Diurnal and dietary-induced changes in cholesterol synthesis correlate with levels of mRNA for HMG-CoA reductase

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Abstract **We determined the extent to which diurnal variation in cholesterol synthesis in liver is controlled by steady-state mRNA levels for the rate-limiting enzyme in the pathway, hydroxymethylglutaryl (HMG)-CoA reductase. Rats 30 days of age and maintained on a low-cholesterol diet since weaning were injected intraperitoneally with ^3H_2O. The specific radioactivity of the whole-body water pool soon became constant, allowing for expression of values for incorporation of label into cholesterol as absolute rates of cholesterol synthesis. In liver, there was a peak of cholesterol synthesis from 8 PM to midnight, a 4-fold increase over synthesis rates from 8 AM to noon. Increases in synthesis were quantitatively in lock step with increases in mRNA levels for HMG-CoA reductase occurring 4 h earlier. In a parallel experiment, rats received 1% cholesterol in the diet from weaning to 30 days of age. Basal levels of hepatic cholesterol synthesis were greatly diminished and there was little diurnal variation of cholesterol synthesis or of levels of mRNA for HMG-CoA reductase. Levels of mRNA for the low density lipoprotein receptor and scavenger receptor-B1 (putative high density lipoprotein receptor) showed little diurnal variation, regardless of diet. This suggests that diurnal variation of hepatic cholesterol synthesis is driven primarily by varying the steady-state mRNA levels for HMG-CoA reductase. Other tissues were also examined. Adrenal gland also showed a 4-fold diurnal increase in accumulation of recently synthesized cholesterol. In contrast to liver, however, there was little corresponding change in mRNA expression for HMG-CoA reductase. Much of this newly synthesized cholesterol may be of hepatic origin, imported into adrenal by SR-B1, whose mRNA was up-regulated 2-fold. In brain, there was no diurnal variation in either cholesterol synthesis or mRNA expression, and no influence of high- or low-cholesterol diets on synthesis rates or HMG-CoA reductase mRNA levels.**—Jurevics, H., J. Hostettler, C. Barrett, P. Morell, and A. D. Toews. **Diurnal and dietary-induced changes in cholesterol synthesis correlate with levels of mRNA for HMG-CoA reductase.** ***J. Lipid Res.*** **2000.** 41: **1048–1053.**

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The existence of a circadian rhythm for hepatic cholesterol biosynthesis is well established (1–5). It is also known that cholesterol synthesis is suppressed by dietary cholesterol (6–8). Much of this metabolic control is exerted at the level of hydroxymethylglutaryl (HMG)-CoA reductase activity, a rate-limiting enzyme in the pathway for cholesterol synthesis (see ref. 9). Elucidation of quantitative aspects of this relationship is complicated by difficulties in obtaining absolute rates of cholesterol synthesis in vivo, as commonly used radioactive precursors are diluted to unknown extents in different body compartments. Exceptions are the studies using ^3H_2O such as those by Edwards, Muroya, and Gould (10) and Jeske and Dietschy (11).

Although HMG-CoA reductase activity can be manipulated in cultured cells, questions arise as to the relative roles of the different control mechanisms in vivo in setting the hepatic circadian rhythm for cholesterol production, and in effecting dietary regulation of cholesterol biosynthesis. HMG-CoA reductase activity is set by steady-state levels of mRNA, as well as by various post-translational mechanisms. The latter include protein synthesis and degradation, as well as balance between cAMP-dependent kinase phosphorylation (inactivation) and phosphatase-catalyzed dephosphorylation of the enzyme (for review, see ref. 12). Specifically, we wished to know the relative roles of steady-state mRNA levels versus that of post-translational mechanisms with respect to control of cholesterol biosynthesis. Our experimental design involved correlating rates of cholesterol synthesis with levels of mRNA for HMG-CoA reductase in various tissues over the course of 24 h in animals on either a low-cholesterol or high-cholesterol diet (because of the possible interplay of circadian rhythm and nutritional status). Cholesterol synthesis rates were determined in vivo; eliminating the possibility that assay of cholesterol synthesis ex vivo does not truly reflect in vivo activity. Our results suggest that much of the variation

Abbreviations: HMG, hydroxymethylglutaryl; SR, scavenger receptor; LDL-R, low density lipoprotein receptor; HDL, high density lipoprotein.

