Treatment With Atorvastatin Ameliorates Hepatic Very–Low-Density Lipoprotein Overproduction in an Animal Model of Insulin Resistance, the Fructose-Fed Syrian Golden Hamster: Evidence That Reduced Hypertriglyceridemia Is Accompanied by Improved Hepatic Insulin Sensitivity

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A novel animal model of insulin resistance, the fructose-fed Syrian golden hamster has been previously documented to exhibit considerable hepatic very–low-denisty lipoprotein (VLDL) overproduction concomitant with the development of whole body insulin resistance. Here, we investigated whether hepatic lipoprotein overproduction can be ameliorated by treatment with a hydroxymethyl glutaryl conenzyme A (HMG-CoA) reductase inhibitor, atorvastatin, using a series of ex vivo experiments. Hamsters were fed a fructose-enriched diet for 14 days to induce a state of insulin resistance, and then continued on a fructose-enriched diet supplemented with or without 40 mg/kg atorvastatin per day for 14 days. Fructose feeding in the first 2 weeks caused a significant increase in plasma total cholesterol and triglyceride levels. There was a significant decline in plasma triglyceride levels following supplementation with the inhibitor (50% to 59%; $P < .05$). Experiments with primary hepatocytes revealed a decreased VLDL-apolipoprotein B (apoB) production (37.4% ± 10.4%; $P < .05$) in hamsters treated with atorvastatin. Interestingly, atorvastatin treatment partially attenuated (by 23%) the elevated hepatic level of microsomal triglyceride transfer protein (MTP) induced by fructose feeding. There was molecular evidence of improved hepatic insulin sensitivity with atorvastatin treatment based on assessment of the phosphorylation status of the insulin receptor and the expression of protein tyrosine phosphatase-1B. The improvement in insulin signaling was not mediated by a change in hepatic triglyceride accumulation as no significant difference was observed in liver triglyceride levels. Taken together, these data suggest that statins can ameliorate the VLDL-apoB overproduction state observed in a fructose-fed, insulin-resistant hamster model, and may potentially contribute to an enhanced hepatic insulin sensitivity.

A MAJOR UNDERLYING mechanism for lipid abnormalities observed in insulin-resistant states appears to be hepatic overproduction of very–low-density lipoprotein (VLDL). Insulin is known to influence the rate of hepatic VLDL production by controlling the rate of apolipoprotein B (apoB) synthesis and/or degradation, or by affecting the supply of free fatty acids to the liver for lipoprotein production.[1] It has been shown that insulin suppresses VLDL production by reducing plasma free fatty acid levels.[2] In the insulin-resistant state, where there is minimal or no response to insulin, VLDL production is no longer suppressed, resulting in an increased hepatic VLDL secretion. Evidence based on cultured HepG2 cells and some primary hepatocyte cultures have indicated that free fatty acids can acutely stimulate the secretion rate of apoB-containing lipoproteins[3,4] and that increased flux of free fatty acids increases the intracellular availability of triglycerides, therefore stimulating the production of VLDL particles.[5,6] This stimulatory effect of free fatty acids on apoB-lipoprotein secretion has not, however, been observed in other primary cell culture systems examined.[7-10]

Inhibition of hepatic hydroxymethyl glutaryl conenzyme A (HMG-CoA) reductase with statins has been shown to reduce the plasma levels of cholesterol and apoB-containing lipoprotein particles in subjects with familial hypercholesterolemia[11,12] or nonfamilial hypercholesterolemia.[13] Statins have also been shown to influence the secretion of both low-density lipoprotein (LDL)- and VLDL-apoB, in patients with combined and moderate hyperlipidemia.[13,14] More recently, statins were also found to be effective in reducing plasma triglyceride levels in patients with hypertriglyceridemia.[15-17] The mechanisms by which HMG-CoA reductase inhibitors lower plasma levels of apoB-containing lipoproteins have not been fully characterized. HMG-CoA reductase inhibitors are known to inhibit intracellular cholesterol biosynthesis, as well as induce the upregulation of LDL receptor expression.[18-20] Statins may also influence the hepatic production and secretion of VLDL, possibly via the reduction in cholesterol synthesis and/or through mechanisms of action on apoB, such as reduced translocation across the endoplasmic reticulum (ER) membrane, increased intracellular degradation, and diminished lipoprotein assembly.[21,22] Atorvastatin, a potent inhibitor of HMG-CoA reductase, was found to decrease the translocational efficiency of newly synthesized apoB in HepG2 cells, enhancing its susceptibility to proteasome-mediated degradation.[21]

Although several reports have recently appeared on statins and their mechanisms of action, no report is available on the mechanisms by which HMG-CoA reductase inhibition influences hepatic VLDL overproduction in insulin-resistant states. In the present study, we employed a novel animal model of insulin resistance, the fructose-fed Syrian golden hamster, to investigate whether statin treatment can hamper or block the

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Submitted March 8, 2001; accepted October 11, 2001.

Supported by operating grants from the Heart and Stroke Foundation of Ontario (T-4301) and Parke-Davis/Pfizer.

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0026-0495/02/5104-0002$35.00/0

doi:10.1053/meta.2002.30954

hepatic apoB overproduction induced by the development of insulin resistance. The Syrian golden hamster is an excellent model to investigate hepatic assembly and secretion of lipoproteins since its lipoprotein metabolism closely resembles that of humans.[10,23,24] Hamsters can be made obese, hypertriglyceridemic, hyperinsulinemic, and insulin-resistant by carbohydrate feeding, particularly fructose feeding.[25] Evidence for the development of insulin resistance in the fructose-fed hamster model was recently documented.[26] The fructose-fed hamster has increased plasma insulin, free fatty acid, and triglycerides, exhibits full body insulin insensitivity, reduced phosphorylation levels of the insulin receptor in hepatocytes, significant overproduction of VLDL-apoB and VLDL-triglyceride, and increased hepatic levels of microsomal triglyceride transfer protein (MTP).[26]

In the present study, we show that the lipoprotein abnormalities developed in the hamster in response to fructose feeding can be partially reversed by statin treatment. The hepatic overproduction of $apoB_{100}$-VLDL in the insulin-resistant hamster model, and the sensitivity of this process to HMG-CoA reductase inhibitors, make the hamster model very useful in investigating the pharmaceutical modulation of hepatic VLDL secretion.

