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TRPC5-mediated podocyte calcium toxicity drives progressive glomerular disease

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AGTR1	Angiotensin II receptor type 1
CI	Confidence interval
CKD	Chronic kidney disease
DMSO	Dimethylsulfoxid
ESRD	End stage renal disease
FBS	Fetal bovine serum
IFN-γ	Interferon-γ
MPT	Mitochondrial permeability transition
PI	Propidium iodide
SD	standard deviation
TG	Transgenic
Thg	Thapsigargin
TRPC5	Transient receptor potential cannel 5
ROS	Reactive oxygen species

1 INTRODUCTION

Chronic kidney disease (CKD) is a major public health problem worldwide. According to the 2014 Center of Disease Control and Prevention estimate more than 10% of the US population – more than 20 million people have CKD. Studies from Europe and Asia show similar numbers (Levey et al., 2007). By 2030 more than 2 million Americans will have progressed to end stage renal disease (ESRD) (Stevens et al., 2006), which with the use of dialysis still has a mortality rate of 20% per year or 5 year mortality of 60-70% (Go et al., 2004). Additionally CKD is associated with a high risk of cardiovascular mortality to an extent that a patient with CKD is more likely to die due to stroke or myocardial infarction than to progress to ESRD (Agrawal et al., 2009).

1.1 Podocyte damage

Damage to podocytes – the cells that form the filter structure in the kidney, plays a central role in the progression of kidney disease (Greka and Mundel, 2012b). To make the filtering process possible, podocytes have a highly specialized cytoskeleton with interdigitating foot processes that cover the blood vessels in the glomerular tufts – the filter units in the kidney (Pavenstadt et al., 2003). To guarantee sustained glomerular filter function there is a sophisticated regulatory network underlying the cytoskeleton in podocytes (Faul et al., 2008). The podocytes response to injury is characterized by a cytoskeletal rearrangement called foot process effacement. This rearrangement leads to breakdown of the filter structure and loss of vital blood proteins like albumin with the urine (Mundel and Shankland, 2002). Albuminuria therefore is one of the earliest and most sensitive markers of filter damage and is a strong and independent predictor of kidney function decline, progression of cardiovascular disease and all-cause mortality (Agrawal et al., 2009). Why foot process effacement is initiated by the podocytes as the stereotypical response to almost all insults is still under debate. According to one theory it is an adaptive strategy to protect podocytes from being lost by detachment from the glomerular tuft. Loss of more than 40% of its podocytes leads to irreversible destruction of the glomerulus called glomerulosclerosis (D'Agati et al., 2011). As podocytes cannot be regenerated in the adult a mechanism to protect those viable cells even at the expense of some filter capacity would not be surprising (Kriz et al., 2013). In agreement with this theory foot process effacement in it self is a reversible process as can be seen in minimal change disease: During active disease with massive proteinuria the only visible change is foot process effacement and after remission no difference can be detected to health kidneys.

1.2 The role of TRPC5 in the initiation of foot process effacement

One of the essential steps in the initiation of foot process effacement is activation of the canonical transient receptor potential cannel 5 (TRPC5) (Schaldecker et al., 2013). TRPC channels are a family of six nonselective cation channels, which are classically activated downstream of phospholipase C activation and generation of phosphatidylinositol-4,5-bisphosphate (PIP_2) (Clapham, 2003). Primarily Ca^{2+} and Na^{+} are conducted by TRPC5 at a permeability ratio of 10:1 (Blair et al., 2009). A range of additional factors including membrane stretching (Lau et al., 2016) and oxidation (Hong et al., 2015) activates TRPC5. Thus the Ca^{2+} influx through TRPC5 is the integrated result of a variety of input signals. Recent work in the Greka laboratory revealed that TRPC5 knock out (KO) mice are unable to initiate foot process effacement in response to toxic substances widely used as established models of acute kidney damage (Schaldecker et al., 2013). Therefore our current understanding is that calcium influx through TRPC5 is activating well-known mediators of cytoskeletal rearrangement to initiate foot process effacement (Clapham, 2007; Faul et al., 2008; Greka and Mundel, 2012a; Hall, 1998).

The question what is causing the final progression from foot process effacement to ESRD remains unresolved (D'Agati et al., 2011). We hypothesized that persistent Ca^{2+} influx through sustained TRPC5 activation in a chronic disease scenario will finally overwhelm the podocytes Ca^{2+} buffering mechanisms and cause irreversible Ca^{2+} toxicity and cell death.

1.3 Calcium toxicity

Loss of Ca^{2+} homeostasis with sustained elevation of cytosolic Ca^{2+} has been known to cause cell death for decades (Schanne et al., 1979). Among the different destructive pathways that get activated by high cytosolic Ca^{2+} mitochondrial permeability transition (MPT) is often decisive in determining the lethal outcome (Dong et al., 2006). Rising levels of cytosolic Ca^{2+} often signal increased energy demand for the cell. Following the electrical gradient of the mitochondrial membrane potential Ca^{2+} is taken up by mitochondria. Increased free Ca^{2+} in the mitochondrial matrix stimulates oxidative metabolism matching ATP production to the increased demand (Pan et al., 2013; Rasola and Bernardi, 2011). A rise in oxidative metabolism is accompanied by a rise in reactive oxygen species (ROS) (Rasola and Bernardi, 2011). If the level of mitochondrial matrix Ca^{2+} or ROS rises too high a cyclophilin D (encoded by the Ppif gene) dependent large conductance pore in the inner mitochondrial membrane opens (Baines et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). Under physiological circumstances the reversible MPT pore opening seems to be an important homeostatic mechanism to deal with strong, transient and local fluctuations in Ca^{2+} concentration (Rasola and Bernardi, 2011). An impressive example is the inability of Ppif KO mice to deal with pressure overload after transaortic constriction and their high mortality in a cardiac performance test

called “swimming exercise” (Elrod et al., 2010). Prolonged MPT pore opening in consequence of sustained high cytosolic calcium or ROS leads to release of matrix Ca^{2+}, loss of the mitochondrial membrane potential with termination of oxidative phosphorylation and mitochondrial swelling up to rupture of the outer membrane releasing even more cell death inducing factors (Rasola and Bernardi, 2011). Apart from being the critical step in ischemia reperfusion injury (Baines et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005) MPT also plays a decisive and detrimental role in chronic settings like Alzheimer’s disease (Du et al., 2008). A similar study in an animal model of heart failure could show that enhanced activity of a Ca^{2+} channel progresses to Ca^{2+} overload causing MPT, cell death and finally organ failure (Nakayama et al., 2007).