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in hepatic cholesterol synthesis, both with respect to diurnal variation and as a function of cholesterol in the diet, is set by regulation of the steady state level of mRNA. Relevant to interpretation of these data, we assayed mRNA levels for two lipoproteins involved in cholesterol trafficking, the low density lipoprotein receptor (LDL-R) and the scavenger receptor B1 (SR-B1; putative HDL-receptor). As a control for organ specific aspects of the relationship between circadian rhythm and cholesterol biosynthesis, we also examined these parameters in brain and adrenal glands of these same animals.

METHODS

Animals

Male Sprague-Dawley rats were either obtained from our own breeding colony or from Charles River Breeding Laboratories (Raleigh, NC). Animals were maintained on a 12-h light/dark cycle (light from 6 AM–6 PM) and retrieved from the animal facility 1 h before each designated time point (light or dark conditions were maintained during transport and subsequent labeling periods; see below). To assess the rate of cholesterol synthesis, rats were injected intraperitoneally with 25 mCi ^3H_2O and maintained for a 2-h labeling period that bracketed each time point (injection 1 h prior to, and killing 1 h after, each plotted time point). Rats were killed every 4 h, beginning with the 12 noon time point. Liver, adrenal glands, and brain were dissected, weighed, and lipids were extracted. Blood samples were also obtained and used to prepare serum for determination of specific radioactivity of body water (assuming 0.95 μl of water/μl of serum). For RNA analysis, tissue samples from parallel groups of animals of the same ages and dietary conditions as described above were dissected, immediately frozen on dry ice, and then stored at −80°C prior to analysis. The animals studied were from litters weaned at 20 days of age onto either a low-cholesterol diet (normal milled Purina lab chow; ≤0.002% cholesterol as reported at www.labdiet.com) or a high-cholesterol diet (milled Purina lab chow supplemented with 1% $^w/_w$ cholesterol; Sigma, St. Louis, MO). The investigation of diurnal cycle was initiated at noon when the animals were 30 days of age. A similar experiment was also carried out with suckling animals, 16 days of age, except without the variable of diet.

Lipid extraction and separation

Lipids were extracted by a modification (13) of the method of Folch, Lees, and Sloane Stanley (14). Sterols were separated from other lipids by reverse-phase HPLC on a C18 column and were eluted isocratically with acetonitrile–isopropanol (97.5:2.5). Absorbance of the eluate was monitored at 210 nm to determine fractions containing cholesterol, desmosterol (a significantly labeled cholesterol precursor in brain), and lanosterol (a significantly labeled cholesterol precursor in liver). Radioactivity in eluted fractions corresponding to the various lipids was determined by evaporating the samples, adding scintillation fluid, and quantitating radioactivity in a liquid scintillation counter.

Assay of cholesterol biosynthesis

Methods for determination of cholesterol synthesis were as outlined in detail previously (15, 16). Briefly, the rate of cholesterol synthesis was determined by measurement of incorporation of radioactivity originating from ^3H_2O into HPLC peaks corresponding to cholesterol (and desmosterol and lanosterol, precursors committed to this biosynthetic pathway). The underlying assumption is that ^3H_2O equilibrates with all body pools of water. Newly synthesized cholesterol incorporates hydrogen from water with a stoichiometry in accordance with the known pathway for cholesterol biosynthesis. As reviewed by Dietschy and Spady (17), 22 hydrogens, either from water directly or from metabolic intermediates whose protons rapidly equilibrate with water, are incorporated into cholesterol. Thus, the amount of cholesterol synthesized during the labeling period can be calculated from radioactivity present in cholesterol (and related precursors), assuming the specific radioactivity of newly synthesized cholesterol is 11 times that of the body pool of water. The smaller amounts of labeled desmosterol and lanosterol, presumably committed to cholesterol biosynthesis, were accounted for by assuming specific radioactivities of 10 and 9 times, respectively, that of body water. Relevant to the present study was the presence of label in liver lanosterol which, depending on conditions, accounted for 2 to 26% of the total label in sterol at 2 h after injection of ^3H_2O. Label in adrenal lanosterol stayed more constant, about 6% under all conditions studied. The fraction of total sterol label present as desmosterol in brain was about 60% under a variety of conditions, as reported previously (16).

