Liver coenzyme A ester content: comparison between Reye's syndrome and control subjects

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Summary

1. The concentrations of the acid soluble and insoluble coenzyme A (CoA) esters were measured in samples of liver obtained at autopsy from Reye's syndrome and control subjects because the long chain fatty CoA compounds which make up the bulk of the acid insoluble CoA esters are known to inhibit a number of mitochondrialy located enzymes, several of which may be affected in Reye's syndrome.

2. Concentrations of the acid insoluble esters varied widely in both control and Reye's liver samples. The difference between the mean values was not statistically significant (1.06 ± SEM 0.33 nmol/g wet weight in Reye's samples vs 0.88 ± 0.21 in control samples).

3. Concentrations of the acid soluble CoA esters, which include the short chain fatty CoA compounds, were higher in Reye's liver samples than in samples from controls. The mean value for Reye's samples was 104.8 ± SEM 29.4 nmol/g of liver compared with 26.4 ± 10.1 nmol/g for control samples (P < 0.05).

4. Studies with rats designed to assess post-mortem change indicate that the liver concentration of the acid insoluble CoA compounds does not change during a 4 h period at 4°C. This finding suggests that the observations made in Reye's liver was probably due to a premorbid abnormality.

5. These findings implicate a block in the β-oxidation of fatty acids and could account for the reported relative increase in the concentrations of the short to medium chain fatty acids in the plasma of Reye's syndrome patients.

Key words: coenzyme A esters, liver, Reye's syndrome.

Introduction

Reye's syndrome is a disorder of unknown etiology characterized by the development of a fatty liver and a rapidly progressive encephalopathy in children recovering from such viral infections as influenza and chicken pox [1, 2]. By morphological and biochemical criteria, the mitochondria of the liver are severely affected [3, 4].

The plasma free fatty acids are increased in affected patients [5, 6] and it is well known that the activated form of the long chain fatty acids in the liver, the coenzyme A (CoA) esters, exert inhibitory effects on mitochondrial function [7, 8]. To explore the possibility of a deleterious role for such compounds in this disorder, the concentrations of the CoA esters were measured in liver samples at autopsy from Reye's and control subjects.

Materials and methods

Clinical. Samples of human liver were collected at autopsy and kept at −70°C until analysis. Summaries of the pertinent clinical features of the subjects from whom samples were obtained are presented in Tables 1 and 2. The diagnosis of Reye's syndrome was confirmed by post-mortem examination. Some of the clinical conditions diagnosed in control subjects are known to affect
TABLE 1. Pertinent clinical data of Reye's subjects

<table>
<thead>
<tr>
<th>Reye's patient no.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Stage at diagnosis*</th>
<th>Serum glutamate-oxaloacetate transaminase (units/ml)</th>
<th>Glucose (mg/dl)</th>
<th>Ammonia (μg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4/12</td>
<td>F</td>
<td>IV</td>
<td>680</td>
<td>540</td>
<td>Not done</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>IV</td>
<td>144</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>M</td>
<td>I†</td>
<td>688</td>
<td>72</td>
<td>200</td>
</tr>
<tr>
<td>4</td>
<td>7/12</td>
<td>F</td>
<td>IV</td>
<td>240</td>
<td>16</td>
<td>653</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>F</td>
<td>III</td>
<td>190</td>
<td>103</td>
<td>198</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>F</td>
<td>III/IV</td>
<td>2184</td>
<td>23</td>
<td>Not done</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>M</td>
<td>III</td>
<td>188</td>
<td>140</td>
<td>550</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>M</td>
<td>III/IV</td>
<td>325</td>
<td>300</td>
<td>Not done</td>
</tr>
<tr>
<td>9</td>
<td>7</td>
<td>F</td>
<td>II/III</td>
<td>430</td>
<td>57</td>
<td>576</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>M</td>
<td>III/IV</td>
<td>340</td>
<td>154</td>
<td>Not done</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
<td>M</td>
<td>III/IV</td>
<td>540</td>
<td>20</td>
<td>980</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>M</td>
<td>III</td>
<td>217</td>
<td>149‡</td>
<td>560</td>
</tr>
<tr>
<td>13</td>
<td>16</td>
<td>M</td>
<td>II/III</td>
<td>160</td>
<td>284‡</td>
<td>370</td>
</tr>
</tbody>
</table>

Mean = 7 years
Normal values: 4-40, 60/105, 18/48

* Staging method according to Lovejoy et al. [36].
† Progressed from stage I–IV within hours.
‡ On 5% glucose intravenously when sample was taken.

