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Insulin-mediated inhibition of apolipoprotein B secretion requires an intracellular trafficking event and phosphatidylinositol 3-kinase activation: studies with brefeldin A and wortmannin in primary cultures of rat hepatocytes

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Insulin inhibition of the secretion of apolipoprotein B (apo B) was studied in primary cultures of rat hepatocytes by using brefeldin A (BFA), an inhibitor of protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus, and by using the phosphatidylinositol 3-kinase (PI 3-K) inhibitor wortmannin. Incubation of hepatocytes with BFA (10 μg/ml) for 1 h inhibited the subsequent secretion of apo B, albumin and transferrin for up to 3 h. BFA treatment resulted in the time-dependent accumulation in cells of [^{14}C]leucine-labelled proteins and apo B. Under conditions where insulin decreased total apo B (cell plus secreted), BFA blocked the insulin-dependent effect. These results suggest that export of apo B from the ER is a prerequisite for the observed insulin effect. Treatment of hepatocytes with wortmannin for 20 min abolished insulin inhibition of apo B secretion, suggesting that the insulin effect on the apo B pathway involves activation of PI 3-K. Enzyme inhibitor studies indicate that chymostatin and (+)-(2*S*,3*S*)-3-[(*S*)-methyl-1-(3-methylbutylcarbamoyl)-butylcarbamoyl]-2-oxiranecarboxylate (E-64-c) partially block insulin effects on apo B compared with leupeptin, which had no discernible effect. The cell-permeable derivative of E-64-c, EST, and *N*-Ac-Leu-Leu-norleucinal (ALLN) were most effective in blocking insulin effects on apo B. These results suggest that insulin action on apo B in primary rat hepatocytes involves (1) vesicular movement of apo B from the ER; (2) activation of PI 3-K and (3) a cellular protease that is either a cysteine- or calcium-activated neutral protease.

INTRODUCTION

Apolipoprotein B (apo B) is a required protein component for assembly of very-low-density lipoproteins (VLDL) [1]. Each VLDL contains one molecule of apo B [2]. Hence, apo B secretion is a measure of the number of VLDL particles secreted. Insulin is a key regulator of VLDL–apo B secretion by rat hepatocytes [3–6] and has been hypothesized to have a role in balancing hepatic and intestinal lipoprotein metabolism during the post-prandial period [7]. Addition of insulin to primary cultures of rat hepatocytes results in reduced apo B synthesis as well as in the failure of a substantial portion of newly synthesized apo B to be secreted, and this portion of apo B is degraded intracellularly [3]. Oleate addition to culture medium of hepatocytes derived from fed rats neither stimulates apo B secretion [8,9] nor overcomes the ability of insulin to inhibit apo B secretion [6], suggesting that insulin-mediated apo B degradation is distinct from that which occurs in the endoplasmic reticulum (ER) in HepG2 cells [10]. Insulin also inhibits apo B secretion by human hepatocytes [11] and by HepG2 cells post-transcriptionally [12,13].

The present study evaluates the ability of specific inhibitors to attenuate insulin-mediated reductions in cell and secreted apo B in primary rat hepatocytes. Brefeldin A (BFA), which blocks transport from the ER to Golgi [14], was used to determine whether vesicular transport out of the ER is required for insulin effects on apo B to be observed. To determine whether the insulin effect is mediated through a pathway involving activation of phosphatidylinositol 3-kinase (PI 3-K), an inhibitor of PI 3-K, wortmannin, was used. Wortmannin has recently been shown to inhibit insulin-stimulated glucose transport and antilipolysis in rat adipocytes [15] as well as glycogen synthesis in 3T3-L1 adipocytes [16]. Because a major effect of insulin on apo B is stimulation of its intracellular degradation [3], we also evaluated the ability of a number of proteolytic enzyme inhibitors to prevent insulin effects on apo B.

MATERIALS AND METHODS

Materials

Sprague–Dawley rats (180–250 g) were obtained from Charles River Laboratories, Wilmington, MA, U.S.A. Brefeldin A was obtained from Epicentre Technologies, Madison, WI, U.S.A. Rabbit anti-rat albumin and anti-rat transferrin were obtained from Cappel Research Products, Organon Teknika Corp., Durham, NC, U.S.A. Immunoprecipitin (Protein A cells) was obtained from Bethesda Research Laboratories, Bethesda, MD, U.S.A. Protein A cells were purified before use as described by the manufacturer. (+)-(2*S*,3*S*)-3-[(*S*)-Methyl-1-(3-methylbutylcarbamoyl)-butylcarbamoyl]-2-oxiranecarboxylate (E-64-c) and its cell-permeable derivative, EST, were kindly provided by Dr. M. Tamai, Taisho Pharmaceutical Co., Japan. Leupeptin, chymostatin and wortmannin were from Sigma Chemical Corp., St. Louis, MO., U.S.A. Calpain inhibitor I, [*N*-Ac-Leu-Leu-

Abbreviations used: ALLN, *N*-Ac-Leu-Leu-norleucinal; Apo, apolipoprotein; BFA, brefeldin A; E-64-c, (+)-(2*S*,3*S*)-3-[(*S*)-methyl-1-(3-methylbutylcarbamoyl)-butylcarbamoyl]-2-oxiranecarboxylate; ER, endoplasmic reticulum; EST, the ethyl ester of E-64-c; HBSS, Hanks' balanced salt solution; IRS-1, insulin receptor substrate-1; LDH, lactate dehydrogenase; PI 3-K, phosphatidylinositol 3-kinase; TGN, trans-Golgi network; α-TSF, α-toluenesulphonyl fluoride; VLDL, very-low-density lipoproteins.

