Distribution of 3-Hydroxy Fatty Acids in Tissues after Intraperitoneal Injection of Endotoxin

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Background: 3-Hydroxy fatty acids (3-OH FAs) with 10- to 18-carbon chain lengths are constituents of the endotoxin [lipopolysaccharide (LPS)] of gram-negative bacteria. We investigated whether these FAs may be used as chemical markers in measuring endotoxin concentrations in mammalian tissue samples.

Methods: We used gas-liquid chromatography–tandem mass spectrometry to measure 3-OH FAs in serum and tissues (heart, liver, and skeletal muscles) of rats after intraperitoneal injection of Escherichia coli LPS. One group of rats (group I) received a single LPS dose of 20 mg/kg of body weight; group II rats received the same total dose but over the course of 10 days (2 mg/kg each day). Rats receiving saline (group III) were used as controls.

Results: 3-OH FAs with chain lengths of 10, 12, 14, 16, and 18 carbons were detected in all studied types of samples. Group I rats had 50-fold and group II rats had 3-fold higher serum concentrations of 3-hydroxytetradecanoic acid (3-OH 14:0, the predominant 3-OH FA of E. coli LPS) than group III rats. Concentrations of 3-OH 14:0 in livers from group I and II rats were similar and fourfold higher than in group III rats, whereas concentrations of the same acid in skeletal and heart tissues did not differ among the three groups of rats. 3-OH 14:0 dominated in heart and liver of group III rats, whereas 3-OH 16:0 (followed by 3-OH 14:0) dominated in skeletal muscles and blood.

Conclusions: 3-OH FAs 10–18 carbons in length, probably originating from endotoxin and mitochondrial \( \beta \)-oxidation, are abundant in rat liver, skeletal muscles, and heart and can also be detected in blood. The widespread presence of these compounds in mammals limits their usefulness as LPS markers for endotoxin in clinical samples.

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Lipopolysaccharide (LPS),4 a constituent of the outer membrane of gram-negative bacteria, is the endotoxin responsible for a variety of pathologic inflammatory reactions that can lead to septic shock and death. Little is known about the translocation of endotoxin into the bloodstream and about its tissue distribution and degradation, mainly because of problems encountered in measuring this potent and multifunctional compound in body fluids and tissues. The Limulus amebocyte lysate (LAL) assay is the most widely used test for endotoxin, but it is not ideal in terms of specificity and reproducibility, despite many attempts undertaken to standardize and optimize the procedures (1, 2).

The lipid A moiety of LPS is composed of a disaccharide of glucosamine with covalently bound 2- and 3-hydroxy fatty acids (2- and 3-OH FAs). The 3-OH FAs (usually of 10- to 18-carbon chain lengths) have been suggested as chemical markers for LPS in mammalian samples. Low concentrations of 3-OH FAs have been found in normal human sera and plasma (3, 4). Recently, the same 3-OH FAs were identified in rat blood, and because the concentrations were approximately the same in germ-free as in conventional rats, they were assumed to originate mainly from mitochondrial \( \beta \)-oxidation of endogenous FAs (5).

In the present study we used a rat model to simulate the likely mechanism of gram-negative sepsis, a condition often thought to be caused by bacteria from the intestine (6), by injecting endotoxin into the peritoneal cavity. The aim was to use 3-OH FA analysis to determine the
distribution of intraperitoneally delivered endotoxin in serum and various organs: liver, heart, and skeletal muscles. State-of-the-art mass spectrometry [gas-liquid chromatography–tandem mass spectrometry (GLC-MSMS)] was used for optimum analytical specificity.

**Materials and Methods**

**LPS**
We used *Escherichia coli* O127:B8 LPS (Sigma Chemicals). The lyophilized preparation was made up in a pyrogen-free solution containing 9 g/L NaCl.

**ANIMALS**
Male Wistar rats (250 g), fed a standard diet and with ad libitum access to water, were used. They were sacrificed by decapitation without previous anesthesia. Blood was collected from neck vessels. After coagulation, the blood samples were centrifuged (870 g for 10 min), and serum was collected and immediately frozen in liquid nitrogen. Similarly, samples of liver, heart, and skeletal muscle tissues were collected and frozen in liquid nitrogen. All samples were stored at −20 °C until analysis.

