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ROCK Inhibition – A New Therapeutic Avenue in Kidney Protection

Stefan Reuter, Dominik Kentrup and Eckhart Büssemaker
Department of Medicine D, University of Münster, Münster
Germany

1. Introduction

Chronic allograft nephropathy (CAN) remains the main cause of renal transplant loss besides the death of patients with a functioning graft. Its prevention and treatment still lacks any significant breakthrough since many years (Meier-Kriesche *et al.* 2004; Pascual *et al.* 2002). Pathological manifestations of CAN include interstitial fibrosis, tubular atrophy, vascular occlusive changes, glomerulosclerosis and a progressive renal dysfunction accompanied by hypertension and proteinuria (Joosten *et al.* 2005; Racusen *et al.* 1999). This histopathologic constellation is now more formally and descriptively referred to as interstitial fibrosis and tubular atrophy (IF/TA) without evidence of any specific aetiology (Solez *et al.* 2007). IF/TA or CAN describe the common final path of different injuries causing renal damage, whereas their precise pathogenesis is complex and only incompletely understood (Joosten *et al.* 2005). The process encompasses multifactorial aetiologies including alloantigen-dependent as well as alloantigen-independent factors (Gottmann *et al.* 2003; Joosten *et al.* 2005; Kerjaschki *et al.* 2006; Nankivell *et al.* 2003; Reuter *et al.* 2010; Tullius & Tilney, 1995). Hence, an effective treatment is not available so far.

Rho effectors Rho-associated, coiled-coil containing protein kinases (ROCK) and their associated signaling pathways have emerged as important players in cardiovascular and renal pathophysiology. Recently, it has been shown that ROCK inhibition is protective in diabetic and obstructive nephropathy, hypertensive nephrosclerosis, ischemia reperfusion injury, and chronic allograft nephropathy (Kanda *et al.* 2003a; Kentrup D. *et al.* 10 A.D.; Kentrup D. *et al.* 2010; Komers *et al.* 2011a; Komers *et al.* 2011b; Liu *et al.* 2009; Nagatoya *et al.* 2002; Nishikimi *et al.* 2004a; Satoh *et al.* 2002; Song *et al.* 2008; Versteilen *et al.* 2011).

ROCKs have been initially identified as downstream targets of the small GTP binding protein Rho. Members of the Rho family include Rho (isoforms A–E, and G), Rac (isoforms 1 and 2), Cdc42 and TC10 (Nobes & Hall, 1994). After translocation to the plasma membrane, GTP-RhoA activates its effectors, including the two isoforms of ROCK, ROCK1 (ROKβ, p160ROCK) and ROCK2 (ROKα, Rho kinase) (Nakagawa *et al.* 1996). Although, ROCK1 and 2 can be differentially regulated under distinct circumstances there is no evidence that ROCK1 and ROCK2 have different functions at present (Nobes & Hall, 1994). ROCKs are protein serine/threonine kinases belonging to the AGC (PKA/PKG/PKC) family (Ishizaki *et al.* 1996; Leung *et al.* 1995; Matsui *et al.* 1996). ROCK activity is involved in actin cytoskeletal organization, stress fiber formation, and cell contraction thereby controlling vascular smooth muscle contraction, endothelial barrier and leukocyte functions (e.g., cellular

motility, migration, adhesion and transmigration) (Loirand, Guerin & Pacaud, 2006; Riento & Ridley, 2003). Both isoforms of ROCK can be the target of effective inhibitors such as TAT-C3, HMG-CoA reductase inhibitors, mTOR inhibitors, angiotensin II antagonists, Rad GTPase, dominant-negative ROCK, and the specific ROCK inhibitors fasudil, hydroxyfasudil, and Y-27632 (Liao, Seto & Noma, 2007; Oka *et al.* 2008). We herein discuss favourable effects of ROCK inhibitors in kidney transplantation-related diseases, and highlight their potential impact on novel therapeutic strategies to improve long-term renal graft survival.

1.1 Rock Inhibition in Diabetic nephropathy

Diabetic nephropathy (DN) is the most common cause of chronic kidney disease (CKD) with a considerable risk of progression to end stage renal disease (ESRD) (Zimmet, Alberti & Shaw, 2001). Thus, it is not surprising that DN is the main cause of patients entering permanent renal replacement programs (dialysis/transplantation) worldwide (Rugg, 2003). When dialysis is initiated, the survival of patients with DN is inferior when compared with other renal diseases. Despite an inferior survival of diabetics, which is primarily due to cardiovascular disease generally present prior to transplantation, kidney transplantation has been established as the renal replacement therapy of choice for these patients (Cosio *et al.* 2008). Because longer waiting times on dialysis negatively impact post-transplant graft survival (Meier-Kriesche *et al.* 2000) and successful transplantation was shown to confess a substantial survival benefit to patients with diabetic-ESRD, transplantation should be performed early (Becker *et al.* 2006; Hirschl, 1996; Son *et al.* 2010; Wolfe *et al.* 1999). As diabetes persists (or occurs, e.g. post-transplant diabetes (Rodrigo *et al.* 2006)) after renal transplantation it contributes to delayed graft function and long-term (re-)graft loss (Arnol *et al.* 2008; Fellstrom *et al.* 2005; Khalkhali *et al.* 2010; Parekh, Bostrom & Feng, 2010; Wilkinson *et al.* 2005). Interestingly, in a study by Wiesbauer et al. maximal glucose, HbA1c, or diabetes treatment did not influence death-censored functional graft survival but mortality (Wiesbauer *et al.* 2010). However, there is evidence that DN can re-occur after transplantation and that reversal or stabilization of the course of DN (still difficult to achieve) may slower the progress to ESRD again (Bhalla *et al.* 2003; Osterby *et al.* 1991; Salifu *et al.* 2004; Wojciechowski, Onozato & Gonin, 2009).

