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EFFECTS OF AMPHETAMINE AND FENFLURAMINE ON THE NET RELEASE OF TRIGLYCERIDES OF VERY LOW DENSITY LIPOPROTEINS BY SLICES OF RAT LIVER

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Abstract—Slices of rat liver, incubated in a medium devoid of very low density lipoproteins (VLDL),† release an average of 0·2 mg of triglyceride (TG)† per 0·1 *g* liver protein in 3 hr at 37°. Liver slices responded qualitatively in the same manner as the perfused liver with regard to TG release, as judged by (a) enhancement by incubation in rat serum, (b) decreased output after fasting, (c) increased output after fasting and re-feeding, (d) inhibition by norepinephrine and (e) increased output when medium FFA† levels were increased, up to 2 mM. Liver slices differed from the perfused liver in that a net output of FFA was observed. During the incubation, liver slices showed a net loss of phospholipid (PL)† and a net synthesis of TG.

The effects of *d*-amphetamine, *dl*-fenfluramine, and *dl*-norfenfluramine on the release of VLDL-TG were studied after *in vivo* injection or *in vitro* addition of these drugs. *In vivo*, the drugs, which elevate serum FFA levels, increased liver TG, decreased serum TG and prevented net synthesis of TG during incubation, but no significant effects on VLDL-TG release were found. When added *in vitro*, all of these compounds inhibited TG release. Norfenfluramine and norepinephrine were effective inhibitors at 10^{-4} M while fenfluramine and amphetamine required higher concentrations. Liver TG synthesis was inhibited by the presence of the drugs *in vitro*. It is concluded that the liver slice system, while unable to perform at the level of the perfused organ, can nevertheless be useful in studies of the effects of drugs on hepatic lipoprotein metabolism.

THE SECRETION of triglycerides (TG)† by the liver occurs in the form of very low density lipoproteins.[1] Amphetamine and some of its derivatives, such as fenfluramine, have been found to decrease serum TG levels in man[2] and rat.[3] In order to study their mechanism of action, we have employed a relatively simple system involving the incubation of slices of rat liver in diluted whole rat serum from which chylomicrons and VLDL had previously been removed. Very little work on lipid release by liver slices has been reported, although this system was the earliest to be used in *in vitro* studies of lipoprotein synthesis.[4,5] For studies of the effects of drugs, slices have some advantages because they can be pooled from several animals and appropriate controls are simplified. Analysis of the incubation medium and of the tissue before and after incubation is readily accomplished. However, tissue slices are metabolically inferior to perfused organs and their ability to carry out the process under study must be carefully evaluated. For these reasons, we have studied VLDL-TG release by liver

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† Abbreviations used: VLDL, very low density lipoproteins ($d < 1{\cdot}006$); TG, triglycerides; PL, phospholipids; FFA, free fatty acids.

slices under various conditions known to affect TG release in the perfused liver and in every case, we have found that they behave in a qualitatively similar manner.

