Diabetic Complications Consortium

Application Title: *Reactive lipids in podocyte homeostasis*.

Principal Investigator: *Krisztian Stadler, PhD*

1. Project Accomplishments:

The major accomplishments include a complete set of novel results in Aim 1 with analysis, and new in vivo experiments in Aim 2. We have successfully demonstrated that a) reactive lipids indeed activate RhoA in podocytes, b) higher levels of reactive lipids are detrimental to podocyte function, c) decoupling the redox sensitivity of RhoA from its activity by experimentally manipulating its cysteine residues preserves podocyte function.

Importantly, the results serve as preliminary data for an R01 application (as purpose of DiaComp guidelines suggest) which has been discussed in its first round, and currently being resubmitted for consideration with a deadline of Nov 5, 2015.

The project has been approved for a no cost extension by NIH until October, 2016 to complete the experiments.

2. Specific Aims:

The Specific Aims have not been modified from the original DiaComp application. We have made significant progress on both Aims.

Specific Aim 1. **We will demonstrate that reactive lipids serve as redox instigators of RhoA and survival mechanisms through Akt and podocyte specific proteins.**

Results: We finished a comprehensive set of experiments to test the hypothesis of Aim 1. These experiments demonstrate that in cultured, conditionally immortalized podocytes, reactive lipids generated from the alkyl radical donor AAPH activate RhoA. We also demonstrate that they influence podocyte function, causing podocyte injury at higher levels. Of note, it is interesting that at lower levels, reactive lipids seem somewhat stimulatory to certain markers such as WT-1, nephrin and the pAkt signaling axis. Further experiments are planned to investigate whether this observation indicates: a) stimulation of podocyte differentiation, b) adaptive hypertrophy where cells adapt to an initial, low level of oxidative stress or c) this is equally pathological and maladaptive, with a certain threshold that is experimentally measurable. Figs. 1-3 summarize the results and show representative pictures of these experiments.

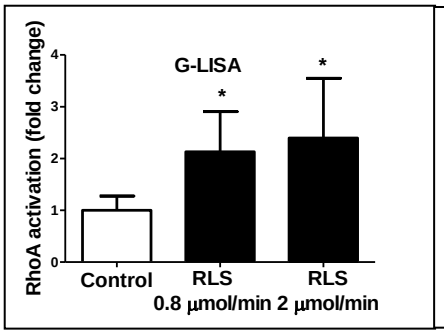

Fig. 1. Generation of reactive lipids trigger RhoA activation in podocytes. Reactive lipids (RLS) activate RhoA in podocytes as shown with an active RhoA specific G-LISA assay. The assay measures only the GTP-bound (active) RhoA using a monoclonal anti-RhoA antibody. RLS (0.8 and 2 µmol/min) were generated from AAPH in differentiated podocytes for 5 minutes. Two independent experiments were performed on 6-well plates, two replicates, 20,000 cells/well, serum-free media. * p<0.05

