Selective Compensatory Induction of Hepatic HMG-CoA Reductase in Response to Inhibition of Cholesterol Absorption

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The effect of the potent inhibitor of cholesterol absorption, ezetimibe, on serum cholesterol levels was tested in diabetic and thyroidectomized male Sprague-Dawley rats. Feeding diets supplemented with 1% cholesterol to the diabetic rats raised serum cholesterol levels from 132 to 514 mg/dl while decreasing hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase protein and mRNA levels. Addition of 10 mg/kg/day of ezetimibe to the diets of these animals lowered the serum cholesterol level to 90 mg/dl and produced a large compensatory increase in hepatic HMG-CoA reductase protein without significantly increasing mRNA levels, indicating a post-transcriptional effect. Hepatic LDL receptor protein levels in these diabetic rats were unaffected by ezetimibe treatment. In contrast, ezetimibe treatment of these young normal Sprague-Dawley rats, known to express high levels of hepatic HMG-CoA reductase, did not lower serum cholesterol levels. In thyroidectomized rats, dietary cholesterol increased serum cholesterol levels from 116 to 135 mg/dl and ezetimibe treatment lowered these elevated cholesterol levels to 85 mg/dl. Cholesterol feeding of thyroidectomized rats severely reduced hepatic HMG-CoA reductase protein, while ezetimibe treatment restored reductase protein to normal levels. Again, hepatic LDL receptor protein levels were unaffected by ezetimibe treatment of cholesterol-fed thyroidectomized rats. The data demonstrate that the cholesterol absorption inhibitor ezetimibe profoundly lowers serum cholesterol levels in animals expressing very low rates of hepatic cholesterol synthesis and produces large compensatory increases in hepatic HMG-CoA reductase expression without significantly affecting expression of hepatic LDL receptors. This indicates that ezetimibe should be most effective in lowering serum cholesterol levels in people with low rates of cholesterol synthesis/high rates of cholesterol absorption.** Exp Biol Med 231:559–565, 2006

Key words: ezetimibe; cholesterol; HMG-CoA reductase; LDL receptor; liver

Introduction

Both endogenous cholesterol biosynthesis and absorption of dietary cholesterol contribute to needs for cholesterol to provide membrane lipid, steroid hormones, and bile acids (1) and to establish serum and tissue cholesterol levels. The relative contribution of each source varies among individuals. In a comprehensive study of the Finnish participants in the Scandinavian Simvastatin Survival Study Group study, it was shown that individuals with the highest rates of endogenous synthesis have the lowest rates of cholesterol absorption and *vice versa* (2). This situation may reflect the known feedback regulation of endogenous cholesterol biosynthesis by the end product, cholesterol (3). Individuals with the highest rates of endogenous cholesterol biosynthesis were found to respond the best to treatment with a statin inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme that catalyzes the rate-limiting step of cholesterol biosynthesis (4). Experimental animals treated with statins exhibit large compensatory increases in expression of hepatic HMG-CoA reductase (5). However, hepatic LDL receptor protein levels are not increased by treatment with the statins (6).

Cholesterol absorption is also being targeted in an effort to further lower LDL cholesterol levels in order to help achieve the newly recommended lower LDL cholesterol levels (7, 8). Ezetimibe, a potent inhibitor of cholesterol absorption, is being used alone and in combination with several statins toward this end (9–19). Ezetimibe inhibits cholesterol absorption by over 90% in experimental animals (20). One might expect that ezetimibe would be most effective in lowering serum cholesterol levels in animals or people who are most dependent upon cholesterol absorption.

In order to test this hypothesis we selected cholesterol-fed diabetic and hypothyroid rats. Animals in both of these

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hormone deficient states have diminished expression of hepatic HMG-CoA reductase and thus must rely heavily on absorption for their cholesterol needs (21). When diabetic or hypothyroid rats are placed on 1% cholesterol diets, their serum cholesterol levels increase over 2-fold (21). Adequate expression of hepatic HMG-CoA reductase is required in order to "buffer" the serum cholesterol-raising action of dietary cholesterol (3). Type I diabetic patients exhibit low levels of cholesterol biosynthesis and elevated rates of cholesterol absorption and frequently have increased serum cholesterol levels and premature atherosclerosis (22, 23). Hypothyroidism is associated with elevated serum cholesterol levels (24, 25).

