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Effects of the lipase inhibitors, Triton WR-1339 and tetrahydrolipstatin, on the synthesis and secretion of lipids by rat hepatocytes

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The lipase inhibitors, Triton WR-1339 and tetrahydrolipstatin, were incubated with rat hepatocytes. Triton WR-1339 increased the recovery of triacylglycerol in the hepatocytes and incubation medium by 31% and 38%, respectively. Tetrahydrolipstatin decreased the accumulation of newly synthesized, and of total triacylglycerol in the medium. This compound might be useful in determining mechanisms involved in intracellular triacylglycerol metabolism and the secretion of very low density lipoproteins.

Lipase; Tetrahydrolipstatin; Triacylglycerol; Verly low density lipoprotein

1. INTRODUCTION

There is great interest in studying the mechanisms that control the secretion of very low density lipoproteins (VLDL) and much of this work is now being done with monolayer cultures of hepatocytes. One potential problem with this kind of experiment is that there may be degradation of secreted triacylglycerol during an extended culture period. This could be caused by extracellular lipolysis due to the secretion of hepatic lipase [1–3]. We therefore undertook experiments to determine the effect of inhibiting extracellular lipase activity on the accumulation of triacylglycerols in the medium of cultured rat hepatocytes.

For this study we chose to use the compound, tetrahydrolipstatin, which is a derivative of microbial lipstatin and which is a specific inhibitor of acylglycerolipases, but not Ca^{2+}-dependent phospholipases [4–6]. Tetrahydrolipstatin forms a covalent bond with the active site serine of the lipases [7]. This inhibitor has been used to inhibit the digestion and absorption of triacylglycerol from the small intestine because of its action in decreasing gastric lipase, carboxylester lipase and pancreatic lipase activities [4–7]. It therefore appeared to be an ideal inhibitor to add to the culture medium of the hepatocytes in order to prevent extracellular degradation of triacylglycerol. We tested whether this compound would increase the recovery of triacylglycerol in VLDL from cultures of rat hepatocytes. As a comparison, we used the well known non-specific lipase inhibitor, Triton WR 1339 [8].

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2. MATERIALS AND METHODS

2.1. *Animals and materials*

The sources of most of the materials have been described previously [9–11]. Radiochemicals were purchased from Amersham International and Triton WR-1339 (Tyloxapol) was from Sigma Chemical Co., St. Louis, MO, USA. Tetrahydrolipstatin was a gift from Dr M.K. Meier, Hoffman-La Roche Ltd, Basel, Switzerland. It was dissolved in dimethyl sulfoxide at a concentration of 240 mM for use in the assays. Male Wistar rats weighing about 200 g were supplied by Charles River, Québec, Canada.

2.2. *Preparation and incubation of hepatocytes*

Hepatocytes were prepared as described previously and approximately 2×10^6 cells were applied to tissue culture dishes that had been previously coated with collagen. The cells were incubated for 1 h in modified Leibovitz L-15 medium [9–11] containing 10% (v/v) newborn calf serum in air at 96% humidity. Unattached and non-viable cells were removed by changing the medium and the cells were incubated for a further 3 h. The medium was changed, but this time it contained 0.03 mM fatty acid poor bovine serum albumin instead of 10% serum, and the incubation was continued overnight. The cells were then incubated for a further 1 h in a similar medium in which the albumin concentration was increased to 0.1 mM. The cells were then incubated in fresh medium containing the additions as indicated. At the completion of the incubation, the supernatant was collected from triplicate dishes, centrifuged at 10000 rpm for 10 min at 4°C to remove detached cells and cell debris. The monolayers of hepatocytes were washed with 2×2.5 ml of 0.15 NaCl and then scraped from the dishes in 1 ml of ice-cold 0.25 mM sucrose containing 0.5 mM dithiothreitol and 10 mM HEPES (pH 7.4). The cell suspensions from triplicate dishes were pooled, sonicated and the viability of the cells was determined by measuring lactate dehydrogenase activity [10,11]. Damage and permeabilization of the cells results in the loss of this enzyme. We were therefore able to ensure that this did not happen in the incubations reported in this paper. The results have been

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expressed relative to the activity of lactate dehydrogenase in the cells to compensate for small differences in the number of hepatocytes per dish. This method is considered superior to the measurement of DNA or protein since non-viable cells which may be still attached to the dishes could contribute to these latter values and the incubations contain relatively high albumin concentrations [10,11]. One dish of cells contained about 1.5 mg of hepatocyte protein.

