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

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Hung, Daniel Y., 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 [3H]palmitate and its low-molecular-weight metabolites in perfused normal and steatotic rat liver were studied using the multiple indicator dilution technique and a physiologically based slow diffusion/bound pharmacokinetic model. The steatotic rat model was established by administration of 17α-ethinylestradiol to female Wistar rats. Serum biochemistry markers and histology of treated and normal animals were assessed and indicated the presence of steatosis in the treatment group. The steatotic group showed a significantly higher alanine aminotransferase-to-aspartate aminotransferase ratio, lower levels of liver fatty acid binding protein, and histology of treated and normal animals were assessed and indicated the presence of steatosis in the treatment group. The steatotic group showed a significantly higher alanine aminotransferase-to-aspartate aminotransferase ratio, lower levels of liver fatty acid binding protein, and cytochrome P-450, as well as microvesicular steatosis with an enlargement of sinusoidal space. Hepatic extraction for unchanged [3H]palmitate and production of low-molecular-weight metabolites were found to be significantly decreased in steatotic animals. Pharmacokinetic analysis suggested that the reduced extraction and sequestration for palmitate and its metabolites was mainly attributed to a reduction in liver fatty acid binding protein in steatosis. 17α-ethinylestradiol; hepatic palmitate disposition

NONALCOHOLIC FATTY LIVER DISEASE is increasingly recognized as a major health burden and probably the most common of all liver disorders (10, 22). The prevalence of excessive hepatic fat accumulation in the general population in the United States and other Western countries has been estimated to be 20–30% (19, 22, 23). In the majority of cases, the condition does not develop into more severe liver disease, although 20–30% of patients at the time of diagnosis show signs of steatohepatitis and are consequently at a higher risk to progress to cirrhosis, liver failure, or hepatocellular hepatoma (1a, 6, 25). The effects of excess of intracellular fatty acid concentrations, oxidant stress, ATP depletion, and mitochondrial dysfunction apparently all contribute in different ways to the subsequent hepatocellular injury, but mitochondrial dysfunction is thought to play an especially important role in the development of the condition (23).

A question remains, however, as to what are the physiological and metabolic changes occurring in the very early (and undiagnosed) stages of fatty liver disease. To date, the hepatic kinetics-associated free fatty acid disposition and metabolism in the presence of lipid deposits in hepatic tissue has been poorly characterized. We have recently related the hepatic disposition kinetics 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 symptomatological aspects of the early stages of fatty liver disease. A physiology-based pharmacokinetic model (slow diffusion/ bound model) that accommodates the changed cytoplasmic permeation 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, [1H]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 nM −1 cm −1 in absorption at peak position (~450 nm) as described by Matsubara and co-workers (20).

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In each liver, a maximum of two injections \( \text{bolus 1: purified } [3H]\text{palmitate (3 \times 10^6 dpm), Evans blue dye (3 mg/ml), and } [14C]\text{sucrose (1.5 \times 10^6 dpm); bolus 2: } [3H]\text{water (3 \times 10^6 dpm)} \) 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]\text{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 \times 20, 4 s \times 5, 10 s \times 5, 30 s \times 4), and aliquots (100 \mu l) were taken for scintillation counting using a MINAXI beta TRI-CARB 4000 series liquid-scintillation counter (Packard Instruments). Aliquots (100 \mu l) were also removed from the outflow samples for absorption spectrophotometric analysis of Evans blue dye at 620 nm using a Spectracount plate counter (Packard)\].

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

Low-molecular-weight metabolites of \([3H]\text{palmitate were separated from the effluent nonextracted samples by an ultracentrifugation method using Millipore Microcon YM-30 filter devices (30,000 MWCO, Millipore) and were counted by a beta liquid-scintillation counter (Packard), and thin layer chromatography assay was used to confirm that the radioactivity measured in the ultrafiltrate did not represent \([3H]\text{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 (AUC_{net}) and MTT metabolites in the liver (MTT_{net}) was determined from the perfusate outflow concentration-time profile of these separated metabolites. MTT_{net} 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]\text{palmitate (3 \times 10^6 dpm) was performed at } 37^\circ C. \text{ Samples were then collected at 0, 5, 10, and 20 min. Unchanged } [3H]\text{palmitate was extracted with trichloroacetic acid as described above. The logarithm of the radioactivity of unchanged } [3H]\text{palmitate remaining in solution was plotted against time to obtain a slope and the extrapolated initial radioactivity at time } 0) \text{. 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 correlation for catheter effects was used to estimate the sinusoidal albumin space \( V_{\text{Palmit;}} \) determined by Evans blue dye) or the sinusoidal sucrose space \( V_{\text{Sucrose;}} \) determined by \([14C]\text{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 \([3H]\text{water.}\)
HEPATIC DISPOSITION OF PALMITATE IN STEATOSIS

\[ F = \frac{Q \cdot AUC}{D} \]  

