Transcriptional regulation of the apoC-III gene by insulin in diabetic mice: correlation with changes in plasma triglyceride levels

May Chen,*, Jan L. Breslow,** Weihua Li,*,† and Todd Leff[1,*,†,**]**

Department of Biotechnology,* Parke-Davis, 2800 Plymouth Road, Ann Arbor, MI 48105; Department of Biological Chemistry,† University of Michigan, Ann Arbor, MI 48104; and Laboratory of Biochemical Genetics and Metabolism,** The Rockefeller University, New York, NY 10021

Abstract Insulin-dependent diabetes mellitus (IDDM) is associated with elevated plasma triglyceride levels that normalize after insulin administration. The observation that overexpression of the apoC-III gene in transgenic mice can cause hypertriglyceridemia and other evidence implicating apoC-III in the regulation of triglyceride levels prompted us to examine whether apoC-III might be involved in the hypertriglyceridemia associated with IDDM. To this end, the regulation of apoC-III gene expression was studied in the streptozotocin-treated mouse model of IDDM. In the insulin-deficient diabetic state, these mice have elevated glucose and triglyceride levels and a 1.4- to 1.5-fold increase in hepatic apoC-III mRNA levels, by Northern analysis as well as quantitative solution hybridization RNase protection assay. Insulin treatment normalized the glucose and triglyceride levels and diminished hepatic apoC-III mRNA levels by 59%. Analysis of transcription rates using the nuclear run-on technique demonstrated that the changes in hepatic apoC-III mRNA levels were the results of changes in the transcriptional activity of the gene. To determine the role of insulin in the regulation of apoC-III transcription, HepG2 cells were transfected with an apoC-III reporter construct, and treated with different insulin concentrations. The results demonstrated that insulin treatment induced a dose-dependent down-regulation of apoC-III transcriptional activity. These data suggest that the apoC-III transcriptional changes seen in animals are caused by differences in insulin concentrations. Assuming that apoC-III mRNA levels reflect the synthesis and secretion of the protein, these results present the possibility that overexpression of the apoC-III gene could contribute to the hypertriglyceridemia observed in IDDM.—**Chen, M., J. L. Breslow, W. Li, and T. Leff.** Transcriptional regulation of the apoC-III gene by insulin in diabetic mice: correlation with changes in plasma triglyceride levels. *J. Lipid Res.* 1994. **35:** 1918–1924.

Supplementary key words apoC-III • triglyceride • lipoproteins • diabetes • gene expression • transcription • insulin

In IDDM poorly controlled patients exhibit elevated plasma triglyceride levels that are normalized with insulin treatment (1–4). This has principally been ascribed to two effects of insulin deficiency. Insulin has been shown to increase the levels of lipoprotein lipase and the insulin-deficient diabetic state has been associated with diminished tissue lipoprotein lipase activity (5–7). As lipoprotein lipase is the principal enzyme that hydrolyzes triglycerides in chylomicrons and very low density lipoproteins (VLDL), these triglyceride-rich particles would accumulate in insulin deficiency and cause hypertriglyceridemia. The second mechanism relates to insulin's ability to inhibit the activity of hormone-sensitive lipase. This enzyme is present in adipose tissue where it hydrolyzes stored triglyceride. The free fatty acids liberated go to the liver where they stimulate the secretion of VLDL triglycerides. Insulin deficiency results in excess hormone-sensitive lipase activity. This floods the liver with free fatty acids causing increased VLDL production and if removal is saturated, as it often is, increases triglyceride levels (8, 9).

In the current study we explore another possible mechanism that might contribute to the hypertriglyceridemia in IDDM. Previous studies with transgenic mice have shown that expression of the human apoC-III transgene can cause hypertriglyceridemia (10, 11). Triglyceride levels were proportional to the level of transgene expression and as little as 30–40% excess apoC-III could double the triglyceride levels. In addition, the apoC-III gene promoter is known to contain a consensus sequence resembling a negative insulin response element of other genes (12). The transgenic mouse results plus the promoter architecture suggests that the hypertriglyceridemia in IDDM may in part be due to the loss of insulin repression of apoC-III gene transcription resulting in overexpression of the gene. Indeed the current study shows that insulin deficiency in the mouse results in in-

Abbreviations: apo, apolipoprotein; IDDM, insulin-dependent diabetes mellitus; VLDL, very low density lipoprotein.