If the incorporation of label were to be interpreted as a rate in absolute terms, it would be necessary to correct for the time-lag before the rate of incorporation of label into sterols (cholesterol and major lipid precursors) became linear with time. This time-lag accounts for equilibration of label from injected water with the body pool of water and subsequent early steps of incorporation leading to sterol biosynthesis. Determination of this time-lag is possible and, for example, for various brain regions it varies from 15 to 20 minutes (16). It was not feasible, however, to determine a time-lag for liver as the diurnal cycle introduces differences in incorporation rate within the same time frame as required to determine whether incorporation of label was linear. Thus, we report the data directly as assayed, without accounting for the time-lag. We reasoned that only the most drastic changes between tissues, as regards time-lag for equilibration of label with precursors, would significantly alter the relative values of rates of synthesis as measured during the diurnal cycle.

Assay of mRNA species

Procedures for analysis of mRNA have been described in detail previously (18). Briefly, total RNA was isolated from frozen tissues by homogenization in guanidine isothiocyanate followed by centrifugation through cesium chloride with subsequent ethanol precipitation (19). RNA species were separated according to molecular weight on denaturing 0.8% agarose gels containing formaldehyde and then transferred to Zeta-Probe nylon blotting membranes (Bio-Rad Laboratories, Richmond, CA). Filters were hybridized with ^{32}P-labeled cDNA probes specific for HMG-CoA reductase (20), LDL-receptor (21), or SR-B1 (22), all synthesized using single-stranded polymerase chain reaction methodology (23). Filters were washed and distribution of RNA was quantitated using a Packard InstantImager electronic autoradiography system. Filters were also exposed to X-ray film to obtain a visual pattern of mRNA levels. To control for variability in sample handling, values obtained were normalized to the amount of ribosomal RNA in each lane, as assayed with a ^{32}P end-labeled oligonucleotide specific to a defined sequence in the 28S subunit.

RESULTS

Synthesis of hepatic cholesterol as a function of diurnal cycle and dietary cholesterol

Incorporation of label from tritiated water into cholesterol (and its precursor, lanosterol) was monitored as a di-

rect correlate of the absolute rate of cholesterol synthesis in vivo. When young adult rats were fed a low-cholesterol diet, there was a 4-fold elevation in the rate of cholesterol biosynthesis, beginning just prior to entry into the dark cycle (**Fig. 1A**). When rats were maintained on a diet containing 1% cholesterol, the basal level of cholesterol biosynthesis was lowered by 60–70% and diurnal variation was suppressed. When suckling rats were examined, there was little diurnal variation in cholesterol biosynthesis, and cholesterol synthesis was even more suppressed, presumably reflecting down-regulation by the high cholesterol content of dam's milk.

For animals on a low-cholesterol diet, the steady-state level of mRNA for HMG-CoA reductase tracked cholesterol synthesis reasonably well, with the observed up-regulation of mRNA levels preceding the increase in synthesis by 4–6 hours (Fig. 1B). This is of interest because the peak mRNA level occurred prior to initiation of the dark period and the consequent marked increase in feeding activity. Up-regulation of HMG-CoA mRNA was severely blunted in animals fed a high-cholesterol diet and was absent in the milk-fed suckling pups. As cholesterol levels in liver are influenced by uptake of cholesterol-containing lipoproteins from the circulation as well as by de novo synthesis, we also assayed mRNA levels for two lipoprotein receptors, LDL-R (Fig. 1C) and SR-B1 (Fig. 1D). There were no noticeable diurnal variations in mRNA levels for either of these two lipoprotein receptors.

Synthesis of brain cholesterol as a function of diurnal cycle and dietary cholesterol

Our data give no evidence for diurnal variation in cholesterol synthesis in brain, regardless of the nutritional status (**Fig. 2A**). Cholesterol synthesis in brains of the 16-day-old suckling animals was approximately 50% higher than that in the 30-day-old rats; this presumably reflects the rapid accumulation of cholesterol-rich myelin in the younger animals. As circulating cholesterol is not available for use by the nervous system, all cholesterol required for myelin must be synthesized locally in brain (16, 24). There was also no significant diurnal variation in brain for mRNA levels coding for HMG-CoA reductase (Fig. 2B) or for the two lipoprotein receptors studied (Fig. 2C, D). In addition, dietary status had no effect on mRNA levels for these proteins.

