The effect of glucagon, norepinephrine, and dibutyryl cyclic AMP on cholesterol efflux and on the activity of 3-hydroxy-3-methylglutaryl CoA reductase in rat hepatocytes

Peter A. Edwards, Donna Lemongello, and Alan M. Fogelman

Division of Cardiology, Department of Medicine, University of California, Los Angeles, CA 90024

Abstract Incubation of rat hepatocytes for 3 hours in a sterol-free medium containing 1.5% albumin resulted in efflux of cellular sterol into the medium and an increased activity of 3-hydroxy-3-methylglutaryl CoA reductase. The secretion of cholesterol was inhibited when cells were incubated with glucagon, norepinephrine, or dibutyryl cyclic AMP. Glucagon and dibutyryl cyclic AMP also inhibited the induction of HMG-CoA reductase. Norepinephrine treatment resulted in a decrease in the synthesis and secretion of proteins but caused an increase in reductase activity. Insulin treatment had no effect either on reductase activity or on sterol efflux from rat hepatocytes.

Supplementary key words insulin • very low density lipoprotein

HMG-CoA reductase is the rate controlling enzyme for cholesterol biosynthesis under most physiological conditions (1). The activity of this enzyme in rat liver (2, 3), freshly isolated rat hepatocytes (4, 5), human leukocytes (6), and in cultured cells (7) has been shown to be determined by the relative flux of cholesterol into and out of the cells. Phospholipid dispersions in vivo (2) and in vitro (4, 7) produced increased efflux of cellular cholesterol and resulted in induction of HMG-CoA reductase activity. Conversely, when cholesterol efflux was blocked by colchicine (8), a drug known to inhibit the secretion of cholesterol-rich lipoproteins (9), reductase activity in rat liver declined. The induction of HMG-CoA reductase in rat hepatocytes was also prevented when the rate of endogenous sterol synthesis, from added mevalonolactone, was equal to the rate of sterol leaving the cells (5).

The direct effect of hormones on the activity of hepatic HMG-CoA reductase and on lipoprotein secretion is poorly understood. Injection of pharmacological doses of cyclic AMP, glucagon, or hydrocortisone into intact rats is reported to inhibit the activity of HMG-CoA reductase (10). Enzyme activities were increased after injection of epinephrine (11), norepinephrine (12), or insulin (13). However, only the catecholamines have been shown to directly affect the activity of the rat liver enzyme (14).

The secretion of triglycerides by the perfused rat liver is reported to be inhibited by dibutyryl cyclic AMP, glucagon (15), or catecholamines (16, 17). However, the effect of these hormones on the secretion of the other components of lipoproteins has not been reported.

MATERIALS AND METHODS

Chemicals

Glucagon, hydrocortisone, and dibutyryl cyclic AMP were from Sigma; norepinephrine bitartrate (Levophed) was from Winthrop Laboratories; insulin was from Eli Lilly; 1-[4,5-3H]leucine (60 Ci/mmol) and Aquasol were from New England Nuclear; and AG1-X8 formate was from Bio-Rad.

Rat hepatocytes

Cells were prepared as previously described (4, 14). Standard incubations contained 1.1 × 10⁷ cells in 10 ml of modified Swim's S-77 medium (14). Hormones were added in volumes of 10–500 µl and replaced equal volumes of medium, keeping the total incubation at 10 ml. Glucagon was dissolved in 0.001 N HCl

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; VLDL, very low density lipoprotein.

1 PAE is an Established Investigator of the American Heart Association.

2 AMF is the recipient of a U.S. Public Health Service Research Career Development Award (HL-00426).
INCUBATION TIME (hr)

Fig. 1. 1.1 × 10⁵ rat hepatocytes were incubated under standard conditions (●), hydrocortisone (5 × 10⁻⁴ M) (○), or glucagon at 1.4 × 10⁻⁵ M ( △) or 1.4 × 10⁻⁶ M (□). At various times the cells were pelleted by centrifugation and sonicated in 3 ml of a phosphate buffer, and tyrosine aminotransferase activity was determined by the method of Diamondstone (33) as previously described (14).

just before addition to the cells. At the end of the incubation, cells were pelleted by centrifugation at 50 g for 3 min. The medium was removed and centrifuged at 10,000 g for 15 min and an aliquot of the supernatant was removed for cholesterol analysis. A 6-ml aliquot of the supernatant was also removed; the density was adjusted to 1.074 g/ml with a 2.0 M NaCl-KBr solution (18) and the aliquot was centrifuged at 100,000 g for 24 hr. The floating lipoprotein fraction and the infranatant were separated and analyzed for cholesterol content.

