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Autophagic degradation of peroxisomes in isolated rat hepatocytes

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Degradation of the peroxisomal enzymes fatty acyl-CoA oxidase and catalase was studied in hepatocytes isolated from rats treated with clofibrate and from control rats. Hepatocytes were incubated in the absence of amino acids in order to ensure maximal flux through the autophagic pathway and in the presence of cycloheximide to inhibit protein synthesis. (1) Degradation of the two peroxisomal enzymes in hepatocytes from clofibrate-fed rats, but not in hepatocytes from control rats, was much faster than that of other intracellular enzymes. This increased degradation of the peroxisomal enzymes was almost completely prevented by 3-methyladenine, an inhibitor of macroautophagic sequestration. (2) The increased degradation of the peroxisomal enzymes was also inhibited by a long-chain (C16:0) and a very-long-chain (C26:0) fatty acid, but not by C12:0, a medium-chain fatty acid, or by C8:0, a short-chain fatty acid. These results provide direct evidence for the proposal that autophagic sequestration can be highly selective [(1987) Exp. Mol. Pathol. 46, 114–122]. It is concluded that preferential autophagy of peroxisomes is prevented when these organelles are supplied with their fatty acid substrates.

Autophagy; Peroxisome; Hepatocyte; Catalase; Fatty acyl-CoA oxidase

1. INTRODUCTION

Hepatic protein degradation mainly occurs by macroautophagy [1,2]. During this process, parts of the cytoplasm are surrounded by a sequestering membrane originating from the endoplasmic reticulum [3]. Autophagosomes thus formed, which may even contain whole organelles, fuse with existing or newly formed lysosomes, and this is followed by degradation of the sequestered macromolecular material. Although autophagic proteolysis of cytosolic proteins is largely non-selective in that they are sequestered in the same ratio as they occur in the cytosol [4,5], some cytosolic proteins may be specifically targeted to the lysosomes because of the presence near the N-terminus of the peptide sequence KFERQ, which acts as an autophagic targeting signal [6]. Morphometric measurements have indicated that different cell compartments turn over with different half lives [7]. Another indication for selective autophagy is that regulation by glucagon of hepatic autophagic RNA and protein degradation proceeds by different mechanisms [8]. Furthermore, administration of antilipolytic agents agents to starved rats results in preferential autophagic breakdown of hepatic peroxisomal enzymes at rates which exceed those of enzymes in other cellular compartments [9,10]. We show here that selective autophagic breakdown of peroxisomal enzymes also occurs in hepatocytes isolated from clofibrate- treated rats and incubated in the absence of amino acids in order to ensure maximal flux through the autophagic pathway. It is further shown that long-chain fatty acids protect peroxisomal enzymes against this degradation.

Abbreviations: C8:0, octanoate; C12:0, laurate; C16:0, palmitate; C26:0, cerotate.

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2. MATERIALS AND METHODS

Rat hepatocytes were isolated from fed male Wistar rats (200–250 g) as described previously [11]. Clofibrate was administered during 3 weeks by mixing this drug with the lab chow by grinding (5 g clofibrate/kg chow) [12]. The rats had free access to the food.

Hepatocytes (5–10 mg dry mass/ml) were incubated at 37°C in closed 25-ml plastic counting vials with Krebs-Henseleit bicarbonate medium containing 1.3 mM Ca^{2+}, 10 mM sodium HEPES (pH 7.4), 20 μM cycloheximide, 20 mM glucose and 1 mM octanoate. The gas phase was 95% O_2 plus 5% CO_2 (v/v) and was refreshed every 30 min.

Enzyme assays were performed on samples of the cell suspension. In order to eliminate the contribution of dead cells to the enzyme activities, the samples were first centrifuged through 35% (m/v) Percoll (Pharmacia) for 10 min at 50 × *g* at 0°C. During this procedure, dead cells remain at the top while intact cells arrive at the bottom of the centrifuge tube [13]. In order to avoid underestimation of enzyme activity due to latency, all assays were performed in the presence of 0.1% Triton X-100. Enzyme activities were measured according to standard procedures: for lactate dehydrogenase see [14]; glutamate dehydrogenase [14]; β-hexosaminidase [14]; catalase [14]; glucose 6-phosphatase [15]. Fatty acyl-CoA oxidase was measured essentially according to Vamecq [16], in the presence of palmitoyl-CoA, FAD, peroxidase, and homovanillic acid, with azide added to inhibit catalase.