Synthesis of adrenal cholesterol as a function of diurnal cycle and dietary cholesterol

For animals on a low cholesterol diet, the diurnal variation in cholesterol biosynthesis in the adrenal gland was similar to that seen in liver (**Fig. 3A**). In contrast to liver, however, the prominent increase in cholesterol synthesis in the middle of the dark phase was not preceded by an increase in HMG-CoA reductase mRNA levels (Fig. 3B). Inclusion of cholesterol in the diet markedly suppressed basal levels of cholesterol synthesis as well as the diurnal increase, again without observable fluctuation in levels of mRNA for HMG-CoA reductase. Interestingly, although neither diet or cholesterol feeding status had much effect on LDL-R mRNA levels, there was an approximately 2-fold

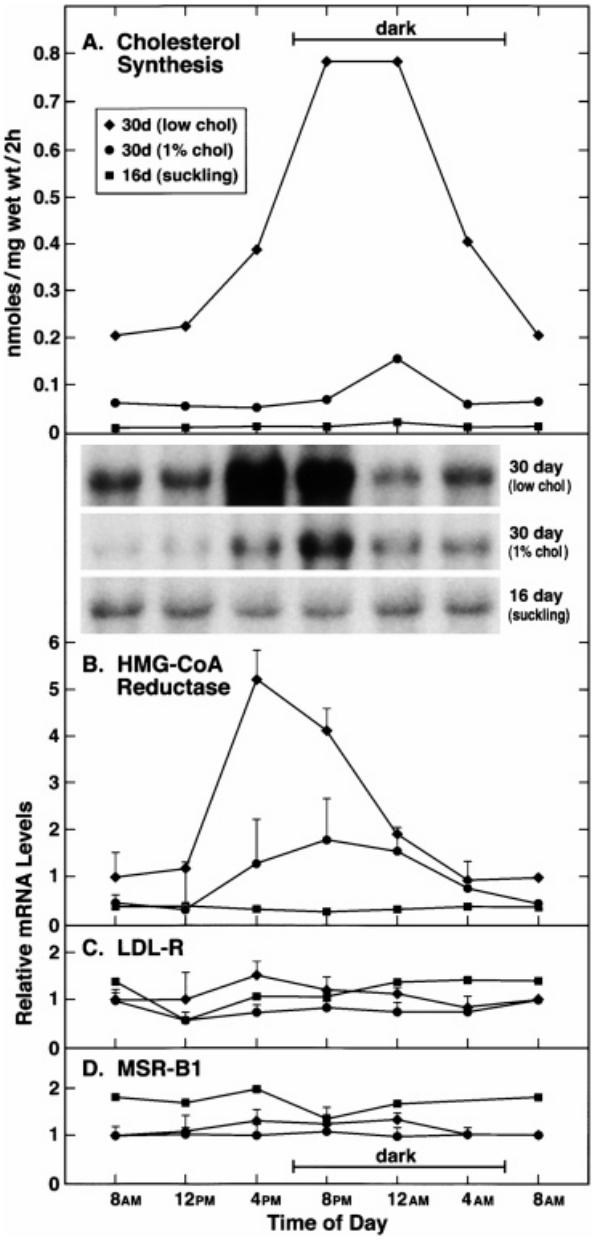

Fig. 1. (A) Diurnal variation of rates of cholesterol synthesis in livers of 30-day-old rats. Animals were kept on either a low-cholesterol diet or on a 1% cholesterol diet for 10 days after weaning. Data for 16-day-old suckling pups are also shown. Absolute in vivo synthesis rates of cholesterol were determined after intraperitoneal injection of ^3H_2O. The assay involved determination of radioactivity incorporated into HPLC peaks corresponding to cholesterol as well as label in precursors committed to the cholesterol biosynthesis pathway (see Methods for details). (B) Diurnal variation in steady-state level of mRNA for HMG-CoA reductase in liver. Northern blots were carried out with a ^{32}P-labeled cDNA probe specific for HMG-CoA reductase, as described in Methods. After rinsing to remove unbound probe, filters were exposed to X-ray film to obtain a visual image of relative mRNA levels. Typical results are shown in the insert. Data such as that shown in the insert were quantified using an electronic autoradiography system, and the results were plotted. Data have been normalized to a constant amount of rRNA, as assessed after hybridization with ^{32}P end-labeled oligonucleotides specific for 28S rRNA. Values are means ± SEM of 3 different samples, except that those for suckling animals were obtained with single samples. (C) Diurnal variation in steady-state level of mRNA for LDL-receptor in liver. Otherwise as in legend to 1B. (D) Diurnal variation in steady-state level of mRNA for the scavenger receptor-B1. Otherwise as in legend to 1B.