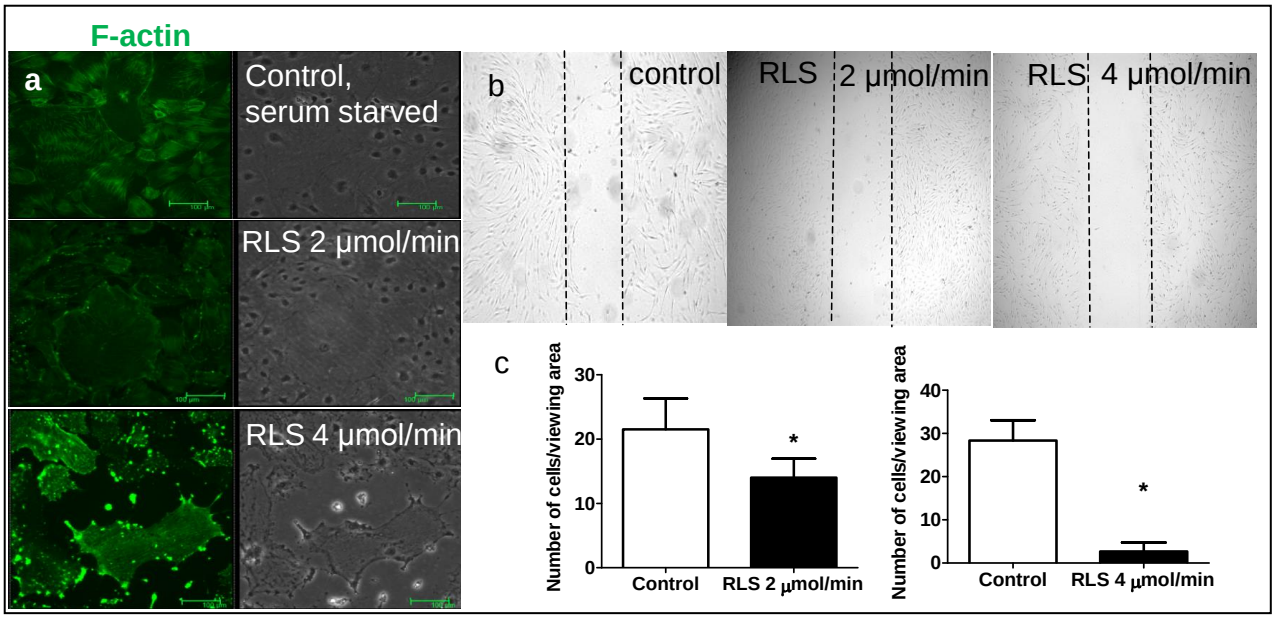

Fig. 2. Reactive lipids (RLS) modulate F-actin and cell migration. (a) Cells seeded at 20,000 cell/well density on 6-well plates were incubated with AAPH for 4 hrs in serum free media to generate 0.8-4 µmol/min RLS. After incubation, cells were fixed with 10% buffered formalin and incubated with a green fluorescent-tagged anti-F-actin antibody. Distribution of F-actin was observed under a fluorescent microscope (20 pictures per well at random viewing areas) and a representative picture is shown. (b) Podocytes treated with AAPH to generate RLS (2-4 µmol/min) under similar conditions were “wounded” with a pipette tip, and the number of cells migrating into the "wound" were counted 48 hrs later and (c) quantified on the bar graphs, *p<0.05

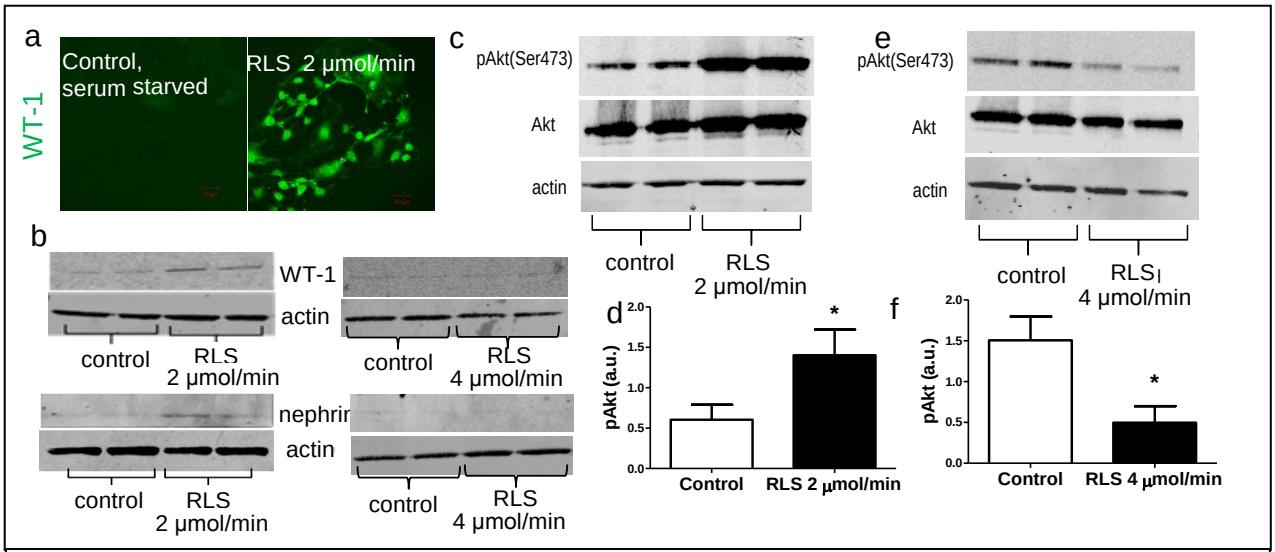

Fig. 3. Reactive lipids affect the levels of Wt-1, nephrin and pAkt. Podocytes were incubated with AAPH in serum free media to generate 2 – 4 µmol/min RLS. (a) Cells were labeled with a WT-1 antibody and a green fluorescent secondary antibody after fixation in 10% formalin. Green staining shows WT-1 in cells. (b) Low levels of RLS increase while higher levels do not stimulate WT-1 and nephrin levels, (c-d): low doses of reactive lipids increase while (e-f) higher levels inhibit Akt phosphorylation as shown on all panels by Western blot analyses of cell lysates. Two independent experiments were evaluated. *p<0.05 vs. control.

Furthermore, the proposed RhoA constructs have been made by Dr. Jason Collier's laboratory. RhoA has two redox sensitive cysteine residues (Cys16 and Cys20) within a redox-sensitive motif in the phosphoryl binding loop. Dr. Collier mutated these cysteines to alanines (C16A and C20A) to deliver the constructs into cells by adenoviral vectors. The mutation of the cysteine residues maintains RhoA GTPase responsiveness to traditional stimuli, but **abrogates the redox sensitivity**. Thus the mutant protein is expected to be an active and functional GTPase, but can no longer be stimulated by reactive lipids. The control vector, wild type RhoA, C16A-, C20A- and C16A/C20A-RhoA double mutant adenoviral vectors have already been constructed, tested for gene delivery by transcript abundance and for RhoA protein production by Western blot analysis. Initial results are shown on Fig. 4 with the C16/20A double mutants.

