Reduced hepatic extraction of palmitate in steatosis correlated to lower level of liver fatty acid binding protein

Daniel Y. Hung, Gerhard A. Siebert, Ping Chang, Frank J. Burczynski, and Michael S. Roberts. Reduced hepatic extraction of palmitate in steatosis correlated to lower level of liver fatty acid binding protein. Am J Physiol Gastrointest Liver Physiol 288: G93–G100, 2005. First published September 2, 2004; doi:10.1152/ajpgi.00196.2004.—Nonalcoholic fatty liver disease is the most common of all liver diseases. The hepatic disposition of palmitate and its metabolites were found to be significantly decreased in steatotic conditions of hepatic steatosis allowed us to successfully characterize and analyze the altered disposition and metabolism pharmacokinetics of palmitate and its low-molecular-weight metabolites with a number of quantitative parameters defining different pathological liver conditions using an in situ rat liver perfusion study (12). In the present study, we sought to examine whether the kinetics of palmitate were affected by fatty liver disease in the rat. In doing so, we also collected a range of biochemical and histological parameters to examine possible correlations. Steatosis in rats was induced by administration of 17α-ethinylestradiol (30). Also, we sought to mimic the hepatic fatty acid accumulation and symptomatology associated with cirrhosis and intrahepatocellular binding processes under the conditions of hepatic steatosis allowed us to successfully characterize and analyze the altered disposition and metabolism pharmacokinetics of palmitate and its low-molecular-weight metabolites in the early steatotic rat liver.

MATERIALS AND METHODS

Materials. All chemicals including fatty acid-free albumin and 17α-ethinylestradiol were purchased from Sigma (St. Louis, MO). [3H]Palmitate, [3H]water, and [14C]sucrose were purchased from New England Nuclear (Boston, MA).

Purification of [3H]palmitic acid. [3H]Palmitic acid was purified by the Borgstrom ethanol extraction procedure as previously described (8).

Fatty liver rat model. All animal studies were approved by the Animal Ethics Committee of the University of Queensland, Queensland, Australia. The steatotic rat model was established using female Wistar rats of ~250 g body weight. The procedure is a modification of the protocol given by Sanchez Pozzi and co-workers (30). Rats were housed and fed under standard conditions and were injected with 17α-ethinylestradiol (2 mg/kg sc) on 8 consecutive days. Hepatic steatosis was biochemically established on day 9. Histology confirmed the existence of microvesicular steatosis (Fig. 1B).

Serum biochemistry determinations. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, triglyceride, HDL, and LDL levels were measured on a Hitachi 747 analyzer (Hitachi, Tokyo, Japan) on day 9.

Analysis of liver fatty acid binding protein, microsomal protein, and cytochrome P-450 tissue levels. Rat liver fatty acid binding protein (L-FABP) sandwich enzyme immunoassay has been described in detail elsewhere (12). The microsomal protein tissue level was determined by the method of Lowry et al. (15). Cytochrome P-450 content in microsomal protein was estimated from the dithionite-reduced difference spectrum of carbon monoxide-bubbled samples using the molar extinction difference of 104·nmM⁻¹·cm⁻¹ in absorption at peak position (~450 nm) as described by Matsubara and co-workers (20).
HEPATIC DISPOSITION OF PALMITATE IN STEATOSIS

