Assessing Steatotic Liver Function after Ischemia-Reperfusion Injury by In Vivo Multiphoton Imaging of Fluorescein Disposition

Camilla A. Thorling, Lu Jin, Michael Weiss, Darrell Crawford, Xin Liu, Frank J. Burczynski, David Liu, Haolu Wang, and Michael S. Roberts

School of Pharmacy and Biomedical Sciences, University of South Australia, Adelaide, Australia (C.A.T., M.S.R.); School of Medicine, University of Queensland, Wolloongabba, Queensland, Australia (C.A.T., M.W., D.C., X.L., D.L., H.W., M.S.R.); Nanjing Drum Tower Hospital Affiliated to the Medical School of Nanjing University, Nanjing, People’s Republic of China (L.J.); Department of Pharmacology, Martin Luther University Halle-Wittenberg, Halle, Germany (M.W.); and Faculty of Pharmacy, University of Manitoba, Winnipeg, Manitoba, Canada (F.J.B.)

Received September 4, 2014; accepted November 7, 2014

ABSTRACT

Ischemia-reperfusion injury, a common complication during liver surgery where steatotic livers are more prone to the injury, may become more prevalent in the growing obese population. This study characterizes liver morphology toward understanding changes in subcellular function in steatotic livers exposed to ischemia-reperfusion injury through quantitative description of fluorescein distribution obtained by minimally invasive in vivo multiphoton microscopy using a physiologic pharmacokinetic model. Rats were fed a high-fat diet for 7 days to induce liver steatosis. Partial ischemia was induced after reperfusion for 4 hours, when fluorescein (10 mg/kg) was injected intravenously. Liver images, bile, and blood were collected up to 180 minutes after injection. Ischemia-reperfusion injury was associated with an increase in alanine transaminase levels and apoptosis. In addition, steatosis featured lipid droplets and an increase in fluorescein-associated fluorescence observed in hepatocytes via multiphoton imaging. Analysis of the hepatic concentration-time profiles has suggested that the steatosis-induced increase in fluorescein-associated fluorescence mainly arises by inducing hepatic fluorescein metabolism. The combination of ischemia-reperfusion with steatosis exacerbates these effects further. This was confirmed by fluorescence lifetime imaging microscopy showing a decreased average fluorescence lifetime of the liver, which is indicative of an increased production of the metabolite. Our results show the potential of noninvasive dye imaging for improving our understanding of liver disease induced by subcellular changes in vivo, providing further quantitative measures of metabolic and biliary liver function, and hence extending the qualitative liver function tests now available.

Introduction

Approximately 5–10% of the general population are diagnosed with cirrhosis, the end result of many types of liver disease. It accounts for 4.4% of all deaths among adults in developed countries (Lim and Kim, 2008), and liver transplantation is the only immediate treatment (Jonas et al., 2001). Fibrosis is treatable in its early stages, so accurate staging of the extent of fibrosis is important for optimal treatment (He et al., 2010). Liver function is traditionally assessed by static measures, which cannot track changes in liver function quickly. Liver biopsy is the gold standard in evaluating fibrosis, but there is a risk of bleeding complications and false-negative results. Liver biopsies provide only a snapshot image of the liver tissue and do not allow dynamic monitoring of morphologic events (Goetz et al., 2008b). Staining efficiency variation, cost, and the impracticality of mass screening are some further disadvantages of liver biopsy (Lin et al., 2012). Performing serial liver biopsies to accurately determine disease progression or monitor treatment effects is not ideal (He et al., 2010). In addition, scoring systems to assess fibrosis use qualitative rather than quantitative measures, so it is difficult to obtain highly reproducible results without a high degree of intraobserver and interobserver discrepancy (as high as 35%) (Tai et al., 2009).