Differentiated podocytes (6-well plates, 20000 cells/well) were transduced with the double mutant RhoA (C16A/C20A) or the wild type RhoA in serum free media and 24 hrs later were stimulated with reactive lipids at 2 µmol/min from AAPH (25 mM) decomposition for 4 hrs. Cells were lysed for GTP-bound RhoA measurements or fixed on the plate for fluorescent staining of F-actin. While wild type RhoA was activated upon reactive lipid stimuli, RhoA bearing mutated cysteines was not redox responsive anymore as indicated by no change in detectable GTP-bound RhoA levels (Figure 4a). F-actin staining revealed that cells with wild type RhoA cortically rearrange their actin fibers with RLS stimuli (Figure 4b left panels), but cells receiving RhoA with both cysteine to alanine substitutions maintain healthy F-actin stress fibers (Figure 4b right panels), despite the presence of reactive lipids.

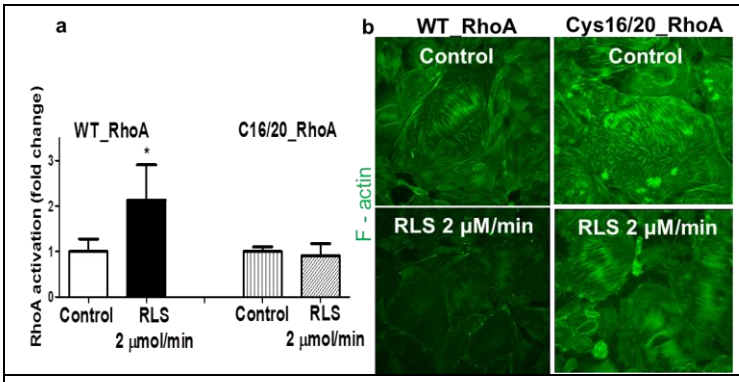

Fig. 4. Double cysteine mutation on RhoA blunts its redox sensitivity and the response in F-actin redistribution. Podocytes transfected with mutated RhoA (Cys16/20 RhoA) were exposed to RLS generated by AAPH as described in the experimental design. (a) GTP-bound RhoA levels were analyzed with a G-LISA assay as described on Fig. 1. (b) F-actin distribution was analyzed as described on Fig. 2. Triplicate experiments, * $p<0.05$.

These results demonstrate that RhoA is no longer activated by reactive lipid treatment when both cysteine residues are mutated, providing definitive evidence that RhoA senses or responds to reactive lipids via these two amino acids. Distribution of F-actin fibers is also regulated by reactive lipids in a RhoA dependent manner.

We will complete the measurements of podocyte markers and pAkt signaling in the no cost extension phase.

Note: the application presented preliminary data with the donor AAPH concentrations (10-50 mM). To demonstrate the actual amount of reactive lipids produced, we have performed calculations based on the decomposition equation of AAPH and results are displayed with these concentrations.

Specific Aim 2. **We will establish that specific scavenging of *excess* lipid radicals restores adaptive redox sensitive signaling in podocytes in vivo.**

Results: We have tested the SHHF rat model for increased lipid radical production, and also explored the effects of a 2-week lipid radical scavenger POBN treatment regimen on their renal phenotype, RhoA activation and podocyte insulin sensitivity. Evaluation of the results, including analyzing the histology stainings from all groups is currently ongoing. Under the no cost extension phase, we will finish these experiments and data analysis, as well as will expand the experiments to the proposed db/db mouse model. IACUC approval has been obtained to include these models in our protocol. We will explore lipid radical production in the kidney of db/db mice and age matched db/m controls, and the potential positive effects of scavenging these radicals by POBN treatment as proposed. We will focus on potential improvement of podocyte insulin signaling, podocyte markers, slit diaphragm proteins and albuminuria as main outcomes.

3. Publications:

Stadler K, Goldberg IJ, Susztak K. (2015) The emerging understanding of the role of lipid metabolism in diabetic kidney disease. *Curr Diab Rep* 15:40 PMID: 25957525.

Wicks SE, Nguyen TT, Breaux C, Kruger C, **Stadler K**. (2015) Diet-induced obesity and kidney disease - In search of a susceptible mouse model. *Biochimie. 2015 Aug* S0300-9084(15) 00248-5. doi: 10.1016/j.biochi.2015.08.001. [Epub ahead of print] PMID: 26248309

A *poster* and an *invited talk* will also be presented on the Annual ASN Meeting (3-8 November, 2015, San Diego) summarizing the research findings on reactive lipids, kidney disease and podocyte function, related to the DiaComp proposal.