Increased Sensitivity to Dietary Cholesterol in Diabetic and Hypothyroid Rats Associated with Low Levels of Hepatic HMG-CoA Reductase Expression

GENE C. NESS[1] AND KAREN R. GERTZ

Department of Biochemistry and Molecular Biology, College of Medicine, University of South Florida, Tampa, Florida 33612

We recently postulated that hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase functions as a cholesterol buffer to protect against the serum and tissue cholesterol raising action of dietary cholesterol. This postulate predicts that diminished basal expression of hepatic HMG-CoA reductase results in increased sensitivity to dietary cholesterol. Because diabetic and hypothyroid animals are known to have markedly reduced hepatic HMG-CoA reductase, these animals were selected as models to test our postulate. When rats were rendered diabetic with streptozotocin, their hepatic HMG-CoA reductase activity decreased from 314 to 22 pmol • min^{-1} • mg^{-1}, and their serum cholesterol levels increased slightly. When the diabetic animals were challenged with a diet containing 1% cholesterol, their serum cholesterol levels doubled, and their hepatic reductase activity decreased further to 0.9 pmol • min^{-1} • mg^{-1}. Hepatic low-density lipoprotein (LDL) receptor immunoreactive protein levels were unaffected in the diabetic rats whether fed cholesterol-supplemented diets or not. In rats rendered hypothyroid by thyroparathyroidectomy, serum cholesterol levels rose from 100 to 386 mg/dl in response to the 1% cholesterol challenge, whereas HMG-CoA reductase activity dropped from 33.8 to 3.4 pmol • min^{-1} • mg^{-1}. Hepatic LDL receptor immunoreactive protein levels decreased only slightly in the hypothyroid rats fed cholesterol-supplemented diets. Taken together, these results show that rats deficient in either insulin or thyroid hormone are extremely sensitive to dietary cholesterol largely due to low basal expression of hepatic HMG-CoA reductase. Exp Biol Med 229:407–411, 2004

Key words: HMG-CoA reductase; liver; insulin; thyroid hormone; serum cholesterol

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[1] To whom requests for reprints should be addressed at Department of Biochemistry and Molecular Biology, College of Medicine, University of South Florida, 12901 Bruce B. Downs Blvd., Tampa, FL 33612. E-mail: gness@hsc.usf.edu

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Differences in sensitivity to dietary cholesterol among individuals is widely suspected if not rigorously established. Among animals, rabbits and hamsters are known to be very sensitive to dietary cholesterol, whereas Sprague-Dawley rats are quite resistant (1). In a recent study of Cynomolgus monkeys (2), resistant and sensitive animals were identified. The resistant monkeys expressed higher levels of hepatic cholesterol biosynthesis. In the Finnish subset of people in the Scandinavian Simvastatin Survival Study, the quartile of individuals with the highest rates of cholesterol synthesis, as reflected by the highest serum levels of sterol precursors, showed the best response to treatment with simvastatin (3). In a study of 35 patients with familial hypercholesterolemia treated with pravastatin, simvastatin, or atorvastatin, those with the highest rates of *in vivo* cholesterol synthesis, as indicated by fasting plasma mevalonic acid levels, exhibited the greatest serum low-density lipoprotein (LDL) cholesterol lowering (4). These correlations have led us to propose that hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase plays a critical role in cholesterol homeostasis by serving as a "cholesterol buffer" (5). Central to this concept is that higher basal levels of hepatic HMG-CoA reductase expression provide a greater extent of feedback regulation in response to a dietary challenge of cholesterol so that serum and tissue cholesterol levels are minimally affected.

Certain hormones, particularly insulin and thyroid hormone (6–12), markedly influence the basal expression of hepatic HMG-CoA reductase. Insulin increases hepatic HMG-CoA reductase activity about 10-fold by acting to increase mRNA and immunoreactive protein levels (11,12). Even larger increases, up to 35-fold, are elicited by thyroid hormone treatment of hypothyroid (hypophysectomized) animals due to increases in both the rate of transcription and stabilization of the mRNA (9).

