Aus der Klinik für Endokrinologie und Nephrologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Regulation of transient receptor potential canonical channels TRPC3 and TRPC6 in kidney diseases

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Ying Liu

aus Shanghai, Volksrepublik China

Gutachter: 1. Prof. Dr. M. Tepel

2. Prof. Dr. med. P. Persson

3. Priv.-Doz. Dr. med. B. Henning

Datum der Promotion: 01.02.2013

Contents

Contents i
Abbreviations ii
Abstract iii
Zusammenfassung v
Introduction vii
Hypotheses viii
Methodology ix
Results xi
Discussion xii
Summary xiv
Anteilserklärung xv
Chapter 1 TRPC channels in proteinuric Munich Wistar Frömter rats 1
Chapter 2 TRPC channels in patients with chronic kidney disease 2
Chapter 3 TRPC channels in syndecan 4 knockout mice 3
Curriculum vitae 4
Complete publications list 5
Selbständigkeitserklärung 8
Acknowledgements 9

Abbreviations

ANOVA	Analysis of variance
BSA	Bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)- dimethylammonio]-1-propanesulfonate
CI	Confidence interval
CKD	Chronic kidney disease
EBA	Evans blue-conjugated albumin
EDTA	Ethylene diamine tetraacetic acid
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
FFA	Flufenamic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid
HUVEC	Human umbilical vein endothelial cells
MALDI-TOF	Matrix assisted laser desorption/ionisation time of flight
mRNA	Messenger ribonucleic acid
MWF	Munich Wistar Frömter
OAG	1-oleoyl-2-acetyl-sn-glycerol
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethane sulfonyl fluoride
RhoGAP	Rho GTPase activating protein
Sdc4	Syndecan 4
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
2-APB	2-aminoethoxydiphenylborane
TFA	Trifluoracetic acid
TRPC	Transient receptor potential canonical
vWF	von-Willebrand-factor
WT1	Wilms tumor protein 1
YFP	Yellow fluorescent protein

Abstract

Transient receptor potential canonical (TRPC) channels are non-selective cation channels mediating cation influx into several cell types. TRPC channels have been associated with kidney diseases. We begin in Chapter 1 where we investigated the expression profile of TRPC channels in tissues from control Wistar rats and Munich Wistar Frömter (MWF) rats showing proteinuria. We used several laboratory approaches including quantitative real-time PCR, immunoblotting, and immunohistochemistry. Our findings suggest that altered TRPC expression pattern in kidney cortex is associated with kidney damage in MWF rats showing albuminuria.

Recent experimental results show that activation of the calcium-sensing receptor affects TRPC expression. To the best of our knowledge, no such data are available for humans. In Chapter 2, we investigate the regulation of TRPC3 channels in patients with chronic kidney disease. Using quantitative in-cell Western assay, we compared the expression of TRPC3 channel protein in monocytes from patients with chronic kidney disease and age- and sex-matched healthy control subjects. TRPC3 channels were identified by immunoblotting and TRPC3 protein was further confirmed by mass spectrometry. This study demonstrates that reduced extracellular calcium concentration up-regulates TRPC3 channel protein expression in patients with chronic kidney disease.

Because previous studies place the type I transmembrane heparan sulfate proteoglycan syndecan 4 (Sdc4) as a major modulator of signal transduction and regulation of channels, we studied the possible link between TRPC channels and Sdc4. In Chapter 3, we propose a knockout mouse model to study the regulation of TRPC channels. Sdc4 knockout ($Sdc4^{-/-}$) mice showed increased glomerular filtration rate, ameliorated albuminuria under baseline conditions and, after bovine serum albumin overload, compared to wild type ($Sdc4^{+/+}$) littermates. Using quantitative real-time PCR and immunoblotting, $Sdc4^{-/-}$ mice showed reduced TRPC6 mRNA by 79% and TRPC6 protein by 82%. $Sdc4^{-/-}$ mice showed an increased RhoA activity by 87% and increased phosphorylation of ezrin in glomeruli by 48%. Sdc4 knockdown in cultured podocytes reduced TRPC6 gene expression, reduced the association of TRPC6 with plasma membrane and TRPC6-mediated calcium influx and currents. Sdc4 knockdown inactivated negative regulatory protein RhoGAP by 33%, accompanied by a 41% increase in RhoA activity and increased phosphorylation of ezrin.

Conversely, overexpression of Sdc4 reduced RhoA activity, increased TRPC6 protein and TRPC6-mediated calcium influx and currents. Taken together, we show that TRPC6 gene expression and function are regulated by Sdc4 via RhoA/ROCK pathway.

Zusammenfassung

Transient receptor potential canonical (TRPC) Kanäle sind nicht selektive Kationen-Kanäle. Sie dienen dem Kationen-Einstrom bei verschiedenen Zelltypen. TRPC Kanäle wurden mit Nierenkrankheiten in Verbindung gebracht.

Zunächst habe ich die Expression von TRPC Kanäle im Gewebe von Wistar Ratten und von Munich Wistar Frömter Ratten verglichen. Munich Wistar Frömter Ratten zeigen eine gesteigerte Proteinurie. Die Expression von TRPC Kanälen wurde mittels quantitativer real-time PCR, Immunobloting und Immunhistochemie untersucht. Meine Ergebnisse legen nahe, dass bei Munich Wistar Frömter Ratten eine veränderte TRPC-Kanal-Expression in der Niere mit einer Nierenschädigung im Sinne einer Albuminurie einhergeht.

