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MINIREVIEW

Feeding-Induced Regulation of Cholesterol Metabolism: A Unified Proposal (42320)

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It is generally agreed that the liver is a key organ in the maintenance of cholesterol homeostasis. Although species variations do exist (1), there is no question that the liver makes a very significant contribution to whole body cholesterol synthesis (2). Indeed, recent studies in the rat suggest that the liver may be responsible for the synthesis of greater than 80% of newly synthesized cholesterol in the body (3). It is also well established that the liver is a major site of uptake of cholesterol from lipoprotein particles (1). The acquisition of cholesterol from lipoprotein particles is a process which occurs via both receptor-independent and receptor-dependent pathways (1). The latter pathway is considered to be more important not only from a quantitative standpoint (1) but also because of the fact that the activity of certain receptors is regulated by the hepatic demand for cholesterol (4). These observations, combined with the fact that the liver is the major site (if not the only site) of synthesis of bile acid, the chief catabolic product of cholesterol (5), suggest that the key hepatic enzymes involved in cholesterol metabolism must be very carefully regulated in order to maintain cholesterol homeostasis.

Hepatic cholesterol 7α-hydroxylase (CH-7A, EC 1.14.13.7) and β-hydroxy-β-methylglutaryl coenzyme A reductase (HMGCoA reductase, EC 1.1.1.34) are the two key enzymes of cholesterol metabolism in the liver (2, 6). HMGCoA reductase is the rate-limiting enzyme in the biosynthesis of cholesterol (2), while CH-7A is considered to be the rate-limiting enzyme in the biosynthesis of bile acids from cholesterol (6). Since cholesterol is the precursor for bile acid biosynthesis, changes in the rate of synthesis of bile acids, in general, affect the rate of biosynthesis of cholesterol (6). Thus, the two enzymes are regulated in a coordinated manner (6).

Consistent with the above is the fact that both the enzymes are also known to have a pronounced diurnal rhythm (7, 8). For example, in *ad libitum*-fed rats kept on a 12-hr light/dark cycle, the diurnal high in enzyme activity occurs at about the midpoint of the dark cycle (7, 8). A nadir in enzyme activity usually occurs at the midpoint of the light cycle (7, 8). It has also been shown that the diurnal variation in enzyme activity is closely linked to the feeding status of the animal (8, 9). This has been shown in rats maintained on the usual light/dark cycle but fed only during the light cycle. Activity of both the enzymes can be shown to increase 4 to 6 hr after feeding (8, 9). Further support for the hypothesis that the increase in enzyme activity is associated with feeding lies in the fact that rats are nocturnal animals and consume about 80% of the total daily food intake during the dark cycle (10). Conversely, fasting has been shown to be associated with a reduction in enzyme activity (2, 7).

The increase in the activity of CH-7A and HMGCoA reductase is related to food intake, and several mechanisms have been proposed to account for the increase in activity of these enzymes subsequent to feeding. However, there is little, if any, information regarding the mechanism, or signal, that triggers these changes. Nevertheless, sufficient evidence has accumulated in recent years to suggest that changes in hepatic "thiol status" are probably responsible for the feeding-related changes in enzyme activity. Thus, the objective of this review is to present the evidence which supports the hypothesis that changes in the hepatic content of reduced glutathione (GSH) may be the missing link in the feeding-related regulation of CH-7A and HMGCoA reductase. This, in turn, will be used to provide a unified concept of the feeding-related regulation of cholesterol metabolism.

Effect of sulfhydryl agents on HMGCoA reductase activity. An indication that sulfhydryl agents may be important in the regulation of

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HMGCoA reductase was first reported by Bucher and McGarrahan in 1956 (11). They found that GSH added to the buffer prior to homogenization of the liver increased the conversion of acetate to cholesterol. Subsequent studies by Durr and Rudney (12) showed that reduced sulfhydryl agents such as GSH, cysteine, and pantetheine had a "protective" effect on the activity of HMGCoA reductase. On the other hand, oxidizing agents such as *p*-hydroxymercuribenzoate inhibited the enzyme activity. Kirtley and Rudney (13) showed that the enzyme activity was stabilized by the addition of mercaptoethanol. However, an intriguing finding was that the enzyme was inhibited by solutions of coenzyme A (CoASH) or by solutions of HMGCoA. This was a curious finding, the basis for which was not understood. Finally, among the earlier studies, Kawachi and Rudney (14) showed that addition of dithiothreitol (DTT) to the incubation medium could stimulate HMGCoA reductase activity.