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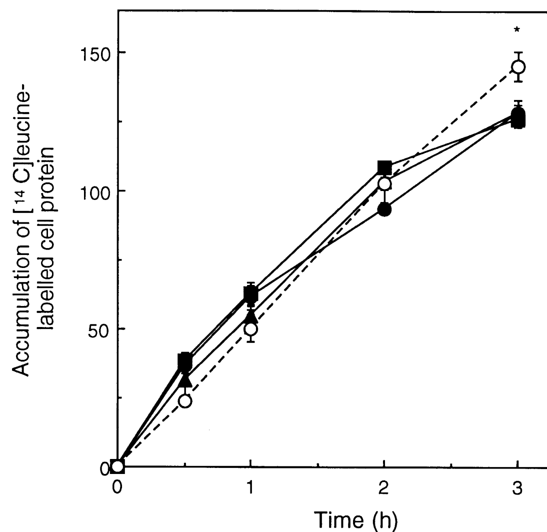

Figure 1 Time-course and dose-dependent effects of BFA on cellular accumulation of L-[^{14}C]leucine-labelled proteins by cultured rat hepatocytes

After overnight culture (12–14 h) in Waymouth's medium, the medium was replaced with RPMI-1640 medium containing 0.2 μM L-leucine and 0.8 μCi/ml L-[U-^{14}C]leucine (specific activity 270 mCi/mmol) and various concentrations of BFA. At indicated times, media were removed and cell lysates were prepared for trichloroacetic acid precipitation to measure cell accumulation of labelled protein. Results are from a single experiment, and are the averages of three dishes for each condition at each time point. The average counts per dish ± S.D. are plotted against time. ●, No BFA; ■, 1 μg/ml BFA; ▲, 5 μg/ml BFA; ○, 10 μg/ml BFA. In some instances the error bars are too small to be visible. $^{*}P < 0.05$.

norleucinal (ALLN)], was obtained from Calbiochem, San Diego, CA, U.S.A. Percoll was obtained from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.

Rat hepatocyte cultures

Perfusions to prepare hepatocytes were performed between 08:00 h and 10:00 h on *ad libitum*-fed rats by using collagenase [3]. Hepatocytes were further purified by centrifugation through Percoll [17]. Purified, viable hepatocytes were washed free of Percoll and were resuspended at a concentration of 10^6 cells/ml in Waymouth's MB/1 medium containing antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml; gentamicin, 50 μg/ml) and 0.2 % (w/v) BSA. This medium is referred to hereafter as Waymouth's medium. Cells were seeded at 2 ml of cell suspension per 60 mm tissue culture dish previously coated with rat-tail collagen. After 2–4 h, medium was removed and cells were washed three times with Hanks' balanced salt solution (HBSS) containing 0.2 % BSA, and hepatocytes were reincubated in 2 ml Waymouth's medium at 37 °C in an atmosphere of air/CO_2, 95:5. Stock solutions of brefeldin A (4 mg/ml), wortmannin (10 mM) and protease inhibitors were prepared in DMSO. Control medium contained an equivalent volume of DMSO, which did not exceed 0.25 % (v/v). At the end of the indicated incubation periods, media were removed and cells were washed three times in HBSS. Cells were scraped into 0.05 M barbital buffer, pH 8.6, containing 0.5 % (v/v) Triton X-100 and 2 mM EDTA, to which 5 mM benzamidine and 2 mM α-toluenesulphonyl fluoride (α-TSF) were freshly added. After brief sonication, homogenates were centrifuged at 2500 ***g*** for 20 min at 4 °C and the resultant supernatant was used for protein and apo B assays.

Apo B assay

Apo B concentrations of cell homogenates and media were determined in triplicate by competitive radioimmunoassay with rat VLDL apo B as a standard and a monoclonal antibody equally reactive to forms of apo B of higher and lower molecular mass [18]. The protein concentrations of cell homogenates from each dish were measured by a modification of the Lowry method [19]. Apo B concentrations of cells and media samples were calculated per mg of cell protein on a per dish basis. For each liver examined, apo B concentrations (cell, medium and total) from four to six dishes per condition were averaged. The average apo B concentration for an experimental condition from each liver was then averaged with corresponding results from other liver preparations.

Protein labelling methods

For leucine labelling studies, hepatocytes were cultured for 12–14 h in Waymouth's medium followed by washing the monolayers in HBSS containing 0.2 % BSA. Medium was replaced with 2 ml RPMI-1640 deficient medium (without L-glutamine, L-leucine, L-lysine, L-methionine and sodium bicarbonate) to which was added (per litre) the following: 350 mg L-glutamine, 250 mg L-lysine-HCl, 50 mg L-methionine, 0.2 μmol L-leucine, 250 mg choline chloride, 2.24 g sodium bicarbonate, 2 g BSA and antibiotics. Labelling medium was adjusted to contain 0.8 μCi/ml L-[U-^{14}C]leucine (specific activity 270 mCi/mmol). After incubation of hepatocytes with labelling medium, cells were washed in HBSS containing 4 mM L-leucine. Cell lysates were prepared by scraping cells into cell solubilizer composed of 0.05 M Tris, 0.15 M NaCl buffer, pH 7.4, containing 1 % (v/v) Triton X-100, 0.5 % SDS, 5 mM EDTA, 5 mM EGTA, and freshly added 2 mM α-TSF and 5 mM benzamidine [3]. After heating the cell lysate for 1 h at 95–100 °C, insoluble material was removed by centrifugation for 20 min at 2500 ***g***. For measurement of [^{14}C]leucine incorporation into total cellular protein, aliquots of clarified supernatants from lysed cells were analysed in triplicate by using trichloroacetic acid precipitation as previously described [3].

For [^{35}S]methionine labelling experiments, hepatocytes after overnight culture (12–14 h) were incubated for 3–4 h in 2 ml RPMI-1640 deficient medium with similar additions to that described, with the exception of L-methionine. Unlabelled L-methionine (0.1 μM) and L-[^{35}S]methionine (50 μCi/ml, specific activity 1000 Ci/mmol) were added to the culture medium. After incubation, cells were washed three times in Waymouth's medium containing 10 mM L-methionine (chase medium), and re-incubated in chase medium with and without addition of BFA (10 μg/ml final) for up to 5 h.

