Insulin Modulation of Hepatic Synthesis and Secretion of Apolipoprotein B by Rat Hepatocytes

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Insulin inhibition of apolipoprotein B (apoB) secretion by primary cultures of rat hepatocytes was investigated in pulse-chase experiments using [35S]methionine as label. Radioactivity incorporation into apoBH and apoBL, the higher and lower molecular weight forms, was assessed after immunoprecipitation of detergent-solubilized cells and media and separation of the apoB forms using sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Hepatocyte monolayers were incubated for 12–14 h in medium with and without an inhibitory concentration of insulin. Cells were then incubated for 10 min with label, and, after differing periods of chase with unlabeled methionine, cellular medium and media labeled apoB were analyzed; >90% of labeled apoB was present in cells at 10 and 20 min after pulse, and labeled apoB did not appear in the medium until 40 min of chase. Insulin treatment inhibited the incorporation of label into total apoB by 48%, into apoBH by 62%, and into apoBL by 40% relative to other cellular proteins. Insulin treatment favored the rapid disappearance of labeled cellular apoBH with an intracellular retention half-time of 50 min (initial half-life of decay \( t_1 = 25 \) min) compared with 85 min in control \( (t_1 = 60 \) min). Intracellular retention half-times of labeled apoBH were similar in control and insulin-treated hepatocytes and ranged from 80 to 100 min. After 180 min of chase, 44% of labeled apoBH in control and 92% in insulin-treated hepatocytes remained cell-associated. Recovery studies indicated that insulin stimulated the degradation of 45 and 27% of newly synthesized apoBH and apoBL, respectively.

When hepatocyte monolayers were continuously labeled with [35S]methionine and then incubated in chase medium with and without insulin, labeled apoBH was secreted rapidly, reaching a plateau by 1 h of chase, whereas labeled apoBL was secreted linearly over 3–5 h of chase. Insulin inhibited the secretion of immunoassayable apoB but not labeled apoB.

Results demonstrate that 1) insulin inhibits synthesis of apoB from [35S]methionine, 2) insulin stimulates degradation of freshly translated apoB favoring apoBH over apoBL, and 3) an intracellular pool of apoB, primarily apoBH, exists that is largely unaffected by insulin. Overall, insulin action in primary hepatocyte cultures reduces the secretion of freshly synthesized apoB and favors secretion of preformed apoB enriched in apoBL.

Very low density lipoprotein is synthesized and secreted by liver after a series of complex intracellular events involving the synthesis of apolipoprotein B (apoB)1 and lipid and their assembly into lipoproteins. apoB is one of the largest mammalian proteins synthesized in liver as a single polypeptide chain having a molecular weight of 512,000 and is an obligate component for very low density lipoprotein assembly and secretion (1, 2). In rats (3–5) and humans (6), apoB exists in two forms which are metabolically distinct which we have designated as apoBH for the higher molecular weight form and apoBL for the lower molecular weight form (3). In rats apoBH- and apoBL-containing lipoproteins are secreted by liver (3–5), whereas in human liver the higher molecular weight form (apoB-100) predominates (7, 8). In both human and rat, apoBH-containing lipoproteins are secreted by intestine (6, 9). Human apoB-100 and apoB-48, corresponding to rat apoBH and apoBL, respectively, are products of a single-copy gene (10, 11). The mechanism of production of both apoB-100 and apoB-48 from a single gene is a result of an RNA-editing process that changes a glutamine codon (CAA) of the mRNA for apoB-100 into a translational stop codon (UAA), thereby generating a shortened protein product, apoB-48 (12–14). A similar mechanism for the formation of apoB48 and apoB100 from a single-copy gene in rat liver has been demonstrated (15, 16) making the apoB forms in rat equivalent to those in humans. As a consequence of this RNA editing mechanism, the amino acid sequence of apoB100 (apoB-48) is identical to that of the corresponding amino-terminal portion of apoB100 (apoB-100).

Numerous studies support the concept that hepatic apoB synthesis and secretion are metabolically regulated. apoB production rates are reduced in fasting (17, 18) and streptozotocin-induced diabetes (19, 20) and are increased in carbohydrate feeding (21). Moreover, there are marked changes in apoBH and apoBL expression in rats through development (22, 23). Even more recent studies have documented that thyroid hormone in vivo influences the RNA-editing process of apoB mRNA in rat liver (24, 25). The specific mechanisms involved in the hormonal regulation of hepatic apoB translation, assembly with lipid and lipoprotein secretion, however, remain largely unknown.

Recent studies using primary cultures of rat hepatocytes

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1 The abbreviations used are: apo, apolipoprotein; apoBH, apo B of higher molecular weight; apoBL, apo B of lower molecular weight; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline.
Indicate there is an insulin receptor-mediated pathway that regulates apoB secretion by rat liver (28-29). This pathway has also been identified in liver perfusion studies (20) and in HepG2 cells (29, 30). Insulin in culture medium stimulates lipid synthesis from acetate (27), causes the accumulation of triglyceride within hepatocytes (26, 27, 29, 31), and reduces triglyceride (26-29, 31) and apoB secretion (20-30). If the mechanism of insulin action were due to inhibition of the apoB secretory pathway the apoB and triglyceride content of hepatocytes would increase concomitantly with the reduction in lipoprotein secretion. As apoB does not accumulate along with the triglyceride within hepatocytes with insulin treatment (27, 32) it is unlikely that the mechanism of insulin action is only to inhibit the lipoprotein secretory pathway.