Three groups of rats were included:

Group I rats (shock model) were subjected to acute endotoxemia via a single intraperitoneal injection of endotoxin (20 mg/kg of body weight). From an original group of 15 animals, 4 died within 12 h and 1 died after 20 h; the remaining 10 were sacrificed after 24 h.

Group II rats (chronic model) were subjected to chronic endotoxemia via a total dose of 20 mg/kg of body weight of intraperitoneally injected endotoxin over 10 days (2 mg/kg each day). All 15 animals in this group survived. They were sacrificed, and samples from 10 of the rats were collected as described above.

Group III rats (controls) received a single intraperitoneal injection of 1 mL of a pyrogen-free solution containing 9 g/L NaCl. All 10 rats in this group were sacrificed after 24 h, and samples were collected.

The project was approved by the Ethical Committee for Animal Research, Poznań, Poland.

**SAMPLE PREPARATION AND ANALYSIS**

We processed 200 μL of serum and 100–200 mg of tissue (liver, heart, and skeletal muscle) as described previously to obtain trimethylsilyl/methyl ester derivatives of the 3-OH FAs (5). In brief, serum samples were heated in 2 mol/L methanolic HCl for 18 h at 80 °C. We then added internal standard (50 ng of pentadeuterated 3-hydroxy tetradecanoic acid methyl ester; a gift from Dr. D. White, Department of Microbiology, University of Tennessee, Knoxville, TN), and the samples were extracted and evaporated. Preparations were purified with use of a silica gel solid-phase extraction column (Varian) and analyzed for 3-OH FAs after derivatization with 50 μL of bis(trimethylsilyl)trifluoroacetamide (Acros Organics) and 5 μL of pyridine (Fluka Chemie).

The (lipid-rich) tissue samples were heated in 2 mol/L methanolic HCl and extracted as described for serum; the organic phase was heated at 80 °C for 30 min in 100 g/L KOH in methanol and shaken with water (2 mL) and heptane (1 mL; Lab-Scan). The lower (water) phase, containing salts of FAs, was acidified and shaken with heptane, and the heptane phase was evaporated and heated in 2 mol/L methanolic HCl for 1 h at 80 °C. After cooling, this preparation (containing FA methyl esters) was extracted with heptane, and the organic phase was collected. Further purification and derivatization steps were performed as described for the serum samples (see above).

GLC-MSMS analyses were in the electron impact mode; the parent ion (m/z 175) was fragmented in non-resonance mode. Conditions for formation of the product ions used for monitoring are described elsewhere (7, 8). Moles of LPS were calculated by dividing the sum of the moles of the 3-OH FAs by 4.

**Results**

Several macroscopically visual changes in the abdomen area were observed in the group I rats, including bowel hyperemia, edema of viscera, and in some cases, peritoneal effusion. None of these symptoms was seen in any of the group II or III rats.

Examples of 3-OH FA profiles of serum and soft tissues from a control rat (group III) are shown in Fig. 1. Distinct peaks at the retention times of derivatized 10-, 12-, 14-, 16-, and 18-carbon chain length 3-OH FAs were observed, and the identity of each was verified by its characteristic MSMS spectrum (8).

3-OH FA COMPOSITION

**Serum.** In the group III rats (controls), all studied 3-OH FAs appeared in almost equal amounts (Table 1). As expected, the high dose of endotoxin given to the group I rats changed the serum profile dramatically, and the chromatograms were strongly dominated by 3-OH 14:0, the main 3-OH FA constituent of *E. coli* LPS (9) (Fig. 2A). Group II rats, which received the same total dose of LPS as the group I rats but in a prolonged manner, also had increased 3-OH FA concentrations in comparison with the controls. As mentioned, all 15 rats in this group survived without any symptoms.