Materials and Methods

Experimental Animals. Young male Sprague-Dawley rats weighing 100–125 g were purchased from Harlan (Madison, WI). The animals were housed in a 12:12-hr reverse light:dark-cycle room at 21 ± 2°C and a humidity of 55% ± 5% with the lights on from 1800 to 0600 daily. The animals were cared for according to the NIH guidelines set forth in the Guide for the Care and Use of Laboratory Animals and according to protocol 2317 approved by the University of South Florida Institutional Animal Care and Use Committee. The animals received Harlan Teklad 22/5 rodent chow and water ad libitum. Thyroparathyroidectomy (Tx) was performed by Harlan. These Tx rats were maintained on 1% calcium gluconate as their drinking water. Diabetes was induced by a subcutaneous injection of 65 mg/kg of streptozotocin in 0.1 *M* sodium citrate, pH 4.5. Diabetes was confirmed by the presence of glucose in the urine as determined with Clinistix (Bayer, Elkhard, IN). The diabetic and Tx animals were fed diets containing 1% cholesterol, 0.01% ezetimibe, both, or chow alone for 4–6 days.

Materials. Streptozotocin, cholesterol, Infinity Cholesterol Reagent, and Colorburst electrophoresis markers were purchased from Sigma Chemical Co., St. Louis, MO. The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL). The ECL Western blotting reagents and PDVF-Plus membranes were from Amersham Biosciences (Piscataway, NJ). Anti-mouse and anti-rabbit HRP conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The A9 monoclonal antibody to HMG-CoA reductase was harvested from A9 hybridoma cells purchased from American Type Culture Collection (ATCC; Manassas, VA) and cultured according to directions from ATCC. Polyclonal antiserum to a C-terminal peptide of the rat LDL receptor was generated in rabbits as previously described (26). Pre-cast 4%–15% Tris-HCl gradient Ready Gels were purchased from Bio-Rad (Hercules, CA). X-Omat film was from Fischer Scientific (Atlanta, GA).

Membrane Isolation. Approximately 2 g of liver was minced in ice-cold 0.25 *M* sucrose to largely free the tissue of blood. The washed mince was then homogenized in 20 ml of 0.25 *M* sucrose with a motor-driven serrated Teflon pestle in a glass homogenizer (Potter-Elvehjem type) from Thomas Scientific (Swedesboro, NJ). The resulting homogenate was centrifuged for 15 mins at 12,000 *g* at 4°C. The supernatant fraction was transferred to a fresh tube and centrifuged at 100,000 *g* for 1 hr at 4°C. The pellet was resuspended in 1 ml of 0.25 *M* sucrose. Membrane protein concentration was determined by the BCA method according to the manufacturer's (Pierce) recommendations.

Western Blotting Analysis. Liver microsomal protein, 25 μg, in 25 μl of Western Sample Buffer (62.5 m*M* Tris-HCl, pH 6.8, 2% SDS, 0.2 *M* sucrose, 7.75 *M* urea, 0.01% bromophenol blue, and 5% β-mercaptoethanol) was denatured by boiling for 5 mins, cooled on ice, and applied to a 4%–15% SDS gel. The separated proteins were electrophoretically transferred to PVDF-Plus membranes. The membranes were blocked by soaking in 5% nonfat dry milk containing phosphate-buffered saline/0.1% Tween 20 (PBST) for 60 to 90 mins. The membranes were then incubated overnight in 5% nonfat dry milk with a 1:2000 dilution of A9 monoclonal antibody to HMG-CoA reductase, 1 μg/μl of purified IgG, or a 1:5000 dilution of the LDL receptor polyclonal antiserum. Three 10-min washes in PBST were then done at room temperature. The blots were incubated with the secondary anti-mouse or anti-rabbit HRP conjugates at 1:50,000 dilutions. HMG-CoA reductase or LDL receptor proteins were detected using ECL reagents as described previously (27).