Anschließend untersuchte ich die Regulation von TRPC3 Kanälen bei Patienten mit chronischer Nierenerkrankung. Mittels quantitativem in-cell Western-Assay habe ich die Expression von TRPC3 Kanal-Proteinen in mononukleären Zellen von Patienten mit einer chronischen Nierenerkrankung sowie von gesunden Kontrollpersonen verglichen. TRPC3 Kanäle wurden durch Immunoblotting und auch durch Massenspektrometrie identifiziert. Ich konnte zeigen, dass eine verminderte extrazelluläre Kalziumkonzentration zu einer Steigerung der TRPC3 Kanal-Protein-Expression bei Patienten mit einer chronischer Nierenerkrankung führt.

Syndecan 4, ein Heparansulfatproteoglycan stellt einen wichtigen Modulator der Signaltransduktion und bei der Regulation von Kanälen dar. Ich habe eine mögliche Verbindung zwischen TRPC Kanälen und Syndecan 4 untersucht. Syndecan 4- Knockout Mäuse ($Sdc4^{-/-}$) hatten eine höhere glomeruläre Filtrationsrate und eine geringere Albuminurie, sowohl unter Baseline-Bedingungen als auch nach Stimulation mit Rinderserumalbumin jeweils im Vergleich zu den Wildtyp Mäusen ($Sdc4^{+/+}$). Quantitative real-time PCR und Immunobloting zeigten bei $Sdc4^{-/-}$ Mäusen um 79% reduzierte TRPC6 Transkripte und um 82% reduziertes TRPC6 Kanal-Protein. $Sdc4^{-/-}$ Mäuse zeigten eine Steigerung der RhoA Aktivität um 87% und erhöhte Phosphorylierung von Ezrin in den Glomeruli um 48%. Sdc4 knockdown führte in kultivierten Podozyten zu einer reduzierten TRPC6 Genexpression, zu einer Verminderung von TRPC6 Kanal-Protein in der Plasmamembran und zu einer Verminderung des TRPC6-vermittelten Calcium-Einstrom und Kationen Strömen (voltage

clamp). Sdc4 knockdown inaktivierte das negativ-regulatorische Protein RhoGAP um 33%. Dies war begleitet von einem 41% Anstieg der RhoA-Aktivität und von einer erhöhten Phosphorylierung von Ezrin. Andererseits führte die Überexpression von Syndecan 4 zur Verminderung der RhoA Aktivität, zur Steigerung von TRPC6 Kanal-Protein, zur Steigerung des TRPC6-vermittelten Calcium-Einstroms und von Kationen-Strömen. Zusammengenommen zeigt meine Untersuchung, dass Syndecan 4 die TRPC6 Kanal-Proteine und deren Funktionen über einen RhoA/ROCK-abhängigen Weg reguliert.

Introduction

The transient receptor potential (TRP) protein superfamily is a group of voltage-independent cation-permeable ion channels. TRP channels are subdivided, based on structural homology, into six subfamilies: C (Canonical, TRPC1-TRPC7), V (Vanilloid, TRPV1-TRPV6), M (Melastatin, TRPM1-TRPM8), A (Ankyrin, TRPA1), P (Polycystin, TRPP1-TRPP3), and ML (Mucolipin, TRPML1-TRPML3). TRP channels are expressed in several tissue and cell types. Many TRP channels are greatly potentiated by phospholipase C activation by G protein-coupled or tyrosine-kinase receptors. The broadly expressed TRP channels are permeable to cations, most resulting in increased intracellular calcium. Subsequently, activation of a number of calcium-dependent signaling pathways affects every aspect of a cell's life and death.

Recent studies indicate that TRPC channels play an essential role in the pathophysiology of human cardiovascular and kidney diseases. An increased TRPC3 expression has been observed in human essential hypertension and animal models of hypertension. Gain-of-function mutations of TRPC6 are associated with familial focal and segmental glomerulosclerosis, which causes proteinuria and chronic kidney disease.

TRPC channels are expressed in kidney podocytes and represent a component of the glomerular filtration barrier. Podocytes are specialized cells in glomerulus that cover the urinary surface of the filtering capillaries, normally preventing protein leakage into the urinary space. Podocyte foot processes and the interposed glomerular slit diaphragm are critical components of the permeability barrier in the kidney. Dysregulated TRPC expression levels and channel function, especially in kidney podocytes, lead to actin reorganization, followed by disruption of the ultrafiltration process at the slit diaphragm, finally contributing to glomerular disease pathogenesis. From recent literature there is evidence that both patients and mice with proteinuric kidney disease show an altered expression of native TRPC channels in podocytes. However, the expression profile of TRPC channels and the mechanisms by which TRPC channels are regulated are only partly known.

Hypotheses

The Munich Wistar Frömter (MWF) rat exhibits significant increase of urinary albumin excretion. We therefore hypothesize, in Chapter 1, that altered TRPC expression pattern in kidney cortex may be associated with such kidney damage observed in MWF rats.