In the above studies, the basis for the effect of reduced sulfhydryl agents on HMGCoA reductase activity was not understood. However, since oxidizing agents could inactivate the enzyme, it was assumed that reduced sulfhydryl agents protected the enzyme against oxidative inactivation.

In 1981 Gilbert and Stewart (15) looked for the basis for the observation by Kirtley and Rudney that solutions of CoASH and HMGCoA inhibited HMGCoA reductase activity. They showed that solutions of coenzyme A disulfide (CoASSCoA) rapidly and reversibly inactivated, while solutions of DTT reactivated, HMGCoA reductase. Addition of solutions of HMGCoA and CoASH also reversibly inactivated the enzyme. The activity could be restored by dialysis of the enzyme against buffer containing DTT. If, however, the solutions of HMGCoA and CoASH were incubated with DTT for 1.5 hr prior to their addition to the HMGCoA reductase solution, then the enzyme was not inactivated. Taken together, the results suggested that the inhibitory effects of HMGCoA and CoASH solutions on HMGCoA reductase (13, 15) were probably due to contamination of those solutions by CoASSCoA. HPLC examination of the solutions of HMGCoA and CoASH showed that to be the case (15). Thus, it was shown that the enzyme could be inhibited by disulfides. These observations—coupled with the demonstration that reagents such as glutathione disulfide (GSSG), Ellman's reagent, and *p*-hydroxymercuribenzoate could also inactivate the enzyme—suggested (15) that the enzyme had sulfhydryl groups which were required to be in the reduced state for optimal enzyme activity.

Some of the more definitive studies on the role of sulfhydryl agents in the regulation of HMGCoA reductase have recently been reported by Shechter and his colleagues (16–19). Dotan and Shechter (16) showed that the enzyme isolated in the absence of sulfhydryl agents was inactive. However, full activity could be restored equally well by DTT or dithioerythritol (DTE) and less so by GSH. They showed that the enzyme could be inhibited by GSSG and that the degree of inhibition was proportional to the GSH/GSSG ratio in the incubation (17). Lack of stimulation of inactive HMGCoA reductase exposed to $NaBH_4$ showed that the activation effect of thiols was due specifically to thiols and not a nonspecific effect of reducing substances. Finally, Dotan and Shechter showed that the thiol-activated enzyme could bind to specific affinity resins, while the inactive enzyme did not, suggesting that activation of the enzyme by thiols involved increasing the affinity of the enzyme for the substrate (16).

Dotan and Shechter (17) subsequently confirmed their earlier observations and in addition showed that DTT, DTE, and GSH affected both K_m and V_{max} values of the enzyme toward HMGCoA and NADPH. They also showed that DTT and GSH probably competed for the same binding site on the enzyme and that diethylene glycol disulfide (ESSE) was a more potent inhibitor of enzyme activity than GSSG (18).

Finally, Roitelman and Shechter (19) have provided evidence for a GSH-dependent allosteric regulation of HMGCoA reductase activity. At low concentrations of GSH (2 to 4 mM), enzyme activity with increasing concentrations of NADPH showed sigmoidal kinetics with a Hill coefficient of 2.01 ± 0.07. Increasing GSH concentrations resulted in a gradual change toward Michaelis–Menten kinetics and a Hill coefficient of 1.0 ± 0.03 for NADPH at 25 mM GSH. Their results sug-

gested that GSH/GSSG ratio-related regulation of HMGCoA reductase was not an "all or none" phenomenon and that the enzyme was responsive to the thiol status of the cell and modulated its activity accordingly.