Hormone status can also greatly affect serum cholesterol levels. A most striking example is the case of a woman with heterozygous familial hypercholesterolemia who also had hypothyroidism (13). She had a serum cholesterol level

of 700 mg/dl, whereas her two heterozygous siblings who were not hypothyroid exhibited serum cholesterol levels of only 300 mg/dl. Some, but not all, diabetic patients exhibit increased serum cholesterol levels. Diabetic rats often exhibit elevated serum cholesterol levels. To test the proposal that relative expression of hepatic HMG-CoA reductase affects the degree of sensitivity to dietary cholesterol, responses of diabetic and hypothyroid rats to a cholesterol challenge were determined.

Materials and Methods

Experimental Animals. Young male Sprague-Dawley (SD) and Wistar-Furth (WF) rats weighing 100 to 125 g were purchased from Harlan (Madison, WI). Thyroparathyroidectomy (Tx) on the SD rats was performed by Harlan (SD Tx rats are commercially available). The Tx rats were maintained on 1% calcium gluconate as their drinking water. Diabetes was induced in WF rats by subcutaneous injection of 65 mg/kg of streptozotocin in 0.1 *M* sodium citrate, pH 4.5. The WF rats tolerate streptozotocin quite well. Diabetes was confirmed by the presence of glucose in their urine as determined with Clinistix (Bayer, Elkhart, IN). All rats were housed in 12:12 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 allowed free access to Teklad mouse/rat diet and water. The animals were cared for and sacrificed according to the NIH guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (1996 edition) and according to protocol 1749 approved by the University of South Florida Institutional Animal Care and Use Committee. The rats were sacrificed between the third and fourth hour of the dark period when hepatic HMG-CoA reductase activity is at its daily peak. Levels of LDL receptor protein do not vary on a daily basis.

Materials. Streptozotocin, Infinity Cholesterol Reagent, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C] HMG-CoA (55.7 mCi/mmol) was purchased from Perkin Elmer/NEN (Boston, MA). Polyclonal antiserum to homogenous rat liver HMG-CoA reductase (catalytic domain; Ref. 14) was generated in rabbits as previously described (15). Polyclonal antiserum to a C-terminal peptide of the LDL receptor was also generated in rabbits as previously described (16). The ECL Western blotting kit was purchased from Amersham (Chicago, IL).

Microsomes. Liver, brain, and testes microsomes essentially free of lysosomes were isolated by differential centrifugation as previously described (15). Protein concentrations were determined by a biuret method (17).

HMG-CoA Reductase Assay. HMG-CoA reductase activity was determined in microsomes by a radiochemical assay as previously described (18). The product [^{14}C] mevalonate was converted to the lactone by acidification of the reaction mixture and isolated by thin-layer chromatography on 750-μm–thick silica gel G plates using benzene:acetone (1:1) as the developing solvent. Enzyme activity is expressed as pmol / min^{-1} / mg^{-1} of microsomal protein.

Cholesterol Determination. Total cholesterol levels were determined in serum by a cholesterol oxidase method using Infinity Cholesterol Reagent. Values are expressed in terms of mg/dl.

Western Blotting Analysis. Microsomal membranes, 25 μg of protein, were added to 25 μl of sample buffer (30 m*M* Tris/HCl, pH 6.8, 1% sodium dodecyl sulfate [SDS], 0.1 *M* sucrose, 8 *M* urea, 5% β-mercaptoethanol, and 0.005% bromophenol blue), boiled for 5 mins, cooled on ice, and applied to 7.5%SDS-polyacrylamide gels (19). Prestained molecular weight markers of 185, 118, 85, 62, 51, 38, 22, 15, 10, and 6 kDa were applied to one lane (catalog no. C4105; Sigma Chemical). The separated proteins were electrophoretically transferred to PDVF-Plus membranes. HMG-CoA reductase and LDL receptor proteins were detected as previously described using the ECL kit (19).

