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# Insulin Decreases the Secretion of apoB-100 from Hepatic HepG2 Cells but Does Not Decrease the Secretion of apoB-48 from Intestinal CaCo-2 Cells

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**Key Words**

Insulin · Apolipoprotein B-48 · Apolipoprotein B-100 · Chylomicron · Very low-density lipoprotein · CaCo-2 · HepG2 · Cholesterol · Kinase

**Abstract**

We compared the acute effect of insulin on the human colonic intestinal epithelial cell line CaCo-2 and the transformed human hepatic cell line HepG2. Over 24 h, 100 n*M* and 10 μ*M* insulin significantly inhibited the secretion of apolipoprotein (apo) B-100 from HepG2 cells to 63 and 49% of control, respectively. Insulin had no effect on the secretion of apoB-48 from CaCo-2 cells. There was no effect of insulin on the cholesterol ester or free cholesterol concentrations in HepG2 or CaCo-2 cells. HepG2 and CaCo-2 cells bound insulin with high affinity, leading to similar stimulation of insulin receptor protein tyrosine kinase activation. Protein kinase C or mitogen-activated protein kinase activity in the presence or absence of insulin was not correlated with apoB-48 production in CaCo-2 cells. Therefore, insulin acutely decreases the secretion of apoB-100 in hepatic HepG2 cells, but does not acutely modulate the production or secretion of apoB-48 from CaCo-2 intestinal cells.



## Introduction

Recent studies in our laboratory have found that viscerally obese men have raised concentrations of chylomicrons and chylomicron remnants in the circulation [19, 25], and several studies have shown that subjects with disturbances in chylomicron metabolism are prone to premature atherosclerosis [21, 30, 38, 48]. The dyslipidemia and hyperlipoproteinemia observed in viscerally obese subjects may be related to several metabolic factors including insulin resistance [11, 36]. Since insulin acutely inhibits very low-density lipoprotein (VLDL) synthesis and secretion in hepatic cell models [first shown in ref. 9 and reviewed in ref. 42], it has been suggested that insulin resistance leads to the overproduction of hepatically derived VLDL in viscerally obese humans [14]. Therefore, the increased concentration of chylomicrons in viscerally obese subjects may partly be a consequence of exaggerated biogenesis of chylomicrons by the intestine due to insulin resistance.

The assembly and secretion of triglyceride-rich lipoproteins is dependent on the availability of apolipoprotein (apo) B as a lipid chaperone. ApoB-48 and apoB-100 are necessary for the correct assembly and subsequent secretion of chylomicrons and VLDL, respectively, and

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there is one apoB molecule present per lipoprotein [49]. Insulin acutely modulates the production of VLDL in the liver via a direct effect on apoB-100 that causes increased degradation of the protein [35, 40]. The assembly of chylomicrons in the intestine occurs via similar processes to that in the liver; however, the factors regulating apoB-48 synthesis and secretion are less well understood. Insulin has been shown to affect the secretion of fatty acids from human fetal intestinal explants [32], and a later study showed a decrease in apoB-48 secretion in the same model [23]. Furthermore, insulin has been found to significantly decrease cholesterol ester synthesis and secretion via a decrease in acyl coenzyme A cholesterol acyltransferase (ACAT) activity in the transformed, human intestinal epithelial cell line, CaCo-2 [20]. Recent data from our laboratory suggest that inhibition of ACAT activity causes a significant decrease in the secretion of apoB-48 from CaCo-2 cells [32]. Therefore, insulin may decrease the secretion of apoB-48 from the intestine by limiting cholesterol supply through its inhibition of ACAT. A study by Black and Ellinas [3] monitored the effect of insulin on chylomicron particle production in jejunal explants of newborn pigs. They found that insulin had no effect on the incorporation of [$^{35}$S]-methionine into apoB-48 and hence no effect on chylomicron production. Due to the discrepancy between the results of Levy et al. [23] and Black and Ellinas [3], the effect of insulin on the production of apoB-48-containing chylomicrons in mature human intestinal cells warrants further investigation.

Several studies have found that insulin significantly decreases the secretion of VLDL from cultured liver cells [reviewed in ref. 42], due to post-translational modification of apoB-100, via activation of insulin-signaling pathways. The two main signaling pathways for insulin are the phosphatidylinositol 3-kinase (PI3-K) and mitogen-activated protein kinase (MAPK) pathways. One study showed that PI3-K activation is necessary for the insulin-stimulated decrease in apoB secretion from rat hepatocytes [35]. Protein kinase C (PKC) is activated downstream of PI3-K, and inhibition of PKC activity also decreased VLDL-apoB secretion from rat hepatocytes to 60% of control [2]. Therefore, inhibiting PKC or PI3-K activity in intestinal cells may alter apoB-48 secretion. Insulin has been shown to affect apoB-100 levels via reuptake and clearance as it increases the expression and activity of the low-density lipoprotein receptor in HepG2 cells [46]. Later studies have suggested that this occurs due to activation of the transcription factor sterol regulatory element binding protein via either the MAPK [22, 37] or PI3-K signaling cascades [4]. In addition, a recent paper by Au et al. [1] showed that insulin downregulates microsomal triglyceride transfer protein (MTP) expression via activation of the MAPK pathway in HepG2 cells. MTP is necessary for the lipidation of apoB-100 and apoB-48 in the liver and intestine, respectively [17], and therefore regulates apoB degradation and secretion. Evidence that chylomicrons are affected by similar phosphorylation pathways comes from a study investigating the effect of okadaic acid (a potent inhibitor of protein serine/threonine phosphatases 1 and 2A) on apoB-48 production [28]. Incubation of CaCo-2 cells with 1 $\mu M$ okadaic acid for 16 h decreased the secretion of newly synthesized apoB-48 by 50%.