MATERIALS AND METHODS

Male Syrian golden hamsters (*Mesocricetus auratus*) were purchased from Charles River (Montreal, Canada). Fetal bovine serum (certified grade), liver perfusion medium, hepatocyte wash medium, liver digest medium, and hepatocyte attachment medium were obtained from Life Technologies (Grand Island, NY). Polyclonal hamster apoB antiserum was raised in rabbit and purified in the laboratory. Anti-bovine MTP antibody was the generous gift of Dr David Gordon (Bristol-Meyers Squibb, New York, NY). Polyclonal insulin receptor β subunit antibody and monoclonal tyrosine phosphorylated protein antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal protein tyrosine phosphatase-1B (PTP-1B) antibody raised against the catalytic domain (Ab-1, cat # PH01) was provided by Oncogene Research Products (Cambridge, MA). Atorvastatin was obtained from Parke-Davis (Ann Arbor, MI) and Nutri-Cal, a palatable dietary supplement used as the vehicle for atorvastatin delivery, was obtained from Evsco (Buena, NJ). Fructose diet (60% fructose) was from Dyets Inc. (Bethlehem, PA). [^{35}S] protein labeling mixture was obtained from Mandel (Guelph, Canada). Protein concentrations were measured using the Pierce Micro bicinchoninic acid (BCA) protein assay (Rockford, IL).

Experimental Design and Statistical Analysis

Two groups of hamsters similar in age/weight were initially selected and fed a fructose-enriched diet for a 2-week period. Blood chemistries were measured at the end of the 2-week feeding protocol and then the hamsters were either continued to receive the fructose-rich diet for an additional 2 weeks or received the fructose-rich diet supplemented with 40 mg/kg body weight of atorvastatin for 2 weeks. Blood chemistries were measured at the end of the study and animals were then subjected to liver perfusion and isolation of hepatocytes.

Comparisons of the means of plasma cholesterol and plasma triglyceride levels at different weeks of feeding were analyzed by paired 1-way analysis of variance (ANOVA) using SPSS version 10.0 for the PC (SPSS, Inc, Chicago, IL). In all other experiments, comparisons of means between different treatment conditions were assessed based on Student *t* tests.

Administration of Atorvastatin

Syrian golden hamsters weighing approximately 95 g (34 to 40 days of age) were used in all studies. Two different protocols of atorvastatin delivery were utilized. In protocol A hamsters were simultaneously started on a 60% fructose diet supplemented with a daily dose of 40 mg of atorvastatin per kilogram of body weight for a period of 3 weeks. In protocol B hamsters were fed a fructose-rich diet for 2 weeks and then treated daily with 40 mg of atorvastatin per kilogram of body weight for an additional 2 weeks in combination with the fructose-rich diet. There was no significant difference in weight of the 2 groups receiving fructose or fructose + atorvastatin (95 ± 1.5 *v* 96.1 ± 1.2, respectively; $P = .57$). Atorvastatin was obtained from Parke-Davis (Pfizer, New York, NY) as a powder. The powder was mixed with approximately 200 mg of Nutri-Cal, a palatable nutrient supplement, and fed to the animals by hand to ensure accurate dose delivery. Control animals received equivalent quantities of Nutri-Cal.

Plasma Measurements

Hamsters were fasted for approximately 12 hours, anesthetized with isofluorane, and blood was sampled from the orbital sinus. Approximately 0.5 mL of blood was collected in EDTA (5 mg/mL final concentration). Plasma was separated by centrifugation (5 minutes, 4°C, $2,300 \times g$), and used to measure plasma glucose, cholesterol, and triglyceride levels on an Ortho Diagnostics (Raritan, NJ) Vitros chemistry analyzer. Free fatty acid concentrations were measured by an enzymatic, colorimetric assay kit from Wako Chemicals GmbH (Neuss, Germany).

Isolation of Primary Hamster Hepatocytes

Male Syrian golden hamsters weighing 100 to 120 g were anesthetized by inhalation of Isofluorane. After achieving complete general anesthesia, the liver was perfused and digested as recently described.[7] Hepatocytes released from digested liver tissue were washed 3 times in hepatocyte wash medium and transferred to culture medium (hepatocyte attachment medium containing 5% fetal bovine serum [FBS], 10 μg/mL insulin, 50 U/mL penicillin G sodium, 50 g/mL streptomycin sulfate). Viability of hepatocytes isolated in this manner ranged from 60% to 75% as measured by trypan blue dye exclusion. Cells were then seeded in collagen-coated dishes (1.5×10^6 living cells per 35-mm dish). After a 4-hour incubation at 37°C, 5% CO_2, attached cells were washed and used for experiments.

Pulse-Chase Labeling and Analysis of VLDL-ApoB Secretion

Pulse-chase labeling experiments were performed as described.[26] For analysis of VLDL-apoB secretion, primary hamster hepatocytes were preincubated in methionine-free minimum essential medium at 37°C for 1 hour and pulsed with 100 μCi/mL of [^{35}S] protein labeling mixture for 2 hours. Media was then collected for VLDL isolation and cells were harvested and lysed in 0.05 mol/L NaOH for protein assay. The density of the culture media was adjusted to 1.006 g/mL and VLDL was isolated by ultracentrifugation in a Beckman (Palo Alto, CA) LE-80K ultracentrifuge (18 hours, 35,000 rpm, SW55 Ti rotor). The VLDL fraction was collected, solubilized, and immunoprecipitated for apoB.

In Vitro Labeling and Analysis of Newly Synthesized Lipids

Freshly isolated adherent hamster hepatocytes were incubated in hepatocyte attachment medium supplemented with 5% FBS, antibiotics, and 0.0015 μg/mL insulin. This media also contained 5.0 μCi/mL [9,10-^{3}H]oleic acid mixed with unlabeled oleic acid and bound to bovine serum albumin (BSA) at a molar ratio of 8:1. The final concentration of oleate in 1 mL of media was 360 μmol/L. Under these conditions, cellular lipid synthesis is significantly upregulated, includ-

ing the incorporation of labeled oleate into triglyceride. Cells were incubated in the presence of the radiolabel for 18 hours, then the cells and media were collected and analyzed for cellular and secreted lipids as described previously.[26]

Immunoprecipitation, SDS-PAGE, and Fluorography

Lysates and media were immunoprecipitated as described.[27] Immunoprecipitates were washed three times and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) essentially as described.[28] The gels were fixed, incubated in Amplify (Amersham, Baie d'Urfé, PQ), dried, and exposed to Kodak X-Omat AR5 film (Eastman Kodak, Rochester, NY) at –80°C for 4 days. To quantitate radiolabled apoB and apoB fragments, the corresponding bands were visualized by fluorography, excised from the gel, digested, and counted in a scintillation counter.

Chemiluminescent Immunoblotting of the MTP 97-kd Subunit and PTP-1B

Cell lysate samples containing equivalent total protein were separated on SDS-PAGE mini-gels (8 × 5 cm) and subjected to immunoblot analysis as described[26] using either a rabbit anti-bovine MTP antiserum or a mouse monoclonal antibody raised against the catalytic subunit of PTP-1B. Quantitative analysis was performed using an imaging densitometer.

