Inhibition of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Activity in Morris Hepatoma 7800 after Intravenous Injection of Mevalonic Acid

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ABSTRACT

The effect of i.v. injection of mevalonate on the activity of microsomal 3-hydroxy-3-methylglutaryl Coenzyme A reductase was studied in livers from non-tumor-bearing rats and in host liver and hepatomas from rats bearing transplantable Morris hepatoma 7800. We confirmed that a single bolus injection of 100 mg of mevalonate in non-tumor-bearing male rats caused a 90% inhibition of hepatic 3-hydroxy-3-methylglutaryl Coenzyme A reductase activity within 2 hr. In two experiments, mevalonate injection caused a 50 to 60% reduction in enzyme activity of hepatomas but no significant decline in the enzyme activity in host livers. Thirty min after injection of [14C]mevalonate in a similarly sized bolus, the ratio of specific activities of cholesterol in liver:hepatoma:kidney:blood was 13:5.6:0.5:1. Thus, both the liver and hepatoma efficiently utilized mevalonate for the synthesis of cholesterol. The precise cause of the inhibition of enzyme activity in the liver in the non-tumor-bearing rats and in the transplantable hepatomas is not clear from this study. However, on the basis of other published reports, we suggest that it resulted from the accumulation of endogenous cholesterol in microsomal membranes. The activity of cholesterol 7α-hydroxylase, the rate-controlling enzyme for bile acid synthesis, was also studied in the hepatoma, but, in general, it did not differ from that in the host liver or control liver.

INTRODUCTION

In normal liver under physiological conditions, the regulation of cholesterol synthesis is finely controlled by the activity of the rate-controlling microsomal enzyme HMG-CoA reductase (1-3). In the rat, the control is easily demonstrated by feeding cholesterol, which results in an increase in cholesterol within chylomicrons and their derivatized remnant particles. These latter particles are rapidly taken up by hepatocytes (1-3), and the included cholesterol decreases the activity of HMG-CoA reductase (22) in as little as 2 hr after feeding the sterol (2). However, this regulation, referred to as dietary cholesterol-mediated feedback inhibition, is totally absent in transplantable hepatomas (14, 33-35, 37). The evidence is quite convincing that at least part of the lost control results from defective uptake of remnant particles (18). However, the intracellular regulation of cholesterologenesis in transplantable hepatocellular carcinomas might still be intact, particularly since we found recently that the level of enzyme activity in primary hepatomas from rats fed cholestyramine, an anionic exchange resin that blocks the intestinal absorption of cholesterol, was significantly higher than that in hepatomas from rats fed cholesterol (23). In order to test this idea further, we determined the level of enzyme activity in hepatomas from rats given i.v. injections of mevalonate. It was our belief that, since the procedure results in the rapid inhibition of hepatic HMG-CoA reductase activity, presumably by increasing the level of newly synthesized cholesterol in the microsomes (24), it might also inhibit enzyme activity in transplantable hepatomas by a similar mechanism. Furthermore, since cholesterol 7α-hydroxylase (EC 1.14.13.17), the rate-controlling enzyme for bile acid synthesis (10, 31), is located in the same submicrosomal fraction as HMG-CoA reductase (13, 25) and since the postulated increase in endogenous cholesterol derived from mevalonate might also be responsible for the reported increase in the former enzyme (24), we also determined its activity in the hepatoma after mevalonate injection.

MATERIALS AND METHODS

Chemicals were obtained from the following sources: DL-[2-14C]mevalonic acid lactone, DL-[2-3H]mevalonic acid lactone, and [4-14C]cholesterol from Amersham Corporation, Arlington Heights, Ill.; 3-hydroxy-3-methyl-[3-14C]glutaric acid from New England Nuclear, Boston, Mass.; mevalonic acid lactone, CoA, NADP, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase from Sigma Chemical Co., St. Louis, Mo.; cholesterol oxidase, cholesterol ester hydrolase, and horseradish peroxidase from Miles Biochemicals, Elkhart, Ind.