1.4 Approach and aim of the study

We hypothesized that sustained TRPC5 activation in a chronic kidney disease setting will cause Ca^{2+} overload with MPT leading to podocyte loss and finally kidney failure. Therefore we wanted to investigate if sustained pharmacological TRPC5 activation causes cell death in cultured podocytes with detectable signs of mitochondrial damage and MPT. If our hypothesis is correct TRPC5 blockade could be a new therapeutic strategy for kidney disease.

2 MATERIAL AND METHODS

2.1 Cell culture

Conditionally immortalized mouse podocytes were provided by Dr. P. Mundel (MGH, Boston, USA) and cultured as previously described (Mundel et al., 1997). In brief podocytes were cultured in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100U/ml penicillin, 100µg/ml streptomycin (all gibco) and 50U/ml mouse interferon-γ (IFN-γ) (eBioscience) at 33°C and 5% CO_2 I. The cells were split when reaching 70% confluency. For differentiation the podocytes were split, thermoshifted to 37°C and cultured in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100U/ml penicillin, 100µg/ml streptomycin (all gibco) without (IFN-γ) for twelve days. Media was changed every other day for differentiating as well as for proliferating cells. All cell culture dishes for podocytes were coated with type I collagen (100µg/ml in 0.02N acetic acid incubated for 1h at 37°C). Only cultures which reached optimal confluency and showed the typical differentiated morphology (Shankland et al., 2007) after 12 days of differentiation were used for experiments.

2.2 Viability assays

2.2.1 CellTiter-Glo® Luminescent Cell Viability Assay

Podocytes were differentiated in 96 well plates and incubated with the indicated concentration of Riluzole dissolved in DMSO for two or three days. Promega's CellTiter-Glo® reagent was added to the cells and viability measured according to the manufacturers instructions. This assay uses cellular ATP to create light with luciferase. Therefore the detected luminescence signal is proportional to cell viability. All experimental conditions were performed at least in quadruplicate and the entire experiment was repeated three times. The data is presented as fraction of viable cells, with DMSO only treated set as 1.

2.2.2 Annexin V / propidium iodide flow cytometry

Annexin V – Alexa Fluor 647 conjugate and propidium iodide (PI) solution (1 mg/ml) were purchased from Molecular Probes™. Annexin V conjugates bind phosphatidylserine in the outer layer of the cell membrane, indicating this early apoptotic change. PI is an intercalating dye, which can only bind cellular DNA after membrane integrity is broken to indicate necrosis. Podocytes were differentiated in 6 well plates and incubated with Riluzole and/ or ML204 as indicated. At the end of the experiment the cells were trypsinized, washed with PBS, stained in annexin binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) with 4µL annexin V conjugate and 0.5 µL PI, kept on ice and analyzed by flow cytometry as soon as

possible. All flow cytometry experiments were performed on a BD LSRFORTESSA Flow Cytometer. Annexin V or PI positive cells were classified as dead. For each condition more than 90000 cells were analyzed in total in three experiments performed in triplicate.

2.3 Mitochondrial Permeability Transition assay

To directly measure mitochondrial permeability transition the MitoProbe Transition Pore Assay Kit for flow cytometry (Molecular Probes) was used according to the manufacturers instructions. In brief after incubating podocytes with Riluzole and/ or ML204 as indicated the cells were trypsinized and stained with Calcein AM and Cobalt in HBSS for 15min. After Calcein AM is taken up into living cells esterases cleave of the AM group resulting in a bright green fluorescence in all living cells. Co^{2+} quenches the Calcein fluorescence everywhere except in mitochondria, as it cannot penetrate through the intact mitochondrial membrane. The reagents were quenched by adding more HBSS, spun down, resuspended in annexin binding buffer with annexin V and PI and analyzed by flow cytometry as soon as possible. For each condition more than 450000 cells were analyzed in total in three experiments performed in triplicate. The data is presented as relative calcein fluorescence, with DMSO only treated set as 1.

2.4 Reactive oxygen species detection

CM-H2DCFDA and Mitosox were bought from Molecular Probes and used according to the manufacturers protocol. In brief after incubating podocytes with Riluzole the cells were stained with reactive oxygen species indicator and analyzed by flow cytometry as soon as possible.

2.5 Mitochondrial morphology

Mitochondria in differentiated podocytes were stained with MitoTracker® Red CM-H2XRos (Molecular Probes). The dye is non-fluorescent until it gets oxidized in live cells and becomes covalently bound to mitochondrial proteins. Because the accumulation of the staining in mitochondria is mitochondrial membrane potential dependent we stained live podocytes before the start of the experiment. After incubating the cells with Riluzole as indicated, images were acquired by confocal microscopy from living podocytes in cell culture media with a 63X water immersion lens. Representative images were acquired by randomly sampling all four quadrants of a dish. To determine the aspect ratio (ratio of major axis to minor axis) (Yu et al., 2006) and a value for the number of swollen mitochondria the images were quantified with custom written ImageJ scripts (see supplement). First, the images were convolved with a 7x7 Mexican hat kernel to separate closely packed mitochondria. The mitochondria were segmented from the background by an automated

thresholding procedure. ImageJ's "analyze particles" function was used to determine the aspect ratio for all segmented objects above a minimum size (to control for background noise artifacts) or to count all swollen mitochondria defined by a minimum size and circularity. To verify the classification the counted objects were visualized separately for each image.

2.6 Statistical analysis

All statistical analysis was done in Graphpad Prism. The type of statistical test is indicated in the figure legend for each experiment. p-Values < 0.05 were considered significant.

3 RESULTS

To test our hypothesis we first wanted to investigate if it is mechanistically possible to induce cell death in podocytes by sustained TRPC5 activation. We used riluzole, a drug used for treatment of amyotrophic lateral sclerosis with unknown mechanism (Bellingham, 2011), which was recently identified as a specific TRPC5 activator (Richter et al., 2014). To be able to investigate the effects of TRPC5 activation specifically in podocytes we used the podocyte cell line established by our collaborator Dr. Mundel (Mundel et al., 1997).

3.1 TRPC5 activation causes podocyte death

TRPC5 activation with riluzole causes cell death in a dose and time dependent manner (**Fig. 1A-C**). The Luminescent Cell Viability Assay measuring ATP content (**Fig. 1A**) and annexin V & propidium iodide (PI) staining measuring membrane integrity (**Fig. 1 B/C**) both showed increasing cell death with increasing dose or time of exposure to riluzole. To verify that the riluzole effects are mediated by TRPC5 activation we used ML204, a specific TRPC5 blocker (Miller et al., 2011). By blocking TRPC5 30min prior to and during the incubation with riluzole we could protect podocytes from riluzole induced cell death. ML204 alone had no effect on survival. To keep the culture condition the same over the time course of the experiment and replenish potentially degraded or metabolized drugs we changed media every 3rd day. Because dead cells disintegrate or loose adherence to the dish during media changes, the number of surviving (Annexin&PI negative) cells is given. (**Fig. 1D**) At 100µM riluzole podocyte death becomes apparent by phase contrast microscopy after three days while podocytes incubated with riluzole and ML204 or ML204 alone look healthy. (**Fig. 1E**) These results establish that sustained TRPC5 activation causes podocyte death.

