

Effect of human recombinant leptin on lipid handling by fully differentiated Caco-2 cells

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Abstract It has been established that leptin displays a number of effects on peripheral tissues. We have investigated the effect of the hormone on lipid synthesis, apolipoprotein biogenesis and lipoprotein secretion in Caco-2 cells. Immunocytochemistry revealed the presence of leptin receptors (Ob-Rb) on the basolateral membrane. Incubation of cells with 200 nM leptin resulted in a decreased export of triglycerides in the basolateral medium without affecting monoglyceride, diglyceride and cholesterol ester lipid classes. It also significantly reduced the output of de novo-synthesized apolipoprotein (Apo)B-100 and ApoB-48 as well as that of newly formed chylomicrons and of low-density lipoproteins. It also enhanced that of ApoA-I, ApoA-IV and ApoE. Our results support the hypothesis that leptin can affect energy balance at the gut level by reducing lipid release into the circulation. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Leptin; Leptin receptor; Lipid; Lipoprotein; Immunohistochemistry; Protein A-gold

1. Introduction

The lipid content of the diet has considerably increased over the past century and has contributed to the epidemics of obesity in Western countries, as most clearly illustrated in populations that have experienced a sudden change in dietary habits, such as Pima Indians, and the Australian Aborigines [1,2]. Due to the high-caloric value of fat nutrients and the relative efficiency of intestinal handling, fat absorption and post-prandial lipemia have become a major focus of obesity research.

It is generally accepted that leptin, a peptide hormone discovered in 1994 by Zhang et al. [3], controls appetite and increases energy expenditure at the hypothalamic level. In humans, its concentrations generally correlate positively with obesity [4–6]. Its importance in human physiology has further been exemplified in patients with multiple endocrine defects and immune dysfunction in whom a missense mutation of the

leptin gene caused its deficiency [7]. However, in the vast majority of obese human subjects elevated leptin concentrations are observed, indicating a leptin-resistant state rather than leptin deficiency [8,9].

In recent years, it has clearly been established that leptin, besides its central role at the hypothalamic level, displays a number of effects on a variety of cells and peripheral tissues. For example, leptin inhibits basal insulin secretion and glucose-stimulated insulin secretion in normal and ob/ob mice [10]. Furthermore, leptin shows a diuretic action in hydrated rodents, probably through specific receptors localized in the internal kidney medulla [11]. On the other hand, its acute administration has been reported to decrease white adipose tissue lipogenesis and increase fat mobilization [12]. Furthermore, although acute treatment of ob/ob mice with recombinant leptin rapidly caused a stimulation of liver long-chain fatty acid synthesis, chronic treatment diminished the de novo fatty acid synthesis [13], thereby impacting on lipoprotein synthesis. Accordingly, other *in vivo* experiments using ob/ob mice have shown impaired liver high-density lipoprotein (HDL)–apolipoprotein which can be reversed by low-dose leptin treatment [14]. Similarly, disturbances occur in ob/ob mouse hepatocytes HDL–lipoprotein binding, degradation and secretion processing which can be corrected by leptin [15].

Closer to the present report, it is interesting to note that the baseline leptin level could be predicted from body mass index and circulating apolipoprotein A-I (ApoA-I) concentration in obese children [16]. Surprisingly, the influence of leptin on the elaboration of apolipoproteins and of HDL by the intestine, an organ contributing 50% of total circulating lipids and lipoproteins, has not been investigated. Morton et al. [17] have reported the presence of functional leptin receptors in the mouse jejunum and in differentiated Caco-2 cells. They have also shown that intravenous leptin administration, 15 min before a fat load, paradoxically reduced jejunal ApoA-IV transcript levels. Aside from these data, and the recent report of Doi et al. [18] who also reported that the simultaneous intravenous administration of leptin and duodenal lipid load attenuated the increase of cellular ApoA-IV content, little information is available, to our knowledge, on the role of leptin in intestinal lipid synthesis, apolipoprotein biogenesis and lipoprotein secretion.