Cholesterol analysis

Aliquots of medium or lipoprotein were saponified after addition of an internal standard (coprostanol) as previously described (5). The infranatant from the centrifugation at density 1.074 g/ml was added to an equal volume of 20% TCA and the pellet was saponified and analyzed as described previously (4). The cholesterol and coprostanol contents of each sample were determined on a Hewlett Packard gas-liquid chromatograph model 5830A using the method of Bates and Rothblat (19).

Protein synthesis from tritiated leucine

The incorporation of [4,5-³H]leucine into proteins and the isolation of the radioactive proteins have been previously described (14).

HMG-CoA reductase assay

HMG-CoA reductase activity in washed microsomes was measured as described previously except that the total volume of the assay was 0.5 ml (14). Activities are given as nmol mevalonate formed per minute per mg microsomal protein. All other methods were as previously described (5).

RESULTS

Effect of hormones on cholesterol efflux

Addition of glucagon, insulin, or hydrocortisone to isolated rat hepatocytes resulted in increased activity of tyrosine aminotransferase (Fig. 1) similar to that reported from in vivo studies (20). These results indicate that the plasma membrane receptors for glucagon and insulin and the resulting mechanisms of enzyme induction are relatively undamaged by the cell isolation procedure. This conclusion is supported by other studies showing that the characteristics of insulin binding and degradation were similar in cells prepared by the current method and by a mechanical isolation procedure (21).

Incubation of freshly isolated rat hepatocytes with either norepinephrine at 59–295 μM or glucagon at 0.027–27 μM resulted in a hormone concentration-dependent decrease in the amount of cholesterol effluxing from the cells into the medium (Fig. 2). The normal increase in medium cholesterol concentration was partially inhibited by hormones as early as 1 hr and was approximately 65% of controls after 3 hr (Fig. 2, Table 1). Addition of dibutyryl cyclic AMP to the hepatocytes also inhibited the efflux of cholesterol, although significant changes were observed only at hormone concentrations greater than 10⁻⁵ M (Table 1).

Isolated rat hepatocytes are reported to secrete VLDL (22). In the present study cells incubated under standard conditions secreted a protein component that had flotation properties of VLDL and was associated with both cholesterol (Table 2) and triglyceride. After cells had been exposed to norepinephrine for 3 hr, the cholesterol content of the VLDL fraction was only 20% of controls (Table 2). The decreased cholesterol content of the medium and of the VLDL fraction were similar (7.3 and 8.8 μg, respectively; Table 2). Moreover, this decline in VLDL cholesterol content cannot be ascribed to the incomplete recovery of cholesterol (Table 2). Even if

all of the cholesterol that could not be accounted for was in the VLDL fraction, there would still have been a minimum decline in VLDL cholesterol content of 51%.

Norepinephrine also inhibited the incorporation of [3H]leucine into both cellular proteins and into proteins secreted by the hepatocytes (Table 3). The magnitude of the decrease in the secretion of tritiated proteins (70%) was similar to that for cholesterol secretion (64%) (Table 3).

**Effect of hormones on HMG-CoA reductase activity**

Previously we showed that the induction of HMG-CoA reductase during a 3-hr incubation of rat hepatocytes was proportional to the amount of cholesterol leaving the cells (4).

Addition of glucagon or dibutyryl cyclic AMP to rat hepatocytes at concentrations that inhibited cholesterol efflux also partially inhibited the normal induction of HMG-CoA reductase during the 3-hr incubation; at hormone concentrations of approximately $10^{-4}$ M the activity of the reductase was only 52% and 56% of controls for glucagon and dibutyryl cyclic AMP, respectively (Table 1). In agreement with a previous report (4), the activity of HMG-CoA reductase increased when cells were incubated with lecithin dispersions (Fig. 3). The phospholipid-induced increase in enzyme activity was inhibited by glucagon or dibutyryl cyclic AMP (Fig. 3). Insulin at levels of 10–2400 microunits per milliliter had no effect on the normal induction of the reductase occurring during a 3-hr incubation. Addition of 2400 microunits of insulin at both the start of a standard incubation and after 1.5 hr had no effect on the efflux of cellular sterol into the medium or on the induction of the reductase measured after 3 hr; the reductase activities were 0.29 in controls and 0.28 in experimentals and the cholesterol content of the medium was 16.1 μg in controls and 15.8 μg in experimentals. In the same experiment, cells incubated with glucagon at $2.7 \times 10^{-3}$ M had a reductase activity of 0.15 and the medium contained 10.5 μg of cholesterol.

