Insulin and Glucagon Modulate Hepatic 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase Activity by Affecting Immunoreactive Protein Levels*

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The question of whether the effects of insulin and glucagon on hepatic 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity are mediated largely by changes in the phosphorylation state of the enzyme or by changes in the quantity of enzyme protein was investigated by measuring enzyme protein and mRNA levels. If phosphorylation/dephosphorylation is responsible for the observed changes in HMG-CoA reductase activity, one would not expect to see changes in immunoreactive protein or mRNA levels in response to induction of diabetes, administration of insulin, or administration of insulin and glucagon. It was found that hepatic HMG-CoA reductase mRNA levels were decreased to 12% of control in diabetic rats. Immunoreactive protein was reduced to essentially undetectable levels. Administration of insulin restored both mRNA and immunoreactive protein levels. Glucagon blocked these effects. Enzyme activity changes were fully accounted for by changes in HMG-CoA reductase mRNA and immunoreactive protein. Fasting caused parallel falls in HMG-CoA reductase activity and immunoreactive protein levels with a lesser effect on mRNA levels. The insulin-mediated changes in HMG-CoA reductase gene expression correlated well with changes in blood glucose levels, indicating a physiological effect. Taken together, these results indicate that insulin and glucagon regulate HMG-CoA reductase gene expression largely at the level of enzyme protein through changes in mRNA concentrations.

The rapidity with which insulin acted to increase HMG-CoA reductase activity suggested that this effect might be mediated by an alteration in the degree of phosphorylation of the enzyme. Using rat hepatocytes and measuring HMG-CoA reductase activity in microsomes isolated in medium containing sodium fluoride, Ingebritsen et al. (5) reported that insulin increased the portion of enzyme present in the active, presumably dephosphorylated form. Glucagon addition to the hepatocytes opposed the effect of insulin. Subsequently, it was reported that significant diurnal changes in the fraction of hepatic HMG-CoA reductase present in the "active" form occurred when rat livers were cold-clamped and microsomes were isolated in fluoride-containing medium (6). These observations served as the basis for concluding that insulin and glucagon modulate HMG-CoA reductase activity by altering the degree of phosphorylation of the enzyme (7-12).

Microsomal HMG-CoA reductase activity was first reported to be inhibited by incubation with MgATP and a cytosolic fraction over 20 years ago (13). Further investigations suggested that HMG-CoA reductase kinase also underwent phosphorylation. Thus, a complex cyclic protein phosphorylation cascade was proposed for regulation of HMG-CoA reductase activity (14). Continued investigations revealed that HMG-CoA reductase kinase was an AMP-activated protein kinase (15). Phosphorylation of the reductase was reported to result in decreased enzyme activity and increased susceptibility to degradation (16). The actual site of phosphorylation was identified as serine 871 (17). Thus, it has been concluded that the AMP-activated protein kinase is the kinase of prime physiological importance for the regulation of HMG-CoA reductase (18).

There are, however, certain problems with the currently accepted view (7-12) that HMG-CoA reductase activity is acutely regulated by altering the degree of phosphorylation of the enzyme. It was recently shown that replacement of serine 871 of hamster HMG-CoA reductase with alanine did not prevent the normal feedback regulation of reductase activity by mevalonate, 25-hydroxysterol, or low density lipoprotein (19). Since this mutant enzyme cannot be phosphorylated, the data convincingly demonstrate that feedback suppression by these agents does not involve alterations in the phosphorylation state of the enzyme in UT-2 cells. Recent immunoblotting studies demonstrated that cholesterol feeding of rats markedly reduced hepatic HMG-CoA reductase protein (20). This observation is also inconsistent with regulation by phosphorylation. These recent findings are in contrast with an earlier conclusion that feedback regulation of hepatic reductase by dietary cholesterol was mediated by phosphorylation (21). It has also been demonstrated that the AMP-activated kinase is inhibited by either NADPH or HMG-CoA (18). Thus, substrates may protect against inactivation. Studies with both substrates present were not carried out (18). Recently, we demonstrated that hepatic HMG-CoA reductase mRNA levels were dramatically decreased.
in diabetic rats and that administration of pharmacological doses of insulin restored these levels within 2 h (22). We also showed that immunoreactive HMG-CoA reductase protein levels changed in a similar fashion (22). These changes fully accounted for the observed changes in enzyme activity. Taken together, these observations question the physiological significance of changes in phosphorylation state mediating short-term effects on HMG-CoA reductase activity.