Noninvasive imaging has the potential to extract extra information over a long period of time, over a larger sampling area, and with minimal morbidity (He et al., 2010). Noninvasive biomedical imaging techniques such as ultrasonography, computed tomography, and magnetic resonance imaging are widely used to detect liver disease, but they do not have sufficient sensitivity, spatial resolution, or specificity to detect or stage liver disease (Lin et al., 2012). By contrast, virtual biopsies obtained via fluorescence imaging can give an overall view of the liver (Goetz et al., 2008a) as well as provide dynamic information (Goetz et al., 2008b). The latter technique remains useful regardless of sample preparation (e.g., frozen or paraffin-embedded tissue), is easy and fast, has no sampling bias (Gailhouste et al., 2010), and may potentially quantify liver diseases such as fibrosis (He et al., 2010). However, to provide functional information regarding uptake, metabolism, and excretion, a fluorescent dye must be administered.

Fluorescein, a nontoxic dye approved by the U.S. Food and Drug Administration for diagnostic angiography of the retina, is mainly excreted by the kidneys and liver (Neumann et al., 2010). Fluorescein has been used to study liver function in humans, permitting visualization

This work was supported by the National Health and Medical Research Council [Grants 569710, APP1002611]. C.A.T. and L.J. contributed equally to this work.

dx.doi.org/10.1124/dmd.114.060848.

ABBREVIATIONS: ALT, alanine transaminase; CL, clearance; FLIM, fluorescence lifetime imaging microscopy; FG, fluorescein monoglucuronide; I/R, ischemia-reperfusion; MPM, multiphoton microscopy.
of hepatocytes, bile ducts, sinusoids, and collagen fibers in vivo (Goetz et al., 2008a). Fluorescein, which rapidly diffuses throughout the body in seconds, is taken up by the hepatocytes, metabolized to fluorescein monoglucuronide (FG), and excreted into the bile by active transport (Sherman and Fisher, 1986). Tissue architecture and vessels can be well visualized, although nuclei are not well seen (Goetz et al., 2008b). Fluorescein and FG have overlapping excitation and emission spectra but different fluorescence lifetimes, which makes fluorescence lifetime imaging to detect these lifetime changes possible. Our group has shown that fluorescence lifetime imaging microscopy (FLIM) can study the metabolism of fluorescein to FG (Thorling et al., 2011a). In this study, we used clinically approved fluorescein to examine liver subcellular function changes induced by liver disease.

Because the obese population has been increasing, the number of individuals with hepatic steatosis has been growing accordingly, which may impact the availability of suitable liver donors (Nativ et al., 2012). Steatotic liver grafts are more susceptible to ischemia-reperfusion (I/R) injury, with increased lipid peroxidation (Vairetti et al., 2009; Ben Mosbah et al., 2010), increased release of proinflammatory mediators (Selzner and Clavien, 2001; Vairetti et al., 2009), and increased neutrophil infiltration (Nakano et al., 1997). Steatosis also increases the cellular volume, resulting in narrowed microvessels and thus reduced blood flow (Nakano et al., 1997; Ben Mosbah et al., 2010). These factors put steatotic donor grafts at great risk of primary nonfunction (Busuttil and Clavien, 2001; Vairetti et al., 2009), and increased neutrophil infiltration (Nakano et al., 1997; Ben Mosbah et al., 2010). These factors put steatotic donor grafts at great risk of primary nonfunction (Busuttil and Clavien, 2001; Vairetti et al., 2009), and increased neutrophil infiltration (Nakano et al., 1997; Ben Mosbah et al., 2010).

Materials and Methods

Chemicals and Reagents. Sodium fluorescein and β-glucuronidase were obtained from Sigma-Aldrich (St. Louis, MO). Ilium xylazine and ketamine hydrochloride were purchased from Bayer Australia (Pymble, New South Wales, Australia) and Parnell Laboratories (Alexandria, New South Wales, Australia), respectively.