Recent experimental results showed that calcium-sensing receptors regulate expression of TRPC channels in rat cardiomyocytes. In Chapter 2, we hypothesize that extracellular calcium may regulate the expression of TRPC channels in patients with chronic kidney disease.

Because previous studies place the transmembrane heparan sulfate proteoglycan syndecan 4 (Sdc4) as a major modulator of channels, we test the hypothesis, in Chapter 3, that TRPC channels may be regulated by Sdc4.

Methodology

We cultured conditionally immortalized mouse podocytes (podocyte cell line E11), human endothelial cell line EA.hy926, human umbilical vein endothelial cells (HUVEC), and human embryonic kidney cells (HEK293). Human monocytes were obtained from heparinized blood using superparamagnetic polystyrene beads coated with a primary monoclonal antibody specific for the CD14 membrane antigen expressed on human monocytes (Invitrogen, Germany).

RNA from cells or tissues was extracted using the RNeasy Midi Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Quantitative real-time PCR was performed using LightCycler 2.0 Instrument with LightCycler Software Version 4.0 (Roche Diagnostics). Immunoblot assay of proteins was performed using the Odyssey infrared imaging system (Licor biosciences). Podocyte membrane proteins were biotinylated and isolated using a commercially available cell membrane protein isolation kit (Pierce Biotechnology, Rockford, IL, USA). The separated proteins were identified using a MALDI-TOF/TOF spectrometer (Ultraflex III; Bruker-Daltonic, Bremen, Germany). The annotated spectra were subjected to a database search (Swiss-Prot, Zurich, Switzerland) utilizing Bruker-Daltonic Bio-Tools (vers. 3.1) and the Mascot search engine (vers. 2.2). Immunohistochemistry, immunofluorescence, and transmission electron microscopy were performed using standard protocols.

Transient transfection in cultured cells was performed using lipofectamine reagent (Invitrogen). Intracellular calcium measurements were performed using the Ca^{2+} indicator fluo-4-AM and the Bio-Rad (Munich, Germany) laser scanning confocal microscope. For patch clamp measurements, currents through the pipette were recorded by an Axopatch 200B amplifier (AxonInstruments; MDS Analytical Technologies Inc, Sunnyvale, CA, USA), filtered at 5 or 10 kHz (Besselfilter), analyzed and adjusted using pCLAMP software (version 10.1; AxonInstruments). RhoGAP or RhoA activity was measured using a RhoGAP assay Biochem Kit or G-LISA RhoA activation assay biochemistry kit (Cytoskeleton, Inc., Denver, CO, USA). Permeability of podocyte monolayer was measured using 3.0 μm-pore Polycarbonate Transwell inserts (Corning, Lowell, MA) in a 12 well plate following the manufacturer's instructions.

For animal studies, 6-month old male Munich Wistar Frömter (MWF) rats and Wistar rats were kept under environmentally controlled conditions with 12 h/12 h light/dark cycle at 20 to 22 ℃, fed a normal diet containing 0.2% NaCl and had free access to food and water. Blood pressure was monitored using tail-cuff plethysmography. Urinary albumin excretion was obtained using the rat specific ELISA technique. In addition, we created syndecan 4 knockout ($Sdc4^{-/-}$) mice by homologous recombination in 129SvJae embryonic stem cells and backcrossed founder mice for ten generations in C57BL/6 mice. We generated all data from male age-matched littermates. To set up the protein overload model in mice, both $Sdc4^{-/-}$ and wild type ($Sdc4^{+/+}$) mice received intraperitoneal injections of endotoxin-free bovine serum albumin for 5 days on a stepwise incremental dosage regimen. Urinary albumin excretion was measured using metabolic cages. Glomeruli were isolated using sieves (Retsch Inc, Haan, Germany) with pore sizes of 125 µm and 75 µm, respectively. Animal studies were conducted according to protocols approved by state and institutional regulations for the care and use of laboratory animals.

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Data were expressed as the mean ± SEM. Comparisons between groups were analyzed using non-parametric Mann-Whitney test, non-parametric Kruskal-Wallis-test with Dunn's multiple comparison post-test, t-test, or ANOVA with Bonferroni's multiple comparison test as appropriate. Relations between variables were investigated using Spearman correlation. A two-tailed probability value less than 0.05 was considered to indicate statistical significance.

Results

In Chapter 1, Munich Wistar Frömter (MWF) rats showed significantly increased systolic blood pressure and significantly higher left ventricle weight (each $p < 0.01$). TRPC3 transcripts were significantly higher, whereas TRPC6 transcripts were significantly lower in kidney cortex from MWF rats compared to Wistar rats ($p < 0.01$). Immunoblotting showed that TRPC3 channel protein expression was also significantly higher in kidney cortex from MWF rats compared to Wistar rats ($p < 0.01$). There was a significant correlation of TRPC3 mRNA and a specific marker for endothelium, von Willebrand factor (vWF; Spearman $r = 0.564$; $p < 0.01$). We observed a significant correlation between the TRPC3 transcripts to TRPC6 transcripts ratio and urinary albumin excretion (Spearman $r = 0.785$, $p < 0.001$).