In situ rat liver perfusions. Fasted female Wistar rats (~300 g body wt) were anesthetized by an intraperitoneal injection of 10 mg/kg xylazine (Bayer, Pymble, NSW, Australia) and ketamin-medoxchloride 80 mg/kg (Parnell Laboratories, Alexandria, NSW, Australia). After laparotomy, animals were heparinized (heparin sodium, David Bull Laboratories, Melbourne, VIC, Australia; 200 U) via the inferior vena cava. The bile duct was cannulated with PE-10 (Clay Adams, Franklin Lakes, NJ). The portal vein was cannulated using a 16-G needle into the flowing perfusate, the injection needle was cleaned with a heptane-wetted tissue to remove any adherent [3H]palmitate on the outside of the stainless steel needle. A stabilization period of 10 min was afforded between injections. Outflow samples were collected using a fraction collector over 3.5 min (1 s × 20, 4 s × 5, 10 s × 5, 30 s × 4), and aliquots (100 µl) were taken for scintillation counting using a MINI beta TRI-CARB 4000 series liquid-scintillation counter (Packard Instruments). Aliquots (100 µl) were also removed from the outflow samples for absorption spectrophotometric analysis of Evans blue dye at 620 nm using a Spectracount plate counter (Packard).

In each liver, a maximum of two injections [bolus 1: purified [3H]palmitate (3 × 10⁶ dpm), Evans blue dye (3 mg/ml), and [14C]sucrose (1.5 × 10⁶ dpm); bolus 2: [3H]water (3 × 10⁶ dpm)] was administered in randomized order. The total perfusion time for each liver was <1 h. Before introduction of the Hamilton syringe injection needle into the flowing perfusate, the injection needle was cleaned with a heptane-wetted tissue to remove any adherent [3H]palmitate on the outside of the stainless steel needle. A stabilization period of 10 min was afforded between injections. Outflow samples were collected using a fraction collector over 3.5 min (1 s × 20, 4 s × 5, 10 s × 5, 30 s × 4), and aliquots (100 µl) were taken for scintillation counting using a MINI beta TRI-CARB 4000 series liquid-scintillation counter (Packard Instruments). Aliquots (100 µl) were also removed from the outflow samples for absorption spectrophotometric analysis of Evans blue dye at 620 nm using a Spectracount plate counter (Packard).

[3H]palmitate and low-molecular-weight metabolites outflow concentration-time profile. [3H]palmitate was separated from any [3H]-labeled fatty acid metabolites by a modification of the Dole procedure (7). Briefly, trichloroacetic acid (10% solution, 50 µl) was added to Eppendorf tubes containing 100 µl of effluent sampled from the first bolus and vortexed. Samples were centrifuged for 4 min at 10,000 g using a microcentrifuge (Sigma Laborzentrifugen, Harz, Germany). The supernatant was removed, and the Eppendorf tube was cut at a level just above the pellet. The tube remnant containing the pellet was placed directly into a scintillation vial, and 2 ml Ready Safe scintillant were added. The pellet was allowed to dissolve overnight, and bovine serum albumin-associated radioactivity (representing [3H]palmitate) was determined the following day. The bolus dose was assayed identically to that of outflow samples. The hepatic extraction ratio (E) of palmitate, retention of palmitate in the liver (MTT), and normalized variance (CV²) of palmitate were determined from the outflow concentration-time profile of these trichloroacetic acid-extracted effluent samples.

Low-molecular-weight metabolites of [3H]palmitate were separated from the effluent nonextracted samples by a urocentrifugation method using Millipore Microcon YM-30 filter devices (30,000 MWCO, Millipore) and were counted by a beta liquid-scintillation counter (Packard), and a thin layer chromatography assay was used to confirm that the radioactivity measured in the ultrafiltrate did not represent [3H]palmitate and precluded high-molecular-weight metabolites (such as VLDL) to be detected in the ultrafiltrate as described previously (12). The production of low-molecular-weight metabolites of palmitate (AUCmet) and MTT metabolites in the liver (MTTmet) was determined from the perfusate outflow concentration-time profile of these separated metabolites. MTTmet is not due to a single metabolite but is a function of the MTTs of several metabolites and their relative transit times in the liver.

