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REGULATION OF THE ACTIVITY OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE BY INSULIN

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In hypophysectomized - diabetic rats full restoration of hepatic HMG-CoA reductase activity required administration of both triiodothyronine and insulin. Giving triiodothyronine alone resulted in full restoration of reductase mRNA and immunoreactive protein but not of enzyme activity. Increasing the ratio of dithiothreitol to microsomal protein resulted in full HMG-CoA reductase activity. With 5mM glutathione, HMG-CoA reductase activity was 5 to 10 fold higher in non-diabetic animals. The data suggest that insulin acts by increasing the portion of HMG-CoA reductase present in its active free sulfhydryl form.

HMG-CoA reductase is regulated by the interplay of several hormones (1). These include: thyroid hormones, insulin, glucocorticoids and glucagon. For example both thyroid hormones and insulin are required to maximally stimulate hepatic HMG-CoA reductase in hypophysectomized - diabetic (HD) rats (2). Although some information has been obtained from studies with inhibitors of RNA and protein synthesis (3), the mechanisms by which these hormones regulate HMG-CoA reductase are not well defined.

Recently, suitable methods for determining levels of HMG-CoA reductase mRNA and immunoreactive protein have become available.

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HD hypophysectomized-diabetic; T_3, triiodothyronine.

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Using these methods we[1] recently demonstrated that stimulation of HMG-CoA reductase activity by triiodothyronine in hypophysectomized rats is associated with a large increase in HMG-CoA reductase mRNA levels. In this study, these techniques were used to investigate the basis of the stimulation of HMG-CoA reductase activity by insulin. Our findings with HD rats suggest that insulin appears to act posttranslationally.

MATERIALS AND METHODS

Materials - Hypophysectomized rats were purchased from Altech Laboratories. Oligo$_2$(dT) cellulose type 7 was purchased from PL Biochemicals. α-^{32}P dCTP and the nick translation kit was obtained from New England Nuclear. Insulin, streptozotocin and T_3 were from Sigma Chemical. The plasmid pRED-10 was a generous gift from Drs. M.S. Brown and J.L. Goldstein.

Animals - The animals were housed in a reverse cycle light-controlled room with a 14 hr light and a 10 hr dark period. Hypophysectomized rats were rendered diabetic by an intraperitoneal injection of streptozotocin, 4 mg per 100 g of body weight in 0.1M sodium citrate pH 5.5. Diabetes was confirmed by detection of urinary sugar. Where indicated, 2.5 units of insulin per 100 g of body weight was injected intraperitoneally 2 hrs prior to death. T_3 was given as a single intraperitoneal injection of 100µg per 100 g of body weight. Rats were killed by decapitation 72 hrs after receiving T_3.

Immunoblotting and HMG-CoA Reductase Activity - The preparation of lysosome-free microsomes, HMG-CoA reductase antisera, determination of microsomal protein and immunoblotting were carried out as recently described (4). HMG-CoA reductase activity was assayed as previously described (4) except that glutathione replaced dithiothreitol in one experiment.

RNA Isolation and Blotting - RNA was isolated from a cytoplasmic fraction of liver homogenate by a phenol extraction procedure (5). Poly A$^+$ RNA was isolated by three cycles through an oligo dT column (6). The Poly A$^+$ RNA was denatured (7) and applied to nitrocellulose using a slot blot apparatus. The relative levels of HMG-CoA reductase mRNA were determined by hybridization (8) using pRED-10 labeled with ^{32}P to a specific activity of about 4×10^8 cpm/ g by nick translation (9).

RESULTS

HMG-CoA reductase activity was essentially undetectable (<0.005 nmol/min/mg) in liver microsomes from HD rats and was not

[1]Sample, C.E., Pendleton, L.C. and Ness, G.C. (1986) manuscript submitted.

increased by an injection of insulin alone. In contrast administration of T_3 alone increased HMG-CoA reductase activity to 0.10 ± 0.05 (7) nmol/min/mg. When both hormones were given HMG-CoA reductase activity was increased to a value of 1.04 0.73 (8).

To determine whether these increases in HMG-CoA reductase activity were due to corresponding increases in mRNA levels, the relative levels of HMG-CoA reductase mRNA were determined by hybridization. HMG-CoA reductase mRNA could not be detected in poly A^+ RNA from HD rats treated with or without insulin. Surprisingly, the levels of HMG-CoA reductase mRNA were nearly identical in poly A^+ RNA from HD rats injected with only T_3 or with both T_3 and insulin, despite a large difference in enzyme activity (Fig. 1).

Fig. 2 presents an immunoblot of microsomes from HD rats treated with insulin or T_3 or both. The levels of the 97 KDa form, which is the active form, of HMG-CoA reductase are essentially identical in HD rats treated with only T_3 (lane 2) or with both T_3 and insulin (lane 3). In contrast no detectable 97 KDa material was present in microsomes from HD rats treated with

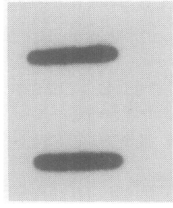
TREATMENT	REDUCTASE ACTIVITY	HYBRIDIZABLE RNA
T_3	0.05	
T_3 + INSULIN	0.78	

Fig. 1. Effects of Triiodothyronine and Insulin on Hepatic HMG-CoA Reductase Activity and Hybridizable mRNA in Hypophysectomized-Diabetic Rats. Livers were divided for the isolation of microsomes and poly A^+RNA. For each sample, 2 µg of poly A^+RNA was immobolized on nitrocellulose prior to hybridization. HMG-CoA reductase activity is expressed as nmol/min/mg.