TABLE 2. Pertinent clinical data of control subjects

<table>
<thead>
<tr>
<th>Control patient no.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>F</td>
<td>Hypertension, cardiovascular disease</td>
</tr>
<tr>
<td>2</td>
<td>10/12</td>
<td>F</td>
<td>Congenital heart disease</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>M</td>
<td>Chronic renal disease</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>M</td>
<td>Congenital heart disease, failure</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>F</td>
<td>Acute cerebral concussion, on respirator for 3 days</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>F</td>
<td>Post-craniopharyngioma/ventriculography</td>
</tr>
<tr>
<td>7</td>
<td>6/12</td>
<td>F</td>
<td>Viral myocarditis</td>
</tr>
<tr>
<td>8</td>
<td>10/12</td>
<td>F</td>
<td>Sickle cell crisis</td>
</tr>
<tr>
<td>9</td>
<td>2/12</td>
<td>M</td>
<td>Congenital heart disease</td>
</tr>
<tr>
<td>10</td>
<td>1/12</td>
<td>F</td>
<td>Congenital heart disease</td>
</tr>
<tr>
<td>11</td>
<td>Foetus</td>
<td>M</td>
<td>Spontaneously aborted</td>
</tr>
</tbody>
</table>

Liver function. All samples were collected within 8 h of death. Most were collected between 3 and 4 h after death. Thirteen Reye's and 11 control samples were analysed. The mean age for the Reye's patients was 7-0 years, and was 7-3 years for the control patients excluding the foetus. The interval between the diagnosis of Reye's syndrome and death was 4-4 days, with a range of 0.5-10 days. This study was approved by the Institutional Review Board.

Liver samples were also obtained from male Sprague-Dawley rats between 100 and 120 g in weight and allowed free access to laboratory chow and water. Rats were killed by cervical dislocation, except where indicated below when intraperitoneal Nembutal was used for anaesthesia before clamping of the visceral attachments of the liver.

Laboratory. Liver specimens were cut from frozen liver samples, weighed, minced while semi-frozen and homogenized in 3-5 vol. of 8% (v/v) perchloric acid in 40% ethanol at 4°C. The method used to separate the long chain versus the short chain CoA esters was based on the insolubility of the latter in such solutions [9]. Corroboration was by the recovery of 92, 99 and 98% of added [3H]acetyl CoA in the supernatant fraction in human control liver homogenates and the nearly 95% recovery of [14C]palmitoyl CoA in the precipitate. Liver samples weighed between 0.5-1 and 0.0 g. After centrifugation at 25 000 g for 10 min, the precipitate was washed successively with 2-5 vol. of 5% HClO₄, 0-6% HClO₄ and water followed by centrifugation and recovery of the pellet. The pellets were kept frozen until further analysis.
For analysis of the acid insoluble CoA esters, 2 vol. of dithiothreitol (10 mmol/l) was added to the pellet and the pH was raised to 12 with KOH solution (1 mol/l), followed by incubation at 55°C for 10 min. The pH was promptly reduced to 5 with 5% HClO₄-triethanolamine/HCl (0.5 mol/l) before centrifugation at 25,000 g for 10 min. The supernatant fraction was analysed for CoA by the method of Allred & Guy [10], after neutralization by measuring the conversion of NAD into NADH through the sequential use of malate dehydrogenase, citrate synthase and phosphotransacetylase at 340 nm. The commercially obtained citrate synthase, phosphotransacetylase and malate dehydrogenase used in this method were purchased from Boehringer–Mannheim and were extensively dialysed with Tris (1 mol/l), pH 7.20, to rid them of contaminating CoA before use. Standard curves of absorbance readings were generated over a range of CoA concentrations from 1 to 128 pmol/l for determination of the unknowns. The method allowed the detection of 0.5 nmol of CoA/ml. Duplicate samples varied within experimental error.

