The Effect of Ethyl Pyruvate Supplementation on Rat Fatty Liver Induced by a High-Fat Diet

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Summary Continuous positive energy imbalance leads to obesity, which increases the risk of developing non-alcoholic fatty liver disease. The hepatoprotective effect of ethyl pyruvate has been revealed in several studies. Therefore, we examined the effect of ethyl pyruvate supplementation on liver cell damage, metabolism, membrane fluidity, and oxidative stress markers in rats fed a high-fat diet. After 6-wk feeding of a control or high-fat diet, Wistar rats were divided into 4 groups: control diet, control diet and ethyl pyruvate, high-fat diet, and high-fat diet and ethyl pyruvate. Ethyl pyruvate was administered as a 0.3% solution in drinking water, for the following 6 wk. Ethyl pyruvate intake attenuated the increase in activities of plasma transaminases and liver TNF-α. However, the supplementation was without effect in the lipid profiles, membrane fluidity or oxidative metabolism in liver induced by the high-fat diet. Our data confirm the potency of ethyl pyruvate against cell liver damage. Nevertheless, prolonged intake did not affect the development of a fatty liver.

Key Words fatty liver, alanine transaminase, aspartate transaminase, tumor necrosis factor α, oxidative stress

Prolonged energy imbalance, caused by an increased intake of high-energy food and decreased physical activity, has led to a global epidemic of obesity. Higher prevalence of obesity and related metabolic disorders contribute to an elevated risk for developing non-alcoholic fatty liver disease (NAFLD). Although the liver normally presents a great metabolic flexibility in order to maintain energy homeostasis, chronic intake of a high-fat diet (HFD) can progressively deregulate this feature (1). Accelerated fatty acid oxidation at the beginning of HFD is subsequently decreased after prolonged HFD treatment (1). Reduced mitochondrial oxidative capacity consequently provokes cytosolic fatty acid accumulation (2). Fatty liver is accompanied by inflammation and oxidative stress (3).

Pyruvate has been used for enhancing mitochondrial function, through either increased metabolite supply (4), or increased mitochondrial biogenesis (5). Since pyruvate may transform into parapyruvate—an inhibitor of tricarboxylic acid cycle (6)—ethyl pyruvate (EtP)—a more stable compound—has been formed. Moreover, EtP is an effective antioxidant and anti-inflammatory agent (7). Acute treatment with EtP ameliorates hepato-cellular injury in mice subjected to alcohol intoxication (8), can reduce liver ischemia-reperfusion damage (9), attenuates the inflammatory response, and decreases hepatic destruction secondary to severe acute pancreatitis (10). Chronic administration of EtP for 10–14 d has been shown to decrease the number of liver tumors in a murine model of metastatic cancer (11). Moreover, studies on rats have demonstrated that 10 wk of EtP administration protects the DNA fragmentation of leukocytes (12) and oxidative damage in membranes (13) induced by chronic alcohol intake.

Therefore, in the present study we examined the effect of EtP supplementation on fatty liver induced by a HFD in rats.

METHODS AND MATERIALS

Animals and diets. Thirty-two male Wistar rats at the age of 7 wk were obtained from the Center of Experimental Medicine at the Medical University of Białystok (Poland). The animal room was maintained at 22±1°C with a 12-h light and 12-h dark cycle. After a 1-wk familiarization period, the rats were divided randomly into 2 groups.

The control group (n=16; 201±4 g) was fed a standard maintenance diet containing 3.06 kcal/g metabolizable energy, with 9% of its energy from fat, 33% from protein, and 58% from carbohydrates (V1534-000 ssniff R/M-H, ssniff Spezialdiäten GmbH, Soest, Germany). The diet group (n=16; 201±3 g) was fed a HFD composed as previously described (14). The HFD contained 4.66 kcal/g metabolizable energy, with 45% of
its energy from fat, 17% from protein, and 38% from carbohydrates (ssniff Spezialdiäten GmbH). The fat was derived from lard (31%), peanut oil (7%), and canola seed oil (7%); carbohydrates from cornstarch (26%) and sucrose (12%). Animals had free access to food and water and were kept at room temperature with a light-dark cycle of 12 h. Since in the previous study mitochondrial respiratory activity was reported higher than for the control after 4 wk of the HFD, and decreased below control values after 8 wk of the HFD (1), we have decided to include EtP treatment after 6 wk of controlled feeding. Both groups were then subdivided into 4 groups: control diet (CC; n=8), control diet and EtP (CP; n=8), HFD (DC; n=8), HFD and EtP (DP; n=8), and EtP was administered for the following 6 wk as 0.3% EtP solution in drinking water (12). During this period, body weight and food intake were evaluated weekly. At the end of the 12th week, the rats were sacrificed and tissue samples were collected. The excised livers were immediately frozen in liquid nitrogen and stored at −70°C until analysis. All procedures were approved by the Local Animal Ethics Committee (Consent No. 47/2006) and performed in accordance with guidelines for animal care.