Male Buffalo rats (100 to 120 g) were given i.m. inoculations in the thighs with Morris hepatoma 7800. They were housed individually in cages and kept in a room with a reversed light-darkness cycle (dark from 9 a.m. to 9 p.m.). They were fed on Lab-Blox rat diet (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.) ad libitum. Water was also provided ad libitum. The rats were killed after 6 weeks when each transplant weighed between 1.5 and 2.5 g.

For rats given i.v. injections, mevalonic acid was prepared according to the method of Mitropoulos et al. (24). The buffered acid (pH 7.4) was injected through the tail vein under light ether anesthesia. Control animals received equivalent volumes of buffer.

The rats were sacrificed by cervical dislocation at 2:30 p.m., the time of peak diurnal activity of HMG-CoA reductase and cholesterol 7α-hydroxylation (8). Blood samples were collected by cardiac puncture under light ether anesthesia just prior to sacrifice. Livers and hepatomas were excised (trimmed of necrotic tissue) and homogenized in a 0.1 M phosphate buffer, pH 7.4, and used to prepare microsomal fractions according to the method described by Carlson et al. (8). Microsomal protein was estimated by a modification of the biuret method.
Tissue cholesterol was determined by the enzymatic method of Carlson and Goldfarb (7). Digitonin precipitates were prepared from the saponified extracts by the procedure of Sperry and Webb (38).

Since one of the goals of this study was to determine the time course of the disappearance of mevalonate in tissues after an i.v. injection, and since mevalonate is itself metabolized, it was necessary to develop a method for determining the precise amount of radioactive mevalonate in the tissues. This was accomplished by injecting [2-14C]mevalonate, adding [2-3H]mevalonate to tissue homogenates as an internal standard (15), and recrystallizing the DBED salt of mevalonate to a constant ratio of 14C:3H.

Liver, kidney, and hepatoma were homogenized in 0.1 M potassium buffer (pH 7.4, 4 ml tissue per g). Aliquots of homogenates and blood were incubated at 37°C for 30 min after adding nonradioactive carrier mevalonate (100 mg), a known quantity of [2-3H]mevalonate, and 0.4 ml of 10 M HCl to lactorize the mevalonic acid. The lactone was extracted into diethyl ether and converted to its DBED salt as described by Hoffman et al. (21). The DBED salt of mevalonolactone was crystallized twice (21), and the radioactivity in 3H:14C in the DBED salt after the first and second crystallizations differed by no more than 1%, thus validating the purity of the crystallized salt of the mevalonate extracted from the tissue.

HMG-CoA reductase activity was measured in microsomal fractions according to the method of Goldfarb and Pitot (15) with minor modification (8). Cholesterol 7α-hydroxylase activity was determined in microsomal preparations by quantitating the percentage of conversion of trace amounts (2 μM) of [4-14C]-cholesterol to 7α-hydroxy[4-14C]cholesterol during a 40-min incubation of 0.7 mg of microsomal protein at 37°C (8). Parallel assays carried out at 100 μM levels of exogenous cholesterol validated the assays carried out at 2 μM of cholesterol (8).

RESULTS

HMG-CoA Reductase and Cholesterol 7α-Hydroxylase Activities after Mevalonate Injection (Table 1). In 2 sets of experiments, the enzymatic activities were assayed 2 hr after i.v. injection of mevalonate. The marked inhibitory effect of i.v. mevalonate on HMG-CoA reductase activity was confirmed in liver of non-tumor-bearing rats in Experiment 1. In both groups of tumor-bearing animals given injections with buffer only, the level of HMG-CoA reductase activity was 6 to 9 times higher in hepatomas than in the host liver. Following mevalonate injection, the level of enzyme activity in the host livers did not change, but that in the hepatomas decreased to levels that were between one-half and one-third of levels in hepatomas from control animals.

No consistent changes in the activity of cholesterol 7α-hydroxylase were noted in either of our experiments. The enzyme levels of the hepatoma were similar to those in the host liver in Experiment 1 and slightly higher in Experiment 2. Mevalonate injection did not alter the activity of cholesterol 7α-hydroxylase in the hepatomas, but it did increase the enzyme activity in the host livers in Experiment 1.