For the acid soluble CoA assays, liver was homogenized in 2 vol. of Tris buffer (1 mol/l), pH 7.2, at 4°C. One-half volume of 16% HClO₄ was then added and the preparation was spun at 25,000 g for 10 min. An aliquot of the supernatant fraction was made alkaline (to pH 12) with KOH solution (1 mol/l), followed by incubation at 50°C for 35 min. The pH was promptly reduced to 5. The CoA content was then determined in both the alkaline-hydrolysed and neutralized sample and the untreated, unhydrolysed sample by the method of Allred & Guy. The treated sample represented the CoA released from ester linkages and was based on the linear portion of a standard curve constructed with hexanoyl CoA in Tris (1 mol/l) subjected to alkaline hydrolysis for 35 min at 50°C, followed by acidification to pH 5.0 and analysed by the method of Allred & Guy. This curve was linear for concentrations of hexanoyl CoA from 0.5 to 10 μmol/l. The untreated sample represented the free CoA present in the sample and was analysed by the Allred & Guy method with CoA as a standard.

Protein was determined by the method of Lowry with bovine serum albumin as a standard. Because of interference with this method by Tris, protein concentration was determined from a separate sample of liver, which was homogenized in water [11].

Results

Autopsied liver from Reye's syndrome and control subjects contained 1.06 ± SEM 0.33 and 0.88 ± 0.21 nmol of acid insoluble CoA compounds/g wet weight respectively. Variation was present in both groups and the mean values were not significantly different (Table 3).

Supernatant fractions of liver homogenates acidified with 0.5 vol. of 16% HClO₄ contained large amounts of free CoA. This free CoA was readily destroyed with alkali at pH 12. Within a 4 min period at 50°C, all of the free CoA is destroyed, as reflected by the failure to convert NAD into NADH in neutralized aliquots of such treated samples. Serial analysis of supernatant fractions of acidified rat liver homogenates exposed to alkali at 50°C at 4 min intervals gave tracings with no detectable CoA after 4–8 min followed by the reappearance of CoA by 28–32 min, presumably as a result of CoA hydrolysed from ester linkages. After 60 min, no CoA is

Table 3. Coenzyme A and CoA ester content of liver at autopsy from Reye's and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>CoA from acid-insoluble fraction after hydrolysis</th>
<th>CoA from acid-soluble fraction after hydrolysis</th>
<th>Free CoA plus acetyl-CoA (nmol/g of liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nmol/g of liver</td>
<td>nmol/g of protein</td>
<td></td>
</tr>
<tr>
<td>Reye's</td>
<td>Mean</td>
<td>1.06</td>
<td>10-89</td>
<td>104-8</td>
</tr>
<tr>
<td></td>
<td>± SEM</td>
<td>0.33</td>
<td>3-34</td>
<td>29.4</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>11</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>Mean</td>
<td>0.88</td>
<td>14-09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.21</td>
<td>4-23</td>
<td>26.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>P values</td>
<td>N.S.</td>
<td>N.S.</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
</tbody>
</table>

N.S., Not significant.
detectable, probably as a result of the complete destruction of the newly released CoA (Table 4). Comparable findings were also noted in several human samples examined.

Every Reye's sample had measurable quantities of acid soluble CoA compounds whereas two of six control samples had no detectable amounts. The mean (±SEM) values found in both groups were 104.8 ± 29.4 nmol/g wet weight for Reye's samples compared with 26.4 ± 10.1 for control samples. The difference between the mean values was significant (P < 0.05) (see Table 3).

The free CoA and acetyl CoA content of the liver homogenates was similar in both groups (mean ± SEM values were 7.2 ± 1.5 vs 8.4 ± 2.5 nmol/g wet weight for Reye's and control samples respectively; see Table 3).

Liver samples were also examined for sampling variability, which might contribute to these findings. In one control and one Reye's liver where the peripheral and proximal margins could be identified, identically-sized samples were removed from three sites, the proximal, middle and peripheral regions, for analysis as described above. Results presented in Table 5 indicate that a 40% difference in content could result from sampling bias for the acid insoluble CoA compounds. Such variability was not as pronounced for the acid soluble CoA compounds (30%). These findings indicate that it is unlikely that the higher tissue content of the acid soluble CoA compounds seen in Reye's liver samples could be entirely due to sampling bias, which consistently selected areas of higher concentration only in Reye's tissues in contrast to control samples.

