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Studies on the Intracellular Localization of Acetyl-CoA Carboxylase

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Since its original discovery as a soluble enzyme (4) AAC has been assumed to be located in the cytoplasm. Earlier work concluded that the activity of the enzyme was not associated with subcellular particles (5), but later reports indicated that activity of ACC could be detected in high speed precipitates of rat-liver homogenates (6-8) and in a so-called mitochondrial fraction of such homogenates (9,10). Furthermore, permeabilization of isolated hepatocytes with digitonin also suggested association of the enzyme with some kind of intracellular structure (11).

The present work was performed to identify the subcellular localization of hepatic acetyl-CoA carboxylase (ACC). Cellular organelles involved in fatty acid oxidation that contain a malonyl-CoA sensitive carnitine palmitoyltransferase (CPT) activity or that are linked to the control of this activity were analysed for the presence of ACC. No significant amount of ACC was observed in the mitochondrial fraction prepared from isolated rat hepatocytes. Furthermore, no association of ACC activity and mass with isolated hepatic peroxisomes could be detected. In cubation of isolated hepatocytes with compounds known to affect the integrity of the cytoskeleton like okadaic acid or taxol indicates that ACC is associated with this subcellular structure of the hepatocyte. Such association may allow for efficient regulation of CPT activity and thus of fatty acid oxidation.

Depending on the physiological state of the animal, the liver is a tissue that can either exhibit high rates of fatty acid biosynthesis or high rates of fatty acid oxidation. Control of the activity of acetyl-CoA carboxylase (ACC) is of special interest in this respect because the product of ACC, malonyl-CoA, is not only a substrate for the cytosolic process of long-chain fatty acid synthesis but is also an inhibitor of the activity of carnitine palmitoyltransferase (CPT), an important rate-limiting enzyme in the process of long-chain fatty acid oxidation (1). As a matter of fact, malonyl-CoA sensitive CPT activity is present both in mitochondrial outer membranes (CPT-I) (2) and in the peroxisomal matrix (3).

Given the functions of malonyl-CoA in or on different organelles of the cell, the enzyme responsible for its production, ACC, may be compartmentalized as well.

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Materials and Methods

Male Wistar rats (250-300 g) which had free access to food and water were used throughout in this study. Hepatocytes were isolated and incubated as described in (18). Acetyl-CoA carboxylase activity, isoflaxin distribution and mass were determined in isolated hepatic mitochondria, isolated hepatic peroxisomes or in digitonin-permeabilized hepatocytes. For isolation of mitochondria from liver tissue, the procedure described by Roman-Lopez et al. (19) was followed. Mitochondria from hepatocytes were isolated by homogenizing cells (4-6 mg of protein) with a loose-fitting Dounce homogenizer in a low
ionic strength, iso-osmotic medium consisting of 0.3 M mannitol and 2 mM Hepes pH 7.4 in the presence of a proteinase inhibitor mixture (17). The homogenate was centrifuged at 1500 × g for 2 min. The pellet was discarded and the supernatant centrifuged for 2 min at 16,000 × g. The resulting supernatant was termed the cytosolic fraction. The pellet was washed once by resuspension in the low ionic strength medium and recentrifugation at 16,000 × g. The resuspended final pellet was termed the mitochondrial fraction. Peroxisomes were isolated from animals fed a standard pelleted diet supplemented with 1% dl(2-ethylhexyl)phthalate in order to proliferate the peroxisomal compartment (20). Treatment of animals and isolation of peroxisomes was performed as in (20). All homogenizing steps and subsequent procedures were performed at 4° C. The isolated fractions were snap frozen in liquid nitrogen and stored at -20° C. To determine the amount of ACC retained in the cell ghosts following incubation of the intact hepatocytes with different cellular effectors, the isolated cells were permeabilized and thoroughly washed prior to harvesting the cell ghosts. Thus, 1.0 ml of hepatocyte suspension was permeabilized with digitonin (ca. 60 μg per mg cell protein) dissolved in a medium containing 50 mM Hepes (pH 7.5), 0.25 M mannitol, 5 mM 2-mercaptoethanol and a proteinase inhibitor mixture as in (17). The resulting mix of cells and permeabilizing medium was gently shaken for 5 s. and rapidly diluted by transfer to tubes containing 40 ml of ice-cold medium without digitonin. Cell ghosts were sedimented by centrifugation at 350 g for 15 s. and pellets were taken up in 1.0 ml ice-cold medium containing 50 μl of the proteinase inhibitor mixture. The resulting suspensions of cell ghosts were kept at -80° C till mass measurements were performed. Determination of total ACC mass retained in cell ghosts following digitonin treatment or associated either with isolated mitochondria or peroxisomes, was performed with an ELISA assay essentially as described by Iversen et al. (21) employing a primary antiserum against rat liver ACC (17). The distribution of isoforms was determined using immunoprecipitation, SDS/PAGE, immunoblotting and autoradiography as in (17). Activities of ACC and fatty acid synthase were monitored exactly as described before (15,17). Sources of chemicals as in (17).

