The effect of glucagon on the synthesis and degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase

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Abstract Incubation of rat hepatocytes with glucagon results in a time- and dose-dependent decrease in the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase. We demonstrate, using immunoprecipitation of radiolabeled enzyme, that 10 nM glucagon inhibits the synthesis of the enzyme by approximately 50%, but that the apparent rate of degradation of the enzyme is not affected by the hormone. We also demonstrate that the intact reductase polypeptide contained phosphoserine. We conclude that glucagon inhibits the activity of the reductase by inhibition of enzyme synthesis. —**Edwards, P. A., S-F. Lan, and A. M. Fogelman.** The effect of glucagon on the synthesis and degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Lipid Res.* 1986. **27:** 398–403.

Supplementary key words phosphoserine • immunoprecipitation • rat hepatocytes

3-Hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase (EC 1.1.1.34) is thought to be an important regulatory enzyme in the biosynthesis of isoprene and its derived products such as cholesterol, ubiquinone, dolichol, and isopentenyl tRNA (reviewed in Refs. 1–3). The activity of the enzyme in intact rats and in cultured cells is affected by the levels of various hormones (reviewed in Refs. 2 and 4). Treatment of rats with glucagon (5–7) or addition of glucagon to isolated rat hepatocytes (8–10) results in decreased reductase activity. The mechanisms responsible for this decreased activity have not been clearly elucidated.

HMG-CoA reductase from rat liver and Chinese hamster ovary fibroblasts has a M_r = 97,200, as determined either from the cDNA (11), or from migration of the enzyme following immunoprecipitation with monospecific antibodies and electrophoresis on denaturing polyacrylamide gels (12, 13). The concentration of reductase mRNA and the rates of synthesis and degradation of the enzyme are rapidly modulated following either administration of mevalonolactone or 25-hydroxysterol to either cultured cells, or to rats in vivo, or by addition of cholestyramine and mevinolin to rat diets (12, 14–17). In contrast, the effect of hormones on the synthesis and degradation of the 97,000-dalton reductase polypeptide is not known.

Numerous studies have established that, in vitro, the activity of HMG-CoA reductase is modulated by phosphorylation (reviewed in Refs. 18–20). A 51,000-dalton, enzymatically active fragment of the enzyme has been isolated after phosphorylation (6, 21–23) and shown to contain two phosphopeptides (22, 23). Keith, Kennelly, and Rodwell (23) recently reported that this enzyme fragment contained phosphoserine. The nature and number of phosphorylated amino acids in the intact 97,000-dalton enzyme subunit is not known.

In the present report we have determined the effect of glucagon on the synthesis and degradation of the intact 97,000-dalton reductase polypeptide using pulse and pulse-chase techniques followed by immunoprecipitation of the enzyme. In addition, we have analyzed the intact immunoprecipitated phosphorylated enzyme and demonstrate that the major phosphoamino acid is phosphoserine.

MATERIALS AND METHODS

Materials

Carrier-free $^{32}P_i$ (285 Ci/mg) was from ICN. The glucagon used in all the studies reported was a gift from Dr. W. Bromer, Eli Lilly. This glucagon (1.05 Units/mg) was contaminated with less than 9 μU insulin/mg (W. Bromer, personal communication.). Glucagon (Eli Lilly) was also obtained from the UCLA pharmacy (1 unit/mg). This latter glucagon had effects on enzyme synthesis, degradation, and phosphorylation similar to the glucagon supplied by Dr. Bromer. The sources of all other materials have been described previously (12, 14, 24).

Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; MEM, Dulbecco's modified Eagle's medium.

Rat hepatocytes

Rats were fed a normal diet unless otherwise stated, and hepatocytes were prepared at the fourth hour of the 12-hr daily dark period as previously described (24). In order to measure enzyme synthesis, cells (1.1×10^6/1.5 ml) in methionine-free minimum Eagle's medium (Flow Lab, Los Angeles, CA) were preincubated in the presence of glucagon for 10 or 90 min before addition of [^{35}S]-methionine (50 μCi; >1100 Ci/mmol) (24). After 15 min the cells were pelleted and the reductase was immunoprecipitated as described (24). The apparent rate of enzyme degradation was determined as previously described (24). In all studies, glucagon, at the indicated concentration, was added to the cells every 60 min.