Limited studies suggest similarities between liver and intestinal lipoprotein assembly, although the two have never been directly compared as they are in this study. We previously put forward the hypothesis that chylomicron biogenesis is less acutely regulated on the basis of a limited increase in apoB-48 concentration after a fat challenge in vivo [27]. In normal healthy humans, there is only about a 25% increase in apoB-48 secretion above basal levels after the consumption of 40 g of fat [25]. The intestine continuously synthesizes chylomicrons, even in the fasted state, and this basal rate of synthesis makes up the bulk of the apoB-48 secretion into plasma. Therefore, insulin resistance in obese subjects may cause a major shift in the basal rates of chylomicron production, leading to an oversecretion of chylomicrons. However, the postprandial increase in chylomicron levels represents an acute exposure of enterocytes to insulin, and this aspect may not be as sensitive to regulation by insulin. To further explore this concept, we compared the acute effects of insulin on apoB-100 production in HepG2 cells and apoB-48 production in intestinal CaCo-2 cells. Both PKC and MAPK are involved in the intracellular signaling of insulin in hepatocytes and can alter the phosphorylation of proteins. Therefore, inhibitors and activators of these kinases were used to assess the role of these pathways in insulin modulation of apoB-48 production from CaCo-2 cells. It has been suggested that apoB-48 exists in a state of hyperphosphorylation under basal media conditions [18, 28], and this perhaps makes it less susceptible to regulation by insulin. Therefore, inhibitors of intracellular kinases were used to explore whether altering the basal phosphorylation status could change the amount of apoB-48 secreted.

## Materials and Methods

*Materials*

The human transformed colonic epithelial CaCo-2 cell line and the human transformed hepatic cell line HepG2 were purchased from the American Type Culture Collection. Cell culture media, reagents and fetal bovine serum (FBS) (certified grade) were from Gibco BRL Life Technologies (Gaithersburg, Md., USA). Costar transwell, polycarbonate microporous cell culture inserts were obtained from Corning (Cambridge, Mass., USA). Flasks for tissue culture were obtained from TPP Techno Plastic Products AG (Switzerland). Bicinchoninic acid protein assay reagents were obtained from Pierce (Rockford, Ill., USA). Human recombinant insulin, oleic acid (sodium salt), fatty acid-free bovine serum albumin, 5α-cholestane, Triton X-100, protease inhibitor cocktail, phorbol 12-myristate 13-acetate (PMA), okadaic acid (sodium salt) and other common laboratory reagents were from Sigma Chemical Co. (St. Louis, Mo., USA). Polyvinylidene fluoride membrane was from Millipore Corporation (Bedford, Mass., USA). Enhanced chemiluminescence detection reagents, hyper-film ECL and donkey anti-rabbit IgG were purchased from Amersham International (England). A14-Tyrosyl[$^{125}$I]iodoinsulin was purchased from Amersham (Australia). Rabbit anti-human apoB antibody was purchased from DAKO A/S (Denmark). PKC inhibitor peptide (peptide 19–31, recognizes 6 epitopes of PKC) was purchased from Upstate Biotechnology, Inc., and MAPK kinase (MEK) inhibitor, PD 98059, was purchased from Calbiochem.

*Culture Conditions*

*CaCo-2 Cells.* The transformed human epithelial CaCo-2 cells were grown in continuous cell culture at 37 °C under 5% $CO_2$, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% (v/v) FBS, 2% penicillin-streptomycin-glutamine (PS) and 1% nonessential amino acids (NEAA) (complete medium). Cells were seeded at a density of $1 \times 10^6$ in 75-cm$^2$ flasks. The medium was replaced every 2 days. Cells were subcultured from flasks at 90% confluency to polycarbonate microporous membranes (transwells, 0.4 μm pore size, inserts of 24.5 mm diameter) and plated at a density of $2 \times 10^4$ cells/24.5-mm-diameter filter. Fully differentiated CaCo-2 cells were used for all experiments (13 days post-subculturing). Serum-free (S-f) medium (see below) was added to the apical well after washing the cells with phosphate-buffered saline (PBS). DMEM supplemented with 2% PS and 1% NEAA was placed in the basolateral well (plain medium). S-f medium consisted of DMEM supplemented with 0.08% (w/v) bovine serum albumin (fatty acid free) complexed to 50 μ*M* oleic acid (sodium salt), 22.2 m*M* glucose, 45.5 m*M* $Na_2CO_3$, 1 m*M* sodium pyruvate, 2% PS and 1% NEAA. CaCo-2 cells incubated with this S-f medium were found to secrete more apoB-48 than cells incubated with complete medium (data not shown). Cells were incubated in S-f medium for approximately 10 h prior to the commencement of experiments. The medium was then replaced and cells were incubated with S-f medium with or without insulin for 24 h. The concentrations of insulin tested corresponded to physiological (1 n*M*), hyperinsulinemic (100 n*M*) and pharmacological (10 μ*M*) human plasma concentrations. Insulin was added to the apical and basolateral well and was replaced every 8 h. Experiments were performed three times, in duplicate. The medium in the apical well was discarded, as apoB-48 is not secreted in this compartment of the transwell [45] (also confirmed in our laboratory). The volume of medium in the basolateral well was measured, and apoB-48 and apoB-100 concentrations were determined both in the medium and intracellularly. For the kinase inhibitor studies, CaCo-2 cells were preincubated for 10 h in S-f medium prior to beginning the experiment. Cells were incubated with PKC (100 n*M*) and MEK inhibitor (50 μ*M*) in the apical well for 4 h prior to the further addition of 10 μ*M* insulin or 100 n*M* PMA [44]. When the insulin and PMA were added to the apical well, the basolateral medium was changed to plain medium, and apoB-48 secretion was measured over the incubation period. Intracellular and secreted apoB-48 were measured as described below. All experimental points were performed in duplicate.

