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BIPHASIC EFFECT OF OLEIC ACID ON HEPATIC CHOLESTEROGENESIS[1]

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SUMMARY: Livers isolated from adult male rats were perfused in vitro with oleic acid (0.6mM) as a complex with bovine serum albumin. Albumin alone was infused in control experiments. Oleic acid exerted a biphasic effect on incorporation of ^3H_2O into cholesterol, which was inhibitory during the first hour of perfusion, but exhibited a net stimulatory effect over a four hour period. No differences were observed in total activity or apparent phosphorylation state of HMG-CoA reductase after one hour of perfusion, with or without addition of oleic acid, implying that some other step limits the rate of cholesterol synthesis during this interval. After four hours of perfusion, HMG-CoA reductase activity was higher in livers perfused with oleic acid than in those perfused in its absence, in agreement with the observed differences in rates of cholesterol synthesis.

INTRODUCTION: In response to the availability of free fatty acids, the liver secretes triglyceride, primarily in the form of the very low density lipoprotein (VLDL), for export to other tissues. Secretion of triglyceride in this form requires simultaneous secretion of other lipid and protein components of the VLDL particle. Free cholesterol is secreted into the circulation primarily as a VLDL surface component. In the isolated perfused rat liver, exogenous fatty acids, by promoting hepatic triglyceride synthesis and secretion, stimulate secretion of cholesterol as well as cholesteryl ester in the VLDL core. (1-4).

We reported previously that exogenous oleic acid stimulates synthesis of cholesterol and augments the activity of the cholesterogenic enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (E.C.1.1.1.34) in the isolated perfused rat liver (2-5). In these studies, the effect of fatty acid on incorporation of radiolabeled precursors into cholesterol was measured

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Abbreviations: HMG-CoA Reductase, 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase; VLDL, Very Low Density Lipoprotein.

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over a period of four hours; HMG-CoA reductase activity was also determined after four hours of perfusion. In contrast, it was reported by other investigators that exogenous oleic acid inhibited cholesterol synthesis in perfused rat liver (6) and hepatocyte suspensions (7,8) in experiments lasting one hour. The present study was undertaken to ascertain the apparent reasons for this dichotomy, to determine if the effect of oleic acid on cholesterogenesis in perfused rat liver is biphasic and whether these effects correlate with changes in the activity of the presumed rate limiting enzyme on this pathway, HMG-CoA reductase.

MATERIALS AND METHODS

Materials: Oleic acid was obtained from Nu-Chek Prep (Elysian, MN). Radiolabeled materials (^3H_2O, [^{3}H]-sodium acetate, [3-^{14}C]-HMG-CoA, [5-^{3}H]-mevalonolactone) were purchased from the New England Nuclear Corporation (Boston, MA). Bovine serum albumin was purchased from the Sigma Chemical Company (St. Louis, MO) and was delipidated prior to use, as described previously (9). Bovine erythrocytes were obtained from a local slaughterhouse, washed, and stored in ice-cold Krebs-Henseleit buffer. Other chemicals were all reagent grade.

Animals: Male Sprague-Dawley rats (250-300g) were obtained from Harlan Industries (Indianapolis, IN) and housed with illumination from 05:00-17:00h. Animals were allowed free access to Purina rat chow and water ad libitum.

Liver Perfusion: Animals in the fed state were anesthetized by intraperitoneal injection of pentobarbital (60mg/ml) between 9:00 and 11:00h. Livers were removed surgically and perfused in a recycling system as described previously (10,11) except that the concentration of bovine serum albumin was 6g/dl in the buffer containing the infusate fatty acid complex. The initial volume was 80 ml, and the hematocrit was 30% washed bovine erythrocytes. In some experiments, as indicated, bovine erythrocytes were omitted. A complex of oleic acid with fatty acid free purified albumin was prepared as described previously (12). After a 20 minute period of equilibration, a fatty acid-albumin complex (oleic acid 1.41 mmol; albumin 6g/dl) was infused at a rate of 166μmol/h (11.7 ml/h), which sustained an average fatty acid concentration in the perfusate of 0.6 mM. Simultaneously a bolus of ^3H_2O (10 mCi) was injected into the perfusion circuit. In control experiments, the same amounts of albumin, without fatty acid, were infused. Samples of liver and perfusate were removed for analysis after one or four hours. In some perfusions of one hour duration, ^3H_2O was omitted and [^{3}H]-sodium acetate was added to the initial perfusion medium and to the infusate at a final concentration of 16.2 mM (5.7 μCi/ml).

Enzyme Assays: At the termination of the perfusion experiments, livers were perfused briefly with ice cold oxygenated 0.9% NaCl. Aliquots were removed and homogenized as rapidly as possible in either buffer A (50 mM potassium phosphate, 30 mM EDTA, 5mM dithiothreitol, 200 mM NaCl, 50 mM NaF, pH 7.0) or buffer B (same as buffer A but with 250 mM NaCl and without NaF). The homogenate was centrifuged at 10,000xg for 10 min and the supernatant fraction was recentrifuged for the same period. The supernatant from the second centrifugation was centrifuged again at 105,000xg for 60 min. The pellet was resuspended in 2 ml of either buffer A or buffer B and the suspension stored at -70° C until assayed. HMG-CoA reductase activity was determined radiochemically by the method of Shapiro et al. (13), except that buffer A or buffer B was used as incubation buffer, as appropriate. Protein concentration was determined by the method of Lowry et al. (14) after alkylation of dithiothreitol with iodoacetate (15).