MATERIALS AND METHODS

Male albino rats, 200 ± 50 g, fed *ad lib.* on laboratory chow, were used. Chylomicrons and VLDL were removed from rat serum, or isolated from the incubation medium, by centrifugation for 16 hr at 4° at 36,000 rev./min in a Spinco 40 rotor. The top 1 ml of each centrifuge tube was removed with a 0·5 ml Lang–Levy micropipet, and constituted the VLDL fraction. The incubation medium consisted of rat serum from which VLDL had been removed, diluted with an equal volume of Krebs–Ringer bicarbonate solution. After decapitation, the rat livers were perfused with 10 ml of 0·15 M NaCl via the portal vein. Four or five slices, about 1 g, 5 mm thick, were prepared with the Stadie–Riggs microtome, washed twice at 0° in Krebs–Ringer bicarbonate medium and placed in 10 ml of incubation medium in 125 ml Erlenmeyer flasks. After gassing with 95% O_2–5% CO_2, the flasks were shaken at 120 strokes/min at 37° for 3 hr. One flask was kept at 0° in order to measure the initial medium VLDL and the initial slice content of glycogen, TG and PL. After incubation, slices and medium were cooled to 0° and centrifuged for 10 min at 4° at 2000 rev./min. Eight and one-half ml of supernatant fluid was removed for VLDL isolation and analysis. Samples of the medium were also analyzed for FFA by the method of Trout.[6] The VLDL fraction was treated with 5 ml of methanol followed by 10 ml of chloroform. After addition of 2 ml of 0·15 M NaCl, the phases were separated and the chloroform phase washed once with 5 ml of 0·15 M NaCl. TG was analyzed by the method of Van Handel and Zilversmit[7] and PL by the method of Svanborg and Svennerholm.[8] The remaining liver slices were resuspended in 0·15 M NaCl at 0° and recentrifuged to remove residual medium protein. They were then macerated with a stainless steel spatula in 5 ml of methanol at room temperature. Five ml of chloroform was then added and the mixture allowed to stand 30 min. After centrifugation, the extraction was repeated and the combined chloroform–methanol extract treated with an equal volume of 0·15 M NaCl and allowed to stand overnight. The lower chloroform phase was used for the analysis of TG and PL as described. The extracted precipitate was washed once by resuspension and centrifugation in 10 ml of methanol and the precipitate extracted with 10 ml of 5 per cent trichloroacetic acid. The "readily extracted" glycogen was estimated by addition of 2 vol. of ethanol and measuring the optical density at 400 nm compared with a glycogen standard. The optical density was linear in the range of 0·2–1·0 mg/ml. The final extracted precipitate was dissolved in 1 N NaOH and the protein content measured by the biuret method.[9] All analytical results are expressed per 100 mg of slice protein. Fenfluramine and norfenfluramine were kindly supplied by Servier Labs, Paris; *d*-amphetamine sulphate by Recordati, Milan.

RESULTS

A survey of the output of VLDL-TG by liver slices in response to several perturbations known to affect TG release in perfused liver is shown in Table 1. In Experiment 1, it is clear that whole serum (free of VLDL) contains a factor which favors the release of VLDL-TG; this observation is in agreement with the work of Roheim, Miller and

TABLE 1. EFFECTS OF SOME EXPERIMENTAL CONDITIONS ON VLDL-TG RELEASE, FFA RELEASE, TG SYNTHESIS AND PL BREAKDOWN BY SLICES OF RAT LIVER

Expt. no.	Variable	VLDL-TG release (μg)	FFA release (μequiv.)	Net change in liver TG (mg)	Net change in liver PL (mg)
1 (a)	Medium: K-R-B	29*	0·0*	n.d.†	n.d.
(b)	Medium: 3% serum albumin in K-R-B	24*	1·9	n.d.	n.d.
(c)	Medium: rat serum minus VLDL	159	2·2	n.d.	n.d.
2 (a)	Fasted 48 hr	0*	n.d.	n.d.	n.d.
(b)	Control	102	n.d.	n.d.	n.d.
3 (a)	Fasted 48 hr	36*	1·5*	−0·22*	−7·7*
(b)	Fasted 48 hr, re-fed 24 hr	216*	2·2	+1·14	−1·2
(c)	Control	139	2·4	+1·11	−1·9
4 (a)	10^{-4} M norepinephrine	127*	2·6	+0·96	−2·6
(b)	Control	240	2·3	+1·07	−2·1
5 (a)	10^{-4} M norepinephrine	32*	2·7	+0·49	−3·3*
(b)	Control	102	2·3	+0·35	−1·9
6 (a)	1·0 μequiv./ml palmitic acid‡	135	−1·6*	+1·29*	−3·5*
(b)	Control (0·25 μequiv. FFA/ml)	153	2·6	+0·86	−6·4
7 (a)	1·7 μequiv./ml palmitic acid‡	174*	−0·9*	+2·23*	−1·8*
(b)	Control (0·31 μequiv. FFA/ml)	141	5·7	+1·27	−3·7
8 (a)	3·0 μequiv./ml palmitic acid‡	393*	n.d.	+3·70*	−2·5
(b)	Control (0·40 μequiv. FFA/ml)	292	n.d.	+0·98	−4·5

* Values marked with an asterisk are beyond the greatest differences from controls found in duplicate analytical determinations. Duplicate slice incubations and measurement of VLDL-TG output agreed to within ± 20 per cent. All values are expressed per 100 mg slice protein.

† Not determined.

‡ The value given for the concentration of palmitic acid represents the total FFA concentration. The FFA concentration of the control medium should be subtracted to give the correct concentration of palmitic acid itself.

