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Induction of eryptosis by uremic toxins

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Summary

Anemia is a major complication of end stage renal disease. The anemia is mainly the result of impaired formation of erythrocytes due to lack of erythropoietin and iron deficiency. End stage renal disease causes enhanced accelerated erythrocyte death (eryptosis). Eryptosis is characterized by cell shrinkage and by cell membrane scrambling with phosphatidylserine-exposure at the erythrocyte surface. Triggers of eryptosis include increase of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$), which could be sensitized by ceramide. Mechanisms involved in the triggering of erpytosis in chronic kidney disease remained enigmatic. The present study explored the effect of two important uremic toxins acrolein and indoxyl sulfate, which are enhanced in chronic kidney disease, on eryptosis.

A 48 hour exposure to acrolein (30 – 50 µM) did not significantly modify $[Ca^{2+}]_i$ but significantly decreased forward scatter and increased annexin-V-binding. Acrolein further triggered slight, but significant hemolysis and increased ceramide formation in erythrocytes. Acrolein (50 µM) induced annexin-V-binding was significantly blunted in the nominal absence of extracellular Ca^{2+}. Acrolein augmented the annexin V binding following treatment with Ca^{2+} ionophore ionomycin (1µM). Furthermore, 48 hour exposure to indoxyl sulfate significantly increased $[Ca^{2+}]_i$ (≥ 300 µM), significantly decreased forward scatter (≥ 300 µM) and significantly increased annexin-V-binding (≥ 50 µM). Indoxyl sulfate did not significantly modify hemolysis of erythrocytes. Indoxyl sulfate (150 µM) induced annexin-V-binding was virtually abolished in the nominal absence of extracellular Ca^{2+}. In addition, indoxyl sulfate increased ceramide formation.

As conclusion, the two uremic toxins acrolein and indoxyl sulfate stimulate suicidal erythrocyte death or eryptosis, an effect at least in part due to increased $[Ca^{2+}]_i$ and stimulation of ceramide formation, which sensitizes for cytoplasmic Ca^{2+}. These results suggest that the uremic toxins acrolein and indoxyl sulfate could participate in chronic kidney disease induced eryptosis and possible subsequent anemia.

Zusammenfassung

Anämie ist eine schwerwiegende Komplikation der chronischen Niereninsuffizienz, welche sich teilweise aufgrund gestörter Erythrozytenbildung durch Erythropoietinmangel und Eisenmangel entwickelt. Der suizidalen Erythrozytentod (Eryptose) ist vermehrt bei chronischer Niereninsuffizienz. Eryptose ist gekennzeichnet von einer Zerhackung der Zellmembran und der Freilegung von Phosphatidylserin an der Zelloberfläche. Eryptose kann durch erhöhte intrazelluläre Kalzium Konzentrationen ausgelöst werden, was durch Ceramid sensibilisiert werden kann. Auslösende Mechanismen der Eryptose bei chronischer Niereninsuffizienz sind großteils unbekannt. Diese Studie untersuchte die Rolle der zwei wichtigen Urämie-Toxine Acrolein und Indoxyl-Sulfat auf den suizidalen Erythrozytenzelltod.

Die Behandlung von Erythrozyten mit Acrolein (30 – 50 µM) beeinflusste die intrazelluläre Kalziumkonzentration nicht, führte aber zu einer verringerten Vorwärtsstreuung und verstärkter Annexin-V Bindung. Acrolein führte zu einer geringen aber signifikanten Hämolyse und Ceramid Bildung. Die Acrolein (50µM) induzierte Annexin-V Bindung war signifikant erniedrigt in der Abwesenheit von Kalzium. Acroleinbehandlung führte zu einer verstärkten Bindung von Annexin-V nach Ionomycinbehandlung (1µM). Indoxylsulfat führte zu einer Erhöhung des intrazellulären Kalziums (≥ 300 µM), Verringerung der Vorwärtsstreuung (≥ 300 µM) und verstärkten Annexin-V Bindung (≥ 50 µM), hatte aber keinen Einfluss auf die Hämolyse. Die durch Indoxylsulfat (150µM) hervorgerufene Annexin-V Bindung war nicht festzustellen in der Abwesenheit von Kalzium. Indoxylsulfat führte zusätzlich zu vermehrter Ceramidbildung.

Die Urämietoxine Acrolein und Indoxylsulfat induzieren suizidalen Erythrozytentod (Eryptose). Diese Effekte sind zumindest teilweise vermittelt durch eine Erhöhung des intrazellulären Kalziums und eine vermehrte Bildung von Ceramid, welches eine kalziumsensibilisierende Rolle hat. Dies weist auf eine mögliche Rolle von Acrolein und Indoxylsulfat bei Niereninsuffizienz assozierter Eryptose hin, mit möglichen Auswirkungen auf die Anämie bei Niereninsuffizienz.

List of Abbreviations

CHF: Congestive heart failure

CKD: Chronic kidney disease

COX: Cyclooxygenase

CRP: C-reactive protein

CVD: Cardiovascular disease

EPO: Erythropoietin

ESAs: Erythropoiesis-stimulating agents

ESRD: End stage renal disease

FGF23: Fibroblast growth factor 23

GFR: Glomerular filtration rate

GIT: Gastrointestinal tract

Hb: Hemoglobin

HEPES: 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid

h: Hour

LVH: Left ventricular hypertrophy

MBD: Mineral bone disorders

min.: Minute

NO: Nitric oxide

OAT: Organic anion transporter

PAF: Platelet activating factor

PGE2: Prostaglandin E2

PKC: Protein kinase C

PLA: Phospholipase A2

pRBC: infected red blood cells with Plasmodium

PTH: Parathyroid hormone

RBCs: Red blood cells

ROS: Reactive oxygen species

SCR: Scramblase

WHO: World health organization

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Chapter 1

Introduction

1. 1. Chronic kidney disease:

The chronic kidney disease (CKD) is a condition described as advanced decrease of renal function over months or years, due to various disease processes or natural aging. Also, it is defined as the existence of kidney damage for more than three months, quantified by glomerular filtration rate (GFR), which appears by abnormal excretion of albumin or decreased kidney function (KDOQI (2006), Levey, Eckardt et al. (2005)). The prevalence of CKD is near to epidemic rates worldwide (Coresh, Selvin et al. (2007), Levey, Atkins et al. (2007)). Thirty percent of Americans suffer from CKD (Whaley-Connell, Bomback et al. 2011)). In the majority of CKD patients, not the failure of the kidney itself, but the early commencement of the common diseases cancer, infection and cardiovascular diseases is the real cause of death (Whaley-Connell, Bomback et al. 2011), Kestenbaum, Sampson et al. (2005)). Depending on estimated glomerular filtration rate (eGFR) CKD is staged as follows (Coresh, Astor et al. (2003)):

- Stage 1, normal eGFR ≥ 90 mL/min per 1.73 m^2 and persistent albuminuria

- Stage 2, eGFR between 60 - 89 mL/min per 1.73 m^2

- Stage 3, eGFR between 30 - 59 mL/min per 1.73 m^2

- Stage 4, eGFR between 15 - 29 mL/min per 1.73 m^2

- Stage 5, eGFR of < 15 mL/min per 1.73 m^2 or (end-stage renal disease)

1. 2. Complications of chronic kidney disease:

Many complications are associated with the progression of chronic kidney disease. These complications include increased incidence of cardiovascular disease (Fig.1), hyperlipidemia, anemia and metabolic bone disease (Thomas, Kanso et al. (2008)). The complications of CKD ultimately lead to a vicious

cycle, which is the underlying cause for the high mortality of the renal failure patients.

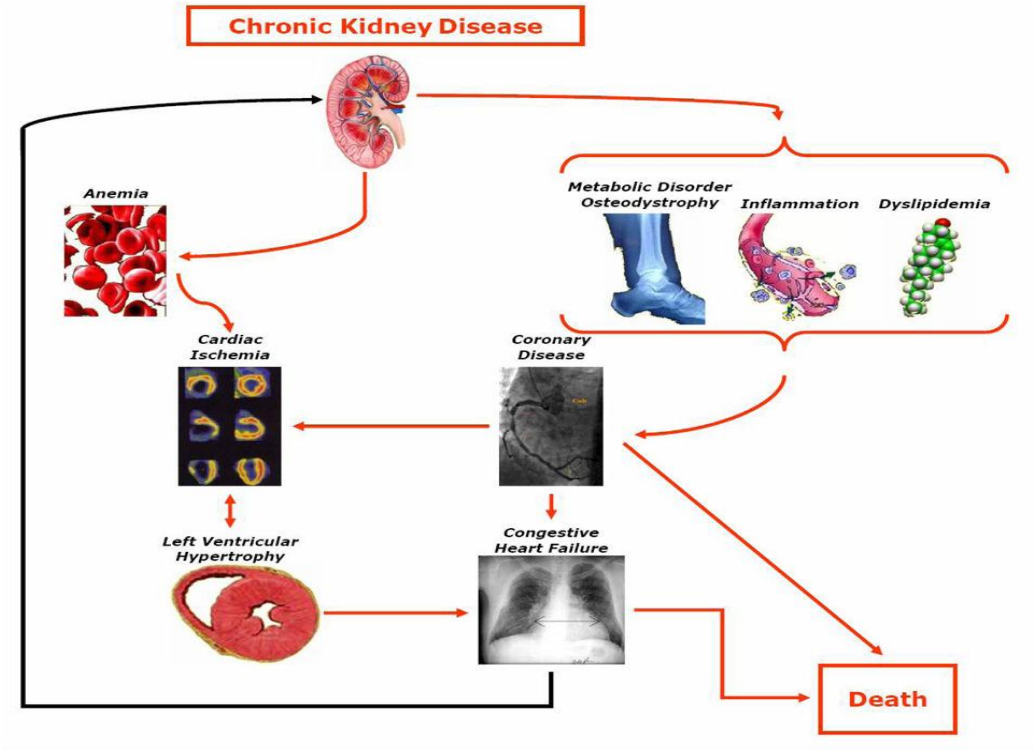

Fig.1. Processes interaction secondary to chronic kidney disease leading to cardiovascular disease and death. Red arrows: Pathogenetic pathways; black arrow: Feedback loop; kidney disease worsened by heart failure (Thomas, Kanso et al. (2008)).

1. 2. 1. Cardiovascular complications and chronic kidney disease:

CKD patients are amongst the highest risk group for cardiovascular disease (CVD). The most important cause of death among long-term hemodialysis patients is cardiac disease, which equals to 44% of overall deaths (Herzog, Ma et al. (1998)). Actually, the dramatically high risk for CVD mortality in hemodialysis patients is due to two possible reasons; the high prevalence of CVD and the high mortality in CVD patients (Foley, Parfrey et al. (1998), Sarnak (2003)). Several studies indicate that hemodialysis patients show greater prevalence of congestive heart failure and clinical ischemic heart disease than the general population (Foley, Parfrey et al. (1998)). In addition, dialysis patients show a high percentage of left ventricular hypertrophy (Foley, Parfrey et al. (1998)). Herzog et al. showed that hemodialysis patients with acute myocardial infarction have a high mortality due to cardiac causes as well as poor long-term survival (Herzog, Ma et al. (1998)). 30% of end stage renal disease (ESRD) patients show clinical evidence of heart failure or ischemic heart disease. Moreover, the cause of death in patients with reduced glomerular filtration rate is more likely due to CVD rather than progressed ESRD (Shulman, Ford et al. (1989)).

There are several pathological forms of CVD that are associated with CKD. The following pathological cardiovascular changes are highly prevalent in CKD patients (Sarnak (2003)):

The change in geometry of the heart includes eccentric left ventricular hypertrophy (LVH), concentric LVH, and associated LV remodeling. The pressure overload secondary to hypertension, aortic stenosis, or arteriosclerosis is considered a risk factor for concentric LVH, while the risk factors for eccentric LVH include; volume overload secondary to fluid retention, arteriovenous fistulae or anemia (Schunkert and Hense (2001)).

In hemodialysis patients, atherosclerosis is the main cause of ischemic heart disease. A study described, that around 50% of hemodialysis patients suffers from significant large-vessel coronary artery disease (Rostand, Kirk et al. (1984)). Coronary artery plaques in dialysis patients differ from non-uremic

patients by a stronger degree of calcification and media thickening (Schwarz, Buzello et al. (2000)).

