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Degradation of HMG-CoA reductase in rat liver is cholesterol and ubiquitin independent

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Abstract In contrast with the accelerated degradation observed in tumor cells in response to sterols, hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase turnover in whole animals was not increased by dietary cholesterol. Furthermore, treating rats with lovastatin to lower hepatic cholesterol levels did not decrease the rate of degradation. The half-life remained in the 6 h range. Co-immunoprecipitation studies revealed that the amount of ubiquitin associated with the reductase was entirely dependent upon the amount of microsomal protein subjected to immunoprecipitation. The results indicate that in liver, neither the rate of reductase protein degradation nor the ubiquitin-proteasome system appear to play roles in mediating changes in HMG-CoA reductase protein levels in response to dietary cholesterol.

Keywords: 3-Hydroxy-3-methylglutaryl coenzyme A reductase; Liver; Protein degradation; Ubiquitin

1. Introduction

The enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), catalyzes the rate-limiting reaction of cholesterol biosynthesis. The expression of this enzyme is regulated by various hormonal and dietary factors and particularly by the end product, cholesterol [1]. The currently proposed mechanism for the sterol-dependent downregulation of HMG-CoA reductase entails increased degradation by the ubiquitin-proteasome system [2] and involvement of the membrane-spanning regions, particularly sterol-sensing domains of the enzyme [3–7]. This mechanism is based on studies of cultured tumor cells grown in the presence of inhibitors of HMGR. Various Chinese hamster ovary cell lines have been used to study the degradation of HMG-CoA reductase [8–12]. Other cells used in these studies include: Met-18b-2, squalene synthase deficient, human embryonic kidney (HEK-293), LP-90, C100, and SV-589 fibroblasts [2,7,13–18]. These studies have been carried out in either tumor cells or immortalized cells grown in lipoprotein depleted media in the presence of inhibitors of HMG-CoA reductase. The conclusion that accelerated degradation of the reductase mediated by sterols appears to involve the ubiquitin-proteasome system has resulted from these studies.

The degradation of HMG-CoA reductase in liver, the major site of sterol-mediated feedback regulation [19], has not been extensively investigated. It has been reported [20] that cholesterol-mediated feedback regulation primarily occurs at the level of translation in liver. The question of whether ubiquitin is involved in the degradation of hepatic HMG-CoA reductase has not been investigated. The data obtained in the present study indicate that the rate of hepatic reductase degradation is not accelerated by dietary cholesterol nor is the amount of ubiquitin associated with the enzyme changed in response to excess cholesterol or depletion of cholesterol caused by an inhibitor of HMG-CoA reductase. Thus, feedback regulation of HMG-CoA reductase in liver fundamentally differs from that previously characterized for the tumor enzyme.

2. Materials and methods

2.1. Experimental animals

Male Sprague–Dawley rats were purchased from Harlan Industries, Madison, WI. At the time of experimentation, the rats were about 200 g. All experiments were conducted in accord with NIH guidelines and as approved by the University of South Florida IACUC, protocol 2317. The rats were housed in a 12 h/12 h reversed cycle light-controlled room at 21 ± 1 °C with a humidity of 45–55% and 10 complete changes of air/hour. The animals were provided Harlan Teklad Global 18% protein rodent chow and water ad libitum. Groups of animals were fed chow, chow supplemented with 1% cholesterol or 0.02% lovastatin or both. Rats were given 2.5 mg/kg of cycloheximide subcutaneously [21] at the fourth hour of the dark cycle and killed 0, 2, 4 or 6 h later. Three groups of four animals each were employed for each condition – normal, cholesterol fed, lovastatin-treated and lovastatin and cholesterol treated. Additional rats were injected subcutaneously with 1 mg/kg of lactacystin in DMSO or were given just DMSO.