In agreement with a previous report (14), the addition of norepinephrine to hepatocyte incubations resulted in stimulation of reductase activity (Table 1).

**DISCUSSION**

The activity of HMG-CoA reductase in a variety of cells has been shown to be directly related to the net loss of cellular cholesterol (2–7). The loss of cholesterol from the liver is particularly high as a result of the secretion of lipoproteins (23). However, the direct effect of hormones on the hepatic secretion of cholesterol and on the activity of HMG-CoA reductase is poorly understood.

We have chosen to study the effect of hormones on the efflux of cholesterol from the cells by quantitative analysis of the cholesterol in the medium. It would...
TABLE 1. Effect of hormones on reductase activity and cholesterol secretion of rat hepatocytes

<table>
<thead>
<tr>
<th>Additions to Incubations</th>
<th>Relative Reductase Activity</th>
<th>Relative Concentration of Cholesterol in Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Norepinephrine (59 μM)</td>
<td>151 ± 28% (6)</td>
<td>66 ± 11.5% (7)</td>
</tr>
<tr>
<td>Norepinephrine (295 μM)</td>
<td>110 ± 13% (4)</td>
<td>58 ± 3.6% (4)</td>
</tr>
<tr>
<td>Norepinephrine (3 × 295 μM)</td>
<td>133 ± 6% (7)</td>
<td>52 ± 4.6% (9)</td>
</tr>
<tr>
<td>db-cAMP (10^-5 M)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>db-cAMP (10^-4 M)</td>
<td>55 ± 15% (5)</td>
<td>64 ± 4.0% (5)</td>
</tr>
<tr>
<td>db-cAMP (10^-3 M)</td>
<td>56 (2)</td>
<td>86 (2)</td>
</tr>
<tr>
<td>db-cAMP (10^-2 M)</td>
<td>67 (2)</td>
<td>94 (2)</td>
</tr>
<tr>
<td>db-cAMP (10^-1 M)</td>
<td>92 (2)</td>
<td>102 (2)</td>
</tr>
<tr>
<td>db-cAMP (10^0 M)</td>
<td>100 (2)</td>
<td>104 (2)</td>
</tr>
<tr>
<td>glucagon (2.7 × 10^-5 M)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>glucagon (2.7 × 10^-4 M)</td>
<td>52 ± 13% (5)</td>
<td>64 ± 8% (6)</td>
</tr>
<tr>
<td>glucagon (2.7 × 10^-3 M)</td>
<td>57 (2)</td>
<td>71 (2)</td>
</tr>
<tr>
<td>glucagon (2.7 × 10^-2 M)</td>
<td>72 (2)</td>
<td>86 (2)</td>
</tr>
<tr>
<td>glucagon (2.7 × 10^0 M)</td>
<td>105 (2)</td>
<td>92 (2)</td>
</tr>
</tbody>
</table>

Rat hepatocytes were incubated for 3 hr under standard conditions or in the presence of the indicated concentrations of hormone. The activity of HMG-CoA reductase and the cholesterol content of the medium were determined as described in Materials and Methods and the values are given as a percentage of the control incubations, mean ± SE. The numbers of experiments are shown in parentheses. In a typical 3-hr cell incubation under standard conditions, the activity of microsomal HMG-CoA reductase increased from 0.1 to 0.50 nmol mevalonate synthesized/min per mg protein and the medium cholesterol increased from 0 to 18.0 μg. Analysis of the original data by Student’s t test using paired variates indicated that the experiments were significantly different from controls.

Approximately 260 μg of cellular cholesterol and, of this, approximately 17 μg of cholesterol effluxed into the medium. However, the net decrease in cellular cholesterol concentration would be less than 17 μg as a result of induction of HMG-CoA reductase and hence increased synthesis of cholesterol.

We report here that glucagon, norepinephrine, and dibutyryl cyclic AMP inhibited the efflux of cholesterol from isolated rat hepatocytes (Table 1, Fig. 2). In addition, norepinephrine decreased both the synthesis and secretion of proteins and decreased the secretion of cholesterol in a fraction with the flotation properties of VLDL (Tables 2 and 3). These results are essentially in agreement with those of Heimberg and Fizette (16) who showed that the secretion of triglycerides by the perfused liver was inhibited by glucagon and dibutyryl cyclic AMP.

The requirement in the present study for supraphysiological levels of glucagon (10^-8 M) to affect both sterol efflux and reductase activity may be due to the rapid degradation of the hormone. The liver is reported to be the major site of catabolism of both insulin and glucagon (24) and we have previously confirmed that isolated liver cells rapidly degrade insulin (21).