In the present report we have investigated the effect of

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human recombinant leptin (hrLeptin) on lipid handling in fully differentiated Caco-2 cells.

2. Materials and methods

2.1. Cell culture

Caco-2 cells obtained from the ATCC (Bethesda, MD, USA) were grown at 37°C with 5% CO₂ in minimum essential medium (MEM; Gibco-BRL, Grand Island, NY, USA) in 175 cm² plastic flasks (Corning, NY, USA) as described in detail previously [19,20]. The medium contained 1% penicillin/streptomycin, 1% MEM non-essential amino acids (Gibco-BRL) and was supplemented with 10% decomplemented fetal bovine serum (FBS; Flow McLean, VA, USA). Cultures (passages 30–40) were split (1:3–1:6) upon reaching 70–90% confluency, using 0.05% trypsin–EDTA (0.5 mM; Gibco-BRL).

For individual experiments, cells were plated at a density of 1×10^6 cells/well on 24.5-mm polycarbonate Transwell filter inserts with 0.4- μ m pores (Costar, Cambridge, MA, USA), in MEM (as above) supplemented with 5% FBS. The inserts were placed into six-well culture plates, permitting separate access to the upper (apical) and lower (basolateral) compartments of the monolayers. Cells were cultured for 20 days, at which time Caco-2 cells were highly differentiated [21]. For a more efficient induction of lipid, lipoprotein and apolipoprotein synthesis, cells were incubated in presence of unlabeled oleic acid, bound to albumin, in a serum-free medium, for 20 h prior to the experiments. Transepithelial resistance, an index of confluence and tight junction integrity, was determined using a Millicel-ERS apparatus (Millipore, Bedford, MA, USA) [22].

2.2. Effect of leptin on the *de novo* lipid synthesis

Radiolabeled [¹⁴C]oleic acid (Amersham, Oakville, ON, Canada) was added to unlabeled oleic acid and solubilized in fatty acid free-bovine serum albumin [23,24]. The final oleic acid concentration was 1 μ mol/well with 0.45 μ Ci of [¹⁴C]oleic acid attached to albumin. 200 nM hrLeptin in 5% FBS-supplemented MEM (R&D Systems Inc., Minneapolis, MN, USA), concentration at which maximal effects were observed by Morton et al. [17] in the same model, was then added to the basolateral compartment. Cells were incubated for a period of 8 h at 37°C, in the presence of [¹⁴C]oleic acid-containing medium added to the apical compartment. At the end of the incubation period, the basolateral medium was collected and the cells were

scraped in cell lysis buffer as described previously [20]. After the addition of anti-proteases (phenylmethylsulfonyl fluoride, pepstatin, leupeptin and trasylol), the lipids contained in the cell lysates and medium were extracted by standard methods [23–25] in presence of unlabeled carrier phospholipids, monoglycerides (MGs), diglycerides (DGs), triglycerides (TGs) free fatty acids (FFAs) and cholesteryl ester (CE).

The various lipid classes were separated by thin-layer chromatography (TLC) using a ternary solvent system consisting of hexane/ether/acetic acid (80:20:3, v/v) as previously described [26]. The area corresponding to each lipid standard, run simultaneously, was scraped off the TLC plates, placed in a vial with Ready Safe[®] counting fluid (Beckman, Montreal, QC, Canada) and counted on a scintillation counter (Beckman LS 5000 TD) to determine its radioactivity content. Proteins were measured by the method of Lowry et al. [27] using bovine serum albumin as standard. Results were reported as disintegration per minute/mg protein.

