The in Vivo Regulation of Rat Liver 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase

PHOSPHORYLATION OF THE ENZYME AS AN EARLY REGULATORY RESPONSE FOLLOWING CHOLESTEROL FEEDING

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Although substantial evidence supports the conclusion that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the major regulatory enzyme in cholesterol biosynthesis, the molecular events involved in the in vivo regulation of this enzyme have remained obscure. In order to study this problem, rats received a single meal consisting of either rat chow or rat chow containing 2% cholesterol. The rats were killed 60 or 120 min after the beginning of feeding, and liver microsomes were prepared by ultracentrifugation. Two phases of inhibition of microsomal HMG-CoA reductase were observed. The first phase of inhibition, observed 60 min after the beginning of cholesterol feeding, was completely reversed by preincubation of the microsomes with purified phosphoprotein phosphatase. By contrast, however, at 120 min after the beginning of cholesterol feeding, the inhibition of rat liver HMG-CoA reductase, produced in vivo, was not reversed by in vitro preincubation of the microsomes with phosphoprotein phosphatase.

EXPERIMENTAL PROCEDURES

Materials—The materials used in this article were obtained from described sources. The 2% cholesterol rat chow diet was obtained from U. S. Biochemicals. Sephadex G-25 columns (PD-10) were obtained from Pharmacia.

Cholesterol Feeding to Rats—Male Sprague-Dawley rats were maintained on a 4 a.m. to 4 p.m. dark cycle (with 4 p.m. to 4 a.m., light) for a minimum of 1 week prior to the experiment. For 3 days before the experiment, the rats (150 to 200 g), housed two/cage, were fed normal rat chow for only 30 min/day (8:30 a.m. to 9:00 a.m.). On the day of the experiment, pairs of rats were fed either rat chow or rat chow containing 2% cholesterol from 8:30 a.m. to 9:30 a.m. (60-min group) or from 7:30 a.m. to 8:30 a.m. (120-min group). All rats were killed at 9:30 a.m.

Preparation of Rat Liver Microsomes—Rats were killed by decapitation. Liver microsomes were prepared in Buffer A (0.1 M sucrose, 0.05 M KCl, 0.04 M potassium phosphate, 0.03 M EDTA, 0.01 M dithioerythritol, at pH 7.2), except where noted. The livers were homogenized with a tight-fitting, motor-driven, Teflon pestle, using 2.0 to 2.5 ml of Buffer A/g of liver. The homogenate was centrifuged at 30,000 g for 15 min, the supernatant was collected, and centrifugation (10,000 g) was repeated. The 10,000 g supernatant was centrifuged at 300,000 g for 30 min. The supernatant was discarded, and the microsomal pellet was resuspended in Buffer A, using a tight-fitting, motor-driven, Teflon pestle. The resulting microsomal suspension was applied to a Sephadex G-25 column (PD-10), eluting with Buffer A-I. Aliquots (100 μl) of the fresh microsomal suspension (see above) were added to incubation tubes; aliquots of purified phosphoprotein phosphatase (50 μg) were added, and the volume of each tube was adjusted to 200 μl with Buffer A-I. NADPH (dissolved in 20 μl of distilled water) was added to each tube (final concentration, 4 mM), and the tubes were incubated in a Dubnoff incubator (with shaking) at 37°C for 20 min, followed by the addition of [14C]HMG-CoA (152,000 dpm, 300 μM final concentration), dissolved in 30 μl of distilled water, to each tube. Incubation was then conducted at 37°C for 20 min. Mevalonate

The present investigation focuses on the processes which change the activity state of HMG-CoA reductase in vivo. In order to study this problem, rats received a single meal consisting of either rat chow or rat chow containing 2% cholesterol. The rats were killed 60 or 120 min after the beginning of feeding and liver microsomes were prepared by ultracentrifugation. The results show that, at 60 min after the beginning of cholesterol feeding, the inhibition of rat liver microsomal HMG-CoA reductase, produced in vivo, can be completely reversed by preincubation of the microsomes in vitro with purified phosphoprotein phosphatase. By contrast, however, at 120 min after the beginning of cholesterol feeding, the inhibition of rat liver HMG-CoA reductase, produced in vivo, was not reversed by in vitro preincubation of the microsomes with phosphoprotein phosphatase.

