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Using quantitative intravital multiphoton microscopy to dissect hepatic transport in rats

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ABSTRACT

Hepatic solute transport is a complex process whose disruption is associated with liver disease and drug-induced liver injury. Intravital multiphoton fluorescence excitation microscopy provides the spatial and temporal resolution necessary to characterize hepatic transport at the level of individual hepatocytes *in vivo* and thus to identify the mechanisms and cellular consequences of cholestasis. Here we present an overview of the use of fluorescence microscopy for studies of hepatic transport in living animals, and describe how we have combined methods of intravital microscopy and digital image analysis to dissect the effects of drugs and pathological conditions on the function of hepatic transporters *in vivo*.

1. Introduction

A primary function of the liver is to regulate the constitution of the blood, mediating the metabolism and excretion of endogenous and exogenous compounds. The critical role of these processes is demonstrated by the serious pathologies associated with mutations in the proteins that mediate hepatic metabolism and transport. Hepatic transport is of particular interest in pharmaceutical development. In addition to mediating the metabolism and clearance of drugs, the liver is frequently the victim of off-target drug effects. In particular, drug-induced liver injury, one of the most expensive and vexing problems in drug development, is associated with drugs that inhibit hepatocyte secretory function [1–3].

The transport of drugs and metabolites is accomplished by proteins that mediate transport from the sinusoid capillaries into the hepatocyte cytosol, from the hepatocyte cytosol to the bile canaliculi or from the hepatocyte cytosol back into the sinusoids. The major uptake transporters include proteins of the Solute Carrier family, e.g. OATP (SLC01A2, SLC21A6 SLC21A8, SLC21A9), OCT (SLC22A1) and NTCP (SLC10A1). Major secretory transporters include proteins of the ATP-binding Cassette transporter family, such as MRP2 (ABCC2), MDR3 (ABCB4) and BSEP (ABCB11), which mediate canalicular secretion) and MRP3 (ABCC3) and MRP4 (ABCC4), which mediate sinusoidal reflux back into the blood). Drugs may impact one or more of these transporters in such a way as to increase or decrease the metabolism and clearance of drugs and metabolites. Furthermore, depending upon the balance of effects on uptake and secretory transporters, a cholestatic drug may induce the accumulation of a drug or toxic metabolite in the hepatocyte cytosol, inducing liver injury. It is thus critical to not only determine that a drug disrupts normal liver transport, but also to determine the site(s) of transport disruption.

While the effects of drugs on the function of specific transporters can be sensitively measured in studies of isolated membrane vesicles, the additional complexity of the *in vivo* situation makes it difficult to extrapolate these measurements to predictions of *in vivo* effects, particularly since local *in vivo* drug concentrations are typically unknown. Transport can also be evaluated in studies of cultured cells. While hepatocytes grown *in vitro* recapitulate many *in vivo* functions, particularly when grown in sandwich culture, results obtained from these systems can also be difficult to extrapolate to the *in vivo* condition. First, depending upon culture conditions, transporter expression levels change to varying degrees after isolation. Second, cultured cells lack the structure of the lobule, a transport unit in itself that is organized such that substrates are sequentially transported and metabolized by hepatocytes with different and independently-regulated populations of transporters [4–8]. Third, these simple systems lack the systemic inputs that dynamically regulate the abundance and distribution of hepatic transporters *in vivo* [9–13], making them incapable of reproducing the complex circumstances of pathological conditions, such as infection.

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Conversely, the standard imaging and biochemical approaches used in animal studies can detect cholestasis, but provide only indirect evidence as to underlying mechanisms. The ideal system for dissecting hepatic transport would provide the spatial and temporal resolution necessary to distinguish the individual steps of transport, but in the relevant physiological context, consisting of differentiated hepatocytes in the multi-cellular environment of the intact liver lobule, with intact vascular, immune and endocrine inputs. In addition to supporting dissection of drug effects under physiological conditions, such a system would support studies of the effects of pathology on liver transport and studies of the effects of drugs in the context of pathology.

Studies of isolated and cultured hepatocytes have demonstrated that fluorescence microscopy provides the spatial and temporal resolution necessary to characterize transport of fluorescent probes [14–17]. The same general approach can be applied to studies of hepatic transport *in vivo* using intravital microscopy of the liver of laboratory animals. As early as 1945, a crude fluorescence microscope system was used to study the effects of various insults on transport of fluorescein in the rat liver [18]. Approximately 40 years later, digital epifluorescence microscopy was used to characterize transport of fluorescein and fluorescein-glycocholate in the livers of rats and hamsters [19,20]. In the past twenty years, investigators have capitalized on the increased reach and resolution of multiphoton fluorescence excitation microscopy to evaluate transport of fluorescein and other fluorescent probes in rats [21–26] and mice [27,28]. Fig. 1 shows an example of a multiphoton fluorescence excitation image collected from the liver of a rat 20 min after intravenous injection of rhodamine dextran (retained in the vasculature) and sodium fluorescein (transported into hepatocytes and bile canaliculi). The inset demonstrates that the resolution provided by multiphoton microscopy is sufficient to clearly resolve sinusoids, hepatocytes and bile canaliculi. Here we describe how we have combined methods of intravital microscopy and image analysis to quantify the effects of drugs and disease on l transport *in vivo*.