1. 2. 2. Endokrine dysregulation (mineral bone disorder) and chronic kidney disease:

An increasing evidence suggests, that the dysregulation of the mineral metabolism in CKD plays an essential role in accelerating cardiovascular abnormalities and disease progression associated with CKD, termed mineral bone disorder (Goodman, Goldin et al. (2000), Haap, Heller et al. (2006)). In patients with CKD, the dysregulation of calcium (Tentori, Blayney et al. (2008)), phosphate (Block, Klassen et al. (2004)) and vitamin D (Teng, Wolf et al. (2005), Wolf, Shah et al. (2007)) balance is independently associated with increasing mortality. The high risk of cardiovascular events is evident even at glomerular filtration rate levels >60 mL/min/1.73 m^2 (Levin, Bakris et al. (2007), Di Angelantonio, Chowdhury et al. (2010)). Vascular calcification is a cardiovascular complication of CKD based on the mineral bone disorder. Arteriosclerosis, the disease of the large vessels, is a process which includes remodeling of the vessel, lack of elasticity, and development of noncompliant vessels (London, Marchais et al. (2002)). This ultimately results in increased pulse pressure, which has been considered as a factor determining CVD outcomes in dialysis patients (Klassen, Lowrie et al. (2002)). In CKD, the mineral bone disorder (MBD) affects vascular calcifications (Moe, Drueke et al. (2007)). Vascular calcification in CKD patients affects the elastic lamellae in the artery and differs from that of atheroschlerotic vascular disease (Mizobuchi, Towler et al. (2009)). High concentration of extracellular phosphate induces the reprogramming of vascular cells into an osteoblastic phenotype to promote vascular calcification (Yoshida, Fujimori et al. (2002)). The dysregulation of the phosphate homeostasis is associated with the FGF23-klotho endocrine system. This endocrine system to control phosphate homeostasis is failing in patients suffering from CKD even at early stages (Hu, Shi et al. (2011),Levey, Atkins et al. (2007)). Fibroblast growth factor 23 (FGF23) acts mainly on renal proximal tubule cells to reduce the expression and /or the apical surface content of type 2

sodium-dependent phosphate co transporter and by decreasing the 1α-hydroxylase enzyme, which converts 1,25-dihydroxyvitamin D 3 to calcitrol to reduce serum phosphate levels (Stubbs, Liu et al. (2007), Galitzer, Ben-Dov et al. (2010), Urakawa, Yamazaki et al. (2006)). FGF23 requires the klotho protein as co receptor (Fig.2) (Tsujikawa, Kurotaki et al. (2003)). The klotho protein is a single-pass transmembrane protein which is expressed mainly in the kidney (Kuro-o, Matsumura et al. (1997)). A syndrome similar to aging in mice can be obseved due to a defect in klotho expression (Kuro-o, Matsumura et al. (1997)). Along these lines, overexpression of klotho results in prolongation of the lifespan in mice (Kurosu, Yamamoto et al. (2005)).

Klotho is also secreted into the systemic circulation and acts as a hormone, the lack of which is presumed to contribute to the cardiovascular complications in CKD (Hu, Shi et al. (2011). The klotho hypomorphic mouse mirrors the cardiovascular complications found in CKD patients (Hu, Shi et al. (2011). In klotho hypomorphic mice, indicators of vascular remodeling reminiscent of CKD has been found in calcified tissue with increased osteogenic programming including increased Pit-1 transcription (Hu, Shi et al. (2011), Takei, Yamamoto et al. (2012)).

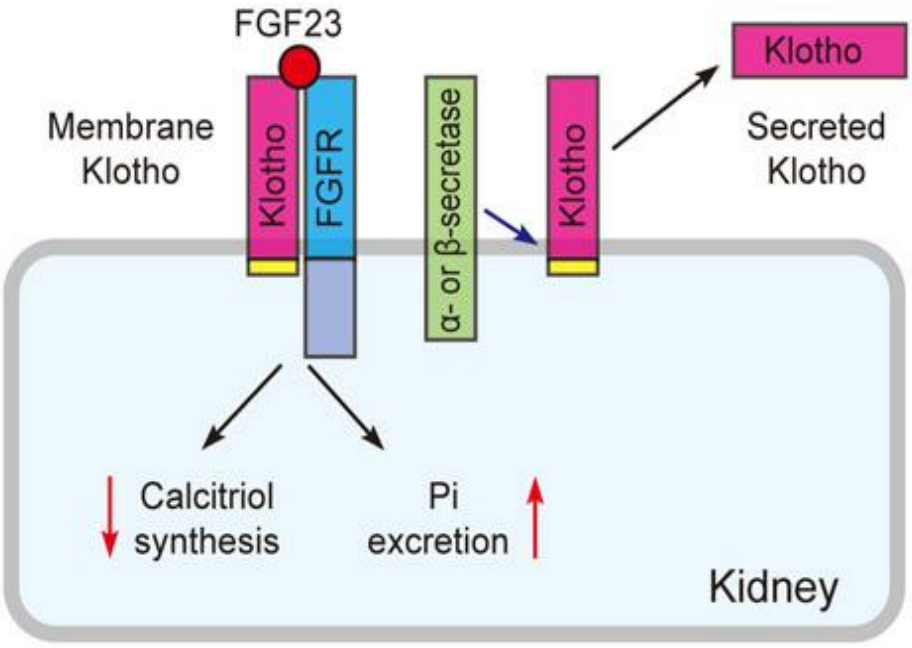

Fig. 2. Membrane Klotho and secreted Klotho. Membrane Klotho forms a complex with the fibroblast growth factor receptor (FGFR) to create a de novo high-affinity binding site for FGF23. Membrane Klotho is subject to ectodomain shedding by α- and β-secretases to release secreted Klotho (Kuro-o (2011)).

1. 2. 3. Anemia as a complication of chronic kidney disease:

Anemia is a term which describes a derangement of the normal function of erythrocytes. It indicates a reduction of red cell count, hemoglobin concentration, or hematocrit. Anemia has been defined by the World Health Organization (WHO) as a hemoglobin level less than 13g/dL in men and post-menopausal women, and less than 12g/dL in pre-menopausal women (WHO (1968)). Progressive CKD is usually accompanied with a normochromic, normocytic anemia (Besarab and Levin (2000). Approximately fifty percent of chronic kidney disease patients suffer from anemia (McClellan, Aronoff et al. (2004)). Although, diagnosis of anemia could be possible at any stage of CKD, the prevalence of anemia is strongly correlated with the severity of CKD. The prevalence of anemia increases with a decline of renal function (McClellan, Aronoff et al. (2004)). CKD-associated anemia increases morbidity and mortality from cardiovascular complications (e. g. left ventricular hypertrophy (LVH) (Besarab and Levin (2000)). Cardiovascular complications may cause further decline of renal function and the establishment of a vicious cycle termed the “cardiorenal anemia syndrome”. End stage renal patients with LVH have lower five years survival than CKD patients without LVH (Levin, Singer et al. (1996)). Also in stable coronary artery disease patients with CKD, anemia is considered as an independent predictor of mortality (Muzzarelli, Pfisterer et al. (2006)).

Several mechanisms are involved in the development of CKD-associated anemia. Anemia in CKD results primarily from the reduction of erythropoietin formation in the kidney and secondary due to shortage lifespan of erythrocytes (Tsagalis (2011)). A major factor contributing to CKD-associated anemia is therefore the reduction of erythropoietin (EPO) synthesis. EPO is the essential stimulus for erythroid maturation during the late stage of erythropoiesis. In the human body, EPO is a hormone that circulates at about one hundredth of the concentration of most of other hormones (Lappin, Maxwell et al. (2002), Maxwell (2002)). Kidney interstitial fibroblasts secrete erythropoietin (Ratcliffe (1993)), which is essential for the growth and differentiation of erythrocytes in bone marrow. A reduction of erythropoietin is considered as a major cause of CKD associated anemia (Nangaku and Eckardt (2006)). EPO is expressed by

hepatocytes during fetal development, while in adults the main site for EPO production are the peritubular fibroblasts in the renal cortex. It is also found in brain, liver, spleen, lung and testis. In the setting of CKD, these organs are not able to substitute the declined renal EPO production (Jelkmann (2011)). The specific stimulus of EPO expression is the low tissue oxygen pressure. Due to hypoxic conditions, EPO production is increased mainly in kidneys and only very minor in other organs like liver and brain (Jelkmann (2011)). Tubular atrophy during CKD generates tubulointerstitial fibrosis, leading to a reduction of erythropoietin synthesis capacity which contributes to CKD associated anemia. Treatment of anemia of CKD patients via recombinant erythropoietin was described as beneficial for those patients (Fink, Blahut et al. (2001)). The usage of erythropoiesis-stimulating agents (ESAs) since the late 1980s to manage renal anemia significantly improved the quality of patient´s life and avoided the need for blood transfusion. Still about 5–10% (Nangaku and Eckardt (2006)) of patients have an inadequate response to ESAs. However, recent studies have shown that treatment with ESAs may have adverse effects. Cardiovascular events were increased when targeting a higher hemoglobin level in CKD patients, which caused a discussion over the uncritical use of erythropoiesis stimulating agents in CKD patients (de Francisco and Pinera (2011)).

Iron is a necessary substrate for erythroid precursors. The liver produces and secrets hepcidin, a key mediator of systemic iron homoeostasis, regulating absorption and utilization of iron. In CKD patients, a variety of interferences in both the iron metabolism and the inhibition of iron release from the reticuloendothelial system are observed. Excessive production of hepicidin caused by pro-inflammatory cytokines contributes to a functional iron deficiency, associated renal anemia and resistance to EPO (Besarab and Coyne (2010), Babitt and Lin (2010), Uehata, Tomosugi et al. (2012)). Raised hepcidin levels caused by inflammatory processes cause iron retention in enterocytes and macrophages. This leads to a reduction in the iron availability for erythropoiesis with subsequently impaired haeme synthesis (Yilmaz, Solak et al. (2011), Beaumont and Karim (2013), Maruyama, Yokoyama et al. (2012)).

Hyperthyroidism may also play a permissive role in the CKD associted anemia (Boxer, Ellman et al. (1977). The high levels of circulating parathyroid hormone (PTH) in CKD patients with secondary hyperparathyroidism unfavorable influence the anemia of CKD patients (Di Iorio, Minutolo et al. (2003)). The possible mechanism for this observation could be either a direct effect of PTH on inhibition of early erythroid progenitors, endogenous EPO synthesis or erythrocytes survival. In addition, an indirect effect of PTH (Brancaccio, Cozzolino et al. (2004), Horl (2004), Gaweda, Goldsmith et al. (2010)) via induction of fibrosis in the bone marrow could be possible (Rao, Shih et al. (1993)). The increase of serum alkaline phosphatase and hyperphosphataemia may also play a role in CKD associated anemia and EPO hyporesponsiveness (Bowry and Gatti (2011), Kalantar-Zadeh, Lee et al. (2009)).

It has become obvious, that anemia in chronic kidney disease is not caused by a single event, but is rather an interplay of various pathophysiological effects due to the renal failure. Multiple mechanisms contribute to the development of anemia in renal disease.

1. 3. Erythrocytes and eryptosis:

Erythrocytes are one of the most abundant cell types in the human body. Erythrocytes account for approximately one quarter of total cell number in the adult human body. The individual erythrocyte has a limited lifespan of approximately 100-120 days. More than 200 billion erythrothytes need to be replaced every day due to the limitation of their lifespan. The senescence of erythrocytes leads to clearance of aged erythrocytes (Bosman, Willekens et al. (2005), Arese, Turrini et al. (2005)).

The disposal of abundant, defected, or potentially harmful nucleated cells can be completed by suicidal cell death (Green and Reed (1998), Gulbins, Jekle et al. (2000)). The features of apoptosis or suicidal cell death (Green and Reed (1998), Gulbins, Jekle et al. (2000)) of nucleated cells are characterized by loss of cellular K^+ with cell shrinkage, nuclear condensation, DNA fragmentation, mitochondrial depolarization, cell membrane blebbing, breakdown of

phosphatidylserine and asymmetry of the plasma membrane (Bortner and Cidlowski (1999) Yu, Canzoniero et al. (2001)). Several factors induce apoptosis including stimulation of receptors such as CD95 (Daniel, Wieder et al. (2001), Lang, Szabo et al. (1999)) or TNFα (Rieger, Frank et al. (2007)). Apoptosis can also be triggered by exposing cells to various stressors including oxidants (Han, Wang et al. (2004), Varela, Simon et al. (2007)), cytostatic drugs (Bachmeier, Nerlich et al. (2007)), radiation (Rosette and Karin (1996)), osmotic shock (Shimizu, Wehner et al. (2006)), alkaline stress (Chen, Wang et al. (2007)), Na^+/H^+ exchanger inhibitors (Konstantinidis, Koliakos et al. (2006)) or bile salts (Becker, Reinehr et al. (2007)). A similar apoptotic process plays an important role in the process of elimination of eryptotic red blood cells (RBC)s (Lang, Lang et al. (2005)). This can be explained by the following; first the apoptotic cells are recognized by macrophages equipped with receptors specific for phosphatidylserine (Fadok, Bratton et al. (2000)). Then the macrophages rapidly engulf and degrade phophtidylserine-exposing cells (Boas, Forman et al. (1998)). Thus, this process could allow the elimination of erythrocytes without release of intracellular proteins, which could cause inflammation (Gulbins, Jekle et al. (2000)).