Eder.[10] It is also evident that there is a net release of FFA and that an acceptor (albumin) in the medium is required for this. In all of the following experiments, rat serum from which VLDL had been removed was used to prepare the incubation medium. In experiments 2 and 3, fasting for 48 hr greatly depressed VLDL release, while fasting followed by re-feeding increased it, in accord with the observations of Windmueller and Spaeth.[11] As expected, slices from fasted rats did not synthesize TG,* broke down more PL, and released less FFA than the controls. In experiments 4 and 5, the addition of 10^{-4} M norepinephrine inhibited VLDL-TG release, as was found in perfusions by Heimberg and Fizette.[12] When palmitic acid was added to the medium (by the method of Spector and Hoak[13]), very little effect on TG release was found until high levels of medium FFA were reached, as shown in experiments 6, 7 and 8. The increased medium FFA resulted in increased TG synthesis and decreased PL breakdown.

* Loss of protein into the medium occurs during the incubation of liver slices. Since our results are based on the amount of protein remaining in the slice, the net synthesis of TG was overestimated. Direct measurement of the loss of protein on a wet weight basis indicated that this loss was about 8 per cent, which produced an average 40 per cent overestimate of TG synthesis. When this correction was applied to the data obtained in 19 experiments, the mean net synthesis of TG in control liver slices was 0·59 ± 0·059 mg/100 mg of protein. The net loss of PL during incubation was similarly *under*estimated, and in 17 experiments, the average loss of PL was 4·1 ± 0·54 mg.

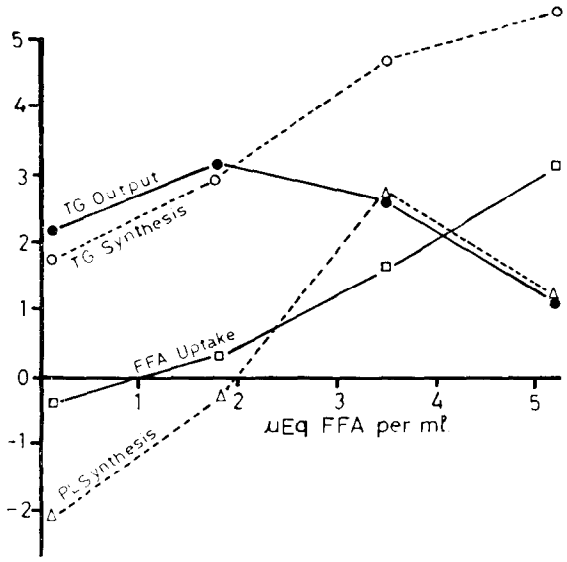

FIG. 1. ●———●, VLDL-TG output, ordinate in mg × 10; ○- - - - -○, liver TG synthesis, ordinate in mg; □———□, liver FFA uptake, ordinate in μeq × 10^{-1}; △- - - - -△, liver PL synthesis, ordinate in mg. All values are expressed per 100 mg slice protein after 3 hr at 37°. Oleic acid complexed to albumin according to Heimberg and Fizette[12] was added in a volume of 1·5 ml of 10 per cent bovine serum albumin (Pentex fraction V) which had been treated with charcoal to remove FFA. This was added to 8·5 ml of regular medium. The abscissa represents the initial concentration of FFA in the medium.

To confirm and extend these observations of the effects of medium FFA concentration, an additional and more complete experiment was performed with oleic acid added to the medium as the albumin complex. This experiment, shown in Fig. 1, indicates that net fatty acid uptake began at some level near 1 mM and was linear up to the highest concentration tested, 5·2 mM. VLDL-TG output increased significantly, but above 2 mM a decline in output occurred. Net synthesis of liver TG increased steadily, while the breakdown of PL was reversed and net synthesis took place above 2 mM. Inhibition occurred at 5 mM, as far as PL synthesis and TG release were concerned. This phenomenon may be related to the effect of very high intracellular levels of fatty acyl CoA which can inhibit many reactions.[14] In perfused liver, 3-fold increases in TG output in response to raising FFA levels have been observed.[15]

The phenomenon of net release of FFA was briefly explored. Kook and Rubinstein[16] had observed earlier a large release of labeled FFA when liver slices from rats pre-injected with labeled fatty acid were incubated *in vitro*. In one experiment, in which about 1 g of slices were incubated in 10 ml of medium, the net release of FFA was 1·2, 1·5, 1·8 and 2·6 μequiv. after 30, 60, 90 and 180 min, respectively. In a duplicate flask, the liver slices were removed after 90 min and incubation continued for an additional 180 min (gas phase, 95% O_2–5% CO_2). An additional 0·95 μequiv. of FFA was released. Therefore, release of hydrolytic enzymes from the slices accounts for about one-third of the FFA released.