*HepG2 Cells.* Human hepatocyte HepG2 cells were grown in continuous cell culture at 37 °C under 5% $CO_2$, in DMEM supplemented with 10% (v/v) FBS and 2% PS (complete medium) [34]. Cells were seeded at a density of $1 \times 10^6$ in 75-cm$^2$ flasks. The medium was replaced every 2 days. Cells were subcultured from these flasks into 25-cm$^2$ flasks and seeded at a density of $4 \times 10^5$. Cells were preincubated in S-f medium for 24 h and then incubated in S-f medium with or without insulin for a further 24 h. HepG2 cells incubated with this S-f medium were found to secrete more apoB-100 than cells incubated with complete medium (data not shown). The concentrations of insulin tested were 1 n*M*, 100 n*M* and 10 μ*M*. Insulin was replaced every 8 h. Experiments were performed three times, in duplicate. The medium was collected from the flasks and the volume measured. The intracellular and media concentrations of apoB-100 were determined.

*Cell Isolation*

CaCo-2 and HepG2 cells were washed three times, harvested and resuspended in PBS following incubation treatments. The cells were solubilized in buffer (1.5% Triton X-100 in PBS with protease inhibitor cocktail) at 4 °C for 3 h. Samples were pelleted at 400 *g* for 5 min at 4 °C, the supernatant was collected and measured, and the cell debris was discarded. The supernatant was used to measure apoB, total and free cholesterol and total solubilized protein using the bicinchoninic acid assay kit. Total cell protein was used to assess whether a treatment compromised cell viability. None of the treatments significantly compromised total cell protein in comparison to control cells (S-f medium alone) (data not shown).

*Sterol Quantification*

Lipid was extracted from solubilized CaCo-2 and HepG2 cells for the analysis of free cholesterol using a modification of the method of Folch et al. [12]. Briefly, 1 ml of chloroform ($CHCl_3$) was added to cells along with internal standard (5α-cholestane) and 25 μl of saturated salt solution to prevent the formation of a fatty acid emulsion. Samples were mixed thoroughly and centrifuged at 200 *g* for 10 min. The top aqueous layer was aspirated and discarded and the lower chloroform layer was dried under a stream of $N_2$. Samples were reconstituted in toluene before gas chromatography (GC) analysis.

To measure total cholesterol in the cells, 1 ml of 1 *M* KOH in methanol was added to an aliquot of cells to saponify cholesterol esters. The tubes were then flushed with $N_2$ and heated at 45 °C for 1 h. After hydrolysis was complete, the solution was diluted with 2 ml of water, internal standard was added and the lipids were extracted twice with hexane (1 ml). The hexane was dried down under a steam of $N_2$ and then samples were reconstituted in toluene before GC analysis.

Quantitation of free and total cholesterol was determined by the peak area ratio between cholesterol and the internal standard. A Perkin Elmer Autosystem XL gas chromatograph was used to determine

free and total cholesterol. A ZB-1 cross-linked methyl silicone column (30 m × 530 μm, 1.5 μm film thickness, Phenomenex) was used with helium as the carrier gas at a flow rate of 6.0 ml/min. An estimate of esterified cholesterol was derived as the difference between total and free cholesterol.

*Competitive Insulin-Binding Experiment*

Confluent HepG2 cells in 12-well trays (Falcon, Becton Dickinson Labware, N.J., USA) and CaCo-2 cells that had been grown in transwells for 14 days were preincubated in S-f medium containing 50 m*M* HEPES, pH 7.4, for 24 and 10 h, respectively. Cells were washed 3 times in cold PBS, and then S-f medium containing unlabeled insulin and approximately 333 Bq of A14-tyrosyl[$^{125}$I]iodoinsulin (74 TBq/mmol) was added to the wells of HepG2 cells and the apical and basolateral wells of transwells containing CaCo-2 cells. After 3 h at 15 °C, cells were washed 3 times in ice-cold PBS. HepG2 cells were scraped into PBS and transferred to 3DT tubes in 300 μl of 1.5% Triton X-100 in PBS. CaCo-2 cells were left on the polycarbonate membranes, which were cut out of the transwell inserts with a scalpel and placed in the bottom of a 3DT tube with 300 μl of 1.5% Triton X-100 in PBS. Samples were counted on a Packard Cobra II Autogamma counter. After counting, aliquots of the cells were used to measure the protein concentration. Experiments were performed in duplicate. The dissociation constant and receptor number were determined using the EBDA and LIGAND programs of McPherson [29], adapted from the Scatchard analysis program of Munson and Rodbard [31].