In Chapter 2, we observed a significant increase of TRPC3 channel protein expression in patients with chronic kidney disease compared to healthy control subjects (normalized expression, 0.42 ± 0.06 vs. 0.19 ± 0.03; $p < 0.01$). Expression of TRPC3 was significantly inversely correlated with estimated glomerular filtration rates (Spearman $r = -0.41$) or serum calcium concentration (Spearman $r = -0.34$). During a hemodialysis session, serum calcium concentrations significantly increased, whereas the expression of TRPC3 channels and calcium influx significantly decreased. In vitro studies confirmed that higher calcium concentrations but not magnesium, barium nor sodium concentrations significantly decreased TRPC3 expression in human monocytes.

In Chapter 3, we observed that Syndecan4 knockout ($Sdc4^{-/-}$) mice showed an increased glomerular filtration rate and under baseline conditions and after bovine serum albumin overload (each $p < 0.05$), ameliorated albuminuria. $Sdc4^{-/-}$ mice showed reduced TRPC6 mRNA by 79% and TRPC6 protein by 82% (each $p < 0.05$). $Sdc4^{-/-}$ mice showed an increased RhoA activity by 87% and increased phosphorylation of ezrin in glomeruli by 48% (each $p < 0.05$). Sdc4 knockdown in cultured podocytes reduced TRPC6 gene expression, reduced the association of TRPC6 with plasma membrane and TRPC6-mediated calcium influx and currents. Sdc4 knockdown inactivated negative regulatory protein RhoGAP by 33%, accompanied by a 41% increase in RhoA activity and increased phosphorylation of ezrin ($p < 0.05$). Conversely, overexpression of Sdc4 reduced RhoA activity, increased TRPC6 protein and TRPC6-mediated calcium influx and currents.

Discussion

In the study of Chapter 1, we observed significantly increased expression of TRPC3 and reduced expression of TRPC6 in Munich Wistar Frömter (MWF) rats which exhibit hypertension and albuminuria compared to control Wistar rats. We also observed a significant association between TRPC3 transcripts to TRPC6 transcripts ratio in kidney cortex and urinary albumin excretion, indicating that the altered TRPC expression pattern in kidney cortex is associated with kidney damage in MWF rats. Reduced nephron number, hypertension and albuminuria which can be observed in MWF rats mirror similar well-known characteristics of patients with hypertension who are at high risk for chronic kidney disease. The present study indicates that increased TRPC3 channels in kidney cortex of MWF rats are associated with hypertension and albuminuria. It should be noted that an increased TRPC3 expression was also observed in peripheral blood cells from patients with essential hypertension and in vascular tissues from animal models of hypertension. Our findings of reduced TRPC6 expression in MWF rats should be considered in view of a recent study showing reduced TRPC6 expression in glomerular mesangial cells exposed to high glucose. Thus, the deficiency of TRPC6 protein might contribute to the impaired calcium signaling of mesangial cells seen in diabetes and diabetic nephropathy. It may be concluded that varying changes of the expression of TRPC channels in different tissue may be characteristic for special types of kidney diseases.

In Chapter 2, our studies performed both *in vivo* and *in vitro* suggested that extracellular calcium concentrations directly affect TRPC3 channel protein expression in humans. In hemodialysis patients, the increase of serum calcium concentrations during the hemodialysis session caused a decrease of TRPC3 channel protein expression. In addition, *in vitro* experiments using several extracellular cation concentrations confirmed that increasing extracellular calcium concentrations reduced TRPC3 expression, whereas magnesium, barium or sodium concentrations showed no significant effects in monocytes. Which mechanisms are responsible for the increased TRPC3 channel protein expression in the presence of low extracellular calcium concentrations? We may speculate that reduced low extracellular calcium may initiate a rapid translocation of TRPC channels from vesicles held in reserve just under the plasma membrane. A rapid translocation of TRPC channels had been observed after activation of human embryonic kidney cells and neurons by growth factors. This speculation is supported by the observation that extracellular calcium affected TRPC3 expression within a

short period of time. Elevation of TRPC3 channel protein expression may thus facilitate the process of capacitative calcium entry, which is essential for sustained calcium influx and replenishment of intracellular calcium stores. Numerous studies have shown that increased TRPC3 expression is linked to several diseases including arterial hypertension. Measures aimed at preventing an increase in TRPC3 expression may ameliorate an important factor for increased blood pressure.