The current study demonstrates that insulin in the medium of primary cultures of rat hepatocytes reduces the incorporation of [35S]methionine label into apoB and apoB10, relative to other cellular proteins and favors the degradation of newly synthesized presecretory apoB. Decreased apoB synthesis and increased apoB degradation stimulated by insulin are responsible for reducing the amount of apoB secreted by hepatocytes incubated in the presence of insulin. Evidence is presented for a slower secretory pool of cellular apoB, mostly apoB10, which continues to be accreted in the presence of insulin in the medium.

**Experimental Procedures**

Materials—Studies were performed in male Sprague-Dawley rats fasted ad libitum and weighing 200-280 g. [35S]Methionine (>800 Ci/mmol) was obtained from Amersham Corp. RPMI 1640 medium, Waymouth's MB 752/1 medium, Amberlite MB-3, Triton X-100, and benzamidine were from Sigma. Immunoprecipitin was purchased from Bethesda Research Laboratories. Human insulin, Triton X-100, and XAR-5 film were from Eastman Kodak Co. Sepharose CL-4B was obtained from Amersham Corp. RPMI 1640 medium, Amberlite MB-3, Triton X-100, and 2 AM EDTA. Labeled apoB was eluted in 150 μl of 0.0625 M Tris-HCl, 0.15 M NaCl, pH 7.4, and 0.5 μl of rabbit antiserum containing added L-methionine (1 μM) [35S]methionine (100 μCi/dish), and 0.1 μM insulin. After incubation, labeled medium was withdrawn, and hepatocyte monolayers were washed three times and reincubated for 0.5, 1, 2, and 3 h (and in some cases 5 h) in 5.0 ml of RPMI 1640 medium containing 0.2% (w/v) BSA and 10 nm L-methionine (chase medium). Immunoprecipitation of hepatocellular and medium [35S]labeled apoB was carried out as described below. The time of chase required for 50% of the maximum newly synthesized labeled apoB to disappear from hepatocytes was determined from disappearance curves in order to calculate intracellular retention half-times as described by Yeo et al. (31).

The half-life of decay of cellular apoB in the cell was determined from curves where bound apoB radioactivity (100%) was set to 0 min and other times during the chase period were adjusted accordingly. The initial half-life was calculated as described by Borchardt and Davis (42). In control studies after 180 min of incubation in chase medium hepatocyte monolayers were washed twice with 5.0 ml of chase medium containing heparin (300 units/ml) or human low density lipoprotein (100 μg/ml) followed by a third wash with chase medium before detergent solubilization and immunoprecipitation of cellular [35S]-labeled apoB.

In pulse-chase 2 studies, after seeding the cells for 2-4 h hepatocyte monolayers were washed three times in methionine-free RPMI 1640 medium containing 0.2% (w/v) BSA and were reincubated for 12-14 h at 37°C in methionine-free RPMI 1640 medium (5 ml/dish) containing added L-methionine (1 μM) [35S]methionine (100 μCi/dish), and 0.1 μM insulin. After incubation, labeled medium was withdrawn, and hepatocyte monolayers were washed three times and reincubated for 0.5, 1, 2, and 3 h (and in some cases 5 h) in 5.0 ml of RPMI 1640 medium containing 0.2% (w/v) BSA and 10 nm L-methionine (chase medium) with and without 100 nm insulin added.