Animals. Male Wistar rats were purchased from the Animal Resource Centre (Perth, Australia), and housed at the Pharmacy Australia Centre of Excellence in the University of Queensland Biologic Resources Facility, where they had unlimited access to food and water. The temperature was maintained at 20 ± 1°C and humidity at 60–75%, with artificial light for 12 hours (7 AM to 7 PM) daily. All animals received human care, and the study was approved by the Animal Ethics Committee at the University of Queensland.

Surgical Procedures. All rats were first anesthetized by an intraperitoneal injection of ketamine hydrochloride (80 mg/kg) and ilium xylazine (10 mg/kg), which was maintained throughout the experiment by administering ketamine (2.2 mg/100 g) and ilium xylazine (0.25 mg/100 g). Their body temperatures were controlled by placing them on a heating pad set to 37°C. Figure 1 shows a schematic flowchart of the experiments.

Ischemia-Reperfusion Injury Model. Ischemia (70%) was induced by using a microvascular clamp to clamp the portal vein, hepatic artery, and bile duct that supply the median and left lobes. After 1 hour, we removed the clamp to allow reperfusion of the liver, and the rats woke during this time under close observation. After the liver had reperfused for 3 hours, the rats were anesthetized again; the jugular vein and carotid artery were cannulated for sodium fluorescein administration—10 mg/kg bolus after 4 hours of reperfusion—and blood collection, respectively. A midline laparotomy was performed to expose the liver. The bile duct was cleared of surrounding tissue and was cannulated for bile collection. All cannulations were performed using PE-10 tubing (Becton, Dickinson and Company, Parsippany, NJ). The left lobe was slightly raised above the intraperitoneal cavity, and 0.9% saline was administered continuously.

Steatosis Model. Steatosis was induced in rat livers by feeding rats a high-fat diet, based on the diet used by Arnault et al. (2003), which was obtained from Specialty Feeds Pty. Ltd. (Glen Forrest, Australia), for 7 days before induction of ischemia.

Tissue Collection. The blood collected after 4 hours of reperfusion was analyzed for alanine transaminase (ALT) by use of a Hitachi 747 analyzer (Hitachi Ltd., Tokyo, Japan). Blood and bile were collected for measurements of fluorescein and FG, respectively, and the liver was collected for histologic assessment.

Histopathologic Analysis. Fixed liver tissue was sectioned onto each slide, stained, and scanned using an AperioScanScope XT slide scanning system (Aperio Technologies, Vista, CA), as previously described elsewhere (Thorling et al., 2011b). The number of monocytes and macrophages were recognized by counting ED1-positive cells in the sinusoids in ten random fields per slide. The number of apoptotic cells was identified by counting Apoptag-positive nuclei in 10 random fields per liver slide per region (periportal, centrlobular, and midzonal) of the liver.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>ALT levels in plasma and inflammatory cell infiltration (monocytes/macrophages) in normal and steatotic livers with and without I/R injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>ED1 (Monocytes/Macrophages)</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Sham</td>
<td>54.3 ± 7.1</td>
</tr>
<tr>
<td>I/R</td>
<td>1330.8 ± 280.2*</td>
</tr>
<tr>
<td>Steatosis</td>
<td>40.3 ± 4.8</td>
</tr>
<tr>
<td>Steatosis + I/R</td>
<td>556 ± 81.3*</td>
</tr>
</tbody>
</table>

*p < 0.05 versus sham.
High-Performance Liquid Chromatography. Concentrations of fluorescein and FG in each collected bile sample were determined by high-performance liquid chromatography analysis (Shimadzu, Kyoto, Japan) as previously described elsewhere (Thorling et al., 2011a). In brief, the mobile phase consisted of 50 mM NaH₂PO₄ and methanol (2:3, v/v). The flow rate was set at 1 ml/min flow through a C18 column (4.6×150 mm, 5 µm; Agilent, Santa Clara, CA). The fluorescence excitation and emission wavelengths were 488 and 515 nm, respectively, with a retention time of 5 minutes.