Assay of HMG-CoA reductase

Cells (1.1×10^7) were pelleted and 3 ml of ice-cold isotonic buffer (1.5 mM Tris, 10 mM KCl, 10 mM $MgCl_2$, and 18 mM EDTA, pH 7.2) was added and after 10 min the cells were disrupted in a Dounce homogenizer. Seven ml of buffer B (100 mM sucrose, 50 mM KCl, 40 mM KH_2PO_4, and 30 mM EDTA, pH 7.2) was added and the sample was then centrifuged at 10,000 *g* for 15 min. The supernatant was removed and centrifuged at 100,000 *g* for 45 min. The microsomal pellet was stored frozen until assayed for reductase activity.

In some experiments, cells were resuspended in ice-cold buffer C (0.25 M sucrose, 15 mM EDTA, 15 mM EGTA, pH 7.2) and immediately sonicated for 10 sec. Microsomal pellets were obtained by the centrifugation protocol described above. Enzyme activities were similar when microsomes were prepared by either method.

Unless otherwise stated, microsomes were thawed and sonicated in the presence of buffer D (0.2 M KCl, 0.16 M KH_2PO_4, 4 mM EDTA, 10 mM DDT, pH 6.8) and assayed in a volume of 0.25 ml as previously described (25).

Isolation and identification of phosphoamino acids

Phosphoamino acid analysis was by a minor modification of a published method (26). Hepatocytes were prepared from rats fed 5% cholestyramine for 4 days (24). Cells (9.9×10^6) were incubated at 37°C for 60 min in 4.5 ml of phosphate-free Dulbecco's minimal Eagle's medium supplemented with $^{32}P_i$ (1 mCi). The cells were pelleted, washed once, lysed in 9 ml of buffer (1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.01% NaN_3, 5 mM EDTA, 0.1 M NaCl, 0.01 M phosphate buffer, pH 7.5) (24) supplemented with 50 mM NaF and 50 μM leupeptin. The lysed cells were centrifuged at 100,000 *g* for 45 min and the supernatant was removed for analysis. Aliquots (900 μl) of the cell lysate were pretreated by incubating the lysate with 50 μl of staphylococci for 60 min at room temperature before centrifugation, and the pellet was discarded (24). This treatment with staphylococci was repeated twice more before addition of 8 μl of anti-reductase serum. After 18 hr at 4°C, the reductase was precipitated and analyzed on SDS/8 M urea/polyacrylamide gels as described (24). The areas containing the 97,000-dalton reductase were identified from the radioautogram and the nine gel slices were excised and homogenized in 2 ml of buffer F (50 mM NH_4HCO_3, 0.1% SDS, 8% β-mercaptoethanol) containing 133 μg BSA/ml and incubated at 37°C for 5 hr. The supernatant was removed and the gel was reincubated with 1 ml of buffer F for 16 hr. The protein in the combined extracts was precipitated with 10% trichloroacetic acid and the pellet was washed three times with acetone at −20°C. HCl (300 μl; 6.0 N) was added to the air-dried pellet and the sample was heated at 100°C for 4 hr. The sample was dried under vacuum and then resuspended in 5 μl of water containing 2.5 μg each of phosphoserine, phosphothreonine, and phosphotyrosine. The sample was applied directly to a cellulose thin-layer plate and two-dimensional electrophoresis was performed by a minor modification of the method of Cooper and Hunter (26); the first dimension electrophoresis was at 400 V for 1.5 hr using a buffer of glacial acetic acid–formic acid–water 78:25:897 (v/v) pH 1.9, and the second dimension was at 400 V for 2.5 hr using a buffer of glacial acetic acid–pyridine–water 50:5:945 (v/v) pH 3.4. Radioactive areas were identified by radioautography and the positions of the standard phosphoamino acids were shown by the use of ninhydrin staining.

RESULTS

A 2-hr incubation of rat hepatocytes normally results in increased activity of microsomal HMG-CoA reductase (**Table 1**) (8, 24). In agreement with previous results (8, 9), the activity of the enzyme was lower in cells incubated with 1 nM–3 μM glucagon as compared to controls (Table 1 and data not shown). The maximal inhibitory effect occurred at 2–10 nM glucagon (Table 1 and data not shown).