In Chapter 3, we observed that Sdc4 syndecan 4 (Sdc4) regulates TRPC6 channels by affecting RhoA/ROCK activity. Recent studies suggest that Sdc4 could participate in kidney diseases. Furthermore, increased expression of TRPC6 in the podocyte slit diaphragm and gain-of-function mutations in TRPC6 have been identified to cause podocyte injury and human kidney disease. Our results demonstrate a specific Sdc4-TRPC6 interaction, which underscores TRPC subtype-specific characteristics of TRPC6 compared to TRPC3. Our results show that Sdc4 selectively affects TRPC6 channel protein expression and membrane association in podocytes. Furthermore, we uncovered a mechanistic link between Sdc4 and TRPC6. Small GTPases have been reported to act downstream of syndecans regulating several cellular functions. In the present study, we found that overexpression of Sdc4 reduced baseline RhoA activity in podocytes. We also observed that overexpression of Sdc4 inhibits RhoA/ROCK activity as confirmed by reduced phosphorylation of ezrin, which is known to link RhoA/ROCK activity with actin cytoskeleton rearrangements. Consistent with our data on podocytes, recent studies showed that Sdc4 reduces RhoA activity in fibroblasts via activation of p190RhoGAP-A, which is a negative regulator of active Rho GTPase. Rho-GTPases have a role in cytoskeletal rearrangement, but also regulate vesicular trafficking. TRPC6 channels are localized in the plasma membrane and in caveolae-related microdomain vesicles subjacent to the plasma membrane. Using confocal laser scanning microscopy and biotinylation assays, we found that the activation of RhoA/ROCK signaling, via inactivation of RhoGAP by Sdc4 knockdown, or via treatment with RhoA/ROCK activator, triggered the translocation of TRPC6 channels from the plasma membrane into the cytoplasm. On the other hand, the inhibition of RhoA/ROCK signaling, via activation of RhoGAP by overexpression of functional Sdc4, or via treatment with RhoA/ROCK inhibitors, increased the plasma membrane pool of TRPC6 channels. The wide tissue distribution of Sdc4 and TRPC6 channels in vasculature and our confirmation of these events in HEK293 cells, strongly suggest that the regulation of TRPC6 channels by proteoglycan Sdc4 via RhoA/ROCK signaling may be a general process.

Summary

In the present study series, we studied the expression profile and regulation of TRPC channels via various laboratory techniques and transgenic animal models. We firstly observed significantly increased TRPC3 gene expression in Munich Wistar Frömter (MWF) rats, which show an inherited nephron deficit and exhibit hypertension and increased albuminuria. We observed a significant association between TRPC3 transcripts to TRPC6 transcripts ratio in kidney cortex and urinary albumin excretion. We conclude that altered TRPC expression pattern in kidney cortex is associated with kidney damage in MWF rats.

We then show that extracellular calcium concentrations affect TRPC3 channel protein expression in patients with chronic kidney disease (CKD). TRPC3 channel protein expression was significantly higher in patients with CKD compared to control subjects. Furthermore, we observed a significant negative correlation between serum calcium concentrations and TRPC3 channel protein expression. We conclude that reduced extracellular calcium concentrations up-regulate TRPC3 channel protein expression in patients with CKD.

Finally, we identified a major regulator of TRPC6 channels. We found that syndecan 4 knockout ($Sdc4^{-/-}$) mice showed reduced expression of TRPC6, reduced albuminuria, and increased RhoA/ROCK activity in kidney cortex compared to wild type ($Sdc4^{+/+}$) littermates. Importantly, knockdown of Sdc4 *in vitro* was able to mimic theses effects, which were reversed by overexpression of functionally intact Sdc4. We conclude that Sdc4 serves as a unique regulator of TRPC6 channels in kidney podocytes.

Anteilserklärung

Herr **Ying Liu** hat seine experimentelle, kumulative Doktorarbeit mit dem Thema „**Regulation von Transient Receptor Potential Canonical Kanälen TRPC3 und TRPC6 bei Nierenerkrankungen**“ angefertigt. Grundlage für die kumulative Promotion sind folgende 3 Publikationen:

Publikation 1

Liu Y, Thilo F, Kreutz R, Schulz A, Wendt N, Loddenkemper C, Jankowski V, Tepel M. Tissue expression of TRPC3 and TRPC6 in hypertensive Munich Wistar Frömter rats showing proteinuria.
Am J Nephrol. 2010;31:36-44.
Impact factor 3.164

In dieser Arbeit wird die Expression von Transient Receptor Potential Canonical Typ 3 (TRPC3) und Typ 6 (TRPC6) Kanälen in Munich Wistar Frömter (MWF) Ratten untersucht. MWF Ratten haben einen genetisch determinierten Mangel an Nephronen in der Niere und entwickeln eine Proteinurie und eine arterielle Hypertonie. In der Arbeit wird die Assoziation von TRPC3 Transkripten in der Niere mit der Albumin-Ausscheindung gezeigt.

Herr Ying Liu hat bei der Erstellung dieser Arbeit in folgenden Bereichen wesentlich mitgewirkt:

1. Bedeutsame Mitwirkung bei Konzeption und detailierter Ausarbeitung der Untersuchungen, Datengewinnung und Durchführung der Experimente und Messungen, sowie Analyse, Auswertung und Interpretation der gewonnenen Daten
2. Erstellung und Revision des Manuskripts einschließlich bedeutsame Mitwirkung bei Einleitung und Diskussion der gewonnenen Resultate
3. Zustimmung zur endgültigen Manuskriptfassung für die Einreichung zur Publikation.

Einzelheiten:

Folgende Experimente und Messungen wurden von Herrn Ying Liu durchgeführt:

Isolation of RNA and cDNA synthesis; Quantitative real-time RT-PCR; Immunoblotting of TRPC;

Bei folgende Experimenten und Messungen hat Herr Ying Liu wesentlich mitgewirkt: Identification of TRPC using matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry; Immunohistochemistry;

Herr Ying Liu hat Analyse, Auswertung und Interpretation der gewonnenen Daten, einschließlich statistischer Auswertung und graphischer Ausarbeitung, wesentlich mitgewirkt. Der Anteil von Herrn Ying Liu an dieser Arbeit ist mit mindestens 90% einzustufen.