Determination of LDL Receptor Half-Life. Normal, diabetic, and thyroidectomized rats were injected subcutaneously with 250 μg/100 g of cycloheximide (20). The rats were sacrificed 0, 2, 4, or 6 hrs later, and liver microsomes were prepared (15). Relative levels of LDL receptor protein were determined by Western blotting analysis. The half-lives were determined from semi-log plots.

Results

Diabetic rats are known to express low levels of hepatic HMG-CoA reductase (11,12). The effects of supplementing the diets of Wistar-Furth (WF) diabetic rats with 1% cholesterol on hepatic HMG-CoA reductase activity and serum cholesterol levels are presented in Figure 1. Serum cholesterol levels were increased more than 2-fold whereas hepatic HMG-CoA reductase activity, which was already very low, was decreased to essentially undetectable levels. Feeding normal WF rats diets containing 1% cholesterol had negligible effects on serum cholesterol levels (21). Immunoblotting analysis (Figs. 2 and 3) showed that induction of diabetes markedly lowers hepatic HMG-CoA reductase protein levels without affecting hepatic LDL receptor protein levels. Adding cholesterol to the diets of WF diabetic rats virtually eliminated hepatic HMG-CoA reductase protein (Fig. 2) but had no effect on hepatic LDL receptor protein levels (Fig. 3). Thus, the marked increase in serum cholesterol levels in these diabetic rats was associated with very low levels of HMG-CoA reductase expression but near normal levels of hepatic LDL receptor protein.

Thyroid deficiency in rats is also known to decrease hepatic HMG-CoA reductase gene expression (7–10). Figure 4 shows that adding cholesterol to the diets of thyroidectomized (Tx) SD rats increased serum cholesterol levels more than 3-fold, whereas HMG-CoA reductase activity was reduced to very low levels. Immunoblotting

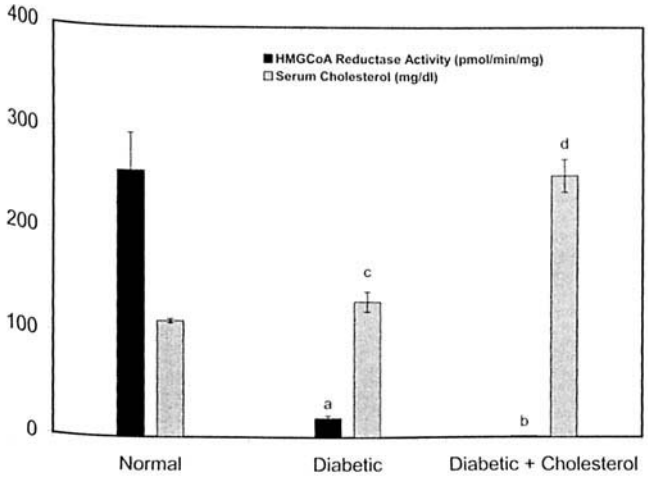

Figure 1. Effect of feeding diets supplemented with 1% cholesterol on hepatic HMG-CoA reductase activity and serum cholesterol levels in diabetic rats. The WF diabetic rats were maintained on either a normal chow diet or a cholesterol supplemented diet for 4 days prior to being sacrificed. Hepatic HMG-CoA reductase activity and serum cholesterol levels were determined as described in "Materials and Methods." Values for normal chow-fed WF rats are presented for comparison purposes. All values are presented as means ± standard deviations for at least 4 animals in each group. For HMG-CoA reductase, $P < 0.001$ for both (a) and (b) compared with the normal. For serum cholesterol, (c) is not statistically significant compared to the normal, whereas for (d), $P < 0.001$ as compared to the normal.