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 concentrations used were expressed in molar equivalents. Hepatic extraction ratio \( (E) = 1 - F \).

\[ MTT = \frac{AUC}{AUC} \]  

where AUMC is the area under the first moment curve.

\[ CV^2 = \frac{\sigma^2}{MTT^2} \]  

where

\[ \sigma^2 = \frac{\int_0^\infty C(t)dt}{MTT^2} \]

Statistical analysis. All data are presented as means \( \pm SD \) 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 \( \pm SD \) \((n = 6)\) liver wet weight of animals used in the perfusion studies was 10.2 \( \pm \) 1.1 g for control animals and 12.5 \( \pm \) 0.9 g for steatotic rats \((P < 0.05)\). During liver perfusion, steatotic rats had significantly lower bile flow \((P < 0.05)\). The mean \( \pm SD \) bile flow \((n = 6)\) was 0.57 \( \pm \) 0.12 \( \mu \)l\( \bullet \)min\(^{-1}\)\( \bullet \)g liver\(^{-1}\) for normal rats and 0.38 \( \pm \) 0.09 \( \mu \)l\( \bullet \)min\(^{-1}\)\( \bullet \)g liver\(^{-1}\) for steatotic rats. The hepatic oxygen consumption for all animals was in the range of 1.39 to 1.75 \( \mu \)mol\( \bullet \)min\(^{-1}\)\( \bullet \)g liver\(^{-1}\), and perfusion pressure was in the range of 10.1 to 12.3 cmH\(_2\)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 \((\text{Fig.} \ 1A)\). Liver slices from 17\( \alpha \)-ethinylestradiol-treated animals showed signs of microvesicular steatosis and a pronounced enlargement of the sinusoidal space \((\text{Fig.} \ 1B)\).

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, triglyceride, 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 metabolic clearance than the normal group, whereas no significant
differences were found in microsomal protein levels between both groups.

Table 3 shows a comparison of nonparametric moments for unchanged [3H]palmitate (extracted with trichloroacetic acid) and low-molecular-weight metabolites of palmitate (separated by ultrafiltration) between normal and steatotic rats. The steatotic group showed significantly decreased E of palmitate, AUCmet, and MTTmet in the liver compared with the normal group, whereas the MTT of unchanged palmitate in the liver of the steatotic group was significantly increased. No significant difference in CV^2 values was found between these two groups.

Figure 3 shows typical normalized concentration-time profiles (normal scale and semilogarithmic scale) for unchanged [3H]palmitate, low-molecular-weight metabolites of [3H]palmitate, Evans blue dye-labeled albumin, [14C]sucrose, and [3H]water in the isolated perfused normal and steatotic rat liver. The normal group had a significantly smaller area under the curve (higher hepatic extraction) of unchanged [3H]palmitate, higher efflux of low-molecular-weight metabolites of [3H]palmitate, shorter mean transit time of Evans blue dye-labeled albumin (smaller sinusoidal albumin space), and also shorter mean transit time of [3H]water (smaller hepatocellular water volume) during the perfusion compared with the steatotic group, whereas the mean transit time of [14C] sucrose did not appear to be different. Figure 4 shows corresponding regression line fits for unchanged [3H]palmitate using a slow diffusion/bound model and a data weighting of 1/\(y_{obs}^2\) 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 [3H]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 [3H]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 [3H]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 17α-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 17α-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 17α-ethynylestradiol-treated group, coincide with the clinical characterization of nonalcoholic fatty liver disease (21, 31). We also observed that 17α-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 17α-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
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 L-FABP/(0.08 + L-FABP) + 0.95 L-FABP, r² = 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
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 \(^{3}H\)palmitate in the steatotic group compared with normal untreated animals result from a reduction in both L-FABP intracellular transport and palmitate metabolism. Although 80% of palmitate taken up by the liver is converted to VLDL or oxidation by the microsommal \(\omega\)-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 \(P-450\) 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 AUC\(_{\text{met}}\) and lower MTT\(_{\text{met}}\) in the steatotic liver (Tables 2–4) compared with normal untreated animals result from a reduction in both L-FABP intracellular transport and palmitate metabolism. Although 80% of palmitate taken up by the liver is converted to VLDL or export, Charlton et al. (4) have shown that the time frame is relatively long (>300 min) compared with other metabolic pathways. The AUC\(_{\text{met}}\) (nonparametric moments result) reported in Table 3 represents a short time frame (experimental observation, 3½ min) of metabolism (VLDL conversion). Comparison of metabolic-related parameters (cytochrome \(P-450\), in vitro metabolic clearance, 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 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 \(^{3}H\)palmitate significantly differs between male vs. clofibrate-treated male and female vs. pregnant female rats. In contrast, hepatic permeability for unchanged \(^{3}H\)palmitate appeared similar between the steatotic and normal groups in this work, suggesting that the effect of steatosis on palmitate disposition is mainly intracellular. 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