Clearance of i.v. Mevalonate from the Blood (Chart 1). The rate of disappearance of [14C]mevalonate from the blood was determined after i.v. injection of 100 mg of mevalonic acid containing 10 μCi [2-14C]mevalonate. Fifty-μl blood samples were collected at various time intervals, and the total 14C radioactivity was determined. The radioactivity in the blood decreased precipitously at first but dropped much more slowly between 1 and 2 hr after injection. Assuming a blood volume of 20 ml in our 250-g rats, after 15 min, the circulating radioactivity had decreased to less than 5% of the injected dose. Radioactivity in circulating digitonin-precipitable sterols was also determined in the plasma after 30 and 120 min. While it accounted for only 4% of the radioactivity at the early time point, it represented two-thirds of the circulating radioactivity at the later time.

Organ and Hepatoma Distribution of Mevalonate (Table 2) and Its Incorporation into Digitonin-precipitable Sterols (Table 3). For this study, groups of rats were also sacrificed at 30 and 120 min following i.v. injection of a 100-mg bolus of mevalonate containing 10 μCi of [2-14C]mevalonate. There were no significant differences between hepatic or hepatoma cholesterol concentrations in rats given injections of mevalonate...
and those given injections of 0.9% NaCl solution (Table 1). The radioactivity in mevalonate- and in digitonin-precipitable sterols was determined in blood, kidney, liver, and hepatoma tissue. For purposes of determining specific activity of cholesterol, it was assumed that all digitonin-precipitable sterols were in the form of cholesterol. The livers and hepatomas were at least 10 times more efficient than were the kidneys in incorporating mevalonate into digitonin-precipitable sterols. These differences were even more impressive in view of the differences in available substrate between 30 min and 2 hr following injection. It was clear that considerable equilibration of the radioactivity in sterols within different pools had occurred during this time, since the specific activity of the digitonin-precipitable sterols in the blood had increased about 6-fold while that in the liver had not doubled.

**DISCUSSION**

In the present study, we observed that mevalonate was efficiently incorporated into cholesterol in Morris hepatoma 7800 after i.v. injection and that it also induced a reproducible and highly significant reduction of HMG-CoA reductase activity in the neoplasm. The idea for our experiments was stimulated by studies which had demonstrated that exposure of suspensions of hepatoma cells to mevalonate induced a 4- to 7-fold increase in cholesterogenesis (27) and by the observation that i.v. administration of a bolus of mevalonate caused a rapid 90% inhibition of microsomal HMG-CoA reductase activity (24).

In order to evaluate the rate of uptake of mevalonate, we developed a dual isotopic-labeling technique for its measurement, and we observed that, while the kidney maintained a high concentration of mevalonate, the concentrations in liver and hepatoma were quite similar to those in blood. This preferential trapping of mevalonate by renal tissue has also been...
observed under physiological conditions (19, 29) where much of it is shunted into a non-sterol-metabolic pathway (12). Furthermore, under our experimental conditions, less than 1% of the radioactivity in renal mevalonate was recovered in digitonin-precipitable sterols. In contrast, cholesterogenesis from mevalonate proceeded very efficiently in liver (19) and hepatoma. The supernormal dose of mevalonate appears to have been disposed of quite rapidly in our rats. Over 95% of the administered dose apparently left the blood compartment within 15 min while after 2 hr, even the minute amount of residual radioactivity was primarily in the form of sterols. It is improbable that the transiently high intracellular concentrations of mevalonate were responsible for a "toxic inhibition" of HMG-CoA reductase, since levels as high as 2 mw were reported recently not to inhibit the purified enzyme (11). Furthermore, the host livers in our study were resistant to the inhibitory effects of the injected mevalonate.