Multiphoton Microscopy. MPM images were recorded by a DermaInspect system (JenLab GmbH, Jena, Germany) with an ultrashort pulsed, modelocked, 80-MHz Titanium:Sapphire MaiTai laser (Spectra Physics, Mountain View, CA). The excitation wavelength was set to 740 nm for liver autofluorescence or 920 nm for fluorescein fluorescence, with an emission range of 350–650 nm. The laser power was set to 20 mW, and the acquisition time was 7.4 seconds per frame. Images were recorded at intervals up to 180 minutes after fluorescein injection, using 10× and 40× objective magnification.

Fluorescence Lifetime Imaging Microscopy. FLIM was performed using a TCSPC830 detection module (Becker and Hickl, Berlin, Germany) characterized by the time resolution of 100 ps. FLIM data of fluorescein and FG were obtained in the emission range of 515 to 620 nm. Fluorescence lifetime images were analyzed using SPCImage (Becker and Hickl, Berlin, Germany). Each pixel of a given fluorescence lifetime image contains photon decay data, to which an exponential function is fitted to retrieve the fluorescence lifetime value(s). These lifetimes characterize the decay and, by extension, the fluorophore(s) present in the sample. Fluorescence lifetimes reported here are the mean ± S.E.M. of the values measured at the highest-intensity pixel in all images.

Fig. 2. Morphologic changes associated with I/R injury in steatosis. (A) Positively stained apoptotic cells in the perportal, centrilobular, and midzonal regions of the liver acinus. Black arrows indicate positively stained cells as measured by ApopTag. Black hollow arrows indicate lipid droplets. (B) MPM images of the liver at low (10×) and high (40×) objective magnification before injection of fluorescein. The white arrow points to a lipid droplet inside the hepatocyte.
Image Analysis and Physiologic Pharmacokinetic Modeling. Liver images were obtained over time. The fluorescence intensity of fluorescein in sinusoids and hepatocytes was determined separately using ImageJ 1.44p (U.S. National Institutes of Health, http://imagej.nih.gov/ij/) and was converted into concentrations using a standard curve prepared in homogenized liver. The fluorescein concentrations in the sinusoids \( [C_{\text{sin}}(t)] \) and hepatocytes \( [C_{\text{hep}}(t)] \) together with the fluorescein amounts excreted into the bile as parent compound fluorescein \( (\text{OF}) \) or as metabolite \( [A_{\text{met},i}(t)] \) were measured over time and were fitted simultaneously into a compartment model (see Fig. 4B) modified from a previous one (Weiss et al., 2013). The uptake clearance \( (\text{CL}_{\text{uptake}}) \) controls transport of fluorescein from sinusoids into hepatocytes; fluorescein elimination from hepatocytes is governed by its metabolism to FG \( (k_{\text{met}}) \) and biliary excretion of fluorescein \( (k_{\text{bile},F}) \) and FG \( (k_{\text{bile,FG}}) \). The time course of the fluorescein amount in sinusoids was described by a biexponential function with the parameters of \( A, B, \alpha, \) and \( \beta \) and used as a fixed forcing function. The differential equations for the model are as follows:

\[
\frac{dA_{\text{sin}}(t)}{dt} = Ae^{-\alpha t} + Be^{-\beta t} - \frac{\text{CL}_{\text{uptake}}}{V_{\text{sin}}} A_{\text{sin}}
\]

\[
\frac{dA_{\text{hep}}(t)}{dt} = \frac{\text{CL}_{\text{uptake}}}{V_{\text{sin}}} A_{\text{sin}} - (k_{\text{met}} + k_{\text{bile,F}}) A_{\text{hep}}
\]

\[
\frac{dA_{\text{hep},F}(t)}{dt} = k_{\text{met}}A_{\text{hep}} - k_{\text{bile,FG}}A_{\text{hep},F}
\]

\[
\frac{dA_{\text{hep},FG}(t)}{dt} = k_{\text{bile,FG}}A_{\text{hep},F}
\]

with \( C_{\text{sin}}(t) = A_{\text{sin}}(t)/V_{\text{sin}} \) and \( C_{\text{hep}}(t) = A_{\text{hep}}(t)/V_{\text{hep}} \).