Proteolysis was measured as the rate of release of valine because this amino acid is not catabolised in rat liver [17,18]. For the measurement of proteolysis, samples of the cell suspension were acidified with

Table I

Enzyme	Activity relative to that in rat fed normal diet		
	Clofibrate treated	Clofibrate then normal diet	Clofibrate then fasted
Lactate dehydrogenase	1.3	0.9	1.0
Glutamate dehydrogenase	0.8	0.7	0.9
Glucose-6-phosphatase	0.8	0.9	0.9
β-Hexosaminidase	1.6	1.2	3.3
Fatty acyl-CoA oxidase	12.8	5.2	3.3
Catalase	1.4	0.9	1.0

Effect of clofibrate feeding and withdrawal on enzyme activities in rat liver. Rats were fed with clofibrate for 3 weeks, after which the rats were either sacrificed, or fed a normal diet without clofibrate for 2 days, or starved for 2 days. Enzyme acitities were measured in hepatocytes isolated from the livers of these rats without further incubation. Enzyme activities are expressed relative to the values for hepatocytes isolated from rats fed a normal diet. Data for each condition are the mean values of 2 hepatocyte preparations.

sulphosalicylic acid (final concentration 4%; m/v). After removal of the protein by centrifugation, the samples were brought to pH 2.2 with 3 M LiOH. The amino acid analysis was carried out on an LKB alpha-plus amino acid analyser using a lithium citrate buffer system.

Cell samples for the determination of ATP were acidified in the cold with $HClO_4$ (final concentration 3; m/v). After removal of the protein by centrifugation, the extract was neutralised to pH 7 with a solution containing 2 M KOH and 0.3 M MOPS. ATP was determined fluorimetrically according to Williamson and Corkey [19].

Enzymes and nucleotides were purchased from Boehringer GmbH (Mannheim, Germany). Clofibrate and fatty acids were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 3-Methyladenine (6-amino-3-methylpurine) was obtained from Fluka AG (Buchs, Switzerland).

The statistical significance of the difference between groups of observations was tested with Student's *t*-test.

3. RESULTS

In order to study degradation of peroxisomes, the rats were first treated with clofibrate in order to induce hepatic proliferation of peroxisomes [20,21]. Activity of long-chain acyl-CoA oxidase increased more than 12-fold by this treatment, while catalase activity increased by 40% (Table I). These data are in agreement with those published by other authors [12,22]. The activity of lactate dehydrogenase, glutamate dehydrogenase and glucose 6-phosphatase, markers for cytosol, mitochondria and endoplasmic reticulum, respectively, were not affected very much, while the lysosomal enzyme β-hexosaminidase increased by 60% (Table I). When after 3 weeks of feeding with clofibrate the drug was either removed from the diet and normal feeding was contin-

Table II

Hepatocytes from:	Parameter	Activity in hepatocytes incubated for 4 h in the absence of amino acids with the following additions					
		none	3-methyl-adenine	C12:0	C16:0	C26:0	α-cyclo-dextrin
Control rats (n=3)	Lactate dehydrogenase	83.1 ± 3.2	101.9 ± 1.2*	82.0 ± 0.8	86.1 ± 2.5	85.8 ± 1.7	80.2 ± 0.8
	Glutamate dehydrogenase	80.6 ± 1.5	99.6 ± 0.4*	80.8 ± 3.6	84.4 ± 1.2	78.9 ± 1.5	79.3 ± 0.6
	Glucose-6-phosphatase	86.1 ± 1.1	99.3 ± 1.8*	85.3 ± 0.5	88.7 ± 5.1	86.5 ± 2.7	87.0 ± 1.2
	β-Hexosaminidase	86.8 ± 2.4	101.6 ± 0.9*	87.8 ± 5.3	89.9 ± 1.9	88.7 ± 3.5	83.7 ± 7.0
	Fatty acyl-CoA oxidase	89.3 ± 1.2	99.7 ± 0.2*	87.3 ± 2.5	94.8 ± 2.9	91.5 ± 1.6	88.4 ± 0.2
	Catalase	87.7 ± 3.0	99.5 ± 1.0*	83.9 ± 1.2	89.2 ± 3.5	88.8 ± 2.1	84.2 ± 1.9
	Valine (nmol·g·dry mass^{-1}·min^{-1})	241 ± 8	122 ± 16*	255 ± 17	250 ± 6	244 ± 4	256 ± 12
	ATP (μmol·g dry mass^{-1})	9.4 ± 1.0	11.4 ± 0.7	10.2 ± 0.4	9.2 ± 0.2	8.4 ± 1.0	9.6 ± 2.2
Clofibrate-treated rats (n=5)	Lactate dehydrogenase	85.4 ± 1.6	99.6 ± 0.9*	82.2 ± 3.0	90.5 ± 1.0	86.0 ± 2.0	84.7 ± 0.2
	Glutamate dehydrogenase	78.8 ± 3.9	96.8 ± 2.8*	84.7 ± 4.3	82.5 ± 1.2	83.8 ± 2.6	83.0 ± 2.1
	Glucose-6-phosphatase	88.8 ± 4.4	98.6 ± 1.3*	88.4 ± 2.7	87.6 ± 3.6	85.7 ± 2.4	91.8 ± 3.8
	β-Hexosaminidase	87.3 ± 4.5	96.6 ± 4.2	84.0 ± 1.8	85.5 ± 1.8	85.7 ± 3.6	84.0 ± 4.0
	Fatty acyl-CoA oxidase	56.6 ± 4.9**	97.7 ± 3.2*	57.2 ± 3.4**	94.5 ± 1.7*	93.3 ± 2.7*	55.8 ± 3.6**
	Catalase	67.8 ± 2.7**	102.4 ± 3.3*	65.5 ± 1.4**	88.4 ± 1.3	90.1 ± 1.1*	69.3 ± 2.5**
	Valine (nmol·g·dry mass^{-1}·min^{-1})	216 ± 32	116 ± 16*	216 (2)	188 ± 27	184 ± 21	204 ± 27
	ATP (μmol·g dry mass^{-1})	9.2 ± 1.7	12.2 ± 1.3*	10.0 (1)	9.0 ± 1.4	8.6 ± 1.4	8.2 ± 1.4