2.2. Materials

The A9 monoclonal antibody to HMG-CoA reductase was harvested from A9 hybridoma cells obtained from American Tissue Culture Collection (ATCC) (cat# CRL-1811) cultured according to directions from ATCC. Ubiquitin monoclonal IgG1 antibody (Ub-(P4D1) cat# C2404) and ubiquitin control protein (Ub (FL-76) cat# sc-4274), a 35 kDa tagged fusion protein corresponding to amino acids 1–76 of human ubiquitin, were purchased from Santa Cruz Biotechnology. Monoclonal anti-β-actin, clone AC-15 (cat# A5441), lactacystin

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Abbreviations: HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HMGR, HMG-CoA reductase; BCA, bicinchoninic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ECL, enhanced chemiluminence; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

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(cat# L-6785) and Colorburst electrophoresis markers (cat# C-4105) were obtained from Sigma. Anti-mouse HRP conjugated secondary antibody was purchased from Jackson ImmunoResearch Laboratories. Enhanced chemiluminence (ECL) Western blotting reagents and Protein G Sepharose-4 Fast Flow were purchased from Amersham Biosciences. Pre-cast, 4–15% Tris–HCl gradient Ready Gels (cat# 161-1104) were purchased from Bio-Rad. Complete protease inhibitor cocktail tablets (cat# 1697498) were from Roche Diagnostics. The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce. Lovastatin was a generous gift from Merck.

2.3. Microsomal isolation

Liver was minced in ice-cold 0.25 M sucrose and then homogenized with a motor-driven serrated Teflon pestle in a Thomas glass homogenizer vessel in 10 volumes of 0.25 M sucrose [22]. The resulting homogenate was centrifuged for 10 min at $12000 \times g$ at 4 °C. The supernatant was transferred to a fresh tube and centrifuged at $100000 \times g$ for 1 h at 4 °C. The pellet was resuspended in 1 ml of 0.25 M sucrose containing 1× complete protease inhibitor per 2 g of original liver. Microsomal protein concentration was determined by the BCA protein assay based on absorbance readings at 562 nm according to the manufacturer's (Pierce) recommendations.

2.4. Immunoblotting

Liver microsomal protein, 25 μg, or immunoprecipitated protein, 25–800 μg, was denatured by boiling in Western sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 0.2 M sucrose, 8 M urea, 0.01% bromophenol blue and 5% β-mercaptoethanol). The proteins were analyzed by SDS–PAGE using 4–15% gradient gels at 100 V for 2 h. The separated proteins were transferred electrophoretically to polyvinylidene difluoride membranes at 100 V for 60 min (overnight). The membranes were blocked by soaking in 5% skim milk containing PBST (phosphate-buffered saline (PBS)/0.1% Tween 20) for 60–90 min. The membranes were then incubated in primary antibody/5% skim milk overnight at 4 °C. Three 10-min washes in PBST were then conducted at room temperature. The blot was then incubated for 60 min with the secondary anti-mouse HRP conjugate antibody solution at a 1:50000 dilution. HMG-CoA reductase or ubiquitin proteins were detected using ECL reagents as previously described [23].

2.5. Immunoprecipitation

Varying amounts of microsomal protein (25–800 μg) were solubilized in 100 μl of immunoprecipitation buffer [100 mM Tris–HCl, pH 7.5, 1% SDS and 20 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)] [24] and then diluted 1:10 with deionized water. The solution was pre-cleared with a Protein G Sepharose bead slurry at a 1:10 ratio of beads to sample and incubated for 30 min at 4 °C. The mixture was spun in a microfuge at $1000 \times g$ for 5 min. The supernatant was transferred to a fresh tube. An excess of A9 mouse anti-HMG-CoA reductase or anti-β-actin monoclonal antibody was added to this pre-cleared microsomal solution, which was then incubated overnight on a rocker platform. Protein G Sepharose bead slurry (100 μl) was then added and the mixture incubated for 2 h at 4 °C on a rocker platform. The reactions were centrifuged for 30 s at $1000 \times g$ and the supernatant discarded. The pelleted beads were washed three times with immunoprecipitation buffer and twice with ice-cold 1× PBS. The beads were then spun at $10000 \times g$ for 5 min and any remaining supernatant was discarded. The beads were resuspended in 50–75 μl of Western blotting sample buffer and centrifuged at $10000 \times g$ for 5 min. The supernatant was subjected to immunoblotting analysis.