*Protein Tyrosine Kinase Activity*

HepG2 cells grown in 12-well trays and CaCo-2 cells grown in transwells were preincubated in S-f medium with 50 m*M* HEPES, pH 7.4 (HEPES buffer), for 24 and 10 h, respectively. Cells were then washed three times in ice-cold PBS and harvested. Cells were spun at 500 *g* for 5 min. The cell pellets were solubilized for 1 h at 4 °C in 0.5 ml of solubilization buffer (20 m*M* HEPES, 5 m*M* EDTA, 50 m*M* NaCl, 50 m*M* NaF, 50 μg/ml aprotinin, 5 μg/ml leupeptin, 30 μ*M* β-glycerophosphate, 1 m*M* PMSF and 1% Triton X-100). The extract was spun down at 11,500 *g* for 10 min, at 4 °C, the supernatant was aspirated and the cell debris was discarded. An aliquot of the cell supernatant was used for protein analysis. The remaining cell extract was incubated overnight at 4 °C with an insulin receptor β 29B4 mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA). A secondary antibody (Dynabeads M-450, sheep anti-mouse IgG, Dynal A.S., Oslo, Norway) was added to the cell extract with the primary antibody and incubated for a further 2 h. After washing the beads 3 times with HEPES buffer, they were resuspended in HEPES buffer, aliquoted into microfuge tubes and then incubated with or without 0.5 μ*M* insulin for 15 min at room temperature. The beads were then incubated for 30 min at room temperature with protein tyrosine kinase (PTK) substrate [0.76 m*M* RRDIFETDYFRK peptide (Auspep, Australia), [$^{32}$P]-ATP (10 μCi/μl), 0.5 m*M* ATP and 1 m*M* sodium vanadate; FYF-Pi] added for 30 min. The reaction was stopped with 7.5 *M* guanidine-HCl (terminating solution), and the extracts were spotted onto Whatman 81 paper. After washing 3 times in acetic acid, twice in water and once in ethanol, the filter paper was dried and the bound $^{32}$P was counted on a Packard Cobra II Autogamma counter for 5 min (17% counting efficiency). Nonspecific binding was measured by addition of the guanidine terminating solution prior to adding the FYF-Pi. Experiments were performed in duplicate.

*ApoB Quantification*

Solubilized cell protein (55 μg), aliquots of medium and purified apoB-48 or apoB-100 standards (previously prepared according to Zilversmit and Shea [50]) were separated by SDS-PAGE using precast NuPAGE 3–8% gradient gels in a Novex Mini-Cell (Novex Instruments, Calif., USA) at 150 V for 1 h as described previously [33]. Separated proteins were electrotransferred at 30 V for 90 min onto a 0.45-μm polyvinylidene fluoride membrane. The membranes were blocked for 1 h at 25 °C in TBST (10 m*M* Tris-HCL buffer, pH 7.4, containing 154 m*M* NaCl) and 10% (w/v) skim milk powder. After washing in TBST, the membranes were incubated with 5 μg/ml rabbit anti-human apoB antibody, which recognizes both apoB-48 and apoB-100. The membranes were incubated with 0.5 μg/ml donkey anti-rabbit IgG linked to horseradish peroxidase in TBST and subsequently washed four times with TBST. Membranes were incubated with enhanced chemiluminescence substrate solution for detection of horseradish peroxidase and exposed to hyper-film ECL. ApoB was determined by densitometric scanning using a UMAX Vista S6E Flatbed Scanner. The coefficient of variation for this assay was < 10% [47].

*Statistical Analysis*

Means were compared using a two-tailed Student's t test or by a univariate analysis of variance (ANOVA) with a post hoc Tukey test as indicated (using SPSS version 10.0). Statistical significance was assigned at $p < 0.05$.

## Results

### *Insulin Modulates apoB-100 Levels in HepG2 Cells, but Not apoB-48 Levels in CaCo-2 Cells*

Insulin caused a dose-dependent decrease in the secreted and intracellular concentration of apoB-100 in HepG2 cells (fig. 1A, B). The concentration of secreted and intracellular apoB-100 was significantly decreased to 49 and 50% of control, respectively, at a concentration of 10 μ*M* insulin ($p < 0.05$). In CaCo-2 cells, a similar decreasing trend was observed for apoB-100, except secretion was decreased to 80% of control with 10 μ*M* insulin (data not shown). In contrast, insulin had no effect on the secreted or intracellular concentration of apoB-48 in CaCo-2 cells over 24 h compared to controls (fig. 1C, D).

There is approximately 7 times as much apoB-100 secreted into the medium from HepG2 cells than apoB-48 from CaCo-2 cells (fig. 1A, C). In addition, the amount of apoB-100 in liver cells is approximately 12 times higher than apoB-48 in CaCo-2 cells (fig. 1B, D).

The effect of different exposure times to insulin on apoB-48 metabolism was examined in CaCo-2 cells. The cells were incubated with or without 10 μ*M* insulin for 2, 3, 4, 6, 12 or 24 h. There was no effect of insulin on apoB-48 secreted into the basolateral medium (fig. 2).