All of the above studies were conducted with either microsomal preparations or preparations of the solubilized enzyme *in vitro*. If the above studies are correct, then it seems reasonable that HMGCoA reductase activity assayed *in vitro* would be affected by physiological variations in hepatic GSH content which occur during fasting or feeding (20). While it is known that HMGCoA reductase activity is decreased upon fasting and increases upon feeding (2), there has been no study which has directly examined the relationship between *in vivo* thiol status and *in vitro* enzyme activity.

Effect of sulfhydryl agents on CH-7A activity. Scholan and Boyd (21) were the first to report that sulfhydryl agents may be involved in the regulation of CH-7A activity. They showed that β-mercaptoethylamine at 10 m*M* in the incubation medium significantly enhanced CH-7A activity (21). Since CH-7A activity was also markedly inhibited by low concentrations (20 μm) of *p*-chloromercuribenzoate, they suggested that CH-7A contained important thiol groups which were required to be maintained in the reduced state.

Data on the role of sulfhydryl agents in the regulation of CH-7A are limited, owing in part to the difficulties in obtaining relatively pure and active preparations of specific cytochrome *P*-450 (CP-450) manifesting the 7α-hydroxylase activity. Recently, Kalles and Wikvall (22) showed that in purified reconstituted systems, DTT stimulated CH-7A activity severalfold. CH-7A activity was sensitive to inhibition by *p*-chloromercuribenzoate, *n*-ethylmaleimide, and iodoacetamide, and the enzyme could be reactivated by treatment with DTT. Further, they showed that CH-7A activity was more sensitive to sulfhydryl agents than 12α- or 25-hydroxylases (22).

In a subsequent report, Danielsson *et al.* (23) showed that in purified reconstituted systems the addition of a specific cytosolic fraction could stimulate CH-7A activity severalfold. However, this stimulation was only achieved in the presence of GSH. The addition of GSSG at low concentrations (0.1 m*M*) virtually abolished the stimulatory action of the cytosolic protein. Interestingly, cysteine and DTT were found to stimulate CH-7A activity whether or not the cytosolic factor was present (23). Thus, these studies confirmed the previous observations and, for the first time, showed that a normally occurring endogenous thiol, GSH, could also modulate CH-7A activity.

If the *in vitro* observations of Danielsson *et al.* (23) are correct, then it seems reasonable that endogenous fluctuations in hepatic GSH levels might affect CH-7A activity. The first study to show that modulation of *in vivo* levels of GSH might affect CH-7A activity was carried out recently by Hassan *et al.* (24). Rats were first treated with diethylmaleate (DEM) to rapidly deplete hepatic GSH. Thirty minutes following DEM treatment, some of the animals were treated with L-cysteine, the rate-limiting amino acid in GSH biosynthesis. Thirty minutes following L-cysteine treatment all the animals were killed, and hepatic GSH content and CH-7A activity were assayed. It was found that the *in vitro* CH-7A activity (measured in microsomes) was dependent on the hepatic GSH content at the time the animals were killed. The dependence of the enzyme activity on hepatic GSH content followed Michaelis–Menten kinetics, and a Lineweaver–Burk transformation of the data yielded a straight line ($r = 0.90$). The calculated V_{max} was 34.4 pmole/mg/min with a half-maximal activity at a hepatic GSH content of 1.9 μmole/g. Since hepatic GSH content in the rat varies between 5 and 7 μmole/g, this study showed that CH-7A would operate at 70 to 80% of the calculated maximal rate under physiological conditions.

If acute changes in hepatic GSH content could affect CH-7A activity, then it is reasonable to hypothesize that under physiological conditions such as fasting or feeding, which affect GSH content, CH-7A activity might also be affected. Indeed, it is well known that fasting reduces CH-7A activity and feeding is associated with an increase (7). This hypothesis has been tested in rats adapted to a 2-hr feeding schedule during the 8 AM to 6 PM light cycle[1]. The rats were adapted to eat between 8 AM and 10 AM for 7 days. On the day of the study,

[1] Manuscript submitted for publication.