The mechanisms involved in the glucagon-mediated inhibition of reductase activity were investigated using immunoprecipitation of radiolabeled enzyme. Cells were preincubated for 120 min in the absence or presence of approximately 10 nM glucagon (glucagon was added every 60 min) and then [^{35}S]methionine was added. After 15 min the cells were pelleted and the M_r = 97,000 reductase polypeptide was isolated by immunoprecipitation and SDS/8 M urea/polyacrylamide gel electrophoresis (24). In seven out of seven experiments, glucagon (10 nM) treatment resulted in a 25–100% increase in the radioactive content of total cellular proteins (Table 1 and data not shown). In every experiment, both the absolute and relative radioactive content of the M_r = 97,000 reductase

TABLE 1. Effect of glucagon on the activity and apparent rate of synthesis of HMG-CoA reductase

Experiment	Addition	Incubation Time	Radioactivity in			Relative Rate of Synthesis of Reductase
			Reductase Activity	Total Cell Proteins	Reductase	
		min	*nmol MVA/min/mg protein*	*cpm* × 10^{-3}	*cpm*	*%*
A		0	0.012			
A		120	0.023	237	150	0.063
A	Glucagon (2 nM)	120	0.013	500	92	0.018
A	Glucagon (10 nM)	120	0.012	551	114	0.021
A	Glucagon (100 nM)	120	0.013	460	105	0.023
B		120	N.D.	549 ± 73	906 ± 39	0.165
B	Glucagon (10 nM)	120	N.D.	685 ± 13	613 ± 22	0.09
C		10	0.083	889	270	0.03
C	Glucagon (10 nM)	10	0.093	1170	353	0.03
C		120	0.12	730	308	0.042
C	Glucagon (10 nM)	120	0.07	1022	228	0.022

Rat hepatocytes were incubated at 37°C for the indicated time in the presence or absence of the indicated concentration of glucagon. Cells were either removed and the activity of microsomal reductase activity was determined, or 50 μCi of [^{35}S]methionine was added to 1.1 × 10^6 cells in 1.5 ml of medium and the incubation was continued for 15 min. The radioactivity present either in total cellular proteins or in the 97,000 dalton reductase polypeptide was determined as described in Materials and Methods. Cells in experiments A, B, and C were from different rats. All assays in A and C were done in duplicate and those in B in quadruplicate, respectively. MVA and N.D. represent mevalonolactone and not determined, respectively.

polypeptide was decreased by a 120-min preincubation of the hepatocytes with glucagon (Table 1). Preincubation of cells with glucagon (10 nM) for 120 min resulted in a 45–70% inhibition of the relative rate of synthesis of HMG-CoA reductase (Table 1). Glucagon had no effect on the apparent rate of enzyme synthesis when the rate was measured after a preincubation of cells with glucagon for 10 min (Table 1, experiment C).

The effect of glucagon on the degradation of HMG-CoA reductase was also investigated. Hepatocytes were radiolabeled with [^{35}S]methionine and then chased in the presence of unlabeled methionine in the presence or absence of 10 nM glucagon. The reductase was immunoprecipitated at different times of the chase (**Fig. 1A**) and the radioactivity in the 97,000-dalton polypeptide was determined after excision of the enzyme polypeptide (Fig. 1B). The apparent half-life of the reductase was 70 min and this value was unaffected by inclusion of glucagon