Immunoprecipitation methods

[^{35}S]Methionine-labelled rat apo B was isolated by immunoprecipitation from cell lysates and media by using a monospecific polyclonal antibody raised in rabbits against SDS-column-purified rat apo B [3]. Immunoprecipitations to isolate [^{35}S]methionine-labelled rat albumin and transferrin were performed similarly with commercial rabbit anti-rat antibodies [20]. Clarified supernatants of cell lysates prepared as described above were diluted as follows: one part supernatant plus 2 parts cell diluent, composed of 0.05 M Tris/0.15 M NaCl, pH 7.4, containing 0.5 % BSA, 2.5 mM α-TSF, 2 mM benzamidine, 5 mM EDTA, 5 mM EGTA, 100 μg/ml soybean trypsin inhibitor,

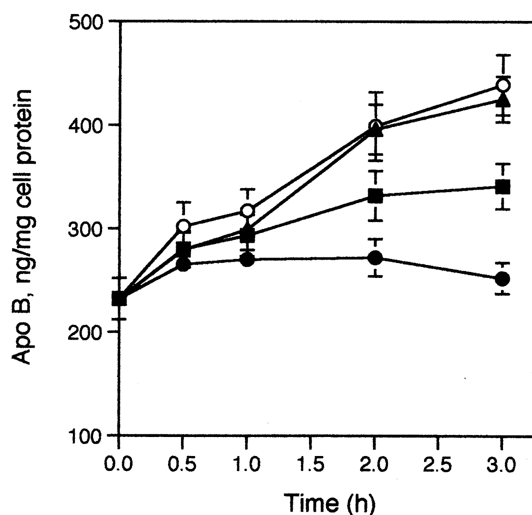

Figure 2 Time-course and dose-dependent effects of BFA on cellular accumulation of apo B in primary cultures of rat hepatocytes

After overnight culture (12–14 h) in Waymouth's medium, hepatocytes were incubated in Waymouth's medium containing various concentrations of BFA. At indicated times, dishes were terminated and cell apo B was measured by radioimmunoassay. Three dishes for each condition and time point were assayed in three independent liver experiments, and the mean cell apo B (ng apo B per mg cell protein) was calculated ± S.E.M. ●, No BFA; ■, 1 μg/ml BFA; ▲, 5 μg/ml BFA; ○, 10 μg/ml BFA. Significant differences were observed at 2 and 3 h with 1, 5 and 10 μg/ml BFA ($P < 0.05$). In some instances the error bars are too small to be visible.

25 μg/ml aprotinin and 30 μg/ml leupeptin. To adjust detergent and inhibitor concentrations of media samples, 1 vol. of cell solubilizer and 1 vol. of cell diluent were added for each vol. of medium. Labelled proteins were immunoprecipitated by addition of rabbit antisera or purified IgG fraction followed by overnight incubation at 4 °C with continuous mixing. Immune complexes were collected by addition of washed Protein A cells and incubation for 1 h at 4 °C. Immunoprecipitates were pelleted by centrifugation at 1500 ***g*** and extensively washed by resuspension six times in 5 ml of cold (4 °C) 0.05 M Tris/0.15 M NaCl, pH 7.4, containing 1 mM α-TSF, 2 mM benzamidine, 0.1% (v/v) BSA, 0.25% (v/v) Triton X-100 and 0.1% *N*-lauroylsarcosine. After removal of the final wash, labelled proteins were eluted into sample buffer composed of 0.0625 M Tris, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol [21], and 10 mM dithiothreitol. The samples were heated to 95 °C for 10 min, and Protein A cells were removed by centrifugation for 20 min at 2500 ***g***. Eluted proteins in the supernatant were radioassayed by β-counting and separated by SDS/PAGE on 3.5–26% (w/v) gradient gels [20,22]. Apo B, albumin and transferrin corresponded to appropriate molecular masses as determined by SDS/PAGE and comparison with ^{14}C-labelled molecular mass marker proteins [3]. For assessment of radioactivity distribution, each lane of the fluorograph was scanned and the percentage distribution determined by using an automatic integrating densitometer (EDC 1376, Helena Laboratories, Beaumont, TX, U.S.A.). To determine the radioactivity of the band of interest, the percentage of the total density for each protein band was multiplied by actual radioassayed counts per min per mg of cell protein in the original sample. More than 95% of the immunoprecipitated radioactivity corresponded with the appropriate molecular-mass protein in the case of albumin and transferrin. For apo B, calculations are based on radioactivity present in bands corresponding to B48 and B100 based on the decile nomenclature [23].

Statistical methods

Unless otherwise indicated, data are presented as the means ± S.E.M. In tabular data, each rat liver preparation involved analysis of multiple dishes of hepatocytes (four to six dishes) for a particular experimental condition. *N* is the number of independent experiments of individual rat livers. Significant differences between means were calculated using Student's *t*-test.

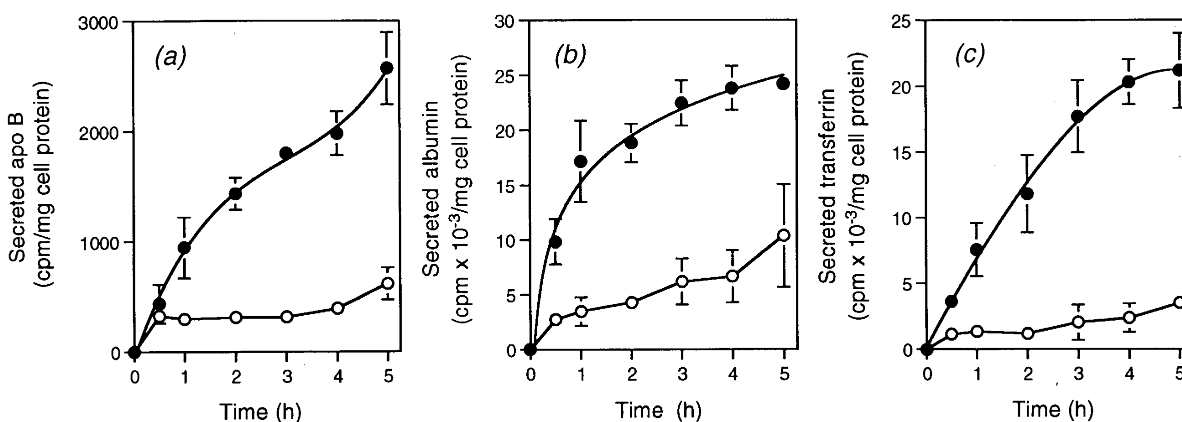

Figure 3 Effects of time and BFA (10 μg/ml) on secretion of L-[^{35}S]methionine-labelled rat (*a*) apo B, (*b*) serum albumin and (*c*) transferrin by primary cultures of rat hepatocytes

