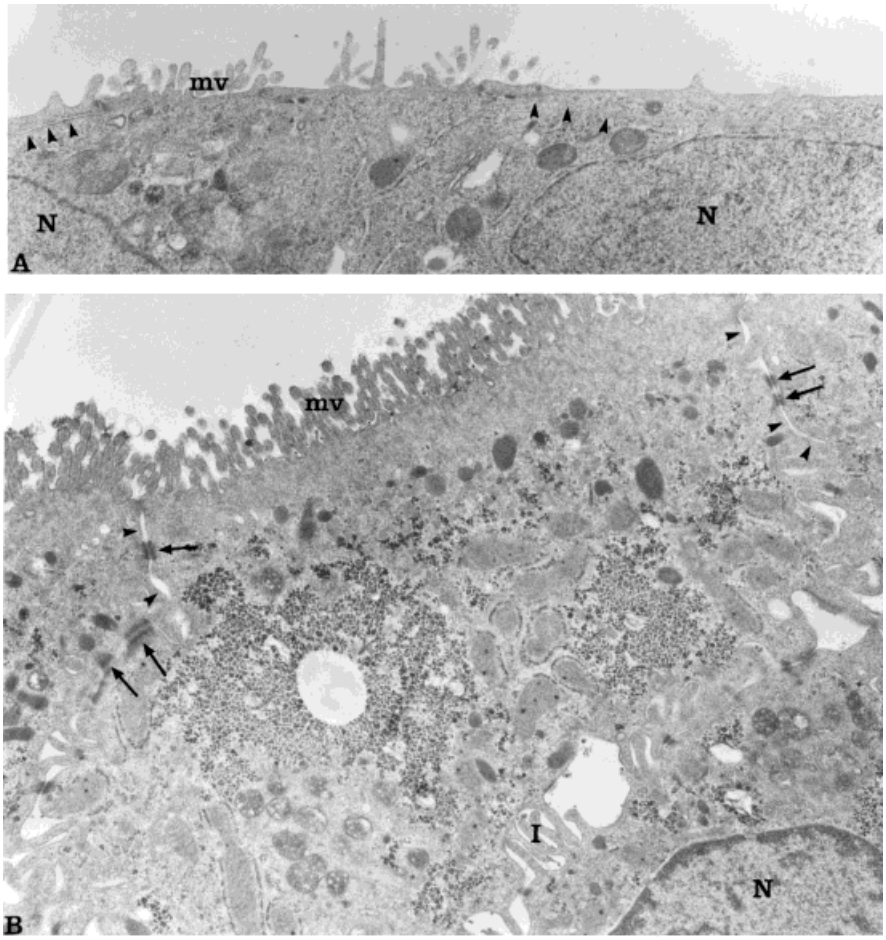

Fig. 1. Morphological aspect of Caco-2 cells at two different stages. Cells were grown on plastic supports for 7d **(A)** and 21d **(B)**. (A) The cells are rather elongated and flat. The nuclei (N) are very close to the cell surface. Microvilli (mv) are few in number and of variable sizes. The intercellular space (arrowheads) runs parallel to the cell surface, while the intercellular junctions are few and small in size. × 15,000. (B) The cells are highly polarized with the nuclei (N) at the base. Microvilli (mv) are numerous, well-developed, and of uniform size. The intercellular spaces (arrowheads) run perpendicular to the surface of the cells and the intercellular junctions (arrows) are well developed. The lateral membrane is characterized by numerous interdigitations (I). Many mitochondria are present in the cell cytoplasm. × 16,000.

DISCUSSION

Two distinct types of FABP are expressed in the enterocyte, namely I-FABP and L-FABP. These cytosolic proteins, which are predominantly 29% homologous, are encoded by two different genes [Sweetser et al., 1987]. In humans, the gene for I-FABP is located in the q28–q31 region of chromosome 4, whereas that of L-FABP is located in the q29–q31 region of chromosome 2. While the binding properties of these proteins have been defined [Lowe et al., 1987; Cistola et al., 1989], their cellular functions remain unknown and it is not understood why the absorptive epithelial cells host two distinct FABP moieties. In an ongoing effort to increase our understanding of the regulatory I- and L-FABP background of the human gastrointestinal tract, we have utilized the Caco-2 cell line that represents a useful model of intestinal epithelial cell lipid transport and lipoprotein metabolism [Levy et al., 1995]. Our results clearly demonstrated that the incubation of Caco-2 cells under different conditions and with various compounds known to modulate lipoprotein assembly and secretion led to a differential regulation of I- and L-FABP: (1) L-FABP was progressively expressed during the cell maturation process, whereas I-FABP was detected only in fully differentiated cells; (2) the considerably elevated L-FABP/I-FABP ratio was due to a 50-fold differentiation-induced increase in the cell content of L-FABP; (3) the supplementation of Caco-2 cells with lipids (butyric acid, oleic acid,