3. Results

3.1. Degradation of hepatic HMG-CoA reductase

The question of whether the relative levels of cholesterol act to alter the stability of liver endoplasmic reticulum HMG-CoA reductase was investigated using cholesterol fed and lovastatin-treated rats as models of cholesterol excess and cholesterol depleted states, respectively. The half-life of hepatic HMG-CoA reductase was determined by following the rate of decline in HMG-CoA reductase immunoreactive protein after administration of the protein synthesis inhibitor, cycloheximide. Western blots of HMG-CoA reductase protein are shown in Fig. 1. As expected reductase protein levels are markedly increased in microsomes isolated from livers of lovastatin-treated animals and severely depressed in the cholesterol fed rats. Animals given both lovastatin and cholesterol exhibited a low level of reductase protein. To determine half-lives, the blots were scanned and the data displayed on semi-log plots (Fig. 2). The half-life for hepatic HMG-CoA reductase from rats fed a normal diet was 5.8 ± 0.6 h. In a previous study with younger animals a half-life of 2.5 h was observed [20]. This likely reflects the higher level of expression of hepatic HMG-CoA reductase in the younger animals [25]. Surprisingly, the half-life was actually increased to over 12 h when 1% cholesterol was added to the diet (Fig. 2B). Lovastatin treatment did not stabilize hepatic reductase protein, as the half-life was 6.1 ± 1.5 h (Fig. 2C). This is in contrast with a previous study in which lovastatin (Mevinolin) and the bile acid binding resin, cholestyramine, were fed. In that study a half-life of about 12 h for hepatic HMG-CoA reductase was observed [26]. Addition of 1% cholesterol to the diets of lovastatin-treated animals did not affect the half-life which was still 6.7 ± 2.7 h (Fig. 2D) despite a large decrease in HMG-CoA reductase protein levels.

3.2. Ubiquitination of hepatic HMG-CoA reductase

In view of the numerous reports [2,15,16,18,27–29] concluding that HMG-CoA reductase in tumor cells becomes ubiquinated and then degraded more rapidly in proteasomes under sterol excess conditions, the degree of ubiquination of hepatic HMG-CoA reductase in high and low states of liver cholesterol levels was investigated. Varying amounts of liver microsomal protein from normal, cholesterol fed, lovastatin-treated and cholesterol and lovastatin-treated rats were subjected to immunoprecipitation using HMG-CoA reductase antibody (Fig. 3). The immunoprecipitates were probed for both ubiquitin and

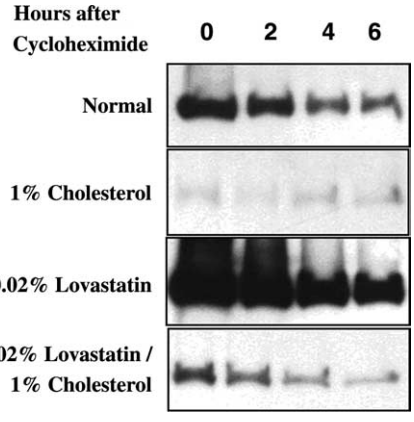

Fig. 1. Immunoblotting of hepatic HMG-CoA reductase. Liver microsomes from rats fed normal, 1% cholesterol, 0.02% lovastatin or 0.02% Lovastatin + 1% cholesterol and killed at 0, 2, 4 or 6 h after inhibiting protein synthesis with cycloheximide were subjected to immunoblotting for HMG-CoA reductase. For the normal, lovastatin and lovastatin + cholesterol samples, 25 μg of protein was applied to each lane. In the case of the samples from cholesterol fed rats, 75 μg of protein was applied to each lane. A 10-min exposure time was used for all. This is a representative experiment.