**Immunoprecipitation**—Dishes were terminated at the times indicated in the figures by washing hepatocyte monolayers three times with room temperature chase medium. Hepatocytes were immediately solubilized by addition of hot (80-90°C) solubilization buffer (1.0 ml/mg cell protein) which consisted of 0.05 M Tris buffer, 0.15 M NaCl, pH 7.4, containing 1 mg/ml 1% Triton X-100 (freshly added), 5.0 mM EDTA, 1% (w/v) Triton X-100, and 0.5% (w/v) SDS. After incubation for 1 h at 65-80°C, the solubilized cells formed a clear solution which was transferred to tubes and reheated for 5 min at 95°C. For immunoprecipitation of cellular apoB, 0.5 ml of the solubilized cell extract was mixed with 0.5 ml of 0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4, and 0.5 μl of rabbit antiseraum to rat apoB diluted in 1% (w/v) BSA/phosphate-buffered saline. For precipitation of apoB from medium samples, 0.5 ml of each medium was mixed with 0.5 ml of solubilization buffer and then with 0.5 ml of diluted rabbit antiseraum. The dilution of antiseraum used was previously shown to optimally precipitate labeled apoB from solubilized cell extracts and media. Immunoprecipitates were collected by centrifugation 30 min at room temperature. Before use, bacterial cells were purified by washing in a solution of 4% (w/v) SDS, 5% (v/v) 2-mercaptoethanol followed by 0.05% (w/v) Triton X-100, 0.15 M NaCl, pH 7.4, containing 1% (w/v) BSA. Immunoprecipitates were collected by centrifugation at 2800 rpm and were washed three to five times in 0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4, containing 0.15% (w/v) SDS, 0.1% (w/v) Triton X-100, and 2 mM EDTA. Labeled apoB was eluted in 150 μl of 0.0625 M Tris-HCl buffer, pH 6.8, containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.01% (v/v) Triton X-100, and incubated with proteinase K at 55°C with heating twice to 95°C for 5 min. Bacterial cells were removed from the eluate by final centrifugation at 2800 rpm for 20 min. Eluted [35S]-labeled apoB was radioassayed and an aliquot was applied to a 3.5-24% Acrylamide/acylamide gradient gel cast on GelBond™ PAGE (Bio-Rad Laboratories).
film. Labeled apoBH and apoBL were separated by electrophoresis for 3-4 h at 250 V (40), and afterwards proteins were fixed in sequential solutions of acetic acid/2-propanol. Fixative was removed from gels by agitation of the gel in 1% (v/v) glycerol for 2-4 h at room temperature and gels were then enhanced by agitation in Autofluor™ for 4 h. Enhanced gels were dried to a thin film in an oven for 30 min at 170 °C and placed in a wafered cassette containing preflashed Kodak XAR 6 film. The film was exposed at -80 °C for appropriate exposure times and were developed using an automatic processor.

For assessment of radioactivity distribution of fluorographs, each lane was scanned three times and the percent distribution was determined using an automatic integrating densitometer (EDC 1376, Helena Laboratories, Beaumont, TX). The average percent apoBH and apoBL in each lane as determined by scanning was multiplied by actual radioassayed disintegrations/min/mg cell protein in order to calculate apoBH and apoBL radioactivity in the original sample.

Analytical Procedures—The apoB content of media and 0.5% (v/v) Triton X-100-solubilized hepatocytes (28) was assayed using a single monoclonal antibody solid phase radioimmunoassay (27, 36). The antibody used was a 125I-labeled mouse monoclonal antibody equally reactive against rat apoBH and apoBL. The apoB content of cell homogenates from individual hepatocyte culture dishes was determined immediately after preparation of the homogenate and each homogenate was assayed in triplicate. For determination of protein synthesis in pulse-chase studies, 0.1 ml of the detergent-solubilized cell extract or medium sample was mixed with 0.4 ml of 1% (w/v) BSA/phosphate-buffered saline and 0.5 ml of 0.1 M 14C-methionine. Afterwards, 1.0 ml of 20% (w/v) trichloroacetic acid was added with mixing and the mixture was heated to 95 °C for 15 min. Precipitated protein was separated by centrifugation at 2800 rpm for 20 min and aliquots of the supernatant were radioassayed to correct for non-trichloroacetic acid-soluble radioactivity.

RESULTS

Insulin Effect on Incorporation of 35S Methionine into ApoB in Pulse-Chase 1 Studies—The capacity of hepatocytes to incorporate labeled methionine into apoB and to secrete newly synthesized apoB was examined using a pulse-chase protocol (Fig. 1A) similar to that described for HepG2 cells (37, 38). Hepatocyte monolayers were cultured for 12-14 h in either control medium (0.1 nM insulin) or in medium containing an inhibitory concentration of insulin (10 nM insulin) prior to pulse labeling. Fig. 1B indicates that reduced secretion of apoB by insulin was maintained for 180 min of the chase period. 35S-Labeled apoBH and 35S-labeled apoBL appeared in the medium at 40 min of chase and continued to be secreted throughout the 180-min chase period (Fig. 2).

Greater than 90% of peak label was present in the cell at 20 min (Table I) and apoB had not been secreted into the medium by 20 min into the chase period. These reasons allowed a direct comparison of the amount of label incorporated into cell apoB during the 10-20 min in control and insulin-treated hepatocytes giving an estimation of freshly synthesized apoB in the two conditions (Table II). To compare experiments, the amount of 35S methionine incorporated into total cellular apoB (apoBH plus apoBL) in control hepatocytes was adjusted to 10,000 dpm/mg cell protein and the same factor was used to adjust the apoB radioactivity in insulin-treated hepatocytes. As shown in Table II, hepatocytes incubated for 12-14 h in medium containing 10 nM insulin incorporated 43% less label into total apoB, 58% less label into apoBH, and 34% less label into apoBL than hepatocytes incubated in control medium.

