Effects of chronic kidney disease on liver transport: quantitative intravital microscopy of fluorescein transport in the rat liver

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Running Head: Effect of chronic kidney disease on hepatocyte transport

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Abstract

Clinical studies indicate that hepatic drug transport may be altered in chronic kidney disease (CKD). Specifically, uremic solutes associated with CKD have been found to alter the expression and/or activity of hepatocyte transporters in experimental animals and in cultured cells. However, given the complexity and adaptability of hepatic transport, it is not clear whether these changes translate into significant alterations in hepatic transport. In order to directly measure the effect of CKD on hepatocyte transport in-vivo, we conducted quantitative intravital microscopy of transport of the fluorescent organic anion fluorescein in the livers of rats following 5/6th nephrectomy, an established model of CKD. Our quantitative analysis of fluorescein transport showed that the rate of hepatocyte uptake was reduced by approximately 20% in 5/6th nephrectomized rats, consistent with previous observations of Oatp down-regulation. However, the overall rate of transport into bile canaliculi was unaffected, suggesting compensatory changes in Mrp2-mediated secretion. Our study suggests that uremia resulting from 5/6th nephrectomy does not significantly impact the overall hepatic clearance of an Oatp substrate.

Key words: chronic kidney disease, uremia, cytochrome P450, hepatic transport, Mrp2, Oatp, sodium fluorescein
**Introduction**

Patients with end-stage renal disease (ESRD) receiving dialysis take on average 12 medications to manage the complications of their renal failure and their comorbid disease which places them at substantial risk for adverse drug events (8, 12, 13).

Currently, modifications in drug dosing to improve safety for ESRD patients have largely been confined to those medications excreted renally. This has likely improved the safety of these medications for the ESRD population, yet it remains an incomplete approach since it does not include the hepatically-metabolized medications. This oversight is significant because evidence increasingly indicates that hepatic drug metabolism and transport is reduced in patients with ESRD (3-6, 14-22, 25-28, 30). Consequently, administering the full dose of a hepatically metabolized medication may place an ESRD patient at similar risk for an adverse drug event as administering the full dose of a renally-eliminated medication.

Experimental evidence suggests multiple mechanisms by which uremic solutes might influence hepatic drug disposition (20, 25). Studies from the Pichette laboratory demonstrated that the activity and expression of cytochrome P450 was reduced in the 5/6\textsuperscript{th} nephrectomy (5/6N) rat model of chronic renal failure (6, 10, 11). In addition, their work elucidated a direct role for uremic solutes with the observation that CYP450 activity and expression were reduced in primary rat hepatocytes incubated with human uremic serum (14). More directly relevant to the effects of CKD on hepatic transport were studies demonstrating that 5/6N induced down-regulation of the uptake transporters Oatp1a1, Oatp1a4 and Oatp1b2 in rats (7, 15). This down-regulation appeared to reflect a direct effect of uremic solutes on hepatocytes, as serum obtained from 5/6N rats reduced the expression of Oatp1b1 in primary rat hepatocytes (15). In addition, serum obtained from ESRD patients attenuated the expression of OATP1B1 and OATP2B1 in
cultured human hepatocytes (27) and diminished the expression of Oatp1a4 in primary rat hepatocytes (18).

Additional studies of cultured cells indicate that uremic solutes not only reduce Oatp expression, but also directly inhibit Oatp activity. Uremic serum and isolated uremic toxins have been found to directly inhibit hepatocyte uptake of the Oatp substrates losartan, pravastatin and erythromycin in cultured human and rat hepatocytes (22, 26-28). Studies of transfected HEK293 cells have also demonstrated that uremic serum inhibits the transport activity of Oatp1b1, Oatp2b1 and Oatp2b2 (22). Together, these studies provide a sensible explanation for the observation of reduced hepatic clearance of drugs in individuals with CKD-diminished hepatic drug clearance which reflects the down-regulation and/or inhibition of Oatp resulting from exposure of hepatocytes to uremic solutes. However, the effects of CKD on hepatic transport have thus far been evaluated only in studies of cultured cells. While cultured cells provide an experimentally tractable system, their physiological relevance is limited by changes in transporter and enzyme expression that accompany hepatocyte isolation. Isolated hepatocytes lack the dynamic, polarized sinusoid-hepatocyte-canalicular structure that determines in situ hepatocyte transport. Furthermore, isolated hepatocytes do not provide the complex physiological context of the intact animal which is particularly important in injury models, such as CKD. Moreover, hepatic transport is highly complex, depending upon the activity of both uptake and secretory transporters whose expression and activity is dynamically regulated, often in compensatory ways. As a result, it is difficult to predict how the effects on Oatp impact overall hepatic transport with existing cell culture methods. In particular, reduced uptake may be compensated by increases in the rates of secretion to the canaliculus or by decreases in the rate of secretion to the sinusoid. Noteworthy in this regard are studies showing up-regulation of Mrp2 in rats
following 5/6N (7, 9, 15). Thus, testing the effect of 5/6N on hepatic transport can only conclusively be evaluated *in-vivo.*