2.3. *Measurement of triacylglycerol*

The mass of triacylglycerol in the cells and medium was determined [12] after extracting lipid by the method of Bligh and Dyer [13]. A standard of triolein was used and the concentration of triacylglycerol was determined with a 'triglyceride GPOP-PAP' test combination reagent purchased from Boehringer Mannheim, Germany. Twenty μl of propan-2-ol were added to each tube in order to solubilize the lipid before adding 350 μl of the color reagent. Triacylglycerol in the medium could not be assayed directly after extraction because of subsequent turbidity caused by fatty acid [14]. In this case, the whole bottom phase (about 2.5 ml) was shaken with 1 g of basic alumina to remove residual oleate [14]. After centrifuging for 30 min and 10000 × *g*, 1.6 ml of supernatant was taken and the solvent was removed before determining triacylglycerol concentrations. The addition of dimethyl sulfoxide, tetrahydrolipstatin and Triton WR-1339 to the medium did not interfere with this analysis.

In other experiments, the incorporation of [1,3-^{3}H]glycerol into lipids was used to determine the accumulation of newly synthesized triacylglycerol in the hepatocytes and secretion into the medium. Lipids were then extracted from the cells and medium as described above. Triacylglycerol was separated from other lipids by thin layer chromatography on silica gel 60 (from Merck, Darmstadt, Germany). Plates were developed with light petroleum bp. 40–60°C/diethyl ether/acetic acid (50:50:1, v/v). The radioactivity in the area containing triacylglycerol was determined after adding 0.5 ml of water and 5 ml of Amersham ACS. This method was devised to give good recoveries of lipids from silica gel. The water inactivates the silica and the lipids dissolve in the detergent and solvent [15].

3. RESULTS AND DISCUSSION

After incubation for 4 h with Triton WR-1339, the amount of triacylglycerol in cells and medium was increased by 31% and 38% (Table I), respectively, when compared to controls. The increase in the recovery in the medium is compatible with an inhibition of extracellular lipase activity. However, the increase in the accumulation of triacylglycerol within the cells indicates an additional interference with intracellular metabolism. Thus although Triton WR-1339 might produce the desired effect of inhibiting extracellular lipases [8], its effect on intracellular triacylglycerol raises serious doubts about its selectivity.

We therefore hoped that tetrahydrolipstatin would be a much more specific inhibitor. However, this compound at 200 μM decreased the accumulation of triacylglycerol in the medium by about 80% (Table I). The recovery of triacylglycerol in the cells was not significantly altered.

In the second series of experiments we determined the effects of tetrahydrolipstatin on newly synthesized triacylglycerol by using [^{3}H]glycerol as a substrate (Fig. 1). Triacylglycerol radioactivity in the medium was progressively decreased as the concentration of tetrahydrolipstatin increased, thus confirming the results in Table I. At the same time, the accumulation of triacylglycerol radioactivity in the cells increased up to a concentration of 500 μM tetrahydrolipstatin. This increase was not reflected in the triacylglycerol mass (Table I). However, the triacylglycerol that is synthesized from [^{3}H]glycerol in presence of 200 μM tetrahydrolipstatin (Fig. 1) accounted for only about 19% of the triacylglycerol in the cells and about 11% of that in the medium compared with the total triacylglycerol mass. This calculation is based upon the specific radioactivity of the [^{3}H]glycerol and there would have been changes in the specific activity of the precursor pool of [^{3}H]glycerol-3-phosphate.

Table I

Relative effect of Triton WR-1339 and tetrahydrolipstatin on the accumulation and secretion of triacylglycerol by rat hepatocytes

	Relative amounts (%)	
	Triton WR-1339 (0.1%)	Tetrahydrolipstatin (200 μM)
Cells	131 ± 2.4**	90 ± 8.8
Medium	138 ± 4.4*	20 ± 3.9*
Cells + medium	132 ± 1.3**	79 ± 7.5

Results are expressed relative to incubations that contained no inhibitor and the values are means ± SE of 4 independent experiments, for Triton WR-1339 and three independent experiments for tetrahydrolipstatin. Absolute amounts of triacylglycerol in control incubations after 4 h were 151 ± 7.6 and 25 ± 1.8 nmol/unit of cellular LDH for the cells and medium, respectively. The significance of the difference relative to the control was calculated by a paired *t*-test and it is indicated by: * $P < 0.005$ and ** $P < 0.001$