Analytical Procedures: Samples of liver or perfusate were extracted with chloroform/methanol (2/1) according to the method of Folch et al. (16). Aliquots of the washed chloroform extracts were resolved into lipid classes by chromatography on silica gel G thin layer plates developed with petroleum ether/ diethyl ether/ acetic acid (85/15/1). Bands of neutral lipids were eluted with chloroform. Portions of extracted liver and perfusate samples were dried in vacuo and saponified with 10% KOH in 80% ethanol (17). Radioactivity was determined by liquid scintillation spectrometry utilizing either single or double isotope quench correction

programs, as appropriate. Cholesterol and triglyceride mass was determined colorimetrically (18,19).

RESULTS AND DISCUSSION

Hepatic secretion of triglyceride and total cholesterol was sustained over the four hour perfusion period (Table 1). Secretion of triglyceride and cholesterol was stimulated by inclusion of oleic acid in the perfusion medium. Incorporation of ^3H_2O into total free and esterified fatty acid (recovered from the liver and perfusate after saponification) was depressed by oleic acid (Figure 1), consistent with the expected inhibitory effect of exogenous fatty acid on lipogenesis. Rates of incorporation were approximately constant over the four hour perfusion period. Incorporation of ^3H_2O into cholesterol (recovered from the liver and perfusion medium after saponification) was significantly stimulated by oleic acid after four hours of perfusion (Figure 1). In contrast, incorporation of ^3H_2O into cholesterol during the first hour of perfusion was depressed by addition of oleic acid. Incorporation of added [^{3}H]-acetate (16.2 mM) into hepatic cholesterol over the first hour was also reduced in the presence of oleic acid (15,920±3352 vs 7612±1305 dpm/g liver, N=4 for each group, $p<0.05$).

No differences were apparent in the activity of HMG-CoA reductase after one hour of perfusion with or without addition of oleic acid to the medium, when assayed in the presence of 50 mM NaF to prevent dephosphorylation of the enzyme after homogenization (Figure 2). Consistent with our previous findings and with the observed relative increase in cholesterol synthesis over four hours of perfusion, HMG-CoA reductase activity after four hours was higher in the presence than in the absence of oleic acid. No significant change in the apparent phosphorylation state of the enzyme was observed.

It is evident from the data presented here and elsewhere (1-5) that fatty acid (oleic acid) stimulates the hepatic synthesis and secretion of triglyceride and cholesterol, and the activity of

Table 1. The effects of oleic acid on cumulative secretion of total perfusate triglyceride and cholesterol by perfused rat liver. Samples of medium were collected hourly from perfusions conducted in the absence or presence of oleic acid. Symbols represent the means ± SEM (μmol/g liver) of 4 perfusions in each group. * Different from control ($p<0.05$).

	Triglyceride		Cholesterol	
Hours	0	18:1	0	18:1
1	0.126±0.028	0.221±0.050	0.06±0.02	0.16±0.02*
2	0.268±0.054	0.428±0.116	0.18±0.02	0.27±0.04*
3	0.359±0.084	0.994±0.269*	0.28±0.02	0.41±0.05*
4	0.682±0.113	1.530±0.262*	0.40±0.03	0.54±0.06*