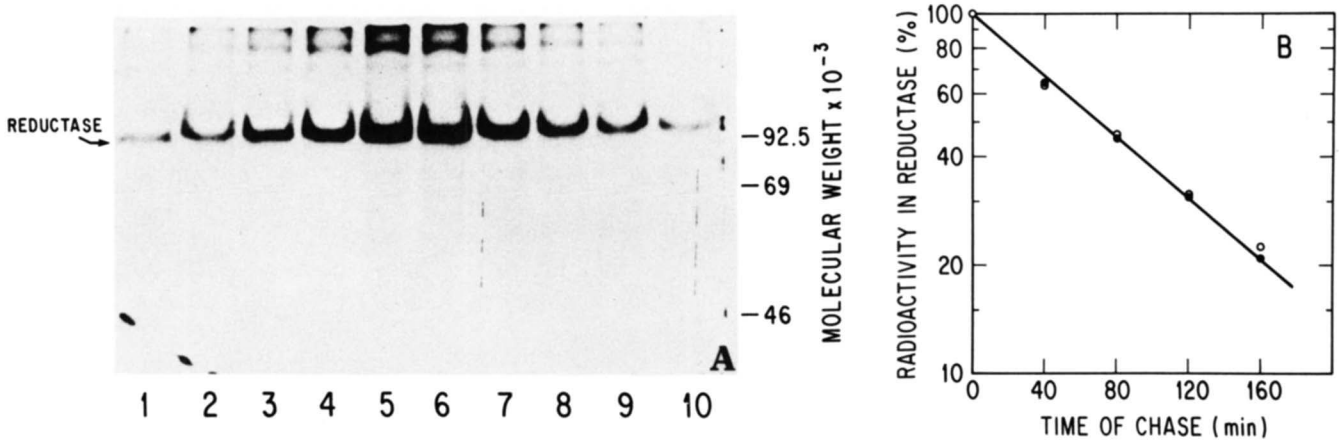

Fig. 1. Effect of glucagon on the degradation of HMG-CoA reductase. Rat hepatocytes (2.4 × 10^7 in 11.0 ml) were incubated at 37°C for 100 min in the presence of 1.1 mCi of [^{35}S]methionine. The cells were washed in media supplemented with 2 mM unlabeled methionine and resuspended in this medium at 2 × 10^5 cells/ml in the presence (●) or absence (○) of 10 nM glucagon. At the indicated times, aliquots (1 × 10^6 cells) were removed and the radioactive content of the M_r = 97,000 reductase polypeptide was obtained after immunoprecipitation, gel electrophoresis, and excision of the band (23). A, The fluorogram of the immunoprecipitated enzyme of M_r = 97,000 from one experiment. Lanes 1–5 correspond to the chase in controls at 160, 120, 80, 40, and 0 min chase and lanes 6–10 correspond to the chase in the presence of glucagon after 0, 40, 80, 120, and 160 min chase. B, The decay of radioactivity in the M_r = 97,000 reductase polypeptide as a function of time. At zero time of the chase the reductase contained 5,300 cpm. The experiment was performed in duplicate. The values at each time point varied by less than 5%. The values shown are the mean value.