RNA Isolation and cDNA Synthesis. Male Sprague-Dawley rats, weighing 125–150 g, were sacrificed using an isoflurane overdose, and total liver RNA was isolated using a Polytron homogenizer (Brinkmann, Westbury, NY) in Tri-Reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions. The concentrations of the resulting samples were determined by measuring absorbance (A_{260}) of a 1:100 dilution of sample at 260 nm. Prior to synthesis of cDNA for use in real-time polymerase chain reaction (PCR), the isolated RNA was DNase treated using TURBO DNA-Free kit (Ambion, Austin, TX). Some modifications of the published protocol were made. Fifty micrograms of RNA was treated using 2 μl of Turbo DNase enzyme in a reaction volume of 30 μl. Next, 5 μg of the isolated and DNase-treated total RNA was used to generate cDNA for use in real-time PCR. The reverse transcriptase reaction was carried out with the Superscript II First Strand Synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the instructions.

Real-Time PCR Analysis. Real-time PCR was conducted according to protocol from iQ SYBR Green Supermix using an iCycler (Bio-Rad) with minor modifications. Total reaction volume was adjusted to 25 μl down from 50 μl. Two microliters of cDNA was used as template for reactions with the HMG-CoA reductase primers, which corresponded to regions of exon 2 of the rat mRNA sequence (GenBank accession # NM_013134). The primers

Table 1. Effect of Ezetimibe on Serum Cholesterol Levels in Cholesterol Fed Diabetic Rats[a]

Condition	Serum Cholesterol (mg/dl)
Diabetic	132 ± 4 (6)
Diabetic + Ez	99 ± 4 (4)[b]
Diabetic + Chol	514 ± 117 (10)
Diabetic + Chol + Ez	90 ± 13 (10)[c]

[a] Values are presented as means ± SEM. Serum cholesterol levels in chow fed controls were 115 ± 5 (12).
[b] Differs from diabetic at $P = 0.00031$
[c] Differs from diabetic + Chol at $P = 0.0057$

were as follows: forward 5′ TGT GGG AAC GGT GAC ACT TA 3′ and reverse 5′ CTT CAA ATT TTG GGC ACT CA. Five microliters of a 1:100 dilution of each cDNA was used for reactions with primers to the rat 18s ribosomal RNA sequences (Genbank accession number X01117), which were as follows: forward 5′ CCA TCC AAT CGG TAG TAG CG 3′ and reverse 5′ GTA ACC CGT TGA ACC CCA TT 3′. Primers were all used at a final concentration of 100 n*M*. The annealing temperature used was 61°C, and 40 amplification cycles were performed. Melting curves were done after each run, and a single distinct peak was obtained for each primer set. The data were then processed by iCycler IQ optical system software 3.0 (Bio-Rad) and analyzed by the ΔΔCT method, using Microsoft Excel statistical programs and SigmaPlot (version 8.0; SPSS Inc., Point Richmond, CA).

Serum Hormones. Free T_3 levels in serum samples were determined using an enzyme-linked immunoabsorbent assay (ELISA) kit from Research Diagnostics, Inc (Flanders, NJ). Free serum T_3 for six normal rats was 4.35 ± 1.14 pg/dl. In three Tx rats the levels were 0.03 ± 0.02 pg/dl. Insulin levels in serum samples were determined using the Ultra Sensitive Rat Insulin ELISA kit from Crystal Chemicals, Inc. (Downers Grove, IL). Insulin levels for six normal rats were 0.74 ± 0.09 pg/dl. In eight diabetic rats, serum insulin levels averaged 0.021 ± 0.020 pg/dl.

Serum Cholesterol. Trunk blood was collected and allowed to clot. The resulting serum was removed with a Pasteur pipette and the total cholesterol levels determined by a cholesterol oxidase method using Infinity Cholesterol Reagent (Sigma). Values are expressed in terms of mg/dl.

Statistical Analysis. Means and standards errors were calculated. The significance of differences between groups was determined by *P*-values calculated using Microsoft Excel's independent samples *t* test.