The amount of 35S methionine incorporated into cell protein during the 10-20 min time interval was measured by trichloroacetic acid precipitation and was somewhat larger in insulin-treated than control hepatocytes (mean percent increase over control ± S.D., n = 3): 25 ± 12%. The increased incorporation into protein corresponded to increased uptake of 35S methionine which was reflected by an increase in

![Fig. 1. Pulse-chase 1 protocol and apoB secretion into chase medium in pulse-chase 1 studies.](http://www.jbc.org/)

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**A. Pulse-chase protocol 1**

| TIME, min | 0 | 5 | 10 | 15 | 20 |
|-----------|---|---|----|----|----|
| SECRETED APO B (mg/10 mg cell protein) | 25 | 15 | 5 | 1 | 0 |

**B. Apo B mass secreted into medium (Pulse-chase 1)**

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**Fig. 1. Pulse-chase 1 protocol and apoB secretion into chase medium in pulse-chase 1 studies.** A, schematic presentation of the pulse-chase 1 protocol. Rat hepatocytes (100-mm dishes) were incubated with Waymouth's MB 752/1 medium containing 0.2% (w/v) BSA and either 0.1 nM insulin (control) or 10 nM insulin (insulin-treated) for 12-14 h. After incubation, medium was withdrawn and hepatocytes were labeled by incubation with medium containing 100 μCi of [35S]methionine/dish for 10 min. The labeled medium was removed and hepatocytes were reincubated in medium containing 0.2% (w/v) BSA and 10 mM L-methionine (chase medium) for 10, 20, 40, 90, and 180 min. B, apoB secretion during the chase period of pulse-chase 1 studies was assayed by monoclonal immunobassay. Control results are adjusted to 150 ng of apoB/mg of cell protein accumulated in medium in 180 min. Results of four rat liver experiments are presented as the mean ± S.D. at each time point. ○ and Δ, apoB secreted by hepatocytes incubated in control medium and in medium containing 10 nM insulin, respectively. *, apoB secreted by insulin-treated hepatocytes is significantly different from that secreted by control hepatocytes (p < 0.05).
Radioactivity (100%) was set to 0 min and other times during the chase period were adjusted accordingly. The initial half-life was calculated as described by Borchardt and Davis (42).

**Fig. 2.** Fluorograph of enhanced SDS-PAGE gels of immunoprecipitated [35S]methionine-labeled apoB100 and apoB100 of detergent-solubilized cells and media. Rat hepatocyte monolayers incubated in control and insulin medium were pulse-labeled and chased as described in the legend to Fig. 1. A (control hepatocytes) and B (insulin-treated hepatocytes) are fluorographs of cell- and medium-immunoprecipitated 35S-labeled apoB from pulse-chase studies at 40, 90, and 180 min of chase as indicated at the top of each lane. S, the beginning of the separating gel; and arrows, molecular weight 205,000, 96,000, and 68,000 marker proteins.

**TABLE I**

| Time | Apolipoprotein B100 | Apolipoprotein B100 |
|------|---------------------|---------------------|
|      | Control             | Insulin             | Control             | Insulin             |
|      | %                   | %                   | %                   | %                   |
| 10 min (3) | 96.8 ± 5.2 | 81.0 ± 12.2 | 99.2 ± 1.3 | 96.2 ± 3.2 |
| 20 min (4) | 94.9 ± 6.2 | 98.2 ± 3.6 | 90.6 ± 16.1 | 91.3 ± 11.4 |

Determined from curves plotted where the peak of cellular radioactivity (100%) was set to 0 min and other times during the chase period were adjusted accordingly. The initial half-life was calculated as described by Borechardt and Davis (42).

**TABLE II**

| Effect of insulin on incorporation of [35S]methionine into apoB |
|-------------------------------------------------------------|
| Hepatocytes (100-mm dishes) were incubated in medium containing 0.1 nM insulin (control) and 10 nM insulin for 12-14 h. Hepatocytes are labeled by incubation with [35S]methionine (100 µCi/dish) for 10 min and reincubated in chase medium containing 10 nM L-methionine. Labeled apoB of detergent-solubilized cells is isolated by immunoprecipitation and radioassayed. The proportion of 35S-labeled apoB100 and 35S-labeled apoB100 in immunoprecipitates is determined by scanning densitometry of fluorographs of enhanced SDS gels. The percent of label in each apoB band is multiplied times the total cellular apoB immunoprecipitated radioactivity per mg of cell protein which is measured by radioassay. Total 35S-labeled apoB in the control hepatocytes (apoB100 plus apoB100, radioactivity) is adjusted to 10,000 dpm/mg of cell protein and the same factor is used to adjust the apoB radioactivity in corresponding insulin-treated hepatocytes. Results are from three rat liver experiments and are expressed as the average ± S.D. |
| ApoB100 | ApoB100 | ApoB100 |
|--------|--------|--------|
| adjusted dpm/mg cell protein* |
| Control | 10,000 | 3,767 ± 428 | 6,233 ± 428 |
| Insulin | 5,669 ± 270 | 1,587 ± 466 | 4,082 ± 319 |
| % decrease | 48 ± 2.7 | 58 ± 9.2 | 34 ± 5.4 |
| relative dpm/mg cell protein* |
| Insulin | 5,168 ± 1,349 | 1,469 ± 632 | 3,700 ± 839 |
| % decrease | 48 ± 13.5 | 62 ± 12.6 | 40 ± 17.3 |

* Adjusted apoB label (dpm/mg cell protein) is the amount of cell synthesized apoB at 10 plus 20 min of the chase period when greater than 90% of the apoB label is intracellular.