Assessment of Insulin Receptor Phosphorylation

In order to detect tyrosine phosphorylation of the insulin receptor β subunit, hepatocytes derived from control, fructose-fed, and fructose plus atorvastatin-fed hamsters were incubated for 5 hours in a serum- and insulin-free medium. Cells were then stimulated with 100 nmol/L insulin for 10 minutes at room temperature followed by lysis with a buffer containing several phosphatase inhibitors (150 mmol/L NaCl, 10 mmol/L tris [hydroxymethyl]aminomethane [pH 7.4], 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 1% Nonidet P-40, 2 mmol/L PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 100 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, and 2 mmol/L sodium orthovanadate). Lysate samples containing equivalent amounts of total protein were subjected to immunoprecipitation with a specific polyclonal antibody against the insulin receptor β subunit. Immunoprecipitates were subjected to immunoblotting with a monoclonal antiphosphotyrosine antibody (1:1,000 dilution) using the enhanced chemoluminescence (ECL) system described above.

RESULTS

Effect of Atorvastatin Feeding on Plasma Lipid Levels in Fructose-Fed Hamsters

Two different protocols of atorvastatin delivery were used to investigate the effect of atorvastatin on plasma total cholesterol and plasma triglyceride levels. Protocol A was designed to determine whether the concurrent administration of atorvastatin and a 60% fructose diet would inhibit the induction of the dyslipidemia associated with the development of insulin resistance. Hamsters were simultaneously started on a fructose-rich diet supplemented with a daily dose of 40 mg of atorvastatin per kilogram of body weight for a period of 3 weeks. Protocol B was designed to investigate whether atorvastatin is effective in reducing elevated plasma triglyceride levels observed in insulin-resistant animals. Hamsters were fed a fructose-rich diet for 2 weeks to fully induce insulin resistance prior to receiving daily doses of 40 mg of atorvastatin per kilogram of body weight for a period of 2 weeks. Weekly or biweekly blood samples were drawn from the orbital sinus and analyzed for plasma glucose, total cholesterol, and triglyceride levels. Control hamsters for both protocols received identical fructose feeding regimes with no atorvastatin supplementation, although the vehicle for atorvastatin delivery, Nutri-Cal, was administered. The timeline of fructose feeding and and dose of atorvastatin were based on optimization experiments and published studies in rodents. In long-term feeding studies, we have determined that the fructose-induced hyperlipidemia occurs over the first 4 to 6 weeks of treatment and is transient in nature. Long-term feeding (>6 weeks) would not be suitable for studies of the effect of hypolipidemic agents such as atorvastatin. For this reason, the present study was perfromed over 2 to 4 weeks of fructose feeding, a period during which significant hypertriglyceridemia is observed in this model. Dose-response studies in which cholesterol and triglyceride levels were determined in hamsters fed different concentrations of atorvastatin were conducted (data not shown). There was no significant decrease in plasma cholesterol levels at doses less than 20 mg/kg body weight. The dose of 40 mg/kg body weight was chosen for subsequent studies to obtain a significant lipid-lowering effect. Generally, small rodents require higher doses of statins than that needed in larger animals to obtain an equivalent lipid-lowering effect.

Figure 1, from protocol A, illustrates the plasma lipid levels for atorvastatin-treated and control hamsters. Figure 1A illus-

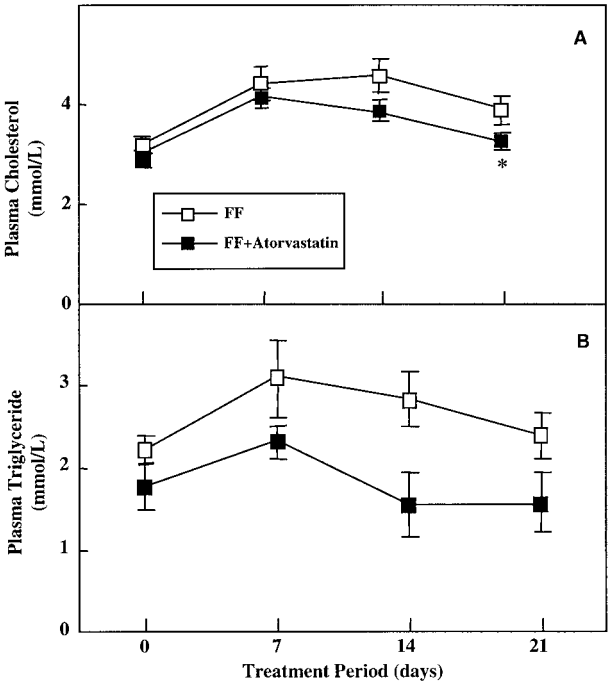

Fig 1. Plasma lipid levels in fructose-fed (FF) hamsters treated with and without atorvastatin (protocol A). Syrian golden hamsters were simultaneously fed a fructose-rich diet with or without atorvastatin (40 mg atorvastatin per kg body weight per day). Weekly blood samples were drawn from the orbital sinus. Plasma levels of cholesterol (A) and triglyceride (B) were measured using an Ortho Diagnostics Vitros chemistry analyzer. Mean ± SEM (8 hamsters per group). *$P < .05$ (based on paired 1-way ANOVA).

trates a significant increase in the initial plasma cholesterol levels taken 1 week after the treatment regimen began. Following 1 week of feeding, control fructose-fed hamsters (day 0: 3.2 ± 0.5 mmol/L; day 7: 4.4 ± 0.9 mmol/L; $P < .05$, n = 8) and fructose-fed hamsters treated with atorvastatin (day 0: 3.0 ± 0.5 mmol/L; day 7: 4.1 ± 0.7 mmol/L; $P < .05$, n = 8) showed 38% and 37% increases in plasma cholesterol levels, respectively. Statistical analysis showed that there was no significant difference in the baseline plasma cholesterol level between the 2 groups. Figure 1B illustrates an increase, although not significant, in plasma triglyceride levels 1 week after the initiation of treatment, while there was no significant difference in baseline levels of triglyceride. Control fructose-fed hamsters showed an increase of 41% (day 0: 2.2 ± 0.5 mmol/L; day 7: 3.1 ± 1.3 mmol/L; n = 8), whereas fructose-fed hamsters treated with atorvastatin showed a 28% increase (day 0: 1.8 ± 0.8 mmol/L; day 7: 2.3 ± 0.6 mmol/L; n = 8).