**Liver.** 3-OH 14:0 dominated strongly over the other 3-OH FAs in all livers (Table 1). The group I and II rats, which received the same total amount of endotoxin (20 mg/kg of body weight), had almost equal amounts of 3-OH 14:0 in their livers. These results are in sharp contrast to the results for the serum, where 3-OH 14:0 concentrations in the group I rats exceeded those of the group II rats by >10-fold. The group III rats (controls) had lower amounts of 3-OH 14:0 than the group I and II rats (Fig. 2B).
Skeletal muscle. The skeletal muscles exhibited a distinct 3-OH FA profile (Fig. 2C). In the control rats (group III), 3-OH 16:0 dominated, followed by 3-OH 14:0, 3-OH 18:0, and 3-OH 12:0. Injection of the endotoxin changed the profile, and in the group I rats, 3-OH 14:0 dominated over 3-OH 16:0, 3-OH 12:0, and 3-OH 18:0. This tendency was even clearer in the group II rats, which had further decreases in 3-OH 16:0 and 3-OH 18:0 (Table 1 and Fig. 2C).

Heart. We observed no changes in the concentrations or profiles of the 3-OH FAs in heart tissue attributable to the intraperitoneal injection of endotoxin (Fig. 2D). As in the liver, 3-OH 14:0 was strongly dominant in all rats (Table 1).

Discussion
The aim of the present study was to evaluate the distribution of 3-OH FAs in rat serum and tissues after intra-

### Table 1. Mean (SD) 3-OH FA concentrations in serum (pmol/mL) and tissues (pmol/mg) of rats (n = 10 for each group).

<table>
<thead>
<tr>
<th></th>
<th>3-OH 10:0</th>
<th>3-OH 12:0</th>
<th>3-OH 14:0</th>
<th>3-OH 16:0</th>
<th>3-OH 18:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Group III (control)</td>
<td>47.2 (26.2)</td>
<td>54.5 (13.1)</td>
<td>63.9 (22.2)</td>
<td>69.6 (11.1)</td>
<td>35.9 (13.9)</td>
</tr>
<tr>
<td>Group II (chronic)</td>
<td>16.9 (11.3)</td>
<td>18.2 (8.9)</td>
<td>216.4 (97.9)</td>
<td>69.6 (8.7)</td>
<td>45.8 (10.6)</td>
</tr>
<tr>
<td>Group I (shock)</td>
<td>15.3 (11.2)</td>
<td>68.0 (32.0)</td>
<td>3540.8 (3105.5)</td>
<td>78.8 (13.7)</td>
<td>58.7 (12.2)</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Group III (control)</td>
<td>0.06 (0.07)</td>
<td>0.24 (0.07)</td>
<td>2.76 (0.40)</td>
<td>0.43 (0.18)</td>
<td>—</td>
</tr>
<tr>
<td>Group II (chronic)</td>
<td>0.00 (0.01)</td>
<td>0.11 (0.06)</td>
<td>10.20 (3.40)</td>
<td>0.14 (0.15)</td>
<td>—</td>
</tr>
<tr>
<td>Group I (shock)</td>
<td>0.05 (0.05)</td>
<td>0.27 (0.14)</td>
<td>9.83 (8.33)</td>
<td>0.64 (0.19)</td>
<td>—</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Group III (control)</td>
<td>0.01 (0.02)</td>
<td>0.10 (0.06)</td>
<td>1.70 (0.43)</td>
<td>2.21 (1.38)</td>
<td>0.48 (0.67)</td>
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<tr>
<td>Group II (chronic)</td>
<td>0.00 (0.01)</td>
<td>0.02 (0.05)</td>
<td>1.55 (0.46)</td>
<td>0.25 (0.30)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>Group I (shock)</td>
<td>0.02 (0.03)</td>
<td>0.19 (0.17)</td>
<td>2.53 (1.07)</td>
<td>1.60 (0.92)</td>
<td>0.07 (0.22)</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Group III (control)</td>
<td>0.02 (0.03)</td>
<td>0.31 (0.16)</td>
<td>6.55 (0.91)</td>
<td>1.14 (0.45)</td>
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<td>Group II (chronic)</td>
<td>0.07 (0.05)</td>
<td>0.41 (0.22)</td>
<td>7.31 (1.13)</td>
<td>0.90 (0.50)</td>
<td>0.42 (0.74)</td>
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<td>Group I (shock)</td>
<td>0.06 (0.04)</td>
<td>0.47 (0.23)</td>
<td>6.86 (0.92)</td>
<td>1.11 (0.56)</td>
<td>0.43 (0.64)</td>
</tr>
</tbody>
</table>

*Not possible to evaluate because of disturbed chromatogram.
peritoneal injection of endotoxin. Most of the injected endotoxin was transported in the portal circulation to the liver, whereas a minor part was taken up by the lymphatic system and subsequently transported to mesenteric lymph nodes and further distributed in the various organs.