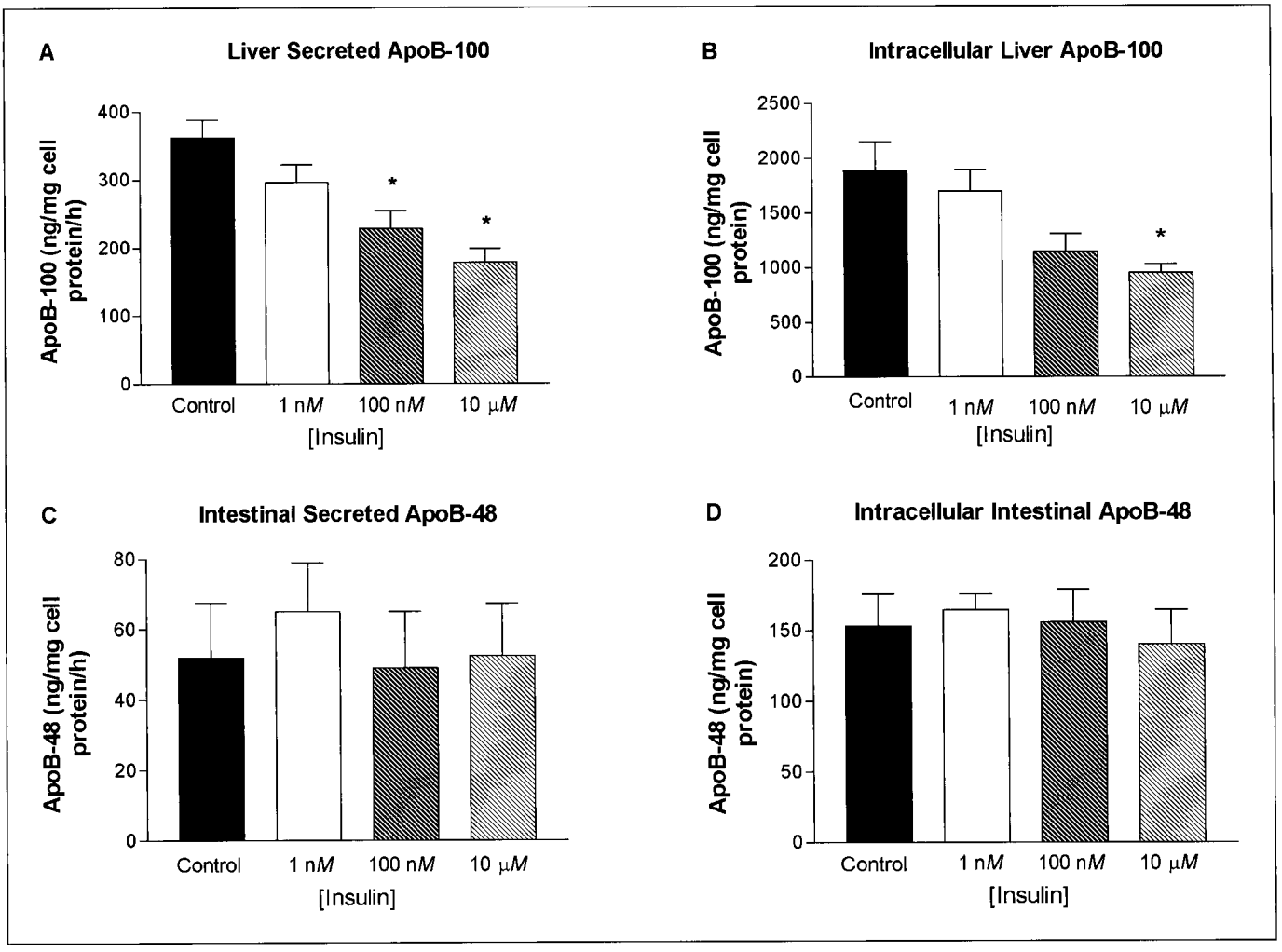


**Fig. 1.** The effect of physiological to hyperinsulinemic doses of insulin on the apoB-100 concentration secreted into the medium (**A**) and within HepG2 cells (**B**) and on the apoB-48 concentration secreted into the medium (**C**) and within CaCo-2 cells (**D**). HepG2 and CaCo-2 cells were incubated in S-f DMEM with or without 1 n*M*, 100 n*M* or 10 μ*M* insulin for 24 h. ApoB-100 and apoB-48 concentrations were measured using Western blotting ECL detection as described in Materials and Methods. ApoB concentrations are expressed as mean ± SEM (ng/mg cell protein/h for the medium and ng/mg cell protein for intracellular concentrations). Three experiments were performed, with duplicate samples. *$p < 0.05$ compared to control. Data were analyzed using a paired Student's t test.

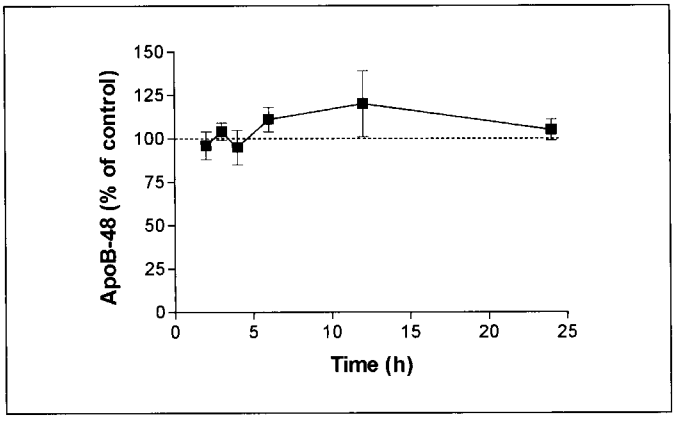


**Fig. 2.** The effect of 10 μ*M* insulin on apoB-48 secretion from CaCo-2 cells over time. CaCo-2 cells were incubated in S-f DMEM with or without 10 μ*M* insulin for 2, 3, 4, 6, 12 or 24 h. ApoB-48 concentration was measured using Western blotting ECL detection as described in Materials and Methods. ApoB-48 concentrations are expressed as percentage of control ± SEM (ng/mg cell protein/h). Three experiments were performed, with duplicate samples. Data were analyzed using a Student's t test.