Autophagy of enzymes in hepatocytes isolated from rats treated with clofibrate and from normal rats. Hepatocytes were isolated and incubated for 4 h under the conditions outlined in Methods and Materials in the absence of amino acids and (where indicated) with 10 mM 3-methyladenine, 400 μM C12:0 fatty acid, 200 μM C16:0 fatty acid, 40 μM C26:0 fatty acid, or 10 mg/ml α-cyclodextrin (the absorbent of C12:0, C16:0 and C26:0 fatty acids). Intracellular ATP was measured after the 4 h incubation period. Enzyme activities are expressed as percentages of the activity in hepatocytes immediately after isolation. Values are the means ± SD of 3 separate (normal fed rats) hepatocyte preparations and 5 separate hepatocyte preparations (clofibrate fed rats).

*Significantly different (P<0.05) from the corresponding control (without addition).

**Significantly different (P<0.05) from the value for lactate dehydrogenase obtained under the same conditions.

ued for two days, or the rats were starved for two days, fatty acyl-CoA oxidase activity greatly decreased while catalase fell back to normal values (Table I). Starvation led to an increase in β-hexosaminidase activity (Table I), in agreement with similar observations by Kovács et al. [23] on cathepsin D, another lysosomal enzyme. These measurements in vivo do not, of course, discriminate between increased rates of enzyme degradation, decreased rates of enzyme synthesis, or a combination if both. In order to study the breakdown of peroxisomal enzymes independently of protein synthesis we isolated hepatocytes from rats treated with clofibrate and incubated the cells in the presence of cycloheximide to inhibit protein synthesis. Control experiments revealed that at the concentration of cycloheximide used (20 μM) protein synthesis was inhibited by more than 95% (not shown, but see [24]). Glucose and octanoate were present to provide the cells with a source of energy. Amino acids were omitted from the medium in order to ensure maximal flux through the autophagic pathway [1,2]. After 4 h of incubation, disappearance of the peroxisomal enzymes fatty acyl-CoA oxidase and catalase was much higher than that of the other four, non-peroxisomal, marker enzymes (Table 2). The rate of disappearance of lactate dehydrogenase (16% in 4 h) was similar to that observed by Kopitz et al. [15] in normal hepatocytes. The disappearance of the peroxisomal enzymes, like that of the non-peroxisomal enzymes, was largely prevented by 3-methyladenine, a strong inhibitor of autophagic sequestration [25] (see Table II). Breakdown of the peroxisomal enzymes, but not that of the other enzymes, was inhibited by a long-chain fatty acid (C16:0) and a very-long-chain fatty acid (C26:0). Addition of α-cyclodextrin, to which these fatty acids were bound, had no effect (Table II). The medium-chain fatty acid C12:0 was not effective (Table II).

Intracellular ATP was not significantly different under the various conditions except that in the presence of 3-methyladenine the concentration of ATP slightly increased, which is in agreement with our previous observations in normal hepatocytes [11]. Overall proteolytic flux, as measured by valine production, was inhibited by about 50% by 3-methyladenine, while the fatty acids C16:0 and C26:0 inhibited flux by 15% (Table II).

Accelerated degradation of peroxisomal enzymes was not observed in hepatocytes isolated from control rats that had not been exposed to clofibrate (Table I).

4. DISCUSSION

In recent reviews on peroxisomes [26–28] emphasis has been placed on the biogenesis of these organelles but no attention was paid to their degradation. This is surprising since the level of peroxisomes in the cell is determined by the relative rates of both synthesis and degradation. At steady state, these rates must be equal. For instance, whereas feeding rats with compounds like clofibrate leads to a remarkable increase in the number of peroxisomes and to a concomitant increase in the rate of synthesis of some peroxisomal membrane proteins (cf. [28] for a review) and of fatty acid oxidation enzymes [29], the large increase in the number of peroxisomes in the liver of rats fed with a high-fat diet is mainly due to depressed degradation of peroxisomes in the liver of rats fed with a high-fat diet is mainly due to depressed degradation of peroxisomes [30].