one group of animals was injected with buthionine sulfoximine (BSO, an inhibitor of γ-glutamylcysteine synthetase) (25), while the control group received an equivalent volume of vehicle. The animals were fed for 2 hr and then killed 2 hr later, and hepatic GSH content and CH-7A activity were assayed. BSO-treated animals had significantly lower CH-7A activity, which was associated with a significantly lower hepatic GSH content relative to controls. Since only the biosynthesis of GSH was blocked, then the results of the study imply that variations in hepatic GSH content due to physiological conditions of feeding or fasting can modulate the activity of hepatic CH-7A. Interestingly, a recent study by Andersson and Bostrom (26) showed that disulfiram (Antabuse), a disulfide-containing drug, could inhibit CH-7A activity both *in vivo* and *in vitro* and that this inhibition could be reversed by DTT. This is further evidence that thiols are involved in the regulation of CH-7A activity.

Feeding status, hepatic GSH content, and enzyme activity. The foregoing discussion on the effect of sulfhydryl agents *in vitro* on HMGCoA reductase taken together with the *in vitro* and *in vivo* studies on the role of sulfhydryl agents on CH-7A activity are strong evidence that both these enzymes are probably regulated by physiological variations in the major intracellular thiol, GSH. It is possible that the diurnal variation in the activity of both the enzymes is linked, at least in part, to concurrent physiological changes in hepatic GSH content. Thus, in order to substantiate this supposition, it is necessary to demonstrate that there is a diurnal variation in hepatic GSH content which is linked to feeding.

In the first study to document a diurnal variation of hepatic GSH content, Beck *et al.* (27) showed that in *ad libitum*-fed mice and rats, the diurnal high in hepatic GSH content occurred in the early morning hours (9:30 AM), while the nadir in hepatic GSH content occurred around 4:30 PM. This pattern held true even in adrenalectomized animals. Beck *et al.* hypothesized that the diurnal variation in hepatic GSH was linked to the increase in food intake during the dark period, thereby increasing the availability of sulfur containing amino acids. Increased availability of cysteine, the rate-limiting amino acid in GSH biosynthesis, would allow for the increase in GSH biosynthesis by the liver for eventual transport to other organs. It is now well established that liver is indeed the major organ of GSH biosynthesis, and there is considerable flux of GSH from the liver to other organs of the body (20, 28).

Subsequent to the study by Beck *et al.* (27), Jaeger *et al.* (29) demonstrated a similar phenomenon and suggested that the diurnal fluctuations in hepatic GSH content may be responsible for the increased toxicity of certain compounds during certain times of the day coinciding with the diurnal low in hepatic GSH content. Several laboratories have recently confirmed the original observations of Beck *et al.* (30–32). Indeed, a recent report has shown fairly convincingly that the diurnal variation in hepatic GSH content is linked to food intake (32). By making food available only during the light cycle, Jaeschke and Wendel (32) were able to reverse the time of the diurnal high and low point in hepatic GSH content normally seen in *ad libitum*-fed mice.

Thus, it is clear that the changes in the activity of hepatic HMGCoA reductase and CH-7A are occurring at the same time as changes in hepatic content of GSH. However, careful examination of the time course of the diurnal variation in hepatic GSH content and activities of hepatic HMGCoA reductase and CH-7A reveals a discrepancy. The activities of both the enzymes reach peak and minimum levels several hours before hepatic GSH content achieves the maximum and minimum values, respectively. This discrepancy exists only if it is assumed that the enzymes respond only to changes in absolute GSH content. If, on the other hand, the enzymes are responsive to the change in the GSH/GSSG ratio as suggested by the studies of Dotan and Shechter (17), then there is no discrepancy. Normally, hepatic GSSG content is very low (20). Thus, small changes in GSH content can result in large changes in the GSH/GSSG ratio. With the onset of feeding, GSH/GSSG ratio would change, triggering the activation of HMGCoA reductase and CH-7A. The activities of these two enzymes would continue to increase with increasing GSH/GSSG ratio up to a point when further increases in GSH/GSSG ratio would not increase enzyme activity any further. This would be the point in time when the peak in enzyme activity would be reached