Significant difference between control versus insulin-treated hepatocytes at a probability level of at least p < 0.02.

Relative apoB label (dpm/mg cell protein) is the adjusted apoB synthesized by insulin-treated hepatocytes calculated relative to the percent change in cellular protein synthesis at 10 plus 20 min of the chase period as determined by trichloroacetic acid precipitation. The increase in label incorporation into cellular protein in insulin-treated cells was (mean ± S.D.): 25 ± 12% for the three experiments. apoB100, apoB100, and apoB100 as a percent of total protein synthesis in control hepatocytes is 1.07, 0.40, and 0.67% and in insulin-treated hepatocytes is 0.48, 0.16, and 0.32%, respectively.

These two calculations differ, in part, because the intracellular retention half-time calculation includes the delay in reaching the peak of maximum radioactivity within the cell. In Figs. 3 and 4 the decay of cellular apoB100 and apoB100 in the cell in control and insulin-treated hepatocytes are compared. The average intracellular retention half-time of 35S-labeled apoB100 in control hepatocytes was significantly longer (85 min) than in insulin-treated hepatocytes (50 min). The half-life of 35S-labeled apoB100 in the cell also differed significantly between control versus insulin-treated hepatocytes, respectively, remained cell-associated (n = 4 rat livers). These results indicate that insulin in the medium stimulated the disappearance of newly synthesized apoB100 from the cell.

In contrast to almost complete disappearance of 35S-labeled apoB100 from hepatocytes, at 180 min of chase, 48 ± 8.0 and 32 ± 15.2% of freshly synthesized 35S-labeled apoB100 in control and insulin-treated hepatocytes, respectively, remained cell-associated (n = 4 rat livers). The intracellular retention half-time of 35S-labeled apoB100 was significantly longer than that of 35S-labeled apoB100 and averaged 140 min in control and 115 min in insulin-treated hepatocytes. The halflives of the initial decay of apoB100 in control and insulin-treated hepatocytes were similar (Fig. 4) and ranged from 80 to 100 min in individual experiments. The proportion of 35S-labeled apoB100 radioactivity in control and insulin-treated hepatocytes at 180
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Fig. 3. Disappearance kinetics of $^{[35]}$S-methionine-labeled cellular apoB, in pulse-chase 1 studies. The curves represent the time course of disappearance of labeled apoB from control hepatocytes (O) and from insulin-treated hepatocytes (O). Hepatocyte apoB radioactivity is the amount of immunoprecipitated $^{35}$S-labeled apoB found in the hepatocytes during the chase period as a percentage of the maximum $^{35}$S-methionine incorporated into hepatocellular apoB (100%) which was adjusted to zero time. Data points are calculated from seven separate liver preparations. A polynomial function described the curves and $R^2$ was 0.96 for control and 0.96 for insulin curves.

Fig. 4. Disappearance kinetics of $^{[35]}$S-methionine-labeled cellular apoB, in pulse-chase 1 studies. The curves represent the time course of disappearance of labeled apoB from control hepatocytes (O) and from insulin-treated hepatocytes (O). Hepatocyte apoB radioactivity is the amount of immunoprecipitated $^{35}$S-labeled apoB found in the hepatocytes during the chase period as a percentage of the maximum $^{35}$S-methionine incorporated into hepatocellular apoB (100%) which was adjusted to zero time. Data points are calculated from six separate liver preparations. A polynomial function described the curves and $R^2$ was 0.91 for control and 0.91 for insulin curves.

The apoB secretory rate assayed by monoclonal immunoassay between 2 and 3 h of incubation was reduced by the presence of insulin in the medium (mean ± S.D., n = 6 livers): 46 ± 7 and 27 ± 7 ng/mg/h, respectively (Fig. 6B).

Distribution of $^{[35]}$S-Methionine-labeled ApoB, and ApoB, at 180 Min in Pulse-Chase 1 Studies—The distribution of $^{35}$S-labeled apoB, and apo B, between hepatocytes and medium at 180 min is seen in Fig. 5. In control hepatocytes an average of 88% of $^{35}$S-labeled apoB, and 84% of $^{35}$S-labeled apoB, were recovered in media plus cells. In insulin-treated hepatocytes an average of 43% of $^{35}$S-labeled apoB, and 57% of $^{35}$S-labeled apoB, were recovered in media plus cells.

