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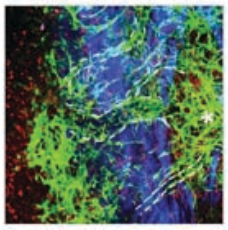

The caspase 3 sensor Phiphilux G2D2 is activated non-specifically in S1 renal proximal tubules

Takashi Hato[a], Ruben Sandoval[a] & Pierre C Dagher[a]

[a] Department of Medicine; Indiana University; Indianapolis, IN USA

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The caspase 3 sensor Phiphilux G2D2 is activated non-specifically in S1 renal proximal tubules

Takashi Hato, Ruben Sandoval, and Pierre C Dagher*

Department of Medicine; Indiana University; Indianapolis, IN USA

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Tubular cell apoptosis is a major phenotype of cell death in various forms of acute kidney injury. Quantifying apoptosis in fixed tissues is problematic because apoptosis evolves over time and dead cells are rapidly cleared by the phagocytic system. Phiphilux is a fluorescent probe that is activated specifically by caspase 3 and does not inhibit the subsequent activity of this effector caspase. It has been used successfully to quantify apoptosis in cell culture. Here we examined the feasibility of using Phiphilux to measure renal tubular apoptosis progression over time in live animals using intravital 2-photon microscopy. Our results show that Phiphilux can detect apoptosis in S2 tubules but is activated non-specifically in S1 tubules.

Introduction

Apoptosis is the most common form of programmed cell death and its role in organ and tissue development and differentiation is well documented. However, apoptosis is also commonly encountered during pathophysiological processes. In the kidney, tubular apoptosis is frequently observed after ischemic, nephrotoxic and septic injury. In fact, we have previously shown that tubular cell apoptosis contributes to kidney injury independently of inflammation.[1]

Pathological apoptosis is a stochastic process that occurs randomly in space and time following injury. Therefore, the quantitation of apoptosis in fixed tissue has always underestimated the magnitude of this form of cell death. This is further confounded by the short life of apoptotic tubular cells as they are shed into the lumen or cleared by phagocytic cells.

We have previously measured apoptosis in the kidney with intravital microscopy.[2,3] This was done with the combined use of Hoechst and propidium iodide. Hoechst easily detected condensed or fragmented nuclei and the vital probe propidium iodide excluded necrotic cells with damaged cell membranes. While this approach was successful in quantifying tubular apoptosis in kidney volumes of live animals, the inherent toxicity of Hoechst and propidium iodide precluded imaging over prolonged periods of time.

Phiphilux is a new generation cell permeable probe which emits fluorescence in the red (G2D2) or green (G1D2) when cleaved by active caspase 3. Its specificity to caspase 3 has been well documented in cell culture studies.[4-7] Its appeal for imaging apoptosis stems from the important fact that it does not inhibit the subsequent activity of caspase 3 once cleaved. Therefore, it offers a unique opportunity to follow the progress of apoptosis over time. We are aware of only one paper that describes the use of Phiphilux with intravital 2-photon microscopy to image apoptosis in the gut villi.[8] In that paper, authors report non-specific background Phiphilux signal in the lumen. To our knowledge, Phiphilux has not been imaged before in the kidney of live animals. We therefore report on the use of Phiphilux to image caspase 3 activity and apoptosis in renal tubules in live mice with 2-photon intravital microscopy.

Brief Methods

All studies were approved by our institutional IACUC. Eight week old C57BL/6J and B6129S1-Casp3^{tm1Flv}/J (caspase 3 knockouts) mice, weighing on average 20 g, were obtained from Jackson labs. We have previously extensively described the techniques of renal intravital imaging.[3] Briefly, live animal imaging was performed using a Bio-Rad MRC-1024MP Laser Scanning Confocal/Multiphoton scanner attached to a Nikon Diaphot inverted microscope with a Nikon 20X or 60X NA 1.2 water-immersion objective.[3] Fluorescence excitation was provided by a Titanium-Sapphire laser (Spectraphysics, Mountain View, CA) 800 nm excitation. In some studies we used an Olympus FV1000-MPE

*Correspondence to: Pierre Dagher; Email: Pdaghe2@iu.edu
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confocal/multiphoton microscope equipped with a Spectra Physics MaiTai Deep See laser and gallium arsenide 12-bit detectors. The system is mounted on an Olympus Ix81 inverted microscope stand with a Nikon 20x and 60x NA 1.2 water-immersion objective. The laser was tuned to 800 nm excitation. The 2 setups showed similar results.[9,10] We estimate that the power at the surface of the kidney was between 2 and 28 mw. Animals were placed on the stage with the exposed intact kidney placed in a coverslip-bottomed cell culture dish (Warner Inst., Hamden, CT) bathed in isotonic saline as we have described.[3] In some animals, unilateral renal ischemia was induced with a 60 min clamp of the renal pedicle. Hoechst was injected ip (80 μg per mouse) 2 hours before imaging. Phiphilux G2D2 (Calbiochem) 570 μl per mouse was injected iv through an external jugular line and the kidney was imaged for periods up to 2 hours.