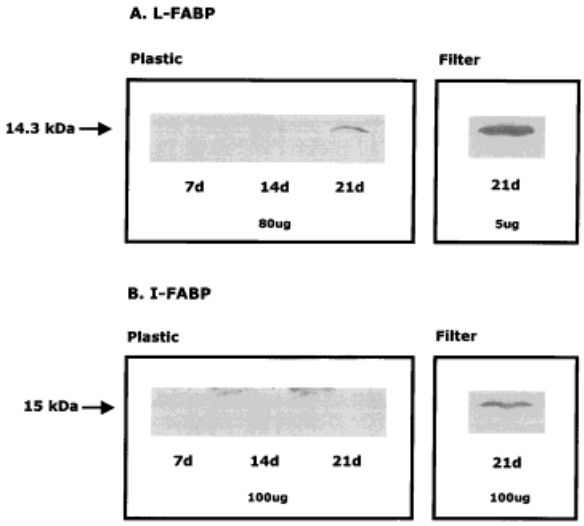

Fig. 2. The effect of the maturation process on I- and L-FABP protein content in Caco-2 cells. Caco-2 cells were grown until 70–90% confluence in 175 cm^2 flasks. Thereafter, there were transferred to plastic support (A) or polycarbonate Transwell filter inserts, and I- and L-FABP content were determined at different postconfluent periods by fractionation on SDS-PAGE, Western blot analysis and chemiluminescence detection.

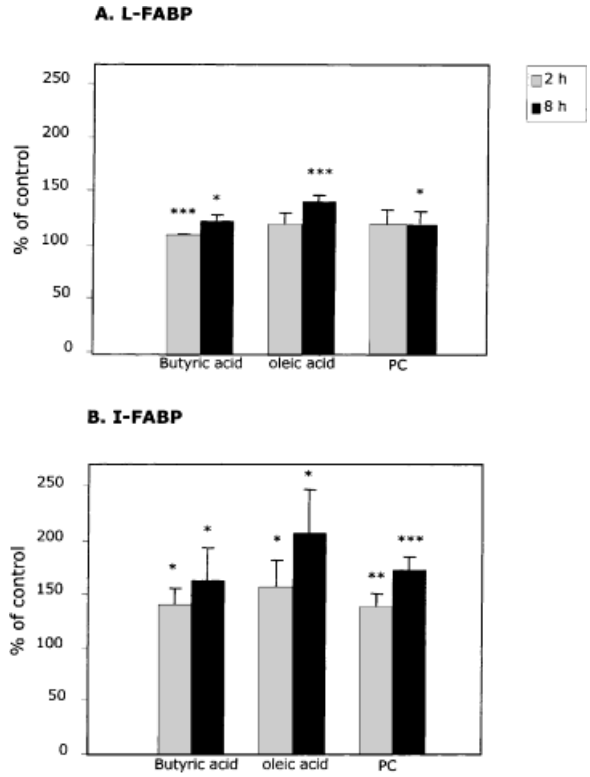

Fig. 3. Fatty acid regulation of I- and L-FABP proteins. Caco-2 cells were cultured for 2 and 8 h with oleic acid (0.5 mM), phosphatidylcholine (0.8 mM), and butyric acid (4 mM). Following the incubation periods, cells were lysed and cytosolic proteins were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, reacted with anti-human I- or L-FABP antiserum, and revealed by chemiluminescence detection. Data represented are mean±standard error (SE) of n=4 experiments and are expressed as % of control. *$P<0.04$, **$P<0.02$, ***$P<0.009$.

PC) and hormones (hydrocortisone and insulin) preferentially upregulated the protein levels of I-FABP; (4) leptin and TNF-α increased the cellular protein amount of I-FABP without affecting L-FABP; and (5) the exposure of Caco-2 cells of IL-6 resulted in a similar, modest induction of I- and L-FABP.