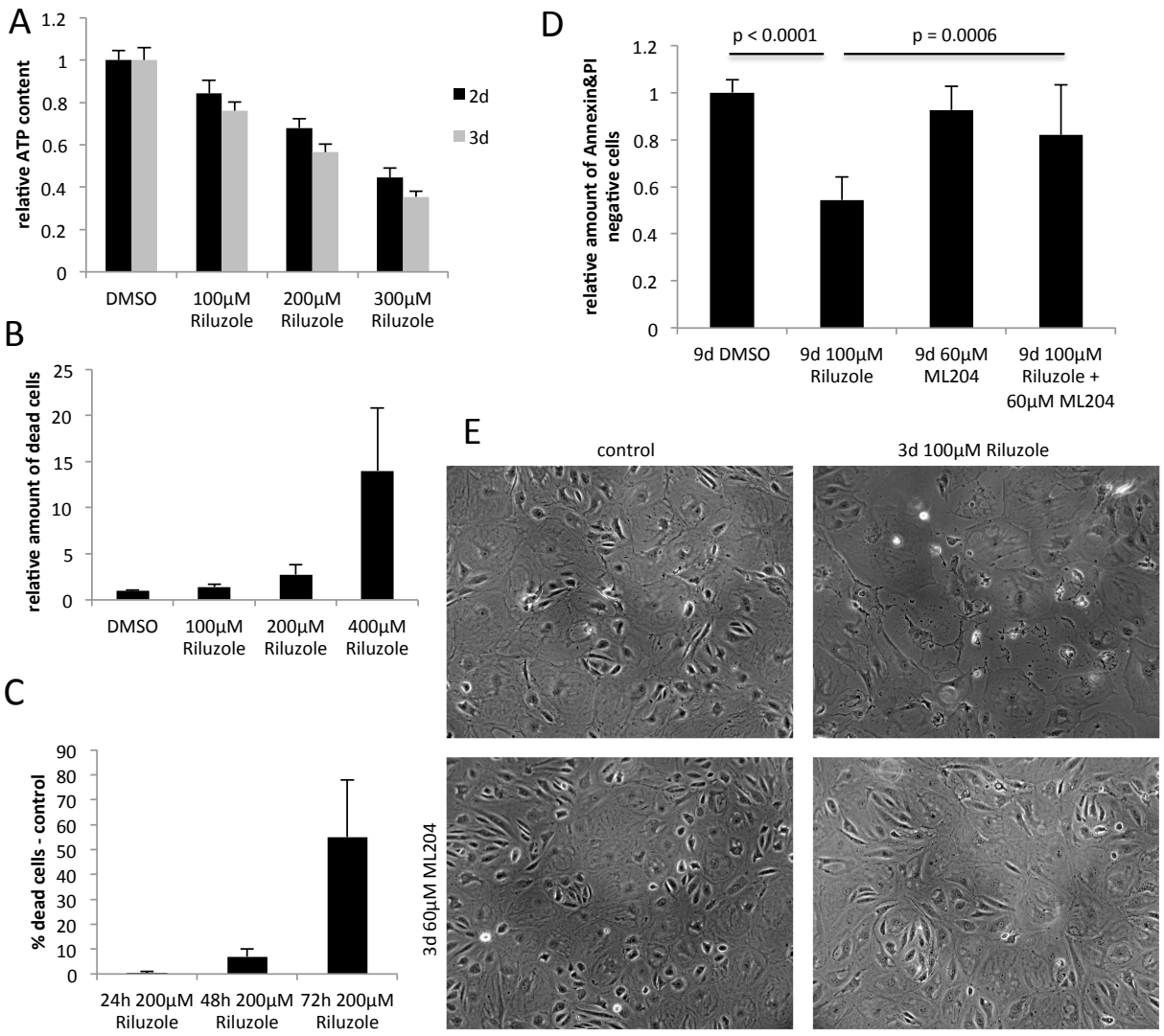

Figure 1: TRPC5 activation induces podocyte death. (**A**) Podocytes were incubated with the indicated doses of TRPC5 activator riluzole for two or three days. Cell survival was measured via ATP content with the Promega CellTiter-Glo® Luminescent Cell Viability Assay according to the manufactures protocol. $p<0.005$ for all differences except 100µM riluzole:2d vs. 100µM riluzole:3d, 100µM riluzole:3d vs. 200µM riluzole:2d and 300µM riluzole:2d vs. 300µM riluzole:3d. Error bars show confidence interval (CI) (**B**) Podocytes were incubated with escalating doses of riluzole for two days. Relative amount of dead (Annexin and/or PI positive) cells was measured by flow cytometry. $p<0.05$ for all differences except 100 vs. 200µM riluzole. Error bars show CI (**C**) Podocytes were incubated for increasing times with 200µM riluzole. Cell death was determined with Annexin&PI by flow cytometry. For each time point the percentage of dead cells in the riluzole sample subtracted by the percentage of dead cells in the control is shown to control for natural turnover. All differences between the groups are significant with $p<0.001$. Error bars show CI (**D**) To show that riluzole is activating TRPC5 specifically we preincubated podocytes with the TRPC5 blocker ML204 followed by parallel incubation with riluzole and ML204 for nine days. Media was changed and replaced with fresh media and drugs every 3rd day. The number of surviving cells measured by flow cytometry shows that TRPC5 blockade is protecting against riluzole induced cell death. Error bars show standard deviation (SD) (**E**) Phase contrast images taken from the same experiment at day 3. Podocytes incubated with riluzole are dying and detaching from the dish while podocytes incubated with riluzole and ML204 are protected. All statistical analysis was done with ANOVA and Tukey post-test.