[1]To whom correspondence should be addressed.

creased hepatic apoC-III mRNA levels that are reversed by insulin treatment, and that the effects are largely at the transcriptional level. These results raise the possibility that apoC-III overexpression may occur in IDDM and could contribute to the hypertriglyceridemia associated with this condition.

MATERIALS AND METHODS

Animals

Three groups of animals were analyzed: normal (no treatment) diabetic (streptozotocin-treated), and treated (diabetic animals treated with insulin), Male C57 BL6 mice (Jackson Laboratories), aged 6–8 weeks, weighing 20–25 g were injected intraperitoneally with 200 mg/kg streptozotocin (Sigma), in 10 mM Na citrate. Streptozotocin was injected within 5 min after being raised in buffer. Control animals were injected with the same volume of buffer alone. Injections were performed between 7 and 9 AM. Animals were given 5% sucrose in their drinking water for the 48 h after the injection. This treatment reduced the mortality from the severe hypoglycemia caused by the destruction of islet cells by streptozotocin (13). Glucose and triglyceride measurements were made 10 and 18 days after injection from plasma samples were obtained by retro-orbital bleeding of lightly anesthetized animals. Animals with fasting glucose levels over 265 mg/dl were included in the diabetic group. Of the 30 animals treated with streptozotocin, 20 animals were included in the final diabetic group.

Twenty days after injection half of the animals in the diabetic group were treated with insulin by subcutaneous injection of 3–4 units of regular pork:isolente insulin (1:1, 10U/ml) (Eli Lily Co.). Diabetic control and normal control animals were injected with equal volumes of saline. After insulin injection, urine glucose levels were measured using colorimetric test strips (Tes-tape, Eli Lily Co.). Animals that responded to the insulin injection with a lowering of urine glucose were included in the insulin-treated group. Of the 21 streptozotocin-treated animals that were injected with insulin, 18 animals were included in the final treated group. All mice were killed 24 h after the insulin injection. Blood samples and livers were collected at killing and analyzed for glucose, triglyceride, hepatic apoC-III mRNA levels, and apoC-III gene transcriptional activity. Glucose was measured by the glucose oxidase/peroxidase method (Boehringer-Mannheim Diagnostics). Triglycerides were measured using a triglyceride enzymatic assay using glycerol kinase (Boehringer-Mannheim Diagnostics), standardized to 100, 300, and 500 mg % triglyceride standards (Sigma).

Determination of mRNA levels/solution hybridization

Riboprobes (labeled with α-^{32}P-UTP) were prepared as described (10) from cDNA clones of mouse actin or mouse apoC-III (gift from Dr. Neil Azrolan). Total RNA was isolated from 1–2 mg of frozen liver using guanidinium isothiocyanate as described (14), and quantitated by optical density at 260 nm. For Northern analysis, 10-μg samples of total liver mRNA were separated on a 1% agarose, 3% formaldehyde gel (14, 15) and transferred to Gene Screen hybridization membrane (DuPont) using a positive pressure blotter (Stratagene), followed by UV-crosslinking (Stratalink, Stratagene). Membranes were prehybridized for 4 h at 65°C in 5 × SSC, 1% SDS, 0.2% powdered milk, 300 μg/ml yeast tRNA, 100 μg/ml sheared single-stranded DNA. Hybridization was carried out in 5 × SSC, 1% SDS, 0.2% powdered milk, 300 μg/ml yeast tRNA, 100 μg/ml sheared single-stranded DNA, 50% formamide, and 1 × 10^6 cpm/ml of specific riboprobe, for 18 h at 65°C. After hybridization, membranes were washed four times at 65°C for 20 min in 2 × SSC, 0.2% SDS, followed by a single 30-min wash at 65°C in 0.2 × SSC, 0.2% SDS. The washed membranes were analyzed in a betascope image analyzer (Betascan) and by autoradiography.

The solution hybridization RNase protection assay was performed essentially as described (10) with 20 μg of total RNA and 5 × 10^5 cpm of specific riboprobe per sample. Each of the RNA samples was analyzed in duplicate. Hybridization was carried out for 3.5 h at 63°C, and was followed by treatment with 5 units RNase A and 3.6 units RNase T1 in 300 μl of 300 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 5 mM EDTA for 45 min at 34°C. Samples were precipitated with 400 μl cold 20% TCA, collected on glass-fiber filter, washed, dried, and counted by liquid scintillation.

