The role of cardiac lysosomal lipases in triacylglycerol cleavage

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1. INTRODUCTION

Erucic acid (EA) or an EA-rich diet in rats induces cardiac lipidosis, with an accumulation of EA-rich triglycerides in heart muscle cells. This cardiac lipidosis shows two distinct phases: a rapid increase in cardiac lipid content during the first 3 days of an EA-rich diet, followed by a continuous slow decrease in lipid content. These phenomena are accompanied by an inflammatory response of the myocardium and by necrosis. Other organs do not accumulate fat significantly or show any pathological changes on exposure to EA (review [1]).

Since the metabolic behaviour of EA is different from that of its lower homologues, it may be conveniently used as a tool in studying the regulation of FA metabolism in the heart. The almost exclusively cardiac lipidosis could not be explained in terms of delayed oxidation of EA by cardiac cells [2], since tissues other than the myocardium (e.g., hepatocytes and skeletal muscle cells) oxidize EA by the same delayed pathway [3]. Using triglyceride (TG) substrates esterified with FA from $C_{12:0}$–$C_{22:1}$, we have shown [4] that heart intracellular lipases cannot cleave trierucate but are able to cleave all other TG esters from $C_{12:0}$–$C_{20:1}$. Skeletal muscle lipases, on the other hand, are able to cleave trierucate, although to a lesser extent than other TG esters. This inability of intracellular lipases to cleave trierucate was postulated as the major cause of EA-induced lipidosis.

Here, we attempt to explain the mechanism by which the cardiac lipid content returns to normal levels during the latter phase of an EA-rich regime. Cultured heart cells were used as a model, since they readily lend themselves to kinetic studies in a controlled environment, and handle EA similarly to the heart in vivo, although probably over a different time span [5,6].

The cultured heart cells serve as the best in vitro system to study events occurring in situ under physiological conditions in an intact cell. Therefore the control of lipid metabolism will be expressed to its full extent and the relative activities of the various lipases may be appreciated under unbiased non-artificial circumstances.

2. MATERIALS AND METHODS

Erucic acid and oleic acid (OA) (Sigma) supplemented with [14-^{14}C]EA (51 mCi/mmol) and [10-^{14}C]OA (54 mCi/mmol), respectively, were prepared as in [5]. All radioactive substrates were purchased from CEA Saclay. Substrate was 0.2 mM. The FA/albumin molar ratio was ~2. Ham F10 culture medium supplemented with 1% FA-free albumin (Sigma) was used as a substrate-free incubation medium and for washing. Chloroquine, cycloheximide and puromycin were purchased from Sigma.

2.1. *Culture preparation*

Cardiac cell cultures from 2-day-old rats of a local strain were obtained as in [7]. Seeding density was 2×10^6 cells/35 mm Petri dish (Falcon 3001),

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at which ≥80% of the cells obtained by day 5 were muscle cells.

To ensure identity in the number and type of cells, each series of experiments was carried out using cultures originating from the same cell batch.

2.2. *Labelling and radioactivity distribution*

On day 5 of culture, labelling was carried out by incubating the cells in either [^{14}C]OA-enriched or [^{14}C]EA-enriched media for varying times at 37°C. The external medium was then discarded and the cells were washed 2–3 times with substrate-free culture medium to eliminate all extracellular absorbed FA.

After labelling and washing, the cells were incubated in substrate-free Ham F10 medium containing 1% albumin for various periods of time. The cells were then washed and scraped off the Petri dishes in 0.5 ml cold NaCl 0.9%. Lipids were extracted according to [8] and the lipid extract evaporated under a nitrogen stream. Lipid classes were separated on 0.25 mm silica-gel thin-layer plastic strips (ICN Pharmaceutics, GmbH) in a solvent system of hexane/ether/acetic acid (70:30:1, by vol.). The radioactive content of the different lipid classes was measured in 1% Triton–toluene using a Packard Tricarb model 2660 performing direct dpm counting.