RESULTS

The present study was undertaken to identify the subcellular localization of ACC. In order to investigate a possible association of ACC with mitochondria—as suggested by Allred and co-workers (9,10)—mitochondria were prepared from isolated hepatocytes. With such a preparation less mechanical force is required to liberate mitochondria as compared to whole tissue as the starring material. Mechanical interference with an association of ACC and mitochondria was further kept to a minimum by using a loose-fitting Dounce homogenizer. To interfere as little as possible with potential electrostatic interactions between enzyme and organelle, a low ionic strength medium was chosen for isolation of the subcellular fractions. In addition, the whole isolation procedure—including subcellular fractionation by centrifugation—was aimed at speed rather than at recovery. Activity measurements for ACC of the resulting subcellular fractions could not be performed because of the presence of malonyl-CoA decarboxylase in mitochondria. The latter enzyme interferes with the assay for ACC activity (15). The subcellular fractions were analysed by SDS gel electrophoresis for the presence of the 265-kDa and 280-kDa isoforms of ACC (ACC-265 and ACC-280, respectively). The two isoforms have been suggested to play different biological roles. In particular, ACC-280 is of interest in this respect because it might be involved in the synthesis of malonyl-CoA for inhibition of CPT-I (22,23). Since the latter enzyme has a mitochondrial localization (24), an association of ACC-265 with mitochondria would be feasible. Fig. 1 shows the analysis of the presence of ACC isoforms in the subcellular fractions from isolated hepatocytes. The results do not reveal a mitochondrial localization of either of the two ACC isoforms. In an attempt to spare potential hydrophobic interactions between ACC and mitochondria, the latter organelles were also prepared from isolated hepatocytes in a high ionic strength medium (0.9% NaCl and 20 mM Tris pH 7.4) exactly as described for the low ionic strength medium. Also in this case no association of any of the ACC-isoforms with the mitochondrial fraction was apparent (data not shown). The analysis of the presence of ACC-isoforms in the mitochondrial fraction was repeated with two different primary antisera also raised against rat-liver ACC. The results were identical to those presented in Fig. 1. (data not shown). The analysis of the presence of ACC isoforms was also performed on subcellular fractions of rat-liver cells from animals in different nutritional states, i.e. starved for 48 h and starved for 48 h followed by refeeding a carbohydrate-rich, fat-poor diet for 48 h. No significant mass of either ACC isoform was observed in the mitochondrial fraction in any of the nutritional states (data not shown).
Hepatic peroxisomes exhibit CPT activity that is sensitive to inhibition by malonyl-CoA (3, 25). To study a possible association of ACC with this organelle, peroxisomes were isolated from liver tissue obtained from rats treated with di(2-ethylhexyl)phthalate (DEHP), a proliferator of the peroxisomal compartment (20). The rats treated with di(2-ethylhexyl)phthalate (DEHP)-Treated Rats resulted in the release of substantially more ACC than in control cells. Interestingly, the OA-induced effect was completely abolished by pretreating the hepatocytes with taxol (Table 2). Likewise, pretreatment of the cells with KN-62, a specific inhibitor of Ca\(^{2+}\)/calmodulin-dependent protein kinase II, also prevented the OA-induced release of hepatic ACC. 5-Aminooimidazole-4-carboxamide ribonucleoside (AICAR), a specific activator of 5'-AMP-activated protein kinase, was without effect on the retention of ACC mass in cell ghosts (Table 2). Incubation of hepatocytes with colchicine did not affect the release of ACC from digitonin-permeabilized cells either. The data obtained with amyloglucosidase (Table 2) suggest that glycogen granules represent a subcellular structure capable of binding ACC. To determine whether the two ACC isoforms will differentially release upon permeabilization, the cell ghosts were also analysed for the presence of ACC-265 and ACC-280. However, irrespective of the incubation condition, the ratio of ACC-265/ACC-280 was identical, i.e. ACC-280 was always about one third of the total ACC mass (data not shown).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control</th>
<th>DEHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>0.83</td>
<td>0.97</td>
</tr>
<tr>
<td>PGI</td>
<td>0.55</td>
<td>0.39</td>
</tr>
<tr>
<td>FAS</td>
<td>1.88 ± 0.11</td>
<td>1.14 ± 0.03</td>
</tr>
<tr>
<td>ACC</td>
<td>0.34 ± 0.03</td>
<td>0.42 ± 0.01</td>
</tr>
</tbody>
</table>

Note. PGI and catalase are marker enzymes for cytosol and peroxisomes, respectively. Data represent means of two control and twode(2-ethylhexyl)phthalate (DEHP)-treated animals. n.d., nondetectable.