Control samples had higher protein concentrations per unit weight of tissue compared with Reye's samples. When the CoA ester content was compared on a protein basis, the difference between Reye's and control samples was further accentuated with respect to the acid soluble CoA compounds (Table 3). The mean protein concentration in Reye's samples was 85.6 ± 3.9 mg/g of tissue compared with 123 ± 18.7 in control samples (P < 0.05).

To explore the possibility that differences were the result of post-mortem changes rather than due to premorbid conditions, the following studies were done: a Sprague-Dawley rat was anaesthetized with intraperitoneal Nembutal, after which the liver was clamped at the vascular and ligamentous attachments with clamp A. Another clamp (B) was used to isolate the right lobe. A third clamp (C) was placed parallel to clamp B and the right lobe was removed by dividing the tissues between clamps B and C. The anaesthetized animal, with clamp A in place, was then placed in the refrigerator (4°C) before removal of the rest of the liver 4 h later. The liver was removed attached to clamp A. Both samples of the liver were frozen at -20°C within minutes of removal from the animal. Each sample was processed and analysed for the acid insoluble CoA ester content as described above. The concentration was 0.231 nmol/mg of protein for the 0 h sample and 0.235 nmol/mg of protein for the 4 h sample. These findings indicate that no significant change in the concentration of the long chain fatty CoA esters occurs 4 h after death when the carcass is kept at 4°C.

**Table 4. Effect of duration of exposure to alkali on CoA concentration of liver preparations**

<table>
<thead>
<tr>
<th>Duration of alkaline hydrolysis (min)</th>
<th>Conc. of CoA (nmol/g wet wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.22</td>
</tr>
<tr>
<td>4</td>
<td>33.83</td>
</tr>
<tr>
<td>8</td>
<td>16.70</td>
</tr>
<tr>
<td>24</td>
<td>11.14</td>
</tr>
<tr>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>36</td>
<td>7.71</td>
</tr>
<tr>
<td>48</td>
<td>22.27</td>
</tr>
<tr>
<td>60</td>
<td>11.93</td>
</tr>
<tr>
<td>64</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 5. Sampling variation in CoA ester content of liver**

All samples were 1-2 g.

<table>
<thead>
<tr>
<th>Sample site</th>
<th>Liver content (nmol/g wet wt.)</th>
<th>Reye's syndrome</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Long chain CoA</td>
<td>Short chain CoA</td>
<td>Long chain CoA</td>
</tr>
<tr>
<td>Proximal</td>
<td>2.83</td>
<td>5.22</td>
<td>0.47</td>
</tr>
<tr>
<td>Middle</td>
<td>1.67</td>
<td>5.23</td>
<td>0.56</td>
</tr>
<tr>
<td>Peripheral</td>
<td>2.58</td>
<td>7.50</td>
<td>0.53</td>
</tr>
</tbody>
</table>

**Discussion**

Fatty acyl CoA compounds are intermediates for both the catabolic as well as the biosynthetic processing of lipids from fatty acids. In the liver, the transport of the long chain fatty CoA compounds into the mitochondria for β-oxidation requires carnitine as a carrier [12, 13]. Carnitine levels have been found to be normal in the plasma in Reye's syndrome [14]. Within the mitochondria, long chain fatty acyl CoA esters are
potent inhibitors of the adenine nucleotide trans-
locase and the tricarboxylic acid carrier systems
[8, 12, 15–18]. At low concentrations, long chain
fatty CoA esters compete and efficiently inhibit
ADP/ATP transport from both sides of the
mitochondrial membrane [19]. The interference
with oxidative phosphorylation seen in starvation,
diabetes and hibernation, which correlate with
changes in high energy phosphate metabolism, is
believed to be due to the effects of the ac-
cumulated long chain fatty CoA esters in the
mitochondria [20]. We had expected the poss-
ibility of an accumulation of the long chain fatty
CoA esters, as reflected by increase in the acid
insoluble CoA compounds in Reye’s liver sam-
plies, which could be incriminated as the toxin
responsible for the morphological and bio-
chemical abnormalities of the mitochondria
which are so prominent in this disorder. Instead,
no significant difference in the total content of
these compounds was found between Reye’s and
control samples.