Plasma biochemical analyses. The blood was centrifuged at 2,000 × g for 10 min at 4°C, and separated plasma samples were frozen at −70°C for later analyses. Plasma total cholesterol (TC), triglycerides (TG), and free fatty acids (FFA) were measured enzymatically with commercial assay kits (Randox Laboratories Ltd., Crumlin, UK). Alanine transaminase (ALT) and aspartate transaminase (AST) activities were measured using a commercially available kits (Emapol LLC. Gdansk, Poland).

Liver lipid extraction. Fragments of liver were used for lipid extraction with a chloroform:methanol ratio (2 : 1, v/v) as described by Folch et al. (15). TG, FFA, and TC concentrations were measured in extracts using test kits (Randox Laboratories Ltd.).

Membrane fluidity measurement. Membrane fluidity was measured as previously described (13). Samples were normalized to a protein concentration of 0.4 mg/mL using the Lowry et al. method (16). The membrane fluidity was measured using the fluorescence technique and two different probes. Laurdan and DPH. The fluorescent probes were added directly to the liver homogenates without isolation of plasma membrane (isolation of the membranes is unnecessary because the employed probes localize only at the membrane level).

The assay was carried out on a 1 mL sample containing the probe (Laurdan or DPH) at a final concentration of 10−6 M. The Hitachi 4500 spectrophotometer (Hitachi Ltd., Tokyo, Japan) was used for fluorescence measurements. The generalized polarization of Laurdan (GP450) was calculated according to Parasassi’s equation (17):

\[ GP_{450} = \frac{(I_R-I_G)}{(I_R+I_G)} \]

where \( I_R \) and \( I_G \) are the intensities at the blue (440 nm) and red (490 nm) edges of the emission spectrum, and correspond to the fluorescence emission maximum in the gel and liquid-crystalline phases of the bilayer, respectively (18). The steady-state fluorescence anisotropy (r) of DPH was calculated using excitation and emission wavelengths of 360 and 430 nm, respectively, according to equation (19):

\[ r = \frac{I_{||}-I_{||g}}{I_{||}+2I_{||g}} \]

where \( g \) is an instrumental correction factor, and \( I_{||} \) and \( I_{||g} \) are the intensities measured on the polarization plane parallel and perpendicular to that of the exciting beam.

Enzymes activities and oxidative stress markers. Prior to the chemical assays, livers were minced and homogenized in an ice-cold buffer that contained 50 mM of potassium phosphate, 1 mM of EDTA, 1 mM of DTT, 0.1 mM BHT at pH 7.4. The homogenates were then centrifuged at 600 × g at 4°C for 10 min to rid them of cellular debris. Enzyme activities and sulphydryl group (SH) concentration were determined in the obtained supernatant using a Super Aquarius CE9200 spectrophotometer (Cecil Instruments Ltd., Cambridge, UK). The carnitine palmitoyl-transferase (CPT) activity was measured as previously described (20). The reaction mixture was composed of 60 mM of Tris HCl at pH 8.0. 1.5 mM of EDTA with 0.05% Triton X-100 and 0.25 mM DTNB (5,5′-dithiobis (2-nitrobenzoic acid)), and 1.67 mM of carnitine. The reaction was started by the addition of 0.025 mM of palmitoyl-CoA, and the increase in absorbance at 412 nm was followed. Fumarase (Fum) activity was measured in the reaction mixture containing 30 mM potassium phosphate, 0.1 mM EDTA at pH 7.4. The reaction was started by the addition of 5 mM L-malate. The increase in absorbance at 240 nm was monitored and the enzyme activity was calculated using a molar absorption coefficient of 2,440 M−1 cm−1 (21).