Results

Diabetic Animals. Ezetimibe treatment of diabetic rats significantly lowered serum cholesterol levels (Table 1). When the diabetic rats were placed on a diet containing 1% cholesterol, their serum cholesterol levels rose to over 500 mg/dl. Ezetimibe treatment lowered these to 90 mg/dl—actually slightly less than normal controls, which were 112 mg/dl. The effects on hepatic HMG-CoA reductase protein are shown in Figure 1. HMG-CoA reductase protein was decreased in the diabetic state and increased to near normal upon treatment with ezetimibe (Fig. 1). Usually reductase protein levels were decreased to 20% of controls in diabetic rats rather than the 50% seen in Figure 1. Placing the diabetic rats on a 1% cholesterol-containing diet caused hepatic HMG-CoA reductase protein to essentially disappear (three lanes on the right of Fig. 1). When ezetimibe (0.01%) was added to the cholesterol-supplemented diet (middle three lanes), hepatic HMG-CoA reductase protein levels were restored to nearly those of the normal control. These findings were repeated several times. This represents a very large compensatory induction in response to the dramatic lowering of cholesterol levels.

HMG-CoA Reductase Levels in Diabetic Rats

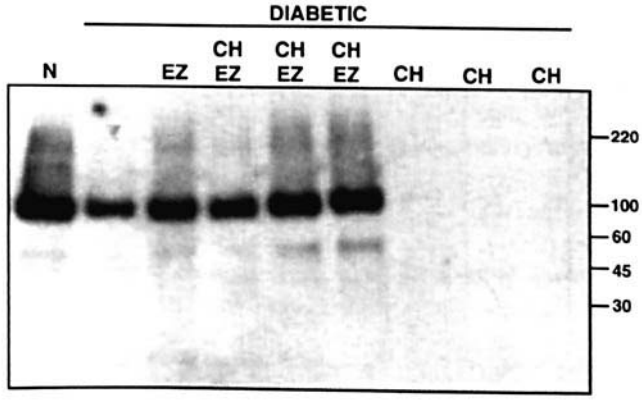

Figure 1. Immunoblot of liver microsomal HMG-CoA reductase from diabetic rats. A lane with liver microsomes from a normal (N) chow-fed rat is on the left. Next are samples from a chow-fed diabetic rat and chow-fed diabetic rat given 0.01% ezetimibe. In the middle three lanes, samples from diabetic rats fed 1% cholesterol (CH) and 0.01% ezetimibe (EZ) are displayed. The three lanes on the right contain samples from diabetic rats fed 1% cholesterol. Twenty-five micrograms of microsomal protein was applied to each lane. Positions of molecular weight markers are displayed on the right side of the blot.

To determine whether this was due to an effect on the rate of transcription, relative hepatic HMG-CoA reductase mRNA levels were determined by real-time RT-PCR. As shown in Figure 2, addition of ezetimibe to the diet of diabetic rats on 1% cholesterol had very little effect on reductase mRNA levels. These remained below 10% of normal controls. Thus, the large compensatory induction of hepatic HMG-CoA reductase protein caused by ezetimibe is due to a post-transcriptional event.

The question of whether inhibiting cholesterol absorption with ezetimibe treatment of cholesterol-fed diabetic rats might also promote a compensatory induction of hepatic LDL receptor was investigated. Ezetimibe treatment did not increase membrane hepatic LDL receptor protein levels (Fig. 3). Also, cholesterol feeding of the diabetic rats did not lower LDL receptor protein levels. The same results were also observed with whole liver samples.

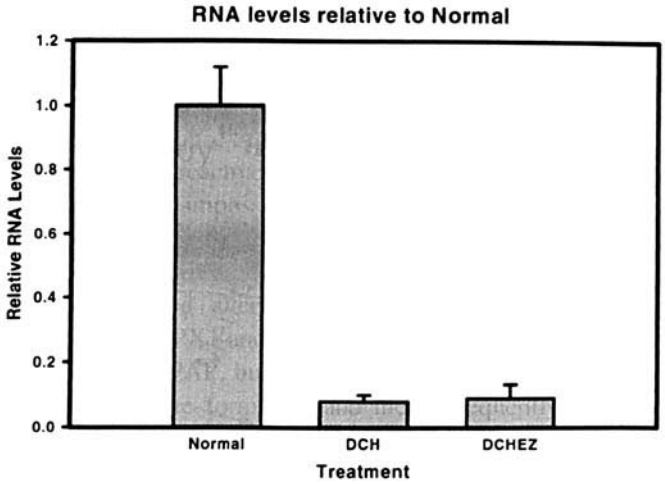

Figure 2. Effect of ezetimibe on hepatic HMG-CoA reductase mRNA levels in cholesterol-fed diabetic rats. Relative mRNA levels were determined by real-time PCR. The values for normal rats are set to 1.0. Values are expressed as means ± SE for at least nine animals in each group. Diabetic rats received either 1% cholesterol (DCH) or both 1% cholesterol and 0.01% ezetimibe (DCHEZ).