Although the initial rise in plasma cholesterol was significant for the drug-treated group, there was a significant decrease (based on paired 1-way ANOVA analysis) in plasma cholesterol following 3 weeks of treatment (day 7: 4.1 ± 0.7 mmol/L; day 14: 3.9 ± 0.7 mmol/L [decrease of 5%]; day 21: 3.3 ± 0.4 mmol/L [decrease of 20%]; $P < .05$, n = 8). Thus, while the effect at 2 weeks was not significant, that at 3 weeks was statistically significant. The control group did not show a significant decrease in plasma cholesterol as time progressed (day 7: 4.4 ± 0.9 mmol/L; day 14: 4.6 ± 0.9 mmol/L; day 21: 3.9 ± 0.8 mmol/L; n = 8). Plasma triglyceride levels did not change to a significant degree in either group following 1 week of treatment. Upon further treatment the plasma triglyceride in the control group remained at a slightly elevated level, with no significant changes over a 3-week period (day 7: 3.1 ± 1.3 mmol/L; day 14: 2.8 ± 0.9 mmol/L; day 21: 2.4 ± 0.7 mmol/L; n = 8). The atorvastatin group did demonstrate a decrease in triglyceride levels, although it did not reach statistical significance (day 7: 2.3 ± 0.6 mmol/L; day 14: 1.6 ± 1.1 mmol/L [decrease of 30%]; day 21: 1.6 ± 0.9 mmol/L; n = 8). These data suggest that atorvastatin may have a minor effect on plasma lipid levels when given concurrently with a diet enriched in fructose. It is likely that development of a hypercholesterolemic state may be required for the drug to exhibit its full hypolipidemic potential. The presence of the drug did appear, however, to hamper the rise in plasma cholesterol level in the fructose-fed hamster particularly at the end of the 3-week treatment protocol.

Figure 2, from protocol B, illustrates the plasma lipid levels for the atorvastatin and control groups. Following this protocol both groups were initially fed a fructose-rich diet and showed a significant increase in both plasma cholesterol (Fig 2A; day 0: 2.8 ± 0.4 mmol/L; day 14: 4.4 ± 1.1 mmol/L [an increase of 57%]; $P < .05$, n = 9 for control hamsters; day 0: 2.9 ± 0.4 mmol/L; day 14: 4.1 ± 0.8 mmol/L [an increase of 41%]; $P < .05$, n = 8 for hamsters to begin atorvastatin treatment) and plasma triglyceride levels (Fig 2B: day 0: 1.6 ± 0.7 mmol/L; day 14: 3.4 ± 0.6 mmol/L [an increase of 113%]; $P < .05$, n = 9 for control hamsters; day 0: 1.7 ± 0.7 mmol/L; day 14: 3.4 ± 1.5 mmol/L [an increase of 100%]; $P < .05$, n = 8 for hamsters to begin atorvastatin treatment). Statistical analysis showed that there were no significant differences in the baseline plasma cholesterol and triglyceride levels between the 2 groups of hamsters. After 2 weeks of fructose feeding, plasma insulin concentrations were significantly increased compared to chow-fed hamsters (chow-fed: 91.7 ± 14.5 pmol/L and fructose-fed: 204.0 ± 27.8 pmol/L; $P = .017$), suggesting the development of an insulin-resistant state.

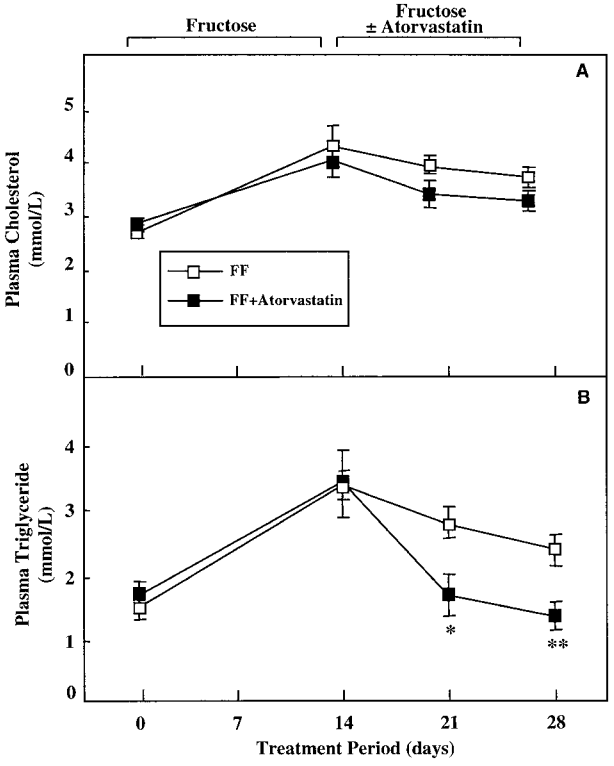

Fig 2. Plasma lipid profiles in fructose-fed (FF) hamsters treated with and without atorvastatin (protocol B). All hamsters were fed a high fructose diet for 2 weeks before the test group was started on daily doses of atorvastatin (40 mg/kg). Control animals were fed the same amount of Nutri-Cal (200 mg), the vehicle for atorvastatin delivery. Blood samples were drawn and analyzed for plasma cholesterol content (A) and triglyceride content (B) as described in Fig 1. Mean ± SEM (8-9 hamsters per group); *$P < .05$, **$P < .01$ (based on paired 1-way ANOVA).

Following initiation of drug treatment, the atorvastatin group showed a slight, but nonsignificant, decrease in plasma cholesterol levels (Fig 2A: day 14: 4.1 ± 0.8 mmol/L; day 21: 3.5 ± 0.7 mmol/L; day 28: 3.4 ± 0.6 mmol/L [a decrease of 17%]). The plasma cholesterol levels of the control group remained at an elevated level (day 14: 4.4 ± 1.1 mmol/L; day 21: 4.0 ± 0.5 mmol/L; day 28: 3.8 ± 0.6 mmol/L). These data suggest that atorvastatin does not significantly suppress elevated plasma cholesterol levels induced in a fructose-fed/insulin-resistant state for the first 2 weeks of treatment; however, a longer period of drug exposure may induce a more significant suppressive effect on plasma cholesterol levels in this model. With respect to plasma triglyceride levels, there was a significant reduction (based on paired 1-way ANOVA analysis) for the drug-treated group (Fig 2B: day 14: 3.4 ± 1.5 mmol/L; day 21: 1.7 ± 1.0 mmol/L [a decrease of 50%]; $P < .05$; day 28: 1.4 ± 0.6

mmol/L [a decrease of 59%]; $P < .01$, n = 8), compared to the control group (day 14: 3.4 ± 0.6 mmol/L; day 21: 2.8 ± 0.7 mmol/L; day 28: 2.4 ± 0.7 mmol/L). These data suggest that treatment with atorvastatin results in a significant decline in elevated plasma triglyceride levels induced by fructose feeding.