Based on our in vitro experiments, it seems that the two forms of FABP did not undergo a parallel and similar control by various effectors. This divergent pattern is in line with the pharmacological profile described by other investigators. Bass and coworkers [1985a, 1985b] reported a two-fold increase in L-FABP protein, but only a 25% increase in I-FABP protein concentration in the jejunum of rats administered clofibric acid. The stimulation gradually decreased along the cephalocaudal axis of the gut, and this was more rapid for I-FABP than for L-FABP. Furthermore, studies of intracellular protein turnover combined with cDNA hybridization analysis of steady-state mRNA abundance documented differences in cytosolic protein levels of I- and L-FABP depending on the gender [Bass et al., 1985a, 1985b]. Along these lines, a number of interest-

TABLE I. Differentiation Characteristics of Caco-2 Cells Grown on Plastic Supports and Polycarbonate Filters

	Plastic	Filter
Sucrase (IU/g protein)	12.5±3.1	18.4±2.7*
Alkaline phosphatase (IU/g protein)	20.4±4.2	29.8±4.7*
Transepithelial resistance (ohm×cm^2)	1630±73	2410±115*

Caco-2 cells were grown on plastic support and permeable polycarbonate filters as described in Methods. Enzyme activities and transepithelial resistance were measured at 21 days after confluence.
*Versus plastic, $P<0.05$ for n=5.

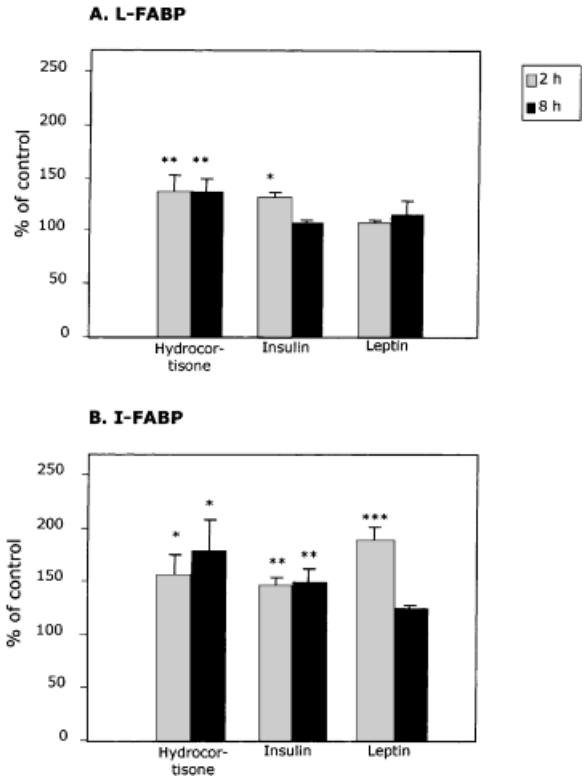

Fig. 4. Hormonal regulation of I- and L-FABP proteins. Caco-2 cells were cultured for 2 and 8 h with insulin (3 mM), hydrocortisone (50 ng/ml) and leptin (50 n M). The Caco-2 cell content of I-and L-FABP was analyzed as described in the legend of Figure 3. Data represented are mean ± standard error of n=4 experiments and are expressed as % of control. *$P<0.04$, **$P<0.02$, ***$P<0.006$.

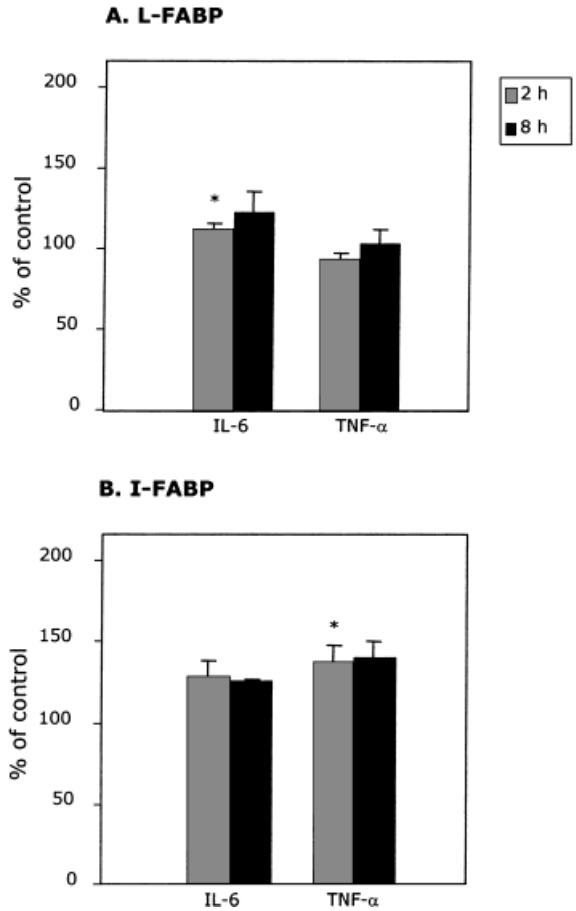

Fig. 5. Cytokine regulation of I- and L-FABP proteins. Caco-2 cells were cultured for 2 and 8 h with tumor necrosis factor (TNF-α, 100 μg/ml) and interleukin 6 (IL-6, 10 ng/ml). The cellular content of I- and L-FABP was analyzed as described in the legend of Figure 3. Data are mean (standard error of n=4 experiments and are expressed as % of content. *$P<0.03$.