Insulin Effect on the Secretion of Immunoassayed ApoB in Pulse-Chase 2 Studies—In order to differentiate insulin effects on newly synthesized apoB versus the cellular apoB pool, we performed pulse-chase 2 studies (Fig. 6A). Hepatocyte monolayers were labeled in medium containing 0.1 nM insulin and $[^{35}]$S-methionine (100 μCi/dish) for 12-14 h to approach steady-state conditions. After the labeling period, medium was withdrawn, and the cells were rinsed and reincubated for 0.5, 1, 2, and 3 h (and in some case 5 h) in chase medium with and without insulin (10 nM). The apoB secretory rate assayed by monoclonal immunoassay between 2 and 3 h of incubation was reduced by the presence of insulin in the medium (mean ± S.D., n = 4 livers): 46 ± 7 and 27 ± 7 ng/mg/h, respectively (Fig. 6B).

Ratio of Cellular $[^{35}]$S-Methionine-labeled ApoB, to $[^{35}]$S-Methionine-labeled ApoB, in Pulse-Chase 2 Studies—After overnight labeling in control medium and before the chase period began, the cellular ratio of labeled apoB, to apoB, was 5.3 ± 2.2 (mean ± S.D., n = 6 livers). A similar ratio was obtained with $[^{35}]$S-[L-leucine] label used in pulse-chase 2 studies where the ratio of cellular labeled apoB, to labeled apoB, averaged 8.6. These results indicate that the cellular pool of apoB is predominantly apoB,.

Secretion of $[^{35}]$S-Methionine-labeled ApoB, and ApoB, in Pulse-Chase 2 Studies—After overnight labeling, the secretion of $^{35}$S-labeled apoB into chase medium containing no insulin or 10 nM insulin was examined at various time points during the chase period. $^{35}$S-Labeled apoB, was secreted rapidly and reached a plateau at about 1 h (Fig. 7). The presence of insulin in the medium delayed secretion of $^{35}$S-labeled apoB, by 1 h (Fig. 8), but afterwards the accumulation rate of $^{35}$S-labeled apoB, in medium paralleled that of control hepatocytes. These results suggest that after an initial readjustment (0-1 h), there is little additional effect of insulin on the secretion of $^{35}$S-labeled apoB, over the 3-h chase period. The secretion of cellular $^{35}$S-labeled apoB, continued to be linear up to 5 h of chase which was the latest time point assayed (results not shown).

Insulin Effect on Cellular ApoB Content—The apoB content...
of hepatocytes incubated in medium containing 0.1 nM insulin (control) and in 10 nM insulin for 12–14 h was measured by radioimmunoassay of freshly prepared cellular homogenates of primary hepatocytes. The averages of at least 5 dishes each of control and insulin-treated hepatocytes were compared from eight separate rat liver preparations. The apoB content of hepatocytes incubated with 10 nM insulin was modestly reduced compared with that of hepatocytes incubated in control medium (mean ± S.D.): 226 ± 36 versus 282 ± 25 ng/mg of cell protein, respectively, p < 0.003. The percent decrease of cellular apoB by insulin in the eight rat liver preparations averaged 19.8 ± 9.3% (mean ± S.D.).

**DISCUSSION**

The current study demonstrates that hepatocytes incubated for 12–14 h in medium containing 10 nM insulin incorporate significantly less \[^{13}S\]methionine into apoB\_TOTAL, apoB\_H, and apoB\_L than hepatocytes incubated in control medium (Table II). We have expressed results relative to cellular protein synthesis to control for precursor availability (41) with the assumption that there is no channeling of amino acid precursor in insulin-treated cells and that both apoB\_H and apoB\_L are derived from the same labeled amino acid pool. The estimate of apoB synthesis is based on the observation that there is a delay in the attainment of the peak of maximum label incorporation into cellular apoB (Table I). The delay is variable and ranges from 10 min in rat hepatocytes (42) to 10–25 min in HepG2 cells (37, 38). The reason for the delay may be due to elongation of preformed labeled apoB on ribosomes (42, 43). We chose to average the 10- and 20-min values as there were some differences in attainment of peak radioactivity; however, the values at 10 and 20 min were similar (Table I), and in both cases more than 90% of apoB radioactivity was cellular. If the calculation were made based on 10-min values, the reduction in apoB synthesis would have been (mean ± S.D., n = 3): 55 ± 10% for apoB\_H, and 42 ± 11% for apoB\_L, compared to 62 and 40% as reported in Table II. Whether apoB mRNA levels change in rat hepatocytes under insulin stimulation remains to be determined. Assuming the mechanism of insulin inhibition of apoB secretion in rat hepatocytes is similar to that recently described in HepG2
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The time course of \(^{35}S\)-labeled apoB\(_{100}\) disappearance from control and insulin-treated hepatocytes differed significantly from that of \(^{35}S\)-labeled apoB\(_{110}\). The intracellular retention half-time of \(^{35}S\)-labeled apoB\(_{100}\) was 140 min in control hepatocytes and 115 min in insulin-treated hepatocytes. After 180 min of incubation in chase medium a significant portion, 44% in control and 32% in the insulin-treated hepatocytes, had not been secreted and remained within hepatocytes. These results suggest that a substantial portion of newly synthesized apoB\(_{100}\) enters a presecretory pool. Movement of freshly synthesized apoB\(_{100}\) through the cell is not dramatically affected by insulin.