After overnight culture (12–14 h) in Waymouth's medium, medium was removed and hepatocytes were incubated in RPMI-1640 medium containing 0.1 μM L-methionine and 50 μCi/ml L-[^{35}S]methionine. After 3–4 h of incubation, labelling medium was removed, and cells were washed and reincubated in Waymouth's medium/10 mM methionine containing either DMSO (●) or 10 μg/ml BFA (○). At the indicated times, media were collected and rat apo B, serum albumin and transferrin were immunoprecipitated. Labelled proteins were separated by SDS/PAGE and secreted protein radioactivity was measured as described in the Materials and methods section. Apo B radioactivity is the average of four independent liver experiments in which apo B was assayed in triplicate and points plotted are the average of averages ± S.E.M. Albumin radioactivity is the average ± S.D. of four independent liver experiments, and transferrin radioactivity is the average ± S.D. of three independent liver experiments. In some instances the error bars are too small to be visible.

RESULTS

Because BFA is rapidly metabolized by rat hepatocytes to an inert form [24], a control experiment was performed to determine the concentration of BFA necessary to inhibit protein secretion maximally for 3 h. A 3 h interval was chosen as this is the earliest time point at which to observe insulin effects on cell and secreted apo B [3,25]. Hepatocytes were cultured in Waymouth's medium for 12–14 h, and medium was replaced with that containing L-[^{14}C]leucine plus various concentrations of BFA (1, 5 and 10 μg/ml) (Figure 1). Inhibition of protein secretion of newly synthesized proteins was assessed by measuring the accumulation of ^{14}C-labelled proteins within cells by using trichloroacetic acid precipitation. BFA at 10 μg/ml produced a linear accumulation of ^{14}C-labelled protein within hepatocytes in 3 h incubations (Figure 1). There was a minor effect of BFA on protein synthesis in the first 1 h of incubation. However, the accumulation rate was essentially linear until 2 h. After 2 h, protein secretion resumed in media at concentrations of BFA below 10 μg/ml (results not shown), accounting for the lower cellular accumulation rates from 2 to 3 h. These results suggest that 10 μg/ml BFA was the appropriate concentration to maximally inhibit overall protein secretion in 3 h, which is similar to results of Vance et al. [26].

The time-course and dose-dependent effects of BFA on the accumulation of cellular apo B (ng/mg of cell protein) were next evaluated. As seen in Figure 2, both 5 and 10 μg/ml BFA were able to cause almost linear accumulation of cell apo B in primary hepatocytes over 3 h of incubation. To confirm the ability of BFA to block protein secretion for 3 h, the effect of 10 μg/ml BFA on the secretion of ^{35}S-labelled rat albumin and transferrin was compared with that of ^{35}S-labelled apo B (Figure 3). Hepatocytes were incubated in medium containing [^{35}S]methionine. After 3–4 h, medium was removed, and hepatocytes were washed and reincubated in Waymouth's medium containing 10 mM methionine with and without 10 μg/ml BFA. At various times after BFA addition, media were collected and ^{35}S-labelled apo B, albumin and transferrin were immunoprecipitated. Labelled proteins were separated by SDS/PAGE, revealed by fluorography after gel enhancement, and the radioactivity of each protein was quantified as described in the Materials and methods section. As shown in Figures 4 and 5, BFA significantly inhibited secretion of rat apo B, albumin and transferrin. Some secretion of labelled proteins occurred during early BFA action (0–30 min) but virtually no additional secretion occurred for up to 3 h. A concentration of 10 μg/ml BFA was chosen for all subsequent experiments because this concentration of BFA (1) maximally inhibited secretion of labelled protein, and caused a constant rate of accumulation of newly synthesized proteins in cells (Figure 1); (2) caused the linear accumulation of apo B within cells (Figure 2); and (3) limited the secretion of ^{35}S-labelled apo B, albumin and transferrin for up to 3 h (Figures 3–5).

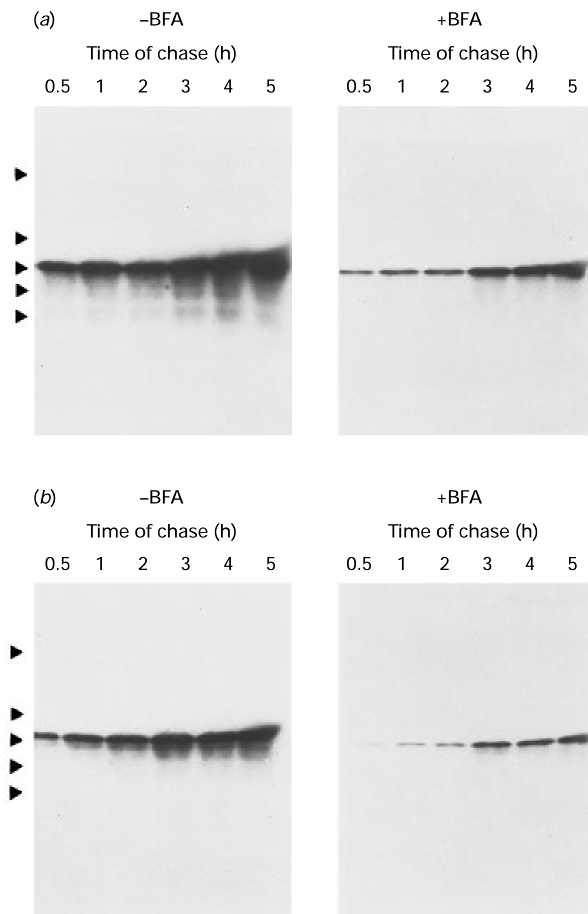

Figure 4 Effect of BFA (10 μg/ml) on the secretion of L-[^{35}S]methionine-labelled rat serum albumin (*a*) and transferrin (*b*)