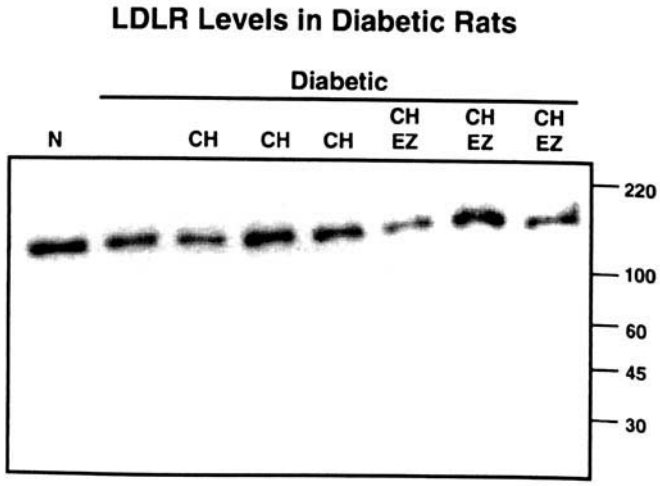

Figure 3. Immunoblot of liver microsomal LDL receptor from diabetic rats. A lane containing liver microsomes from a normal (N) chow-fed rat is on the left. A lane with microsomes from a chow-fed diabetic rat is next. Three lanes were loaded with microsomes from diabetic rats fed 1% cholesterol (CH), and then three lanes were loaded with microsomes from diabetic rat rats fed 1% cholesterol (CH) and 0.01% ezetimibe (EZ). Twenty-five micrograms of microsomal protein was applied to each lane. Positions of molecular weight markers are displayed on the right side of the blot.

Thyroidectomized Animals. Treatment of Tx rats with ezetimibe lowered serum cholesterol levels significantly, to values actually less than controls (Table 2). Feeding 1% cholesterol to the Tx rats increased serum cholesterol levels, but not as dramatically as in the diabetic rats (Table 2). Again, treatment with ezetimibe lowered serum cholesterol levels to values less than those of control animals. Hepatic HMG-CoA reductase protein levels were markedly reduced in the Tx state (Fig. 4). Feeding the Tx rats diets containing 1% cholesterol further reduced reductase protein levels (middle three lanes of Fig. 4), but they were still detectable. Strikingly, adding ezetimibe to the cholesterol diet fully restored HMG-CoA reductase protein (two lanes on the right of Fig. 4). This represents a very large compensatory increase in hepatic HMG-CoA reductase expression. On the other hand, hepatic LDL receptor expression was not increased by ezetimibe treatment in the cholesterol-fed Tx rats (Fig. 5). Actually, ezetimibe treatment caused a slight decrease.

Table 2. Effect of Ezetimibe on Serum Cholesterol Levels in Cholesterol Fed Thyroidectomized Rats[a]

Condition	Serum Cholesterol (mg/dl)
Tx	116 ± 1.5 (3)
Tx + Ez	76 ± (4)[b]
Tx + Chol	135 ± 4.5 (7)
Tx + Chol + Ez	85 ± 6.5 (6)[c]

[a] Values are presented as means ± SEM. Serum cholesterol levels in sham operated chow fed controls were 108 ± 6 (3).
[b] Differs from Tx at $P = 0.00013$.
[c] Differs from Tx + Chol at $P = 0.00021$.

Discussion

Hypocholesterolemic Effect of Ezetimibe. The present data demonstrate that inhibition of cholesterol absorption by ezetimibe will markedly lower serum cholesterol levels under conditions where hepatic HMG-CoA reductase expression (cholesterol synthesis) is low and the system is highly dependent upon absorption to meet cellular needs for cholesterol. The decrease in serum cholesterol levels from over 500 mg/dl to less than 100