2.3. Effect of leptin on the *de novo* apolipoprotein synthesis

As previously mentioned, cells were incubated in presence of non-labeled oleic acid (1 μ M) attached to albumin to stimulate apolipoprotein synthesis. After 20 h, cells were carefully washed with methionine-free MEM and incubated for 3 h in the presence of [³⁵S]methionine (150 μ Ci/well, 50 mCi/mmol; Amersham, Oakville, ON, Canada). At the end of the labeling period, the medium of the basolateral compartment was collected and cells scraped in a lysis buffer. Aliquots of cell homogenates and basolateral medium were precipitated with 5% trichloroacetic acid (TCA). The precipitates were washed (three times) with 5% TCA before counting the radioactivity on a Beckman liquid scintillation spectrometer. Other centrifuged aliquots were reacted with excess monoclonal antibodies directed against ApoA-I, Apo-IV, ApoB and ApoE for 18 h at 4°C. Following the addition of Pansorbin (Calbiochem, San Diego, CA, USA), the mixture was incubated at 20°C for 60 min and the immunoprecipitate extensively washed before being submitted to gradient (4–15%) polyacrylamide electrophoresis as already described [28–30]. Gels were sectioned into 4-mm slices and counted after overnight incubation at 20°C with 1 ml of BTS-450 (Beckman) and 10 ml of Ready Solve[®] liquid scintillation fluid.

2.4. Effect of leptin on the *de novo* lipoprotein synthesis

For the determination of the newly synthesized and secreted lipo-

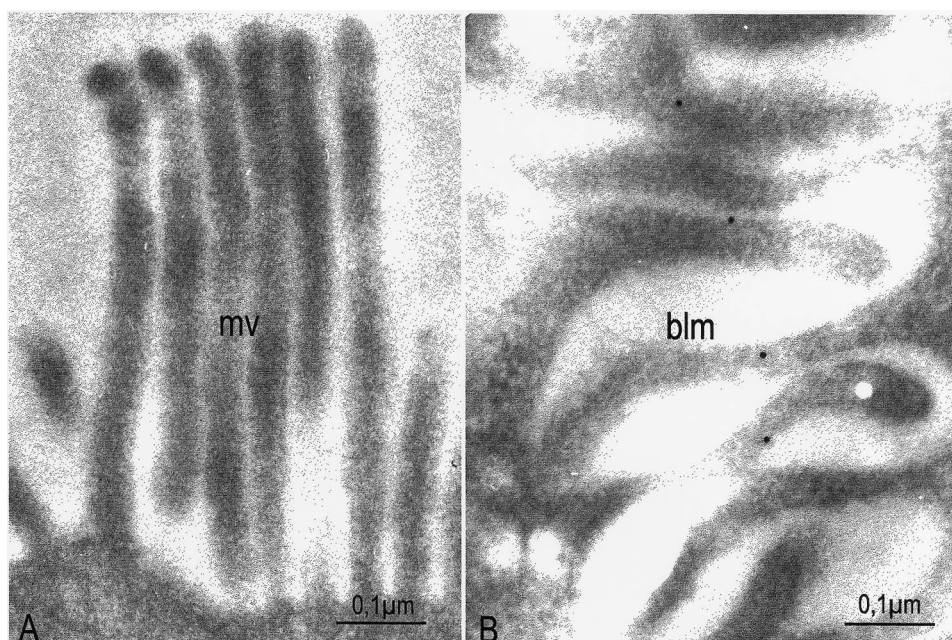


Fig. 1. Protein A–gold labeling of differentiated Caco-2 cells for the specific functional leptin receptor Ob-Rb. Confluent cells were fixed in situ with 1% glutaraldehyde, processed for embedding in Lowicryl at –20°C and processed by the immunogold to reveal the Ob-Rb receptor. The labeling by colloidal gold particles is present along the basolateral membrane (blm) of the Caco-2 cells, while the apical membrane lining the microvilli (mv) is devoid of labeling.