3.2 TRPC5 activation leads to mitochondrial permeability transition

To investigate the role of MPT in the riluzole induced podocyte death we used the calcein and cobalt (Co^{2+}) staining (Vaseva et al., 2012). Co^{2+} is quenching the calcein fluorescence everywhere except in the mitochondrial matrix because it is not able to permeate through the intact inner mitochondrial membrane. If the MPT pore opens, Co^{2+} diffuses into the mitochondria causing a decrease in calcein fluorescence. We used Life Technologies MitoProbe Transition Pore Assay Kit and its adapted protocol for our experiments. Incubation with 200µM riluzole for 24h decreased calcein fluorescence to the level of the positive control for MPT (**Fig. 2A**). This suggests a lethal loss of mitochondrial membrane integrity. When the MPT pore is open, an increase in ROS either as cause or consequence of MPT has been observed (Rasola and Bernardi, 2011). Using the ROS indicator H_2DCFDA we could see an increase after podocyte treatment with Riluzole (**Fig. 2B**). Additionally we could confirm this result using the more specific mitochondrial superoxide indicator Mitosox Red (**Fig. 2C**). Similar to rates of cell death due to Riluzole (Fig. 1A/B and F), MPT was observed in a dose-dependent manner. (**Fig. 2D**). To show the riluzole induced MPT is specifically mediated by TRPC5 we used ML204 to block TRPC5. As shown in **Fig. 2 D and E** the calcein fluorescence is rescued after ML204 treatment. These data suggest MPT as an important mechanism for podocyte death induced by sustained TRPC5 activation.

3.3 TRPC5 activation induces mitochondrial swelling and fragmentation.

Swelling of the mitochondrial matrix is a morphologic characteristic of MPT (Rasola and Bernardi, 2011). Fragmentation is a common mitochondrial response to damage ranging from ischemia reperfusion injury to high glucose (Brooks et al., 2009; Yu et al., 2006). Mitochondria fuse or fragment in response of different environmental stresses. Hyperfusion is seen in response to increased energy demand. The idea is similar to an electrical grid. A long interconnected network is efficiently distributing energy in the cell. Mitochondria use a proton gradient over their inner membrane to produce ATP. This gradient can be established anywhere along the membrane. Thus protons can be pumped out of the mitochondrial network in the periphery of the cell where necessary oxygen is abundant and used to produce ATP closer to where its needed in more central regions of the same network (Tondera et al., 2009; Westermann, 2010; Youle and van der Bliek, 2012). Fragmentation occurs in consequence of direct mitochondrial damage. If only parts of the mitochondrial network are damaged this is a way to facilitate quality control by separating and degrading the damaged mitochondria (Westermann, 2010; Youle and van der Bliek, 2012). While working with thapsigargin (Thg) to establish our assays for mitochondrial morphology we were able to confirm this. Low doses of Thg (3µM) cause a strong and finally fatal unfolded protein response. In this experiment even podocytes close to death after three days of exposure showed no fragmentation but highly elongated, hyperfused mitochondria. In contrast podocytes incubated with

20µM Thg for 24h, which causes direct mitochondrial damage with MPT (Korge and Weiss, 1999) showed extensive swelling and fragmentation (**Fig. 3**).

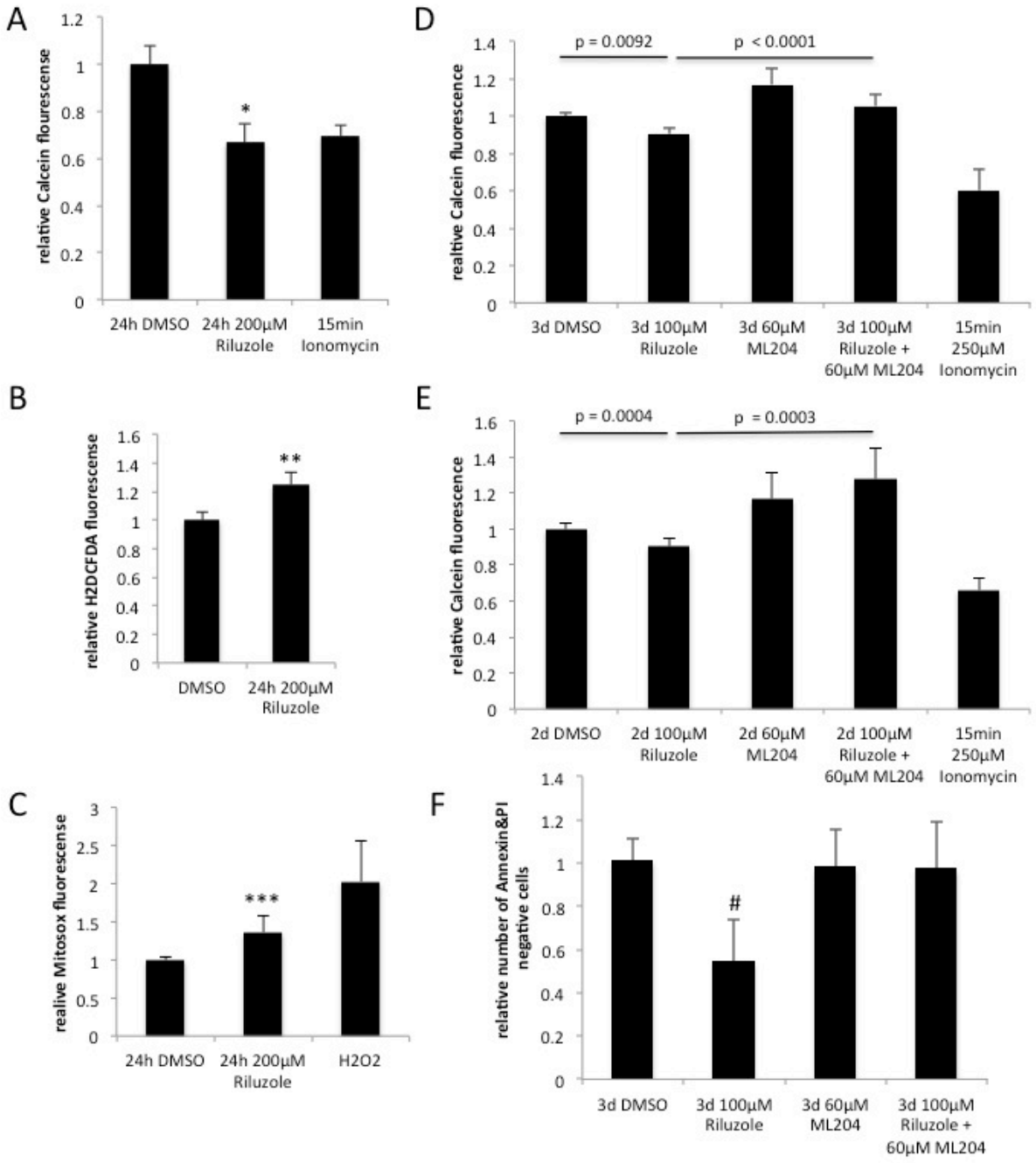

Figure 2: TRPC5 activation induces Mitochondrial Permeability Transition (MPT) in podocytes. (**A**) Podocytes were incubated for 24h with 200µM riluzole before being stained with calcein and Co^{2+} and analyzed by flow cytometry. A decrease in calcein fluorescence means the MPT pore has opened and Co^{2+} can get into the mitochondria quenching the calcein fluorescence there. The ionophore ionomycin was used as positive control to induce MPT by maximally increasing cytosolic Ca^{2+}. The value of each sample is the average calcein fluorescence of 30000 to 50000 cells measured by flow cytometry. Pooled relative values of three experiments done in triplicate are shown. Error bars indicate SD. *p=2.8E-07, t-test (**B**) Riluzole is increasing reactive oxygen species (ROS). Increased fluorescence of the ROS indicator H_2DCFDA was measured by flow cytometry. Tert-butyl hydroperoxide was used as positive control (data not shown because of scale at 8.5±1.6) Error bars indicate CI. **p=6E-05, t-test (**C**) Riluzole is increasing mitochondrial superoxide. Increased