<table>
<thead>
<tr>
<th></th>
<th>Periportal</th>
<th>Centrilobular</th>
<th>Midzonal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>7.4 ± 2.5</td>
<td>2.0 ± 0.6</td>
<td>5.2 ± 1.7</td>
</tr>
<tr>
<td>I/R</td>
<td>17.8 ± 4.9</td>
<td>43.2 ± 12.8( ^b )</td>
<td>89.5 ± 19.5( ^b )</td>
</tr>
<tr>
<td>Steatosis</td>
<td>8.6 ± 4.2</td>
<td>2.0 ± 0.5</td>
<td>5.3 ± 2.1</td>
</tr>
<tr>
<td>Steatosis + I/R</td>
<td>7.9 ± 3.2</td>
<td>25.5 ± 6.7( ^a,b )</td>
<td>84.5 ± 21.5( ^a,b )</td>
</tr>
</tbody>
</table>

\( ^aP < 0.05 \) versus sham.

\( ^bP < 0.05 \) versus steatosis.

Data Analysis. A population approach was used to estimate the various rate constants, the uptake clearance, and other parameters used to describe the model \( (A, \alpha, B, \beta, \text{CL}_{\text{uptake}}, k_{\text{bile,F}}, k_{\text{met}}, k_{\text{bile,FG}} \) and \( V_{\text{hep}} \) (see Fig. 4A) using the package ADAPT 5 (D’Argenio et al., 2009). The maximum likelihood expectation maximization program available in the package provides estimates of the population mean and intersubject variability as well as of the individual subject parameters (conditional means). Model parameters were assumed to be log-normally distributed. The standard deviation of measurement error was assumed to be a linear function of the measured quantity. All data sets including \( C_{\text{sin}}(t), C_{\text{hep}}(t), A_{\text{bile}}(t) \) and \( A_{\text{bile,met}}(t) \) were fitted simultaneously to estimate all parameters.

Statistical Analysis. An unpaired, nonparametric \( t \) test (Mann-Whitney) was used to compare the population mean of each parameter to evaluate the steatosis and I/R injury effect. \( P \leq 0.05 \) was considered statistically significant.

Results

ALT and Morphologic Changes. Serum ALT levels were significantly elevated in normal \( (1330.8 ± 280.2 \) U/l) and steatotic...
(556 ± 81.3 U/l) rats subjected to I/R injury compared with sham (54.3 ± 7.1 U/l) as shown in Table 1.

Apoptotic cells were counted in each of three different regions of the liver acinus (periportal, centrilobular, and midzonal) (Fig. 2B; Table 2). Black arrows indicate positively stained cells. The number of positively stained cells was significantly increased in the centrilobular and midzonal regions in normal (89.5 ± 19.5) and steatotic (84.5 ± 21.5) livers exposed to I/R injury (P < 0.05) compared with sham (5.2 ± 1.7) and steatosis (5.3 ± 2.1). In addition, hollow black arrows show lipid droplets, characteristic of steatotic livers. These lipid droplets were also

Fig. 4. Physiologically based pharmacokinetic model. (A) Schematic overview of compartmental model describing hepatic uptake and elimination kinetics of fluorescein. (B) Plasma concentration-time profiles of fluorescein in normal (sham) rats and rats with steatotic livers with and without I/R injury. The symbols represent mean values, and the error bar represents S.E.M. (n = 3–4). (C) Fluorescein concentration-time profiles in the sinusoid and hepatocyte compartments. (D) Corresponding accumulation of fluorescein in the sinusoid and hepatocyte compartments.
seen in the MPM images (white arrows, Fig. 2B). Figure 2B shows a representative imaged liver for each group, at original objective magnification 10× and 40×, respectively. The sham livers had well-defined liver acinus and hepatocytes, but the structure became less defined when exposed to I/R injury. In steatotic livers, we began to find lipid droplets (white arrows), which are better seen in the higher magnification images.