Nuclear run-on assay

Isolation of nuclei and the run-on transcription assay were performed as described (16–18) with modifications. Frozen liver tissue was weighed and combined with 2 volumes of 0.25 M sucrose in TKM buffer (5 mM Tris-HCl, pH 7.5, 2.5 mM KCl, 5 mM $MgCl_2$). The tissue was disrupted with 15–20 strokes in a Dounce homogenizer. Two volumes of 2.3 M sucrose in TKM buffer was added to the homogenate (final sucrose concentration 1.62 M). Nuclei were separated by sucrose gradient ultracentrifugation using the Beckman Ti40 swing-out rotor. Approximately 30% of the centrifuge tube was layered with 2.3 M sucrose in TKM buffer, and the remaining 70% was overlaid with homogenate. Tubes were spun at 60,0000 *g* at 4°C for 1 h. The supernatant was removed and the pellet containing the nuclei was resuspended in TKM buffer with 40% glycerol. Nuclei were quantified by counting in a hemocytometer, and stored at −80°C. For the nuclear run-on assay, approximately 200 μg nuclei was resuspended on ice, in 200 μl of transcription buffer (10 mM NaCl, 20 mM HEPES, pH 7.5, 5 mM, pH 8.0, 16% (v/v) glycerol, 2 mM DTT, 200 μCi of ^{32}P-α-UTP (300 Ci/mmol, Amersham), and 400

μM each of ATP, GTP, and CTP). Nuclei were incubated with agitation at 26°C for 20 min. The reaction was terminated at this time by addition of 4 M guanidinium isothiocyanate. The solution was passed 8 times through a 19-gauge needle to reduce viscosity and the RNA was isolated by the guanidinium isothiocyanate method as described above.

Labeled RNA prepared from normal, diabetic, and insulin-treated diabetic animals was hybridized to slot blots containing 5 μg of single-stranded anti-sense cDNA for either apoC-III, actin, or the pGEM vector alone. Hybridization was carried out essentially as described (19), by prehybridizing the membrane for 4 h at 65°C in 0.02 M Tris-HCl, pH 7.5, 0.6 M NaCl, 0.02 M EDTA, pH 8.0, 1% SDS (w/v), 1 mg/ml yeast tRNA, 10% dextran sulfate), and hybridizing for 48 h at 65°C in 0.024 M Tris-HCl, pH 7.5, 0.7 M NaCl, 0.024 M EDTA, 1% SDS (w/v), 1.5 mg/ml yeast tRNA, 10% dextran sulfate and 1×10^6–10^7 cpm labeled RNA from the nuclear transcription reaction. Filters were washed three times for 10 min each in 0.5 M NaCl, 0.01 M Tris-HCl, pH 7.5. RNase A was added to a final concentration of 125 μg/ml and filters were incubated for 30 min at 37°C with shaking. Finally, filters were washed at 52°C, twice in 2 × SSC, 0.1% SDS for 15 min, and once in 0.5 × SSC, 0.1% SDS for 15 min. Filters were autoradiographed and the film was quantified in a BioImage image analyzer (Millipore). The data for each hybridization were analyzed by subtracting the background hybridization (to the vector alone), and then taking the ratio of the apoC-III signal to the actin signal.

Cell culture and transfection

HepG2 cells were plated at about 20% confluence in 48-well plates. Plasmid DNA (1.375 μg/well) and lipofectin (4 μg/well) were diluted in serum-free MEM separately then mixed together and incubated at room temperature for 15 min. The cells were then rinsed with serum-free medium and incubated with plasmid/lipofectin mixture for 4 h at 37°C. The cells were returned to MEM + 10% fetal bovine serum for 48 h, and then treated in serum-free media with or without insulin for 6 h. Cells were lysed with 50 μl lysis buffer (Luciferase assay kit, Promega Inc.), and an aliquot of 20 μl was taken for luciferase assay, which was performed according to the manufacturer's instructions (Promega Inc.). Protein concentrations were determined using the Bio-Rad protein assay system.