steady-state pharmacokinetics in our previous work (12). In the steady state, outflow concentration of palmitate from the liver can be described as

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

(6)

where \(C_{\text{out,SS}}\) and \(C_{\text{in,SS}}\), respectively, are the outflow and inflow concentration of palmitate in the steady state, \(f_B\) is the extracellular time density of no-permeating reference (in this study Evans blue dye-labeled albumin), \(k_{in}\), \(k_{out}\), \(k_d\), and \(k_{out}\) are the influx, efflux, elimination, and diffusion rate constants for \(^{3}H\)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 addition to fatty acid disposition in the liver being defined by the processes of membrane transport, intracellular 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 microsommal \(\omega\)-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 \(P-450\) 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 AUC\(_{\text{met}}\) and lower MTT\(_{\text{met}}\) in the steatotic liver (Tables 2–4) compared with normal untreated animals result from a reduction in both L-FABP intracellular transport and palmitate metabolism. Although 80% of palmitate taken up by the liver is converted to VLDL or export, Charlton et al. (4) have shown that the time frame is relatively long (>300 min) compared with other metabolic pathways. The AUC\(_{\text{met}}\) (nonparametric moments result) reported in Table 3 represents a short time frame (experimental observation, 3½ min) of metabolism (VLDL conversion). Comparison of metabolic-related parameters (cytochrome \(P-450\), in vitro metabolic clearance, 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 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 \(^{3}H\)palmitate significantly differs between male vs. clofibrate-treated male and female vs. pregnant female rats. In contrast, hepatic permeability for unchanged \(^{3}H\)palmitate appeared similar between the steatotic and normal groups in this work, suggesting that the effect of steatosis on palmitate disposition is mainly intracellular. 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

![Fig. 5. Rectangular hyperbola relationship between liver fatty acid binding protein (L-FABP) level and hepatic extraction ratio (E) of \(^{3}H\)palmitate in the various animal models.](image-url)

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

<table>
<thead>
<tr>
<th>Animal Model</th>
<th>Cytoplasmic diffusion constant, per min</th>
<th>Ratio of palmitate influx to efflux rate constants</th>
<th>Permeability surface area product, (\text{ml} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1})</th>
<th>(^{a})Metabolic clearance, (\text{ml} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1})</th>
<th>(^{b})Sinusoidal sucrose space, ml/g liver</th>
<th>(^{c})Sinusoidal albumin space, ml/g liver</th>
<th>(^{d})Total water space, ml/g liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.01 ± 0.33</td>
<td>2.35 ± 0.82</td>
<td>6.30 ± 1.44</td>
<td>14.0 ± 1.73</td>
<td>0.37 ± 0.06</td>
<td>0.41 ± 0.07</td>
<td>0.86 ± 0.08</td>
</tr>
<tr>
<td>Steatosis</td>
<td>1.66 ± 0.71†</td>
<td>2.14 ± 0.95*</td>
<td>4.71 ± 0.45*</td>
<td>10.9 ± 1.79*</td>
<td>0.32 ± 0.03</td>
<td>0.41 ± 0.07*</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 \(^{3}H\)water. There were significant differences between the steatotic and normal group for all kinetic values (\(* P < 0.05\); †\(P < 0.01\)).
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, sinusoidal and hepatocellular volumes, and membrane transport (13), are slightly lower in the steatotic liver. It is therefore apparent that palmitate distribution in steatotic liver is mainly determined by L-FABP, which is significantly lower in the steatotic group (Table 2). 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 ± 0.11) 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 MTTₘₑₙ 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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