In vitro metabolism of microsomal protein. To assess the effect of steatosis on metabolism, an in vitro study with a microsomal protein preparation (buffer containing 0.35 mg/ml microsomal protein from normal or steatotic rat livers) incubated with [3H]palmitate (3 × 10⁶ dpm) was performed at 37°C. Samples were then collected at 0, 5, 10, and 20 min. Unchanged [3H]palmitate was extracted with trichloroacetic acid as described above. The logarithm of the radioactivity of unchanged [3H]palmitate remaining in solution was plotted against time to obtain a slope and an extrapolated initial radioactivity (at time 0). The in vitro metabolic clearance was estimated as the product of the slope and the dose divided by the extrapolated initial radioactivity.

Data analysis. A detailed description of the underlying mathematical model and analysis has been reported previously (12). Briefly, a mixture of two inverse Gaussian density functions with correction for catheter effects was used to estimate the sinusoidal albumin space \( V_{P_{\text{PAL}};} \) (determined by Evans blue dye) or the sinusoidal sucrase space \( V_{P_{\text{SU}};} \) (determined by [14C]sucrose). A barrier-limited plus space-distributed liver model with correction for catheter effects was used to estimate the total water space \( V_{W;} \) (determined by [3H]wa-
ter), V\textsubscript{W} then being used to estimate the cellular water space (V\textsubscript{C}),
defined as \([V\textsubscript{W} - V\textsubscript{V,conc}]\). The outflow concentrations for unchanged
\([\textsuperscript{3}H]palmitate were presented as outflow fraction per milliliter. A
physiologically based pharmacokinetic model (slow diffusion/bound
model, illustrated in Fig. 2) was used to estimate the disposition
kinetics parameters for hepatocellular influx (\(k\text{in}\)), efflux (\(k\text{out}\)), elimi-
nation (\(k\text{e}\)), and diffusion (\(k\text{d}\)) for \([\textsuperscript{3}H]palmitate (12). Under the
assumption that diffusion in hepatocytes is a rate-limiting function, if
only the bound drug is contributing significantly to diffusion, the

The nonparametric estimate of hepatic availability (F) of
\([\textsuperscript{3}H]palmitate in the various models was determined from the outflow
concentration (C) vs. time (t) profile from Eq. 2 using the parabolas-
through-the-origin method (extrapolated to infinity) with the assistance
of the Moments Calculator 2.2 program (University of Otago,
Dunedin, New Zealand) for Macintosh computers.

\begin{equation}
\dot{f}(s) = \frac{k\text{out}}{k\text{out} + \sqrt{k\text{in}s + k\text{e}}} \tanh(\sqrt{s + k\text{d}}/k\text{d})
\end{equation}

where \(\tanh\) is the effective thickness of the hepatocytes.

\begin{equation}
F = \frac{Q \cdot \text{AUC}}{D}
\end{equation}

where AUC = \(\int_0^\infty C(t)dt\) is the area under the solute concentration
vs. time curve and \(D\) is the dose of solute administered. All concent-
trations used were expressed in molar equivalents. Hepatic extraction
ratio (E) = 1 - F.

\begin{equation}
\text{MTT} = \frac{\text{AUMC}}{\text{AUC}}
\end{equation}

where AUMC is the area under the first moment curve.

\begin{equation}
CV^2 = \frac{\sigma^2}{\text{MTT}^2}
\end{equation}

where

\begin{equation}
\sigma^2 = \int C(t)dt - \int C(t)dt
\end{equation}

Statistical analysis. All data are presented as means ± SD unless
otherwise stated. Statistical analysis was performed using the Student’s \(t\)-test and regression analysis where appropriate. Statistical
significance was taken at the level of \(P < 0.05\).

RESULTS

The mean ± SD (\(n = 6\)) liver wet weight of animals used in
the perfusion studies was 10.2 ± 1.1 g for control animals and
12.5 ± 0.9 g for steatotic rats (\(P < 0.05\)). During liver
perfusion, steatotic rats had significantly lower bile flow (\(P <
0.05\)). The mean ± SD bile flow (\(n = 6\)) was 0.57 ± 0.12
\(\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}\) for normal rats and 0.38 ± 0.09
\(\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}\) for steatotic rats. The hepatic oxygen
consumption for all animals was in the range of 1.39 to 1.75
\(\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}\), and perfusion pressure was in
the range of 10.1 to 12.3 cm\textsubscript{H}\text{O}. These parameters were comparable
with those reported previously (5, 12).