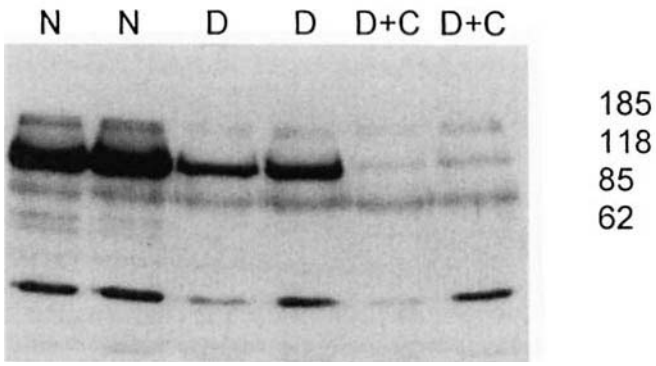

Figure 2. Immunoblotting analysis of HMG-CoA reductase in hepatic microsomes from normal, diabetic, and WF diabetic rats fed a 1% cholesterol diet. The positions of molecular-weight markers are shown on the right side. The major HMG-CoA reductase band is at 100 kDa. Liver microsomal samples, 25 μg, from normal (N), diabetic (D), and diabetic rats fed 1% cholesterol (D+C) from 2 different animals each is shown.

analysis shown in Figure 5 revealed that hepatic HMG-CoA reductase protein was significantly decreased in Tx rats and that feeding cholesterol enriched diets caused a further decrease. In contrast, hepatic LDL receptor protein levels were not significantly decreased in Tx rats and were only slightly reduced in Tx rats given diets enriched in cholesterol (Fig. 6). Thus, increased serum cholesterol levels seen in thyroidectomized rats are associated with decreased expression of hepatic HMG-CoA reductase but not decreased LDL receptor protein levels. In contrast, hypophysectomized rats exhibit decreased hepatic LDL receptor protein levels (16). This may reflect the removal of thyroid stimulating hormone in these animals, which is still present in Tx animals. LDL receptor protein is only modestly increased by T_3 in livers of Tx rats (22).

We have previously shown that treatment with inhibitors of HMG-CoA reductase or feeding dietary cholesterol alters the functioning of hepatic LDL receptors by changing the rate of cycling as reflected by changes in receptor half-life (20). Thus, hepatic LDL receptor half-life was determined in normal (WF and SD), diabetic, and thyroidectomized rats. In all cases, the half-life was approximately 8 hrs. Thus, the effects of insulin and T_3 on hepatic LDL receptor expression appear to differ from those of statins and cholesterol.

Discussion

The concept that hepatic HMG-CoA reductase expression serves as a "cholesterol buffer" (5) was tested using animals deficient in either insulin or thyroid hormone. Supplementing the diets of diabetic or thyroidectomized rats with cholesterol resulted in large 2- to 3-fold increases in serum cholesterol levels in the face of markedly reduced hepatic HMG-CoA reductase expression. In contrast, feeding cholesterol-enriched diets to either WF or SD normal rats has negligible effects on serum cholesterol levels (21). These rats express high levels of hepatic HMG-CoA reductase. Diabetic and thyroidectomized rats given replacement doses of hormone also express high levels of hepatic HMG-CoA reductase (7–12). It was not feasible to examine intermediate replacement hormone levels because the dose curves are so steep. We previously found that 0.1 unit of insulin/200 g gives no increase whereas 0.3 units/200 g gives full induction of hepatic HMG-CoA reductase mRNA (12). Also, 5 or 10 μg/100 g of T_3 gives very little response whereas 25 μg/100 g gives a full response (23).

The increase in serum cholesterol levels observed in diabetic and Tx rats fed the cholesterol-enriched diets could result, at least in part, from decreased expression of the hepatic LDL receptor. However, as shown in Figures 3 and 6, hepatic LDL receptor immunoreactive protein levels are not significantly decreased in either the diabetic or Tx animals. In a recent study (24), we demonstrated that the hepatic LDL receptor is located in the cholesterol-rich caveolae membranes. An increase in caveolar cholesterol content might slow the rate of cycling of the LDL receptor and hence its activity. In a previous study (20), we demonstrated neither treatment with statins nor feeding normal SD rats cholesterol-enriched diets significantly altered levels of hepatic LDL receptor protein. However, the half-life of the receptor was markedly changed. This suggested that the functioning (rate of cycling) of the LDL receptor appeared to be increased by statin treatment and decreased by feeding cholesterol (20). Thus, we determined the effect of hormone deficiency of the half-life of the LDL receptor. No difference was found.