EFFECT OF DRUGS

Liver slices were pooled from rats which had been injected 4 hr previously with doses of amphetamine or fenfluramine which produced significant decreases in serum TG levels. The results in comparison with controls injected with 0·15 M NaCl are given in Table 2. No effect on TG release was observed. In additional experiments, no effects were seen 1 or 2 hr after the drug was given *in vivo*. In these experiments, VLDL-PL release was also measured and the TG/PL ratio of 5 agreed with the analytical results of Koga, Horwitz and Scanu[17] for this fraction. Administration of these drugs tended to elevate liver TG and PL levels and no net synthesis of TG was observed, while PL breakdown was increased. No significant effects on FFA release or initial glycogen content were noted.

TABLE 2. EFFECT OF AMPHETAMINE OR FENFLURAMINE *in vivo* ON VLDL RELEASE AND OTHER PARAMETERS BY SLICES OF RAT LIVER*

Parameter	Control ± S.E.M.	Drug-treated ± S.E.M.
VLDL-TG release (μg)	221 ± 34	195 ± 28
VLDL-PL release (μg)	44 ± 12	42 ± 10
FFA output (μequiv.)	3·17 ± 0·48	3·26 ± 0·52
Liver TG (mg)	2·74 ± 0·17	4·12 ± 0·70†
Net increase in TG (mg)	0·96 ± 0·17‡	0·19 ± 0·57
Liver PL (mg)	20·4 ± 0·70	24·6 ± 1·8
Net decrease in PL (mg)	2·8 ± 0·30‡	5·2 ± 1·4†
Liver glycogen (mg)	11·1 ± 1·9	11·3 ± 2·6

* Slices were incubated 4 hr after intraperitoneal injection of either 20 mg/kg *dl*-fenfluramine-HCl (two experiments, three rats each) or 5 mg/kg *d*-amphetamine sulfate (two experiments, three rats each). No differences greater than 15 per cent were observed between the two groups of treated rats, and the results are pooled in this table. In these experiments, the drugs produced an average decrease of 42 ± 8·8 per cent in serum TG levels.

† Significantly different from the mean of the corresponding controls only when paired differences in each experiment were examined ($P < 0.05$).

‡ Significantly different from zero ($P < 0.001$).

Direct addition of amphetamine, fenfluramine, or norfenfluramine (one of the metabolites of fenfluramine[18]) to the incubation medium at appropriate concentrations inhibited VLDL-TG release and liver TG synthesis (Table 3). No significant effects on FFA release, glycogen content* or PL breakdown were seen in these experiments. To achieve inhibitory levels of approximately two-thirds for each drug, it can be seen from Table 3 that the order of potency is norfenfluramine > fenfluramine > amphetamine. No attempt was made to establish a dose–response relationship for these drugs. In two experiments with each drug, it was found that 10^{-5} M norfenfluramine, 10^{-4} M fenfluramine, and 5×10^{-4} M amphetamine did not produce significant inhibition of VLDL-TG output or hepatic TG synthesis. Increasing the concentration

* However, it cannot be concluded that these drugs have no significant effect on glycogen breakdown from the fact that no significant effects were observed in the seven experiments shown in Table 3, because in a total of 13 experiments in which the average inhibition of VLDL-TG output was 58 ± 6·7 per cent there was a small decrease in final glycogen content of −1·4 ± 0·453 mg, which is significantly different from zero ($P < 0.01$).

TABLE 3. EFFECTS OF *d*-AMPHETAMINE, *dl*-FENFLURAMINE, and *dl*-NORFENFLURAMINE ON VLDL-TG RELEASE AND TG SYNTHESIS BY SLICE OF RAT LIVER*

Expt. No.	Drug	Concentration M	VLDL-TG output (% change from paired control)	TG Synthesis M
1	*d*-Amphetamine sulfate	1×10^{-3}	−66	−78
2	*d*-Amphetamine sulfate	1×10^{-3}	−67	−30
3	*dl*-Fenfluramine HCl	5×10^{-4}	−78	−83
4	*dl*-Fenfluramine HCl	5×10^{-4}	−66	−80
5	*dl*-Norfenfluramine HCl	1×10^{-4}	−76	−57
6	*dl*-Norfenfluramine HCl	1×10^{-4}	−42	−20
7	*dl*-Norfenfluramine HCl	1×10^{-4}	−64	−54

* The absolute values of TG output and synthesis did not differ significantly from those given in Table 2.

of norfenfluramine to 5×10^{-4} M and of fenfluramine to 10^{-3} M increased the inhibition of TG output to 82 ± 6·2 per cent.