Light microscopic analysis of liver slices taken from the
control animals showed the typical hepatic architecture (Fig.
1A). Liver slices from 17\(\alpha\)-ethinylestradiol-treated animals showed signs of microvesicular steatosis and a pronounced
enlargement of the sinusoidal space (Fig. 1B).

Biochemical liver parameters also confirmed the presence of
steatosis in the 17\(\alpha\)-ethinylestradiol-treated animals. Table 1
shows a comparison of serum ALT, AST, cholesterol, trigly-
cone-ride, HDL, and LDL levels between normal and steatotic
animals. Differences were significant between steatotic and
normal animals for all serum biochemistry levels. The steatotic
group showed a significantly higher ALT-to-AST ratio than the
normal group (\(P < 0.05\)).

The L-FABP, microsomal protein, cytochrome P-450 tissue
levels, and in vitro metabolic clearance for each animal group
are shown in Table 2. The steatotic animals had significantly
lower L-FABP, cytochrome P-450 levels, and in vitro meta-
biologic clearance than the normal group, whereas no significant
appear to be different. Figure 4 shows corresponding regres-
sion line fits for unchanged \(^{[3]H}\)palmitate using a slow diffusion/bound model and a data weighting of 1/\(\text{Vol}_{\text{obs}}\) for isolated perfused normal and steatotic rat data (logarithmic scale). All data appeared to be adequately fitted by the model (using Evans blue dye-labeled albumin as sinusoidal reference). The steatotic group showed a slower decline curve (longer hepatic retention time and larger hepatic availability of unchanged \(^{[3]H}\)palmitate) than that of the normal group.

In Table 4, a comparison of the disposition kinetic parameters derived from the slow diffusion/bound model for extracted \(^{[3]H}\)palmitate between normal and steatotic animals is presented. The values of metabolic clearance, permeability-surface area product, partition ratio of influx to efflux rate constant, and cytoplasmic diffusion constant for unchanged \(^{[3]H}\)palmitate were significantly lower in steatosis compared with normal animals. However, the steatotic animals had a significantly larger sinusoidal albumin space and total water space. The sinusoidal sucrose spaces were not significantly different between the two groups.

**DISCUSSION**

Histologically evident hepatic microvesicular steatosis (Fig. 1B) was used to characterize the extent of steatosis induced following repeated 17\(\alpha\)-ethynylestradiol administration to female Wistar rats. Associated with histological evidence of steatosis, there were significantly higher serum ALT, AST, triglyceride, and LDL levels and lower cholesterol and HDL levels in the 17\(\alpha\)-ethynylestradiol-treated group than in the normal group (Table 1). These observations, together with a pattern of an elevated ALT-to-AST ratio in the 17\(\alpha\)-ethynylestradiol-treated group, coincide with the clinical characterization of nonalcoholic fatty liver disease (21, 31). We also observed that 17\(\alpha\)-ethynylestradiol treatment produced a reliable and consistent steatosis model in the rat. However, the ballooning hepatocytes and light microscopic signs of inflammation commonly seen in human nonalcoholic liver disease (11, 27) could not be demonstrated in the livers of 17\(\alpha\)-ethynylestradiol-treated animals.