RESULTS

To study the effect of the diabetic state and insulin treatment on apoC-III gene expression, three groups of mice were studied: control, diabetic (streptozotocin-treated), and insulin-treated diabetic animals. As shown in **Fig. 1,** at the time of killing these animals had fasting glucose levels of 170 ± 6, 446 ± 26, and 46 ± 5 mg/dl, respectively, and triglyceride levels of 118 ± 10, 180 ± 13, and 73 ± 11 mg/dl, respectively (all differences among the groups were highly significant, see legend). These results confirm that the streptozotocin treatment caused hyper-

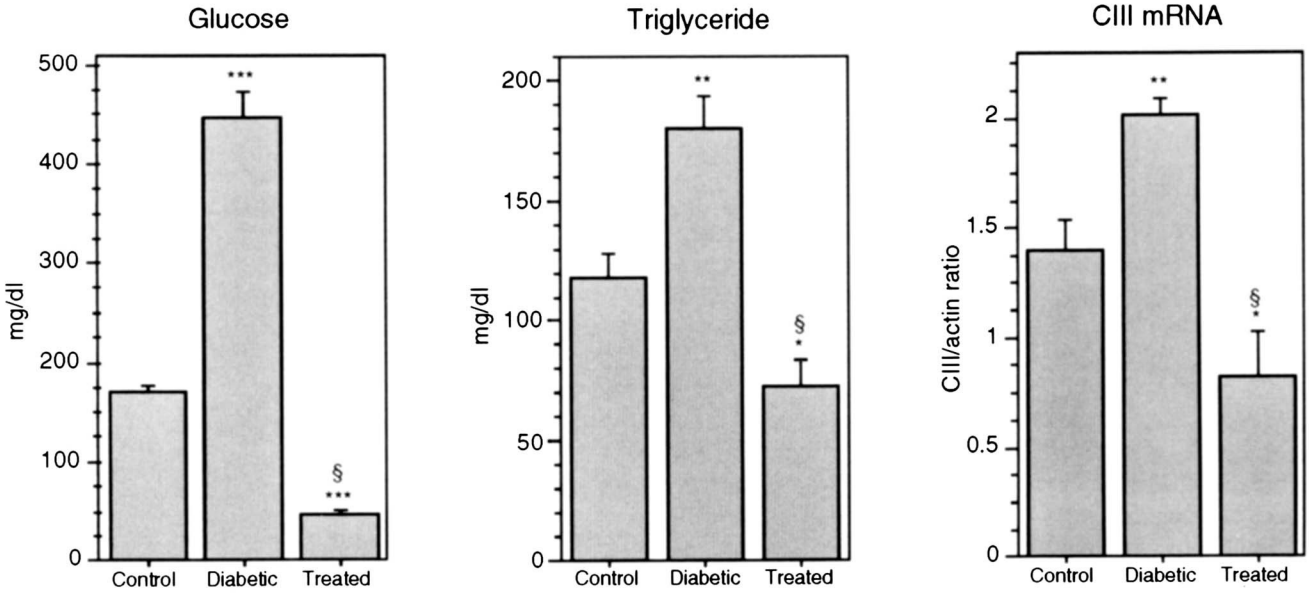


Fig. 1. Comparison of plasma glucose, plasma triglyceride and hepatic C-III mRNA levels in levels in control, diabetic, and insulin-treated mice. Glucose, triglyceride, and C-III mRNA levels were measured in 16 control animals, 20 diabetic (streptozotocin-treated) animals, and 18 treated (insulin-treated diabetic) animals. Mean and standard error measurements are given for each of the three groups. Statistical significance was determined by *t*-test (two-tailed); $^{*}P < 0.02$, $^{**}P < 0.001$, $^{***}P < 0.0001$ relative to control; $^{\S}P < 0.0001$ relative to diabetic.

glycemia and hypertriglyceridemia and this was well controlled by insulin treatment.