Most newly synthesized apoB\(_{110}\) (88%) and apoB\(_{110}\) (84%) were recovered at 180 min following pulse-labeling in medium and cells in control hepatocytes (Fig. 5). In hepatocytes incubated in medium containing 10 nM insulin only 43% of \(^{35}S\)-labeled apoB\(_{100}\) and 57% of \(^{35}S\)-labeled apoB\(_{110}\) were recovered. Low apoB recoveries in pulse-labeling studies were also obtained by Borchardt and Davis (42) using hepatocytes which were cultured in medium containing 1 \(\mu\)g/ml insulin (167 nM). They found only 36% of apoB\(_{100}\) and 60% of apoB\(_{110}\) were recovered in cells plus medium. In the current study an average of 45% of \(^{35}S\)-labeled apoB\(_{100}\) and 27% of \(^{35}S\)-labeled apoB\(_{110}\) degradation was insulin-dependent. In fluorographs of SDS-PAGE of \(^{35}S\)-labeled apoB of detergent-solubilized cellular and medium immunoprecipitates few labeled bands are present in gel regions corresponding to proteins smaller than intact apoB (Fig. 2). Smaller pieces of apoB are seen in cellular immunoprecipitates in the studies of Reuben et al. (15) and Davis et al. (46). Quantitatively, these pieces (<5% of the total apoB) are minor which suggests that degradation is rapid and relatively efficient. Although these fragments constitute a small proportion of total cell immunoprecipitates of apoB they may be important metabolically. Recent studies suggest that apoB degradation may occur early in the endoplasmic reticulum (46) and degradation of freshly translated apoB and its stimulation by insulin may regulate the proportion of apoB which enters the secretory pathway.

Although apoB degradation is a potential hypothesis to explain the lack of apoB recovery, an alternative hypothesis is that \(^{35}S\)-labeled apoB\(_{100}\) may have served as a precursor to \(^{35}S\)-labeled apoB\(_{110}\). This is unlikely as disappearance curves of apoB\(_{100}\) and apoB\(_{110}\) from the cell are not consistent with a precursor-product relationship. In addition, a significant portion of \(^{35}S\)-labeled-apoB\(_{100}\) is also degraded indicating that the process is not selective. Furthermore, the mechanism for generation of apoB\(_{110}\) and apoB\(_{100}\) in rat liver is not believed to be a result of proteolytic cleavage of apoB\(_{100}\) but rather the translation from two distinct mRNAs (15, 16).

Triglyceride but not apoB accumulates within insulin-treated hepatocytes (20–30) coincident to enhanced apoB degradation now reported. Our results suggest that insulin may prevent or uncouple lipid assembly with apoB rendering the unassembled and presumably membrane-bound nascent apoB polypeptide chain more susceptible to degradation in the endoplasmic reticulum. Alternatively, insulin action may directly target apoB for degradation. A protein phosphorylation-dephosphorylation mechanism is an obvious possibility considering the known role of insulin in the dephosphorylation of regulatory enzymes in intermediary metabolism and in the activation of cellular protein kinases (47). In this context, rat apoB has been shown to be phosphorylated on serine (40, 48) and tyrosine residues (40 and vanadate, a phosphotyrosine phosphatase inhibitor, can mimic insulin action by inhibiting apoB secretion by primary cultures of rat hepatocytes (32). Moreover, in primary cultures of hepatocytes from streptozotocin-induced diabetic rats, apoB secretion and cellular content of apoB is markedly reduced (19) and more highly phosphorylated forms of apoB may be present (40). The extent to which insulin alters the phosphorylation state of apoB and/or its fragments is currently being investigated.

In pulse-chase 2 studies where hepatic apoB is prelabeled, the presence of insulin in the chase medium has little effect on the 3 h accumulation of \(^{35}S\)-labeled apoB\(_{100}\) and apoB\(_{110}\) in the medium. The time course of secretion of labeled rat apoB\(_{100}\) was similar to that of prelabeled cellular apoB-100 by HepG2 cells (28). In both studies, the secreted of labeled apoB continues to be secreted even as long as 5 h after the start of the chase period. By the 3rd h the apoB secretory rate (2–3-h interval) as estimated by monoclonal immunoassay is significantly inhibited by the presence of insulin in the chase medium. Lack of synchrony between \(^{35}S\)-labeled apoB secretion and immunoassayable apoB reflects the contribution of newly synthesized apoB to the system. This is because during the chase period newly synthesized and secreted apoB is unlabeled due to the large molar excess of methionine in the chase medium whereas both labeled and unlabeled apoB are detectable by immunoassay. We have interpreted the lack of synchronous secretion of label and mass to be an indication of the predominant effect of insulin on newly synthesized apoB (unlabeled apoB) compared with a small effect of insulin on the secretion of prelabeled cellular apoB. Consistent with this interpretation is the implication that the hepatic pool of apoB (mostly apoB\(_{100}\)) is available for lipoprotein secretion and
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is not affected acutely by the presence of insulin in the medium.