Effect of Atorvastatin Feeding on Plasma Free Fatty Acids and Hepatic Triglyceride Accumulation

Plasma free fatty acid levels were also measured in samples drawn at the end of the feeding protocols. Free fatty acid levels were similar with or without atorvastatin treatment (fructose-fed, n = 6: 2.17 ± 0.68 mmol/L; atorvastatin-treated, n = 7: 2.51 ± 1.5 mmol/L, $P = .60$).

Liver tissue harvested from fructose-fed animals treated with and without atorvastatin were subjected to lipid extraction by homogenization in the presence of chloroform/methanol (3/2).[29] Extracted lipids were dried under vacuum and resuspended in phosphate-buffered saline (PBS) containing 1% Triton X-100. Triglyceride content of tissue total lipid extracts was measured by a colorimetric assay (Randox, Crumlin, UK). There was no significant difference in triglyceride content in the 2 groups (fructose-fed: 2.90 ± 1.11 mg/g tissue, fructose + atorvastatin: 2.68 ± 0.55 mg/g tissue; $P > .05$; 3 hamsters per group; 2 liver specimens from each hamster analyzed in triplicate). These data suggest that a 2-week treatment with atorvastatin does not alter the hepatic accumulation of triglyceride in fructose-fed hamsters.

Atorvastatin Feeding Inhibits Hepatic VLDL-ApoB and Lipid Production in Fructose-Fed Hamsters

Fructose feeding has been shown to significantly increase hepatic production and secretion of VLDL-apoB in the hamster model.[26] To investigate the effect of atorvastatin on the production of hepatic VLDL-apoB from fructose-fed hamsters, a series of in vitro labeling experiments was conducted. Primary hamster hepatocytes from fructose-fed hamsters treated with and without atorvastatin (protocol B) were obtained using the liver perfusion/digestion protocol. Primary hepatocytes were labeled with [^{35}S] protein labeling mix for 2 hours and secreted VLDL was isolated by ultracentrifugation. VLDL-associated apoB was immunoprecipitated and quantitated.

Figure 3A illustrates hepatic VLDL-apoB secretion in control and fructose-fed hamsters treated with atorvastatin, with the VLDL-apoB secreted by the control hamster hepatocytes taken as 100%. Hepatic VLDL-apoB secretion was significantly suppressed with atorvastatin feeding. Hepatocytes from fructose-fed hamsters treated with atorvastatin secreted 46.6% less VLDL-apoB than control fructose-fed hepatocytes (VLDL-apoB level in fructose + atorvastatin–fed hamster hepatocytes was 53.4% ± 27.2% [$P < .01$, n = 6] of that in fructose-fed hamster hepatocytes). These data support the hypothesis that atorvastatin is effective in inhibiting the overproduction of hepatic VLDL-apoB found in fructose-fed hamsters.

Hepatocytes isolated from fructose-fed animals treated with and without atorvastatin were also analyzed for synthesis and secretion of triglyceride, cholesterol, and cholesterol ester. Cellular triglyceride synthesized in drug treated hamster hepatocytes did not significantly differ from that in fructose-fed

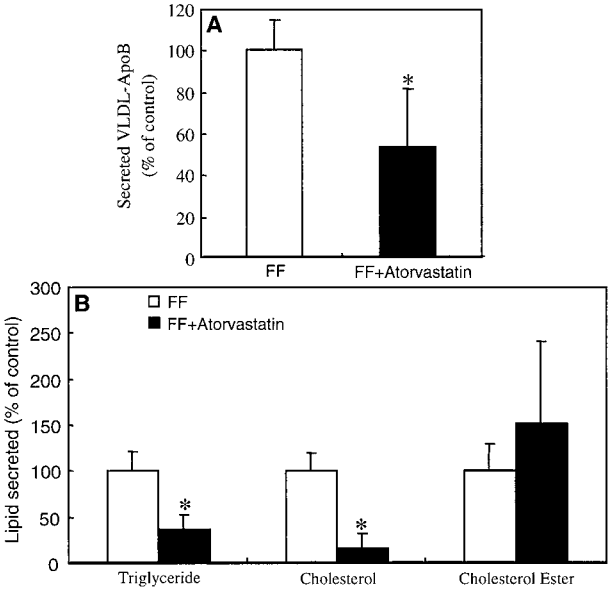

Fig 3. Ex vivo hepatic production of VLDL-apoB and lipids in response to atorvastatin treatment. (A) Primary hamster hepatocytes were pulsed for 2 hours with [^{35}S] protein labeling mixture. The density of the culture media was adjusted to 1.006 g/mL and VLDL was isolated by ultracentrifugation. VLDL fraction was collected and apoB was immunoprecipitated. The immunoprecipitates were analyzed by SDS-PAGE and fluorography (7 hamsters per group), *$P < .05$. (B) Cellular lipid secretion was evaluated by exposing cells to [^{3}H]acetate or [^{3}H]oleate, lipid extraction from culture medium, separation by thin-layer chromatography, and quantitation by liquid scintillation counting. Data are represented as a percentage of lipid secreted from fructose-fed (FF) cells. Mean ± SD (6 hamsters analyzed in triplicate). **$P < .001$.

hamster hepatocytes (87,876 ± 18,502 counts per minutes [cpm] *v* 99,154 ± 30,790 cpm in fructose-fed and fructose + atorvastatin–fed hamster hepatocytes; n = 6). The synthesis of cholesterol and cholesterol ester were both slightly reduced by drug treatment but neither reached statistical significance (data not shown). However, atorvastatin feeding significantly reduced triglyceride secretion by 63% ± 15% (Fig 3B). While there was no significant effect on cholesterol ester secretion, the secretion of free cholesterol was also significantly reduced with atorvastatin treatment (Fig 3B).

Effect of Atorvastatin Feeding on Total ApoB Secretion and Intracellular Stability in Cultured Hepatocytes

To investigate the effect of atorvastatin on intracellular apoB stability in fructose-fed hamsters, a series of pulse-chase labeling experiments was conducted. Primary hamster hepatocytes from fructose-fed hamsters treated with and without atorvastatin, as per protocol B, were obtained using the liver perfusion/digestion protocol, pulsed for 45 minutes, and then chased for 0, 1, and 2 hours. Cell lysates and media were collected and apoB was isolated by immunoprecipitation.