ApoC-III expression was initially assessed by Northern blotting analysis. As shown in **Fig. 2,** hepatic apoC-III mRNA levels were approximately 1.5-fold higher in diabetic mice when compared to controls. After insulin treatment in the diabetics, apoC-III mRNA levels were reduced approximately 3-fold. Liver specimens were also analyzed by a quantitative solution hybridization RNase protection assay. These results were expressed as a ratio of apoC-III mRNA to β-actin mRNA. As shown in Fig. 1, control, diabetic, and insulin-treated diabetic mice had relative apoC-III mRNA levels of 1.40 ± 0.14, 2.01 ± 0.21, and 0.82 ± 0.08, respectively. These results confirm those of the Northern blotting analysis, and suggest that apoC-III mRNA levels are elevated in diabetes and are down-regulated by insulin treatment.

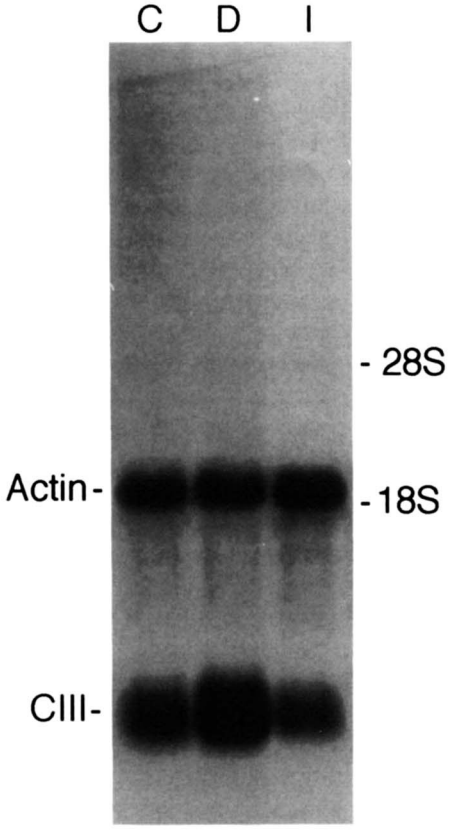


Fig. 2. Northern blot analysis of hepatic apoC-III mRNA levels in representative control, diabetic, and insulin-treated mice. Total liver mRNA was isolated from representative animals from each of three treatment groups, as described in Materials and Methods. RNA samples were subjected to electrophoresis in an agarose-formaldehyde gel, transferred to membrane and hybridized with ^{32}P-labeled riboprobes made from mouse β-actin and mouse apoC-III cDNA clones. Radioactivity in each band was quantified on a betascope image analyzer. The ratios of the C-III band to the actin band are 1.2 for the control (C), 1.6 for the diabetic (D), and 0.6 for the insulin-treated diabetic.

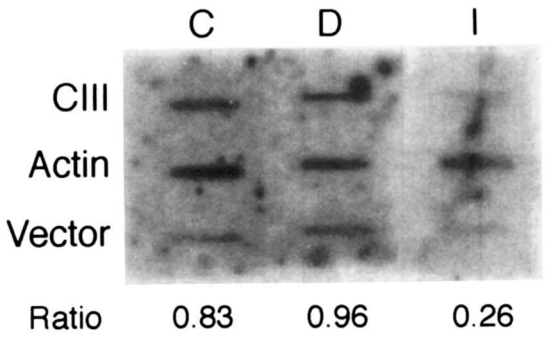
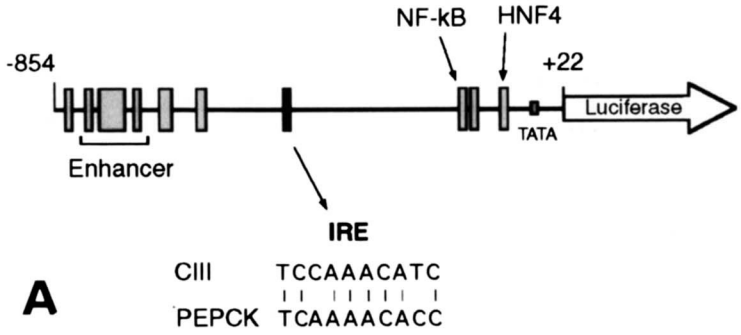


Fig. 3. Nuclear run-on analysis of apoC-III transcriptional activity. Nuclei were prepared from pooled liver samples from each of the three groups (6–12 animals in each group). Labeled RNA was synthesized, purified, and hybridized to filters containing single-stranded apoC-III, β-actin, or vector DNA. The blot was autoradiographed and the film was subjected to densitometric scanning. The apoC-III to β-actin ratio is given on the bottom.