Several critical elements are necessary for the cellular apoptosis machinery, most importantly nuclei and mitochondria. Both of them are missing in erythrocytes. Therefore erythrocytes rely on a different mechanism for an apoptotic process. When erythrocytes are exposed to the Ca^{2+} ionphore ionomycin, this leads to typical features similar to apoptosis in nucleated cells: cell shrinkage, membrane blebbing and phosphatidylserine exposure (Berg, Engels et al. (2001), Daugas, Cande et al. (2001)). Activation of Ca^{2+} sensitive K^+ channels leads to cell shrinkage (Lang, Kaiser et al. (2003)), the phospholipid scrambling of the cell membrane forms the phosphatidylserine exposure, which theoretically may be due to activation of Ca^{2+} sensitive scramblase (Dekkers, Comfurius et al. (2002), Zhou, Zhao et al. (2002)) and/or inhibition of Ca^{2+}sensitive and ATP-dependent aminophospholipid translocase (Seigneuret and Devaux (1984). Due to the differences and similarities to

apoptosis, the term eryptosis has been considered to describe the suicidal erythrocyte cell death (Lang, Lang et al. (2005)).

1.4. Signaling in the stimulation of eryptosis:

A key factor in triggering eryptosis is Calcium. Eryptosis may be triggered by enhanced cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) (Foller, Sopjani et al. 2009, Lang, Qadri et al. 2012)). The increase of $[Ca^{2+}]_i$ may be caused by Ca^{2+} entry through Ca^{2+}-permeable cation channels (Foller, Sopjani et al. 2009, Lang, Qadri et al. 2012), which are activated by oxidative stress (Lang, Qadri et al. 2012). Increasing the activity of cytosolic Ca^{2+} stimulates eryptosis, which leads to cell membrane vesiculation (Allan and Michell (1977)) and stimulates scrambling of cell membrane, resulting in phosphatidylserine exposure at the cell surface (Akel, Hermle et al. (2006), Nicolay, Schneider et al. (2006)). In addition, Ca^{2+} has an effect on degrading the cellular cytoskeleton, via stimulation of cysteine endopeptidase calpain, and thus facilitate blebbing of the membrane (Fig. 3) (Pant, Virmani et al. (1983)).

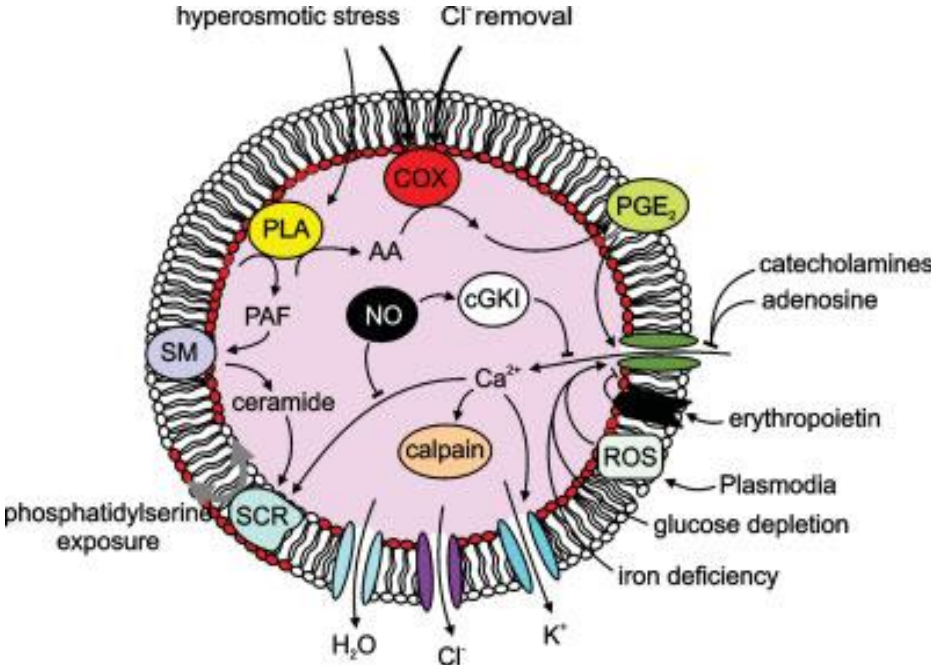

Fig. 3. Signaling of eryptosis. Mechanisms involved in stimulation and inhibition of Ca^{2+} entry and ceramide formation and thus governing cell volume, phosphatidylserine exposure and activation of calpains. cGKI, cGMP-dependent protein kinase type I; COX, cyclooxygenase; NO, nitric oxide; PAF platelet activating factor; PGE2, prostaglandin E2; PLA phospholipase A2; ROS, reactive oxygen species; SCR, scramblase (Foller, Huber et al. (2008)).

Ca^{2+} may also enter into the cell via non-selective cation channels (Lang, Kempe et al. (2005), Kaestner and Bernhardt (2002), Kaestner, Tabellion et al. (2004)). Those channels, at least in part, are made up by TRPC6 (Foller, Kasinathan et al. (2008)). Osmotic cell shrinkage (Lang, Duranton et al. (2003), Huber, Gamper et al. (2001)), oxidative stress (Lang, Duranton et al. (2003), Duranton, Huber et al. (2002)) and Cl^- removal (Lang, Kempe et al. (2005), Huber, Gamper et al. (2001), Duranton, Huber et al. (2002)) are major factors activating cation channels. At least partially, stimulation of cation channels is secondary to prostagalndin E2 (PGE2) formation (Lang, Kempe et al. (2005). Stimulation could be partially reversed by the phospholipase-A2 inhibitors quinacrine and palmitoyl-trifluoromethyl-ketone and the cyclooxygenase inhibitors acetylsalicylic acid and diclophenac ((Lang, Kempe et al. (2005)).

Whenever the cytosolic Ca^{2+} concentration in the erythrocyte is increased, this is followed by subsequent stimulation of Ca^{2+}-sensitive K^+ channels (Bookchin, Ortiz et al. (1987),Franco, Palascak et al. (1996)). The changes of K^+ hyperpolarize the cell membrane, which leads to Cl^- exit parallel to K^+ (Lang, Warskulat et al. (2003)). The shrinkage occurs due to cellular loss of KCL with osmotically obliged water, which further increases the stimulation of cell membrane scrambling (Lang, Warskulat et al. (2003)).

Ceramide enhances the Ca^{2+} sensitivity of cell membrane scrambling, which, comparable to increased cytosolic Ca^{2+} activity, increases phosphatidylserine exposure (Lang, Myssina et al. (2004)). Platelet activating factor (PAF) stimulates ceramide formation, which ultimately leads to breakdown of sphingomyelin through activation of sphingomyelinase (Lang, Kempe et al. (2005)). Accordingly, sphingomyelinase inhibitor 3,4-dichloroisocoumarin, genetic knockout of PAF receptors and by the PAF receptor antagonist ABT491 blunt eryptosis following osmotic shock (Lang, Kempe et al. (2005)). High concentrations of cytosolic Ca^{2+} are not necessary for PAF induced eryptosis, PAF at least partially accounts for Ca^{2+} independent eryptosis (Lang, Kempe et al. (2005)). Beside its effect on cell membrane scrambling, PAF also activates Ca^{2+} sensitive K^+ channels (Garay and Braquet (1986)). PAF exerts these effects by sensitizing the channels for the stimulating

effects of cytosolic Ca^{2+} (Rivera, Jarolim et al. (2002)). Conversely, in erythrocyte progenitor cells PAF is released onto increase Ca^{2+} activity (Dupuis, Levasseur et al. (1997)).

Additionally, energy depletion stimulates eryptosis, via activation of protein kinase C (PKC) and PKC-dependent phosphorylation of membrane proteins with later phosphatidylserine exposure and cell shrinkage (Klarl, Lang et al. (2006)). The stimulation of PKC with phorbolesters or inhibition of protein phophatases such as okadaic acid both mimic the effect of energy depletion (Klarl, Lang et al. (2006)). Stimulation of entry of Ca^{2+} into erythrocyte could be as a result of activation of protein kinase C (PKC) (Andrews, Yang et al. (2002)) and phosphatidylserine exposture (de Jong, Rettig et al. (2002)). PKCα, PKCι, PKCμ and PKCξ are protein kinases expressed by erythrocytes (Govekar and Zingde (2001)). These kinases phosphorlate cytoskeletal proteins such as band 4.1, 4.9 and adducin (Danilov and Cohen (1989)) as well as the human Na^{+}/H^{+} antiporter NHE 1 (Bourikas, Kaloyianni et al. (2003)).

Triggering of eryptosis can furthermore be induced by oxidative stress or due to defects on anti oxidative defense (Bilmen, Aksu et al. (2001), Mavelli, Ciriolo et al. (1984)), partially by stimulating Ca^{2+} entry via activation of the Ca^{2+} permeable cation channels (Duranton, Huber et al. (2002)). Further, oxidative stress activates Cl^{-} channels (Huber, Uhlemann et al. (2002) andTanneur, Duranton et al. (2006)), which play a role in erythrocyte shrinkage and thus also participate in the triggering of eryptosis (Myssina, Lang et al. (2004)). Oxidative stress stimulated eryptosis is paralleled by activation of aspartyl and cysteinyl proteases (Matarrese, Straface et al. (2005)).

Eryptosis is further triggered by activation of caspases (Foller, Huber et al. 2008, Foller, Mahmud et al. 2009, Lau, Chen et al. 2011, Maellaro, Leoncini et al. 2011, Lang, Qadri et al. 2012). Oxidant-sensitive caspases are expressed by erythrocytes (Bratosin, Estaquier et al. (2001), Mandal, Baudin-Creuza et al. (2003)), which cleave the anion exchanger band 3 (Mandal, Baudin-Creuza et al. (2003)) and promote phosphatidylserine exposure of erythrocytes (Mandal, Moitra et al. (2002)). Activation of caspases is not required when eryptosis is induced by ionomycin or hyperosmotic shock (Berg, Engels et al. (2001), Lang,

Myssina et al. (2004), Weil, Jacobson et al. (1998)). Eryptosis is modified by several kinases, such as AMP activated kinase AMPK (Foller, Sopjani et al. 2009), cGMP-dependent protein kinase (Foller, Feil et al. 2008), Janus-activated kinase JAK3 (Bhavsar, Gu et al. 2011), casein kinase (Kucherenko, Huber et al. 2012, Zelenak, Eberhard et al. 2012), p38 kinase (Gatidis, Zelenak et al. 2011), as well as sorafenib (Lupescu, Shaik et al. 2012) and sunifinib (Shaik, Lupescu et al. 2012) sensitive kinases.

1. 5. Anemia and eryptosis:

Accelerated suicidal erythrocyte death may contribute to the anemia of several clinical disorders and eryptosis may be triggered by a wide variety of xenobiotics (Zappulla 2008, Felder, Hoelzle et al. 2011, Ghashghaeinia, Toulany et al. 2011, Lang, Jilani et al. 2011, Qadri, Bauer et al. 2011, Qadri, Kucherenko et al. 2011, Abed, Towhid et al. 2012, Abed, Towhid et al. 2012, Bottger, Multhoff et al. 2012, Gao, Cheung et al. 2012, Jilani, Lupescu et al. 2012, Jilani, Lupescu et al. 2012, Kucherenko and Lang 2012, Lang, Qadri et al. 2012, Lang and Qadri 2012, Lupescu, Jilani et al. 2012, Lupescu, Jilani et al. 2012, Polak-Jonkisz and Purzyc 2012, Shaik, Lupescu et al. 2012, Shaik, Zbidah et al. 2012, Weiss, Cytlak et al. 2012, Zbidah, Lupescu et al. 2012, Zbidah, Lupescu et al. 2012, Zelenak, Pasham et al. 2012).

Severe anemia is a complication of chronic renal failure, which is at least in part explained by compromised renal erythropoietin formation and release combined with subsequent decrease of erythropoiesis (Fishbane and Nissenson 2010, Atkinson and Furth 2011, Parfrey 2011). Moreover, weak formation of erythrocytes in chronic renal failure may result from iron deficiency (Besarab and Coyne 2010, Attanasio, Ronco et al. 2012).