Figure 4 illustrates the effect of atorvastatin on intracellular apoB levels and on secreted apoB levels after 0, 1, and 2 hours chase. The intracellular apoB levels at the end of the pulse period showed a significant difference. Atorvastatin feeding

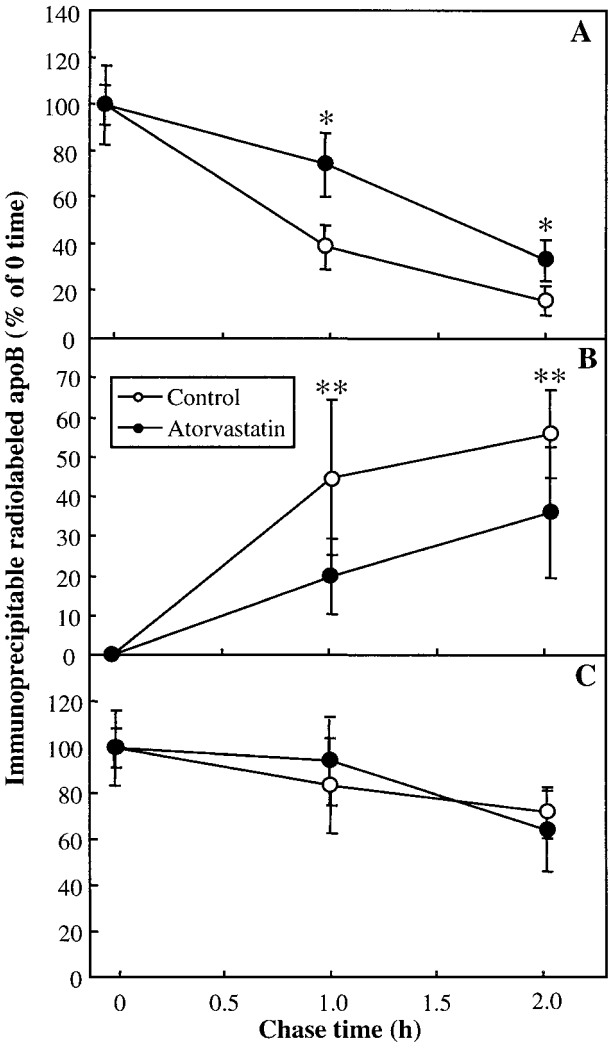

Fig 4. Ex vivo synthesis and secretion of apoB in response to atorvastatin treatment. Primary hamster hepatocytes were pulsed for 45 minutes with [^{35}S] protein labeling mixture and chased for 1 and 2 hours with excess unlabeled methionine. Cell lysates and media were collected for apoB immunoprecipitation, SDS-PAGE, and fluorography. (A) Cells; (B) media; (C) total apoB remaining in cells + media. Mean ± SD (5 hamsters per group). *P < .002. **P < .05. (Student t test).

resulted in hepatocytes accumulating 49% less apoB within a 45-minute labeling period (control: 8,264 ± 3,493 cpm/mg protein; atorvastatin: 4,222 ± 3,313 cpm/mg protein; P = .019, n = 5). The reduction in apoB accumulation during the pulse period may be attributed to either a reduction in apoB synthesis rate or an increase in rapid, cotranslational degradation of apoB. Further studies are needed to determine the exact mechanism underlying this effect. In order to analyze potential differences in cellular and secreted apoB during the chase period, all data were normalized to cellular apoB content at the beginning of the chase and expressed as a percentage of this value. This analysis demonstrated that the cellular apoB content after both 1 hour (P = .002) and 2 hours (P = .0004) of chase was significantly lower in control cells (Fig 4A). The opposite was true in media samples collected, which reflected relatively greater secretion of apoB from control cells after 1 hour (P = .034) and 2 hours (P = .014) of chase (Fig 4B).

The amount of newly synthesized apoB that undergoes degradation can be inferred by comparing the sum of apoB detected in cells and media at each chase time point to the total detected at the beginning of the chase. Figure 4C illustrates that there was no significant difference in degradation of apoB in cells derived from control or atorvastatin-treated hamsters.

Atorvastatin Feeding Alters MTP Protein Levels in Livers of Fructose-Fed Hamsters

We have recently shown that fructose feeding over a 2- to 3-week period induces an increase in the hepatic level of MTP protein[26] and MTP mRNA (unpublished observation, October 1999). It was thus important to determine whether atorvastatin feeding of fructose-fed hamsters attenuates hepatic MTP overproduction, thereby contributing to its lowering of hepatic apoB secretion. Hepatocytes from control, fructose-fed, and fructose + atorvastatin–fed hamsters were used to perform immunoblotting experiments with an anti-bovine MTP antibody. Figure 5 shows the level of MTP protein mass detected in hepatocytes of animals fed either fructose alone or fructose + atorvastatin. Similar to our previous observations, fructose feeding of hamsters resulted in a significant elevation of MTP protein levels in the liver. However, MTP protein mass in hepatocytes derived from atorvastatin treated hamsters decreased 24% relative to fructose-fed hamsters, although it was not restored to the level observed in control hepatocytes.

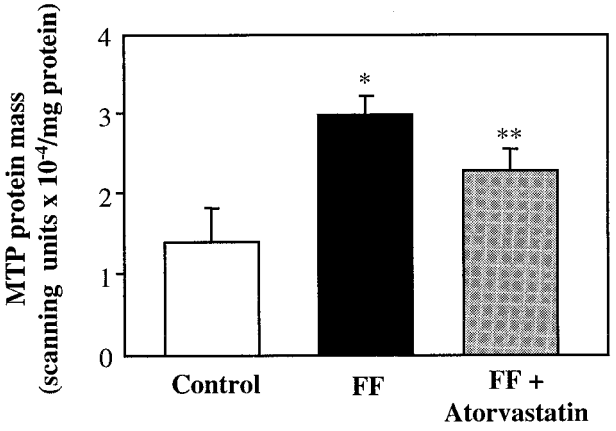

Fig 5. Effect of atorvastatin treatment on hepatic MTP protein levels in fructose-fed (FF) hamsters. Hepatocytes obtained from control, FF, and FF + atorvastatin–fed hamsters were solubilized and equal amounts of cell protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was conducted to detect the 97-kd MTP subunit using an anti-bovine MTP antiserum. MTP bands were quantitated by densitometric scanning and the net band intensity is expressed per μg of cell protein. Mean ± SEM (6 liver specimens from 2 hamsters per group). *Significantly different from control (P = .04). **Significantly different from FF animals (P = .027), but not different from control (P = .18).