We have recently developed methods of quantitative intravital microscopy capable of dissecting hepatocyte transport *in-vivo* (1). Time-lapse multiphoton fluorescence microscopy of the liver of living rats following intravenous injection of fluorescent transport substrates is combined with methods of quantitative digital image analysis to quantify the kinetics of transport into the cytosol of individual hepatocytes and into bile canaliculi. The ability to dissect the individual steps of transport makes this method capable of resolving the effects of CKD on hepatocyte uptake as well as on overall rates of transport to bile canaliculi. Here we apply this approach to evaluate the effects of CKD on the hepatic transport of fluorescein, a fluorescent substrate specific for Oatp and Mrp2 transporters (2, 29) and to test the hypothesis that Oatp mediated transport is reduced in the 5/6N resulting in reduced hepatic transport of organic anions.

**Materials and Methods**

5/6N Rat Model of CKD

The 5/6N rat model is commonly used as a disease model to study CKD in rats (10). The 5/6N and sham-operated rats (8-10 weeks of age, all male) were purchased from Charles River and housed individually in the Indiana University School of Medicine Laboratory Animal Resource Center. The rats were maintained on a diet of Purina rat chow and water *ad libitum.* An acclimatization interval of at least 3 days was allowed prior to the performance of any animal experiments. All animal experiments were approved and conducted according to the Institutional Animal Care and Use Committee guidelines. Briefly, 5/6N rats were prepared in a two-part surgery. In the first, surgeries were conducted on a male Sprague-Dawley rat during which a ventral incision is made and 2/3 of the right kidney is removed. After 5-7 days of recovery, a dorsal incision just below the rib cage is made and the left kidney was removed. For sham-operated
controls, rats were treated to the same anesthesia and surgical processes without the
removal of kidneys.

Renal Function Analyses

Forty two days following the 5/6N or sham surgery, rats were placed in metabolic
cages for 24 hours to collect urine samples. Afterwards, 0.2 ml blood samples were
collected from the tail vein and analyzed for creatinine and blood urea nitrogen (BUN)
levels. Prior to imaging, the weights of the rats were recorded.

Multiphoton Intravital Microscopy

Our overall approach for analyzing hepatic transport involves quantitative
multiphoton microscopy of transport of sodium fluorescein in the intact liver of a living
rat. Techniques of quantitative intravital microscopy follow those that we previously
described (1). After 24 hours in a metabolic cage, the rats were anesthetized with
130mg/kg of Inactin given intraperitonally. Subsequently, a venous catheter was
placed in the right jugular vein and 2mg/kg of Hoechst 33342 (Invitrogen) was injected
I.V. for the labeling of the nuclei. For access to the liver, a 4 cm ventral incision was
placed just 1.5 cm below the rib cage. The liver was carefully placed onto wet gauze
and secured to a glass bottom-plate. A pre-injection of 0.2mg/kg of sodium fluorescein
(Fluka Analytical) was administered prior to imaging to facilitate positioning of the liver
for kinetic studies. The rat was then placed ventral side down on an inverted Olympus
FV1000 multiphoton microscope, and an appropriate field of the rat liver was identified.
A series of image volumes (6 focal planes, spaced at 1 micron apart) were then
collected continuously just before and for ten minutes following injection of a 2 mg/kg
solution of sodium fluorescein. A high-resolution mosaic, consisting of 9 contiguous
volumes was then collected.
Quantification of fluorescein transport

The image volume from each time point was projected into a maximum-projection image, a procedure that ensures collection of images of the canaliculi even in the presence of minor vertical motion. The resulting projections were then assembled into a time-series of images for analysis. Hepatocyte uptake was measured as the increase in the fluorescence measured over time in 10-pixel regions located over the hepatocyte cytosol. Hepatocyte secretion was measured as the increase in fluorescence in 10-pixel regions located over canaliculi. Measurements were repeated for between 20 and 35 hepatocytes for each animal. Measured values were corrected for background by subtraction of the measurements obtained just prior to addition of fluorescein perfusion. The rate of uptake was calculated from the increase in mean cytosolic fluorescence, measured during the initial interval of linear uptake (in this case, the first 30 seconds). The rate of secretion was calculated from the increase in canalicular fluorescence, measured during the initial interval of linear secretion (in this case, the subsequent 90 seconds).