ing physical differences have been found between I- and L-FABP. ^{13}C-NMR and fluorescence spectroscopic analyses have indicated that the FA carboxylate group may lie close to the surface of L-FABP, whereas it is buried in the interior of the I-FABP binding pocket [Lowe et al., 1987; Cistola et al., 1989; Storch et al., 1989]. It has also been shown that I-FABP is less stable than L-FABP to thermal denaturation [Muga et al., 1993; Hsu and Storch, 1996]. In addition, its FA transfer is modulated by acceptor membrane properties, while the transfer from L-FABP is largely independent of membrane characteristics [Muga et al., 1993; Hsu and Storch, 1996]. Overall, these findings show divergent regulation and distinct physicochemical characteristics and they backup the premise that the two FABPs play separate roles in lipid binding and intracellular lipid metabolism.

Intestinal lipoprotein production is markedly influenced by the nutritional state. In vivo studies have shown a proportional increase in triglyceride-rich lipoproteins with the amount of fat in the diet [Windmueller and Levy, 1968]. Many investigators have also demonstrated a marked elevation in apo accumulation in Caco-2 cell culture in the presence of oleate [Dashti et al., 1990; Field et al., 1988; Stan et al., 1999]. In particular, oleic acid and PC increased the synthesis of the essential apo B component of triglyceride-rich lipoproteins by protecting nascent apo B from intracellular degradation [Mathur et al., 1996]. Alternatively, if core lipids are limiting, as is the case in the fasting state, apo B-100 rapidly degrades and the assembly of apo B-containing lipoproteins does not take place [Traber et al., 1987]. Thus, the extent of the current upregulation of FABP in response to oleic acid and PC may have a direct

impact on apo-lipid assembly and export. Since a PPAR-responsive element has been reported for the L-FABP promotor, but not for I-FABP [Veerkamp and Maatman, 1995], the observed different modulation of the two forms of FABP points to different mechanisms of action. Besides, FABP does not bind short chain fatty acids. We therefore surmise that the induction exerted by butyric acid is due to its known potential effects on post-translational modifications [Toscani et al., 1988; Veerkamp and Maatman, 1995; Bernlohr et al., 1997].

Although emerging information suggests that hormones may play distinct roles in lipid binding and intracellular lipid metabolism, their direct effect on the regulation of intestinal lipid transport by FABP was only given a cursory examination. The present investigation showed that insulin and hydrocortisone enhanced both I- and L-FABP in Caco-2 cells. Besides, the addition of these hormones to the organ culture of the human fetal intestine was shown to inhibit apo B synthesis and reduce TG-rich lipoprotein production [Loirdighi et al., 1992, 1997; Levy et al., 1996]. Studies in rat hepatocytes also indicated significant degradation of apo B mediated by insulin [Borchardt and Davis, 1987; Dixon et al., 1991]. Similarly, a decrease in apo B synthesis was observed in insulin-treated HepG2 lysates due to a lower translational efficiency of apo B mRNA [Adeli and Theriault, 1992]. It seems, therefore, that there is no coordinated regulation of FABPs and apo B-containing lipoproteins. However, apo B findings may not be directly applicable to Caco-2 cells, since the modulation of apo B biogenesis by insulin may depend on both the stage of development and the species studied [Black and Ellinas, 1992].

Cytokines, important mediators of inflammation, have been shown to disturb intestinal lipid handling [Mehran et al., 1995]. TNF-α and IL-6 markedly affected the synthesis and secretion of apo B-containing lipoproteins [Mehran et al., 1995; Murthy et al., 1996]. These cytokines stimulated I- and L-FABP in the current experiments, whereas they had decreased the rate of synthesis and secretion of triglycerides and apo B in previous studies [Mehran et al., 1995]. Therefore, the lack of coordination between FABP and the assembly of lipoproteins was also revealed by the utilization of TNF-α and IL-6.

Recently, intestinal epithelium has been shown to be a direct target of leptin action mediated through the OB-Rb receptor and a STAT5 mechanism [Morton et al., 1998]. Our data also established that leptin supplementation to Caco-2 cells enhanced I-FABP levels. The mechanisms of leptin-mediated increase needs to be defined.

In conclusion, our data showed that many effectors, including lipids, cytokines, and hormones, can modulate the cellular amount of cytosolic FABPs. In addition, our results also reveal that leptin and TNF-α have a greater modulatory effect on I-FABP than L-FABP expression.

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