After overnight culture (12–14 h) in Waymouth's medium, medium was removed and hepatocytes were incubated in RPMI-1640 medium containing 0.1 μM L-methionine and 50 μCi/ml L-[^{35}S]methionine. After 3–4 h of incubation, labelling medium was removed, and cells were washed and reincubated in Waymouth's medium/10 mM methionine containing either DMSO or 10 μg/ml BFA. At the indicated times, media were collected and rat serum albumin and transferrin were immunoprecipitated. Proteins in the immunoprecipitates were separated by SDS/PAGE, and revealed after gel enhancement by fluorography. The location of co-electrophoresed ^{14}C-labelled molecular mass protein markers are indicated by the arrowheads and included myosin (H-chain, 200 kDa), phosphorylase *b* (97.4 kDa), BSA (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa). Results are from a single experiment representative of four similar experiments for albumin and three similar experiments for transferrin.

To examine the effect of BFA on insulin-mediated inhibition of apo B secretion, rat hepatocytes were incubated for 3 h with and without insulin in the presence and absence of 10 μg/ml BFA. In two independent experiments, there was little lactate dehydrogenase (LDH) release (less than 2 % of total cellular LDH) in the presence or absence of BFA, indicating that hepatocyte viability was not adversely affected by BFA. At the end of the 3 h incubation period, apo B was measured by radioimmunoassay (Table 1). The presence of insulin in the medium significantly reduced cellular and secreted apo B by 3 h, as has been previously reported [3]. In hepatocytes incubated with insulin plus BFA, the total apo B in the system (cell plus secreted) was similar to hepatocytes incubated with BFA alone, suggesting that BFA blocked insulin-dependent reduction of apo B in rat hepatocytes. The total apo B in cells and medium after 3 h of incubation with and without BFA was similar (463 ± 50 compared with 427 ± 53), indicating essentially complete recovery of apo B in the presence of BFA. Similarly, in label-incorporation experiments using cells labelled for 3–4 h with [^{35}S]methionine, there was recovery of 78.7 ± 3.7 % of apo B after 3 h of chase in controls, compared with recovery of 88.9 ± 7.0 % of apo B in the presence of BFA (four independent liver experiments).

Insulin action in adipocytes involves activation of PI 3-K [27,28]. PI 3-K activity is inhibited by wortmannin, which has been shown to block many metabolic effects of insulin [15,16]. To determine whether PI 3-K activation is also involved in insulin effects on apo B, we evaluated the ability of wortmannin to attenuate the insulin-dependent decrease in cell and secreted apo B in rat hepatocytes. Wortmannin had minimal effects on cell

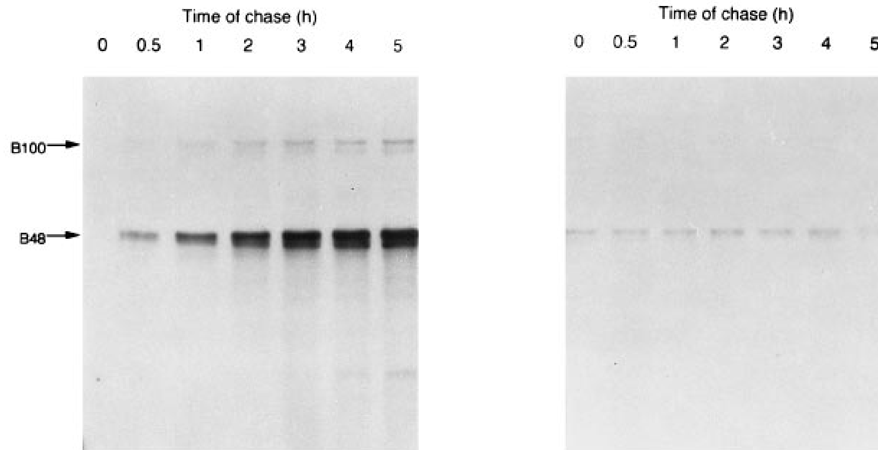

Figure 5 Effect of BFA (10 μg/ml) on the time course of L-[^{35}S]methionine-labelled apo B secretion into medium

After overnight culture (12–14 h) in Waymouth's medium, medium was removed and hepatocytes were incubated in RPMI-1640 medium containing 0.1 μM L-methionine and 50 μCi/ml L-[^{35}S]methionine. After 3–4 h of incubation, labelling medium was removed and cells were washed and reincubated in Waymouth's medium/10 mM methionine containing either DMSO (left panel) or 10 μg/ml BFA (right panel). At the indicated times, media were collected and rat apo B was immunoprecipitated. Labelled B48 and B100 were separated by SDS/PAGE and revealed after gel enhancement by fluorography. The locations of B100 and B48 are indicated by arrows. Results are from a single experiment representative of four similar experiments.

Table 1 Effect of BFA on insulin action on apo B in primary rat hepatocytes

After overnight culture, hepatocytes were incubated in Waymouth's medium with and without added BFA (10 μg/ml) in the presence and absence of 100 nM insulin. At the end of 3 h, media were collected; cell homogenates were prepared and apo B was assayed by radioimmunoassay (four dishes for each condition). Results for each condition were averaged, and the mean was averaged with corresponding results from other liver preparations. Results are expressed as the average of averages ± S.E.M. from four liver preparations. Values marked * indicate significant differences between control and insulin-treated hepatocytes ($P < 0.05$) with the paired Student's *t*-test.