fluorescence of the specific mitochondrial superoxide indicator Mitosox was measured by flow cytometry. H_2O_2 was used as positive control. Error bars indicate SD. ***p=0.0002, t-test (**D**) 100µM riluzole is causing a 10% decrease in calcein fluorescence after three days. The much more delicate phenotype is correlating well with the observed different timeframe between 200 and 100µM riluzole in causing cell death (almost all cells dead after 3-4 days at 200 vs. 50% surviving after 9 days at 100, Fig. 1). Error bars indicate SD. p-values are calculated by ANOVA with Tukey post-test (**E**) The same 10% decrease in calcein fluorescence can been seen already after two days by incubating the cells with riluzole during the staining process. Calcein fluorescence is higher with ML204. p-values are calculated by ANOVA with Tukey post-test (**F**) The number of surviving cells counted in parallel to the MPT assay at three days shows a significant decrease with riluzole. #p<0.005 to all others, all other conditions show no statistically significant differences. ANOVA with Tukey post-test, Error bars show CI.

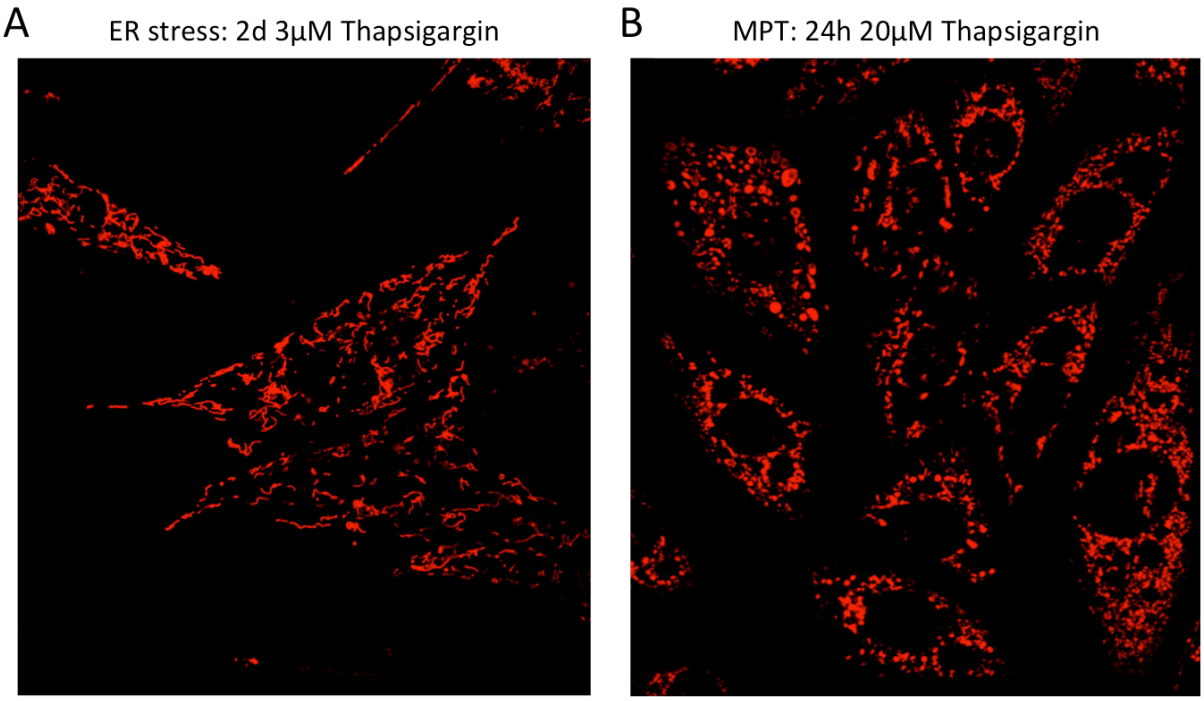

Figure 3 Mitochondrial morphology changes in response to stress. (**A**) Podocytes were incubated with 3µM thapsigargin (thg) for three days to cause a lethal unfolded protein response. While the cells are close to death at this time point their mitochondria maintain elongated hyperfused morphology. (**B**) To introduce direct mitochondrial damage and MPT podocytes were incubated with 20µM thg for 24h. In this condition the mitochondria show characteristic swelling and fragmentation.

Podocytes incubated with 200µM riluzole for 24h showed mitochondrial swelling and fragmentation (representative images are shown in **Fig. 3A**). To be able to visualize mitochondrial dynamics in living cells we used MitoTracker® Red CM-H2XRos to stain mitochondria before starting the experiments. After incubating with riluzole for the indicated times we took the podocytes right out of the incubator to the microscope without any further manipulation. This is necessary because we noticed the morphologic change is reversible within a short timeframe. Live imaging revealed that mitochondrial swelling and fragmentation is reversed in the absence of riluzole within 30min. (**Fig. 3B**). However this only seems to be the case for up to 48h with

200µM riluzole. After 24h incubation with 300µM riluzole the podocytes already had crossed a point of no return and their mitochondria stayed swollen and fragmented even after incubating them with fresh media (**Fig. 3C**). These observations by live microscopy further corroborate that sustained TRPC5 activation causes mitochondrial damage leading ultimately to podocyte death.

3.4 TRPC5 activation leads to podocyte loss in vivo

To validate these in vitro findings, we sought to study an animal model of progressive proteinuric CKD. Rats that overexpress the human Angiotensin II receptor type 1 (hAGTR1) specifically in podocytes (AGTR1 TG rats) spontaneously develop proteinuria, nephrotic syndrome and podocyte loss resulting in glomerulosclerosis, independent of changes in blood pressure, at the age of 15 weeks. They finally die from renal failure at 60 weeks (Hoffmann et al., 2004). TRPC5 is activated downstream of AGTR1 to initiate cytoskeletal rearrangements (Tian et al., 2010). Therefore we hypothesized that sustained TRPC5 activation in the AGTR1 TG rats is causing similar effects to the observed sustained TRPC5 activation with riluzole in vitro. Podocyte loss in the AGTR1 TG rat may be due to Ca^{2+} overload and mitochondrial damage with MPT, thus we specifically hypothesized that it should thus be beneficial to block the Ca^{2+} influx through TRPC5 in vivo.
In an initial study, we treated AGTR1 TG rats with ML204 for two weeks after onset of disease marked by increasing albuminuria. The albuminuria remitted significantly under ML204 treatment.
This study reveals TRPC5 activity as critical mediator of disease progression in CKD and suggests TRPC5 blockade as new therapeutic strategy for acquired and progressive proteinuric kidney disease.