Atorvastatin Feeding Enhances Phosphorylation of the Insulin Receptor in Hepatocytes Isolated From Fructose-Fed Hamsters

Fructose feeding of hamsters induces the development of whole body insulin resistance,[26] as well as inhibiting insulin action in the hamster liver as documented by a reduced level of insulin receptor and insulin receptor substrate-1 (IRS-1) phosphorylation, reduced phosphoinositol-3 kinase activity, as well as enhanced expression of PTP-1B.[30] Here we examined whether atorvastatin feeding affects insulin signaling in hepatocytes from fructose-fed animals by assessing the phosphorylation status of the insulin receptor. Hepatocytes isolated from control, fructose-fed, and fructose + atorvastatin–fed hamster livers were incubated in serum-free media for 5 hours and then stimulated in vitro with 100 nmol/L insulin. Cells were then lysed and subjected to immunoprecipitation for the insulin receptor. Immunoprecipitates were run on SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with an antiphosphotyrosine antibody. Figure 6A shows the phosphorylation level of hepatic insulin receptor under various treatments. Fructose feeding reduced phosphorylation of the insulin receptor by 75.6% ± 0.7% ($P = .027$) relative to control hepatocytes, suggesting the induction of insulin resistance at the molecular level. Treatment of fructose-fed hamsters with atorvastatin increased the phosphorylation level of the insulin receptor significantly ($P = .031$) relative to untreated fructose-fed hamsters and restored it to the levels of phosphorylation in control hamster hepatocytes (112.3% ± 11.2%).

PTB-1B Levels Are Suppressed by Atorvastatin Feeding

PTP-1B has recently been implicated in the induction of insulin resistance[31,32] and appears to specifically inactivate the insulin receptor and IRS-1.[33,34] The effects of both fructose feeding and atorvastatin treatment on expression of PTP-1B were evaluated in this model. Figure 6B depicts the mass of PTP-1B detected in cell lysates harvested from control animals fed chow diet as well as fructose-fed animals and animals fed both fructose and atorvastatin according to protocol B. Intracellular mass of PTP-1B was significantly increased by fructose feeding (51% increase, $P < .0001$). However, atorvastatin treatment significantly reduced the expression level ($P = .0003$) to a value lower than that seen in chow fed animals (63% of control, $P = .046$).

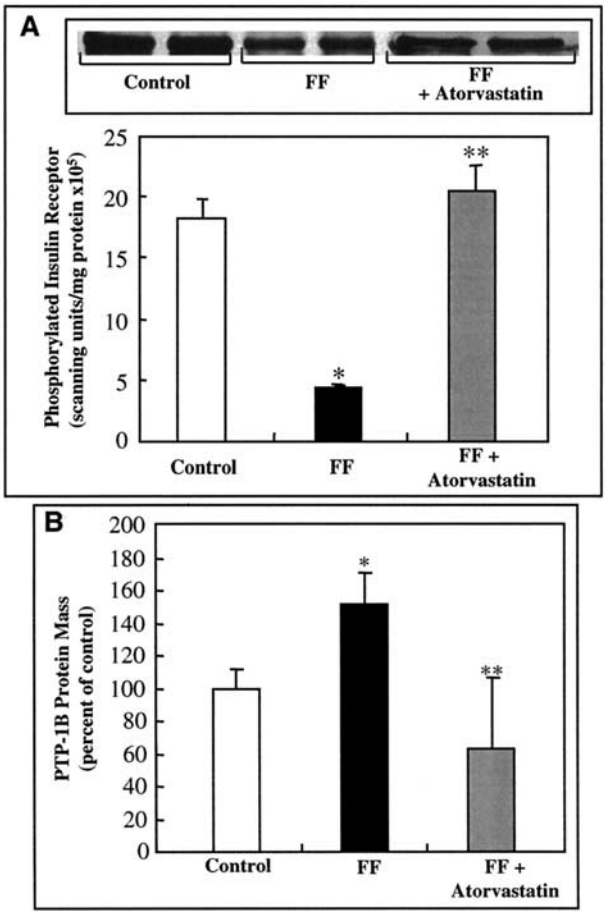

Fig 6. (A) Effect of atorvastatin treatment on insulin receptor phosphorylation in fructose-fed (FF) hamster hepatocytes. Primary hamster hepatocytes obtained from control, FF, and FF + atorvastatin–fed hamsters were incubated in a serum- and insulin-free medium for 5 hours and then stimulated with 100 nmol/L insulin for 10 minutes. Cells were solubilized and subjected to immunoprecipitation with a rabbit anti-human insulin receptor β subunit antiserum. Immunoprecipitates were subjected to immunoblotting with a mouse monoclonal antibody raised against phosphotyrosine residues. The tyrosine-phosphorylated insulin receptor bands (representative blot shown in inset) were quantitated by densitometric scanning and the net intensity of the bands was normalized to cellular protein. Mean ± SEM (4 samples analyzed from 2 hamsters per group). *Significant difference relative to control, $P = .027$. **Significant difference relative to FF, $P = .031$. (B) Effect of atorvastatin treatment on PTP-1B protein mass. Hepatocytes obtained from control, FF, and FF + atorvastatin–fed hamsters were solubilized and equal amounts of cell protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was conducted to detect the protein mass of PTP-1B using a monoclonal antibody. PTP-1B bands were quantitated by densitometric scanning and the net band intensity was expressed per μg of cell protein. Mean ± SEM (9 samples analyzed from 3 hamsters per group). *Significantly different from control, $P < .0001$. **Significantly different from control ($P = .046$) and FF ($P = .0003$).

DISCUSSION

Increasing evidence from apoB kinetic studies in both experimental animals and humans indicates that HMG-CoA reductase inhibitors lower plasma apoB concentrations by decreasing hepatic VLDL-apoB secretion.[35,36] These in vivo observations are supported by in vitro evidence from studies involving statin-treatment of cultured hepatocytes.[37,38] The intracellular mechanisms mediating statin-induced inhibition of hepatic VLDL secretion have been the subject of intense investigation in recent years. Statin treatment (eg, atorvastatin) appears to inhibit the assembly and secretion of apoB-containing lipoproteins by enhancing apoB intracellular degradation and reducing its translocation across the ER membrane.[21,22] however, it is unknown whether the process of VLDL synthesis and secretion is sensitive to statin-mediated inhibition in the state of insulin resistance, a condition in which there is considerable stimulation of hepatic VLDL production.