Theoretically, anemia in renal insufficiency could result in addition from accelerated death of circulating erythrocytes. Erythrocytes may undergo apoptosis-like suicidal death or eryptosis, which is characterized by cell membrane scrambling (Nguyen, Wagner-Britz et al. 2011, Lang, Qadri et al. 2012), and could foster increased erythrocyte removal. The spleen is a highly complex organ. It is responsible for selectively filtration and destroying

senescent erythrocytes, infectious organisms as well as infected erythrocytes with Plasmodium (pRBC) (Bowdler (2002)). The filtration capacity of the spleen is related to its complex structures. It consists of (a) the white pulp with lymphoid tissue and (b) the red plup, with a reticular meshwork. The senescent erythrocytes, aberrant RBCs and pRBCs destruction occurs in the red pulp, and (c) a marginal zone between the two plups which is responsible for the elimination of inert particles, bacteria and viruses (Martin-Jaular, Ferrer et al. (2011)). During their life span, erythrocytes pass repeatedly through the capillaries of the vascular bed and interendothelial slits of venous sinus of red pulp. The dimension of the erythrocytes is bigger than the vascular bed and interendothelial slits diameters (Chen and Weiss (1973)). To pass repeatedly and safely, erythrocytes must be able to undergo repeated, extensive, and reversible deformations. The ion and water permeability changes due to the repeated major deformation of the erythrocytes (Larsen, Katz et al. (1981), Hebbel and Mohandas (1991), Johnson (1994), Dyrda, Cytlak et al. (2010)). The shape of erythrocytes allows them to deform while maintaining a constant surface area (Mohandas, Clark et al. (1980),Clark, Mohandas et al. (1983) Clark, Sterrett et al. (2000), Murdock, Reynolds et al. (2000)).

Wide range of conditions may shorten the survival of the RBCs. Depending on the changes in the membrane, severe changes result in intravascular lysis, while less acute and milder membrane changes lead to recognition, ingestion and, ultimately, destruction by phagocytes of the reticulo-endothelial system. Removal of RBCs from circulation involves several mechanisms. Oxidation of band 3 causes binding of naturally occurring antibodies which mediate opsonization for phagocytosis, and programmed or suicidal cell death ((Lang, Lang et al. 2006), Lutz (2004), Bosman, Willekens et al. (2005)). In Red blood cells there is no nucleus or mitochondria, therefore they do not follow the classical apoptosis feature. The features of RBCs apoptosis (eryptosis) include cell shrinkage, decreased cell deformability, vesiculation and membrane phospholipid scrambling and phosphatidylserine exposure ((Lang, Lang et al. 2006)). Loss of membrane phospholipids is a key trigger that leads to recognition and extravascular removal of senescent and

disordered RBCs as well as transfused RBCs which have been stored for a long time (Kuypers and de Jong (2004), Boas, Forman et al. (1998), Dasgupta, Abdel-Monem et al. (2008)). Finally, this process initiates recognition and removal of RBCs in the spleen and liver by phagocytes (Fens, van Wijk et al. (2012)). Opsonization by lactadherin is essential, for recognition of exposed phosphatidylserine in apoptotic nucleated cells and suicidal RBCs by macrophages and subsequent phagocytosis (Dasgupta, Abdel-Monem et al. (2008),Hanayama, Tanaka et al. (2002)). Subsets of macrophages (Hanayama, Tanaka et al. (2004)) in as well as around blood vessels express lactadherin (Fens, Mastrobattista et al. (2008)), Silvestre, Thery et al. (2005)). Enhanced eryptosis could therefore contribute to the development of anemia in chronic kidney disease.

The percentage of phosphatidylserine exposing erythrocytes is enhanced in chronic renal failure (Myssina, Huber et al. 2003). Klotho has been considered a biomarker for chronic kidney disease and is involved in adverse complications of chronic kidney disease (Asai, Nakatani et al. (2012),Koh, Fujimori et al. (2001)). Along these lines, the klotho hypomorphic mouse shows an increased rate of eryptosis (Kempe, Ackermann et al. (2009)). Little is known, however, about mechanisms stimulating eryptosis in end stage renal disease. Eryptosis is known to be stimulated by vanadate (Foller, Sopjani et al. 2008) and methylglyoxal (Lang, Qadri et al. 2012), both substances increased in uremic plasma (Foller, Sopjani et al. 2008, Lang, Qadri et al. 2012)).

1. 6. Uremic toxins:

Several complications accompany renal failure and CKD. Uremia is considered to be a result of accumulation of organic waste products which are normally cleared by kidneys (Vitetta and Gobe (2013)). The human mucosal and extra mucosal tissues like gastrointestinal tract (GIT) is colonized by bacteria after birth, thereby giving rise to site specific unique microbiota families (Dominguez-Bello, Costello et al. (2010)). Human distal GIT represent the largest microbial community in the body. Reports suggests that intestinal microbiota as well as bacterial load might affect pathogenesis of diseases

(Mazmanian, Round et al. (2008), Wu, Lin et al. (2005)). CKD is particularly related to these bacteria. Several uremic toxins could be produced by GIT bacteria: advanced glycation end products (Hegab, Gibbons et al. (2012)), phenolic compounds and indoles (e.g., indoxyl sulfate) (Macfarlane and Macfarlane (2012)). Also GIT fermentation of amino acids phenylalanine and tyrosine leads to production of *p*-cresol and phenol (Bone, Tamm et al. (1976), Macfarlane and Macfarlane (2012)). The biological effect of these products is due to induction of proinflammatory responses, leukocyte stimulation and endothelial dysfunction (Charney, Walton et al. (1993),Dou, Bertrand et al. (2004)). Hence the excessive production of proinflammatory molecules in the GIT may play a significant role in inflammatory states (Macfarlane and Macfarlane (2012)). In case a dysbiotic and inflamed GIT ensues (Vitetta, Briskey et al. (2012)), a possible spread of these bacteria increases the likelihood of uremic toxin overload (Hida, Aiba et al. (1996)). There are three major classes of uremic retention solutes depending on their removal pattern by dialysis (Vanholder, De Smet et al. (2003)); small water-soluble compounds, middle molecules and protein-bound uremic toxins that are difficult to remove by dialysis due to their protein binding.

Uremic toxins are known to affect cellular homeostasis in chronic kidney disease and their accumulation leads to detrimental effects. Uremic toxins are contributing to the complications of chronic kidney disease (Duranton, Cohen et al. (2012)). Two important uremic toxins are acrolein (Igarashi, Ueda et al. 2006, Thompson and Burcham 2008)) and indoxyl sulfate (Niwa and Ise (1994)).

1. 6. 1. Acrolein:

Acrolein is a highly reactive aldehyde with cytotoxic properties (Tanel and Averill-Bates (2007)). Acrolein is best known as a carcinogen and produces protein and DNA adducts in high concentration exposures (Liu, Zhu et al. (2010), Zhang, Balbo et al. (2011)). The major factors responsible for cellular damage are reactive oxygen species (ROS) such as superoxide anion radical, hydrogen peroxide and hydroxyl radical (Hensley, Robinson et al. (2000)).

Acrolein is more toxic than hydrogen peroxide (Sharmin, Sakata et al. (2001)) and shows a more distinct toxicity than hydroxyl radical 3 (Yoshida, Tomitori et al. (2009)) in vitro. Although human exposure to acrolein may result from food or most importantly tobacco smoking, acrolein can also be generated endogenously from polyamine degradation (Daugas, Cande et al. 2001)). Acrolein is involved in the detrimental effects of smoking (Stevens and Maier (2008)). In the united states cigarette smoking is the most important source of preventable morbidity and mortality (Boyle (1997), McBride (1992)). Besides the risk of smoking as a key-factor for atherosclerosis and cancer, a suggestion has been made by epidemiological studies, that cigarette smoking increases the risk for CKD progression and also accelerates the rate of renal disease progression among patients suffering from diabetes (Orth (2000), Rossing, Hougaard et al. (2002), Stegmayr (1990)) and hypertension (Bleyer, Shemanski et al. (2000), Horner, Fliser et al. (1996), Mimran, Ribstein et al. (1994), Regalado, Yang et al. (2000)). A correlation in clinical studies was found between smoking and development of proteinuria in polycystic kidney disease patients (Chapman, Johnson et al. (1994)), as well a deterioration of renal function in patients with lupus nephritis (Ward and Studenski (1992)), polycystic kidney disease, and glomerulonephritis (Orth, Stockmann et al. (1998), Stengel, Couchoud et al. (2000)).

Acrolein is also believed to be produced from unsaturated fatty acids by ROS (Uchida, Kanematsu et al. (1998)). The more effectively production of acrolein is from degradation of polyamines. Polyamines (Tomitori, Usui et al. (2005), Saiki, Park et al. (2011)) are essential for eukaryotic cell growth (Igarashi and Kashiwagi (2010)). Polyamines have been discovered more than one century ago in seminal fluids. The correct spermatogenesis is dependend onto polyamines. Also, polyamines are important for embryo implantation, in deciduation and the formation and function of placenta (Lefevre, Palin et al. (2011)). In mammalian physiology polyamines are important as signal transducers. Polyamines bind to chromatin and RNAs and also they are essential to keep synthesis of macromolecules and cell survival (Ruiz-Chica, Medina et al. (2001), Medina, Urdiales et al. (2003). Metabolism of polyamines

is very tightly regulated since both, lack and excess of polyamine level can be fatal for cells (Rodriguez-Caso, Montanez et al. (2006)). Acrolein may be generated endogenously from the polyamines spermine and spermidine (Sakata, Kashiwagi et al. 2003, Becker, Reinehr et al. 2007)). Acrolein is spontaneously formed from 3-aminopropanal produced from spermine by spermine oxidase (Cervelli, Polticelli et al. (2003)), and less effectively from 3-acetamidopropanal produced from spermine and spermidine by spermidine/ spermine N1-acetyltransferase and acetylpolyamine oxidase (Sharmin, Sakata et al. (2001), Wang and Casero (2006)).

The pathophysiology of anemia in renal failure has been considered to involve polyamines (Bachmeier, Nerlich et al. 2007, Varela, Simon et al. 2007). The polyamine degradation product acrolein is strongly involved in the harmful effects of polyamines (Sakata, Kashiwagi et al. (2003)). In chronic renal failure the plasma amine oxidase activity and plasma concentration of acrolein are increased, whereas the plasma concentrations of spermidine and spermine are decreased (Sakata, Kashiwagi et al. 2003, Becker, Reinehr et al. 2007). Cigarette smoking is particularly harmful in chronic kidney disease (Rosette and Karin 1996). A part of this study therefore explored, whether eryptosis is triggered by the uremic toxin acrolein (Igarashi, Ueda et al. 2006, Thompson and Burcham 2008)).

1. 6. 2. Indoxyl sulfate:

A subset of toxins derived from microbial metabolism have been associated with progression of CKD and development of CKD-related complications (Evenepoel, Meijers et al. (2009)). These toxins have been suspected to influence progression of renal failure, accelerated cardiovascular disease, and uremic bone disease (Niwa (2010)). Indoxyl sulfate is a protein-bound uremic solute (Niwa (2011)). It is produced by the bacterial metabolism (such as *Escherichia coli*) of metabolizing dietary tryptophan to indole in the colon. Many foods, including turkey, chicken, beef, brown rice, nuts, fish, milk, eggs, cheese, fruit, and vegetables (Niwa (2010), Glorieux and Vanholder (2011), Niwa (2011)) supply the human body with the essential amino acid

tryptophan. After absorption of indole in the gut it is further metabolized in the liver to indoxyl sulfate. The clearance of indoxyl sulfate is normally facilitated by means of renal proximal tubular secretion. However, impaired renal function in CKD patients leads to its accumulation (Schulman (2006), Watanabe, Miyamoto et al. (2011)). The metabolism of indoxyl sulfate is shown in (Fig. 4) (Niwa (2011)). Both the organic anion transporter 1 (OAT1) and (OAT3), are responsible for mediating cellular transport of indoxyl sulfate in the proximal tubules (Enomoto, Takeda et al. (2002), Wikoff, Nagle et al. (2011)). Experimentally, using models of renal failure, OAT1 and OAT3 expression are reduced along with the clearance of indoxyl sulfate, which ultimately leads to the accumulation of indoxyl sulfate (Enomoto, Takeda et al. (2002), Hosoya and Tachikawa (2011)). Also indoxyl sulfate and other toxins can be accumulated due to inhibition of the transport activities of efflux pumps expressed in renal proximal tubule cells (Mutsaers (2010), Mutsaers, van den Heuvel et al. (2011)). As indoxyl sulfate is bound highly to serum albumin, it is improperly removed by hemodialysis (Niwa (2011), Watanabe, Miyamoto et al. (2011), Niwa, Takeda et al. (1988),Niwa (2011)). A clinical study showed that high blood levels of indoxyl sulfate are an independent predictor of cardiovascular disease (Barreto, Barreto et al. (2009)). Indoxyl sulfate has been reported to induce vascular toxicity and nephrotoxicity (Niwa (2010)). Also indoxyl sulfate has been considered as uremic toxin by its accelerating effects on CKD progression (Niwa and Ise (1994)). It may also act as vascular toxin (Dou, Jourde-Chiche et al. (2007), Faure, Dou et al. (2006), Dou, Bertrand et al. (2004)). Barreto et al. confirmed that indoxyl sulfate may be involved in vascular disease and in the high mortality observed in CKD patients (Barreto, Barreto et al. (2009)).