Table 1
Density of labeling for Ob-Rb in plasma membranes of confluent and differentiated Caco-2 cells

| Caco-2 cell plasma membrane domains | Anti-Ob-Rb/protein A–gold | Protein A–gold |
|-------------------------------------|---------------------------|----------------|
| Apical membrane | 0.06 ± 0.02 | 0.04 ± 0.01 |
| Basolateral membrane | 0.60 ± 0.10* | 0.05 ± 0.01 |

Confluent cells were fixed in situ with 1% glutaraldehyde and processed for embedding in Lowicryl at -20°C . Thin sections were mounted on Parlodion and carbon-coated nickel grids and processed for immunocytochemistry using the immunogold approach as described in Section 2. For the quantitative evaluations, the plasma membrane profiles of 25 cells were recorded and printed to a final magnification of $\times 50\,000$. The density of labeling present over the apical and basolateral membranes was evaluated by measuring the length of the membranes and the number of particles being aligned on such membranes. The results are expressed as the number of gold particles per length (μm) of membrane. The significance of the difference in gold particle density between the basolateral and apical membranes was assessed by the Student *t*-test for unpaired variants. * $P < 0.01$.

proteins, cells were incubated for a prolonged period of 20 h in presence of 200 nM hrLeptin and 1.0 μmol /well of unlabeled oleic acid containing 0.9 μCi of [^{14}C]oleic acid so as to detect an appreciable amount of lipoproteins secreted into the basolateral medium. At the end of the incubation period, the basolateral medium was collected and supplemented with protease inhibitors and a plasma carrier in the ratio (2.0/0.6, v/v) [31]. Lipoproteins were then isolated by sequential ultracentrifugation using a TL-100 Beckman ultracentrifuge as per our described method [23]. Each lipoprotein fraction was exhaustively dialyzed against 0.15 M NaCl, 0.001 M EDTA, pH 7.0, at 4°C for 245 h before being counted.

2.5. Immunocytochemistry

The antibody against the leptin receptor (Ob-Rb) was purchased from R&D Systems Inc. This rabbit polyclonal IgG [Ob-Rb (H-300)] is directed against the epitope corresponding to amino acids 541–840 mapping within a cytoplasmic domain of the human Ob-Rb [32]. Confluent cells were fixed in situ with 1% glutaraldehyde and processed for embedding in Lowicryl at -20°C as described previously [33]. Thin sections were mounted on Parlodion and carbon-coated nickel grids and processed for immunocytochemistry using the immunogold approach [33]. In short, the sections were incubated overnight at 4°C with the anti-Ob-Rb antibody diluted 1:20. The sections were then rinsed with 0.01 M phosphate-buffered saline and incubated with the protein A–gold complex for 1 h at room temperature. The protein A–gold complex was prepared with 10 nm gold particles according to the previously reported protocols [33]. Control experiments were performed to assess the specificity of the labeling. The sections were incubated with a normal serum followed by protein A–gold or with protein A–gold alone, omitting the first antibody. For the quantitative evaluations, the plasma membrane profiles of 25 cells were recorded and printed to a final magnification of $\times 50\,000$. The density of labeling present over the apical and basolateral membranes was evaluated by measuring the length of the membranes and the number of particles being aligned on such membranes [33]. The results are expressed as the number of gold particles per length (μm).

2.6. Statistical analysis

Conventional methods were used for calculation of means and standard error. Statistical significance for differences in variables between control and hrLeptin-treated cells was assessed with the Student's *t*-test.