### DISCUSSION

Several studies performed by others (6-10) as well as by our group (11) led to the suggestion of association of ACC with this organelle, peroxisome, and in isolated peroxisomes from liver tissue obtained from rats treated with di(2-ethylhexyl)phthalate (DEHP), a proliferator of the peroxisomal compartment (20). The data in Table 1 indicate that there is no association of ACC activity with peroxisomes from both control and DEHP-treated animals. Likewise, no significant association of ACC to peroxisomes was observed on the basis of measurements of enzyme mass (data not shown).

**TABLE 2**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Percentage of total ACC mass retained in cell ghosts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>53.2 ± 18.0</td>
</tr>
<tr>
<td>OA</td>
<td>20.6 ± 8.0*</td>
</tr>
<tr>
<td>Taxol + OA</td>
<td>55.5 ± 6.6</td>
</tr>
<tr>
<td>KN-62 + OA</td>
<td>48.5 ± 10.5</td>
</tr>
<tr>
<td>AICAR</td>
<td>46.0 ± 19.5</td>
</tr>
<tr>
<td>Colchicine</td>
<td>64.5 ± 8.7</td>
</tr>
<tr>
<td>Taxol + colchicine</td>
<td>67.9 ± 1.0</td>
</tr>
<tr>
<td>Control*</td>
<td>15.8 ± 2.8</td>
</tr>
<tr>
<td>Control + amyloglucosidase*</td>
<td>4.6 ± 1.0*</td>
</tr>
</tbody>
</table>

Note. Hepatocytes were preincubated for 20 min with or without 10 μM taxol or 30 μM KN-62. Incubations were continued for 15 additional min with or without 0.5 mM 5-aminooimidazole-4-carboxamide ribonucleoside (AICAR) or 0.5 mM of colchicine. Subsequently, cell ghosts were prepared as described in Materials and Methods. ACC retained in the cell ghosts was quantified by avidin-based ELISA analysis using as the probing antibody a primary antiserum against rat-liver ACC (17). These results are from two sets of cell ghosts of control cells resuspended and incubated for 15 additional min with or without 50 U amyloglucosidase. Results represent the mean ± S.D. of 3 different hepatocyte preparations. The amount of ACC present in intact hepatocytes was set at 100%. Values of OA are significantly different (P < 0.01) versus its control using the Student's t test. This also applies to amyloglucosidase (P < 0.01) versus its control.
ACC to a subcellular organelle. However, after careful isolation, no association of ACC with either mitochondria or peroxisomes could be observed in the present study. Nonetheless, in the intact cell an association may exist that is not firm so that the enzyme can escape into the supernatant during the biochemical preparation procedure. The present approach of using the technique of permeabilizing isolated hepatocytes rather than homogenizing the cells revealed an association of ACC with the cytoskeleton. The implication of this cytoskeletal connection of both ACC (this study) and CPT-I (26) is that it allows for an efficient regulation of fatty acid oxidation through malonyl-CoA-induced changes in CPT-I activity. Furthermore, the different digitonin-release-pattern of ACC following incubation of hepatocytes with OA as compared to the control situation raises the possibility that ACC may translocate from one compartment to another, depending on the situation of the cell, and thus efficiently control the activity of CPT-I.

It could be argued that the release pattern of ACC following digitonin permeabilization is a reflection of its state of aggregation and that OA by favoring the monomeric state of the enzyme would cause less ACC to be retained by the cell ghosts. However, the identical ACC release pattern following incubation of hepatocytes with insulin or glucagon—putative effectors of the aggregation state of ACC (30)—is not in line with this reasoning (unpublished data of the authors). The inability of AICAR to alter ACC retention by cell ghosts may also indicate that there is no relation between phosphorylation—at least by 5'-AMP-activated protein kinase—and retention of the enzyme by cell ghosts.

The antagonistic effect of KN-62 and taxol on OA-induced release of ACC is quite similar to the antagonistic effect of the two former compounds on the OA-induced stimulation of CPT-I activity (26,31). Data of KN-62 are of special interest since this compound also prevents quite effectively the OA-induced inhibition of ACC activity as measured in a permeabilized-cell assay (unpublished data of the authors). This implicates Ca²⁺/calmodulin-dependent protein kinase II in the control of both ACC and CPT-I and is at odds with the opinion that 5'-AMP-activated protein kinase is the most important—if not unique—protein kinase involved in the control of ACC in intact hepatocytes (32). In addition, phosphorylation of cytoskeletal components—as shown by the effects of KN-62 and OA—may be required for ACC to be released from its anchoring place on the cytoskeleton. Interestingly, colchicine was without effect just like in the case of CPT-I activity (26) suggesting that specific protein-protein interactions between the two enzymes and cytoskeletal components and not the mere disruption of the cytoskeleton may be involved in the intracellular behavior of the two enzymes and their control.

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