Each experiment was performed in duplicate or triplicate

2.3. *Inhibition of lysosomal lipolytic activity*

Prior to pulse labelling, cells were preincubated for 1 h with culture medium to which chloroquine (10^{-4} M) had been added. Pulse-labelling of cells was performed with media containing fatty acid and chloroquine. Post-pulse incubations with substrate-free media determination of radioactivity in the different lipid classes were then carried out as described above.

3. RESULTS AND DISCUSSION

The intracellular kinetics of OA and EA can be studied by following their radioactive distribution between the different lipid classes. Major radioactive variations were observed between FFA, phospholipids (PL) and TG; we therefore report data for these 3 lipid classes only. The radioactivity in diglycerides was high during the first 30 min (not shown) and decreased to a constant low level after 1 h. The radioactivity in cholesterol esters was generally of a low constant level and is taken into account in the calculations, but not reported. After a 'pulse' labelling, the gradual disappearance of radioactivity from the TG pool reflects the cleavage of TG by lipases.

The extent of substrate removal from the extracellular medium was similar for both acids (e.g., after 5 h 28% for OA, 30% for EA), indicating identical rates of FA uptake. Complete FA disappearance is observed after 24 h [9].

Furthermore, the largest part of the newly taken up fatty acid is immediately esterified [10] whereas only a small fraction of it is channeled for immediate oxidation. The bulk fatty acid to be oxidized in the mitochondria originates from the complex lipids primarily via lipolysis of triglycerides [11]. We would like to emphasize that in cultured cells from heart, < 10% of the total fatty acid that was taken up is directed to mitochondrial oxidation [12].

The results presented in table 1 indicate that, in contrast to OA, the release of esterified EA from glycerides by lipolysis was delayed for several hours.

The considerable delay in the cleavage of erucate-containing TG raises the possibility that the breakdown of EA esters is an adaptive mechanism related to the synthesis of new proteins (e.g., new molecules of lipases with specificity towards EA containing esters). The termination of this delay could conceivably be brought about by the synthesis of a new intracellular lipase(s) able to cleave EA-rich triglycerides. If this were so, the inhibition of protein synthesis would prolong the delay in the cleavage of EA esters but would have no effect on the cleavage of oleic acid esters. However, assays carried out after preincubating cell cultures with puromycine (1 μg/ml) and cycloheximide (0.5 mM) for 1 h before and during 'pulse' labellings with either EA or OA, showed that these 2 protein inhibitors did not affect the rate of radioactivity flow with both acids and consequently failed to prolong the delay of TG cleavage in EA-incubated cultures.

Another possible mechanism for the clearing of EA-rich TG in cardiac muscle cells could be through the activity of lysosomal lipases. To test this hypothesis we used chloroquine, a known inhibitor of lysosomal activity [13]. If the hypothesis that lysosomal lipases are involved is correct, then chlo-

Table 1

Radioactivity distribution between the different lipid classes after pulse labellings for 1 h and 24 h with [10-^{14}C]OA or [14-^{14}C]EA, followed by various incubation periods with substrate-free medium

Pulse labelling/duration		1 h				24 h	
Incubation time after 1 h pulse labelling:		0	1	3	5	0	10
[10-^{14}C]Oleic acid radioactivity (%)	PL	48	57	78	78	64	66
	FFA	9	4	1	1	1	1
	TG	36	38	19	17	30	28
	TG/PL	0.8	0.7	0.2	0.2	0.47	0.42
[14-^{14}C]Erucic acid radioactivity (%)	PL	21	31	29	50	31	32
	FFA	30	1	5	6	3	1
	TG	42	67	64	38	59	62
	TG/PL	2	2.2	2.2	0.76	1.9	1.9

After 1 h incubation the total radioactivity inside the cells of each petri dish was $> 10^4$ dpm; 70–80% of this radioactivity remains inside the cell for 6 h after 'post-pulse' incubation. The loss of 20–30% of radioactivity occurred mainly during the first hour of 'post-pulse' incubation. This could be explained by an exit of the intracellular pool of FFA to the albumin containing medium or to a certain degree of cell loss due to damages

roquine should prolong the delay in radioactivity flow from TG to PL for EA-incubated cultures but not for OA-incubated cultures.