**Fluorescein Disposition in Normal and Steatotic Livers—In Vivo Multiphoton Imaging.** Images of fluorescein disposition were continuously recorded in vivo after bolus injection of fluorescein to monitor uptake and excretion of the dye (Fig. 3). The distribution of fluorescein at 2 minutes was irregular in the steatosis group compared with sham. At 40 minutes, the fluorescence intensity was higher in steatotic livers with or without I/R injury and at 180 minutes the distribution of fluorescein was higher in the diseased groups compared with sham.

**A Physiologic Pharmacokinetic Model of Fluorescein Disposition in Normal and Steatotic Livers after Ischemia-Reperfusion Injury.** The various substructures in the liver were represented as subcompartments, as seen in Fig. 4A. The plasma concentrations of fluorescein did not differ significantly between the four groups (Fig. 4B). The concentrations of fluorescein in the sinusoidal and hepatocyte subcellular compartments are shown in Fig. 4C. The fitting curve shows the goodness of the fit. The corresponding area under the curve in sinusoids and hepatocytes showed no significant change (Fig. 4D). However, the rate constant for metabolism in the liver cytosol, $k_{met}$ was significantly increased in steatotic livers with and without I/R injury compared with sham and compared with I/R injury in the steatosis + I/R group (Table 3). Additionally, the average fluorescence lifetime ($\tau_{av}$) was significantly decreased in steatotic livers at 1 minute and 40 minutes after injection of fluorescein compared with sham and in steatotic livers exposed to I/R injury at 20 minutes after injection compared with sham and I/R injury (Fig. 5A). This decrease was also seen in the color-coded images in Fig. 5B, where the decrease in lifetime was indicative of an increase in blue color (red arrow).

**Fluorescein Biliary Excretion in Normal and Steatotic Livers after Ischemic Reperfusion Injury.** The biliary excretion rate of fluorescein was significantly decreased in steatotic livers at 40 minutes after injection and in steatotic livers exposed to I/R injury at 30, 40, 50, and 60 minutes after injection compared with sham (Fig. 6A). The total recovery of fluorescein in bile was also significantly lowered in steatotic livers at 60 and 120 minutes after injection and in steatotic livers exposed to I/R injury at 40, 50, 60 and 120 minutes after injection compared with sham (Fig. 6B). However, the biliary excretion rate and cumulative amount recovered in bile for the FG metabolite were not significantly different between sham and the other groups (Fig. 6, A and B). The average bile flow rate up to 180 minutes after injection was significantly decreased ($P < 0.05; n = 3–6$) in steatotic livers with I/R injury (0.008 ± 0.001 ml/min) compared with sham (0.02 ± 0.004 ml/min), steatosis (0.02 ± 0.001), and I/R of normal livers (0.02 ± 0.004 ml/min) (Fig. 6C).

**Discussion.** A number of studies have reported the noninvasive subsurface imaging of the liver after laparotomy both in animals and in human patients. One of the earliest animal studies used fluorescence microscopy with fluorescein as an indicator (Hanzon, 1952). In this work, we have gone a step farther in evaluating the changes in the subcellular function of the liver in steatosis with I/R injury by using multiphoton subsurface in vivo imaging in combination with physiologic pharmacokinetic modeling.