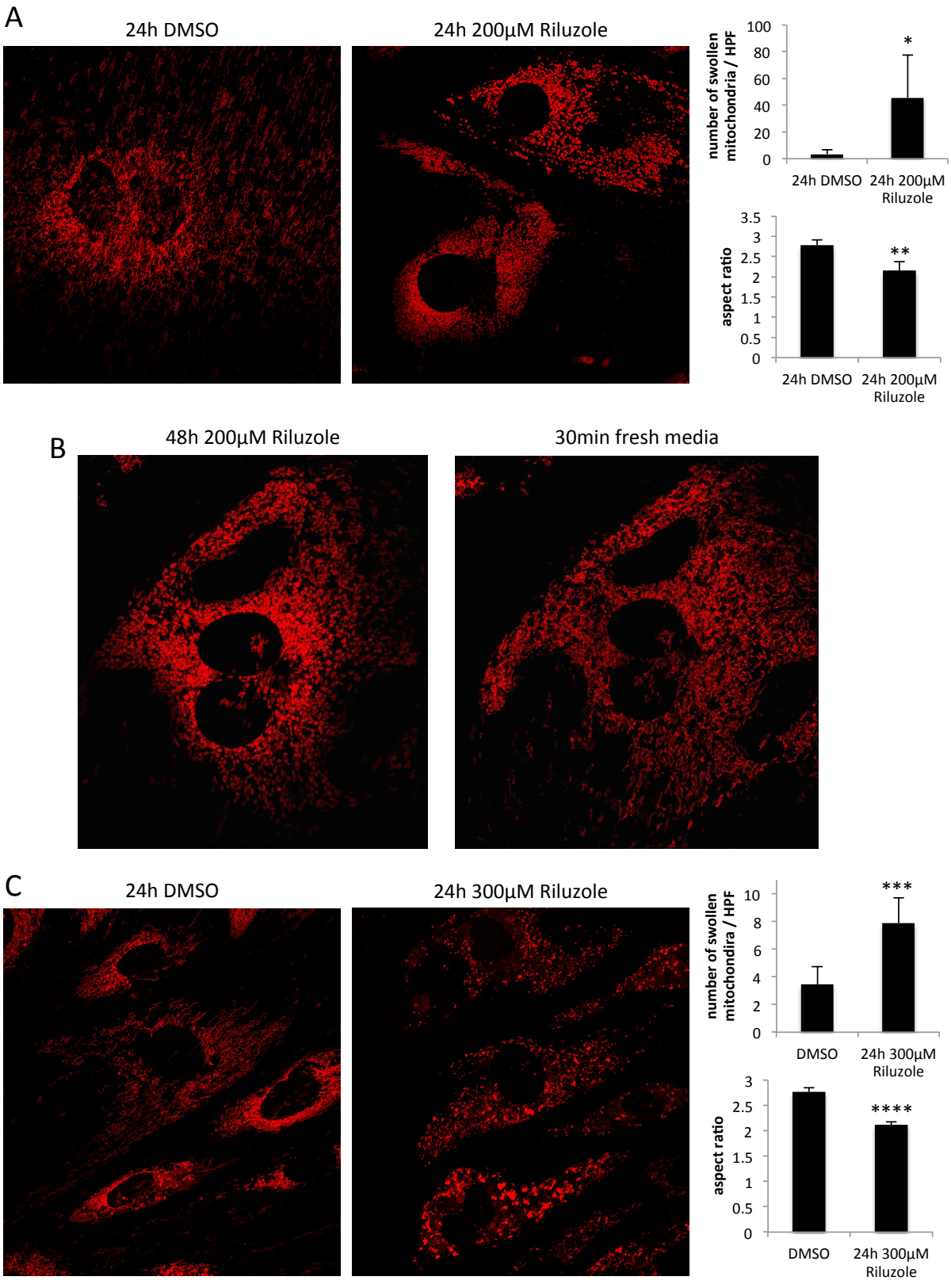

Figure 4: Riluzole induces mitochondrial swelling and fragmentation. Mitochondria were stained using MitoTracker Red before the start of the experiment. The dye forms conjugates with proteins inside mitochondria, which result in stable staining for up to three days in podocytes. All images were acquired by confocal microscopy from living podocytes in cell culture media with a 63X water immersion lens. (**A**) Podocytes incubated for 24h with 200µM riluzole show characteristic mitochondrial swelling and fragmentation. We quantified mitochondrial swelling by counting all mitochondria above certain minimum size and circularity. To quantify fragmentation we used the

aspect ratio (ratio of minor axis to major axis of the best fitting ellipse) for all mitochondria. All images are at the same magnification and were analyzed with ImageJ. *p=0.02, ** p=0.0007, t-test, error bars show SD. (**B**) Even after 48h incubation with 200µM riluzole mitochondrial swelling and fragmentation is still reversible within a short timeframe. Two representative images of the same podocyte are shown. After taking the first image the riluzole containing media was replaced with fresh media without riluzole. Within 30min the mitochondrial structure seemed almost normal. (**C**) In contrast cells incubated with 300µM riluzole for 24h sowed irreversible changes mitochondrial shape. In this experiment we acquired the images in fresh media without riluzole. The pictures were then analyzed with the same algorithms as used in (A). ***p=0.0001, ****p=4.2E-21 Error bars show CI. The graphs show pooled data of three experiments done in triplicate with four pictures taken randomly for each sample. This equals several thousand measured mitochondria for each condition.

4 DISCUSSION

In this study we show that TRPC5 activation with riluzole causes podocyte death by Ca^{2+} toxicity initiating mitochondrial damage and MPT. We could show in two independent assays that TRPC5 activation with riluzole leads to podocyte death in a dose and time dependent manner. Further we show that it is possible to protect podocytes from the detrimental effects of chronic TRPC5 activation with a novel TRPC5 blocker ML204. Podocyte loss is the critical step in the development of irreversible glomerulosclerosis (D'Agati et al., 2011). We suspect that continuous TRPC5 activation in a chronic disease scenario leads to podocyte loss to a point of no return with irreversible glomerulosclerosis. Therefore blocking TRPC5 might prove to be a valuable therapeutic strategy.