To address this question and to explore the potential mech-

anisms, we employed a recently characterized model of diet-induced insulin resistance, namely, the fructose-fed Syrian golden hamster. This animal model was recently shown to exhibit the prominent features of the insulin resistance syndrome including elevated plasma insulin, free fatty acid, and triglyceride concentrations, and whole body insulin insensitivity (as documented by a euglycemic hyperinsulinemic clamp technique).[26] Recent unpublished studies in our laboratory have also confirmed hepatic insulin resistance in fructose-fed hamsters based on evidence including suppressed phosphorylation status of the insulin receptor and IRS-1, as well as reduced activity of phosphotyrosine-associated phosphoinositol 3-kinase activity (manuscript submitted). The mechanism by which fructose induces peripheral and hepatic insulin resistance is unknown, although it has been suggested that fructose interferes with glucose metabolism in these tissues.[39] In the present study, elevated plasma insulin and triglyceride levels suggested the development of insulin resistance in animals after 2 weeks of feeding with fructose. Although we did not perform clamp studies in the hamsters used in the present study, we have found and reported[26] that fructose feeding for a 2-week period consistently results in the development of whole body insulin insensitivity. Our observation of reduced phosphorylation of the insulin receptor in fructose-fed animals further supports the induction of insulin resistance in animals used in the current study. Evidence for development of insulin resistance in fructose-fed hamsters has also been reported elsewhere.[25]

Two protocols were employed to test the effect of atorvastatin treatment on dyslipidemia found in fructose-fed hamsters. Protocol A involved simultaneous feeding with both fructose and atorvastatin for a 3-week period while protocol B animals were fed fructose for 2 weeks followed by supplementation of the fructose-enriched diet with atorvastatin for a further 2-week period. Hamsters fed a diet enriched with both fructose and atorvastatin developed slightly lower (not statistically significant) plasma cholesterol levels and considerably lower triglyceride levels (statistically significant) over 3 weeks compared to those fed fructose alone. This suggests that atorvastatin feeding may interfere with the development of hypertriglyceridemia normally induced by fructose feeding. Indeed, triglyceride levels did not change significantly in animals fed both fructose and atorvastatin when compared with those fed fructose alone (which had a higher triglyceride level at weeks 1 and 2 but not at week 3). Atorvastatin did not affect the significant rise in plasma cholesterol levels found after fructose feeding but did cause an eventual decline in plasma cholesterol such that it reached basal levels after 3 weeks of treatment. In protocol B we attempted to induce the state of insulin resistance and then initiate treatment with atorvastatin in order to observe any potential hypolipidemic effects of this drug in animals with established insulin resistance. Fructose feeding increased plasma levels of both cholesterol and triglyceride but only triglyceride levels reverted to basal following a 2-week treatment with atorvastatin. Thus statin treatment was successful in reversing the hypertriglyceridemia induced by fructose feeding. Protocol B was chosen for subsequent studies on hepatic VLDL production.

The statin-induced reduction in plasma levels of triglyceride in our animal model is consistent with recent observations in hypertriglyceridemic patients undergoing statin therapy.[15-17,40] The decline in plasma triglyceride can be attributed to either an increased clearance of triglyceride-rich lipoprotein particles or a reduced secretion of apoB-containing lipoprotein particles. Examining isolated hepatocytes from livers of drug-treated animals appeared to support the latter possibility as there was a significant suppression of hepatic triglyceride secretion. We did not examine the clearance rate of triglyceride-rich lipoproteins in this study and it is thus possible that plasma clearance may also be enhanced with atorvastatin treatment, as statins are known to induce expression of the LDL receptor.[41,42]

Atorvastatin feeding significantly reduced the ex vivo secretion of VLDL-apoB from hepatocytes cultured from livers of fructose-fed hamsters. Surprisingly, there was no significant difference in apoB stability. Although there was a trend toward enhanced degradation in hepatocytes derived from atorvastatin-treated hamsters, it did not reach statistical significance. Statin-induced stimulation of apoB degradation has previously been reported in HepG2 cells and normal primary hepatocytes treated in vitro with atorvastatin.[21,22,37,38] Overall, the atorvastatin-induced inhibition of VLDL-apoB secretion supports the hypothesis that the VLDL overproduction found in insulin-resistant states can potentially be reversed with statin treatment.

It was also important to determine whether atorvastatin-induced reduction in hepatic VLDL-apoB secretion is related to an indirect effect on MTP expression. We have recently reported[26] that fructose-fed hamsters have a greater expression of MTP as indicated by increased MTP protein mass (as well as MTP mRNA levels, unpublished observations, October 1999). The recent finding that the MTP gene promoter contains a sterol response element and can potentially be regulated by alterations in intracellular cholesterol concentrations[43] further supports the possibility that statin feeding may alter the expression of hepatic MTP. In accord with this hypothesis, our present experiments showed a significant reduction in hepatic MTP protein levels in response to atorvastatin feeding. It is presently unknown whether this statin-mediated reduction in MTP levels is a direct effect via changes in intracellular sterol pools and the sterol regulatory element binding protein (SREBP) pathway, or is secondary to other effects of statin treatment (eg, reduced intracellular triglyceride availability).

Previous reports on the effects of statins on metabolic control/insulin sensitivity in non–insulin-dependent diabetes mellitus (NIDDM) patients appear contradictory. Paolisso et al,[44,45] in 2 separate studies using simvastatin and atorvastatin, reported that statins improved insulin action in NIDDM patients as measured directly (euglycemic, hyperinsulinemic clamp) and indirectly (homeostasis model assessment indexes). In contrast, Farrer et al[46] and Ohrvall et al[47] did not observe any beneficial effects of statins on insulin action in NIDDM patients. To address this question in our study we measured insulin receptor phosphorylation status in hepatocytes of fructose-fed hamsters treated with or without atorvastatin. Interestingly, atorvastatin treatment of fructose-fed animals appeared to ameliorate hepatic insulin resistance based on assessment of the phosphorylation status of the insulin receptor in isolated hepatocytes. We also found that atorvastatin reduced the intracellular level of PTP-1B, a protein tyrosine phosphatase, which is enhanced in response to fructose feeding. The mechanism(s)

by which treatment with an HMG-CoA reductase inhibitor in hamsters influences hepatic insulin signaling and insulin sensitivity is currently unknown. It has recently been proposed that triglyceride accumulation (steatosis) in non-adipose tissues such as the liver and muscle may be an important causative factor in the induction of insulin resistance in these tissues. However, there was no evidence of a change in triglyceride accumulation in the livers of fructose-fed animals, suggesting that the beneficial effect on insulin signaling may not be related to ectopic triglyceride accumulation in the liver. Nevertheless, we cannot rule out the possibility that longer fructose feeding may induce hepatic triglyceride accumulation in this model.

In summary, our current experiments provide evidence supporting the effectiveness of statins in treating hypertriglyceridemia and hepatic VLDL overproduction observed in insulin-resistant states. Inhibition of HMG-CoA reductase appears to exert this beneficial effect at least in part by blocking hepatic assembly and secretion of apoB-containing lipoproteins. A further benefit of attenuated hypertriglyceridemia may be improved insulin sensitivity.

ACKNOWLEDGMENT

We thank Dr M. Haidari for help with the statistical analysis.

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