The animal model of steatosis with I/R injury was developed on the advice of our liver surgeons, who were seeking to better understand the impact of steatosis and I/R injury on their human liver transplantation procedures. Before any human studies, we needed to define the likely outcomes in an animal model. The ischemic damage associated with this model was similar to that previously reported (Thorling et al., 2011b, 2013) and was characterized with significantly elevated serum ALT, an increased number of DNA-damaged hepatocytes, and a lower bile flow. In this study we found that the liver acinus and hepatocytes are less defined when exposed to I/R injury in both normal and steatotic livers. This could be an effect of mitochondrial depolarization, leading to depleted ATP, which could result in apoptosis and necrosis. This is confirmed by a significantly increased number of apoptotic cells in normal and steatotic livers exposed to I/R injury compared with sham, but not in the group of rats that had steatosis without I/R injury (as shown in Table 2).

The salient new observation in this work is the irregular distribution of fluorescein in the in vivo images of steatosis 2 minutes after injection, relative to normal livers (Fig. 3). I/R injury in steatotic livers appears to dampen this irregularity but is associated with a loss of the distinctive sinusoidal structures and the appearance of lipid droplets. The lipid droplets are still visible 40 minutes and 180 minutes after injection in steatotic livers exposed to I/R injury. These results are consistent with traditional morphology (as seen in Fig. 2A) and are in concordance with another study showing lipid droplets in steatotic livers (Hui et al., 1994). Our images showed a higher fluorescence intensity in the liver 180 minutes after fluorescein injection in all diseased groups compared with sham (Fig. 3). These results indicate

**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>I/R</th>
<th>Steatosis</th>
<th>Steatosis + I/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{met}$ (min⁻¹)</td>
<td>0.07 ± 0.06</td>
<td>0.1 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td>$k_{bile,F,G}$ (min⁻¹)</td>
<td>0.001 ± 0.002</td>
<td>0.004 ± 0.003</td>
<td>0.005 ± 0.004</td>
<td>0.0003 ± 0.0004</td>
</tr>
<tr>
<td>$k_{bile,F}$ (min⁻¹)</td>
<td>0.08 ± 0.08</td>
<td>0.08 ± 0.06</td>
<td>0.3 ± 0.08</td>
<td>0.9 ± 0.6</td>
</tr>
<tr>
<td>$k_{FG}$ (min⁻¹)</td>
<td>1.1 ± 1.0</td>
<td>2.3 ± 1.1</td>
<td>0.9 ± 0.6</td>
<td>1.0 ± 0.8</td>
</tr>
</tbody>
</table>

$CL_{uptake}$—uptake clearance into hepatocytes; $k_{met}$—rate constant of biliary excretion; $k_{bile,F,G}$—rate constant describing biliary excretion of fluorescein monoglucuronide; $k_{bile,F}$—rate constant describing biliary excretion of fluorescein; $k_{FG}$—rate constant of metabolism.

$^aP < 0.05$ versus sham.

$^bP < 0.01$ versus sham.

$^cP < 0.001$ versus I/R.
an impaired excretion of fluorescein or change in metabolism. Our previous in vivo MPM studies also showed that the excretion of fluorescein was delayed in livers with I/R injury (Thorling et al., 2013).

To better define the changes in liver function, we fitted the sinusoidal and hepatocyte concentrations of fluorescein acquired from the images and the amount of drug excreted in the bile to a physiologically based pharmacokinetic model (Fig. 4B) to obtain the kinetic parameters characterizing hepatic transporter and metabolism function. Intravital MPM imaging allows the direct observation of fluorescent compound concentrations in both space and time simultaneously under in vivo conditions (Liu et al., 2012). The model allows the fitting of naive data from all rats simultaneously (Weiss et al., 2013).

Our results show a significantly increased rate constant of fluorescein metabolism ($k_{\text{met}}$) in steatotic livers with and without I/R injury compared with sham and also in the steatosis + I/R injury group compared with I/R injury of normal livers, which indicates an increased metabolism of fluorescein within the hepatocyte (Weiss et al., 2013). Because fluorescein is mainly metabolized in the hepatocyte to FG through glucuronidation, conjugating to a single glucuronic acid molecule (Chahal et al., 1985), it is likely that the increased metabolism (increased $k_{\text{met}}$) reflects an increased glucuronidation of fluorescein in the diseased liver.

