Function and dysfunction of the mitochondrial and peroxisomal beta-oxidation systems in human cells and their functional interaction

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CHAPTER 2

RESOLUTION OF THE SUBCELLULAR SITE OF VERY-LONG-CHAIN FATTY ACID ß-OXIDATION IN HUMAN SKIN FIBROBLASTS.

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Jakobs BS, Wanders RJA (1991) Conclusive evidence that very-long-chain fatty acids are oxidized in peroxisomes in human skin fibroblasts. Biochem Biophys Res Commun 178, 842-847

SUMMARY
We have investigated the contribution of peroxisomes and mitochondria to the β-oxidation of palmitate and cerotate in intact human skin fibroblasts. In the presence of the respiratory chain inhibitors, rotenone plus antimycin and KCN, we found a decrease, although to a different extent, in the β-oxidation of both fatty acids. However, further investigations showed that the use of these inhibitors resulted in lowering of cellular ATP levels and an increase in the cytosolic redox-state. When POCA was used to specifically block CPT I, palmitate β-oxidation was inhibited almost completely whereas cerotate β-oxidation was not affected. Other cellular processes were not affected, as reflected in normal ATP levels and a normal cytosolic redox state. Since CPT I is essential for the oxidation of fatty acids in mitochondria this result provides conclusive evidence that oxidation of very-long-chain fatty acids, such as cerotate, is initiated in peroxisomes and not in mitochondria.

INTRODUCTION
Lazarow and de Duve [1976] were the first to report the presence of a β-oxidation system in rat-liver peroxisomes. As in mitochondria, β-oxidation in peroxisomes proceeds via successive steps of dehydrogenation, hydration, dehydrogenation and thiolytic cleavage. These reactions are catalyzed by enzymes which are different from their mitochondrial counterparts. These enzymes include acyl-CoA oxidase, bifunctional protein with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity and peroxisomal thiolase. The functional significance of the peroxisomal β-oxidation system remained obscure until recently it became clear that peroxisomes are involved in the β-oxidative chain-shortening of a distinct set of substrates [Wanders et al., 1990b]. Singh and coworkers [Singh I et al., 1984] suggested that peroxisomes were the primary site of the β-oxidation of very-long-chain fatty acids, at least in rat liver. This was concluded from experiments with peroxisomes and microsomes which were separated via sucrose-density-gradient centrifugation. Subsequent studies by others [Singh H et al., 1987a] supported this suggestion. The finding that oxidation of very-long-chain fatty acids is strongly impaired in fibroblasts from patients suffering from the Zellweger syndrome in which peroxisomes are known to be deficient, was taken as evidence that oxidation of very-long-chain fatty acids is also primarily peroxisomal in human fibroblasts. It should be stressed, however, that mitochondrial abnormalities have also been described in these patients (see [Lazarow and Moser, 1989] for a review).
A number of authors [Veerkamp and Moerkerk, 1986; Reubsaet et al., 1989 and 1991; Singh H et al., 1989] disputed the primary role of peroxisomes in the oxidation of very-long-chain fatty acids. From experiments with homogenates of liver, heart and muscle, Veerkamp and Moerkerk [1986] and Reubsaet et al. [1989] concluded that lignocerate oxidation occurs predominantly (>70%) in mitochondria. The same investigators also performed studies in control human skin fibroblasts using rotenone and antimycin, to inhibit respiratory chain activity, which in turn blocks mitochondrial β-oxidation, and found that the β-oxidation of lignocerate was severely decreased. Furthermore, they found that oxidation of lignocerate proceeded at near normal rates, in Zellweger fibroblasts, which led them to conclude that lignocerate oxidation is also largely mitochondrial in cultured skin fibroblasts [Reubsaet et al., 1991]. Moreover, experiments performed by Singh and coworkers [Singh H et al., 1989] suggested that very-long-chain fatty acid oxidation in rat brain homogenates occurs also primarily in the mitochondria.
Identification of the site of oxidation of very-long-chain fatty acids is important, especially since there is a group of inherited diseases in man in which very-long-chain fatty acids accumulate (e.g. X-linked adrenoleukodystrophy). Therefore, we addressed this question by investigating the ß-oxidation, redox-state and energy charge in intact fibroblasts, either in the presence of respiratory chain inhibitors or of POCA, a specific inhibitor of carnitine-palmitoyltransferase I (CPT I).