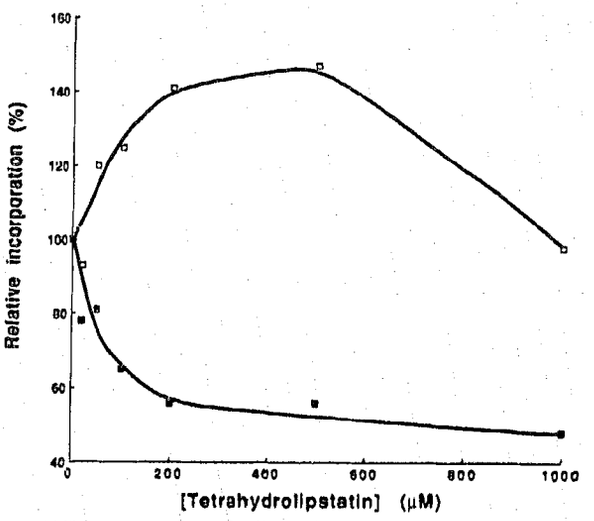

Fig. 1. Effect of tetrahydrolipstatin on the accumulation and secretion of newly-synthesized triacylglycerol in rat hepatocytes. Hepatocytes were incubated for 8 h with 0.1 mM fatty acid poor bovine serum albumin, 1 mM [^{3}H]glycerol (9 Ci/mol), 100 μM choline and tetrahydrolipstatin as indicated. The results are means from two independent experiments. The absolute values for the incorporation of [^{3}H]glycerol into triacylglycerol in the absence of tetrahydrolipstatin were 18.0 and 1.2 nmol/unit of cellular lactate dehydrogenase for the cells (□) and medium (■), respectively.

It is therefore concluded that tetrahydrolipstatin could not be used to enhance the accumulation of secreted VLDL triacylglycerol in the incubation medium. In fact, it had an opposite effect of decreasing the amount of VLDL triacylglycerol. The reason for this is not certain at this stage. Tetrahydrolipstatin is absorbed from the intestine and it appears in urine and bile [6]. It is therefore possible that it could enter the hepatocytes and interfere with VLDL secretion by inhibiting the mobilization of the intracellular triacylglycerol pool via lipolysis. This is likely to be a prerequisite for subsequent secretion of this triacylglycerol that is temporarily stored in the hepatocytes [16–18]. Such an effect is compatible with the accumulation of newly synthesized triacylglycerol that was labelled from [^{3}H]glycerol and a decrease in its appearance in the medium. The failure of tetrahydrolipstatin to increase the mass of triacylglycerol in the hepatocytes is partly explained by the fact that only about 17% of the total triacylglycerol is secreted during the course of the experiment (Table I). Alternatively, tetrahydrolipstatin might block the assembly or secretion of VLDL by an action unrelated to the effect on lipolysis.

It is not yet established whether tetrahydrolipstatin can inhibit intracellular lipase activities therapeutically and decrease VLDL secretion in addition to its effects in decreasing fat absorption. Further investigations of the mode of action of tetrahydrolipstatin in hepatocytes might provide valuable information about intracellular triacylglycerol metabolism and VLDL secretion.

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REFERENCES

[1] Durrington, P.N., Newton, R.S., Weinstein, D.B. and Steinberg, D. (1982) J. Clin. Invest. 70, 63–73.
[2] Parkes, J.G., Chan, P. and Goldberg, D.M. (1986) Biochem. Cell Biol. 64, 1147–1152.
[3] Kasim, S.E., Khilmani, S. and Peterson, W.D. (1986) Clin. Res. 34, A896.
[4] Hadváry, P., Lengsfeld, H. and Wolfer, H. (1988) Biochem. J. 256, 357–361.
[5] Borgström, B. (1988) Biochim. Biophys. Acta 962, 308–316.
[6] Fernandez, E. and Borgström, B. (1989) Biochim. Biophys. Acta 1001, 249–255.
[7] Hadváry, P., Sidler, W., Meister, W., Vetter, W. and Wolfer, H. (1991) J. Biol. Chem. 266, 2021–2027.
[8] Borensztajn, L., Rone, M.S. and Kotlar, T.J. (1976) Biochem. J. 156, 539–543.
[9] Cascales, C., Mangiapane, E.H. and Brindley, D.N. (1984) Biochem. J. 219, 911–916.
[10] Pittner, R.A., Fears, R. and Brindley, D.N. (1985) Biochem. J. 225, 445–462.
[11] Martin-Sanz, P., Vance, J.E. and Brindley, D.E. (1990) Biochem. J. 271, 575–583.
[12] Graham, A., Zammit, V.A. and Brindley, D.N. (1988) Biochem. J. 249, 727–733.
[13] Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[14] Graham, A., Bennett, A.J., McLean, A.M., Zammit, V.A. and Brindley, D.N. (1988) Biochem. J. 253, 687–692.
[15] Sanchez, M., Nichols, D. and Brindley, D.N. (1973) Biochem. J. 132, 697–706.
[16] Mooney, R.A. and Lane, M.D. (1981) J. Biol. Chem. 256, 11724–11733.
[17] Fukuda, N. and Ontko, J.A. (1984) J. Lipid Res. 25, 831–842.
[18] Francone, O.L., Kalopissis, A.-D. and Griffaton, G. (1989) Biochim. Biophys. Acta 1002, 28–36.