Conditions		Apo B (ng/mg of cell protein)		
Insulin	BFA	Cell	Secreted	Total
−	−	266 ± 32	161 ± 22	427 ± 53
+	−	193 ± 26*	116 ± 20*	309 ± 43*
−	+	410 ± 54	53 ± 16	463 ± 50
+	+	375 ± 47	41 ± 15	416 ± 45

viability as measured by LDH release. In three independent experiments, wortmannin caused release of less than 1.6% of total cellular LDH (mean ± S.D.): 29 ± 11 units/litre per mg of cell protein compared with 13 ± 5 in the control. To determine whether wortmannin was able to block insulin effects on apo B, hepatocytes were incubated in Waymouth's medium containing 1 μM wortmannin for 20 min, after which insulin was added to half the dishes and incubation was continued for 5 h. Apo B secreted into the media at various time points after insulin addition was analysed by radioimmunoassay. As seen in Figure 6 for a representative experiment, pretreatment of hepatocytes with wortmannin prevented the decreased secretion of apo B mediated by insulin. In Table 2, results of four similarly performed experiments are summarized demonstrating that 1 μM wortmannin not only blocked the ability of insulin to inhibit apo B secretion, but also prevented insulin-mediated reduction in cell apo B. These results suggest that insulin action on apo B is mediated through activation of PI 3-K. As seen in Table 2, cell apo B (ng/mg of cell protein) averaged 238 ± 8 in hepatocytes incubated with wortmannin compared with 259 ± 7 in control hepatocytes. Secreted apo B averaged 282 ± 18 in hepatocytes incubated with wortmannin compared with 255 ± 23 in control hepatocytes. These results indicate that wortmannin alone had little effect on cell or secreted apo B.

To partially characterize the intracellular protease(s) involved in insulin-mediated apo B degradation, various protease inhibitors were used to determine whether they could alter insulin effects on apo B. Leupeptin was used as an inhibitor of serine proteases and the cysteine proteases, cathepsins B and L [29]. Leupeptin is also a potent inhibitor of calpains (Ca^{2+}-activated neutral thiol proteases), which are predominantly cytosolic. However, because leupeptin is positively charged, it does not readily permeate the plasma membrane but enters cells by pinocytosis [30], favouring localization of the inhibitor to the endosome–lysosome compartment. Chymostatin inhibits chymotrypsin and most cysteine proteases with inhibition of lysosomal degradation by about 45% [31]. Chymostatin also inhibits non-lysosomal protein degradation by more than 50% [31]. E-64-c and its cell-permeant derivative, EST, are epoxysuccinate compounds that form thioethers with thiols, and are potent inhibitors of calpains and cathepsins B and L. E-64-c and EST do not inhibit serine proteases [32]. ALLN is also a cell-permeable inhibitor of calpains, and in addition inhibits the activity of lysosomal cathepsins B and L. Hepatocytes were incubated for 12–14 h in Waymouth's medium containing 0.1 or 100 nM insulin in the presence of each inhibitor. After incubation, cell and secreted apo B were radioimmunoassayed and total apo B was calculated (Table 3). No significant differences in protein content of hepatocytes with and without inhibitor were observed at the end of the incubation period, suggesting that inhibition of proteolytic activity did not have major effects on the adherence of hepatocytes to plates and cell viability (results not shown). To determine whether an inhibitor altered insulin effects on apo B, basal and insulin-induced conditions in the presence of inhibitor were compared. As shown, leupeptin was ineffective in blocking insulin-mediated decreases in cell and secreted apo B, suggesting that apo B degradation mediated by insulin does not involve

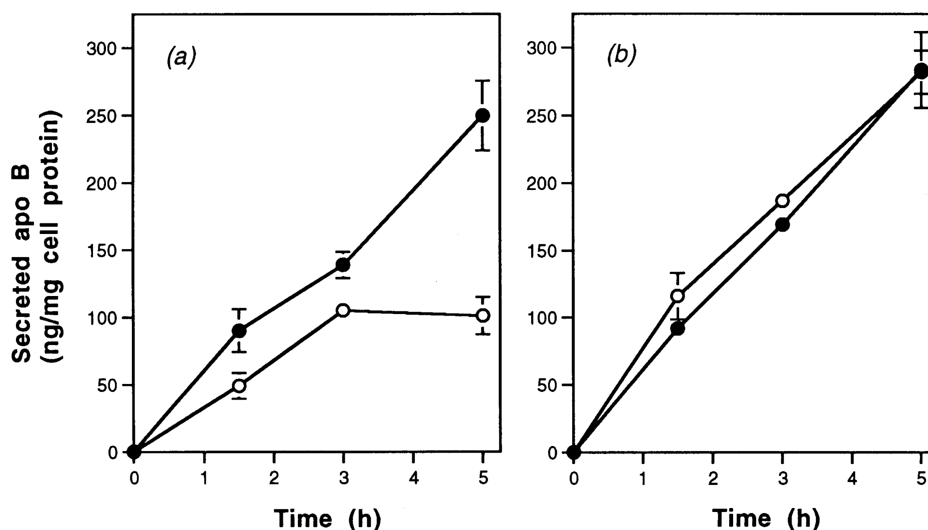

Figure 6 Wortmannin blockade of insulin-mediated inhibition of apo B secretion by rat hepatocytes

Rat hepatocytes were incubated with (***a***) DMSO or (***b***) 1 μM wortmannin dissolved in DMSO. After 20 min of incubation, insulin (final concentration 100 nM) was added to half of the dishes (○) while buffer (●) was added to the other half. Hepatocytes were then reincubated for 1.5, 3 and 5 h. Media were collected from three dishes for each time point, and secreted apo B was measured by radioimmunoassay. Results are from a single representative experiment of four similar experiments. In the figure, the average ± S.D. of triplicate determinations is plotted against time. In some instances the error bars are too small to be visible.