MATERIALS AND METHODS

Cell lines and culture conditions:
Skin fibroblasts from control subjects were cultured in Dulbecco's Modified Eagle's Medium (Flow Laboratories, Irvine, UK) supplemented with 7.5% (v/v) fetal calf serum and 7.5% (v/v) newborn calf serum plus antibiotics [Wanders et al., 1987a]. Confluent cells were harvested by trypsinisation according to standard procedures and finally suspended in PBS and kept on ice until further use.

Preparations of substrates for enzyme assays:
[1-14C]palmitate and [1-14C]cerotate were prepared as 100 μM stock solutions as follows: the solvent, either benzene or toluene was evaporated in a glass tube under N2 and 100 mM Tris-HCL (pH 8.5) containing 10 mg/ml α-cyclodextrin, was added. 25 μl of either [1-14C]palmitate or [1-14C]cerotate was used for each assay.

Assay of fatty acid oxidation:
The reaction mixture consisted of PBS, 25 mM glucose, and fibroblasts (0.5-1.0 mg/ml protein) in a total volume of 250 μl. When added, rotenone, antimycin, KCN or POCA were added from stock solutions in DMSO. The final concentration of DMSO never exceeded 1% (v/v). Fatty acid oxidation was started by the addition of 10 μM [1-14C]palmitate or [1-14C]cerotate. The reactions were allowed to proceed for 60 min. at 37 °C, whereafter the radioactivity of 14CO2 and [14C]water-soluble products was assayed as described [Wanders et al., 1987b].

Formation of lactate and pyruvate from glucose and determination of cellular ATP levels:
The formation of lactate and pyruvate from glucose by human skin fibroblasts was determined as described [Wijburg et al., 1989] with minor modifications. After removal of the culture medium and subsequent addition of 2.5 ml Krebs-Henseleit bicarbonate buffer, containing 5 mM glucose and 10 μM cerotate to fibroblast monolayers, rotenone, antimycin, KCN and POCA were added. After 4 h at 37 °C, 1 ml of this incubation medium was transferred to a tube containing 200 μl of 2 M PCA. After centrifugation and neutralization [Wijburg et al., 1989] lactate and pyruvate levels were measured using standard spectrophotometric and fluorometric assays [Williamson and Corkey, 1969]. The incubation medium remaining in the culture flask (1.5 ml) was subsequently acidified by adding 45 μl of 11.6 M PCA and kept on ice. Cells were scraped from the bottom of the culture flask with the aid of a rubber policeman. The acidified fluid was removed and centrifuged (15000 x g, 4 °C, 5 min.). The pellet was used for protein measurement whereas the supernatant was neutralized with MOPS-KOH and centrifuged. The ATP content in the supernatant was measured fluorimetrically according to [Williamson and Corkey, 1989]. The cell pellet was dissolved in 1 ml of 0.2 N NaOH and used for protein measurement with the Pierce BCA protein assay reagent using HSA as a standard.

Materials:
Radiolabelled fatty acids were obtained from the Radiochemical Centre, Amersham, UK. Antimycin, rotenone and α-cyclodextrin were from Sigma Ltd. (St. Louis, Missouri, USA). All other reagents were of analytical grade. POCA was a generous gift from BYK Gulden Pharmazeutica, Konstanz, F.R.G.