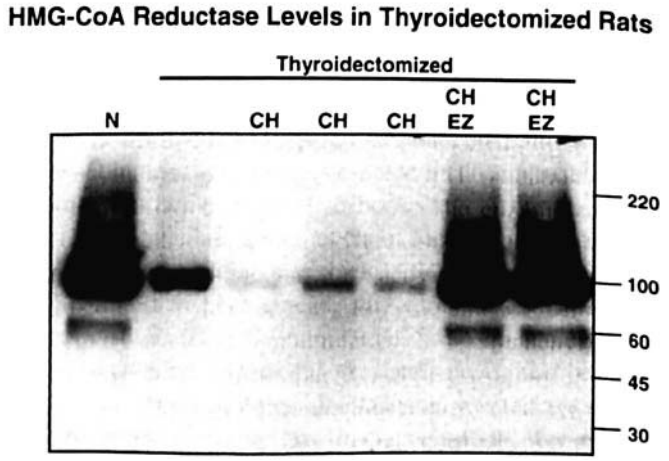

Figure 4. Immunoblot of liver microsomal HMG-CoA reductase from thyroidectomized rats. A sample from a normal (N) chow-fed rat was loaded on the left side. The next lane contains a sample from a chow-fed Tx rat. Three samples from Tx rats fed 1% cholesterol (CH) and two samples from Tx rats fed 1% cholesterol (CH) and 0.01% ezetimibe (EZ) are in the next lanes. Twenty-five micrograms of microsomal protein was applied to each lane. Positions of molecular weight markers are displayed on the right side of the blot.

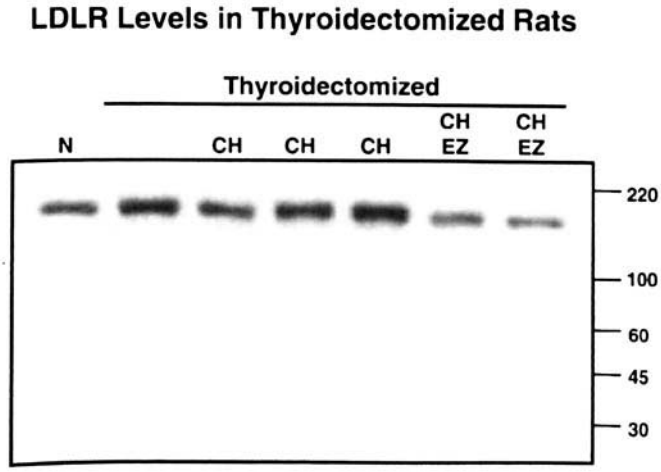

Figure 5. Immunoblot of liver microsomal LDL receptor from thyroidectomized rats. A sample from a normal (N) chow-fed rat was loaded on the left side. The next lane contains a sample from a chow-fed Tx rat. Three samples from Tx rats fed 1% cholesterol (CH) and two samples from Tx rats fed 1% cholesterol (CH) and 0.01% ezetimibe (EZ) are in the next lanes. Twenty-five micrograms of microsomal protein was applied to each lane. Positions of molecular weight markers are displayed on the right side of the blot.

mg/dl in cholesterol-fed diabetic rats is a dramatic example (Table 1). This effect of ezetimibe was greater than that previously observed in $LDLR^{-/-}$ mice fed cholesterol and olive oil where plasma cholesterol was only lowered 18% (20). In contrast, ezetimibe was without effect on serum cholesterol levels (115 vs. 112 mg/dl) in normal chow-fed young Sprague Dawley rats that are known to express high levels of hepatic HMG-CoA reductase and cholesterol synthesis and are resistant to the serum cholesterol-raising action of dietary cholesterol (28).

Compensatory Induction. Administration of ezetimibe to cholesterol-fed diabetic or Tx rats effectively blocked absorption of both dietary and biliary cholesterol so that resulting serum cholesterol levels are actually lower than those in chow-fed normal control rats. Either an increase in endogenous synthesis or hepatic uptake by LDL receptors could compensate. Clearly, increased endogenous synthesis resulting from induction of hepatic HMG-CoA reductase is the mechanism triggered by inhibiting cholesterol absorption. Hepatic HMG-CoA reductase protein levels increase from barely detectable to normal levels, while LDL receptor protein levels are not significantly affected. In a recent investigation, ezetimibe treatment increased HMG-CoA reductase mRNA levels 9-fold in $LDLR^{-/-}$ mice fed a diet enriched with 0.2% cholesterol and 10% olive oil (20). Also, ezetimibe treatment of ABCG5/$ABCG8^{-/-}$ mice increased hepatic HMG-CoA reductase mRNA levels over 2-fold (29). (ABCG5 is ATP-binding cassette, subfamily G, member 5.) These studies did not measure hepatic HMG-CoA reductase protein levels.