Indoxyl sulfate exerts a variety of effects including a promoting effect on the development of anemia (Duranton, Cohen et al. 2012)), which is at least partially due to the suppression of erythropoietin production (Chiang, Tanaka et al. 2011)). Furthermore, indoxyl sulfate down-regulates Klotho (Niwa and Shimizu 2012)), induces oxidative stress (Lang, Szabo et al. 1999, Niwa and Shimizu 2012)) and up-regulates NFκB (Niwa and Shimizu 2012)). Indoxyl sulfate also is involved in aortic calcification and aortic wall thickening (Niwa

and Shimizu 2012)), interference with wound repair (Rieger, Frank et al. 2007)), triggering of cell senescence (Niwa and Shimizu 2012)), stimulation of cardiac and renal fibrosis (Arese, Turrini et al. 2005, Niwa and Shimizu 2012)) and acceleration of renal disease progression (Niwa and Shimizu 2012)). Moreover, indoxyl sulfate induces apoptosis in nucleated cells (Daniel, Wieder et al. 2001)). To this end, indoxyl sulfate might exert also detrimental effects on erythrocytes and trigger eryptosis.

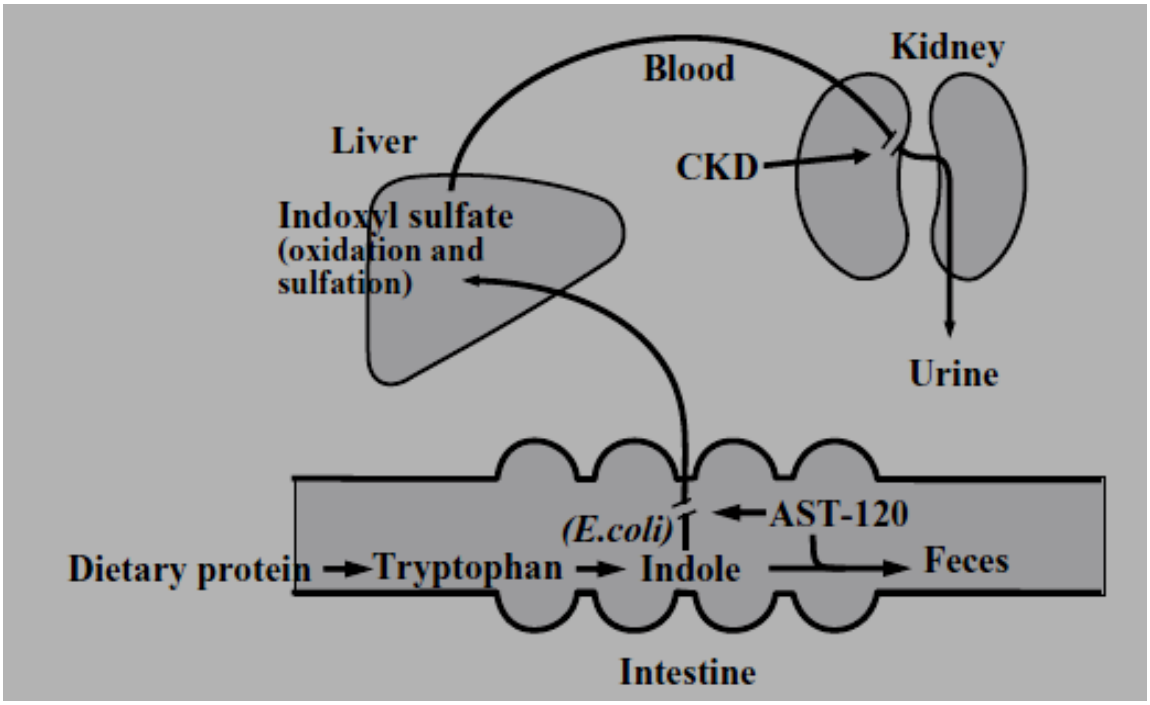

Fig. 4. Metabolism of indoxyl sulfate. Dietary tryptophan metabolizes to indole in the colon by E.Coli. After absorption of indole it is metabolized to indoxyl sulfate in the liver. The excretion is facilitated in the renal proximal tubule, which is impaired in chronic kidney disease. Reducing indole absorption by AST-120 shows beneficial effects in CKD.

CKD, chronic kidney disease; E.coli, Escherichia coli. Modified from (Niwa (2011)).

1. 7. Aim of study:

This study aimed to investigate the effects of the two prominent uremic toxins acrolein and indoxyl sulfate on red blood cells. More specifically, the potential direct effects of acrolein and indoxyl sulfate on the induction of suicidal erythrocyte death (eryptosis) was investigated.

Chapter 2

Materials and methods

2. 1. Erythrocytes, solutions and chemicals:

Leukocyte-depleted erythrocytes were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003V). The volunteers providing erythrocytes gave informed approval.

Isolation of human erythrocytes was performed using the following steps:
1) To prepare a 500 ml erythrocyte concentrate an OptiPure RC quadruple blood pack set with a leukocyte depletion filter was used.
2) 500 ml blood was mixed with 70 ml of CDP-buffer.
3) Centrifugation for 10 minutes at room temperature at 4795g.
4) The blood components were separated and filled in special blood packages.
5) The packaging procedure included addition of SAG-M stabilizing solution to the erythrocytes.
6) Blood was filtered through an integrated leukocyte depletion filter at room temperature.
7) The purified erythrocyte concentrates (30-90%) were stored at 4 °C until experiments were performed.

Erythrocytes were then incubated *in vitro* at a hematocrit of 0.4% in Ringer solution, an isotonic solution which contains several salts dissolved in water. It contains (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$; at pH 7.4. The Ringer solution was filtered using a sterile filter (Millipore, Cork, Ireland). Incubation of erythrocytes was performed at 37°C for 48 h. Where indicated, erythrocytes were exposed to uremic toxins (Acrolein and Indoxylsulfate potassium salt) (Sigma-Aldrich, Steinheim, Germany) at the indicated concentrations. In Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was substituted by 1 mM glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA).

2. 2. FACS analysis of annexin-V-binding and forward scatter:

FACS technique is used for the quantative and qualitive analysis of microscopic particles such as chromosomes or cells. In this study FACS analysis was performed to define several parameters of eryptosis. Primarily, cells-containing fluid is targeted by a laser which then encrypts fluorescence intensity and scatter on an electronic detection apparatus. Four different types of fluorescence channels have been classified based on their emission wavelengths:

1) The FL1 channel with green fluorescence (515-545 nm).
2) The FL2 channel with orange fluorescence (564-606 nm).
3) The FL3 channel with red fluorescence (>670 nm).
4) The FL4 channel with red fluorescence (653-669 nm).

For cell volume determination by the forward scatter (FSC) can be used without requirement of staining. In the present study, FSC was measured simultaneously with annexin V-binding.

After incubation under the respective experimental condition, 50 µl cell suspension was washed in modified Ringer solution (Ringer solution containing 5 mM $CaCl_2$ instead of 1mM $CaCl_2$) and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany). Staining was conducted in this solution at 37°C for 20 min under protection from light. Parallelly, the forward scatter (FSC) of the cells was determined, and annexin-V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

2. 3. Measurement of intracellular Ca^{2+}:

After incubation erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo-3/AM. The cells were incubated at 37°C for 30 min and washed twice in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

2. 4. Measurement of hemolysis:

For the determination of hemolysis the samples were centrifuged (3 min at 400 g, room temperature) after incubation, and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatant was determined photometrically at 405 nm on microplate reader. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis. Accordingly a standard curve (0%, 2.5%, 5%, 7.5%, 10%, 20% and 100%) was made to define the percentage of hemolysis in the samples.

2. 5. Determination of ceramide formation:

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 h at 37°C with 1 µg/ml anti-ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in phosphate buffer saline (PBS) containing 0.1% bovine serum albumin (BSA) at a dilution of 1:5. The samples were washed twice with PBS-BSA. Subsequently, the cells were stained for 30 minutes with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. The samples were then analyzed by flow cytometric analysis in FL-1.

2. 6. Statistics:

Data are expressed as arithmetic means ± SEM. As indicated in the figures, statistical analysis was made using ANOVA and *t* test as appropriate. N denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Chapter 3

Results

3. 1. Acrolein:

As acrolein is increased in renal failure patients, we explored the possibility that acrolein may be capable to trigger eryptosis. A first series of experiments elucidated the potential effect of acrolein on erythrocyte cell volume. To this end, forward scatter as a measure of cell volume was determined utilizing flow cytometry. The experiments were performed after exposing human erythrocytes to 0, 1.5, 15, 30 and 50 µM of Acrolein for 48 hours. As illustrated in Fig. 5, a 48 hours exposure to acrolein was followed by a decrease of forward scatter, an effect reaching statistical significance at 30 µM acrolein concentration. Accordingly, acrolein decreased erythrocyte volume.

Fig.5

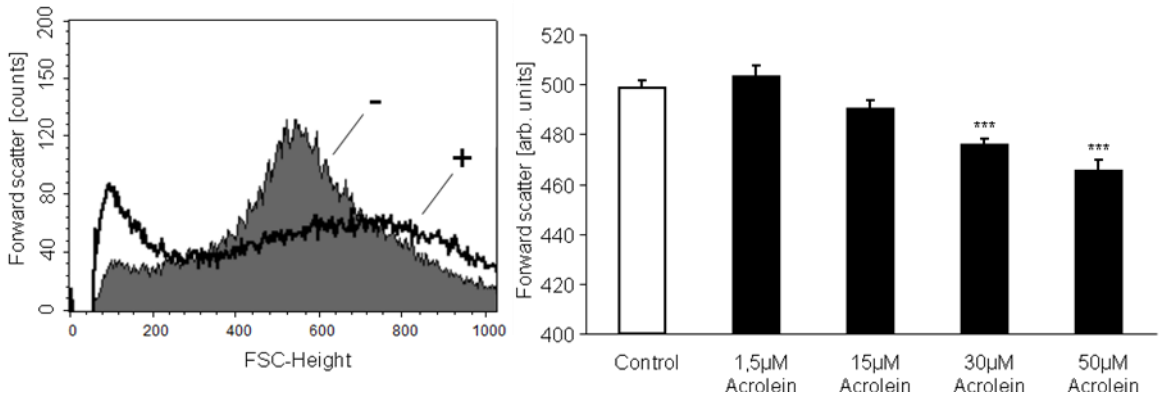

Fig. 5. Effect of acrolein on erythrocyte forward scatter:

A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (-, grey) and with (+, black) presence of 50 µM acrolein.

B. Arithmetic means ± SEM (n = 18 - 19) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) acrolein (1,5 - 50 µM). *** ($p<0.001$) indicate significant difference from the absence of acrolein (ANOVA).

In a second series of experiments cell membrane scrambling was analysed by determination of phosphatidylserine abundance at the cell surface. Phosphatidylserine exposing erythrocytes were identified by annexin-V-binding in FACS analysis. As illustrated in Fig. 6, a 48 h exposure to acrolein dose dependently increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 30 µM acrolein concentration. Accordingly, acrolein exposure was followed by erythrocyte cell membrane scrambling with phosphatidylserine exposure at the cell surface. For comparison, effects of the precursors spermine and spermidine on annexin-V-binding have been tested. The administration of spermine (0, 1.5, 15 and 150 µM) did not significantly modify the percentage of annexin-V-binding erythrocytes (2.18 ± 0.36%, 2.49 ± 0.32%, 2.31 ± 0.49%, and 1.77 ± 0.40%, respectively, n = 5). Moreover, the administration of spermidine (0, 1.5, 15 and 150 µM) did not significantly modify the percentage of annexin-V-binding erythrocytes (2.78 ± 0.42%, 2.30 ± 0.41%, 2.31 ± 0.44%, and 2.05 ± 0.35%, respectively, n = 5).