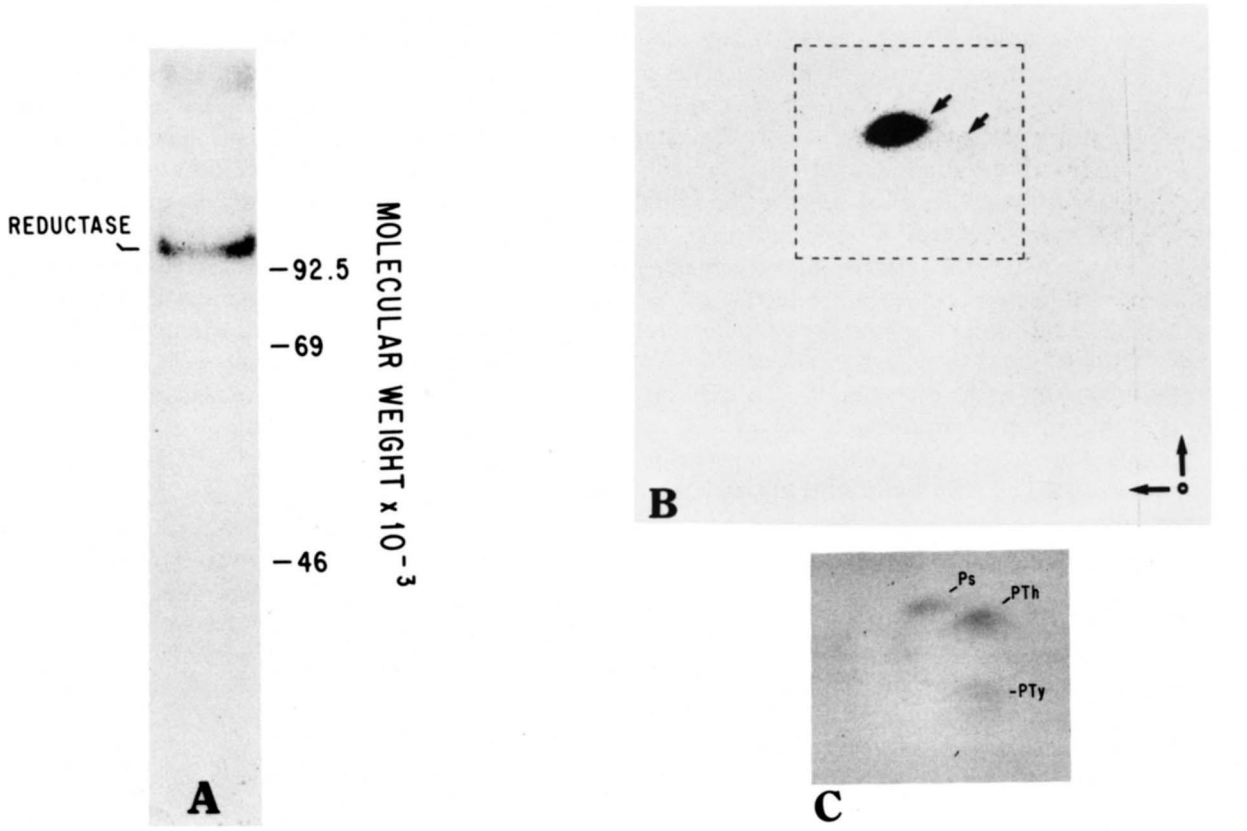

Fig. 2. Electrophoresis of phosphoamino acids. A, ^{32}P-Labeled reductase was isolated by immunoprecipitation and analyzed on SDS/urea/polyacrylamide gels as described in Materials and Methods. Molecular weight markers are shown. B, The 97,000 dalton polypeptide was excised from the gel and the phosphoamino acids were obtained after acid hydrolysis of the 97,000 dalton polypeptide. Unlabeled phosphoserine, phosphothreonine, and phosphotyrosine were added and the phosphoamino acids were separated by two-dimensional electrophoresis as described in Materials and Methods. The first and second dimensions are indicated. The only ^{32}P-labeled spots that developed after radioautography are shown by arrows. C, The area in Fig. 2B enclosed in the dotted line is shown after ninhydrin staining (Fig. 2C). The position of phosphoserine (PS), phosphothreonine (PTh) and phosphotyrosine (PTy) standards are indicated. The two radioactive spots in Fig. 2B comigrate exactly with the phosphoserine and phosphothreonine shown in Fig. 2C.

during the chase (Fig. 1B). The lack of effect of glucagon on the apparent half-life is not due to an inability to shorten the value below 70 min, since in other studies a half-life of 17 min was obtained after addition of 25-hydroxycholesterol to hepatocytes (data not shown).

Immunoprecipitation of HMG-CoA reductase from cells incubated in media supplemented with $^{32}P_i$ indicated that the 97,000-dalton reductase polypeptide was phosphorylated (**Fig. 2A**). The gel-purified 97,000-dalton reductase subunit was shown, on phosphoamino acid analysis, to contain phosphoserine with a minor trace (less than 5%) of phosphothreonine (Fig. 2B). Ninhydrin staining of the standard phosphoamino acids is shown in Fig. 2C. The radioactive spots on Fig. 2B comigrated exactly with the phosphoserine and phosphothreonine shown in Fig. 2C.