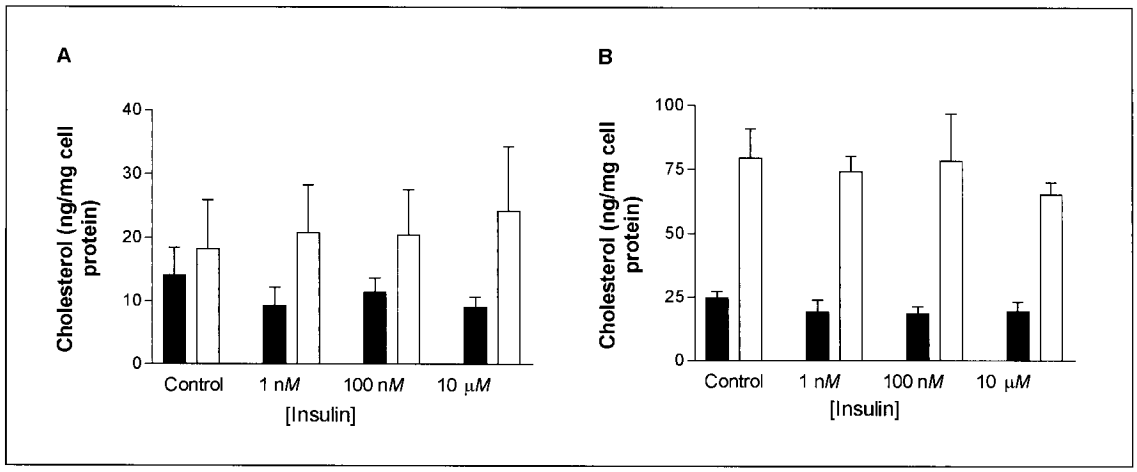


**Fig. 3.** The effect of physiological to hyperinsulinemic doses of insulin on cholesterol and cholesterol ester concentrations within HepG2 (**A**) and CaCo-2 (**B**) cells. Cells were incubated with or without 1 n*M*, 100 n*M* or 10 μ*M* insulin for 24 h in a background of S-f medium. Intracellular total, free (closed bars) and esterified cholesterol (open bars) were quantified by GC as described in Materials and Methods and are expressed as mean ± SEM (ng/mg cell protein). Three experiments were performed, with duplicate samples. Data were analyzed using a Student's t test.

*Insulin Does Not Modulate the Cellular Concentrations of Cholesterol and Cholesterol Ester in HepG2 and CaCo-2 Cells*

On the basis that the intracellular pool of cholesterol may regulate apoB secretion [7, 32], we determined the effects of insulin on cholesterol homeostasis in HepG2 and CaCo-2 cells. The intracellular concentration of total cholesterol, free cholesterol and cholesterol ester was measured by GC after exposure of cells to 1 n*M*, 100 n*M* and 10 μ*M* insulin in S-f medium for 24 h. In both HepG2 and CaCo-2 cells, the intracellular concentrations of total cholesterol, free cholesterol and cholesterol ester were not influenced at any dose of insulin ($p > 0.05$) (fig. 3A, B).

*Insulin Stimulates the Insulin Receptor and Leads to Tyrosine Kinase Activation in HepG2 and CaCo-2 Cells*

HepG2 and CaCo-2 cells bound insulin with high affinity, and the dissociation constants were similar ($3.5 \times 10^{-9}$ and $2.9 \times 10^{-9}$ *M*, respectively) (fig. 4A). HepG2 cells expressed more insulin receptors than did CaCo-2 cells (69,000 and 44,000 receptors/cell, respectively).

The level of PTK activity within cells was measured as a marker of insulin receptor activation. HepG2 and CaCo-2 cells showed similar levels of insulin-stimulated PTK activity ($p = 0.47$) (fig. 4B).

*PKC and MAPK Do Not Modulate apoB-48 in CaCo-2 Cells*

Insulin has been shown to modulate apoB-100 in hepatocytes via PKC- and MAPK-associated pathways [2, 22]. A univariate ANOVA showed that there was no effect of PKC inhibitor in the presence or absence of insulin, MEK inhibitor in the presence or absence of insulin, insulin alone or PMA on apoB-48 secreted into the medium or within CaCo-2 cells ($p > 0.05$) (fig. 5), indicating that PKC and MAPK do not play a role in the basal regulation of apoB-48.

## Discussion

In this study, we confirmed that insulin at plasma, hyperinsulinemic and pharmacological concentrations significantly decreased the accumulation of apoB-100 in the medium of HepG2 cells over a 24-hour period [reviewed in ref. 42]. A similar trend towards an insulin-dependent decrease in apoB-100 secretion was observed in CaCo-2 cells. However, the same concentrations of insulin did not regulate the production or secretion of apoB-48 from intestinal CaCo-2 cells after 24 h of exposure. Time course experiments excluded the possibility that 10 μ*M* insulin stimulation was inducing a rapid response that had returned to baseline by 24 h. The use of CaCo-2 and HepG2 cells as a model to investigate the