Table 2 Effect of wortmannin on insulin action on apo B in primary rat hepatocytes

Hepatocytes were incubated with Waymouth's medium containing DMSO or 1 μM wortmannin dissolved in DMSO and, after 20 min, insulin (100 nM) was added where appropriate. Cells were reincubated for an additional 5 h, after which media were collected and cell homogenates were prepared. Apo B present in medium and cells was measured by radioimmunoassay (three to five dishes per condition) and the average for each condition was then averaged with corresponding results from other liver preparations. Results are expressed as the average of averages ± S.E.M. from four liver preparations. Values marked * indicate significant differences between control and insulin-treated hepatocytes ($P < 0.03$) with the paired Student's *t*-test.

Conditions		Apo B (ng/mg of cell protein)		
Insulin	Wortmannin	Cell	Secreted	Total
−	−	259 ± 7	255 ± 23	514 ± 24
+	−	205 ± 6*	150 ± 17*	355 ± 20*
−	+	238 ± 8	282 ± 18	520 ± 11
+	+	236 ± 13	259 ± 44	495 ± 52

lysosomal degradation. Both chymostatin and E-64-c blocked insulin-mediated declines in cell apo B; however, insulin effects on apo B secretion were still present. In contrast, EST and ALLN blocked the ability of insulin to decrease both cell and secreted apo B. Considering the specificities of EST and ALLN, results suggest that insulin-mediated apo B degradation may involve a cysteine protease or calcium-activated neutral protease. Apo B secretion was not increased with any of the tested protease inhibitors. E-64-c alone did not change the amount of total apo B (results not shown). There was, however, a minor reduction in total apo B with leupeptin, chymostatin and EST (Table 3). Both cell and secreted apo B were significantly decreased with ALLN (down by 58 ± 12.4 %), indicating an effect of ALLN on apo B synthesis.

Table 3 Effect of protease inhibitors on insulin-mediated inhibition of apo B secretion by rat hepatocytes

Rat hepatocytes were incubated in Waymouth's medium containing 0.1 nM insulin (B) or 100 nM insulin (I) in the presence of inhibitors at the indicated concentrations. After 12–14 h, medium was collected, and cell and secreted apo B were radioimmunoassayed (four to six dishes per condition). Apo B results are expressed as ng apo B per mg of cell protein, and are the average of averages ± S.E.M. of *N* independent liver experiments. Values marked * indicate significant differences between basal (B) and insulin (I) conditions for each inhibitor at a probability level of at least $P < 0.05$.

Inhibitor		*N*	Apo B (ng/mg of cell protein)		
			Cell	Secreted	Total
Leupeptin	B	3	252 ± 25	790 ± 133	1042 ± 155
(50 μg/ml)	I		194 ± 16*	485 ± 166*	679 ± 177*
Chymostatin	B	6	241 ± 17	611 ± 67	852 ± 73
(50 μg/ml)	I		233 ± 19	414 ± 49*	647 ± 59*
E-64-c	B	4	300 ± 34	846 ± 89	1146 ± 114
(50 μg/ml)	I		313 ± 42	563 ± 101*	876 ± 89*
EST	B	3	354 ± 37	464 ± 129	818 ± 166
(50 μg/ml)	I		366 ± 57	313 ± 134	679 ± 158
ALLN	B	3	169 ± 38	433 ± 141	602 ± 166
(100 μg/ml)	I		147 ± 30	300 ± 89	447 ± 93

DISCUSSION

There are three findings in the current study. First, by preventing transport of apo B from the ER, BFA blocks the insulin-mediated decrease of apo B in cells and media. Secondly, inhibition of PI 3-K by wortmannin prevents the insulin effect on apo B, suggesting involvement of insulin-activated PI 3-K in this pathway. Thirdly, the degradation of apo B under the influence

of insulin does not seem to involve lysosomal degradation but may involve a cysteine protease or calcium-activated neutral protease.

A concentration of BFA of 10 μg/ml was employed in the current studies, a concentration 10-fold greater than that used in HepG2 cells to study transport from ER to Golgi [33–35]. A higher concentration of BFA was necessary for sustained and maximal inhibition of apo B secretion for 3 h, probably because BFA is rapidly metabolized by rat hepatocytes [24]. This same concentration was used to study intracellular trafficking of newly synthesized phosphatidylethanolamine [26]. We have previously demonstrated that a concentration of 10 μg/ml BFA consistently alters the immunofluorescence staining pattern for apo B in rat hepatocytes for up to 3 h from a pattern of Golgi and ER staining to an entirely reticular-staining pattern, consistent with BFA action [36]. Lower concentrations of BFA allow resumption of apo B secretion presumably because of reassembly of the Golgi complex and re-establishment of ER to Golgi transport [24].

Degradation of apo B before secretion has been proposed as a regulatory event in VLDL secretion by liver. Studies in rat hepatocytes indicate significant degradation of apo B [37] and this degradation is probably mediated by insulin as there is little degradation under similar culture conditions when insulin is absent [3]. In rat hepatocytes oleate has little effect on VLDL–apo B secretion, in contrast with HepG2 cells where oleate stimulates apo B secretion presumably by overcoming early intracellular degradation [10], which has been demonstrated in the ER [33]. Insulin-mediated degradation is blocked by BFA in rat hepatocytes, indicating that vesicular transport to a post-ER compartment is a requirement. Recovery of most of the apo B in labelling studies suggests little ER degradation, in contrast with HepG2 cells. Insulin-mediated apo B degradation is not inhibited by leupeptin, a lysosomal inhibitor indicating a non-endosomal process; however, localization to a proteolytic site such as the proteasome requires further study.