In other experiments we investigated whether exposure of ^{32}P-labeled hepatocytes to glucagon for 3–11 min would affect the phosphorylated state of the immunoprecipitated reductase. In four studies, performed in duplicate or triplicate, hepatocytes were preincubated for 60 or 90 min in phosphate-free minimal Eagle's medium supplemented with $^{32}P_i$ before the addition of 10 nM glucagon. Zero, 3, 5, or 11 min after the addition of glucagon, the cells were lysed and the enzyme was immunoprecipitated and analyzed on urea-polyacrylamide gels. In all four studies the ^{32}P content of the 97,000-dalton enzyme was not significantly affected by the inclusion of glucagon (data not shown). In each experiment the reductase contained approximately 0.013% of the radioactivity incorporated into trichloroacetic acid-precipitable proteins. We concluded that exposure of hepatocytes to glucagon, at levels that inhibit enzyme synthesis, had no significant effect on the degree of phosphorylation of the intact native enzyme.

DISCUSSION

In the present study we demonstrate that addition of glucagon to rat hepatocytes results in both decreased syn-

thesis and decreased activity of HMG-CoA reductase (Table 1). The hormone had no significant effect on the apparent rate of degradation of the enzyme (Fig. 1). It seems likely that these same mechanisms regulate the enzyme in vivo since studies with the intact animal (5–7) and with isolated cells (8–10) have shown that glucagon inhibits the activity of HMG-CoA reductase.

The current study demonstrates that the major phosphoamino acid in the 97,000-dalton reductase polypeptide is phosphoserine (Fig. 2). A minor trace of phosphothreonine was also observed (Fig. 2). Keith et al. (23) have previously reported that the 51,000-dalton enzyme fragment contains only phosphoserine.

The role of phosphorylation in the physiological regulation of HMG-CoA reductase has been controversial. In earlier studies, Beg, Stonik, and Brewer (6) reported that injection of rats with glucagon resulted in a twofold increase in the ^{32}P content of the 51,000-dalton enzyme fragment isolated from rat liver after partial purification of the enzyme. In preliminary studies we found no evidence for increased phosphorylation of the non-proteolyzed 97,000-dalton reductase within 11 min of the addition of glucagon to cells. These differences may result from either the very different methodologies used to isolate the enzyme, the different times of exposure to glucagon, or to the use of rats or hepatocytes.

In a variety of studies (reviewed in refs. 18, 20), the role of phosphorylation of the reductase has been assessed indirectly by determination of the enzyme activity in microsomes isolated in the presence of fluoride or phosphatase. The corresponding activities have been termed "expressed" and "total", respectively (18, 20, 27). It would now appear to be important to demonstrate directly that changes in the ratio of expressed activity:total activity that are observed under various physiological conditions in vivo are associated with altered levels of phosphorylation of HMG-CoA reductase per se. Prior determination of the specific amino acids that are phosphorylated on the 97,000-dalton reductase polypeptide might prove useful in such studies.

Recently Liscum et al. (28) have reported that HMG-CoA reductase from a Chinese hamster ovary cell line, termed UT-1, has a number of structural similarities with rhodopsin, the photoreceptor protein of retinal rod disks. The C terminus of this latter protein is reported to contain approximately seven phosphoserines, plus phosphothreonines, that appear to be involved in a light-dependent reaction (29). It now appears that both HMG-CoA reductase (Fig. 2) and rhodopsin (29) contain phosphoserine and phosphothreonine. Further studies will be required to determine the locale and number of the phosphorylated amino acids in the 97,000-dalton reductase polypeptide.

It is not known whether glucagon plays a role in the synthesis and activity of the reductase under normal physiological conditions. The low level of the reductase in starved animals (2) could, in part, result from the elevated glucagon levels observed in such animals. Further studies are also required to determine whether the low rates of cholesterol synthesis that occur following a portacaval shunt result in part from the effects of the extremely high glucagon levels that occur in this condition (0.6 nM) (30). Other mechanisms have also been proposed to account for the hypolipidemia that is observed after portacaval shunts (31). However, a physiological role of glucagon in controlling plasma cholesterol levels in man is consistent with damage of pancreatic alpha cells producing hypercholesterolemia (32) and glucagon injection producing hypocholesterolemia (33).

We thank Terry Laipari for advice in the analysis of the phosphoamino acids, Dr. Thomas Ingebretson for useful discussions, and Therese Sonnenfeld for typing the manuscript. This work was supported by grant USPHS HL-30568, a grant from the American Heart Association, Greater Los Angeles Affiliate (649-PS), and the Laubisch Fund.

Manuscript received 3 June 1985.

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