Mosaic analysis

As described above, a mosaic of 9 contiguous volumes (each 224 by 224 by 15 microns) was collected for each rat. Each volume was projected into a single image (using Metamorph™, Sunnyvale CA) and the resulting images were assembled into a single square image (using Photoshop™, Adobe Inc., San Jose, CA), representing a hyper-field approximately 650 microns on a side. Using Metamorph™, images were scaled to 8 bits and smoothed (2x2, low pass filter). A median image (using a 24 by 24 region) was subtracted from this image, and the quotient binarized (using a threshold of 50). The binarized image was skeletonized, and single pixels removed, resulting in a binary image that reproduced the distribution of canaliculi in the original image. The
volume density of canaliculi was quantified as the fraction of image pixels occupied by
the binarized canaliculi.

Statistical analyses

Graphical and statistical analysis was conducted using Kaleidagraph 4.03
(Synergy, Reading, PA). Data are presented as means plus or minus standard error of
the mean. Significance testing was conducted using one-tailed Student’s t-test, and
random probability (P values) provided as exact values.

Results

Chronic kidney disease in a 5/6N rat

Studies were conducted using the 5/6N rat model of CKD (10), using male Sprague
Dawley rats. Consistent with previous studies, the characteristics of CKD were
apparent 42 days after 5/6N, as evidenced by reduced weight, increased BUN and
increased creatinine (Table 1).

Fluorescein transport in the livers of 5/6N rats

The organic anion fluorescein has been widely used as a fluorescent probe in
studies of hepatic transport (2, 24) and has recently been identified as a specific
substrate of human and rat organic anion (OATP/Oatp) transporters (2). In previous
studies (1) we demonstrated that quantitative intravital microscopy of the livers of rats
injected with fluorescein can be used to sensitively detect alterations in Oatp function in
vivo, demonstrating a 70% inhibition in the rate of hepatocyte uptake in rats treated with
the Oatp inhibitor, rifampin. In order to evaluate the functional consequences of the
5/6N on hepatic transport, we conducted similar quantitative intravital microscopy
studies of the liver of 5/6N and sham-operated rats following IV injection of sodium
fluorescein.

Figure 1A shows example images from a series of images collected from the liver
of a sham-operated rat during and following IV injection of sodium fluorescein. Initially
restricted to the sinusoids, fluorescein starts to appear in the hepatocytes and even in
the canaliculi within one minute of injection, reflecting rapid transport by Oatp and Mrp2,
respectively. The speed of transport is apparent in the graph shown in the far-right
panel of Figure 1A, which shows the mean fluorescence levels in the cytosol, canaliculi
and adjacent sinusoids measured for 22 hepatocytes.

A comparable study conducted in a 5/6N rat yielded very similar results (Figure
1B), suggesting that 5/6N modestly reduces organic anion transport in rats. The
sensitivity of the technique is demonstrated in studies of a rat treated with the cholestatic
agent tauroliothocholate (23) which profoundly reduces the rate of fluorescein uptake and
essentially blocks secretion into canaliculi (Figure 1C).

These studies were repeated for five sham and five 5/6N rats, and
measurements were obtained for between 22 and 36 hepatocytes for each rat. These
studies demonstrate that 5/6N had a modest effect on the rate at which fluorescein
accumulated in the hepatocyte cytosol (Figure 2A). Quantification of the rate of uptake
during the initial linear period of accumulation, demonstrated that the 5/6N decreased
the rate of hepatocyte uptake of fluorescein (20.7%), an effect that was nonetheless
statistically significant (P = .024, one tailed t-test) (Figure 2C). In contrast, 5/6N had no
detectible effect on the rate of fluorescence accumulation in canaliculi (Figure 2B), and
had no significant effect on the rate of canalicular secretion (P = 0.47, one-tailed t-test)
(Figure 2D).