Catalse (CAT) activity was measured in the mixture containing 50 mM potassium phosphate, 5 mM EDTA, and 0.01% Triton at pH 7.4. The reaction was started by the addition of H2O2. The kinetic of H2O2 decomposition was followed in time at 240 nm, and CAT activity was calculated using a molar absorption coefficient of 43.6 M−1 cm−1 (22). Superoxide dismutase (SOD) activity was assayed using standard test kit (Randox Laboratories Ltd.). The SH group concentration was determined according to Ellman’s method (23). Briefly, samples were incubated with 100 μM DTNB at room temperature for 60 min. Absorbance was determined at 412 nm. Protein content was evaluated by the Lowry et al. method (16).

Liver TNF-α detection. The livers were homogenized in an ice-cold lysis buffer that contained 50 mM Tris, 1 mM of EDTA, 1.15% of KCl, and 0.1% Triton at pH 7.0. TNF-α levels were determined using a commercially available ELISA Kit for Rats (BioVendor Inc., Brno, Czech Republic) according to the manufacturer’s protocol. Absorbance was read at 450 nm using an ELISA reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). The concentrations were calculated with reference to the standard curve.

Chemicals. All reagents were obtained from Sigma-Aldrich, unless otherwise stated.

Statistical analyses. All results are expressed as the mean±standard error (SE). Two-way analyses of vari-
 ance (ANOVA) with the Tukey post-hoc test were per-
formed using STATISTICA 9.0 (Statsoft Inc., Tulsa, OK, USA) software. The cut-off for signifi-
cance was set at \( p < 0.05 \).

**RESULTS**

**Body weight and food intake**

The HFD induced a significant increase in rat body weight (main effect \( p < 0.05 \)). However, 6 wk of EtP systemic consumption (0.083 ± 0.002 g/d in CP group vs. 0.063 ± 0.002 g/d in DP group) did not influence the weight in either group of rats (Table 1).

**Plasma biochemical analyses**

There were no significant effects on plasma TG or FFA, but TC was significantly increased in HFD groups. Plasma lipids were similar between the groups supple-

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**Table 1. Body weight, food and EtP intake of treated rats (means±SE).**

<table>
<thead>
<tr>
<th>Groups</th>
<th>CC</th>
<th>CP</th>
<th>DC</th>
<th>DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>201±8</td>
<td>201±3</td>
<td>201±6</td>
<td>201±4</td>
</tr>
<tr>
<td>Mid body weight (g)</td>
<td>365±11</td>
<td>365±8</td>
<td>380±9</td>
<td>381±8</td>
</tr>
<tr>
<td>Final body weight (g)(^p)</td>
<td>423±14</td>
<td>417±9</td>
<td>454±9</td>
<td>453±12</td>
</tr>
<tr>
<td>Food intake (g/d/rat)(^\ddagger)</td>
<td>23.8±0.6</td>
<td>22.8±0.5</td>
<td>17.0±0.4</td>
<td>17.2±0.6</td>
</tr>
<tr>
<td>EtP intake (g/d/rat)</td>
<td>—</td>
<td>0.083±0.002</td>
<td>—</td>
<td>0.063±0.002</td>
</tr>
</tbody>
</table>

\(^p\) \( p < 0.05 \) main effect of the diet.

\(^\ddagger\) \( p < 0.001 \) main effect of the diet.

**Table 2. Plasma and liver TC, TG and FFA in rats after 12 wk of treatment (means±SE).**

<table>
<thead>
<tr>
<th>Groups</th>
<th>CC</th>
<th>CP</th>
<th>DC</th>
<th>DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC (mmol/L)(^p)</td>
<td>1.5±0.1</td>
<td>1.4±0.1</td>
<td>1.9±0.1</td>
<td>1.7±0.1</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.0±0.2</td>
<td>1.2±0.1</td>
<td>0.9±0.2</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>FFA (mmol/L)</td>
<td>0.9±0.0</td>
<td>0.8±0.1</td>
<td>1.0±0.1</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC ((\mu)mol/g wet tissue)</td>
<td>5.5±0.2</td>
<td>4.7±0.3</td>
<td>5.5±0.3</td>
<td>5.4±0.3</td>
</tr>
<tr>
<td>TG ((\mu)mol/g wet tissue)(^\ddagger)</td>
<td>10.4±0.8</td>
<td>11.8±1.0</td>
<td>26.5±5.6</td>
<td>30.9±6.0</td>
</tr>
<tr>
<td>FFA ((\mu)mol/g wet tissue)(^\ddagger)</td>
<td>0.8±0.2</td>
<td>1.1±0.2</td>
<td>3.3±0.3</td>
<td>3.8±0.7</td>
</tr>
</tbody>
</table>

\(^p\) \( p < 0.05 \) main effect of the diet.