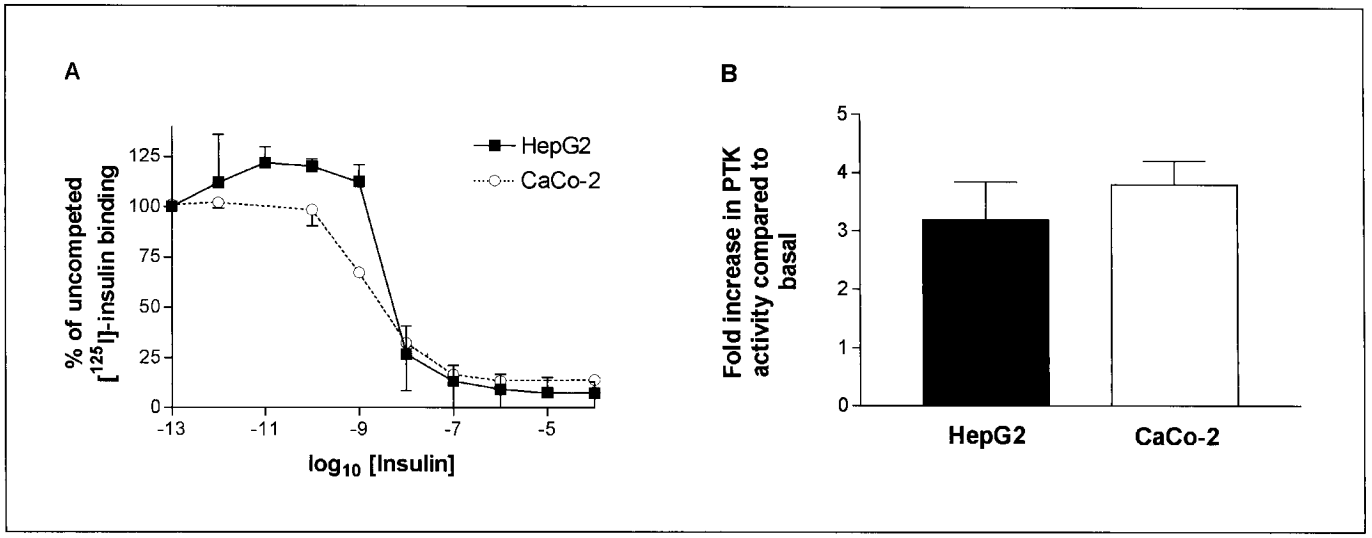


**Fig. 4.** Competitive insulin binding to HepG2 and CaCo-2 cells (**A**) and activation of PTK activity in HepG2 and CaCo-2 cells exposed to insulin (**B**). **A** Cells were incubated with A14-tyrosyl[$^{125}$I]iodoinsulin (74 TBq/mmol) and increasing concentrations of unlabelled insulin ($1 \times 10^{-13}$–$1 \times 10^{-4}$ *M*) for 3 h at 15 °C. The dissociation constant, receptor number and nonspecific binding were determined following computer-aided nonlinear regression analysis. Data shown are the mean ± standard deviation for each cell type. Two experiments were performed. **B** PTK activity was determined following incubation of HepG2 and CaCo-2 cells with 0.5 μM insulin for 15 min. Data are expressed as the fold increase compared to controls and are the mean ± SEM. Three experiments were performed. Data were analyzed using a Student's t test.

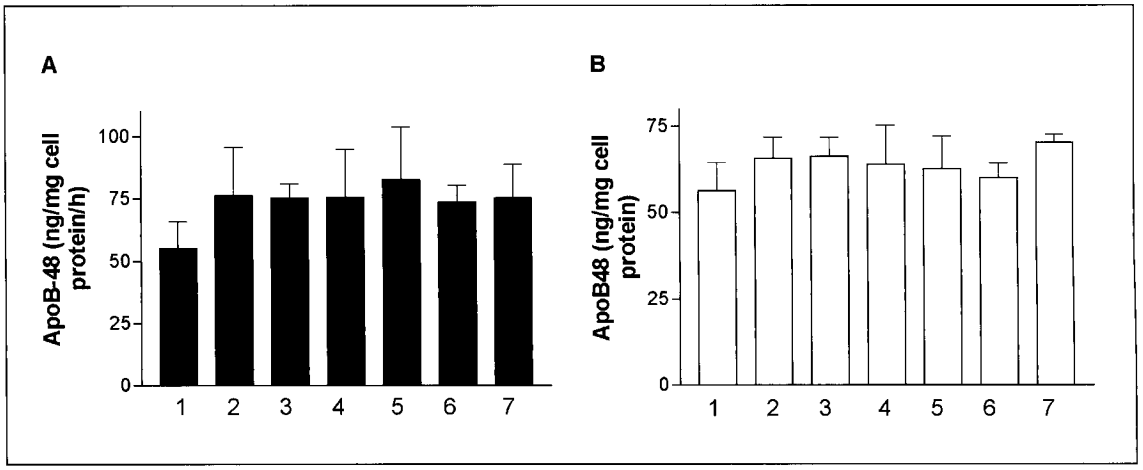


**Fig. 5.** The effect of a PKC inhibitor or activator on the apoB-48 concentration secreted into the medium (**A**) or in CaCo-2 cells (**B**). Cells were incubated with the PKC or MEK inhibitor for 4 h in a background of S-f medium. The basolateral medium was replaced and cells were incubated with the following: (1) no treatment; (2) PKC inhibitor (100 n*M*); (3) PKC inhibitor (100 n*M*) + insulin (10 μ*M*); (4) MEK inhibitor (50 μ*M*); (5) MEK inhibitor (50 μ*M*) + insulin (10 μ*M*); (6) insulin (10 μ*M*), or (7) PMA (100 n*M*). ApoB-48 was measured using Western blotting ECL detection as described in Materials and Methods. ApoB-48 concentrations are expressed as mean ± SEM (ng/mg cell protein/h in the medium and ng/mg cell protein for intracellular concentrations). Three experiments were performed, with duplicate samples. Data were analyzed using a univariate ANOVA and a post hoc Tukey test.

acute effect of insulin was supported by the fact that they secrete a physiological ratio of apoB-48 to apoB-100 [26]. There was no effect of insulin on the intracellular free cholesterol or cholesterol ester concentrations in either HepG2 or CaCo-2 cells. The differential effect of insulin on apoB-100 and apoB-48 was not due to differences in insulin binding or intracellular signaling. HepG2 and CaCo-2 cells displayed high-affinity binding of insulin, and HepG2 cells expressed slightly more insulin receptors compared to CaCo-2 cells. When exposed to insulin, the initial intracellular cascade of insulin signaling determined as PTK activity was activated to a similar extent in HepG2 and CaCo-2 cells. Furthermore, inhibiting or activating the intracellular PKC or MAPK pathways did not affect the intracellular or secreted concentration of apoB-48 in CaCo-2 cells. Collectively, our results suggest that there is differential regulation of apoB-100 in liver cells and apoB-48 in intestinal cells, when exposed to insulin. We provide evidence that in the transformed human intestinal epithelial cell line CaCo-2, insulin does not acutely regulate the production and secretion of apoB-48, and PKC and MAPK do not play a significant role in modulating apoB-48 in CaCo-2 cells.