Publikation 2

Liu Y, Krueger K, Hovsepian A, Tepel M, Thilo F. Calcium-dependent expression of transient receptor potential canonical type 3 channels in patients with chronic kidney disease.
Arch Biochem Biophys. 2011;514:44-49.
Impact factor 3.022

In dieser Arbeit wurde die Expression von TRPC3 Kanalproteinen in Blutzellen von Patienten mit chronischer Niereninsuffizienz untersucht. Es zeigte sich eine Assoziation von TRPC3 Kanalproteinen mit der Serum-Calcium-Konzentration. Es ergab sich die Schlussfolgerung, dass erniedrigte Serum-Calcium-Konzentrationen möglicherweise eine Steigerung der TRPC3 Kanalproteine bewirken können.

Herr Ying Liu hat bei der Erstellung dieser Arbeit in folgenden Bereichen wesentlich mitgewirkt:
1. Bedeutsame Mitwirkung bei Konzeption und detailierter Ausarbeitung der Untersuchungen, Datengewinnung und Durchführung der Experimente und Messungen, sowie Analyse, Auswertung und Interpretation der gewonnenen Daten
2. Erstellung und Revision des Manuskripts einschließlich bedeutsame Mitwirkung bei Einleitung und Diskussion der gewonnenen Resultate
3. Zustimmung zur endgültigen Manuskriptfassung für die Einreichung zur Publikation.
Einzelheiten:
Bei folgende Experimenten und Messungen hat Herr Ying Liu wesentlich mitgewirkt: Preparation of cells; Immunoblotting of TRPC channels from human monocytes; Identification of TRPC using matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry; Quantitative in-cell Western assay for TRPC channel protein expression; Measurements of cytosolic calcium
Herr Ying Liu hat bei Analyse, Auswertung und Interpretation der gewonnenen Daten, einschließlich statistischer Auswertung und graphischer Ausarbeitung, wesentlich mitgewirkt. Der Anteil von Herrn Ying Liu an dieser Arbeit ist mit mindestens 60% einzustufen.

Publikation 3

Liu Y, Echtermeyer F, Thilo F, Theilmeier G, Schmidt A, Schülein R, Jensen BL, Loddenkemper C, Jankowski V, Marcussen N, Gollasch M, Arendshorst WJ, Tepel M. The Proteoglycan Syndecan 4 Regulates Transient Receptor Potential Canonical 6 Channels via RhoA/ROCK Signaling.
Arterioscler Thromb Vasc Biol. 2012;32:378-385.
Impact factor 7.235

In dieser ausführlichen und methodisch sehr überzeugenden Arbeit wird die Regulation der Expression von TRPC6 Kanälen in Podozyten durch Syndecan 4 erstmalig beschrieben. In dieser Arbeit werden sowohl ex vivo Messungen an knockout Mäusen als auch in-vitro Experimente an kultivierten Podozyten eingesetzt. Die äußerst eleganten Untersuchungen belegen, dass Syndecan 4 über den RhoA/ROCK Signaltransduktionsweg die Expression von TRPC6 Kanälen an der Oberfläche der Podozyten reguliert, was dann die Albumin-Ausscheidung beeinflusst.

Herr Ying Liu hat bei der Erstellung dieser Arbeit in folgenden Bereichen wesentlich mitgewirkt:

1. Bedeutsame Mitwirkung bei Konzeption und detailierter Ausarbeitung der Untersuchungen, Datengewinnung und Durchführung der Experimente und Messungen, sowie Analyse, Auswertung und Interpretation der gewonnenen Daten
2. Erstellung und Revision des Manuskripts einschließlich bedeutsame Mitwirkung bei Einleitung und Diskussion der gewonnenen Resultate
3. Zustimmung zur endgültigen Manuskriptfassung für die Einreichung zur Publikation.

Einzelheiten:

Folgende Experimente und Messungen wurden von Herrn Ying Liu durchgeführt:

Cell culture; siRNA knockdown of Sdc4 or TRPC6; Isolation of RNA and cDNA synthesis; Quantitative real-time RT-PCR; Overexpression of Sdc4; Immunoblotting of proteins and co-immunoprecipitation; Quantitative in cell Western assay of proteins; Intracellular cation measurements using fluorescence spectrophotometry; Rho GTPase activating protein (RhoGAP) activity assay; RhoA activation assay; Podocytes membrane protein biotinylation and isolation; Transwell permeability assay; Isolation of glomeruli;

Bei folgende Experimenten und Messungen hat Herr Ying Liu wesentlich mitgewirkt:

Immunofluorescence; Visualization of green fluorescent protein (GFP)-tagged-TRPC6 or yellow fluorescent protein (YFP)-tagged-TRPC6 in podocytes and HEK cells using confocal

laser scanning microscopy; Intracellular calcium measurements using laser scanning confocal microscopy; Matrix assisted laser desorption/ionisation time of flight mass spectrometry of isolated TRPC6 and Sdc4; Patch clamp measurements; Electron microscopy;
Herr Ying Liu hat bei Analyse, Auswertung und Interpretation der gewonnenen Daten, einschließlich statistischer Auswertung und graphischer Ausarbeitung, wesentlich mitgewirkt.
Der Anteil von Herrn Ying Liu an dieser Arbeit ist mit mindestens 90% einzustufen.