If insulin and glucagon act to alter HMG-CoA reductase activity largely via phosphorylation/dephosphorylation of the enzyme, then one would expect to see little effect on levels of mRNA or immunoreactive protein relative to enzyme activity. Thus, we have investigated the effects of diabetes and treatment with insulin with and without glucagon on levels of HMG-CoA reductase mRNA, protein, and activity. We have examined, in particular, whether these effects are of physiological importance.

**EXPERIMENTAL PROCEDURES**

**Animals**—Male Sprague-Dawley rats weighing 125–150 g were purchased from Harlan Industries (Madison, WI). They were housed in a reverse cycle light-controlled room with a 14-h dark period followed by a 10-h light period. The animals were fed Purina rodent laboratory chow and water ad libitum. Rats were rendered diabetic with a subcutaneous injection of streptozotocin (65 mg/kg) given during the light cycle. Blood glucose levels were determined using a glucose oxidase procedure (23). Those rats with blood glucose levels in excess of 400 mg/dl were considered diabetic. Rats were used within the first 2 days after administration of streptozotocin. This avoids the secondary effects of muscle wasting and weight loss seen 2 weeks after induction of diabetes. Rats were given insulin (Humulin R) subcutaneously in doses ranging from 0.1 to 5 units/animal 2 h prior to being killed. Some animals were also given glucagon subcutaneously at a dose of 150 μg/rat. All rats were killed by decapitation at the 4th h of the dark period to control for diurnal variation in HMG-CoA reductase activity.

**Materials**—The sources of the cDNA probes were previously given (24). Streptozotocin and the glucose oxidase reagents were purchased from Sigma. Humulin R and glucagon from Lilly were generous gifts of the supplier. Humulin R was used because of its purity. 

**Enzyme Assays**—HMG-CoA reductase activity was determined in liver microsomes using the thin layer chromatography procedure for isolation of product as described previously (24). Protein concentrations were determined by a biuret method (24). Enzyme activities are expressed in terms of nanomoles/minute/milligram of microsomal protein.

**RESULTS**

**Effects of Diabetes and Insulin Treatment**—The effects of induction of diabetes and insulin treatment on hepatic HMG-CoA reductase mRNA, immunoreactive protein, and enzyme activity levels were determined. As shown in Fig. 1, induction of diabetes rapidly lowered hepatic HMG-CoA reductase mRNA levels. Within 18 h after administration of streptozotocin, relative mRNA levels had fallen to 12% of controls and remained at this level. Administration of insulin increased relative HMG-CoA reductase mRNA levels ~8-fold, restoring these to normal (Fig. 2). A dose of 0.3 unit of insulin/animal was sufficient. Higher doses caused no greater effect. The magnitude of these changes in hepatic HMG-CoA reductase mRNA is similar to that previously reported for enzyme activity (4).

To determine whether the changes in HMG-CoA reductase mRNA levels correlated with similar changes in immunoreactive protein levels, liver microsomes were subjected to immunoblotting analysis. As shown in Fig. 3, HMG-CoA reductase protein levels were diminished to the point of being barely detectable in livers from 42-h diabetic rats. Administration of insulin restored immunoreactive reductase protein to levels comparable to the controls. Again, the 0.3-unit dose was sufficient. A good correlation between immunoreactive protein levels and enzyme activity was observed.

**Effects of Glucagon**—Glucagon administration is known to inhibit the insulin-induced increase in HMG-CoA reductase activity (4). It was of interest to determine its effect on levels of immunoreactive protein. As shown in Fig. 4, glucagon administration markedly attenuated the increase in immunoreactive protein levels caused by insulin. HMG-CoA reductase enzyme activity was corresponding low and correlated with the level of immunoreactive protein. Northern blotting analysis revealed that glucagon treatment also eliminated the rise in HMG-CoA reductase mRNA levels (data not shown). These results, showing opposing effects of insulin and glucagon on HMG-CoA reductase protein levels, are similar to the hormones' effect on phosphenolpyruvate carboxykinase gene expression (28, 29), with the exception that in the present case, insulin causes increased protein levels.