Results and Discussion

Intravital 2-photon imaging of sham mouse kidneys reveals 2 populations of proximal tubules easily distinguished by their green autofluorescence (**Fig. 1A**). Using freely filtered fluorescently labeled inulin, we have previously determined that the tubules with bright green autofluorescence are the downstream S2. Tubules with dim autofluorescence (occasionally with a brown yellow hue) are S1 (**Fig. 1A**).[9] Collecting ducts show minimal, if any, autofluorescence and have a bright nuclear Hoechst signal. Within 1 min after Phiphilux injection, the probe exhibited significant red fluorescence exclusively in S1 tubules near the brush border (**Fig. 1B**). Phiphilux fluorescence continued to increase in S1 tubules over a 30 min time period (**Fig. 1C and 1D**). At these later time points, Phiphilux signal could be seen in a small punctate subapical pattern that partially co-localized with the green autofluorescence signal (inset in **Fig. 1D**). Because the latter is thought to emanate from endosomes, this possibly indicates early endocytosis of the probe.

To determine whether this activation of phiphilux was related to the presence of constitutionally active caspase 3 in S1, we imaged the kidney of a sham caspase 3 KO mouse. As shown in **Figure 2**, Phiphilux was also activated in S1 tubules. Like in wild-type mice, S2 tubules exhibited no Phiphilux signal.

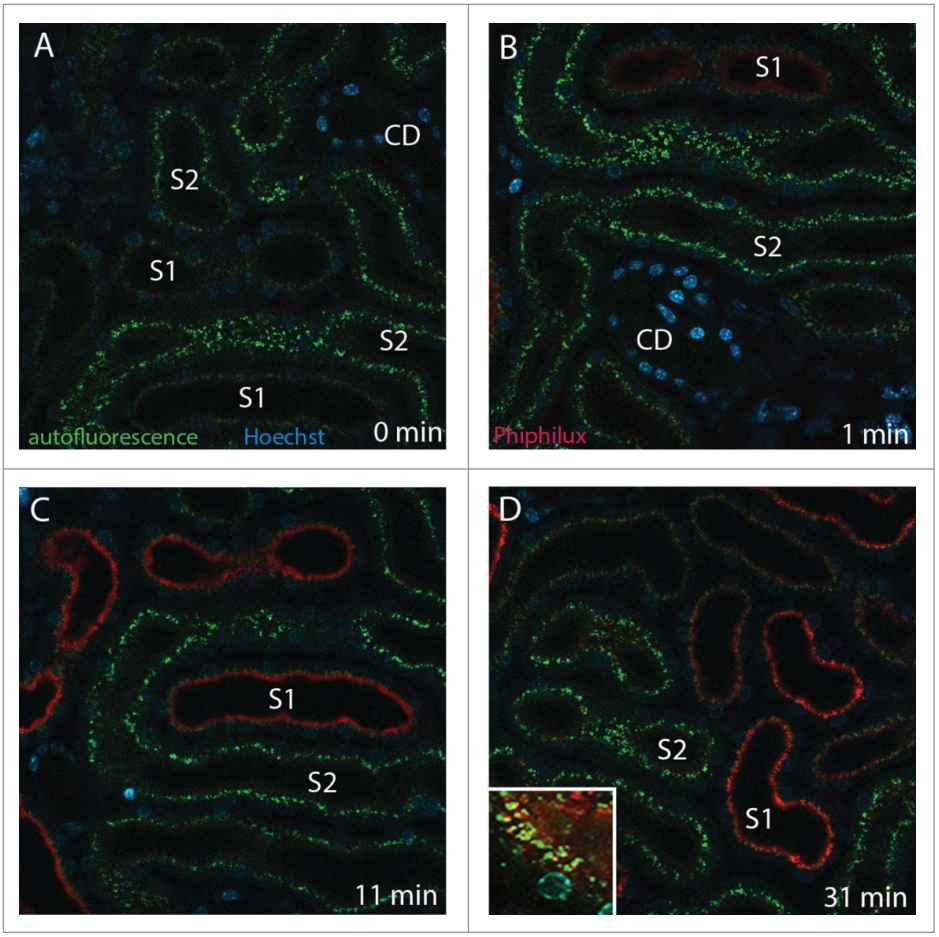

Figure 1. Intravital imaging of a sham wild-type mouse kidney before (**A**) and after (**B-D**) Phiphilux injection. Green color is tubular autofluorescence. Blue represents nuclear Hoechst. Phiphilux fluorescence is red. CD, collecting ducts. Inset in panel (**D**) is a 4X zoom of a random S1 subapical field. All indicated times are minutes after Phiphilux injection.