The results of the current study are compatible with the presence of a cellular pool of apoBn. Several lines of evidence support this finding. First, in pulse-chase 1 studies, 44% of newly synthesized $^{35}$S-labeled apoBn in control hepatocytes is not affected acutely by the presence of insulin in the medium. This finding is consistent with the results of previous studies using liver perfusions. Second, in overnight labeling studies (pulse-chase 2) the ratio of $^{35}$S-labeled apoBL to $^{35}$S-labeled apoBn is 5.3 to 1. The calculated molar ratio of apoBL to apoBn (based on reported molecular weights) is on the order of 11 to 1, which supports the idea that apoBL forms a larger pool than apoBn. Furthermore, in pulse-chase 2 studies $^{35}$S-labeled apoBL continues to be secreted over 5 h compared with $^{35}$S-labeled apoBn, which is rapidly secreted. Consistent with the presence of a cellular apoBL pool in liver are studies by Swift et al. (49) who demonstrated the differential labeling of plasma very low density lipoprotein apoBn and apoBL in pulse-labeling studies using rat liver perfusions.

If the reduction in apoB secretion by insulin were due to simple inhibition of the apoB secretory pathway the apoB content of hepatocytes would theoretically increase with concomitant reduction of apoB secretion. Previous studies from our laboratory suggested that cellular apoB was somewhat reduced (27) or relatively unchanged (32) by insulin in the medium. These previous results were consistent with the studies of Patsch et al. (26) in that the total apoB in the system (medium plus cells) was reduced with insulin treatment. Considering that the current study demonstrates that insulin in the medium leads to a 48% decrease in label incorporation into total apoB and a substantial increase in apoB degradation, we were interested in determining whether cellular apoB levels were altered. In more rigorously controlled experiments using eight liver preparations and multiple dishes of hepatocytes we were able to show that there is a 20% reduction in cellular apoB with insulin considering the magnitude of the alterations in both synthesis and degradation; however, we do not know the factors that regulate entry into or exit out of the cellular pool.

If the amino acid sequence of apoBn and the amino-terminal portion of apoBL are identical, what accounts for differences in protein movement through the cell? What signal allows apoBn to be rapidly secreted and/or degraded and apoBL to be delayed in secretion? Differential rates of secretion of various hepatic secretory proteins have been reported (39, 43, 50) which have been attributed to the variability in rate of transport from the endoplasmic reticulum to the Golgi (39) and to differences in retention of specific proteins within the Golgi (39). Specific transport receptors and conversely specific retention signals have been postulated mechanisms to explain differences in protein transport through the cell. The current study implicates the carboxyl-terminal domain of apoBn as important in intracellular transport. ApoBL, which lacks this domain is not as rapidly transported as apoBn and forms the majority of cellular apoB. The nature of the signals for specific transport and degradation is the subject of ongoing studies.

In summary, our results demonstrate that insulin inhibits hepatic synthesis of apoBn and apoBL while stimulating intracellular degradation of apoB, a process which favors apoBL. The hepatic pool of apoB, which is predominantly apoBL, is relatively resistant to insulin and continues to be secreted. The overall result is a reduction in apoB-containing lipoprotein secretion with secretion of particles enriched in apoBL. We speculate that under insulin stimulation apoBL is the preferred particle secreted by the liver.

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In this section, we discuss the effects of insulin on rat hepatocyte ApoB. Several studies have investigated the role of insulin in modulating the synthesis and secretion of ApoB, a major component of very low density lipoprotein (VLDL). Insulin is known to stimulate the production of VLDL particles, and this effect is mediated, in part, by an increase in the transcription of the ApoB gene.

While the direct mechanism by which insulin acts on the liver to increase ApoB synthesis is still not fully understood, it is clear that insulin plays a crucial role in lipid metabolism. In the liver, insulin promotes the uptake of free fatty acids and glucose, which are then used for the synthesis of triglycerides and VLDL particles. This process is regulated by a complex interplay between insulin, glucagon, and other hormones, as well as by dietary factors.

Some of the key studies that have contributed to our understanding of the insulin-ApoB relationship include those by: Davidson, N. O., Carlos, R. C., Drewel, M. J., and Parmer, T. G. (1988) J. Lipid Res. 29, 1511-1522. Patsch, W., Franz, S., and Schonfeld, G. (1983) J. Clin. Invest. 71, 1161-1174. Sparks, C. E., Sparks, J. D., Bolognino, M., Salhanick, A., Strumph, P. S., and Amatruda, J. M. (1986) Metabolism 35, 1138-1136. Patsch, W., Gatto, A. M., Jr., and Patsch, J. R. (1986) J. Biol. Chem. 261, 9603-9606.

These studies, along with many others, have provided important insights into the molecular mechanisms underlying the regulation of ApoB synthesis in response to insulin. However, further research is needed to fully understand the complex interplay between insulin, metabolic signals, and the transcriptional regulation of the ApoB gene.
Insulin modulation of hepatic synthesis and secretion of apolipoprotein B by rat hepatocytes.
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