DISCUSSION

The present experiments demonstrate the net release of VLDL-TG and VLDL-PL by slices of rat liver. In 3 hr at 37°, 0·19 ± 0·012 mg (25 experiments) of TG was released per 100 mg of liver protein, or about 0·3 mg/g liver. Using the identical medium, we have found[19] that perfused livers released about 0·7 mg/g/2 hr. The slices released about 80 per cent of the VLDL-TG in 2 hr, so that the perfusion system released TG at approximately three times the rate found with slices. The difference may not be quite this great, however, since in the slice experiments the VLDL fraction was actually isolated and some losses undoubtedly occurred, apart from the possible conversion of some VLDL to a higher density lipoprotein fraction during incubation. Since it is known that liver slices consume less oxygen than the perfused organ,[20] the output of VLDL found in the present experiments appears to be of the expected order of magnitude.

The liver slices responded well to physiological variables, such as fasting and refeeding. The only major quantitative differences we have found in comparison with the perfused organ concern the release of FFA at low levels of medium FFA and the relatively poor response of VLDL-TG output to high levels of FFA. These may in fact be related phenomena; higher levels of medium FFA are required to produce net uptake in slices and, as we have seen, very high levels produce inhibitory effects. Rose, Vaughan and Steinberg[21] studied the uptake of labeled FFA and glycerol into TG and PL by liver slices in a phosphate medium. At the lowest level of medium FFA tested, 0·4 mM, they did not find an increase in medium FFA pool size, but found net uptake of FFA at an initial level of 1·76 mM. As mentioned earlier, Kook and Rubinstein[16] observed large increases in labeled FFA in the medium in which slices were incubated following labeled palmitate injection. The liver slice system also shows the enhancement of TG release by a factor present in whole serum which was found in liver perfusions by Roheim *et al.*[10]

To the best of our knowledge, the net synthesis of TG during the incubation of liver slices has not previously been reported. It is implicit in the experiments of Rose *et al.*,[21] who found that incorporation of labeled fatty acid into TG increased progressively as the FFA concentration in the medium was raised. Livers from fed rats contain abundant glycogen, and free fatty acids are available in the incubation medium and also from endogenous PL breakdown. In agreement with the results of Rose *et al.*,[21] liver TG synthesis is preferred over PL synthesis. PL breakdown occurs readily in slices, although very little PL breakdown seems to occur in perfused livers.[15,22] Heimberg *et al.*[15] found no increases in liver PL after infusion of 500 mg of oleic acid over a 2 hr period in perfused liver, but this amount of fatty acid did not raise the medium concentration above 1 mM.

It is difficult to say from our experiments whether the inhibition of TG release, seen only when the drugs were added to the medium and not in liver slices from rats pre-treated with these drugs, plays a role in their effects on serum TG levels *in vivo*. The concentrations required for inhibition by amphetamine, for example, about 10^{-3} M, are high in comparison with an effective *in vivo* dose of 5–10 mg/kg, but it is possible that the perfused organ and the organ *in vivo* is more sensitive to the drug. In addition, a relatively small degree of inhibition applied over a 4-hr period to a constituent with a turnover rate as high as that of VLDL-TG might produce significant decreases in serum TG levels. We can say, however, that if significant metabolic inhibition does occur *in vivo*, it can be readily reversed, since it is easily lost by incubation of liver slices in a drug-free medium. Nevertheless, some changes were seen in liver from rats given the drugs *in vivo*, because net TG synthesis was not observed. This may be related to the higher initial level of liver TG, resulting from the elevation of serum FFA produced by amphetamine or fenfluramine.[23]

We conclude from our experiments that VLDL release occurs in slices of rat liver and that the technique is a valid and useful one for the study of the action of drugs on lipid and lipoprotein metabolism. The technique is particularly useful for the study of the relative potency of drugs affecting hepatic lipid metabolism.

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