**Fig. 5.** Average fluorescence lifetime in the liver. (A) Average fluorescence lifetime ($\tau_m$) obtained from in vivo fluorescence lifetime imaging microscopy of the liver over time after injection of fluorescein intravenously (mean ± S.E.M., $P < 0.05$, $n = 3$). (B) Color-coded images showing change in average fluorescence lifetime. Red arrows indicate the increased blue showing decreased average fluorescence lifetime.
liver. This result is consistent with the previous report of the increased glucuronidation in obese humans (Hanley et al., 2010) and in hepatic I/R injury (Parasrampuria et al., 2012).

Moreover, the increased metabolism of fluorescein in diseased rat livers, as found in our study, coincides with a significant increase in intrinsic metabolic clearance (CL\text{int}) of other compounds, as found in a recent study using another model of steatosis, namely, nonalcoholic steatohepatitis (Li et al., 2011). CL\text{int} represents the metabolic activity of the hepatocytes (Barbare et al., 1985), similar to $k_{\text{met}}$.

To further confirm these results, we tested our previously published model of measuring average fluorescence lifetime to determine whether metabolism of fluorescein to FG was increased (Thorling et al., 2011a). Fluorescein and FG have two distinct lifetimes, but our analysis yielded one single average lifetime ($\tau_m$), which is a combination of the lifetime of fluorescein and FG. Because the lifetime of FG is significantly lower than that of fluorescein in vitro, 2.3 ns and 3.8–4.1 ns, respectively, the average lifetime ($\tau_m$) decreases when more FG is formed in the liver.

Our results show a significantly decreased average lifetime in the liver in steatosis with and without I/R injury, which confirms the pharmacokinetic model of increased $k_{\text{met}}$ presented here.

In addition, we report a decreased rate of excretion of fluorescein in this work. The rate of excretion ($R$) is dependent on clearance into the bile (CL\text{bile}) and concentration of drug ($C$) in the hepatocytes ($R = CL\text{bile} \times C_{\text{hep}}$) (Yamazaki et al., 1997). Because the metabolism is increased (increased $k_{\text{met}}$), the concentration of fluorescein in the hepatocytes is decreased, hence a decrease of the excretion rate is expected.

Although in vivo imaging has the potential to replace or guide biopsies, it has been restricted to the surface or the internal organs after invasive surgery. Thus, microscopy is a better choice, combining intravital microscopy and miniature endoscopy (Kim et al., 2008), sometimes in combination with a rodlike gradient-index lens.

In conclusion, our work shows a novel method of quantitatively assessing liver subcellular function in liver steatosis by noninvasive in vivo multiphoton imaging, supplemented by a physiologic pharmacokinetic model to interpret the liver functional changes. The model
enabled the precise estimation of changes in uptake, metabolism, and excretion resulting from liver steatosis alone or with I/R injury, and it can offer more quantitative measures of liver function. Administering a fluorescent probe in combination with microendoscopy or gradient-index lenses opens up the door for more in-depth information regarding liver functionality and has the potential to replace the traditional static liver biopsy methodology currently used.

Acknowledgments

The authors thank Clay Winterford and colleagues at Queensland Institute of Medical Research for their histology and immunohistochemistry preparation, and Goce Dimeski and colleagues at Queensland Pathology Services, Prince Alexandra Hospital, for conducting the ALT measurements.

Authorship Contributions

Participated in research design: Thorling, Roberts, Crawford, Burczynski, Liu, Wu, Weiss.


Contributed new reagents or analytic tools: Thorling, X. Liu, Jin.

Performed data analysis: Thorling, Jin, D. Liu, Weiss.

Wrote or contributed to the writing of the manuscript: Thorling, Jin, X. Liu, Roberts, Crawford, Burczynski, Weiss, Wang, D. Liu.

References