The effect of chloroquine on the kinetic changes in radioactivity distribution and on the breakdown of TG is shown in table 2. Cells pulse-labelled for 1 h with [10-^{14}C]OA showed a small but significant increase in TG breakdown. However, in cells 'pulse'-labelled with [14-^{14}C]EA the delay in TG cleavage, shown in table 1 was prolonged further in the presence of chloroquine. After labelling for 24 h (table 2), the delay in EA-rich TG cleavage due to chloroquine was more pronounced than after pulse labelling for 1 h. For OA-incubated cultures a 24 h pulse also causes a delay in TG breakdown and the TG/PL ratio is inversed with respect to that reported in table 1.

A comparison between the results presented in table 1 and 2 shows that in cultures which were pulse-labelled with OA for 1 h, the breakdown of TG was actually accelerated in the presence of chloroquine (10^{-4} M). Erucate cleavage, on the other hand, was delayed even further, indicating the involvement of lysosomal lipases in EA-rich TG cleavage. It is noteworthy, however, that in 24 h pulse-labelled cultures, in which all the supplemented FA was removed from the culture medium through cell uptake, chloroquine (10^{-4} M) delayed TG cleavage in oleate as well as in erucate-incubated cultures (cf. tables 1 and 2). Furthermore, a moderate accumulation of fat droplets within cardiac myocytes was reported during prolonged incubation of cultured heart cells with palmitic acid [6] indicating that a slight lipid accumulation occurs in cultured cells consecutive to prolonged incubation periods.

In [13], with perfused hearts from rats fed with EA for 3 days prior to assay, the importance of lysosomal lipase in TG cleavage was shown and lysosomal lipases concluded to be the sole agents of endogenous TG cleavage. Our findings indicate, however, that while EA-rich TG are always cleaved by lysosomal lipases, OA-rich TG are cleaved by lysosomal lipases only during massive FA uptake; during moderate FA uptake OA–TG cleavage is done by intracellular non-lysosomal lipases. The

Table 2

Effect of 10^{-4} M chloroquine on radioactivity flow in cell cultures pulse-labelled for 1 h and 24 h with [10-^{14}C]OA or [14-^{14}C]EA, followed by incubation with substrate-free chloroquine containing medium for various times

Pulse labelling duration		1 h			24 h	
Incubation time after pulse labelling (h):		0	1	5	0	10
[10-^{14}C]Oleic acid radioactivity (%)	PL	56	71	83	38	29
	FFA	7	3	1	1	1
	TG	32	23	6	56	61
	TG/PL	0.6	0.3	0.07	1.5	2.1
[14-^{14}C]Erucic acid radioactivity (%)	PL	16	30	39	16	11
	FFA	29	3	2	2	2
	TG	42	59	49	78	80
	TG/PL	2.6	2.0	1.25	4.5	7.3

conflicting view expressed in [10] could probably be ascribed to differences in their experimental system since these workers used perfused hearts from rats maintained for 3 days on an EA-rich diet, thus using an FA-invaded tissue as a model.

On the basis of these findings, we suggest that the role of lysosomal lipases is to cleave TG only during conditions of lipid accumulation in myocardial cells. Such conditions occur during uptake of an 'abnormal' FA such as EA or during massive invasion of the tissue by a 'normal' FA such as oleic acid.

The histopathological findings observed in the myocardium during EA uptake by the heart [1] occur mainly during the phase in which the intracardial lipid content returns to normal and may possibly also be explained in terms of the intervention of lysosomal enzymes during EA-induced cardiac lipidosis, which may damage cytoplasmic structures.

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