Table 2
Effect of hrLeptin on lipid synthesis by confluent and differentiated Caco-2 cells

| | Compartment | | | | |
|-----------|---------------|-----------|----------------|------------|----------------|
| | MG | DG | TG | CE | Total |
| Cells | | | | | |
| C | 61 658 ± 2213 | 1861 ± 99 | 186 793 ± 6340 | 2072 ± 171 | 253 193 ± 4267 |
| T | 62 922 ± 1484 | 1899 ± 67 | 194 297 ± 6597 | 2078 ± 120 | 261 938 ± 6953 |
| % Control | 102 ± 2.4 | 103 ± 5 | 103 ± 2.5 | 100 ± 3.2 | 103 ± 2.3 |
| BL medium | | | | | |
| C | 936 ± 158 | 161 ± 24 | 10 645 ± 1330 | 164 ± 37 | 12 828 ± 1806 |
| T | 791 ± 102 | 141 ± 12 | 8045 ± 787* | 127 ± 21 | 10 305 ± 1153 |
| % Control | 86 ± 8.1 | 90 ± 7.8 | 68 ± 6.8* | 85 ± 7.9 | 82 ± 8.1 |

Cells were incubated with 200 nM hrLeptin for 8 h and lipids were analyzed as described in Section 2. Cells: intracellular content; BL medium: basolateral medium content; C: control cells ($n = 3$); T: hrLeptin-treated cells ($n = 6$). Values are expressed as dpm/mg protein and as means ± S.E.M. The significance of the difference between the treated and control cells was assessed by Student's *t*-test. * $P < 0.01$.

3. Results

In order to reveal the distribution of leptin receptors, sections of Caco-2 cells were exposed to the anti-Ob-Rb antibody followed by the protein A–gold complex. The gold particles were particularly present at the level of the basolateral membrane (Fig. 1A), while only few particles localized over the apical membrane (Fig. 1B). Furthermore, specific labeling appeared to be absent, with only few particles distributed in the cytoplasm, mitochondria and nuclei. Quantitative evaluations confirmed the presence of a specific higher labeling at the basolateral membrane (Table 1). The absence of labeling with protein A–gold in the absence of the primary antibody confirmed the specificity of our immunocytochemical method (Table 1).

As shown in Table 2, incubation of fully differentiated Caco-2 cells with 200 nM hrLeptin on the basolateral side for 8 h did not affect cell content of any of the de novo-synthesized lipid fractions. It resulted, however, in a decreased export of TG ($68 \pm 15\%$, $P < 0.01$) in the basolateral medium, compared to controls, without affecting MG, DG and CE lipid classes.

Fig. 2A,B illustrates the effects of hrLeptin on the de novo synthesis of apolipoproteins assessed after a 3-h incubation with [^{35}S]methionine as a tracer. Under these conditions, hrLeptin significantly reduced the cell content of de novo-synthesized ApoB-100 and ApoB-48 by 32% ($P < 0.05$) and 22% ($P < 0.01$), respectively (Fig. 2A). Similarly, the presence of the hormone diminished the output of ApoB-100 and ApoB-48 by 33% ($P < 0.01$) and 40% ($P < 0.05$), respectively, in the basolateral medium (Fig. 2B). Besides, ApoA-I, ApoA-IV and ApoE de novo synthesis and secretion were substantially enhanced. Of particular note was the induction of ApoA-IV (400%, $P < 0.01$).