even though absolute hepatic GSH content had not achieved the maximum. The reverse would hold true for the decline in the activities of the enzymes to their diurnal nadir. Isaacs and Binkley (33) have shown that there is a diurnal variation in GSH/GSSG ratio. Thus, it appears that both HMGCoA reductase and CH-7A activities may be regulated by the "thiol/disulfide" status of the liver similar to that described by Gilbert (34) for phosphofructokinase. Since (i) NADPH is a cofactor in the reaction catalyzed by both HMGCoA reductase and CH-7A, and (ii) NADPH is utilized in the reduction of GSSG to GSH, then linking of the enzyme activities to the GSH/GSSG ratio also suggests that both cholesterol and bile acid biosynthesis are tightly coupled to the reductive state of the cell.

The final question in the GSH/GSSG ratio-linked regulation of HMGCoA reductase and CH-7A concerns the nature of the change(s) which might be induced in the two enzymes by changes in the GSH/GSSG ratio. With regard to HMGCoA reductase, there is convincing evidence that the diurnal increase in "activity" is due, at least in part, to an increase in enzyme content (35).

Clarke *et al.* (36) showed that there was a diurnal variation in the HMGCoA reductase mRNA content which coincided with the reductase protein content and activity. There is also very convincing evidence that the diurnal variation in reductase activity may be due to changes in the phosphorylation status of the enzyme (37, 38). Phosphorylation of the reductase reduces its activity, while dephosphorylation activates the enzyme (37, 38). Using a "cold clamping" technique, Easom and Zammit (39) have shown that the diurnal variation in enzyme activity can be accounted for by the phosphorylation status of the enzyme. At the diurnal high point, most of the enzyme is in the active form (active/total ratio, 0.8), whereas at the diurnal low point most of the enzyme is inactive (active/total ratio, 0.30). The transition between these two points is gradual and, therefore, allows for the possibility of regulation by physiological stimuli such as food intake. Since (i) hepatic cAMP content also varies diurnally, low during the dark cycle and high during the light cycle (40), and (ii) cAMP is involved in the phosphorylation of HMGCoA reductase (37), then it is possible that the diurnal variation of reductase activity is due to both changes in content and activity. It may be hypothesized that the diurnal variation in GSH/GSSG ratio modulates the synthetic activity of the reductase while cAMP levels modulate the phosphorylation status of the reductase. Thus, during the dark cycle, more reductase is synthesized, most of which is not phosphorylated, the combination accounting for the high "active/total" ratio found by Easom and Zammit (39).

With respect to CH-7A, it is not clear how changes in GSH/GSSG ratio modulate its activity. An important reason for the lack of information is the fact that procedures involved in the purification of the specific c*P*-450 have only been described recently (41). However, it is known that CH-7A has a very short half-life ($2\frac{1}{2}$ to 3 hr) (42). Thus, it is possible that changes in GSH/GSSG ratio affect the rate of synthesis of CH-7A.

With regard to phosphorylation/dephosphorylation status on CH-7A activity, there is some controversy. In microsomal preparations several groups have been able to demonstrate that phosphorylation of the enzyme increases the activity (43, 44). However, in purified reconstituted systems, there is no convincing evidence that phosphorylation/dephosphorylation has an important regulatory role in the modulation of CH-7A activity (45). Since cAMP levels are decreasing at a time when CH-7A activity is increasing, it does not seem likely that phosphorylation status of the enzyme is an important *in vivo* modulator of CH-7A activity.

Finally, GSH/GSSG ratio may have allosteric effects on CH-7A similar to that described for HMGCoA reductase. It is likely that more information about the regulation of CH-7A will be forthcoming because it is now possible to obtain the specific CP-450 in fairly pure form.