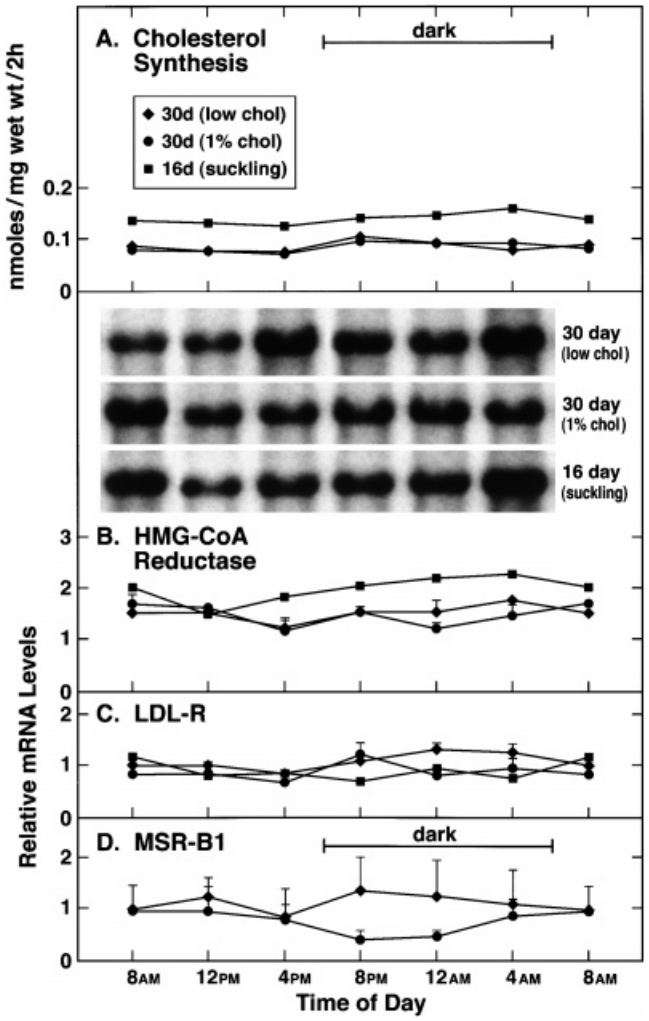

Fig. 2. Diurnal variation of rate of cholesterol synthesis, and of mRNA levels, in brains of the same rats described in the legend to Fig.1. All sections of that legend apply with the substitution of "brain" for "liver."