The caspase 3-independent signal of Phiphilux in S1 tubules was indicative of non-specific activation by protease activity in S1 during probe uptake in this highly endocytic tubular segment. In **Figure 3**, we followed the Phiphilux signal in a sham wild-type kidney volume over a 2 hour time period, imaging intermittently every 30 minutes. We noted a decrease in the Phiphilux signal in S1 and an increase in Hoechst fluorescence in various tubules indicative of photodamage. S2 segments did not exhibit any Phiphilux signal over that time period but also showed some photodamage as evidenced by a change in their typical autofluorescence pattern. At later time points, Phiphilux signal could be easily detected in the lumen of collecting ducts. At no time did S1 nuclei show condensation or fragmentation suggestive of apoptosis.

Ischemia reperfusion is known to induce caspase 3 activation and apoptosis in S2 and S3 renal tubules.[11] To determine whether this can be detected by Phiphilux, we imaged the kidney of a wild-type mouse after 40 minutes of ischemia. Immediately following reperfusion, Phiphilux fluorescence was again seen in S1

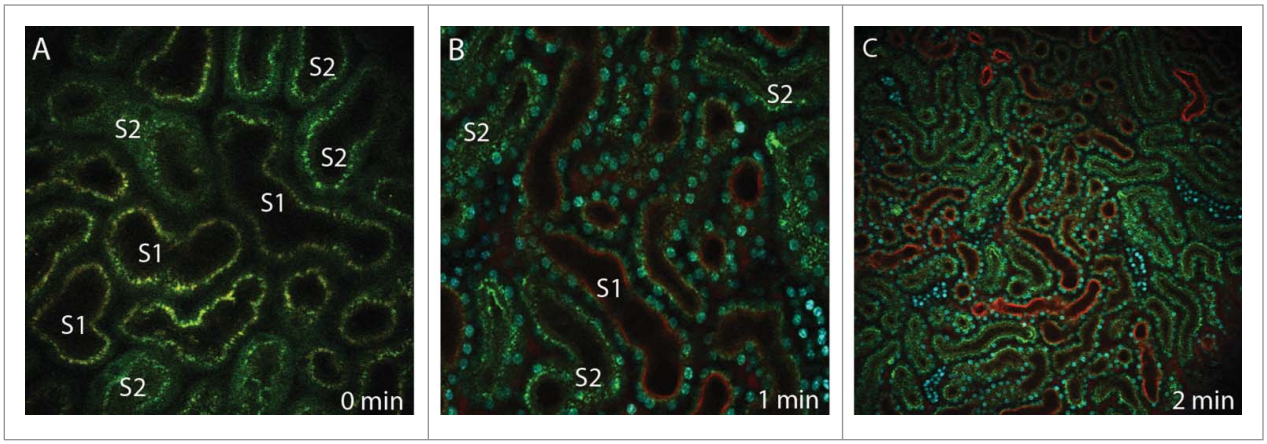

Figure 2. Intravital imaging of a sham caspase 3 KO kidney before (**A**) and after (**B, C**) Phiphilux injection. (**A and B**) are 60x magnification. (**C**) is 20x magnification.

tubules but not S2 (**Fig. 4A**). However, within 30 minutes after reperfusion, some Phiphilux signal could be detected in S2 tubular cells (**Fig. 4B**). Sixty minutes following reperfusion, Phiphilux positive S2 cells with condensed nuclei suggestive of apoptosis could be easily detected in random fields (**Fig. 4C1, 4C2 and 4C3** represent various planes from a single z stack). **Figure 4D** shows the persistence of non-apoptosis-related Phiphilux signal in S1 next to a clearly apoptotic Phiphilux positive S2 cell. Occasionally, Phiphilux signal was detected in damaged S2 cells shed into the S2 lumen (**Fig. 4E**). Interestingly, Phiphilux signal (presumably indicating caspase 3 activity) preceded nuclear condensation in these early shed apoptotic cells. At no time did we observe apoptotic nuclei or diffuse cytoplasmic Phiphilux signal in S1. Indeed, most ischemia-induced apoptosis in the kidney is confined to S2 and S3 segments.