4.1 Transient receptor potential channels in chronic kidney disease

We show further evidence that Ca^{2+} signaling in podocytes plays a major role in kidney disease progression. Activating mutations in TRPC6 (a Ca^{2+}-channel closely related to TRPC5) are known to cause hereditary FSGS in patients (Winn et al., 2005). What role which channel plays in kidney disease progression will need further genetic studies. A major problem with TRPC6 as potential therapeutic target is its prominent role in the cardiovascular system (Dietrich et al., 2005). Interestingly while the TRPC6 KO mouse suffers from elevated systemic blood pressure, overexpressing TRPC6 in mouse hearts causes pathological remodeling culminating in lethal heart failure (Kuwahara et al., 2006). This highlights an important homeostatic role of TRPC6. In podocytes this homeostatic role of TRPC6 is increasingly apparent as well (Wieder and Greka, 2016). TRPC5 is known to regulate the cytoskeletal rearrangement leading to foot process effacement in podocytes (Schaldecker et al., 2013). If and how foot process effacement progresses to glomerulosclerosis is still under debate (D'Agati et al., 2011; Kriz et al., 2013). It could already be shown in cancer cells and neurons that sustained activation of TRPC5 leads to calcium toxicity and cell death (Akbulut et al., 2015; Carson et al., 2015; Hong et al., 2015). Our data shows this for podocytes. Notably the TRPC5 blocker ML204 was able to improve outcome in a Huntington's disease mouse model (Hong et al., 2015). To get an idea of possible side effects off a TRPC5 blocking therapy it is worth to look at the TRPC5 knockout mouse. The only initially apparent phenotypes were restricted to the central nervous system resulting in a diminished innate fear response (Riccio et al., 2009). However, a recent publication unveiled that the TRPC5 knockout mouse has a defunct baroreceptor response leading to severe blood pressure fluctuations and an increased mean arterial blood pressure. Even though the baroreceptor response was abnormal it was not abolished. The authors conclude that TRPC5 is only one of multiple mechanosensors in baroreceptor neurons (Lau et al., 2016). Additionally all chronic kidney disease patients who could

benefit from TRPC5 blockade should receive an ARB (angiotensin receptor blocker) or ACE-I (angiotensin-converting enzyme inhibitor) in any case and most of them will be on additional anti-hypertensive medication as well as part of the standard of care (Stevens et al., 2013). Therefore we believe TRPC5 blocking drugs could be a valuable addition to the treatment regimen for chronic kidney disease.

4.2 Mitochondrial damage in chronic kidney disease

Mitochondrial function is increasingly recognized as one of the critical pillars of podocyte homeostasis. Maintaining the podocytes sophisticated adaptive cytoskeletal structure in the mechanically highly demanding environment of the glomerulus requires a lot of energy. This energy is almost exclusively provided by the mitochondrial respiratory chain in podocytes (Muller-Deile and Schiffer, 2014). Similar to the well-known mutations in slit diaphragm and cytoskeletal proteins, mutations in mitochondrial proteins lead to early onset steroid resistant nephrotic syndrome (Ashraf et al., 2013). Calcium influx through TRPC5 is capable of initiating several different cell death pathways. Thus for instance based on work on TRPC5 initiated calcium toxicity in neurons an additional activation of the calpain-caspase pathways seems likely (Hong et al., 2015). However, knowing that mitochondrial damage in podocytes leads to kidney disease in humans (Malaga-Dieguez and Susztak, 2013), makes us confident that our observation of sustained TRPC5 activation causing mitochondrial damage and podocyte death is relevant for human disease.

4.3 Cell-to-cell variability

TRPC5 activation by 100µM riluzole produced a slow but continuous rate of podocyte death. This in vitro finding may be relevant to chronic, incremental disease progression in vivo. Interestingly, our data suggest that only a fraction of the podocyte population undergoes MPT at any given time after TRPC5 activation with 100µM riluzole. This can be explained by cell-to-cell variability (Spencer et al., 2009). In this publication the authors show that, within hours of their division the response of two daughter cells to an apoptotic stimulus differs significantly. Genetically and epigenetically those cells are identical because they originate from one mitosis. In further experiments the authors show that small differences in protein concentrations can explain this effect. All the observed differences in protein concentrations were within the expected distributions so that it proved to be impossible to predict the cells response based on single protein concentration measurement. Thus the seemingly binary process of cells surviving or dying is spread out over a long timeframe for the population with only a small number of cells dying at any given time point after the insult. In a similar fashion the Ca^{2+} buffering capacity is higher in some podocytes than others, allowing them to survive longer, even during prolonged periods of TRPC5 activation. Thus while the average number of surviving cells drops below 55% after incubation with 100µM Riluzole only a small fraction of cells undergoes

MPT at any given time. This together with the fact that a lot of the early effects we see after TRPC5 activation are reversible makes us hopeful that TRPC5 blockade can be a successful therapeutic strategy in early-diagnosed acquired proteinuric kidney disease.

4.4 TRPC5 blocking agents in vivo

The encouraging results of this study resulted in a trial of TRPC5 blocking agents in a rat model of CKD. Rats overexpressing the human angiotensin receptor type 1 (AT1R) specifically in podocytes show progressive signs of CKD including proteinuria and podocyte loss before they die of kidney failure at an age of 60 weeks (Hoffmann et al., 2004). TRPC5 activity increased in concert with disease progression and treatment with TRPC5 blockers in rats with established disease ameliorated proteinuria and prevented podocyte loss. These data indicate that in vivo TRPC5 is viable target for CKD treatment.

4.5 Conclusion

Our data provide the first evidence that TRPC5 blockers may be a successful therapeutic strategy for proteinuric CKD. In our hands, ML204 treatment showed no significant adverse effects even at high doses, in line with past findings in TRPC5 KO mice, which primarily showed a diminished fear response (Riccio et al., 2009). Given that 20 million Americans and millions more worldwide (Levey et al., 2007) are suffering from CKD, an anti-proteinuric drug with putative anxiolytic side effects appears attractive.