L-FABP has been reported to be the most important factor determining the hepatic uptake of fatty acids (3, 16, 18, 33). A greater hepatic transmembrane permeation, diffusion, and

<table>
<thead>
<tr>
<th>Table 1. Comparison of serum biochemistry levels of ALT, AST, cholesterol, triglyceride, HDL, and LDL between the normal and 17(\alpha)-ethynylestradiol-induced steatotic rat model</th>
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</thead>
<tbody>
<tr>
<td><strong>Serum Marker</strong></td>
</tr>
<tr>
<td>ALT (U/I)</td>
</tr>
<tr>
<td>AST (U/I)</td>
</tr>
<tr>
<td>Cholesterol, mM</td>
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<tr>
<td>Triglyceride, mM</td>
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<td>HDL, mM</td>
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<td>LDL, mM</td>
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Values are means ± SD; \(n = 6\). There were significant differences between the steatotic and normal group for all serum biochemistry markers (*\(P < 0.05\); †\(P < 0.01\); ‡\(P < 0.001\)). The steatotic group showed a significantly higher alanine aminotransferase (ALT) to-aspartate aminotransferase (AST) ratio than the normal group (\(P < 0.05\)).

<table>
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<tr>
<th>Table 3. Comparison of nonparametric moments for (^{[3]H})palmitate (extracted with trichloroacetic acid) and low-molecular-weight metabolites (separated by ultrafiltration) between the normal and 17(\alpha)-ethynylestradiol-induced steatotic rat model</th>
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<tbody>
<tr>
<td><strong>Moment Parameters</strong></td>
</tr>
<tr>
<td>E</td>
</tr>
<tr>
<td>MTT, s</td>
</tr>
<tr>
<td>CV2</td>
</tr>
<tr>
<td>AUCmet*</td>
</tr>
<tr>
<td>MTTmet, s</td>
</tr>
</tbody>
</table>

Values are means ± SD; \(n = 6\). There were significant differences between the steatotic and normal animals for all moment parameters (*\(P < 0.05\); †\(P < 0.01\); ‡\(P < 0.001\)). The elimination of normal variance (CV2) (\(P > 0.05\)). E, extraction ratio; MTT, retention of unchanged palmitate in the liver; AUCmet, production of palmitate low-molecular-weight metabolites; MTTmet, retention of palmitate metabolites in the liver.

Table 2. Comparison of L-FABP, microsomal protein, cytochrome P-450 tissue levels, and in vitro metabolic clearance between the normal and 17\(\alpha\)-ethynylestradiol-induced steatotic rat model

<table>
<thead>
<tr>
<th><strong>Protein</strong></th>
<th><strong>Normal</strong></th>
<th><strong>Steatosis</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>L-FABP, mg/g liver</td>
<td>0.27 ± 0.11</td>
<td>0.15 ± 0.06*</td>
</tr>
<tr>
<td>Microsomal protein, mg/g liver</td>
<td>9.03 ± 2.55</td>
<td>7.93 ± 2.84</td>
</tr>
<tr>
<td>Cytochrome P-450, nmol/mg protein</td>
<td>0.55 ± 0.11</td>
<td>0.38 ± 0.08*</td>
</tr>
<tr>
<td>In vitro metabolic clearance, ml·min⁻¹·mg microsomal protein⁻¹</td>
<td>0.37 ± 0.12</td>
<td>0.22 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SD; \(n = 6\). There were significant differences between the steatotic and normal group for lower fatty acid binding protein (L-FABP) and cytochrome P-450 tissue levels (*\(P < 0.05\)), whereas no significant differences were found for the respective microsomal protein tissue levels.
palmitate metabolic clearance in clofibrate-treated male rats and pregnant female rats compared with male control rats has been related with the different L-FABP levels (12). In the present study, we found that the disposition and metabolism of palmitate and its low-molecular-weight metabolites in a 17α-ethinylestradiol-induced steatotic rat model could also be described by the physiologically based slow diffusion of bound palmitate in the liver model (12, 16–18). This model provided better fits for palmitate disposition in hepatic steatosis than other potential models that have been used to describe the hepatic disposition of palmitate. We had previously reported an increased palmitate extraction ratio being associated with an increased L-FABP in clofibrate-treated and pregnant rat models (12). In contrast, this study showed that a lower E of palmitate in steatotic animals compared with untreated animals and that L-FABP was lower in steatosis than in normal rats.