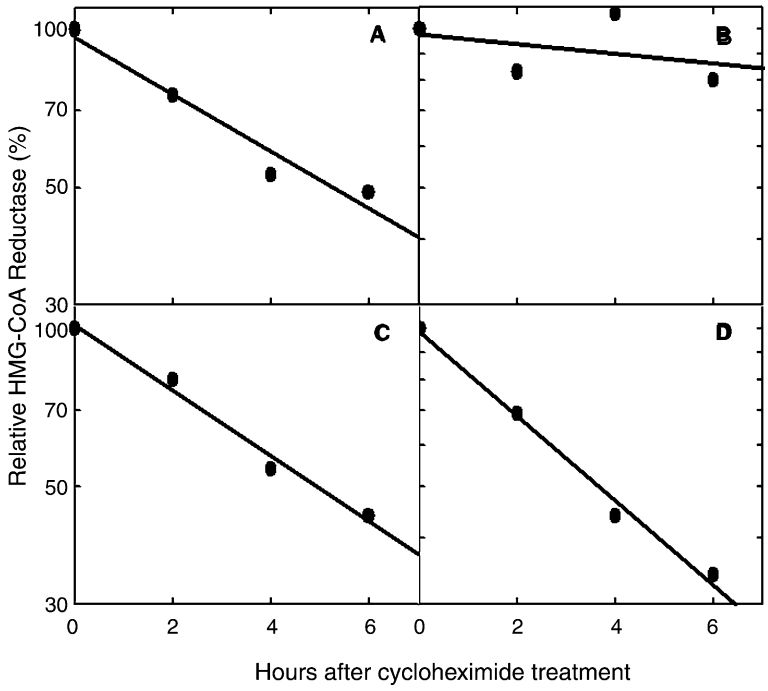

Fig. 2. Determination of hepatic HMG-CoA reductase half-life. The relative amounts of HMG-CoA reductase protein remaining after cycloheximide treatment is displayed on semi-log plots to determine half-lives. Results obtained for normally fed (A), 1% cholesterol fed (B), 0.02% lovastatin-treated (C) and lovastatin-treated and 1% cholesterol fed (D) rats are presented. In all cases, the zero time value is assigned as 100%.

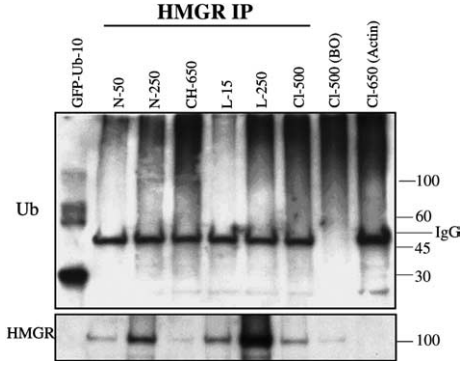

Fig. 3. Ubiquitin content in hepatic microsomes immunoprecipitated with HMG-CoA reductase antisera. Varying amounts of microsomal protein indicated as 15, 50, 250, 500 or 650 μg from normally fed (N), 1% cholesterol fed (CH), 0.02% lovastatin-treated (L) or 0.02% lovastatin-treated and fed 1% cholesterol (CL) were subjected to immunoprecipitation with the A9 monoclonal HMG-CoA reductase antibody. The lane on the left contains 10 μg of green fluorescent protein-ubiquitin as a control. Lanes without HMG-CoA reductase antibody with beads only (BO) and in which β-actin antibody (Actin) was used in place of the HMG-CoA reductase are displayed on the right. The upper panel was developed with ubiquitin antibody while HMG-CoA reductase antibody was used for the lower panel. The arrow indicates the position of the IgG heavy chain.

HMG-CoA reductase (Fig. 3). Ubiquitin in these immunoprecipitates migrated as smears from the top of the gels. This is the same behavior as previously observed by several investigators [2,18,27,30,31]. The apparent amount of ubiquitin associated with HMG-CoA reductase was proportional to the amount of microsomal protein used rather than uniquely increased in samples from cholesterol fed animals. This likely reflects non-specific trapping, despite very stringent washing conditions, as a beads only lane (Fig. 3) had a similar amount of ubiquitin as a corresponding lane with HMG-CoA reductase antibody. Additionally, antibody to β-actin gave the same result as the HMG-CoA reductase antibody.

3.3. Lack of proteosome involvement

To more directly access the involvement of the proteosome in degradation of hepatic HMG-CoA reductase, an experiment was conducted in which the proteosome inhibitor, lactacystin was administered to normally fed rats. If the proteosome was responsible for degradation of the reductase, one would expect to observe an increase in HMG-CoA reductase protein levels as compared with the DMSO control. As shown in Fig. 4, the levels of liver microsomal HMG-CoA reductase were nearly the same, certainly not increased, in the lactacystin treated animals as in controls indicating that proteosome inhibition does not decrease the rate of HMG-CoA reductase degradation in normal animals.

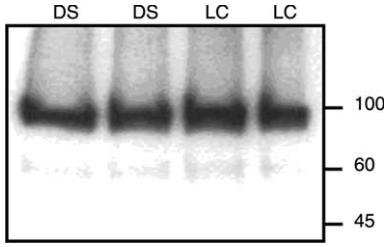

Fig. 4. Effect of lactacystin on hepatic HMG-CoA reductase protein. Rats were injected with 1 mg/kg of lactacystin in DMSO (LC) or with only DMSO (DS) and killed 5 h later. A Western blot of liver microsomal HMG-CoA reductase is shown. Molecular weight markers are on the right side. Each lane contained 25 μg of protein.