We found 50-fold higher concentrations of 3-OH 14:0 in sera of the group I rats (shock model) compared with the control rats. The group II rats (chronic model) had moderately higher concentrations than the controls. Interestingly, livers from the group I and group II rats contained almost equal amounts of 3-OH 14:0, which illustrates the detoxification activity of the liver, including its ability to continuously remove endotoxin from the body.

Septic shock is known to produce increased resistance against insulin in skeletal muscles followed by increased catabolic activity. This mechanism may explain the decreases in the concentrations of longer chain 3-OH FAs (3-OH 16:0 and 3-OH 18:0) and the increases in the shorter chain 3-OH FAs (3-OH 12:0 and 3-OH 14:0) in the group I rats (shock model). The concentrations of 3-OH 16:0 and 3-OH 18:0 were further decreased in the group II rats (chronic model; Fig. 2, C and D; Table 1). We cannot exclude, however, that at least part of the 3-OH 14:0 in skeletal muscle may stem from the endotoxin injected. No influence of injected endotoxin on the 3-OH FA concentrations was observed in samples of heart muscle. This may be attributable to the fact that heart muscle does not respond to changes in insulin and hormone concentrations that promote the catabolism of FAs, or it may be the result of a lack of endotoxin receptors on the cellular surface (10).

It is unlikely that all of the detected 3-OH FAs originated from LPS; rather, some or most may stem from mammalian FA metabolism (5). Mitochondrial β-oxidation of FAs is a fundamental energy-producing pathway, particularly in such tissues as skeletal and cardiac muscle (10). Thus, when glycogen reserves are exhausted, e.g., during fasting, increased muscle activity, or febrile illness, β-oxidation becomes the principal energy supply (11, 12). In such situations, the endogenous FAs are mobilized from adipose tissue stores, converted into CoA esters in the mitochondrion, and undergo β-oxidation (10). 3-OH FAs appear as intermediates and are detectable in the plasma and/or urine, especially in patients with metabolic disorders attributable to inborn deficiencies of the enzymes involved. Analysis of plasma free FAs and 3-OH FAs can be used to diagnose such disorders (12, 13).

Fig. 2. 3-OH FA concentrations (pmol/mL of serum or mg of tissue) in rat serum (A), liver (B), skeletal muscle (C), and heart (D). [group III (controls); ■ group II (chronic); ▣ group I (shock)].
Many sepsis patients with endotoxemia fail to show positive blood cultures for gram-negative bacteria, suggesting the persistence of endotoxin after clearance of the bacteria, leakage of endotoxin from the gut, or deficiencies in blood culture technology. Analysis of plasma and cerebrospinal fluid for meningococcal LPS by measurement of 3-OH 12:0, the specific 3-OH FA of Neisseria (9), was suggested as a possible alternative to the LAL activity assay (4, 14). Interestingly, Limulus activity in plasma from patients with meningococcal septicemia showed a close quantitative association with the 3-OH FA results. In earlier studies (3, 15), convincing dose–response relationships were found between serum concentrations of 3-OH FAs and added amounts of LPS. However, it should be noted that these studies were performed using GLC-MS analysis in the selected-ion monitoring mode, a method that provides high sensitivity but may lack specificity in sample matrices that are chemically complex.

To the best of our knowledge, this is the first report describing the presence of 10- to 18-carbon chain length 3-OH FAs in various tissues of healthy mammals. The origin of these compounds, the identities of which were confirmed by full MSMS spectra, is unknown, although we speculate that they may stem mainly from mitochondrial β-oxidation. The described analytical method could be useful in research on mammalian lipid metabolism and in studies on in vivo tissue uptake and degradation of lipid A of different bacterial sources. However, it must be taken into account that 3-OH FAs detected in mammalian samples may originate from both bacterial and nonbacterial sources. To evaluate the applicability of the method to monitoring of endotoxemia, 3-OH FA concentrations should be correlated to culture, LAL results, and antibiotic treatment effects.

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References