**Effects of Fasting**—Fasting is known to lower insulin levels while raising glucagon levels, resulting in a decreased insulin/glucagon ratio. It is also known that fasting markedly reduces HMG-CoA reductase activity. Thus, it was of interest to study the effects of fasting on HMG-CoA reductase immunoreactive protein levels. As shown in Fig. 5B, HMG-CoA reductase immunoreactive protein was somewhat reduced after 1 day of fasting and then fell to undetectable levels after 2 days of fasting.
Insulin and Glucagon Alter HMG-CoA Reductase Protein Levels

**FIG. 2.** Effect of insulin dose on hepatic HMG-CoA reductase mRNA levels in diabetic rats. Diabetic rats, 40 h after receiving streptozotocin, were given the indicated doses of insulin (units/rat) and killed 2 h later. The relative levels of HMG-CoA reductase (HMGR) mRNA, corrected for β-actin, were determined and are presented at the bottom of each lane.

<table>
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<th>0</th>
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<td>0.1</td>
<td>1.5</td>
<td>1.7</td>
<td>0.6</td>
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**FIG. 3.** Correlation of HMG-CoA reductase immunoreactive protein levels with enzyme activity in diabetic rats treated with insulin or insulin and glucagon. An immunoblot of microsomal HMG-CoA reductase is shown. Diabetic rats were given 5 units of insulin with (+I+G) or without (+I) 150 pg of glucagon 2 h prior to killing. Twenty μg of microsomal protein was applied to each lane of an SDS gel. Corresponding HMG-CoA reductase enzyme activity is shown at the bottom of each lane in nmol/min/mg.

**DISCUSSION**

The data presented show that induction of diabetes in rats markedly reduces hepatic HMG-CoA reductase immunoreactive protein and mRNA levels, thereby largely accounting for the observed decrease in enzyme activity. Administration of physiological doses of insulin, based on blood glucose-lowering effects, restored levels of HMG-CoA reductase mRNA, protein, and activity. Glucagon effectively opposed these increases caused by insulin. If insulin and glucagon acted to alter hepatic HMG-CoA reductase via phosphorylation/dephosphorylation of the enzyme, as is commonly thought (7–12), one would not have expected to see substantial changes in immunoreactive protein levels. Thus, it appears that possible alterations in the degree of phosphorylation do not account for the effects of insulin and glucagon.

Despite the widespread belief (7–12) that insulin and glucagon affect hepatic HMG-CoA reductase activity via altering the phosphorylation state of the enzyme, considerable evidence has been reported in the literature to the contrary (30–34). In a recent review (34), it was stated that the AMP-activated protein kinase (HMG-CoA reductase kinase) does not appear to be involved in mediating the response to hormones. This statement was based, in part, on the lack of a diurnal variation in fasting. This correlated very well with microsomal HMG-CoA reductase activity. Hepatic HMG-CoA reductase mRNA levels also fell during fasting, but not to the same extent as protein levels or activity (Fig. 5A). This is suggestive of a significant degree of post-transcriptional regulation.

**Correlation with Blood Glucose Levels**—To evaluate whether the observed effects of insulin administration to diabetic rats on hepatic HMG-CoA reductase gene expression were physiological, the effects on blood glucose levels were compared with those on enzyme activity. As shown in Fig. 6, a significant increase in HMG-CoA reductase activity was seen with the 0.3-unit dose of insulin, which did not fully normalize blood glucose levels. In general, HMG-CoA reductase activity increased as blood glucose levels fell. The only exception to this correlation occurred with the 3-unit dose. This suggests that physiological doses of insulin act to increase HMG-CoA reductase gene expression.
Insulin and Glucagon Alter HMG-CoA Reductase Protein Levels

Effect of fasting on hepatic HMG-CoA reductase mRNA, immunoreactive protein, and enzyme activity levels. Rats were fasted for 1 or 2 days. In A, hepatic poly(A)+ RNA was isolated and probed for HMG-CoA reductase (HMGR) mRNA. The relative levels of HMG-CoA reductase mRNA were determined and are presented at the bottom of each lane. In B, a portion of an immunoblot is shown. Twenty μg of microsomal protein was applied to each lane of an SDS gel. Corresponding HMG-CoA reductase enzyme activity is shown at the bottom of each lane in nmol/min/mg.

Changes in blood glucose levels and hepatic HMG-CoA reductase enzyme activity in diabetic rats as a function of insulin dose.

REFERENCES
15. Clarke, P. R., and Hardie, D. G. (1990) EMBO J. 9, 2439-2446
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