The data presented above show that Phiphilux should be used with caution when imaging renal apoptosis with intravital 2-photon microscopy. The non-specific activation of this probe in S1 segments was clearly not related to caspase 3 and can be confounding in the overall

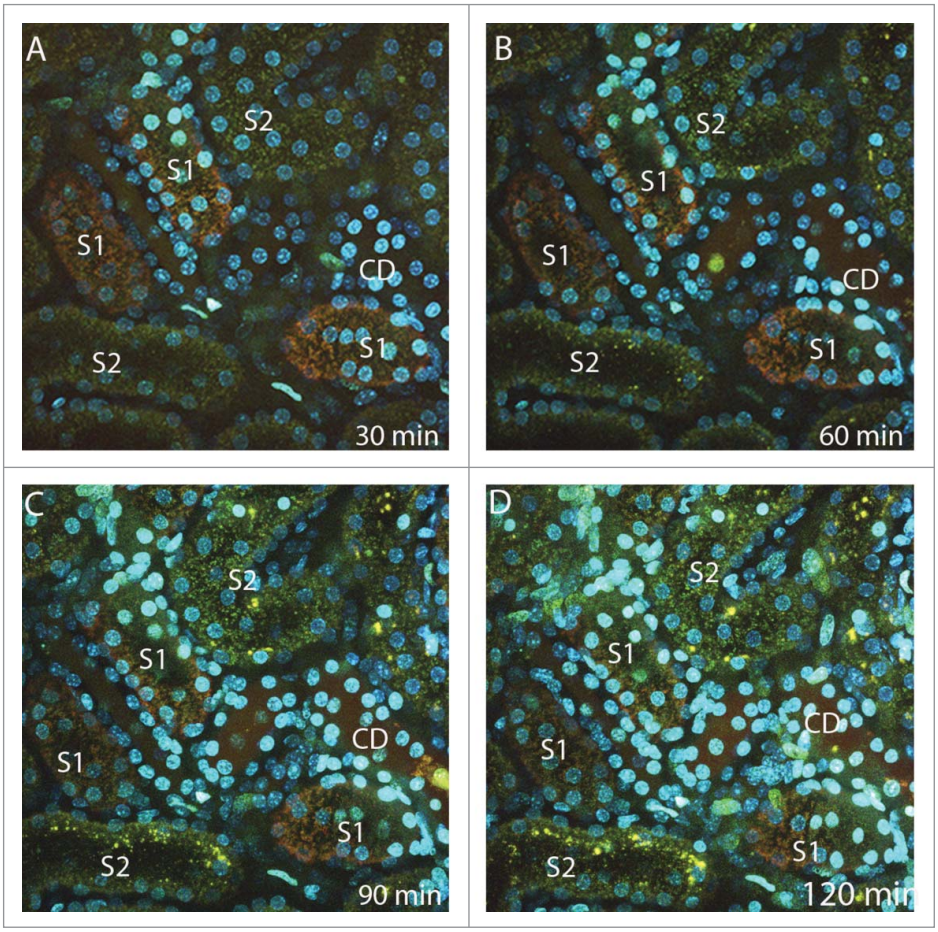

Figure 3. Intravital imaging of a sham wild-type mouse kidney volume after Phiphilux administration. Each of the 4 panels represents projection of a z stack (120 × 120 × 15 cubic micrometers). The same volume was imaged 4 times (at 30, 60, 90 and 120 minutes after Phiphilux administration. The mouse was kept under anesthesia throughout this 2 hour period.