It has been suggested that autophagy plays a role in the degradation of peroxisomes [7,9,10,20,21,31–33], although the extent to which it influences degradation is still uncertain [34]. For instance, the involvement of autophagy in the turnover of peroxisomes in fibroblasts from patients with the Zellweger syndrome recently has been established by us [35] and evidence is accumulating that under certain conditions autophagy of peroxisomes may be selective in that the degradation rate of these organelles is more rapid than that of other cellular structures [7,9,10,30].

In our experimental system, i.e. hepatocytes isolated from rats treated with clofibrate selective degradation of peroxisomal enzymes was, indeed, observed. The fact that 3-methyladenine completely prevented the decrease in activity of both fatty acyl-CoA oxidase and catalase indicates that macroautophagy must have been responsible for the observed rapid removal of peroxisomes from the hepatocytes. The rate of autophagic breakdown of the peroxisomes was extremely high, about 10% per hour. Of course, we deliberately chose conditions under which flux through the autophagic pathway was maximal, i.e. in the absence of amino acids, while protein synthesis was simultaneously inhibited. However, also in vivo the rate of autophagic breakdown of peroxisomes was considerable: two days after withdrawal of clofibrate from the diet 60–70% of fatty acyl-CoA oxidase disappeared. This value agrees well with a reported half life of peroxisomes of 1.4 days in the livers of rats under similar conditions [21,29] and of 2 days in rats switching from a high-fat diet (which also causes an increase in the number of peroxisomes) back to a normal diet [30].

Extremely intriguing is the finding that autophagic breakdown of the peroxisomal enzymes in our experimental system was sensitive to inhibition by a long-chain (C16:0) and a very long-chain (C26:0) fatty acid, but was not inhibited by a medium-chain fatty acid (C12:0). Presumably, there was no inhibition by octanoate (C8:0). The media used for incubating the hepatocytes always contained octanoate in order to ensure an adequate ATP supply, ATP being required at several steps in the autophagic pathway [36,37].

The selective effect of the long-chain and very-long-chain fatty acid on peroxisome degradation was also reflected in their effects on overall proteolysis. While inhibition by 3-methyladenine was about 50% after 4 h

of incubation, the inhibition caused by the long-chain and very-long-chain fatty acids was only 15% (Table II). Because these fatty acids only inhibited the degradation of peroxisomes, but not that of other cell components (Table II), it can be concluded that in hepatocytes from rats treated with clofibrate 100 × 15/50 = 30% of the total cell protein degraded by macroautophagy was derived from the peroxisomes. In the experiments described in this paper, inhibition by 3-methyladenine of overall proteolysis was less than the 60–70% inhibition usually observed in hepatocytes from normal rats [11,25]. The explanation for this could be that proteolysis in hepatocytes isolated from rats treated wih clofibrate proceeded with a lag period of about 1 h, in contrast to a lag of 20 min observed in normal hepatocytes (results not shown).

Our results are in complete accordance with, and provide an explanation for, the data obtained for a situation in vivo by Horie and Suga [30], who showed that hepatic peroxisomal proliferation after feeding rats a high-fat diet is mainly due to inhibition of peroxisome degradation, which the authors ascribe to inhibition of lysosomal functions. Apparently, long-chain and very-long-chain fatty acids, which are substrates for peroxisomes, protect peroxisomes against autophagic degradation.

We did not observe selective degradation of peroxisomes in hepatocytes isolated from control rats. However, this may have been due to protection by endogenous long-chain fatty acids. Interestingly, it was found by Locci Cubeddu et al. [9] that long-term starvation of rats did not affect the activity of peroxisomal fatty acyl-CoA oxidase in vivo, even though the liver weight decreased by 45%, which is known to be due to macroautophagy [1]. However, administration of antilipolytic agents did result in hepatic autophagic peroxisome degradation [9,10]. Although these effects were ascribed to the increased glucagon/insulin ratio caused by these agents [9,10], our results indicate that the decreased supply of endogenous fatty acids per se may have been responsible for the induction of peroxisome degradation under these conditions.

As to the mechanism by means of which long-chain and very-long-chain fatty acids are able to protect peroxisomes against autophagic degradation one can only speculate. A possibility is that one of the peroxisomal membrane proteins contains a peptide-signal sequence, in analogy to the KFERQ sequence reported by Wing et al. [6], which is rendered inactive by acylation and which becomes deacylated, and thus susceptible for autophagy, when the concentration of long-chain fatty acids falls.

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