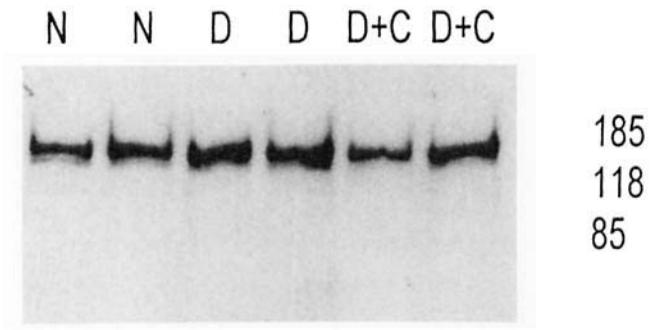

Figure 3. Immunoblotting analysis liver LDL receptor from normal, diabetic, and WF diabetic rats fed a 1% cholesterol diet. The LDL receptor migrates at 160 kDa. The samples are the same as in Figure 2.

Although it may, on the surface, seem paradoxical, the hormone-deficient rats (diabetic and Tx) that express extremely low levels of hepatic HMG-CoA reductase were found to be far more susceptible to dietary cholesterol than normal animals expressing high rates of hepatic reductase activity. Hamsters express much less hepatic reductase than rats and are more susceptible to dietary cholesterol (21). Inbred strains of rats that express lower levels of hepatic HMG-CoA reductase are more susceptible to dietary cholesterol (21). As rats age, their serum cholesterol levels rise, whereas their hepatic HMG-CoA reductase activity significantly declines (25). Taken together, these observations indicate that high levels of hepatic HMG-CoA reductase expression serve as an effective cholesterol buffer.

The relationship between the decrease in hepatic HMG-CoA reductase and increase in serum cholesterol in response to a cholesterol challenge is not a simple linear one as shown in the accompanying paper (21). With a drop to 30% as much reductase activity (SD rat vs BN rat), serum cholesterol levels rise perhaps 20% whereas a drop to 2%

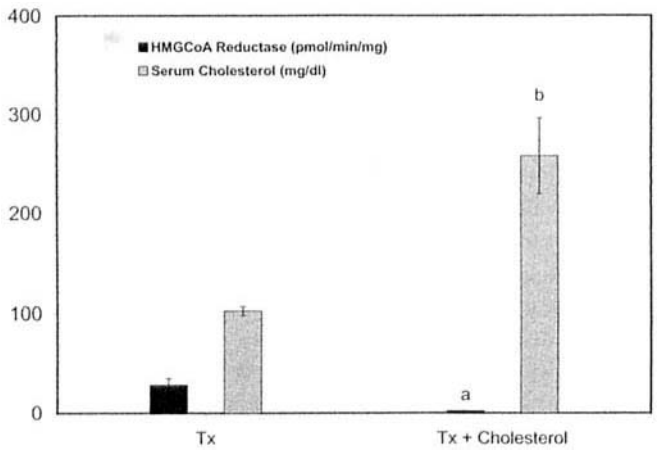

Figure 4. Effect of feeding diets supplemented with 1% cholesterol for 4 days on serum cholesterol levels and hepatic HMG-CoA reductase activity of thyroidectomized rats. Values are given as means ± SD of four or more animals. For HMG-CoA reductase, (a) differs from Tx at $P < 0.001$, and for serum cholesterol, (b) differs from Tx at $P < 0.001$.