Regression analysis showed that a rectangular hyperbola relationship exists between the E of unchanged [3H]palmitate and the L-FABP level [Fig. 5; $E = 0.39 \text{ L-FABP/(0.08 + L-FABP)} + 0.95 \text{ L-FABP, } r^2 = 0.79, n = 36$] in various animal models (male, clofibrate-treated male, normal female, pregnant female, and steatotic female). The relationship between E and L-FABP must be a rectangular hyperbola, because E is limited by 1. The results from the present study using the steatotic liver reiterated the previously reported role of L-FABP level as a major determinant in the hepatic disposition kinetics of palmitate.

It is generally recognized that the transmembrane uptake of fatty acids in the liver is driven by plasma membrane fatty acid transporters [membrane proteins for fatty acid translocase (FAT)/CD36; membrane FABP; and fatty acid transport protein (FATP)] and the cytosolic transport of fatty acids associated with cytosolic FABP or caveolin-1 (1, 3, 9, 12, 18, 39). These hepatic transport processes appear to be key rate determinants of palmitate hepatic extraction. Luxon and co-workers (18) reported that 1) increasing the cytosolic concentration of L-FABP caused a parallel increase in the rate of cytoplasmic palmitate transport in clofibrate-treated male rats; and 2) whereas higher levels of membrane transporters may explain the increased rates of influx and efflux in clofibrate-treated male rats, L-FABP could also directly catalyze transfer of fatty acids to and from the inner surface of the plasma membrane through a collisional mechanism. Weisiger and Zucker (35) have recently proposed a simple kinetic model for cytoplasmic diffusion and suggested that “membrane-active” FABPs are the soluble FABP that enhance faster cytoplasmic diffusion of fatty acids. However, hepatic extraction of palmitate and other solutes may also be influenced by the albumin contents in the perfusate, perfusion flow rate, and vascular dispersion (28, 34).

We have developed an equation approximating the model for the slowest step and comparing the effects of the relative magnitudes of the various parameters on hepatic extraction in
steady-state pharmacokinetics in our previous work (12). In the steady state, outflow concentration of palmitate from the liver can be described as

\[
\frac{C_{\text{out,SS}}}{C_{\text{in,SS}}} = \tilde{f}(0) = \frac{k_{in} - k_{out}k_{in}k_{out}}{(k_{in} + k_{out}) \tanh(k_{out}k_{in})}
\]  

(6)

where \(C_{\text{out,SS}}\) and \(C_{\text{in,SS}}\), respectively, are the outflow and inflow concentration of palmitate in the steady state, \(\tilde{f}_{B}\) is the extracellular time density of no-permeating reference (in this study Evans blue dye-labeled albumin), and \(k_{in}, k_{out}, k_{c}\), and \(k_{d}\) are the influx, efflux, elimination, and diffusion rate constants for \([3H]\)palmitate, respectively. From this steady-state analysis, we concluded that 1) the parameters for metabolism, permeability, and cytoplasmic diffusion are all rate-limiting determinants; and 2) the parameters for metabolic rate and cytoplasmic diffusion may, in reality, be interrelated, increasing the importance of cytoplasmic diffusion as a determinant of extraction. In this study, the smaller cytoplasmic diffusion constant of unchanged \([3H]\)palmitate in the steatotic group compared with the normal group (Table 4) with an associated lower \(E\) (Table 3) reinforces palmitate diffusion bound to L-FABP as the key determinant of its hepatic extraction.