Lipoprotein production was assessed by following the

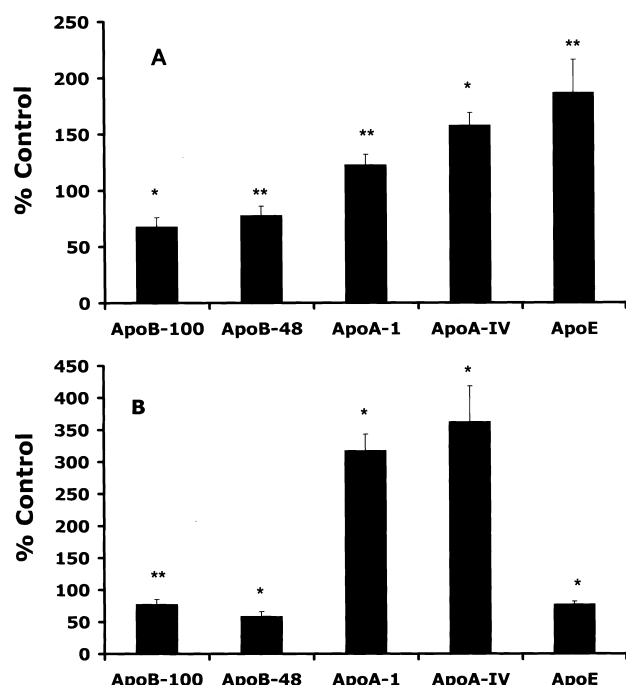


Fig. 2. Effect of hrLeptin on cellular content (A) and on basolateral secretion (B) of de novo-synthesized apolipoproteins in fully differentiated Caco-2 cells. Cells were incubated with 200 nM hrLeptin for 3 h in presence of [35 S]methionine and apolipoproteins were analyzed as described in Section 2. The results are the means \pm S.E.M. of six control and 6–10 leptin-treated cell incubations. Mean values for the control experiments, expressed as dpm/mg proteins for each apolipoprotein in the cell compartment were: ApoB-100: 16596; ApoB-48: 9847; ApoA-I: 3843; ApoA-IV: 16078 and ApoE: 7209. In the basolateral medium, the values were: ApoB-100: 19125; ApoB-48: 9748; ApoA-I: 335; ApoA-IV: 437 and ApoE: 838. The significance of the difference between the treated and control cells was assessed by Student's *t*-test. * $P < 0.01$; ** $P < 0.05$.

[14 C]oleic acid incorporation into the different density fractions collected by ultracentrifugation: 0.95 (chylomicrons, CM), 1.006 (very low-density lipoproteins, VLDL), 1.063 (low-density lipoproteins, LDL) and 1.21 g/ml (HDL). As shown in Table 3, hrLeptin inhibited the secretion of CM ($89 \pm 9\%$, $P < 0.03$) and of LDL (78 ± 18 , $P < 0.05$). VLDL and HDL remained unaffected.

Of interest, when cells were incubated in presence of rhLeptin on the apical side, at the same concentration, no effects were observed on lipid esterification, apolipoprotein biogenesis or lipoprotein assembly (data not shown).

4. Discussion

Leptin, a cytokine-like peptide known to regulate food intake at the hypothalamus level [34], exerts its effect through

the activation of the Janus kinase signal transducer and activator of transcription (STAT) signaling cascade [17,35]. Although Ob-Rb distribution was originally described in the hypothalamus, its expression has subsequently been observed in peripheral tissues as diverse as pancreatic islets [10], hematopoietic stem cells and, more recently, in the stomach [36,37]. Morton et al. [17] have shown, by RT-PCR, that Ob-Rb was expressed in Caco-2 cells and that leptin, at concentrations similar to ours, caused the induction of immediate-early genes and activation of STAT3 and STAT5. They did not, however, address its specific function in intestinal lipid metabolism.

In the present report, using established immunohistochemical methods, we demonstrate that the leptin receptor (Ob-Rb) is specifically located on the basolateral membrane of fully differentiated Caco-2 cells (Fig. 1A and Table 1). We also show that leptin inhibits the TG secretion (Table 2), the biosynthesis of ApoB-100 and ApoB-48 (Fig. 2A) as well as the output of CM and LDL (Table 3). Although the concentration of leptin used is high, it may be that the concentration in the receptor microenvironment is much less due either to a low diffusion of the peptide, its degradation with time or adsorption on the plastic dish. However, the fact that the effects are observed only when the cytokine is present in the basolateral medium militates in favor of a specific action. Furthermore, other studies performed in our laboratory revealed that neither of the two other cytokines, transforming growth factor- β or interferon- γ , affected lipid metabolism (unpublished results).