The abbreviation used is HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.
formation was linear for the incubation period employed. Mevalonate formation was assayed as described (5).

Purification of Rat Liver Phosphoprotein Phosphatase—Rat liver phosphoprotein phosphatase (phosphorylase phosphatase) was purified by a modification (see below) of the method of Brandt et al. (6). Liver microsomes (2 g) of rats was the starting material for this purification, using Buffer I (0.05 M imidazole, 5 mM EDTA, 0.5 mM dithioerythritol, at pH 7.45). The following purification steps (6) were conducted: (i) ammonium sulfate precipitation, (ii) ethanol precipitation, and (iii) ion exchange chromatography, employing a DEAE-Sephadex A-50 column, using 0.24 M sodium chloride in Buffer I to elute the phosphoprotein phosphatase. The specific activity was 600 to 750 units/mg of protein (6).

Phosphoprotein Phosphatase Assay—The method used for measuring phosphoprotein phosphatase activity was based on the method of Thomas and Wright (7) for glycogen phosphorylase. Bovine serum albumin, in the same amount as the phosphoprotein phosphatase, was incubated with phosphorylase a as a control.

RESULTS

Fig. 1 and Table I show the results of an experiment in which rats were fed a single meal of either rat chow (control) or rat chow containing 2% cholesterol. Feeding was begun at 8:30 a.m. and the animals were killed 60 min later at 9:30 a.m. Liver microsomes were prepared by homogenization and centrifugation (see "Experimental Procedures"). Microsomal HMG-CoA reductase was assayed after preincubation of the microsomes with varying quantities of purified phosphoprotein phosphatase.

Fig. 1 and Table I show a substantial inhibition of microsomal HMG-CoA reductase activity (26.7 ± 3.3%), observed 60 min after the beginning of cholesterol feeding. This inhibition was statistically significant (p = 0.00004). Furthermore, this inhibition was completely reversed by preincubation of the microsomes with purified phosphoprotein phosphatase (p = 0.47).

Fig. 2 and Table II show the results of an experiment in which rats were fed a single meal of either rat chow (control) or rat chow containing 2% cholesterol. Feeding was begun at 7:30 a.m. and continued until 8:30 a.m. The animals were killed at 9:30 a.m., 120 min after the beginning of feeding. Liver microsomes were prepared (see "Experimental Procedures"), and microsomal HMG-CoA reductase was assayed after preincubation of the microsomes with varying quantities of purified phosphoprotein phosphatase.

Table I

| HMG-CoA reductase specific activity (nmol/min/mg microsomal protein) |
|------------------------|------------------------|
| 0 μg                  | 8 μg                  |
| Control                | 1.12 ± 0.02            | 1.37 ± 0.02 |
| 60 min after the beginning of cholesterol feeding | 0.62 ± 0.04*           | 1.38 ± 0.03* |

* Amount of phosphoprotein phosphatase added.
* Compared to the control (no phosphoprotein added), p = 0.00004.
* Compared to the control (8 μg of phosphatase added), p = 0.47.
**Table II**

Reactivation of rat liver microsomal HMG-CoA reductase by preincubation of the microsomes with purified phosphoprotein phosphatase

| Time after开始 of cholesterol feeding | 0 pg* | 20 pg* |
|--------------------------------------|-------|--------|
| Control                              | 1.12 ± 0.03 | 1.49 ± 0.06 |
| 120 min                              | 0.57 ± 0.06 | 0.76 ± 0.05 |

* Amount of phosphoprotein phosphatase added.
\* p = 0.00016
\* p = 0.000010

microsomal HMG-CoA reductase activity (49.5 ± 4.4%) was observed 120 min after the beginning of cholesterol feeding, than was observed at 60 min. The inhibition observed at 120 min was statistically significant (p = 0.00002) and this inhibition was not reversed by preincubation of the microsomes with phosphoprotein phosphatase (p = 0.00001).

**DISCUSSION**

The pioneer study of Schoenheimer and Breusch (8), based upon a sterol balance technique, provided evidence that the addition of cholesterol to the diet significantly reduced the ability of an animal to synthesize cholesterol. The demonstration that the addition of cholesterol to the diet inhibits the conversion of [14C]acetate to cholesterol in liver slices was first made by Gould et al. (9–11), and it was confirmed by the studies of Tomkins et al. (12, 13), Langdon and Bloch (14), Frantz et al. (15), and Siperstein and Guest (16, 17). Siperstein and co-workers inferred that the principal biosynthetic step regulated was the conversion of HMG-CoA to mevalonic acid (16, 18, 19). The first direct demonstration that cholesterol feeding blocked HMG-CoA reductase was made by Linn (20) and was later confirmed by Rodwell (21).