In addition to fatty acid disposition in the liver being defined by the processes of membrane transport, intrahepatic mobilization via L-FABP, palmitate is metabolized in the liver by a number of pathways. Acyl-CoA oxidase is the rate-limiting enzyme in the peroxisomal \(\beta\)-oxidation pathway (37). Fatty acids are also eliminated in the liver through conversion to VLDL or oxidation by the microsomal \(\omega\)-oxidation (cytochrome P-450 4A-mediated hydroxylation) and mitochondrial \(\beta\)-oxidation system (26, 32). Feeding mice with a high-fat methionine- and choline-deficient diet for 5 wk produced steatohepatitis and was associated with a downregulation in hepatic fatty acid turnover and reduced mRNA levels for L-FABP and acyl-CoA oxidase (14). In this work, both L-FABP and cytochrome P-450 levels were reduced in steatotic animals (Table 2). It is therefore apparent that the smaller palmitate metabolic clearance (in vitro and in situ) reduced \(\text{AUC}_{\text{met}}\), and lower \(\text{MTT}_{\text{met}}\) in the steatotic liver (Tables 2–4) compared with normal untreated animals result from a reduction in both L-FABP intrahepatic transport and palmitate metabolism. Although 80% of palmitate taken up by the liver is converted to VLDL for export, Charlton et al. (4) have shown that the time frame is relatively long (~300 min) compared with other metabolic pathways. The \(\text{AUC}_{\text{met}}\) (nonparametric moments result) reported in Table 3 represents a short time frame (experimental observation, 3½ min) of metabolism (\(\beta\)- and \(\omega\)-oxidation). It seems likely that a negligible amount of radiolabeled VLDL would be produced during this short time frame. In contrast, the model-derived metabolic clearance reported in Table 4 represents a long time frame (theoretical prediction) of metabolism (VLDL conversion). Comparison of metabolic-related parameters (cytochrome P-450, in vitro metabolic clearance, \(\text{AUC}_{\text{met}}\), and model-derived metabolic clearance) between the steatotic and normal group (0.38/0.55 = 0.69 for CYP, 0.22/0.37 = 0.6 for in vitro metabolic clearance, 0.008/0.013 = 0.62 for \(\text{AUC}_{\text{met}}\), 10.9/14 = 0.78 for model-derived metabolic clearance) shows that steatosis has a higher impact on long-term metabolism (VLDL conversion) than on relatively short-term metabolism (\(\beta\)- and \(\omega\)-oxidation).

We have previously shown (12) that hepatic permeability for unchanged \([3H]\)palmitate significantly differs between male vs. clofibrate-treated male and female vs. pregnant female rats. In contrast, hepatic permeability for unchanged \([3H]\)palmitate appeared similar between the steatotic and normal groups in this work, suggesting that the effect of steatosis on palmitate disposition is mainly intrahepatocellular. It is possible that a difference exists with the intrinsic permeability in the steatotic group being lower but is being masked by a larger surface area in steatosis, because the steatotic group had a significantly larger sinusoidal albumin space than the normal group as estimated by kinetic analysis (Table 4), and this was also

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Table 4. Comparison of kinetic parameters derived from the slow diffusion/unbound model fitting for extracted \([3H]\)palmitate with trichloroacetic acid between the normal and \(17\alpha\)-ethynylestradiol-induced steatotic rat model

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>Normal</th>
<th>Steatosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic diffusion constant, per min</td>
<td>3.01±0.33</td>
<td>1.66±0.71†</td>
</tr>
<tr>
<td>Ratio of palmitate influx to efflux rate constants</td>
<td>2.35±0.82</td>
<td>2.14±0.95*</td>
</tr>
<tr>
<td>Permeability surface area product, (\text{ml} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1})</td>
<td>6.30±1.44</td>
<td>4.71±0.45*</td>
</tr>
<tr>
<td>(\text{AUC}<em>{\text{met}}), (\text{AUC}</em>{\text{met}})</td>
<td>14.0±1.73</td>
<td>10.9±1.79*</td>
</tr>
<tr>
<td>(\text{MTT}<em>{\text{met}}), (\text{MTT}</em>{\text{met}})</td>
<td>0.41±0.06</td>
<td>0.38±0.09</td>
</tr>
<tr>
<td>(\text{Vc}), (\text{Vc})</td>
<td>0.32±0.03</td>
<td>0.41±0.07*</td>
</tr>
<tr>
<td>(b_{\text{sinusoidal sucrose space}}, \text{mL/g liver})</td>
<td>0.86±0.08</td>
<td>1.19±0.19*</td>
</tr>
</tbody>
</table>