\(^\ddagger\) \( p < 0.001 \) main effect of the diet.

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![Fig. 1](https://example.com/fig1.png)

Fig. 1. Plasma Alt (grey bars) and Ast (black bars) activities in control diet (CC), control diet and EtP (CP), HFD (DC), HFD and EtP (DP). Values are means±SE. * \( p < 0.05 \) as compared to DC.
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Table 3. Rat liver membrane fluidity after 12 wk of treatment (means±SE).

<table>
<thead>
<tr>
<th>Groups</th>
<th>CC</th>
<th>CP</th>
<th>DC</th>
<th>DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP (340)‡</td>
<td>-0.118±0.012</td>
<td>-0.124±0.010</td>
<td>-0.169±0.008</td>
<td>-0.171±0.010</td>
</tr>
<tr>
<td>Anisotropy (r)</td>
<td>0.148±0.011</td>
<td>0.150±0.005</td>
<td>0.137±0.007</td>
<td>0.138±0.008</td>
</tr>
</tbody>
</table>

‡ p<0.001 main effect of the diet.

Table 4. Activities of oxidative metabolism and antioxidant enzymes in liver homogenates after 12 wk of treatment (means±SE).

<table>
<thead>
<tr>
<th>Groups</th>
<th>CC</th>
<th>CP</th>
<th>DC</th>
<th>DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidative metabolism</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPT (mU/mg protein)‡</td>
<td>7.35±0.18</td>
<td>7.06±0.17</td>
<td>6.78±0.18</td>
<td>6.77±0.17</td>
</tr>
<tr>
<td>Fum (mU/mg protein)†</td>
<td>86.0±2.7</td>
<td>88.1±3.4</td>
<td>77.9±2.9</td>
<td>78.4±2.4</td>
</tr>
<tr>
<td>Antioxidant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAT (U/mg protein)†</td>
<td>134±9</td>
<td>127±4</td>
<td>103±8</td>
<td>117±6</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>35.5±0.6</td>
<td>34.6±0.8</td>
<td>36.4±1.0</td>
<td>35.0±0.3</td>
</tr>
</tbody>
</table>

‡ p<0.05 main effect of the diet.
† p<0.005 main effect of the diet.

![Liver TNF-α in control diet (CC), control diet and EtP (CP), HFD (DC), HFD and EtP (DP). Values are means±SE. * p<0.05 as compared to DC.](image)

The elevation of plasma Alt and Ast activities in the DC group demonstrated liver damage induced by the HFD. Such changes were not observed in the DP group (Fig. 1).

Liver lipids and membrane fluidity

The HFD resulted in a greater accumulation of TG and FFA in the liver, but TC was not modified by the diet. EtP supplementation did not affect liver lipids in either the control or in the diet groups (Table 2). The higher Laurdan GP340 fluorescence indicated a decrease (p<0.001) in the fluidity and polarity of the plasma liver membranes obtained from rats consuming the HFD compared to the control groups. Plasma membrane fluidity was not affected by the EtP supplementation. Changes in the anisotropy indexes were neither observed as the effect of the HFD nor the EtP treatment (Table 3).

Enzymes activity and oxidative stress markers

Lower activities of oxidative metabolism enzymes in the liver were observed in both the HFD groups: control and supplemented with EtP (Table 4). Moreover, the decrease in CAT activity was related to HFD. EtP supplementation did not have an effect on these enzymes. No changes were detected in liver SOD activity or SH...
levels. Liver SH levels were 1.87±0.10, 1.65±0.16, 1.62±0.03 and 1.68±0.14 mmol/mg protein in the CC, CP, DC and DP groups respectively.