RESULTS

The ß-oxidation of palmitate and cerotate in cultured human skin fibroblasts, measured as production of 14CO2 plus [14C]water-soluble products [Wanders et al., 1987b] was found to proceed linearly for 2 h (data not shown). Subsequently, the effect of addition of respiratory chain inhibitors to block mitochondrial ß-oxidation was tested. In intact mitochondria, ß-oxidation stops when respiration is inhibited, whereas peroxisosmal ß-oxidation is not directly coupled to the activity of the respiratory chain. Accordingly, inhibitors of mitochondrial distinguish between
peroxisomal and mitochondrial β-oxidation [see e.g. refs Veerkamp and Moerkerk, 1986; Reubsaet et al., 1989 and 1991; Singh H et al., 1989; Bremer et al., 1981; Lazarow, 1981; Mannaerts et al., 1979]. Based on the work of Mannaerts [Mannaerts et al., 1979] and Reubsaet [Reubsaet et al., 1991] we selected rotenone plus antimycin at concentrations of 10 plus 5 μg/ml respiration are frequently used to and 10 plus 20 μg/ml, respectively and KCN at a concentration of 2 mM [Bremer et al., 1981; Mannaerts et al., 1979].

Table 1. Effect of rotenone plus antimycin and cyanide on β-oxidation of palmitate and cerotate in human skin fibroblasts.

<table>
<thead>
<tr>
<th>Addition to medium</th>
<th>Fatty acid oxidation rate in pmol<em>h⁻¹</em>mg protein⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Palmitate</td>
</tr>
<tr>
<td>None</td>
<td>435 ± 93</td>
</tr>
<tr>
<td>Rotenone (10 μg/ml) plus Antimycin (5 μg/ml)</td>
<td>112 ± 48</td>
</tr>
<tr>
<td>Rotenone (10 μg/ml) plus Antimycin (20 μg/ml)</td>
<td>94 ± 6</td>
</tr>
<tr>
<td>KCN (2 mM)</td>
<td>72 ± 13</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.D. for 3-5 separate experiments.

Table 1 shows that rotenone plus antimycin, at either concentration, affected palmitate β-oxidation much more than cerotate β-oxidation, but also that KCN inhibited β-oxidation of palmitate and cerotate to the same extent. These results clearly do not allow unequivocal conclusions about the subcellular site of cerotate β-oxidation.

Table 2. Effect of rotenone plus antimycin, cyanide and POCA on cellular ATP levels and cytosolic redoxstate.

<table>
<thead>
<tr>
<th>Addition to medium</th>
<th>Cellular ATP level (nmol*mg protein⁻¹)</th>
<th>Lactate/Pyruvate ratio</th>
<th>Calculated NADH/NAD⁺ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.7 ± 0.9 (6)</td>
<td>17.9 ± 2.6 (6)</td>
<td>2.0 ± 0.3 (6)</td>
</tr>
<tr>
<td>R/A (10/5 μg/ml)</td>
<td>2.0 ± 0.8 (6)</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>R/A (10/20 μg/ml)</td>
<td>0.7 ± 0.5 (5)</td>
<td>83.9 ± 3.1 (5)</td>
<td>9.3 ± 0.3 (5)</td>
</tr>
<tr>
<td>KCN (2 mM)</td>
<td>2.7 ± 1.0 (6)</td>
<td>68.7 ± 25.7 (5)</td>
<td>7.6 ± 2.9 (5)</td>
</tr>
<tr>
<td>POCA (25 μM)</td>
<td>3.6 ± 1.0 (2)</td>
<td>22.5 ± 16.7 (4)</td>
<td>2.5 ± 1.9 (4)</td>
</tr>
</tbody>
</table>

Cultured human skin fibroblasts were incubated in Krebs-Henseleit bicarbonate buffer containing 10 μM cerotate and 5 mM glucose for 4 h followed by measurement of cellular ATP and cytosolic lactate and pyruvate. Results are expressed as mean ± S.D. The number of separate experiments is given between parentheses. Abbreviations used: N.D. = not done; R/A = Rotenone/Antimycin.
More importantly, there are considerable conceptual problems associated with the use of inhibitors of respiratory chain activity to assess the contribution of peroxisomes and mitochondria to overall β-oxidation. First, cellular ATP levels can be expected to decrease due to inhibition of oxidative phosphorylation. This was, indeed, found experimentally (Table 2): rotenone plus antimycin at concentrations of 10 and 5 μg/ml decreased cellular ATP levels by about 50% and a similar decrease in ATP levels was observed with 2 mM KCN. Another consequence of the use of inhibitors of respiratory chain activity in intact cells is that the oxidation of mitochondrial and cytosolic NADH might be blocked. This was also observed in the experiments (Table 2): the presence of respiratory chain inhibitors during β-oxidation resulted in an elevation of the lactate/pyruvate ratio, which directly reflects the cytosolic NAD-redox state. The findings described above led us to develop a different approach to block mitochondrial β-oxidation. We selected a powerful inhibitor of CPT I, i.e. POCA, which is taken up by the cell, metabolized to its CoA-ester [Turnbull et al., 1984] and subsequently blocks CPT I irreversibly. Table 2 shows that POCA had little effect on cellular ATP levels and that the cytosolic NAD-redox state was almost unaffected. The effect of increasing concentrations of POCA on the β-oxidation of palmitate and cerotate is shown in Fig. 1.