Total protein synthesis and secretion in rat hepatocytes are not decreased by insulin. In contrast, apo B [3,25] and VLDL triglyceride [6] secretion are reduced by insulin. This shows that there is specificity for the insulin effect in metabolic control of release of VLDL and its associated lipids from liver. Apo B in rat hepatocytes is present in VLDL, as a transmembrane form [37,38] and as a partly lipidated lipoprotein [39]. It is not known at what site insulin acts. However, it is likely from the studies of Davis et al. [37] that insulin acts at an early stage of VLDL assembly with triglyceride, possibly at the stage of translocation of apo B into the ER. Oleate has been shown to protect partly lipidated forms of B100 in HepG2 cells from ER degradation and to stimulate apo B secretion [10]. In contrast, oleate does not protect apo B from insulin-mediated degradation and does not stimulate apo B secretion by rat hepatocytes [8,9] in the presence of insulin [6]. These findings suggest that insulin-mediated apo B degradation is distinct from ER degradation described in HepG2 cells.

Apo B secretion occurs during the first hour of BFA treatment. Considering that it requires only 15 min of BFA treatment to cause morphological changes [24], it is likely that the apo B secreted under these conditions is derived largely from an immediate presecretory compartment, i.e. *trans*-Golgi network (TGN), which is BFA-resistant [40]. Theoretically, the percentage of apo B present in the TGN can therefore be estimated from Table 1 as the apo B secreted under BFA blockade (53 ng/mg of cell protein) divided by the amount of apo B initially present in cells before BFA blockade (266 ng/mg of cell protein). This calculation suggests that the amount of apo B present in the TGN in primary rat hepatocytes is approx. 20 % of total cellular apo B.

Evidence is accumulating that PI 3-K plays a central role in insulin regulation of cellular metabolism. Insulin binding to its receptor stimulates the receptor's intrinsic tyrosine kinase activity that results in multiple tyrosine phosphorylation of the insulin receptor and other intracellular proteins, including insulin receptor substrate-1 (IRS-1) [41]. Phosphorylated tyrosine residues of IRS-1 then bind directly to cytoplasmic proteins containing Src homology 2 domains including PI 3-K [42,43]. After binding of IRS-1 to the regulatory subunit of PI 3-K, there is stimulation of both the serine/threonine and lipid kinase activities of the p110 catalytic subunit [44,45]. Wortmannin inhibits insulin-induced enhancement of both enzyme activities [15,46], and this inhibitor has been used to demonstrate the essential role of PI 3-K activation in insulin-stimulated glucose transport and anti-lipolysis in rat adipocytes [15,46]. Inhibition of PI 3-K activation in 3T3-L1 adipocytes also blocks insulin activation of glycogen synthesis [16], translocation of GLUT 1 and GLUT 4 glucose transporters [47], and stimulation of pp70 S6 kinase and DNA synthesis [48]. In the current study we demonstrate that wortmannin completely abolished the ability of insulin to reduce cell apo B and to inhibit apo B secretion in 3 h incubations, suggesting that activation of PI 3-K is also necessary for insulin effects on apo B. Wortmannin alone had negligible effects on cell and secreted apo B, suggesting that basal PI 3-K activity is not essential for apo B synthesis or secretion.

The consequences of PI 3-K activation by insulin in terms of effects on apo B in hepatocytes have not previously been reported. Current results imply that insulin post-receptor signalling events may extend to the secretory compartment involved in VLDL assembly and secretion. Consistent with this is the observation in adipocytes that PI 3-K activation by insulin occurs predominantly in low-density microsomes enriched in intracellular and Golgi membranes [27,28]. Studies of the yeast vps-34 gene product, which has significant sequence similarity to the catalytic subunit of mammalian PI 3-K [49] and exhibits both protein and lipid kinase activities [50], have demonstrated that deletions and point mutations result in severe vacuolar protein sorting defects [51,52], suggesting that this gene product may be important in intracellular trafficking events. If it were analogous to the proposed role for the vps-34 gene, PI 3-K activation by insulin on intracellular membranes might regulate the movement of vesicles containing apo B to a post-ER location where degradation takes place. A role for an intracellular trafficking event in insulin effects on apo B is also supported by results obtained with BFA. Interestingly, BFA action is a result of inhibition of binding of cytosolic coat proteins that regulate membrane targeting [53] (reviewed in [14]). It has been postulated that enrichment of membranes with the highly charged phospholipid products of PI 3-K may trigger specific membrane interactions with vesicle coat proteins [52] that control budding and transport vesicle formation. Alterations in membrane phospholipid composition can also affect the efficiency of apo B translocation. Enrichment of microsomal membranes with phosphatidylmonomethylethanolamine has recently been shown to impede apo B translocation, and inhibition of apo B translocation leads to intracellular apo B degradation [54,55]. Considering that PI 3-K is also a protein kinase [44], an activity that is also inhibited by wortmannin [46], alternative mechanisms may relate to protein phosphorylation of apo B [56] or other related proteins. Extending studies from adipocytes to hepatocytes is an important avenue by which to explore insulin regulation of hepatic secretory events.

We propose that insulin-mediated reduction of VLDL–apo B

functions to balance hepatic and intestinal triglyceride delivery during the transition from the fed to the fasted state [7]. In support of this proposal are studies *in vivo* by Moir and Zammit [57] that demonstrate a decrease in hepatic triglyceride secretion after the start of refeeding. In insulin-resistant states, there is loss of insulin regulation of hepatic VLDL-apo B production in rat hepatocytes [58,59]. In obese humans, resistance to insulin inhibition of hepatic VLDL and apo B has also been demonstrated [60]. It is further proposed that insulin resistance in the regulation of hepatic VLDL secretion contributes to the associated hypertriglyceridaemia in insulin-resistant states such as obesity. Studies relating insulin resistance to alterations in PI 3-K or IRS-1 need to be performed to provide a mechanistic understanding of the current findings. In summary, results of the current study suggest that the insulin-mediated decrease in cell and secreted apo B in primary rat hepatocytes involves (1) movement of apo B from the ER, (2) activation of PI 3-kinase and (3) activation of a cellular protease that is likely to be an EST-sensitive cysteine protease or calcium-activated neutral protease.

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