Further experiments tested, whether acrolein exposure is followed by hemolysis. To this end, the percentage of hemolysed erythrocytes was quantified by determination of hemoglobin release into the supernatant. As illustrated in Fig. 6, exposure of erythrocytes for 48 h to acrolein significantly increased the hemoglobin concentration in the supernatant, an effect, however, affecting only a relatively small percentage of erythrocytes (Fig. 6).

Fig.6

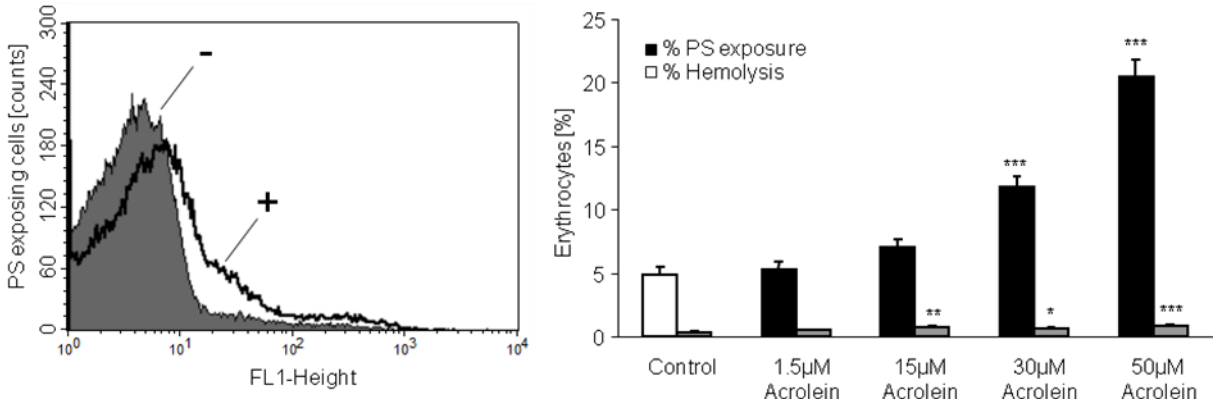

Fig. 6. Effect of acrolein on phosphatidylserine exposure and hemolysis:

A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (-, grey) and with (+, black) presence of 50 µM acrolein.

B. Arithmetic means ± SEM (n = 18 - 19) of erythrocyte annexin-V-binding following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of acrolein (1.5 - 50 µM). For comparison, arithmetic means ± SEM (n = 6) of the percentage of hemolysis is shown as grey bars. *,**,*** ($p<0.05$, 0.01, 0.001) indicates significant difference from the absence of acrolein for the respective measurements (ANOVA).

Both, cell shrinkage and cell membrane scrambling could have resulted from an increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). Thus, further experiments were performed to elucidate whether acrolein increases $[Ca^{2+}]_i$. To this end, erythrocytes were exposed to Ringer solution without or with added acrolein (1.5 - 50 µM) for 48 hours. In the following the erythrocytes were loaded with Fluo3-AM and Fluo3 fluorescence determined in FACS analysis. As illustrated in Fig. 7, following a 48 hours exposure of human erythrocytes up to 50 µM acrolein remained without significant effect on Fluo3 fluorescence. Accordingly, at the concentrations tested, acrolein did not significantly increase cytosolic Ca^{2+} concentration.

Fig.7

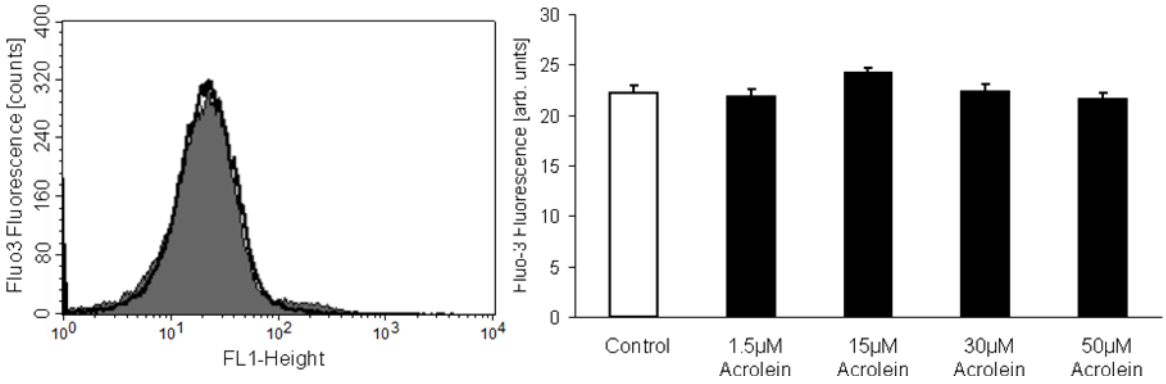

Fig. 7. Effect of acrolein on erythrocyte cytosolic Ca^{2+} concentration:

A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (grey) and with (black) presence of 50 µM acrolein.

B. Arithmetic means ± SEM (n = 18-19) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) acrolein (1.5 - 50 µM).

To further elucidate the potential role of $[Ca^{2+}]_i$, erythrocytes were exposed to 50 µM acrolein for 48 hours either in the presence of extracellular Ca^{2+} (1 mM) or in the nominal absence of Ca^{2+} and presence of the Ca^{2+} chelator EGTA (1 mM). As illustrated in Fig. 8, the effect of acrolein on annexin-V-binding was significantly decreased in the nominal absence of extracellular Ca^{2+}. However, even in the absence of extracellular Ca^{2+}, acrolein still significantly increased the percentage of annexin V binding erythrocytes. Thus, the effect of acrolein was mainly, but not exclusively, dependent on Ca^{2+}.

Fig.8

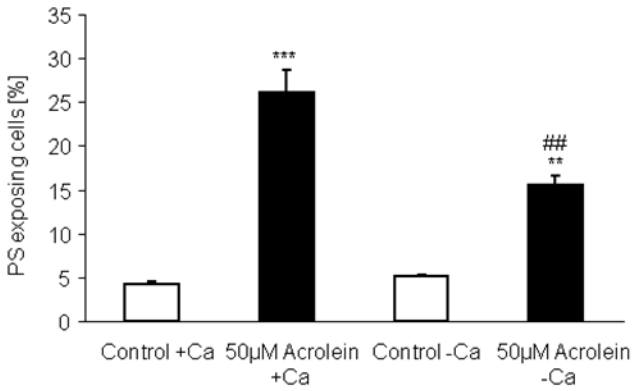

Fig. 8. Effect of Ca^{2+} withdrawal on acrolein- induced annexin-V-binding:

Arithmetic means ± SEM (n = 4) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 50 µM acrolein in the presence (left bars, + Ca) and absence (right bars, - Ca) of calcium. **,*** ($p<0.01$, 0.001) indicates significant difference from respective control (absence of acrolein) (ANOVA) ## ($p<0.01$) indicates significant difference from the respective values in the presence of Ca^{2+}.

In view of the Ca^{2+} sensitivity of acrolein-induced eryptosis and the absence of an increase of $[Ca^{2+}]_i$ following acrolein exposure, additional experiments were performed to test whether acrolein increases the Ca^{2+} sensitivity of cell membrane scrambling. To this end erythrocytes were exposed to the Ca^{2+} ionophore ionomycin (1 µM) in the absence and the presence of acrolein (50 µM). As illustrated in Fig. 9, exposure of erythrocytes to the Ca^{2+} ionophore ionomycin (1 µM) was followed by a marked increase of the percentage phosphatidylserine exposing erythrocytes, an effect significantly more pronounced in the presence than in the absence of acrolein (50 µM). Accordingly, acrolein augments the cell membrane scrambling effect of cytosolic Ca^{2+}.

Fig.9

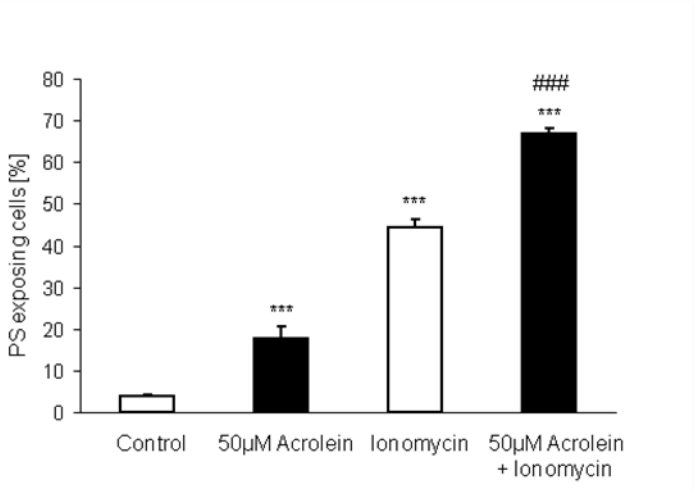

Fig. 9. Effect of Ca^{2+} ionophore ionomycin on annexin-V-binding in absence and presence of acrolein:

Arithmetic means ± SEM (n = 5) of the percentage of annexin-V-binding erythrocytes after a a 48 h pretreatment with Ringer solution without (white bar) or with (black bars) 50 µM acrolein followed by a 30 minutes treatment in the absence (left bars, -Iono) and presence (right bars, +Iono) of the Ca^{2+} ionophore ionomycin (1 µM). *** ($p<0.001$) indicates significant difference from control (absence of acrolein and ionomycin) (ANOVA), ### ($p<0.001$) indicates significant difference from the ionomycin treated erythrocytes in the absence of acrolein.

As ceramide is known to enhance the sensitivity of cell membrane scrambling to cytosolic Ca^{2+}, a further series of experiments was performed to define the effect of acrolein on formation of ceramide. Ceramide abundance at the cell surface was elucidated utilizing FITC-labeled anti-ceramide antibodies. As shown in Fig. 10, a 48 hours exposure of erythrocytes to 50µM acrolein significantly increased ceramide-dependent fluorescence.

Fig.10

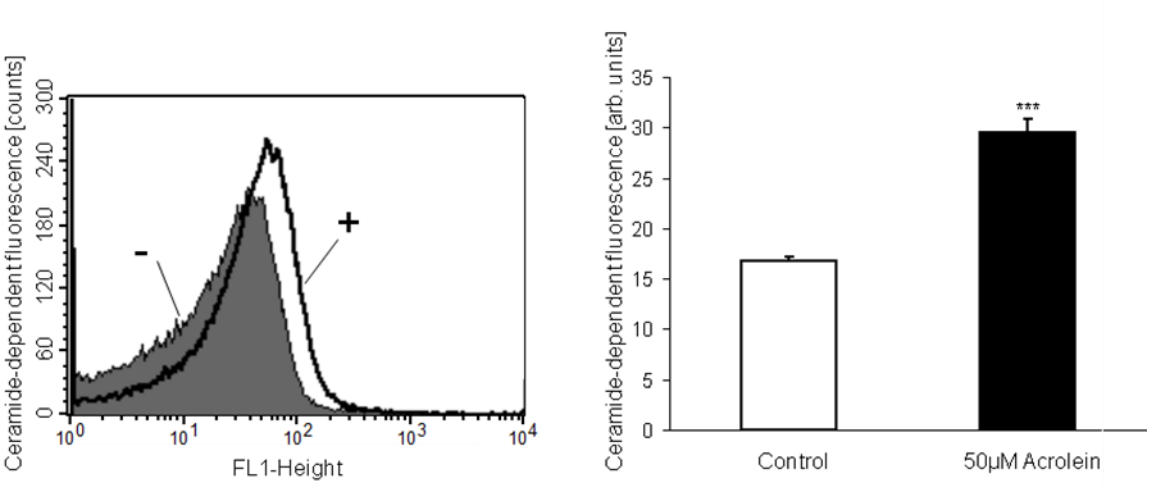

Fig. 10. Effect of acrolein on ceramide formation:

A. Original histogram of anti-ceramide FITC-fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-, grey) and with (+, black) presence of 50 µM acrolein.

B. Arithmetic means ± SEM (n = 6) of ceramide abundance after a 48 h incubation in Ringer solution without (white bar) or with (black bars) acrolein (50 µM). *** (p <0.001) indicates significant difference from control (absence of acrolein) (t test).