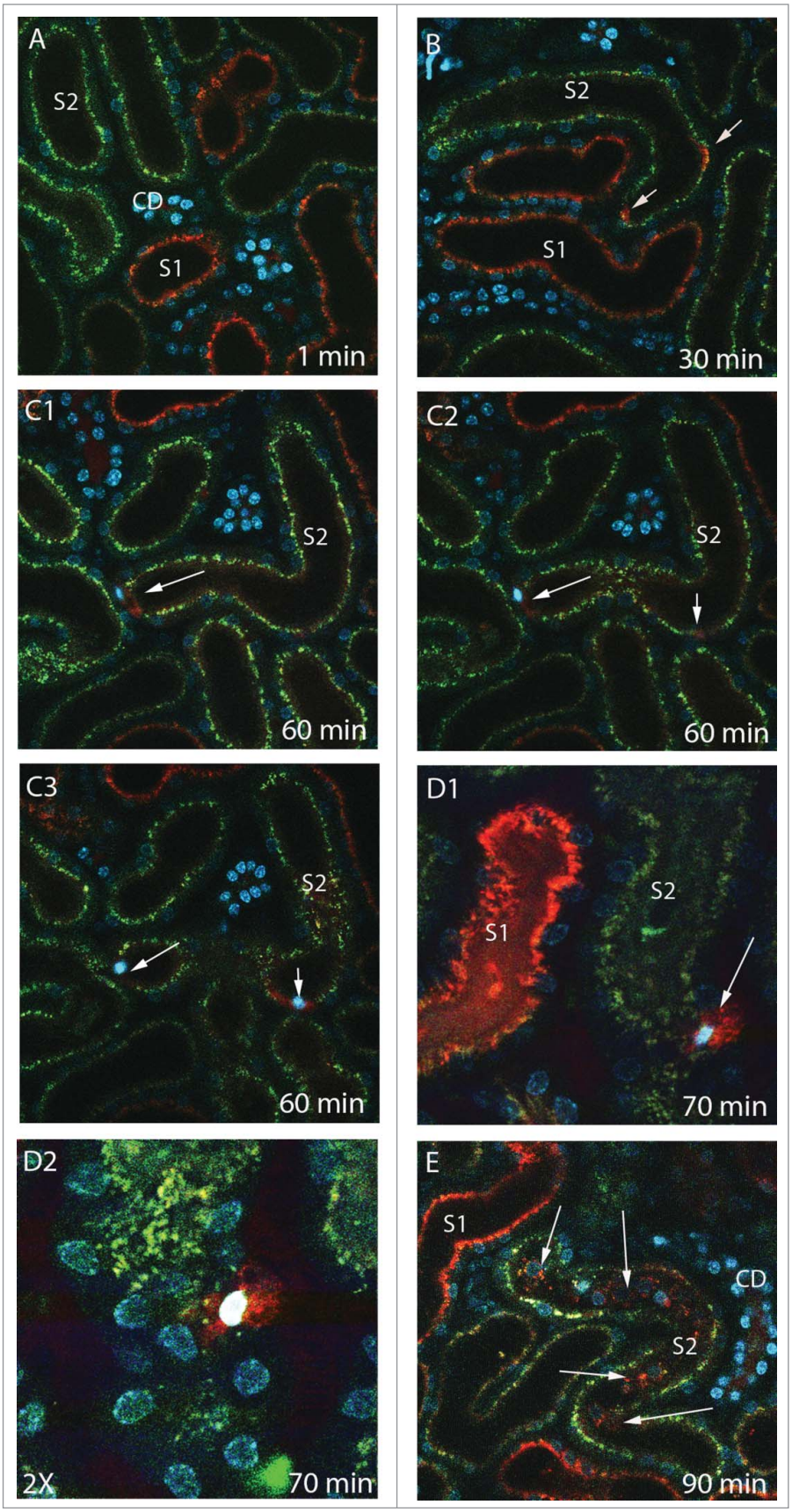

Figure 4. Intravital imaging of a wild-type ischemic mouse kidney. Thirty minutes after Phiphilux injection, the left renal pedicle was clamped for 40 minutes. Clamp was then released and the kidney imaged. Indicated times in panels are minutes post reperfusion. C1, C2 and C3 panels are various focal planes of a single z stack taken at 60 minutes. Arrows in (**B, C**) and (**D**) panels point to S2 cells exhibiting red Phiphilux signal with and without nuclear condensation. D2 is a 4X zoomed view of the apoptotic Phiphilux-positive S2 cells in D1 but in a different focal plane. In (**E**), arrows point to Phiphilux-positive cells in the S2 lumen.

estimation of apoptosis. The mechanism of non-caspase 3 -related activation of Phiphilux in S1 is unclear. Non-caspase proteases such as calpains could be involved in this process.[12,13] However, their distribution and activity in various tubular sub segments are unknown. In contrast, we believe that Phiphilux detected true apoptosis in S2 cells following ischemia. That this Phiphilux signal in apoptotic S2 cells is indeed a read-out of caspase 3 activity could theoretically be confirmed by performing the ischemia reperfusion experiment in caspase 3 KO mice. However, many groups have shown that with inhibition or absence of caspase 3, apoptosis at these early time points is greatly reduced if present at all.[14,15]

We have previously described the profound biological differences between S1 and S2 tubules.[9,10] The non-caspase 3 – related activation of Phiphilux in S1 tubules underscores this important segmental difference in the kidney. The utility of Phiphilux in reporting caspase 3 activation with intravital microscopy in other organs and tissues remains to be determined.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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