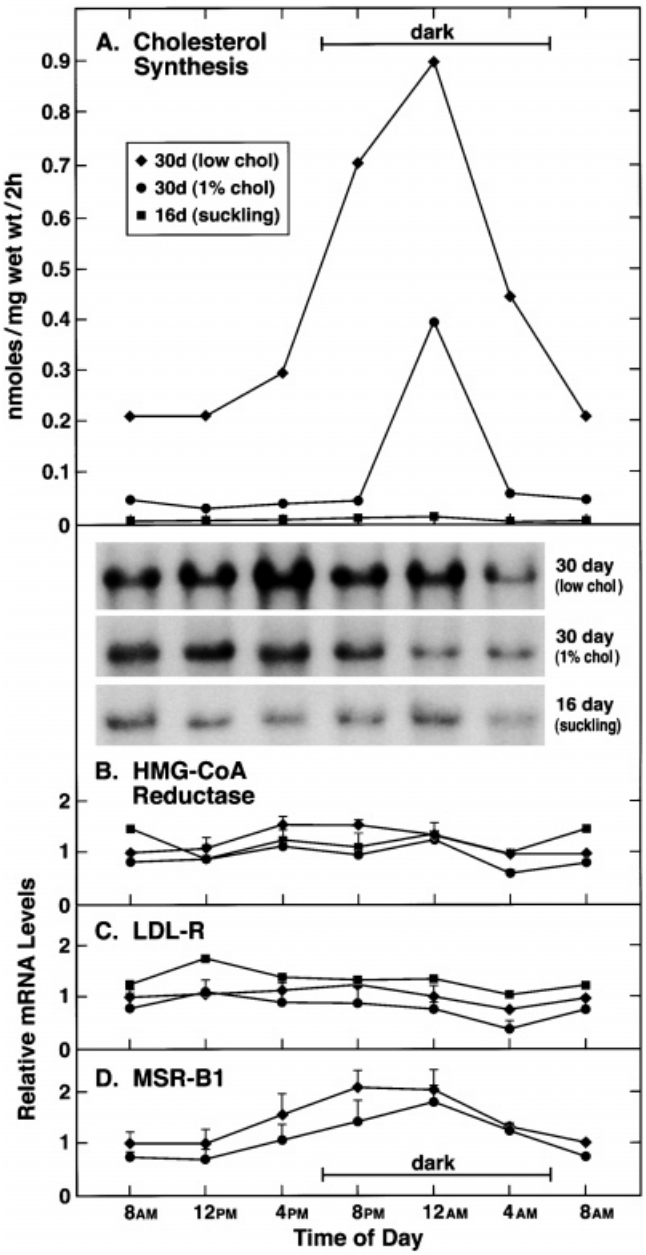

Fig. 3. Diurnal variation of rate of cholesterol synthesis, and of mRNA levels, in adrenal glands of the same rats described in the legend to Fig. 1. All sections of that legend apply with the substitution of "adrenal gland" for "liver."

diurnal increase in mRNA levels for SR-B1, and this increase was present in animals on both the low- or high-cholesterol diets (Fig. 3C, D); see Discussion regarding a role for this receptor in uptake of circulating cholesterol by adrenal gland. Levels of synthesis of cholesterol by adrenal gland in suckling pups was very low, presumably due to the presence of maternally derived hormones that suppress growth and maturation of the adrenals (25).

Relative levels of mRNA expression in liver, brain and adrenal glands

A comparison of relative levels of mRNA for HMG-CoA reductase, LDL-R, and SR-B1 in various tissues of 30-day-old rats on a low cholesterol diet at 4 PM (time of maximal mRNA expression for HMG-CoA reductase in liver) is shown in **Fig. 4.** For each of the three mRNA species examined, levels were highest in adrenal gland. Levels of mRNA for HMG-CoA reductase in liver and brain were approximately 60% and 25% of those in adrenal, respectively. For both LDL-R and SR-B1, mRNA levels in liver and brain were much lower than in adrenal gland.

DISCUSSION

The diurnal 4-fold variation in hepatic cholesterol synthesis is similar to that reported previously (10, 26, 27). Our data correlating this variation with that for mRNA levels for HMG-CoA reductase suggest that much of the control for activity of this rate-limiting enzyme of cholesterol biosynthesis involves alterations in steady-state mRNA levels. Dietary cholesterol clearly suppresses the dark-phase elevation of cholesterol synthesis and the preceding rise in HMG-CoA reductase mRNA. We conclude that the increased hepatic synthesis of cholesterol in rats fed cholesterol-poor diets, relative to those on cholesterol-rich diets, is driven

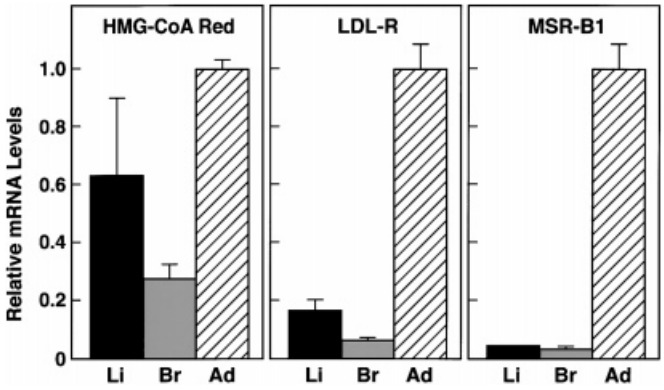

Fig. 4. Relative levels of mRNA expression for HMG-CoA reductase, LDL-R, and SR-B1 in liver, brain, and adrenal gland. Data shown are from tissues taken from animals on a low-cholesterol diet, at the 4 PM time-point (maximal level of mRNA for HMG-CoA reductase in liver). Data were normalized to a constant amount of rRNA in each sample and are plotted relative to maximal values for each mRNA species. Values are means ± SEM of samples from 3 separate animals.