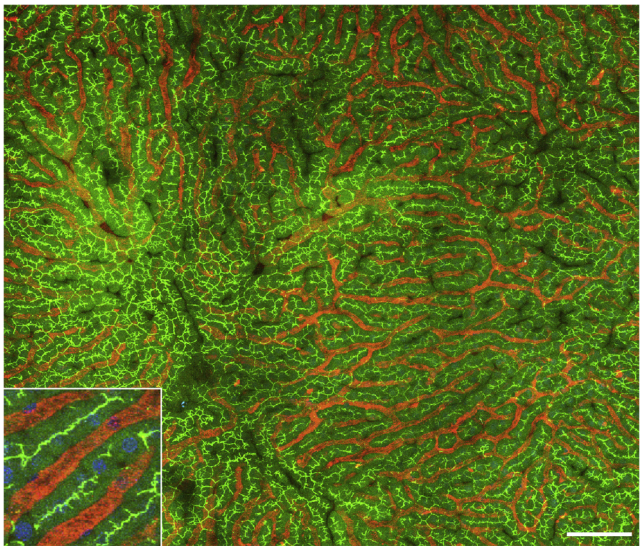

Fig. 1. Intravital multiphoton microscopy of the liver of a living rat. Montage of nine image volumes collected from the liver of a living rat 20 min after injection of 6 mg/kg 2 M MW rhodamine dextran (red), and 2 mg/kg sodium fluorescein (green). Each of the nine panels of this mosaic is a maximum projection of 15 images collected over a depth of 15 μm. Scale bar depicts 100 μm length. Inset shows a 3× magnification of a maximum projection of a 6 μm thick volume, showing the clear resolution of the sinusoids (red, labeled with rhodamine dextran), the hepatocyte cytosol (dim fluorescein fluorescence) and bile canaliculi (bright fluorescein fluorescence). Inset image also shows nuclei, labeled with 2 mg/kg Hoechst 33342 (blue). Figure adapted from cover figure published in Intravital, 2013, Volume 5, number 3. Used with permission. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Approach

2.1. Overview

The intravital microscopy transport assays described here are based upon quantitative analysis of images collected in time series from the liver of a living rodent following intravenous injection of fluorescent transport substrates, largely following previously described approaches [22,23]. Images collected in this way demonstrate that fluorescein uptake and secretion proceed very rapidly. Fig. 2 shows that within a minute of perfusion, detectible levels of fluorescein are present not only in the hepatocyte cytosol, but also in bile canaliculi. Within 6 min of perfusion, a substantial amount of fluorescein has already been transported into canaliculi. Panel F shows that fluorescein secretion into canaliculi is essentially blocked in rats treated with the cholestatic agent taurolithocholate (TLC). The 10 min time course of fluorescein transport in the presence or absence of TLC is shown in Video 1.

2.2. Surgical preparation and presentation of the rat liver on the microscope stage

All studies described here were approved and conducted according to the Institutional Animal Care and Use Committee guidelines of Indiana University, and adhere to the guide for the care and use of animals [29].

Experimental animals are typically acclimatized for a period of at least 4 days and, depending upon the study, fasted for 16 h prior to studies. Adult Sprague Dawley (or Wistar) rats, weighing between 180 and 400 g, are sedated with 5% Isoflurane, weighed and 130 mg/kg Inactin is administered intraperitoneally for anesthesia. Anesthesia is monitored by evaluation of responses to gentle pinching of ears and feet. The rat is placed on a heating pad to maintain body temperature. Body temperature is monitored using a rectal thermometer, and heart rate and respiratory rate are monitored visually.

Once anesthetized, a 3 × 1.5 cm, L-shaped incision is made 1 cm to the right of the ventral midline in the neck. A jugular cannula is then placed using PE 50 tubing filled with sterile 0.9% saline and attached to a Luer stub adapter and 1 mL syringe. The neck is sutured with 3-0 black silk sterile suture. At this time, a bolus of Hoechst 33342 (Invitrogen, 2 mg/kg) diluted in 0.9% sterile saline to a total volume of 0.4 mL is injected into the jugular line to label cell nuclei [30].

The liver is exposed for imaging by making a 4 cm incision across the torso 1–2 cm below the middle of the rib cage. A wet (0.9% saline) 2 × 2 gauze sponge is gently placed below the left lateral liver lobe. In order to minimize tissue motion for microscopy, the liver is tethered to the bottom of a 50 mm Willco coverslip-bottomed dish (GWST-5040, Warner Instruments, Hamden, Ct). Tape is placed on the periphery of the glass window of the dish, and cyanoacrylate glue is applied to the tape. The glass window is pressed to the liver and the gauze on either side of the liver is gently pressed and glued to the tape using cotton-tipped applicators. Alternatively, the liver itself may be glued directly to the coverglass along the periphery of the window. Sterile 0.9% saline is then placed in the coverslip-bottomed dish to keep liver moist throughout the imaging session. A small dose (0.2 mg/kg) of sodium fluorescein (Fluka Analytical) is administered IV in order to generate dim labeling of bile canaliculi that can be used to facilitate positioning of the liver at the beginning of the study.