5 SUPPLEMENT

5.1 Aspect ratio ImageJ script:

```
macro "mitoAR [F6]" {
run("Convolve...", "text1=[0 0 -1 -1 -1 0 0\n0 -1 -3 -3 -3 -1 0\n-1 -3 0 7 0 -3 -1\n-1 -3 7 24 7 -3 -1\n-1 -3 0 7 0 -3 -1\n0 -1 -3 -3 -3 -1 0\n0 0 -1 -1 -1 0 0\n] normalize");
setAutoThreshold("Default dark");
setThreshold(100, 255);
run("Analyze Particles...", "size=0.5-Infinity circularity=0.00-1.00 show=[Bare Outlines] display exclude summarize in_situ");
}
```

5.2 Mitochondrial swelling ImageJ script:

```
macro "mitoswelling [F5]" {
run("Convolve...", "text1=[0 0 -1 -1 -1 0 0\n0 -1 -3 -3 -3 -1 0\n-1 -3 0 7 0 -3 -1\n-1 -3 7 24 7 -3 -1\n-1 -3 0 7 0 -3 -1\n0 -1 -3 -3 -3 -1 0\n0 0 -1 -1 -1 0 0\n] normalize");
setAutoThreshold("Default dark");
//run("Threshold...");
setThreshold(101, 255);
run("Analyze Particles...", "size=1.1-Infinity circularity=0.55-1.00 show=Masks display exclude include summarize in_situ");
}
```

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7 POSTER ABSTRACT

for the American Society of Nephrology Kidney Week 2014 Annual Meeting, November 13-16 in Philadelphia:

TRPC5-mediated podocyte calcium toxicity drives progressive glomerular disease

Frank Dubois, Philip Castonguay, Sookyung Kim, Jonas Sieber, Astrid Weins & Anna Greka

Background: Podocyte injury, with proteinuria as the earliest sign, is the critical step in various forms of kidney disease. Podocyte-specific expression of the human angiotensin receptor type 1 (AT1R) in rats (AT1R TG rats) causes progressive podocyte damage and loss, leading finally to kidney failure and death after 40-50 weeks. Our laboratory established that deletion or pharmacological blockade of TRPC5 channels is protective in acute models of filter barrier damage, but the role of TRPC5 in in disease progression remains unknown. Previously we have also shown that AT1R activation promotes TRPC5-mediated cytoskeletal disruption in podocytes.
Methods: We investigated the influence of TRPC5 on disease progression in AT1R TG rats by 2-week treatment with the TRPC5 inhibitor ML204, starting after proteinuria had developed. For mechanistic studies, we activated TRPC5 pharmacologically in conditionally immortalized mouse podocytes after 11 days of differentiation. Annexin V/PI labeling was used as cell death assay, and mitochondrial permeability transition (MPT) was measured with Calcein/Co^{2+}. Analysis was done by flow cytometry.
Results: Treatment with the TRPC5 blocker ML204 decelerated disease progression in the AT1R TG rats. Specifically, albuminuria remitted under ML204 treatment. *In vivo,* podocytes showed signs of recovery following treatment. Mechanistically, we show that sustained TRPC5 activation leads to podocyte calcium toxicity via MPT and finally cell death, in a dose and time dependent manner. These effects can be blocked effectively by ML204.
Conclusion: This study reveals sustained TRPC5 activation as a driving force in progressive glomerular disease. Mitigated disease progression by ML204 treatment in AT1R TG rats is an important proof of concept study for the potential of TRPC5 blockade as a therapeutic strategy in progressive proteinuric kidney diseases

8 KEY WORDS

chronic kidney disease
proteinuria
podocyte
ion channels
mitochondrial permeability transition

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10 SUMMARY

Chronic kidney disease is a major cause of mortality and morbidity worldwide. Damage to the filter barrier formed by the podocytes in the kidney is a critical step of kidney disease. Podocytes are terminally differentiated cells, which cannot be replaced in adults. If enough podocytes are lost the kidney function breaks down. The complex cytoskeletal arrangement necessary to create the filter structure is regulated by calcium currents through transient receptor potential channels. One of earliest clearly visible sign of podocyte damage is the cytoskeletal rearrangement known as foot process effacement. Transient receptor potential channel 5 (TRPC5) has recently been show to play an essential role in the initiation of this process. However, the role of TRPC5 in disease progression remains unknown. We hypothesized that the continuous calcium influx caused by sustained TRPC5 activation, as in a chronic disease setting, could lead to calcium toxicity and podocyte death.

To test this hypothesis, we activated TRPC5 pharmacologically in conditionally immortalized mouse podocytes. We used cell viability assays, confocal microscopy, and flow cytometry to examine the relationship between TRCPC5 activation and cell death and mitochondrial damage typical for calcium toxicity.

Our results showed that sustained TRPC5 activation can lead to irreversible podocyte injury and death in a dose and time dependent manner. Exploring the underlying mechanisms we observed signs of mitochondrial damage, as a result of sustained TRPC5 activation. These included mitochondrial swelling, fragmentation, increased production of reactive oxygen species and mitochondrial permeability transition leading to irreversible damage to the podocytes.

This study revealed that sustained TRPC5 activation leads to podocyte death by Calcium toxicity initiating mitochondrial damage and mitochondrial permeability transition. Further we show that it is possible to protect podocytes from the detrimental effects of chronic TRPC5 activation with a novel TRPC5 blocker ML204. Podocyte loss is the critical step in the development of irreversible glomerulosclerosis causing chronic kidney disease. We suspect that continuous TRPC5 activation in a chronic disease scenario leads to podocyte loss up to a point of no return with irreversible glomerulosclerosis. Therefore blocking TRPC5 might prove to be a valuable therapeutic strategy. One of the major advantages of TRPC5 as therapeutic target is the fact that it is only expressed in the kidney and in the nervous system. The TRPC5 knockout mouse therefore has no major health impairment. A TRPC5 blocking drug, which could stop the progression of chronic kidney disease, thus seems a promising possibility.

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EDUCATION

Charité Berlin, (*05/2017 – present),* Medical School, final year practical training

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SCHOLARSHIPS

03/2012 – present,	German National Academic Foundation scholarship
08/2013 – 07/2014	Boehringer Ingelheim Fonds MD fellowship for experimental biomedical research
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RESEARCH EXPERIENCE

03/2016 – 08/2016	**Massachusetts General Hospital**, Department of Radiology**,** Laboratory of Umar Mahmood, MD, PhD Examined androgen receptor imaging for prostate cancer therapy monitoring and contributed to establishing Granzyme B PET Imaging as a Predictive Biomarker of Immunotherapy Response

01/2015 – 02/2016	**Heidelberg University,** Interdisciplinary Center for Neurosciences, Laboratory of Prof. Hilmar Bading Established CRISPR-Cas9 genome engineering for induced pluripotent stem cell derived neurons
08/2013 – 07/2014	**Harvard Medical School**, Kidney-NExT, Laboratory of Anna Greka, MD, PhD Examined TRPC5-mediated podocyte calcium toxicity in progressive glomerular disease

PUBLICATION

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03/2015 – 02/2016	**Heidelberg University,** Molecular Biosciences Master's program at the Interdisciplinary Center for Neurosciences, Lecturer for CRISPR-Cas9 genome engineering
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