**Figure 1. Effect of increasing concentrations of POCA on the β-oxidation of palmitate (C16:0) and cerotate (C26:0) in human skin fibroblasts.**

Fibroblasts from control subjects were incubated as described in Materials and Methods and the oxidation of palmitate and cerotate was measured in the presence of different concentrations of POCA as indicated in the figure.

The oxidation of palmitate was progressively inhibited by increasing concentrations of POCA, whereas oxidation of cerotate was not affected (see also Table 3). From these results, we conclude that the initiation of cerotate β-oxidation takes place in peroxisomes rather than in mitochondria.

**DISCUSSION**

Since peroxisomal β-oxidation is not directly linked to respiration, inhibitors of respiratory chain enzymes have been frequently used to block mitochondrial β-oxidation selectively [Lazarow and de Duve, 1976; Lazarow, 1978;
Hashimoto, 1982] in order to distinguish peroxisomal and mitochondrial β-oxidation. However, our experiments show that secondary effects of inhibition of respiration can severely interfere with peroxisomal β-oxidation, which makes it complicated to draw definite conclusions. In view of this, we have used intact cells to elucidate the site of very-long-chain fatty acid oxidation, employing POCA, a specific inhibitor of mitochondrial β-oxidation at the level of CPT I, to discriminate between mitochondrial and peroxisomal β-oxidation. POCA did not affect the cytosolic redox state and we observed no drop in cellular ATP levels (Table 2), in contrast to inhibitors of the respiratory chain. The presence of respiratory chain inhibitors in intact cells does not only stop re-oxidation of mitochondrial NADH and FADH₂ (which will inhibit mitochondrial oxidation), but also leads to lowered cellular ATP levels and an increase in cytosolic NADH/NAD⁺ ratio (Table 2).

Table 3. Effect of POCA on palmitate and cerotate β-oxidation in human skin fibroblasts.

<table>
<thead>
<tr>
<th>Addition to medium</th>
<th>Palmitate (pmol h⁻¹ mg protein⁻¹)</th>
<th>Cerotate (pmol h⁻¹ mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>435 ± 93 (3)</td>
<td>233 ± 40 (3)</td>
</tr>
<tr>
<td>POCA (25 μM)</td>
<td>27 ± 10 (3)</td>
<td>252 ± 59 (3)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.D. with the number of different experiments between parentheses.

A drop in cellular ATP may well contribute to inhibition of peroxisomal β-oxidation, especially since the concentration of ATP required to give half-maximal rates of lignocerate β-oxidation is known to be high (9.6 mM) [Wanders et al., 1987b]. In addition KCN, antimycin and rotenone are well known and often used inhibitors of respiratory chain activity but their site of action might not be restricted to the mitochondrial respiratory chain. It has been suggested [Vamecq et al., 1987] that antimycin is an inhibitor of acyl-CoA oxidase which by itself may cause lowered rates of peroxisomal β-oxidation. Furthermore, cyanide is known to bind NAD⁺ [Metzler, 1977] and to inhibit catalase activity [Nichols and Schonbaum, 1963]. Consequently, accumulation of H₂O₂ within peroxisomes may lead to suppression of peroxisomal β-oxidation [Hashimoto and Hayasi, 1987].

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