We found that the specific activity of newly synthesized cholesterol was about 6 times higher than that in blood. Clearly then, the radioactive sterol was synthesized in the neoplasm and did not merely accumulate there by physical exchange with cholesterol in the blood compartment. This is of particular importance since 2 recently published findings suggest that free cholesterol accumulates in microsomes after i.v. injection of mevalonate (24). These include: (a) alterations in the $K_m$ and Arrhenius plots that closely resemble those induced by feeding cholesterol; and (b) a decrease in the specific activity of $\Delta_7$-hydroxycholesterol in an assay using exogenous $[^3H]$cholesterol, suggesting dilution by an enlarged endogenous pool of cholesterol (24). Moreover, our inability to demonstrate an accumulation of cholesterol in hepatomas after i.v. injection of mevalonate does not argue against endogenous mevalonate-derived cholesterol acting as a feedback regulator since, in one study, dietary cholesterol induced a 99% inhibition of hepatic cholesterogenesis without causing a demonstrable increase in tissue cholesterol (36). Thus, even though we have not demonstrated accumulation of the "feedback inhibitor," our data are at least consistent with the interpretation that a product of mevalonate metabolism induced the inhibition of HMG-CoA reductase activity in the hepatoma.

Recent studies suggest that the uptake of chylomicron remnant particles by hepatocytes is receptor mediated (6, 9). This process is apparently quite different from that mediating the uptake of low-density lipoprotein by fibroblasts (4), since it is not dependent on the rate of cellular cholesterogenesis (32). While the precise features of this control are not understood, the mechanism of this regulation is severely deranged in transplantable hepatomas. In this regard, administration of $[^3H]$cholesterol by stomach tube was shown to cause the rapid accumulation of the sterol in the host liver but not in the hepatomas of tumor-bearing rats (18). Thus, the specific activity of cholesterol in the host liver was twice that in the rat blood after only 12 hr, while the level rose very slowly in the hepatoma, equilibrating with that in the blood only after 96 hr, presumably primarily by a process of a physical exchange of cholesterol (18). Until now, the inability of hepatocellular carcinomas to remove remnant particles from the blood has made it difficult to study the in vivo process of intracellular control of cholesterogenesis in these neoplasms. However, a number of published reports support the notion that control of cholesterogenesis is as least partially intact in hepatocellular carcinomas. These include the recent study documenting a degree of feedback control in primary hepatomas (23), the demonstration that hepatoma cells in culture manifest an increase in cholesterol synthesis, the activity of HMG-CoA reductase when exposed to delipitated serum (3), and the finding that a degree of diurnal rhythmicity of HMG-CoA reductase, synchronous with that of the host liver, is maintained in transplantable hepatomas (14). In addition, we now suggest that the decrease in HMG-CoA reductase activity that follows i.v. administration of mevalonate is evidence for the existence of at least some of the feedback control of cholesterol synthesis in transplantable hepatocellular carcinomas.

The inability of mevalonate injection to inhibit host hepatic HMG-CoA reductase activity is still unexplained by this study. In part, this may have resulted from the relatively low preinjection level of host HMG-CoA reductase activity, a host-tumor effect that was also noted in a previous study (14). Many other examples of unusual and often unexplained effects of transplantable hepatomas on the metabolic regulation of host liver have been documented (16). Since the patterns of enzyme response to hormonal or dietary manipulation often resemble those seen in fetal liver (20), it would be of interest to determine whether fetal liver is also unresponsive to injection of mevalonate in pregnant rats.

One additional aspect of these studies warrants comment. We found in 2 experiments conducted over a period of several months that the activity of HMG-CoA reductase in Morris hepatoma 7800 varied by 250%. In the past, the unique growth rate (26) and enzyme phenotypic characteristics of each individual Morris hepatoma have been emphasized (28). While it is true that, with few exceptions, Morris hepatomas maintain a constant rate of growth on serial transplantation for periods of up to several years (26), there are no comprehensive studies showing that enzyme phenotypes of hepatomas remain stable for extended or even short periods of time. Therefore, in view of our own observation of varied basal level of HMG-CoA reductase activity, investigators should be cautioned against assuming that the enzyme activities of individual neoplasms remain stable during the course of serial transplantations.

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