Feeding-related regulation of cholesterol metabolism. Figure 1 shows the overall feeding-related regulation of cholesterol metabolism. Initiation of feeding or an increase in feed intake after a period of relative fasting results in an increase in hepatic GSH content and, therefore, a change in GSH/GSSG ratio. An increase in GSH/GSSG ratio triggers an increase in the activities of CH-7A and HMGCoA reductase. Increase in CH-7A ac-

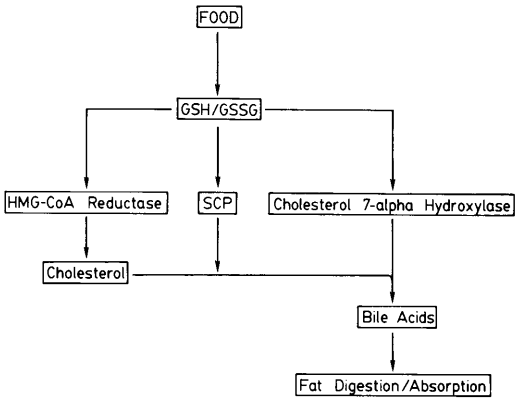

FIG. 1. Feeding-related regulation of hepatic HMGCoA reductase and cholesterol 7α-hydroxylase. Feeding leads to an increase in hepatic GSH content and, therefore, an increase in GSH/GSSG ratio. This change in GSH/GSSG ratio is hypothesized to result in an increase in the activities of HMGCoA reductase and cholesterol 7α-hydroxylase. The increase in activity of these enzymes may be due to (i) increased synthesis, (ii) changes in phosphorylation status, and (iii) allosteric modifications induced by the change in GSH/GSSG ratio. At the same time, the change in GSH/GSSG ratio is hypothesized to increase the synthesis of sterol carrier protein (SCP) or SCP_2. Increased conversion of cholesterol to bile acids leaves a "sink" for cholesterol which is filled by increased biosynthesis of cholesterol. SCP is believed to stimulate the synthesis of bile acids presumably by making the substrate, cholesterol, more accessible to the enzyme, cholesterol 7α-hydroxylase. Overall result is an increase in bile acid biosynthesis presumably to maximize the efficiency of fat digestion and absorption.

tivity "drains" the endogenous hepatic cholesterol pool and provides a "sink" for cholesterol which is "filled" by the increase in the rate of cholesterol biosynthesis. In this context, it is of interest to note that a recent report by Seltman *et al.* (46) showed that a specific cytosolic sterol carrier protein (SCP_2) stimulated CH-7A activity *in vitro*. While the mechanism for the effect of SCP_2 on CH-7A activity is not known, it is believed that SCP_2 may function in making the substrate, cholesterol, more accessible to the enzyme. If this is indeed the mechanism of action of SCP_2 on CH-7A, then it is also of interest to note that Dempsey and others have recently shown that there is a diurnal variation in the hepatic content of a sterol carrier protein (SCP) (47, 48). The amount increases approximately sevenfold during the dark cycle and returns to basal levels by the beginning of the light cycle. They have shown that this diurnal change in SCP content is not due to an increase in SCP mRNA content but due to an increased rate of translation of the SCP mRNA (48). They have also shown that the diurnal increase in SCP content is related to food consumption and associated hormonal events, rather than the photoperiod (49). It is not known whether the Dempsey SCP has the same effect on CH-7A as SCP_2. Comparison of amino acid residues of the 2 proteins reveal significant differences (50). However, the Dempsey SCP has been shown to stimulate 12α-hydroxylase activity (51). While further studies are necessary to elucidate the properties of Dempsey SCP and SCP_2, the above studies provide a logical addition to the feeding-related regulation of cholesterol metabolism. That is, it may be hypothesized that the food-induced change in GSH/GSSG also triggers, in some unknown way, an increase in the rate of translation of SCP (or SCP_2) mRNA leading to an increase in SCP (or SCP_2) content at the same time when HMGCoA reductase and CH-7A activities are also increasing. The increase in SCP (or SCP_2) content would facilitate the "delivery" of cholesterol, the substrate for CH-7A.

Finally, one may ask a teleological question concerning the purpose behind the food-related simultaneous increase in the activities of HMGCoA reductase, CH-7A, and SCP content. I propose that the system is geared toward maximizing the digestion and absorption of fat. Clearly, the net result of these processes occurring simultaneously would be an increase in the synthesis of bile acids which are required for efficient fat digestion and absorption (52). Since fat has the highest caloric density (9 kcal/g), it "behooves" the animal to be able to maximize the digestion and absorption of fat.

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