4. Discussion

4.1. Degradation rate

Physiologically, feedback regulation of the endoplasmic reticulum enzyme, HMG-CoA reductase, occurs mainly if not solely in liver [19]. Thus, it is most appropriate to study feedback regulatory mechanisms in liver. As shown in Fig. 2, feeding cholesterol to raise liver cholesterol levels did not accelerate the rate of degradation of hepatic HMG-CoA reductase. Actually, stabilization was observed in the present study. This may reflect the lower initial level of hepatic HMG-CoA reductase in these cholesterol fed animals; so that reductase protein levels cannot drop further. In a previous study in which cholesterol feeding had lowered reductase protein levels to about 50% of control, the half-life was the same as in the normal chow fed animals [20]. This finding is in contrast with results obtained in CHO tumor cells where the half-life of HMG-CoA reductase decreased from 6 to 2 h upon addition of 25-hydroxycholesterol and cholesterol to the culture media [9]. Additionally, hepatic HMG-CoA reductase protein was not stabilized when liver cholesterol levels were lowered as a result of lovastatin treatment. Furthermore, feeding cholesterol to lovastatin-treated animals did not significantly decrease the half-life of reductase protein (Fig. 2). It does not appear that feedback regulation of hepatic HMG-CoA reductase expression involves alterations in the rate of degradation of the enzyme. Rather, feedback regulation by cholesterol in liver appears to primarily involve regulation at the level of translation as we have previously demonstrated [19,32]. Perhaps, tumor cells no longer express a protein that binds to HMG-CoA reductase mRNA to slow its rate of translation in response to cholesterol or a metabolite. Thus, feedback regulation of HMG-CoA reductase in liver and tumor cells appears to occur by distinctly different mechanisms.

This is not surprising as over 30 years ago it was demonstrated that feedback regulation of HMG-CoA reductase is lost in all hepatomas [33–36]. In general HMG-CoA reductase activity was found to be higher in various hepatocellular carcinomas. This may reflect the need for more cholesterol synthesis for membranes in a population of rapidly dividing cells. It was shown that of the 17 rat tissues examined, cholesterol feeding only decreased cholesterol synthesis in liver [37]. In normal or host livers, feeding cholesterol enriched diets typically lowers hepatic HMG-CoA reductase to 1–2% of controls while in hepatomas reductase activity is increased 3–4-fold as compared with host liver [33–35].

4.2. Ubiquitination

In order to determine whether alterations in the degree of ubiquitination might play a role in the turnover of hepatic HMG-CoA reductase, immunoprecipitation experiments were performed. Our initial results were similar to those observed previously [2], showing an apparent trail of ubiquitin extending down from the top of the gel. This was most pronounced with liver microsomal samples from cholesterol fed rats. Further experiments demonstrated that the ubiquitin present was proportional to the amount of microsomal protein used. Considerably more protein was taken from cholesterol fed samples because of very low levels of reductase protein. The ubiquitin trail was also seen without addition of HMG-CoA reductase antibody (beads only) and in samples in which β-actin antibody was added (Fig. 3). The ubiquitin appears to be trapped; however, additional washes did not remove it. Thus, ubiquitin associated with the enzyme does not appear to explain the differences in hepatic HMG-CoA reductase expression observed in these studies.

4.3. Proteosome

It is well established that the proteosome degrades misfolded, mutated or damaged proteins as well as the normal turnover of cellular proteins [38]. The several studies with immortalized cells or tumor cells that reported proteosomal involvement in the degradation of HMG-CoA reductase have all added a statin, such as compactin (mevastatin) to the media, in order to induce high expression of HMG-CoA reductase [2,15,16,18,27–29]. HMG-CoA reductase from animals treated with a statin is predominantly in a 100 kDa monomer form rather than the usual disulfide-linked dimer [39,40]. This monomer form might be recognized as abnormal, become ubiquinated and then degraded by the proteosome. In immortalized SV 589 human fibroblasts grown in lipoprotein-deficient serum with compactin and mevalonate, lanosterol, 24,25-dihydrolanosterol and 27-hydroxylanosterol were found to bind to the reductase and potently stimulate ubiquitination of the reductase [41]. If this were the case in rats treated with lovastatin; one would expect to see increased ubiquination and turnover of liver microsomal HMG-CoA reductase. This was not observed (Figs. 2 and 3). Perhaps the difference between liver and tumor cells relates to the lost of normal feedback regulation of HMG-CoA reductase by cholesterol in tumor cells [33–36].

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