To determine whether the changes in hepatic apoC-III mRNA levels are due to changes in the rate of apoC-III gene transcription, a nuclear run-on assay was performed. Nuclei were prepared from equal amounts of liver from animals in each of the three groups and in vitro transcription was carried out in the presence of radioactive ribonucleotides under conditions that allow elongation but prohibit reinitiation by RNA polymerase (see Materials and Methods). Labeled RNA was isolated and the amount of apoC-III specific RNA was measured by hybridization to slot blots containing excess single-stranded anti-sense apoC-III cDNA. β-actin RNA was measured as the internal standard. The data presented in **Fig. 3** demonstrate that the transcriptional activity of the apoC-III gene is slightly elevated in diabetic mice compared to controls (0.96 vs. 0.83 apoC-III/actin ratio). Although the accuracy of the run-on assay is not great enough to draw a specific conclusion, the 1.2-fold increase in apoC-III transcriptional activity is consistent with the 1.4-fold increase observed in apoC-III mRNA levels. It remains a possibility that posttranscriptional processes also play a role in determining apoC-III mRNA levels in the diabetic state. Insulin treatment of diabetic mice strongly suppressed apoC-III transcriptional activity (0.26 vs. 0.96, treated vs. untreated diabetic mice). This 73% reduction in apoC-III transcriptional activity can account for the entire 59% decrease in apoC-III mRNA levels observed in the diabetic mice treated with insulin.

Although it is clear from the data presented above that the transcriptional activity of the apoC-III gene is different in low versus high insulin states, it is difficult to know whether this is a direct effect of insulin, or whether it is the indirect result of general metabolic changes brought about by insulin treatment of diabetic animals. To determine whether apoC-III transcription can be directly regulated by insulin, we examined the insulin responsive-



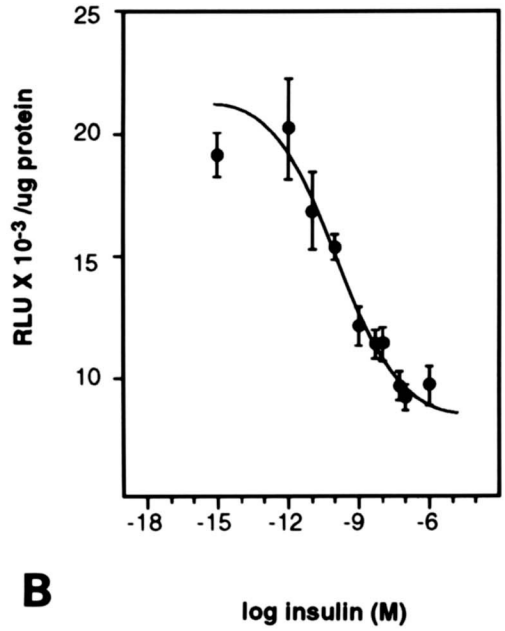


Fig. 4. Repression of apoC-III transcription by insulin in HepG2 cells. A: A schematic diagram of the apoC-III promoter as it appears in the plasmid pL854. ApoC-III promoter sequences from −854 to +22 were inserted immediately upstream of the luciferase coding region of the plasmid pGL-basic (Promega Inc.). Stippled boxes represent previously identified transcription factor binding sites in the apoC-III promoter (30, 31). The sequence element (−462 to −455) that is similar to the insulin response element found in the PEPCK gene is shown (IRE homology). B: The plasmid pL854 was transfected into HepG2 cells as described (Materials and Methods). The transfected cells were treated with the indicated concentrations of insulin for 6 h in serum-free media. The cells were harvested and luciferase activity in cell extracts was determined and normalized to protein concentration. Data points represent the means (± SE) of three independent experiments each done in quadruplicate (n = 12).

ness of the apoC-III promoter in HepG2 cells. The plasmid pL854, containing the apoC-III promoter (−854 to +22) upstream of the luciferase reporter gene (**Fig. 4A**), was transfected into HepG2 cells subsequently treated with different concentrations of insulin. The results (Fig. 4B) demonstrate that apoC-III transcriptional activity is repressed by insulin in a dose-dependent manner, with an EC_{50} of about 1×10^{-10} M insulin. An insulin concentration of 1×10^{-8} M resulted in a 60% reduction of apoC-III transcriptional activity. This is comparable to the reduction observed when diabetic animals were treated with insulin (73%, Fig. 3). These results corroborate the data from the nuclear run-on studies and suggest that insulin is in fact a regulator of apoC-III gene transcription in diabetic animals.