Studies from several laboratories are consistent with the conclusion that cholesterol feeding may suppress HMG-CoA reductase activity by decreasing the quantity of enzyme protein present (22–26) or by the inhibition of pre-existing enzyme (24–26). In a recent study, we described the immunotitration of HMG-CoA reductase with HMG-CoA reductase antisemur (3). It was found that regulation of HMG-CoA reductase, following cholesterol feeding for 7 days, occurred both by a change in the concentration of the enzyme and by a change in the activity state of the enzyme.

Although in vitro evidence exists supporting the concept that HMG-CoA reductase activity is inhibited by phosphorylation and activated by dephosphorylation (27–36), there has been a lack of evidence that this mechanism actually is physiologically relevant and is operative in vivo. In fact, the study of Brown et al. (37) seemed to exclude phosphorylation/dephosphorylation of HMG-CoA reductase as an in vivo regulatory phenomenon, when liver microsomes were examined 12 to 72 h after a variety of physiological manipulations (fasting, cholesterol-feeding, cholestyramine-feeding, etc.).

However, it is clear from the present study that the reason that Brown et al. did not observe evidence for the in vivo regulation of HMG-CoA reductase by phosphorylation was the long time interval used (12 h) between the regulatory intervention, e.g. cholesterol-feeding and the killing of the animals. In the experiments conducted in the present investigation, we demonstrate that cholesterol feeding produces: (i) a rapid inactivation in vivo of liver HMG-CoA reductase, observed 60 min after the beginning of cholesterol-feeding, which can be completely reversed by in vitro preincubation of the microsomes with phosphoprotein phosphatase (Fig. 1 and Table I) and (ii) a further inactivation of HMG-CoA reductase, observed 120 min after the beginning of cholesterol feeding, which is not reversed by in vitro preincubation of the microsomes with phosphoprotein phosphatase (Fig. 2 and Table II). Thus, the timing of the experiments is crucial. These results are consistent with the conclusion that phosphorylation of HMG-CoA reductase is the first step in a series of in vivo regulatory events which produce inactivation and, ultimately, degradation of the enzyme.

In a related investigation, we studied the effect of a single dose (100 mg) of mevalonolactone, administered to rats by intragastric tube, on liver microsomal HMG-CoA reductase (38, 39). This experimental model allows the study of the effect of a sudden pulse of newly synthesized sterol on the regulation of HMG-CoA reductase (40, 41). When microsomes were isolated in the absence of KF (38, 39), a 39 ± 2.1% inhibition of HMG-CoA reductase activity was observed 20 min after mevalonolactone administration (p = 0.000002, compared to control). Furthermore, this inhibition was completely reversed by preincubation of the microsomes with purified phosphoprotein phosphatase. When microsomes were prepared in the presence of 50 mM KF, a 35 ± 2.8% inhibition of HMG-CoA reductase was observed 20 min after mevalonolactone administration (p = 0.0001, compared to control). As above, this in vivo inhibition was reversed (after removal of the KF) by treatment of the microsomes with phosphoprotein phosphatase (38, 39). Therefore, it made no difference, as far as the per cent inhibition of HMG-CoA reductase, whether the microsomes were prepared in the presence or absence of KF.

As in the present study with cholesterol feeding, two phases of inhibition were observed with mevalonate feeding (38, 39). As mentioned above, the first phase of inhibition, observed 20 min after mevalonolactone administration, was completely reversed by preincubation of the microsomes with purified phosphoprotein phosphatase. The second phase of inhibition, observed 60 min after mevalonolactone administration, was not reversed by phosphoprotein phosphatase. The reactivation of liver microsomal HMG-CoA reductase by phosphoprotein phosphatase, in vitro, was blocked by either potassium fluoride or by phosphoprotein phosphatase inhibitor.

In summary, the findings obtained in the present article are consistent with the conclusion that phosphorylation of rat liver HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, is an early in vivo regulatory response, following cholesterol feeding, and that this initial response is followed by other as yet unknown regulatory events.

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