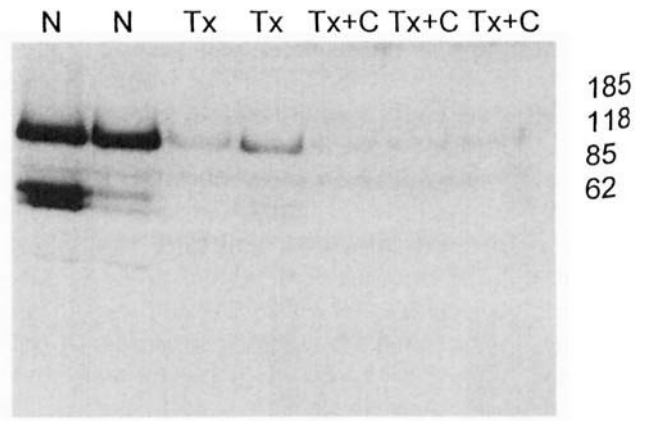

Figure 5. Immunoblotting analysis of liver HMG-CoA reductase from normal, thyroidectomized, and SD thyroidectomized rats fed a 1% cholesterol diet. The positions of molecular-weight markers are shown on the right. The major HMG-CoA reductase band is at 100 kDa. The lower bands are proteolysis products. Liver microsomal samples, 25 μg, from normal (N), thyroidectomized (Tx), and Tx fed cholesterol (Tx+C) SD rats were applied.

causes increases of more than 5-fold (SD rat vs hamster). It appears that serum cholesterol levels rise significantly in response to a cholesterol challenge only when basal hepatic HMG-CoA reductase is quite low so that even complete feedback downregulation cannot provide sufficient compensation for the incoming cholesterol.

It is likely that the 2- to 3-fold elevations in serum cholesterol observed in these diabetic and Tx rats fed a cholesterol-supplemented diet could eventually result in lesions in the aorta. In one experiment, we maintained the Tx rats on the 1% cholesterol for 15 days but did not observe a further increase in serum cholesterol levels. It seems that a new steady state was reached within 4 days. This is consistent with the 2.5-hr half-life of HMG-CoA reductase (26) and the 8-hr half-life of the LDL receptor (20). In apo E knockout mice, extensive aortic lesions are observed especially in the mice on a "Western type diet" with serum cholesterol levels of over 1800 mg/dl (27). Chow-fed apo E -/- mice have serum cholesterol levels of only 400 mg/dl and much less aortic involvement. The

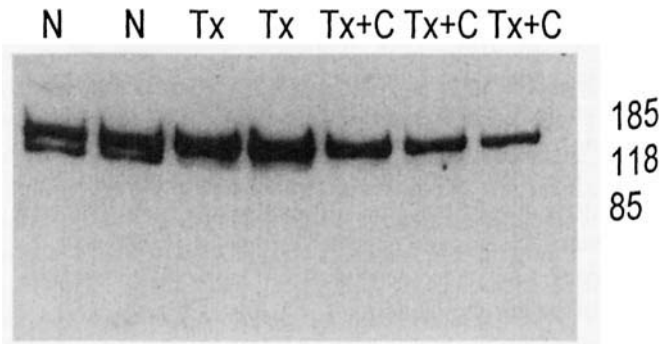

Figure 6. Immunoblotting analysis of liver LDL receptor from normal, thyroidectomized, and SD thyroidectomized rats fed a 1% cholesterol diet. The LDL receptor migrates at 160 kDa. The samples are the same as those in Figure 5.

genetic background of the apo E knockout mice also affects the atherosclerotic lesion area. C57 block mice have much greater involvement than FVB mice (28). This appears to be due to lower levels of apo A-I in C57 block mice. Interestingly, LDL receptor knockout mice only have 2-fold elevations in serum cholesterol levels (29). In humans with LDL receptor defects (familial hypercholesterolemia), serum cholesterol levels can range from 400 to more than 1100 mg/dl in homozygotes (30). Correspondingly, the age of onset of clinical symptoms varies from 4 to over 40 years of age. This is due to the existence of more than 350 different mutations in the LDL receptor gene.

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