The finding that the normal induction of HMG-CoA reductase in rat hepatocytes was inhibited by glucagon and dibutyryl cyclic AMP (Table 1, Fig. 2) is consistent with the inhibitory effect of these hormones on HMG-CoA reductase activity in vivo (10). Hence the inhibition observed in vivo may be due to a direct effect of the hormones on the liver. However, the stimulation of reductase activity in vivo following injection of pharmacological doses of insulin (13) may result from an indirect effect of this hormone since addition of insulin to rat hepatocytes in the present study did not affect reductase activity or sterol efflux.

TABLE 2. Effect of norepinephrine on secretion of VLDL

<table>
<thead>
<tr>
<th>Additions to Incubations</th>
<th>Cholesterol Content of Medium</th>
<th>Cholesterol Content of VLDL</th>
<th>Recovery of Cholesterol after Ultracentrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg</td>
<td>μg</td>
<td>μg</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Norepinephrine (6)</td>
<td>16.02 ± 0.9</td>
<td>10.89 ± 0.8</td>
<td>2.18 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>8.68 ± 0.6</td>
<td>2.08 ± 0.2</td>
<td>3.43 ± 0.2</td>
</tr>
</tbody>
</table>

Rat hepatocytes were incubated for 3 hr under standard conditions or 0.59 μmol of norepinephrine was added to the 10-ml incubation at time 0, 30, and 60 min of incubation. After separation of the medium from cells, an aliquot of the medium was removed for determination of total cholesterol. Another aliquot of the medium was adjusted to density 1.074 g/ml and the VLDL and VLDL infranatant were prepared and analyzed for cholesterol as described in Materials and Methods. The values given are the mean ± SE and the number of incubations is shown in parentheses. The recovery of cholesterol in the VLDL and VLDL infranatant was calculated as a percentage of the value obtained from direct analysis of the medium.
In apparent contrast to the present study, Geelen and Gibson (25) have reported that addition of insulin to isolated rat hepatocytes increased the rate of incorporation of acetate into nonsaponifiable lipids. However, the activity of HMG-CoA reductase was not determined in this latter study.

The direct inhibition of both cholesterol synthesis and secretion by glucagon in rat hepatocytes is consistent with experiments made in the whole animal. Following cobaltious chloride treatment, rat pancreatic alpha cells were damaged, glucagon levels declined, and the animals became hypercholesterolemic (26). In man, glucagon treatment is reported to lower the plasma cholesterol concentration (27). Moreover, after portacaval shunt surgery in a patient with homozygous familial hypercholesterolemia, the plasma glucagon levels rose to more than 2000 pg/ml and there was a concomitant decrease in the rate of both lipoprotein and cholesterol synthesis, and a dramatic decline in the plasma cholesterol concentration (28).

While our studies and those cited above suggest a causal relationship between the increase in the glucagon levels and the decline in the cholesterol levels following portacaval shunt, only direct experimentation can determine if the relationship is indeed causal.

The inhibition of HMG-CoA reductase activity by glucagon appears to be in direct contradiction to the results and conclusions of Raskin, McGarry, and Foster (29), who perfused rat livers for 30 or 60 min and found that glucagon had no significant effect on reductase activities. However, the animals were killed at the time of maximum reductase activity, a time when the synthesis of HMG-CoA reductase was already completely inhibited (30, 31). We found that glucagon had no significant effect on reductase activity during the first 60 min of hepatocyte incubation, but the inhibitory effect was observed after 3 hr of incubation under conditions that promoted HMG-CoA reductase induction (i.e., synthesis). Hence it is not surprising that Raskin et al. (29) did not observe an effect of glucagon on the reductase under their experimental conditions. Our observation that norepinephrine stimulated reductase activity is in agreement with previous studies with both isolated rat hepatocytes (14) and intact rats (12). However, the inhibition of cholesterol and lipoprotein secretion by norepinephrine was a novel and unexpected observation. The paradoxical finding that with norepinephrine treatment the reductase levels increased while there was a decreased loss of cholesterol from the cells is consistent with the proposal of Weis and Dietschy that the activity of HMG-CoA reductase is controlled by multiple mechanisms (32).

These studies were supported by United States Public Health Service Research Grants HL 19063, 20807, and 22474 and from Grant 522 from the American Heart Association, Greater Los Angeles Affiliate, and the Edna and George Castera Fund at UCLA.

Manuscript received 25 January 1978; accepted 14 June 1978.

### REFERENCES