Herr Ying Liu ist an weiteren Publikationen beteiligt, indem er weitere molekular-biologische Untersuchungen etabliert und durchgeführt hat.

Prof. Dr. med. Martin Tepel	Ying Liu

Chapter 1

TRPC channels in proteinuric Munich Wistar Frömter rats

The results of this study have been published in:

http://www.ncbi.nlm.nih.gov/pubmed/19887786

Liu Y, Thilo F, Kreutz R, Schulz A, Wendt N, Loddenkemper C, Jankowski V, Tepel M. Tissue expression of TRPC3 and TRPC6 in hypertensive Munich Wistar Frömter rats showing proteinuria. Am J Nephrol. 2010;31(1):36-44. [Impact factor 3.164]

Chapter 2

TRPC channels in patients with chronic kidney disease

The results of this study have been published in:

http://www.ncbi.nlm.nih.gov/pubmed/21802402

Liu Y, Krueger K, Hovsepian A, Tepel M, Thilo F. Calcium-dependent expression of transient receptor potential canonical type 3 channels in patients with chronic kidney disease. Arch Biochem Biophys. 2011;514(1-2):44-49. [Impact factor 3.022]

Chapter 3

TRPC channels in syndecan 4 knockout mice

The results of this study have been published in:

http://www.ncbi.nlm.nih.gov/pubmed/22155451

Liu Y, Echtermeyer F, Thilo F, Theilmeier G, Schmidt A, Schülein R, Jensen BL, Loddenkemper C, Jankowski V, Marcussen N, Gollasch M, Arendshorst WJ, Tepel M. The Proteoglycan Syndecan 4 Regulates Transient Receptor Potential Canonical 6 Channels via RhoA/ROCK Signaling. Arterioscler Thromb Vasc Biol. 2012;32(2):378-385. [Impact factor 7.235]

Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Complete publications list

Liu Y, Echtermeyer F, Thilo F, Theilmeier G, Schmidt A, Schülein R, Jensen BL, Loddenkemper C, Jankowski V, Marcussen N, Gollasch M, Arendshorst WJ, Tepel M. The Proteoglycan Syndecan 4 Regulates Transient Receptor Potential Canonical 6 Channels via RhoA/ROCK Signaling.
Arterioscler Thromb Vasc Biol. 2012;32(2):378-385.
Impact factor 7.235

Liu Y, Krueger K, Hovsepian A, Tepel M, Thilo F. Calcium-dependent expression of transient receptor potential canonical type 3 channels in patients with chronic kidney disease.
Arch Biochem Biophys. 2011;514(1-2):44-49.
Impact factor 3.022

Liu Y, Thilo F, Kreutz R, Schulz A, Wendt N, Loddenkemper C, Jankowski V, Tepel M. Tissue expression of TRPC3 and TRPC6 in hypertensive Munich Wistar Frömter rats showing proteinuria.
Am J Nephrol. 2010;31(1):36-44.
Impact factor 3.164

Liu Y, Xu Y, Thilo F, Friis UG, Jensen BL, Scholze A, Zheng J, Tepel M. Erythropoietin increases expression and function of transient receptor potential canonical 5 channels.
Hypertension. 2011;58(2):317-324.
Impact factor 6.908

Thilo F, **Liu Y**, Krueger K, Foerste N, Wittstock A, Scholze A, Tepel M. Do cysteine residues regulate transient receptor potential canonical type 6 (TRPC6) channel protein expression?
Antioxid Redox Signal. 2012;16(5):452-457.
Impact factor 8.209

Thilo F, **Liu Y**, Loddenkemper C, Schuelein R, Schmidt A, Yan Z, Zhu Z, Zakrzewicz A, Gollasch M, Tepel M. VEGF regulates TRPC6 channels in podocytes.
Nephrol Dial Transplant. 2012;27(3):921-929.
Impact factor 3.564

Maier A, **Liu Y**, Scholze A, Westhoff TH, Tepel M. Green urine following exposure to flupirtine.
Am J Kidney Dis. 2010;56(5):1014-1015.
Impact factor 5.242

Thilo F, **Liu Y**, Schulz N, Gergs U, Neumann J, Loddenkemper C, Gollasch M, Tepel M. Increased transient receptor potential vanilloid type 1 (TRPV1) channel expression in hypertrophic heart.
Biochem Biophys Res Commun. 2010;401(1):98-103.
Impact factor 2.595

Thilo F, Suess O, **Liu Y**, Tepel M. Decreased expression of transient receptor potential channels in cerebral vascular tissue from patients after hypertensive intracerebral hemorrhage.
Clin Exp Hypertens. 2011;33(8):533-537.
Impact factor 1.142