ApoB-100 and apoB-48 protein expression is regulated at multiple levels. ApoB-100 and apoB-48 are synthesized from the same gene, except that post-transcriptional mRNA editing occurs in the human intestine to produce apoB-48, which is 48% of the full-length apoB-100 [6, 13]. The variation in apoB protein length may have effects on the stability and secretion of the various apoB isoforms. Studies using McArdle RH7777 rat hepatocytes expressing recombinant human apoB variants ranging in size from apoB-15 (15% of full length apoB-100) to apoB-100 showed that intracellular translocation and stability of apoB is inversely related to the length of the protein [5]. In addition, in vitro data on apoB-100 and apoB-48 suggest that the stability of both isoforms can be affected by altering the phosphorylation status of these proteins, where apoB-48 exists in a hyperphosphorylated state compared to apoB-100, and is perhaps more stable [10, 18, 28, 35, 43]. Inhibition of PKC and MAPK had no effect on apoB-48 in CaCo-2 cells, suggesting that these kinases are not associated with regulation of the basal phosphorylation status of apoB-48. In contrast, insulin acts through PI3-K, PKC and MAPK to alter apoB-100 metabolism pathways in hepatocytes [2, 4, 22, 35]. Although we did not investigate the effect of PI3-K inhibition in CaCo-2 cells, it appears as though insulin does not alter apoB-48 production in CaCo-2 cells. In rat hepatocytes, insulin downregulates apoB-100 secretion to a greater extent compared to apoB-48 secretion, implying that insulin may affect apoB-100 stability more than apoB-48 [41]. Our results mirror the findings of Sparks and Sparks [41] and suggest that the differential effects of insulin on apoB-100 and apoB-48 may not be cell type specific, but rather specific to the particular apoB isoform. It may be of interest to investigate the effect of PI3-K inhibition on the production of apoB-100 in CaCo-2 cells to further establish and confirm this idea.

Our previous studies have demonstrated that a decrease in cholesterol ester availability in CaCo-2 cells inhibits apoB-48 secretion, while increasing apoB-48 degradation [32]. ACAT (the enzyme responsible for the conversion of cholesterol to cholesterol ester) activity is significantly decreased in CaCo-2 cells following 3 h of exposure to insulin, and at 24 h, there was less incorporation of $^{14}$C-oleic acid into cholesteryl oleate [20]. Although we did not measure ACAT2 (the isoform of ACAT in the intestine [39]) activity in this study, we found that 4 h of exposure to insulin did not alter apoB-48 secretion compared to controls. Furthermore, in both HepG2 and CaCo-2 cells, there was no effect of insulin on intracellular free cholesterol or cholesterol ester concentrations after 24 h. The cells in the study of Jiao et al. [20] were grown on transwell inserts for 7 days and therefore may have been at a different stage of differentiation compared to our CaCo-2 cells, which were grown for 14 days. A study by Dashti et al. [8] suggests that CaCo-2 cells do not begin to secrete apoB into the medium until about day 6 after confluence, and thus the cells in the study of Jiao et al. [20] may still have been undergoing replication and not yet secreting apoB lipoproteins. As insulin decreased apoB-100 in HepG2 cells without affecting cholesterol or cholesterol ester levels, its effect is probably independent of cholesterol and more likely due to changes in protein phosphorylation.

Consistent with other studies, we observed a significant decrease in the intracellular and secreted concentrations of apoB-100 from HepG2 when incubated with insulin for 24 h [reviewed in ref. 42]. The decrease in hepatic apoB-100 secretion was possibly mediated via a direct effect of insulin on apoB-100 stability or degradation, and recent work suggests that upregulation of low-density lipoprotein receptor expression [4] and a decrease in MTP expression [1, 24] are closely linked to this regulation in HepG2 cells. We showed for the first time that in the human intestinal cell line CaCo-2, apoB-48 is not regulated by insulin over a 24-hour period. Also, we extend these findings and suggest that changing the phosphoryla-

tion status of CaCo-2 cells with inhibitors and activators of PKC and MAPK does not alter apoB-48 production.

Collectively, our data suggest that apoB-48 production and secretion by human intestinal cells is not acutely regulated by insulin. By extrapolation of our observations to a clinical setting, we would predict that postprandial dyslipidemia in insulin-resistant subjects is not regulated by the rapid release of insulin which occurs in response to a mixed meal [15]. Rather, hyperchylomicronemia in insulin-resistant subjects might more accurately reflect increased basal rates of chylomicron secretion, a pathway that might be chronically regulated by endocrine homeostasis. Overproduction of apoB-48 has been observed in an insulin-resistant hamster model via greater rates of enterocytic de novo lipid synthesis and increased apoB-48 lipidation as a consequence of exaggerated MTP activity [16]. It is possible that increased basal rates of chylomicron biogenesis in insulin-resistant subjects may occur via similar mechanisms, and this warrants further investigation.


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