Hepatic TNF-α

TNF-α level indicated pro-inflammatory modifications in the liver in the DC group (Fig. 2). Moreover, TNF-α in the DP group was lower than in the DC group (p<0.05).

Discussion

We observed that the HFD induced, in rats, body weight gain and fatty liver associated with higher plasma transaminases activity, dysfunction of mitochondrial metabolism, mild oxidative stress, decreased fluidity of the plasma membrane, and increased hepatic TNF-α. EtP supplementation for 6 wk attenuated the increase of Alt and Asp leakage into the blood and pro-inflammatory cytokine levels, but had no effect on metabolism or plasma membrane fluidity.

The hepatoprotective effect of EtP has been indicated in earlier studies (8–11, 24); however, in all these studies EtP was injected intravenously (9, 24), or intraperitoneally (8, 10, 11). In a previous study with a supplementation protocol, calcium pyruvate was used for 5 wk (25). The weight gain of the supplemented obese Zucker rats was lower compared with an ad libitum-fed control group and a pair-fed control group. Moreover, acceleration of the resting metabolic rate was observed in pyruvate-treated rats (25). Since the higher availability of pyruvate could accelerate gluconeogenesis, the process of glucose synthesis causing increased utilization of high energy phosphate bonds, the futile cycle was proposed as the mechanism responsible for elevated energy expenditure (26). However, no change in body weight due to EtP treatment was noted in our study. In addition, the liver lipids were not modified by the EtP supplementation either in the control or in the diet groups. Lipid accumulation in the liver may be associated with decreased mitochondrial content (27). It has recently been shown, that 8 wk of a HFD down-regulate mitochondrial biogenesis (1). On the other hand, the presence of sodium pyruvate enhanced mitochondrial biogenesis (5). Lower activities of enzymes regulating mitochondrial fatty acid oxidation observed after 12 wk of HFD were in line with the previous results (1, 27). However, systemic consumption of EtP did not influence either CPT, the enzyme involved in long-chain fatty acid transport across the mitochondrial membrane, or Fum, the enzyme of the tricarboxylic acid cycle.

Despite the absence of modification in fatty liver, the mean level of the circulating transaminases in the DP group was significantly lower than in the DC group. Similar findings were noted in a study where Ringer’s ethyl pyruvate solution reduced liver injury induced by an acute ethanol treatment (8). Previous reports (28, 29) have shown that hepatocyte membrane integrity is strongly associated with plasma membrane fluidity. Additionally, EtP supplementation effectively abolished the negative effect of ethanol consumption on erythrocyte membrane fluidity (13). Therefore, we assumed that an increased transaminase leakage into the blood in DC group was the result of modification in plasma membrane fluidity. However, decreased plasma membrane fluidity, as measured by Laurdan, in HFD rats was not prevented by EtP.

Plasma ALT activity was related to TNF-α gene expression in hepatic tissues (8). An increased TNF-α level is one of the earliest events in hepatic inflammation (30). The importance of this cytokine in NAFLD is supported by the observations that its elevation is associated with development of insulin resistance (30), whereas neutralization of TNF-α can improve insulin sensitivity in rats fed a high-fat diet (31). Insulin sensitivity was not measured; however, our results indicate that EtP supplementation attenuated the rise of TNF-α induced by the diet. This observation is in agreement with the previous studies indicating that EtP down-regulates the secretion of multiple pro-inflammatory proteins, including TNF-α, and high mobility group box 1 (32).

Inflammation status appeared to be interacting with oxidative stress in the obese condition. Moreover, reactive oxygen species production has been considered as one of several adverse cellular responses to nutrient excess in obesity (33). Since EtP has been demonstrated to be an effective antioxidant (34, 35), the markers of oxidative stress have been determined. Decreased CAT activity as a consequence of HFD was noted, similarly to previous studies (33, 36). CAT inactivation under oxidative stress condition, related to damage in protein moiety and heme release (37), was protected against by pyruvate (38). However, in the present study we were unable to notice any effect of EtP on CAT inactivation.

In summary, EtP consumption can reduce hepatic cell damage. Our data did not confirm the concept that the observed effect of EtP supplementation was related to changes in liver metabolism or plasma membrane fluidity. However, the obtained data are of practical importance because EtP might be used as a food additive (JECA No. 938) (39). Therefore, further studies will be performed to measure the influence of EtP in various cell types.

Acknowledgments

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