Intestinal epithelial cells have the unique ability to elaborate CMs, the major vehicle for the transport of dietary fat [38]. In our investigation we document the influence of leptin on the sequential events leading to the lipid output into the basolateral compartment. We show that the administration of the hormone, at the same concentration (200 nM) as that used by Morton et al. [17], results in the decrease of TG secretion without affecting cell content, pointing to a lack of hormonal effect on the esterification process. The concomitant diminution of CM and LDL delivery to the basolateral compartment indicates, on its part, that the lipoprotein assembly process is the target of leptin action. This hypothesis is supported by the reduction of de novo-synthesized ApoB cell content and presence in the basolateral medium. These data, in turn, hint to ApoB being the limiting factor for CM delivery. Various reports have indeed demonstrated that the post-translational translocation of the apolipoprotein across the endoplasmic reticulum (ER) membrane is the rate-limiting step in ApoB-containing lipoprotein formation [21,39].

The ER MTP/PDI heterodimer is crucial for the lipidation and protection of ApoB from proteolysis as well as for the enrichment of lipoprotein particles with TGs [40]. In view of our data documenting a lack of effect of hrLeptin on MTP

Table 3
Effect of hrLeptin on lipoprotein secretion in the basolateral medium by confluent and differentiated Caco-2 cells

| | CM | VLDL | LDL | HDL |
|-----------|--------------------|---------------------|------------------|-----------------|
| C | 40 595 \pm 6053 | 51 043 \pm 14 398 | 9230 \pm 1996 | 4952 \pm 1031 |
| T | 33 236 \pm 3590* | 44 305 \pm 7511 | 7064 \pm 1086* | 5440 \pm 579 |
| % Control | 89 \pm 3.9* | 114.5 \pm 5.7 | 78 \pm 7.4* | 99 \pm 3.7 |

Cells were incubated with 200 nM hrLeptin for 20 h and lipoproteins were analyzed as described in Section 2. C: Control cells ($n = 3$); T: hrLeptin-treated cells ($n = 6$). Values are expressed as dpm/mg protein and as means \pm S.E.M. The significance of the difference between the treated and control cells was assessed by Student's *t*-test. * $P < 0.05$.

activity (data not shown), we suggest that this protein complex is not implicated in the modulation of lipoprotein assembly and exocytosis. These results are similar to those of Macri et al. [41] who have recently shown, in HepG2 cells, that inhibition of MTP did not affect the translocation of ApoB, but facilitated its proteasome-mediated degradation, thereby impacting on the assembly and secretion of ApoB particles. Therefore, additional investigation is required to understand the mechanisms by which leptin affects lipoprotein metabolism in Caco-2 cells.

Data on the direct effect of leptin on enterocyte apolipoprotein synthesis are scarce. Two reports address the effect of leptin on enterocyte apolipoprotein synthesis in *in vivo* models. In the first one [17], the authors have shown that an intravenous injection of leptin, 90 min after a fat load, caused a two-fold reduction in ApoA-IV transcript levels in the jejunum of leptin-deficient mice (ob/ob). In the second, Doi et al. [18] have recently reported a rapid (4 h) increase in ApoA-IV immunoreactivity in rat jejunum and ileum following an intraduodenal lipid infusion. This stimulatory effect was, however, paradoxically attenuated by a simultaneous leptin infusion. We show that, in Caco-2 cells fully differentiated as enterocytes, ApoA-I, ApoA-IV and ApoE biosynthesis and secretion were enhanced by leptin (Fig. 2). In terms of ApoA-IV, the variance of our results with the aforementioned reports could be due to the model used and the different experimental conditions.

Our results indicate that leptin has a role in the absorptive and metabolic functions of the enterocyte and could possibly play an early role in limiting fat absorption. It could be conceived that disruption of the leptin signaling pathway is acquired in the obese state, leading to an impaired control of intestinal lipid synthesis and export. This 'resistance' state could then possibly lead to a more important and prolonged post-prandial lipid load. Our results also support the hypothesis that leptin can affect energy balance through mechanisms prior to and possibly independent of those described at the hypothalamic level.

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