3. 2. Indoxyl sulfate:

Indoxyl sulfate is increased in patients with chronic kidney disease, and is furthermore known to accelerate the progression of renal disease in the CKD patients (Niwa and Ise (1994)). We therefore hypothesized, that indoxyl sulfate is capable to trigger eryptosis and could contribute to increased eryptosis in CKD. To this end, we incubated erythrocytes with concentrations of 0-600µM indoxyl sulfate. A first series of experiments elucidated the potential effect of indoxyl sulfate on cell volume. To this end, forward scatter as a measure of cell volume was determined utilizing flow cytometry. As illustrated in Fig. 11, a 48 hours exposure to indoxyl sulfate was followed by a decrease of forward scatter, an effect reaching statistical significance at 300 µM indoxyl sulfate concentration. Accordingly, indoxyl sulfate decreased erythrocyte volume.

Fig. 11

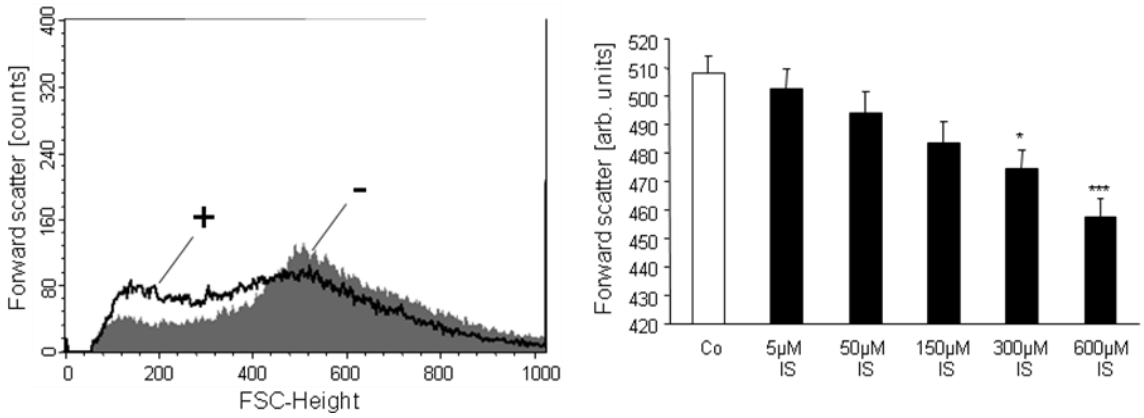

Fig. 11. Effect of indoxyl sulfate on erythrocyte forward scatter:

A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (-) and with (+) presence of 600 µM indoxyl sulfate.

B. Arithmetic means ± SEM (n = 24-25) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) indoxyl sulfate (5 - 600 µM). *** ($p<0.001$) indicate significant difference from the absence of indoxyl sulfate (ANOVA).

In a second series of experiments cell membrane scrambling was analyzed by determination of phosphatidylserine abundance at the cell surface. Phosphatidylserine exposing erythrocytes were identified by annexin-V-binding in FACS analysis. As illustrated in Fig. 12, a 48 h exposure to indoxyl sulfate dose dependently increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 50 µM indoxyl sulfate concentration. Accordingly, indoxyl sulfate exposure was followed by erythrocyte cell membrane scrambling with phosphatidylserine exposure at the cell surface.

Further experiments tested, whether indoxyl sulfate exposure is followed by hemolysis. To this end, the percentage of hemolysed erythrocytes was quantified by determination of hemoglobin release into the supernatant. As illustrated in Fig. 12, exposure of erythrocytes for 48 h to indoxyl sulfate, despite the induction of cell membrane scrambling, did not significantly modify the hemoglobin concentration in the supernatant. (Fig. 12).

Fig.12

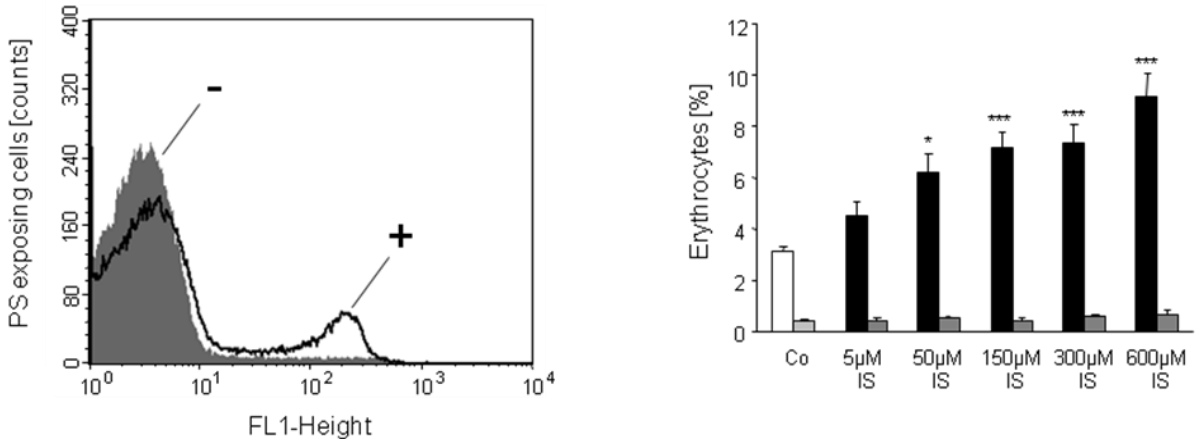

Fig. 12. Effect of indoxyl sulfate on phosphatidylserine exposure and hemolysis:

A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (-) and with (+) presence of 600 µM indoxyl sulfate.

B. Arithmetic means ± SEM (n = 24 - 25) of erythrocyte annexin-V-binding following incubation for 48h to Ringer solution without (white bar) or with (black bars) presence of indoxyl sulfate (5 - 600 µM). For comparison, arithmetic means ± SEM (n = 5) of the percentage of hemolysis is shown as grey bars. *,**,*** ($p<0.05$, 0.01, 0.001) indicates significant difference from the absence of indoxyl sulfate for the respective measurements (ANOVA).

Both, cell shrinkage and cell membrane scrambling could have resulted from an increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). Thus, further experiments were performed to elucidate whether indoxyl sulfate increases $[Ca^{2+}]_i$. To this end, erythrocytes were exposed to Ringer solution without or with added indoxyl sulfate (5 - 600 µM). In the following the erythrocytes were loaded with Fluo3-AM and Fluo3 fluorescence determined in FACS analysis. As illustrated in Fig. 13, following a 48 h exposure of human erythrocytes with 300 µM indoxyl sulfate significantly increased Fluo3 fluorescence. Accordingly, indoxyl sulfate did increase cytosolic Ca^{2+} concentration.

Fig.13

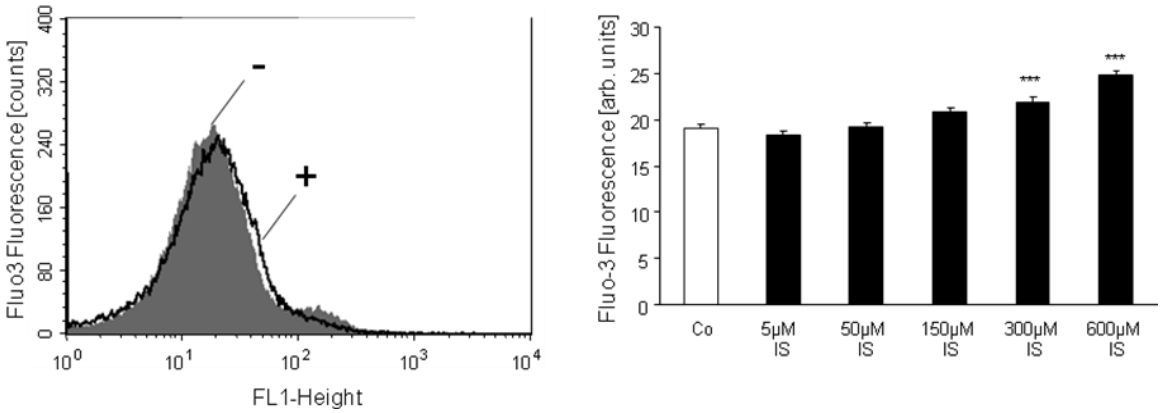

Fig. 13. Effect of indoxyl sulfate on erythrocyte cytosolic Ca^{2+} concentration:

A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-) and with (+) presence of 600 µM indoxyl sulfate.

B. Arithmetic means ± SEM (n = 20) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) indoxyl sulfate (5 - 600 µM). *** ($p<0.001$) indicates significant difference from the absence of indoxyl sulfate for the respective measurements (ANOVA).

To further elucidate the potential role of $[Ca^{2+}]_i$, erythrocytes were exposed to 150 µM indoxyl sulfate for 48 h either in the presence of extracellular Ca^{2+} (1 mM) or in the nominal absence of Ca^{2+} and presence of the Ca^{2+} chelator EGTA (1 mM). As illustrated in Fig. 14, the effect of indoxyl sulfate on annexin-V-binding was significantly decreased in the nominal absence of extracellular Ca^{2+}. Thus, the effect of indoxyl sulfate to induce eryptosis required the presence of Ca^{2+}.

Fig. 14

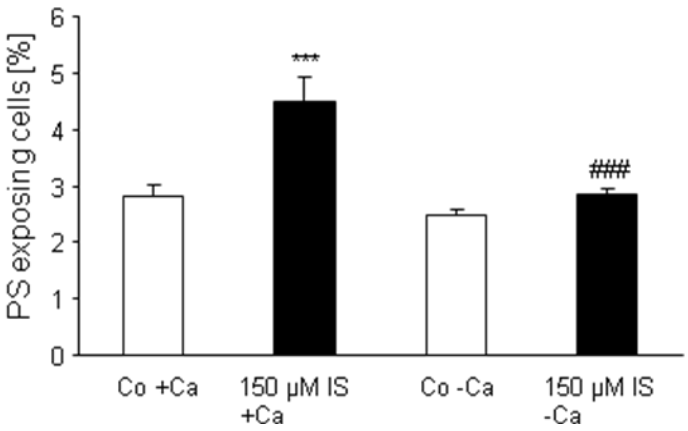

Fig. 14. Effect of Ca^{2+} withdrawal on indoxyl sufate- induced annexin-V-binding: Arithmetic means ± SEM (n = 10) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 150 µM indoxyl sulfate in the presence (left bars, + Ca) and absence (right bars, - Ca) of calcium. *** (<0.001) indicates significant difference from respective control (absence of indoxyl sulfate) (ANOVA) ### (p<0.001) indicates significant difference from the respective values in the presence of Ca^{2+}.

Ceramide is known to enhance the sensitivity of cell membrane scrambling to cytosolic Ca^{2+} and is a key factor in sucidal cell death. A further series of experiments was performed to define the effect of indoxyl sulfate on formation of ceramide. Ceramide abundance at the cell surface was elucidated utilizing FITC-labeled anti-ceramide antibodies. As shown in Fig. 15, a 48 hours exposure of erythrocytes to 150µM indoxyl sulfate significantly increased ceramide-dependent fluorescence.

Fig. 15

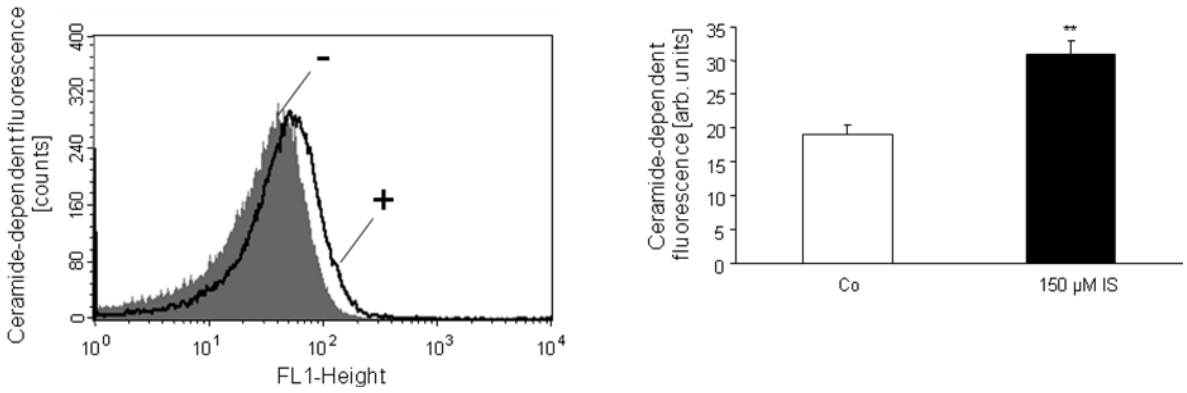

Fig. 15. Effect of inoxyl sulfate on ceramide formation:

A. Original histogram of anti-ceramide FITC-fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-, grey) and with (+, black) presence of 150 µM indoxyl sulfate.