Thilo F, Vorderwülbecke BJ, Marki A, Krueger K, **Liu Y**, Baumunk D, Zakrzewicz A, Tepel M. Pulsatile Atheroprone Shear Stress Affects the Expression of Transient Receptor Potential Channels in Human Endothelial Cells.
Hypertension. 2012;59(6):1232-1240.
Impact factor 6.908

Oral presentations and poster presentations

Liu Y, Thilo F, Kreutz R, Tepel M:
TRPC expression in tissue from hypertensive Munich-Wistar-Frömter (MWF) rats (Oral presentation)
37. Rostocker Gespräch über kardiovaskuläre Funktion und Hypertonie
Rostock, Germany, June 20, 2009

Liu Y, Thilo F, Tepel M:
Erythropoietin increases transient receptor canonical TRPC5 channel expression in endothelial cells
1. Jahrestagung der Deutschen Gesellschaft für Nephrologie

Göttingen, Germany, September 26-29, 2009

Liu Y, Thilo F, Kreutz R, Tepel M:
Tissue TRPC expression in hypertensive Munich-Wistar-Frömter rats
33. Wissenschaftlicher Kongress der Deutschen Hochdruckliga
Lübeck, Germany, November 19-21, 2009

Liu Y, Thilo F, Tepel M:
Increased transient receptor canonical TRPC5 channel expression might be responsible for hypertension during erythropoietin therapy (Oral presentation)
XLVII European Renal Association, European Dialysis and Transplant Association (ERA-EDTA) Congress and 2nd Congress of Deutsche Gesellschaft für Nephrologie
München, Germany, June 25-28, 2010

Liu Y, Tepel M:
Erythropoietin-induced TRPC5 Expression and Hypertension
34. Wissenschaftlichen Kongress der Deutschen Hochdruckliga e. V. DHL® – Deutschen Hypertonie Gesellschaft
Berlin, Germany, December 9-11, 2010

Liu Y, Thilo F, Echtermeyer F, Jensen BL, Gollasch M, Tepel M:
Syndecan4 selectively modulates TRPC6-mediated calcium signaling in podocytes
35. Wissenschaftlichen Kongresses der Deutschen Hochdruckliga e.V. DHL® - Deutschen Gesellschaft für Hypertonie und Prävention finden industriegetragene Symposien
Köln, Germany, November 24-26, 2011

Liu Y, Thilo F, Echtermeyer F, Jensen BL, Gollasch M, Tepel M:
Blockade of the proteoglycan syndecan 4 ameliorates proteinuric kidney disease (Oral presentation)
49th European Renal Association, European Dialysis and Transplant Association (ERA-EDTA) Congress
Paris, France, May 24-27, 2012

Selbständigkeitserklärung

„Ich, Ying Liu, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: Regulation von transient receptor potential canonical Kanälen TRPC3 und TRPC6 bei Nierenerkrankungen selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe."

Datum Unterschrift

Acknowledgements

The writing of this medical dissertation has been a precious journey in my academic life. I could not have traveled this far without the passionate and continued support of advisors, colleagues, and family.

First and foremost I would like to express my heartfelt gratitude to Professor Dr. med. Martin Tepel, my main advisor and supervisor of this medical dissertation. Over the past three and half years, he has been the major pillar of my academic life at Charité Universitätsmedizin Berlin. His patience, insightful comments, and constructive criticism were invaluable and enabled me to not only constantly improve and refine my doctoral project but also complete several publications in peer-reviewed journals. He has enlightened me in so many aspects about medical statistics, experimental design, academic presentations, and life in general. He is also an understanding and encouraging friend who has stood by me in times of difficulties. I feel very privileged to have been one of Professor Dr. med. Martin Tepel's medical doctoral students. One simply could not wish for a better or friendlier supervisor.

Dr. Florian Thilo and Dr. Katharina Krüger have helped in so many ways to enable and shape the laboratory work while continue to provide firm support along the way. For that, I will always be grateful. I am deeply indebted to Professor Maik Gollasch and Dr. Lena Löhr, both of whom have offered much advice and insight throughout my work on electrophysiological systems. Dr. Antje Schmidt from Max-Delbrück-Center of Molecular Medicine and Professor Ralf Schülein from Leibniz-Institute for Molecular Pharmacology have provided the support and equipment I have needed to produce and complete my thesis. I offer my sincerest gratitude to them. I am greatly indebted to Dr. Alexandra Scholze who has devoted hours to proofreading my manuscripts.

I would also like to thank Dr. Frank Echtermeyer and Professor Gregor Theilmeier, both from the Medical University Hannover, for providing syndecan 4 knockout mice; Professor Alexander Dietrich (Institut für Pharmakologie und Toxikologie, Universität Marburg) for providing green fluorescence protein-tagged TRPC6; Professor Michael Schaefer (Institut für Pharmakologie und Toxikologie, Universität Leipzig) for providing yellow fluorescence protein-tagged TRPC6; Professor Christoph Loddenkemper for immunohistochemistry

experiments; Professor Reinhold Kreutz and Dr. Angela Schulze for providing Munich Wistar Frömter rats; and Dr. Vera Jankowski for MALDI-TOF mass spectrometry experiments.

Finally, I thank my parents for supporting me throughout all my studies at university and wish to express my deepest appreciation for all you have given me.