DISCUSSION

The results reported here demonstrate that hepatic apoC-III mRNA levels are elevated in insulin-deficient diabetic mice and that these levels are lowered after insulin treatment. Assuming that mRNA levels correlate with apoC-III synthesis and secretion rates, this would suggest that apoC-III is overproduced in the hypertriglyceridemic insulin-deficient diabetic state and that production is diminished in the normotriglyceridemic insulin-treated diabetic mice. This association of apoC-III production and triglyceride levels may, in fact, be a cause and effect relationship, as in human apoC-III transgenic mice apoC-III gene expression, as measured by plasma human apoC-III concentration, is highly correlated with triglyceride levels (10, 11).

The changes in VLDL metabolism causing hypertriglyceridemia in humans with IDDM have been studied. In acute insulin deficiency the rapid increase in free fatty acid mobilization from adipose tissue causes increased hepatic VLDL triglyceride secretion (8, 9). However, with prolonged insulin deficiency the liver increasingly converts free fatty acids to ketone bodies and VLDL triglyceride secretion decreases (reviewed in ref. 20). In more chronic insulin deficiency there is a persistent decrease in the fractional catabolic rate of VLDL particles and this is thought to be the major metabolic mechanism causing hypertriglyceridemia in IDDM. The mechanism of the hypertriglyceridemia has also been studied in human apoC-III transgenic mice. In these animals VLDL accumulation was similarly shown to be due primarily to decreased VLDL fractional catabolic rate (11). Thus the major mechanism of hypertriglyceridemia in both IDDM and the human apoC-III transgenic mouse are similar. This suggests that the decrease in VLDL fractional catabolic rate in IDDM previously ascribed to diminished lipoprotein lipase activity might just as easily be due to apoC-III overexpression.

In addition to the current study and the transgenic mouse reports, a number of other lines of evidence link apoC-III to hypertriglyceridemia. In vitro high concentrations of apoC-III inhibit lipoprotein lipase activity (21) and, in perfused liver, apoC-III inhibits the uptake of triglyceride-rich VLDL remnants (11, 22). In humans, apoC-III levels and production rates correlate with plasma triglyceride levels (23–25). Finally, association studies have indicated three classes of apoC-III alleles with regard to hypertriglyceridemia, susceptible, neutral,

and resistant (12, 26–28). In view of all of the above, the case for a role of apoC-III in hypertriglyceridemia has become quite compelling.

The data presented here show that the decrease in apoC-III mRNA levels caused by insulin treatment of diabetic mice results from suppression of apoC-III gene transcription. In whole animal experiments one cannot determine whether this is a direct effect of insulin or due to some other metabolic change brought about by insulin treatment of the diabetic mice. However, the transfection studies, which demonstrated a direct effect of insulin on apoC-III transcriptional activity in cultured cells (Fig. 4), supports the possibility that insulin is the mediator of the changes in apoC-III transcription in diabetic animals. Although the mechanism by which insulin treatment regulates apoC-III transcription is not known, the apoC-III gene promoter contains within it an insulin response element located between −462 to −455 from the start site of transcription (Fig. 4A). This 10-bp region shares sequence homology with the insulin response element in the PEPCK gene which mediates a negative effect of insulin on transcription (29). The presence of this element in the apoC-III promoter is consistent with the cell culture results that demonstrate a direct role for insulin in the regulation of apoC-III transcription. The modulation of apoC-III transcription by insulin may ultimately be accomplished by altering the amount or activity of a specific transcription factor (or factors) yet to be identified.

In summary, these studies demonstrate that apoC-III gene transcription is regulated by insulin in animals and in cultured cells, and that the apoC-III gene is overexpressed in IDDM. The specific contribution of apoC-III gene expression to hypertriglyceridemia in diabetes remains to be determined. Our findings present the possibility that the hypertriglyceridemia associated with IDDM is due, in part, to overproduction of apoC-III, which would decrease VLDL clearance rates resulting in elevated plasma triglyceride levels.

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