Transcription-Independent Induction. Despite the large increase in hepatic HMG-CoA reductase protein levels that occurred in ezetimibe-treated cholesterol-fed diabetic rats, no increase in reductase mRNA levels was observed (Fig. 2). This finding indicates that insulin is required for transcription of hepatic HMG-CoA reductase and that decreasing cholesterol levels by inhibiting the uptake of dietary cholesterol cannot substitute, even though reductase protein levels are returned to normal. We have recently demonstrated that insulin acts to rapidly increase transcription of hepatic HMG-CoA reductase (30). We have also previously shown that feeding dietary cholesterol does not decrease hepatic HMG-CoA reductase mRNA levels (31) or the rate of transcription (32). Feeding increased amounts of dietary cholesterol also does increase the rate of degradation or extent of ubiquitination of hepatic HMG-CoA reductase (33, 34). Dietary cholesterol acts to markedly decrease the rate of translation of hepatic HMG-CoA reductase mRNA (33) and decrease its association with polysomes that are actively engaged in protein synthesis (35). Thus, altering hepatic cholesterol levels does not affect transcription but rather exerts its effects on translation. The present results, showing that ezetimibe treatment markedly increases hepatic HMG-CoA reductase protein levels independent of an effect on mRNA levels, agree with and provide further support for these previous findings (31–35). These results are in contrast with the transcriptional regulation of HMG-CoA reductase *via* sterol response element binding proteins observed in investigations of tumor cells grown in lipid depleted media in the presence of a statin and by studying transgenic and knockout mice (36–39). In liver, alterations in cholesterol content appear to regulate HMG-CoA reductase gene expression at the level of translation.

Interrelationships. People with type 1 diabetes, who exhibit low rates of cholesterol synthesis and high rates of cholesterol absorption (23), have low expression of ABCG5 and ABCG8. These half-transporters function as heterodimers to limit cholesterol absorption by promoting efflux in the intestine and biliary secretion from the hepatocyte (40, 41). Could the decreased expression of these transporters in diabetes indirectly cause decreased expression of hepatic HMG-CoA reductase in diabetes? In ABCG5/ABCG8-deficient mice, noncholesterol sterols are increased 30-fold (42). This could provide a link between synthesis and absorption of cholesterol. It is well established that individuals who exhibit high rates of cholesterol synthesis have low rates of cholesterol absorption and *vice versa* (2, 43, 44). Individuals with relatively high rates of cholesterol absorption and low rates of cholesterol synthesis can be identified by a relatively high ratio of plasma plant sterols to noncholesterol sterols (2, 43, 44). These individuals would be predicted to respond quite well to cholesterol absorption inhibitors.

Recent investigations have now identified Niemann-Pick C1 Like 1 (NPC1L1) protein as a key molecule involved in the process of intestinal cholesterol absorption (45–47). The most recent study demonstrated binding of ezetimibe to recombinant NPC1L1 protein and the elimi-

nation of ezetimibe binding to enterocyte membranes from NPC1L1 knockout mice (48). Further investigations showed that cholesterol absorption and ezetimibe binding were unaffected in caveolin knockout mice (49). This rules out caveolin-1/annexin-2 heterocomplex involvement in intestinal cholesterol absorption, as had been suggested earlier (50). A possible relationship between NPC1L1 (cholesterol absorption) and hepatic HMG-CoA reductase expression (cholesterol synthesis) is suggested by the present studies, which demonstrate very large increases in HMG-CoA reductase expression in ezetimibe-treated cholesterol-fed diabetic and Tx rats.

There are current efforts to identify individuals who are "high" or "low" cholesterol absorbers based on measurements of their ratio of plasma plant sterols, such as campesterol, to cholesterol precursor, such as lathosterol (51). Interestingly, nonsynonymous sequence variations in NPC1L1 were five times more common in low absorbers than in high absorbers. The low absorbers also had significantly lower plasma LDL cholesterol levels.

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