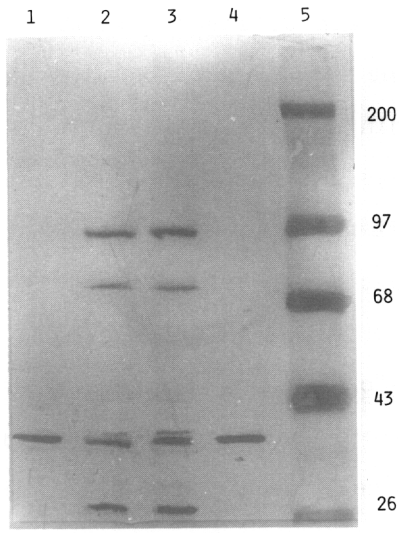


Fig. 2. Immunoblot of Hepatic Microsomal HMG-CoA Reductase from Hypophysectomized-Diabetics Rats Treated with Triiodothyronine and Insulin. Each lane contains 50 µg of microsomal protein from hypophysectomized-diabetic rats, (lane 1), injected with T_3 alone (lane 2), T_3 and insulin (lane 3), or insulin alone (lane 4). Prestained protein standards are shown in lane 5. Molecular weights $x10^{-3}$ are indicated.

or without insulin (lanes 4, 1). Only the 35 KDa immunoreactive species, apparently an intermediate in the degradation of HMG-CoA reductase which accumulates in microsomes from hypophysectomized rats[1], was present in these cases.

Recently, we (10) demonstrated by radiation inactivation and immunoblotting analysis that HMG-CoA reductase exists in an active free sulfhydryl monomeric form and a less active disulfide-linked form which may or may not be a dimer. Thus in an attempt to explain the discrepancy between levels of 97 KDa HMG-CoA reductase and enzyme activity in HD rats treated with only T_3, we considered the possibility that a greater portion of the enzyme might be present as the less active disulfide-linked 200 KDa form. If this were the case, then treatment with dithiothreitol should result in activation of the enzyme. We found that when the ratio of dithiothreitol to microsomal protein in the preincubation mixture was increased from 2.5 to 25 µmol/mg,

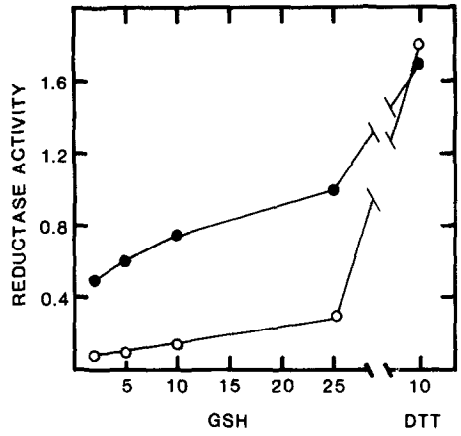


Fig. 3. Effect of Diabetes on the Response of Hepatic Microsomal HMG-CoA Reductase from Hypophysectomized Rats treated with T_3 to Glutathione. Reaction mixtures contained the indicated mM concentrations of glutathione or dithiothreitol and an NADPH regenerating system with 500 µM NADP in a final volume of 300 µl. After allowing the NADP to be convertd to NADPH (5 min), the reactions were started by the addition of 0.24 mg of microsomal protein. HMG-CoA reductase activity in liver microsomes from hypophysectomized rats treated with T_3 (●——●), or hypophysectomized-diabetic rats treated with T_3 (○——○) is expressed in terms of nmol/min/mg.

HMG-CoA reductase activity increased from 0.03 to 0.87 nmol/min/mg. Such an increase in the ratio of dithiothreitol to microsomal protein did not increase enzyme activity in microsomes from HD rats treated with both T_3 and insulin.

Although treatment with sufficient dithiothreitol produces high levels of HMG-CoA reductase activity in HD rats treated with only T_3, the question of the level of enzyme activity under thiol conditions more closely approximating those encountered *in vivo* remained. The question was addressed by the experiment depicted in Fig. 3. At physiological thiol levels, about 5 mM glutathione, HMG-CoA reductase activity was at least 5 times greater in the nondiabetic animal.

The possibility that the low levels of HMG-CoA reductase activity in microsomes from HD rats treated with T_3 might be due to phosphorylation of the enzyme was considered. However incubation with alkaline phosphatase failed to increase enzyme activity.

DISCUSSION

The data presented in this communication suggest that insulin acts to stimulate HMG-CoA reductase by increasing the portion of enzyme present in the more active free sulfhydryl form. Whether this is due to cleavage of inter- or intramolecular disulfide linkages cannot be stated at this time. We previously demonstrated that in mevinolin and colestipol rats the 100 KDa form predominated suggesting that cleavage of intermolecular disulfide linkages may have occurred. However in the present situation, the 200 KDa species was the major form in both diabetic and normal animals and the amount of the 100 KDa form was too small to be able to demonstrate any changes in it. This is similar to the situation seen in fasted rats (11).

The mechanism by which various dietary factors or insulin alter the amount of HMG-CoA reductase protein in the active form now becomes a topic for investigation. It would seem unlikely that changes in the ratio of reduced to oxidized glutathione would be responsible as large changes in this ratio are not observed in various physiological states (12). A more likely possibility might be activation of a thiol exchange enzyme (13).

It should be pointed out that the suggestion that HMG-CoA reductase activity might be regulated by sulfhydryl status first came from the studies of Tormanen and Scallen (14). These investigators demonstrated an inverse relationship between the level of microsomal HMG-CoA reductase activity and the concentration of thiol required for half-maximal enzyme activity. The present observations are quite consistent with a recent report that insulin treatment of diabetic rats did not increase the quantity of HMG-CoA reductase protein present despite a significant increase in enzyme activity (15). The authors concluded that insulin must exert some type of posttranslational effect.

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