B. Arithmetic means ± SEM (n = 5) of ceramide abundance after a 48 h incubation in Ringer solution without (white bar) or with (black bars) indoxyl sulfate (150 µM). ** (p <0.01) indicates significant difference from control (absence of indoxyl sulfate) (t test).

Chapter 4
Discussion

4.1 Eryptosis in CKD associated anemia:

The complications of chronic kidney disease are of crucial importance, as these complictations are decisive for the survival of the patients**.** The early onset of common diseases cancer, infection and cardiovascular diseases is the real cause of death in CKD patients, while these complications develop based on the failure of the kidney (Whaley-Connell, Bomback et al. 2011), Kestenbaum, Sampson et al. (2005)). A major complication of CKD is the CKD associated anemia.

Approximately fifty percent of chronic kidney disease patients suffer from anemia (McClellan, Aronoff et al. (2004)). Advanced CKD is normally escorted with a normochromic, normocytic anemia (Besarab and Levin (2000). The prevalence of anemia increases when the renal function declines (McClellan, Aronoff et al. (2004)). CKD associated anemia develops by multiple mechanisms, the anemia in CKD results at least partly from the reduction of erythropoietin formation in the kidney, iron deficiency and secondary due to shortage lifespan of erythrocytes (Tsagalis (2011)). CKD associated anemia could be treated by recombinant erythropoietin, but the uncritical use of EPO has been questioned due to possible adverse effects (de Francisco and Pinera (2011)).

Eryptosis is observed in many diseases which are associated with anemia (e.g. sepsis (Kempe, Akel et al. (2007)), malaria (Brand, Sandu et al. 2003)), iron deficiency (Kempe, Lang et al. 2006), Wilson's disease (Lang, Schenck et al. (2007)), and haemolytic uremic syndrome (Lang, Beringer et al. (2006)). A wide range of factors may trigger eryptosis (Foller, Huber et al. (2008). Theoretically, anemia in renal insufficiency could results from accelerated death of circulating erythrocytes. Erythrocytes may undergo apoptosis-like suicidal death or eryptosis, which is characterized by cell membrane scrambling (Nguyen, Wagner-Britz et al. 2011, Lang, Qadri et al. 2012)). At least in theory, the stimulation of eryptosis could contribute to the accelerated erythrocyte death in chronic renal failure. Phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood (Lang, Qadri et al. 2012, Lang and Qadri 2012)). In renal insufficiency, the accelerated loss of erythrocytes cannot be fully

compensated by enhanced formation of new erythrocytes and anemia develops (Lang, Qadri et al. 2012)). The percentage of phosphatidylserine exposing erythrocytes is enhanced in chronic renal failure and klotho hypomorphic mice (Kempe, Ackermann et al. (2009), (Myssina, Huber et al. 2003)). Eryptosis may therefore be a contributing factor to CKD associated anemia. Little is known, however, about mechanisms stimulating eryptosis in end stage renal disease. Theoretical, some uremic toxins may stimulate eryptosis. Eryptosis is known to be stimulated by vanadate (Foller, Sopjani et al. 2008)) and methylglyoxal (Lang, Qadri et al. 2012)), both substances increased in uremic plasma (Foller, Sopjani et al. 2008, Lang, Qadri et al. 2012)). We show here, that the two uremic toxins acrolein and indoxyl sulfate, which are known for adverse effects in CKD patients, may cause eryptosis.

4. 2. Acrolein:

Acrolein is formed in renal failure due to increased spermine degradation by serum amine oxidase (Becker, Reinehr et al. 2007). Acrolein is increased in end stage renal disease and partially removed by hemodialysis (Konstantinidis, Koliakos et al. 2006). Increased acrolein levels are also observed in nephropathy models (Chen, Wang et al. 2007). In a rat model of CKD, acrolein was lowered by an oral charcoal absorbent, a treatment associated with beneficial cardiovascular effects (Shimizu, Wehner et al. 2006).

We could disclose a novel effect of acrolein, i.e. the stimulation of erythrocyte cell membrane scrambling, a typical feature of suicidal death or eryptosis. The concentrations of acrolein required for statistically significant stimulation of cell membrane scrambling (30 µM) are similar to those (25 -100 µM) previously shown to trigger death of nucleated cells (Liu-Snyder, McNally et al. 2006, Wang, Sun et al. 2011)). In nucleated cells acrolein can induce both, necrotic and apoptotic cell death (Boas, Forman et al. 1998, Daugas, Cande et al. 2001, Liu-Snyder, McNally et al. 2006, Wang, Sun et al. 2011)). According to the present observations the extent of hemolysis is clearly smaller than the percentage of cell membrane scrambling suggesting that the erythrocytes die from

eryptosis rather than hemolysis. Nevertheless, the effect of acrolein on forward scatter is mininal contrasting the marked decrease of forward scatter following stimulation for eryptosis by other small molecules (Lang, Qadri et al. 2012, Lang and Qadri 2012)).

The weak effect of acrolein on forward scatter may be related to the lack of effect on cytosolic Ca^{2+} activity. Eryptotic erythrocyte shrinkage results from activation of Ca^{2+} sensitive K^+ channels (Lang, Qadri et al. 2012)) with subsequent K^+ exit, cell membrane hyperpolarisation, Cl^- exit and thus cellular loss of KCl with osmotically obliged water (Lang, Kaiser et al. 2003)). Activation of those channels is probably weak.

The acrolein induced cell membrane scrambling is thus not the result of increased cytosolic Ca^{2+} activity. Nevertheless, the presence of extracellular Ca^{2+} is required for full stimulation of cell membrane scrambling. Accordingly, acrolein is effective by increasing the Ca^{2+}sensitivity of cell membrane scrambling. Along those lines, acrolein increases the scrambling effect of the Ca^{2+} ionophore ionomycin.

As observed earlier (Lang, Qadri et al. 2012)), the Ca^{2+} sensitivity of the erythrocyte cell membrane scrambling is enhanced by ceramide. Acrolein treatment indeed resulted in an increase of ceramide formation. Thus, acrolein triggers cell membrane scrambling at least in part by increasing the formation of ceramide, which in turn increases the Ca^{2+} sensitivity of cell membrane scrambling. Besides its effect on erythrocyte cell membrane scrambling (Lang, Qadri et al. 2012)), ceramide is known to stimulate apoptosis of nucleated cells (Morad and Cabot 2013)). To the best of our knowledge, an effect of acrolein on ceramide formation has never been shown previously.

4. 3. Indoxyl sulfate:

In addition, the uremic toxin indoxyl sulfate has been examined to uncover a novel effect of indoxyl sulfate, i.e. the stimulation of erythrocyte cell membrane scrambling, a typical feature of suicidal death or eryptosis. The concentrations of indoxyl sulfate required for statistically significant stimulation of eryptosis is in the

range of those encountered in uremic plasma (Barreto, Barreto et al. (2009), Duranton, Cohen et al. (2012)).

According to the present observations, no profound effect of indoxyl on hemolysis was observed. The extent of cell membrane scrambling suggesting that the erythrocytes die from eryptosis rather than hemolysis. Nevertheless, similar to the effects of acrolein, the effect of indoxyl sulfate on forward scatter is minimal contrasting the marked decrease of forward scatter following stimulation of eryptosis by other small molecules (Lang, Qadri et al. 2012, Lang and Qadri 2012)).

The observed erythrocyte shrinkage following indoxyl sulfate treatment presumably resulted from increase of cytosolic Ca^{2+} concentration with subsequent activation of Ca^{2+} sensitive K^+ channels (Bookchin, Ortiz et al. 1987, Brugnara, de Franceschi et al. 1993)), K^+ exit, hyperpolarization, Cl^- exit, cell membrane hyperpolarisation, Cl^- exit and thus cellular loss of KCl with osmotically obliged water (Lang, Kaiser et al. 2003)).

The observed indoxyl sulfate induced cell membrane scrambling was similarly due to increased cytosolic Ca^{2+} activity. Accordingly, the presence of extracellular Ca^{2+} is required for full stimulation of cell membrane scrambling. Nonetheless, indoxyl sulfate also increased ceramide formation in erythrocytes and could therefore sensitize towards the efefcts of Ca^{2+}.

Indoxyl sulfate can cause anemia (Duranton, Cohen et al. 2012), which is at least partially caused by suppression of erythropoietin production (Chiang, Tanaka et al. 2011)). Our results showed an induction of eryptosis after indoxyl sulfate exposure. To this end, indoxyl sulfate may augment the anemia in renal insufficiency, by the interaction with suppression of erythropoietin production together with trigger of eryptosis (Chiang, Tanaka et al. 2011). Further effects of indoxyl sulfate include downregulation of Klotho (Niwa and Shimizu 2012)), which may play a role in chronic kidney disase. Also indoxyl sulfate accelerates the renal disease progression (Niwa and Shimizu 2012)). Indoxyl sulfate is generated by colonic microbes (Yu, Canzoniero et al. 2001)) and accumulates

in blood, if renal excretion is impaired (Niwa and Shimizu 2012)). Moreover, indoxyl sulfate induces apoptosis, the suicidal death of nucleated cells, an effect involving activation of ERK1/2 and p38 MAP kinase (MAPK) (Daniel, Wieder et al. 2001)). In accordance to these observations, Indoxyl sulfate stimulates eryptosis.

4.4. Clinical implications:

Theoretically, the stimulation of eryptosis by indoxyl sulfate could be synergetic with to acrolein, which in turn could contribute to the accelerated erythrocyte death in chronic renal failure. Both acrolein and indoxyl sulfate, are not the only substances stimulating eryptosis in renal insufficiency. Further substances have been reported to stimulate eryptosis and to be enhanced in renal insufficiency, which are vanadate (Foller, Sopjani et al. 2008)) and methylglyoxal (Lang, Qadri et al. 2012)). Along those lines, iron deficiency, which contributes to anemia in chronic renal failure (Besarab and Coyne 2010, Attanasio, Ronco et al. 2012)) is a known trigger of eryptosis (Kempe, Lang et al. 2006)). It can be hypothesized, that additional uremic toxins will be identified, which stimulate eryptosis and thus contribute to the anemia of patients with end stage renal disease.

These results suggest, that several effects coincide in chronic kidney disease, which facilitates an environment which could promote eryptosis in these patients. It still remains unclear though, how much the eryptotic death of erythrocytes contributes to the anemia in chronic kidney disease. As CKD patients require treatment with relatively high doses of EPO to adjust the hematocrit levels and to avoid the need for blood transfusions (Besarab, Bolton et al. (1998), Eschbach, Abdulhadi et al. (1989), Singh, Szczech et al. (2006)), a contribution of eryptosis to the anemia might be possible. Further studies are required to address this question. Nonetheless, blocking eryptosis might be a possible treatment option for anemia in kidney disease.

Phosphatidylserine exposing erythrocytes may adhere to endothelial CXCL16/SR-PSO of the vascular wall (Borst, Abed et al. 2012) thus

compromising microcirculation. Accordingly, the adhering erythrocytes may interfere with blood flow (Wood, Gibson et al. 1996, Andrews and Low 1999, Closse, Dachary-Prigent et al. 1999, Gallagher, Chang et al. 2003, Pandolfi, Di Pietro et al. 2007, Borst, Abed et al. 2012)). Klotho hypomorphic mice resemble partly the complications of chronic kidney disease and show increased eryptosis (Kempe, Ackermann et al. (2009)). Similarily, erthrocytes from Klotho deficient mice show increased adhesion to endothelial cells in-vitro (Abed, Towhid et al. (2013)). Moreover, phosphatidylserine exposing erythrocytes may stimulate blood clotting and thus trigger thrombosis (Andrews and Low 1999, Zwaal, Comfurius et al. 2005, Chung, Bae et al. 2007)). The attempt to fully compensate the accelerated loss of erythrocytes by eryptosis may result in a high turnover of erythrocytes with increased numbers of phosphatidylserine exposing erythrocytes in circulating blood. Accordingly, uncritical use of erythropoietin or other erythropoiesis stimulating agents (Singh 2010)) may jeopardize microcirculation.

4.5. Conclusion:

As conclusion of these studies, the uremic toxins acrolein and indoxyl sulfate trigger cell membrane scrambling and cell shrinkage and thus suicidal death of human erythrocytes. The effect can be partly attributed to increased cytosolic Ca^{2+} by indoxyl sulfate and to enhanced formation of ceramide with subsequent sensitization of cell membrane scrambling to cytosolic Ca^{2+}. The uremic toxins acrolein and indoxyl sulfate might be a contributing factor to enhanced eryptosis in renal failure patients and possibly foster CKD associated anemia.

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