On the other hand, a striking accumulation of
the acid soluble CoA esters was found in Reye’s
samples. This class of esters includes the short
chain fatty CoA compounds. On a molar basis,
these esters are present at concentrations nearly
doubling those in control samples. In normal
liver, β-oxidation in the mitochondria is complete
with the C2 fragment entering the citric acid cycle
for the even-carbon numbered fatty acids and the
C3 fragment entering the propionic acid pathway
for the odd-carbon numbered fatty acids [21].
The accumulation of the acid soluble CoA
compounds, which include the short chain fatty
CoA esters in the liver, is indicative of a block in
the further oxidation of the long chain fatty acids
in the mitochondria. These findings could ac-
count for the presence of a relative increase in the
proportions of the medium to short chain fatty
acids observed in the plasmas of many Reye’s
patients [5, 6, 22]. Increases in the concentra-
tions of short chain fatty CoA compounds in the
liver presumably occur in such genetically
determined disorders as propionic acidemia and
methylmalonic acidemia [23, 24]. Reports of
quantitative measurements of the CoA esters of
the compounds involved have not yet come to
our attention, but the CoA esters are believed to
be responsible for the toxic effects of these
metabolic conditions for the following reasons:
the free acid in methylmalonic acidemia was
without clinical effects when administered to
animals, and the administration of valine, a
precursor of methylmalonic acid, to a vitamin
B12-deficient pig resulted in toxicity (vitamin B12
is a cofactor for the enzyme involved in methyl-
malonic acidemia) [25, 26]. Interestingly,
evidence of mitochondrial dysfunction as re-
lected by interference with urea synthesis is a
feature common to Reye’s syndrome as well as to
patients with propionic acidemia and methyl-
malonic acidemia [23, 24].

Fatty acyl CoA compounds of long carbon
chain lengths exert negative effects on the
catalytic rates of a number of enzymes [27, 28].
Detergent-like physical properties have been
demonstrated for these compounds [28] and
critical concentrations for micelle formation
appear to occur within the concentration ranges
which inhibit several of these enzymes [28].
Several groups have, however, shown that the
effects of fatty acyl CoA on enzymes may also be
specific [27, 29]. It is possible that these
compounds found in Reye’s samples may not be
detrimental to the catalytic activities of enzymes
by detergent-like mechanisms since the CoA
esters of fatty acids of shorter chain lengths than
ten carbon atoms are less deleterious. However,
specific effects of such esters may still occur.
Work is currently in progress to identify the chain
lengths of these compounds. It also remains to be
shown whether the accumulated short chain
fatty CoA esters are limited to the mitochondria
or are also present outside the mitochondria.
Lazarow & de Duve [30] demonstrated the
capability of rat liver peroxisomes to oxidize long
chain fatty CoA esters by β-oxidation. Unlike the
mitochondrial system peroxisomal β-oxidation is
not coupled to phosphorylation. Like the mito-
chondrial system, acetyl CoA is generated
through successive steps by dehydration, hyd-
rination, dehydrogenation and thiolytic cleavage
[30]. Whereas mitochondrial β-oxidation is nor-
mally complete, peroxisomal β-oxidation is not.
Only five acetyl CoA equivalents were obtained
per equivalent of palmitoyl-CoA by the peroxi-
somal system. Furthermore, the isolated peroxi-
somes failed to oxidize the CoA esters of short
chain fatty acids [31]. Although the relative rate
of the peroxisomal oxidation of fatty acids was
found to proceed at only 10% of the mito-
chondrial rate [32], this system may be important
in Reye’s syndrome because of the significant
increase in the numbers of these microbodies
observed in liver biopsies [33, 34]. It may thus be
important to examine the subcellular distribution
of these CoA esters of the fatty acids in the livers
of affected subjects.

In conclusion, our findings indicate that an
accumulation of the acid soluble CoA esters,
probably largely due to medium and short chain
fatty acyl CoA compounds, occurs in the liver of
Reye’s syndrome patients. This finding implicates
a block in the β-oxidation of the fatty acids. It might be expected that the shorter chain fatty acids would be found in the plasma compartment since their transport out of the mitochondria does not require the carnitine carrier [35]. The possibility exists that the peroxisomes, which are increased in number in Reye's syndrome livers, may also play a role in the accumulation of these esters. The impact of these accumulations on enzymes and membranes could contribute to the pathophysiology of this disease.

Acknowledgments

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References