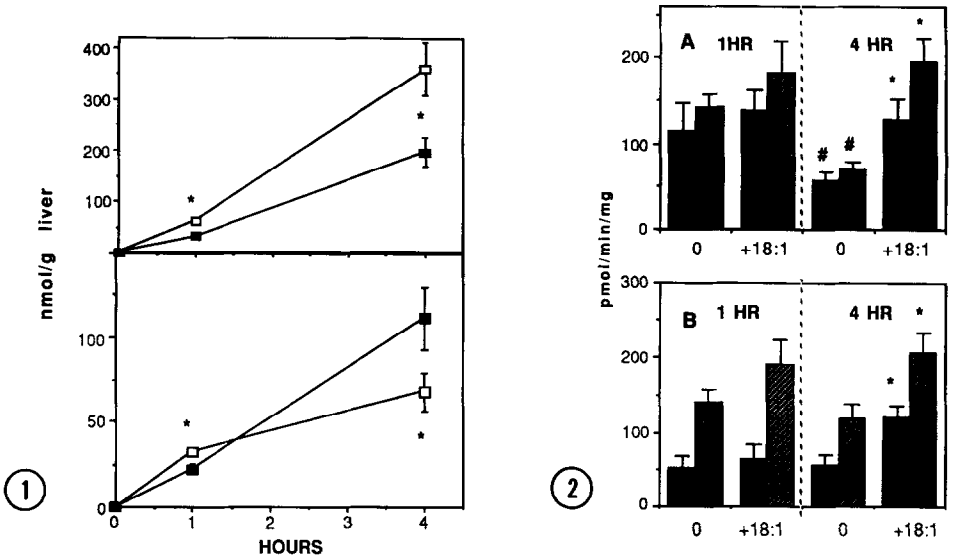

Figure 1. The effects of oleic acid on incorporation of ^3H_2O into total fatty acid and cholesterol of liver and perfusate. Experiments were carried out in the absence (open symbols) or presence (closed symbols) of oleic acid. Samples of liver and perfusion medium were saponified, lipids extracted with chloroform and methanol, and fatty acid and cholesterol separated by thin layer chromatography as described in the text. Absolute rates of synthesis were calculated from the measured incorporation of radioactivity into fatty acid and cholesterol, respectiviely and the specific activity of the medium in each sample at the end of the perfusion (27,28). Symbols represent the means±SEM of four perfusions in each group. * Different from control ($p<0.05$).

Figure 2. The effects of oleic acid on the activity of hepatic HMG-CoA reductase. Perfusions conducted with (panel A) or without (panel B) bovine erythrocytes in the medium, for either 1 or 4 hours. Solid bars represent HMG-CoA reductase activity (means ± SEM) in microsomes isolated and incubated in the presence of 50 mM NaF; hatched panels represent activity in microsomes isolated and incubated without NaF. Numbers enclosed in the bars represent the number of samples analyzed. # Different from perfusion for one hour ($p<0.05$). * Different from perfusion for four hours in the absence of oleic acid ($p<0.05$).

HMG-CoA reductase in the long term, although oleic acid has been reported to decrease synthesis of cholesterol in perfused liver and in hepatocytes in shorter exposures (6-8). It has been suggested that the inhibitory effect of oleic acid on cholesterol synthesis in suspensions of rat hepatocytes is due to reduction of the flux of substrate (acetyl-CoA) into the cytoplasm, either due to inhibition of pyruvate dehydrogenase or cytosolic citrate lyase (7). It is unlikely that this is the only factor responsible for the observed inhibitory effect of oleic acid in perfused liver, since incorporation of added [^{3}H]-acetate (16.2 mM) into hepatic cholesterol was also blunted. In rat liver, activation of exogenous acetate occurs in the cytosol (21); if contraction of the cytosolic pool of acetyl-CoA were the sole basis for reduced generation of cholesterol, incorporation of the exogenous precursor should have, if anything, been increased.

Phosphorylation by enzymes in rat liver cytosol of HMG-CoA reductase, the enzyme catalyzing the rate limiting step for sterol synthesis under most circumstances, is accompanied by

loss of catalytic activity. Fatty acyl-CoA has been reported to augment phosphorylation and activation of rat hepatic HMG-CoA reductase kinase, a 5'-AMP dependent protein kinase that catalyzes phosphorylation of serine residues on both HMG-CoA reductase and acetyl-CoA carboxylase(22) . Oleic acid has also been reported to stimulate protein kinase C in vitro (23), another kinase capable of phosphorylating HMG-CoA reductase (24). However, it does not appear that increased phosphorylation of HMG-CoA reductase accounts for the temporary reduction in cholesterol synthesis after exposure to oleic acid. The possibility that the initial inhibitory effect of the fatty acid is exerted at a step following formation of mevalonate is under current investigation in our laboratory.

The apparent phosphorylation state of HMG-CoA reductase after perfusion without red blood cells is significantly higher than after perfusion with red cells present. Since the liver contains a 5'-AMP dependent protein kinase capable of phosphorylating HMG-CoA reductase, it is possible that steady state levels of the nucleotide were higher during perfusion in the absence of erythrocytes. Indeed, elevation of 5'-AMP during hypoxia has been implicated in producing artifactually high estimates of phosphorylation states in previous studies, when precautions were not taken to keep the tissue well cooled prior to homogenization of liver samples (25). In the present study, we attempted to minimize this risk by perfusing the liver with ice-cold oxygenated saline at the end of the experimental period and by homogenizing samples immediately upon dismounting the liver from the perfusion apparatus. That oxygen tensions within the liver may have been lower during perfusion in the absence of erythrocytes is a possibility. It should however be noted that the same effects of oleic acid on cholesterol synthesis, lipogenesis, and lipid secretion were observed whether perfusions were performed with or without added red blood cells.

In these experiments cholesterol synthesis in the absence of oleic acid was not sustained at its initial rate. This decline in the rate of cholesterol formation cannot be attributed to a decrease in the pool of acetyl-CoA, ATP, or NADPH available for this purpose, since the rate of net lipogenesis, requiring the same factors, was not diminished.

The stimulation by oleic acid of HMG-CoA reductase activity in the long term is correlated with the synthesis and secretion of triglyceride and cholesterol in the VLDL and is consistent with the apparent requirement of cholesterol for secretion of the lipoprotein (26). The mechanism of the inhibitory action of oleate in the short term on incorporation of ^3H_2O into cholesterol without a concommitant decrease in the activity of HMG-CoA reductase remains to be determined.

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