largely by increased levels of mRNA for HMG-CoA reductase. This view contrasts with that of Ness, Keller, and Pendleton (28) as stated in the title of their paper "Feedback regulation of hepatic 3-hydroxy-3-methylglutaryl-CoA reductase activity by dietary cholesterol is not due to altered mRNA levels." These authors utilized a 14-h light/10-h dark daily cycle, and obtained samples at or just past the midpoint of the dark cycle (roughly equivalent to our midnight time-point). The discrepancy may arise because Ness et al. (28) selected a single time-point during the day for study, possibly so far into the dark cycle that, although near the peak of cholesterol synthesis, mRNA levels had already returned to near-normal levels (see Figs. 1A and B). As indicated in Fig. 1B, missing the approximately 8-h period of markedly elevated mRNA levels would give a result suggesting that mRNA levels do not vary much as a function of diet. Our study does not challenge the several papers from this group (e.g., 29) indicating that both diurnal variation and cholesterol feeding status bring about changes in the amount of HMG-CoA reductase as immunoreactive protein; rather it suggests a different (or additional) control point for effecting this change.

Our conclusions may also restrict somewhat the generalization of Spady and Cuthbert (30) who state that "in vivo cholesterol synthesis is predominantly controlled by postranscriptional regulation of HMG-CoA reductase activity." Their relevant data demonstrated that levels of hepatic HMG-CoA reductase mRNA were reduced by only 50% while total cholesterol synthesis was suppressed by 98% by feeding of high levels of bile salts as well as cholesterol. We assume that this more vigorous manipulation and the greater age of the animals they studied (about 4 months old) might present a more extreme case in which compensatory mechanisms available at the level of transcription are exceeded and other mechanisms, noted in the Introduction, come into play.

Our study also adds to what is known regarding regulation of LDL-R mRNA levels. In cultured cells, regulation of steady-state mRNA levels for LDL-R correlate well with those for HMG-CoA reductase (31). The correlation is weaker in vivo in the mouse, where a diet-induced 10-fold increase in hepatic mRNA for HMG-CoA reductase produced only a 2-fold increase in mRNA for LDL-R (32). Our results for the rat also indicate that, under normal circumstances, the correlation is weak (our data cannot exclude a 10 to 2 correlation). Again, if the regulatory mechanism is more severely strained, as with bile salt feeding, a coordinate regulation of mRNA for these two genes in vivo can be demonstrated (30).

Results for brain were very different from those obtained with liver. These experiments, the initial focus of the present study, were to determine whether synthesis of cholesterol by brain was subject to diurnal variation. Were this the case, it would complicate our ongoing studies of coordination of metabolic events involved in regeneration of myelin, a cholesterol-enriched membrane, after metabolic insult. The negative result for diurnal variation in brain also serves as a control for the positive results obtained with liver.

The data for adrenal gland also show a 4-fold diurnal variation in accumulation of newly synthesized cholesterol, of about the same magnitude and time-course as in liver. This is as expected from the well-known diurnal rhythm of HMG-CoA reductase enzyme activity shown in the adrenal gland of the rat (33, 34). Our data suggest that, unlike the situation in the liver, the control for this cycle is not at the level of a proportional increase in level of mRNA for HMG-CoA reductase. This is not unexpected in view of the demonstration that the steady-state level of the mRNA is higher than that achieved by liver at any time during the diurnal cycle. We note that our observation of lack of diurnal cycle for this mRNA species is not completely compatible with a previous investigation (34) in which a 2-fold variation of mRNA levels for this enzyme was demonstrated.

Another interpretation of the data is that much of the labeled cholesterol accumulating in adrenal glands may be of hepatic origin and may arrive through the circulation. It is known that the adrenal glands obtain appreciable amounts of newly synthesized cholesterol from the circulation (35, 36). The scavenger receptor SR-B1 has been reported to play a prominent role in delivering cholesterol from the circulation to adrenal gland, where its expression is coordinately regulated with steroidogenesis (22, 37–39). In fact, steady-state mRNA levels for this receptor were much higher in adrenal than in either liver or brain. The moderate increase in SR-B1 mRNA levels during the dark feeding period supports a role for this receptor in uptake of cholesterol by the adrenal, regardless of the dietary state.

In summary, our data suggest that alteration of steady-state levels of mRNA for HMG-CoA reductase can account for diurnal variation in hepatic cholesterol synthesis. Furthermore, we have shown that, contrary to expectations from the literature, basal levels of mRNA coding for HMG-CoA reductase in liver are altered by cholesterol feeding.

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