Discussion
Emerging evidence indicates that chronic kidney disease attenuates the non-
renal clearance of medications. Experimental animal models have demonstrated that
CKD is associated with reduced hepatic expression of CYP450 (6, 10, 11) and Oatp
transporters (7, 15). Similar results have been obtained in studies of cultured cells,
which have also demonstrated that uremic solutes directly inhibit Oatp-mediated transport (18, 22, 26-28). Taken together, these studies suggest that the reduced non-renal drug clearance associated with CKD may reflect reduced Oatp transport function. The studies described here represent the first direct, in vivo measurement of the impact of uremia on the hepatic transport of fluorescein, an Oatp substrate. Our study identified a 20.7% decrease in the rate of fluorescein uptake in hepatocytes of 5/6N rats, a modest decrease that did not translate into a detectable decrease in the overall rate of fluorescein secretion into canaliculi. Although speculative, the lack of an effect on overall transport may reflect up-regulation of Mrp2, consistent with previous studies of 5/6N rats (7, 9), supporting the idea that increased Mrp2 function may compensate for reduced Oatp activity in CKD (7, 25). Regardless of the mechanism, our study indicates that uremia resulting from a 5/6th nephrectomy does not significantly impact the overall hepatic clearance of fluorescein, an Oatp substrate.

**Perspectives and Significance**

This research provides further evidence of the complex, integrated relationship between the kidney and liver in the clinically important area of CKD and drug disposition. The significance of our findings transcends earlier research which merely demonstrated a decrement in hepatic transport of substrates in CKD. Our research corroborated this reduction in hepatic transport, but more significantly did not find a significant difference in the overall hepatic clearance of an Oatp substrate in CKD. This finding suggests that the reduced Oatp function in CKD may not be a clinically important factor in drug disposition. This is a compelling finding since it may indicate an adaptive response on the part of the liver to ameliorate the deleterious effect of the uremic milieu. Future research in our laboratory will focus on further elucidating hepatic transport in CKD with a specific focus on the efflux of substrates and this potential adaptive response.
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Disclosures:

All authors listed have contributed sufficiently to the project to be included as authors. No financial or other conflicts of interest exist.
References


Table 1: Renal function in 5/6N and Control Rats. At day 42 post 5/6N surgery (CKD) or sham surgery (Control), blood creatinine and BUN levels were measured (mg/dL). The creatinine (p=0.00102, one-tailed Student’s T-test) and BUN (p=0.0384, one-tailed Student’s t-test) levels were significantly higher in CKD rats when compared to control rats.

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<tr>
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<th>CKD Rats</th>
<th>Control Rats</th>
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<tr>
<td>Creatinine (mg/dL)</td>
<td>0.65 ± 0.278</td>
<td>0.20 ± 0.107</td>
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<tr>
<td>BUN (mg/dL)</td>
<td>42.03 ± 25.76</td>
<td>18.63 ± 2.35</td>
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<tr>
<td>Weight (g)</td>
<td>355.63 ± 17.12</td>
<td>472.38 ± 13.63</td>
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<tr>
<td>Urine (mL)</td>
<td>48.13 ± 3.80</td>
<td>23.25 ± 4.83</td>
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Figure 1: Results of studies of fluorescein transport in the livers of rats 42 days following sham surgery (A) or 5/6N (B). Micrographs show projections of image volumes collected from the livers of living rats at the indicated times after perfusion. Imaged areas are 224 microns across. Graphs show quantifications of mean fluorescence (plus or minus SEM) measured in the cytosol (open circles), canaliculi (crosses) and adjacent sinusoid (closed circles) of 22 hepatocytes (top, sham-operated) and 31 hepatocytes (bottom, 5/6th nephrectomy). Assay sensitivity is demonstrated in panel C, which shows results obtained 15 minutes after IV injection of taurolithocholate, an established inhibitor of hepatocyte transport. As shown in both the micrographs and the associated graph, uptake is profoundly slowed, and secretion is essentially blocked, with canalicular fluorescence exceeding cytosolic levels only slightly and slowly.
Figure 2: Kinetics of the increase in fluorescence measured in the cytosol (A) and canaliculi (B), measured in images collected from the livers of living rats 42 days following either 5/6N (red symbols) or sham surgery (blue symbols). Each point represents the mean (plus or minus SEM) of the measurements collected from 5 rats, each the mean of measurements of between 22 and 32 hepatocytes. Overall transport kinetics are very similar for the two conditions. Panel C shows the mean initial rates of uptake (measured during the first 30 seconds following perfusion), and Panel D shows the mean rates of secretion (measured during the subsequent 90 second interval). The mean rate of uptake was reduced by 20.7% in the 5/6th nephrectomy group (p = 0.024, one tailed Student’s t test). The mean rate of secretion did not differ between the two groups (p = 0.470, one tailed Student’s t test).