Values are means ± SD; \(n = 6\). *Given by product of elimination rate constant \(k_{e}\) and cellular volume of distribution \(V_{c}\). †sinusoidal reference space determined by Evans blue dye; ‡total water space determined by \([3H]\)water. There were significant differences between the steatotic and normal group for all kinetic values (*\(P < 0.05\); †\(P < 0.01\)).

Fig. 5. Rectangular hyperbola relationship between liver fatty acid binding protein (L-FABP) level and hepatic extraction ratio (E) of \([3H]\)palmitate in the various animal models [○: pregnant female (data from Ref. 12); ▲: normal female (data from Ref. 12); ▽: normal female; ●: steatotic female; ◇: normal male; ◆: clofibrate-treated male].
confirmed by histology (Fig. 1B). In this work, permeability has not been adjusted for fraction unbound in perfusate as described previously (12), because there have been suggestions that perfusate albumin facilitates palmitate uptake (2, 24).

The MTT of unchanged palmitate in the liver of the steatotic group was significantly increased (Table 3). Hepatic distribution of palmitate may be expected to be influenced by the presence of existing fatty deposits in steatosis (Fig. 1B), especially because there was a larger liver weight, sinusoidal albumin space, and hepatocellular water volume in the steatotic group (Table 4). However, estimates of hepatocellular-to-sinusoidal palmitate partition coefficients, based on the ratio of influx-to-efflux rate constants, and cytoplasmic diffusion constant of palmitate were significantly lower in the steatotic group (Table 5). Nevertheless, the expression of other plasma membrane fatty acid transporters (FAT/CD36 and FATP) may also be reduced in steatosis and hence influence the distribution of palmitate in liver.

The CV² for a solute in the liver is a function of three terms: 1) due to vascular dispersion, 2) due to diffusion in tissue, and 3) due to tissue distribution clearance (29, 36). The CV² values for [³H]palmitate reported in this work (normal = 2.66 ± 0.25, steatosis = 2.53 ± 0.25) are much larger than that of [¹⁴C]sucrose (~0.5), which only dispersed in the vascular plus Disse space. Therefore, a larger CV² value means the hepatic disposition of [³H]palmitate was determined not only by vascular dispersion but also by diffusion and distribution clearance in the tissue.

In conclusion, 17α-ethinylestradiol treatment was found to reliably produce steatosis in rat livers that reflected some of the early symptoms of human nonalcoholic fatty liver disease such as changed serum biochemistry levels, higher ALT-to-AST ratio compared with normal, light microscopically visible microvesicular steatosis. Furthermore, the steatotic group showed significantly lower L-FABP, cytochrome P-450 levels, and in vitro metabolic clearance. 17α-ethinylestradiol treatment resulted in significantly decreased E of palmitate, decreased production of low-molecular-weight metabolites, and a lower MTTₘₚₑₜ in the steatotic liver. The model-derived parameters of metabolic clearance, permeability-surface area product, ratio of influx to efflux rate constants, and cytoplasmic diffusion constant, were significantly decreased in the 17α-ethinylestradiol-treated group. Fatty acid transport in steatotic livers appears to be compromised by the reduction in L-FABP being found in the (early) 17α-ethinylestradiol-induced steatosis. Consistent with our previous work in male, clofibrate-treated male, female, and pregnant rats, L-FABP therefore appears to be a predictor of the hepatic ratio of palmitate extraction.

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GRANTS

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