What happens in DN? The mechanistic driving force of DN still remains undetermined. Only 30-50% of people with diabetes develop overt nephropathy over a lifetime. This suggests that other factors besides diabetes are required to share in for the progression of DN (Nakagawa *et al.* 2011; Rugg, 2003). One important parameter for the development of DN identified is glomerular hypertension due to (intra renal) vascular alterations (Johnston *et al.* 1998). Vascular damage of the glomerular capillaries causes leakage of albumin and other proteins across the filter into the urine. It was postulated that the degree of DN correlates to the amount of urinary albumin excretion. However, others have found that DN is an independent risk factor for the development of microalbuminuria (Chang *et al.* 2011a). In contrast, progressive renal failure in diabetics can also occur in the absence of proteinuria even though histology present with typical signs of DN (Caramori, Fioretto & Mauer, 2003). Thus, it was questioned whether the decline of renal function is linked to proteinuria or whether both simply appear in parallel (Perkins *et al.* 2007) (reviewed in (Jefferson, Shankland & Pichler, 2008)). Because classical cardiovascular risk factors like hyperglycemia, hypertension, and hyperlipidemia are well-described to promote DN it would stand to reason that mechanisms causing vascular damage, such as endothelial dysfunction, oxidative stress, advanced glycation end products

(AGEs), and angiogenesis, are involved in the pathogenesis of DN (Brownlee, 2001; Chang *et al.* 2011b; Jansson, 2007). It was suggested that hyperglycemia accelerates the polyol and the hexosamine pathway, activates protein kinase C (PKC), and induces nonenzymatic glycosylation AGEs (Brownlee, 2001; Schena & Gesualdo, 2005). Especially AGEs lead to fibrosis via accumulation of interstitial collagens. AGEs also induce oxidative stress which activates NF-kappa b. NF-kappa b and PKC-activation are associated with the release of (proinflammatory) cytokines and growth factors such as vascular endothelial growth factor (VEGF), tumor necrosis factor a (TNFa), fibroblast growth factor (FGF), tissue factor, transforming growth factor-beta (TGF-ß), Interleukin 1 (IL-1), IL-6 and IL-18 (Brownlee, 2001; Johnston *et al.* 1998). PKC activation is also related to hemodynamic changes (predominantly through the activation of the renin-angiotensin aldosterone system (RAAS) which in term promotes hypertension, oxidative stress, and fibrosis.

Therefore, current renoprotective treatment strategies for DN are rather classical and include the control of blood glucose, blood pressure, lipids (notably, many cholesterol-independent or "pleiotropic" effects of statins are mediated by ROCK inhibition (Liao, 2007)) and body weight as well as RAAS blockade and physical training (Alicic & Tuttle, 2010; Van Buren & Toto, 2011). The interest emerged on ROCK inhibitors when it was observed that Rho/ROCK play important roles in hypertension/cardiovascular system and in the kidney in models of diabetes (Arita *et al.* 2009; Gojo *et al.* 2007; Kawamura *et al.* 2004; Kolavennu *et al.* 2008; Miao *et al.* 2002; Peng *et al.* 2008; Rikitake & Liao, 2005). Besides hyperglycemia, further factors of the diabetic milieu, such as reactive oxygen species (ROS), oxidized LDL, acceleration of the hexosamine pathway, and AGEs, can activate the Rho/ROCK pathway in vascular and renal cells (Komers, 2011). Interestingly, among other actions AGEs can increase endothelial permeability/hyperpermeability of vessels through the RAGE/Rho signaling pathway which can be inhibited by application of Y-27632 (Hirose *et al.* 2010). Moreover, Rho/ROCK have been identified as key mediators of VEGF-induced endothelial cell hyperpermeability (Zeng *et al.* 2005). As mentioned above, VEGF expression is increased in response to hyperglycemia. Because albuminuria, due to glomerular leakage, is a common feature of DN, ROCK activity might be a mechanism involved in renal proteinuria in these patients. In addition, the Rho/ROCK pathway can be activated by hormones or cytokines involved in DN pathophysiology, like AngII, aldosterone, TGF-ß, and VEGF (Komers, 2011). It was stated that this activation of ROCK-dependent pathways, e.g. due to production of osteopontin, plasminogen activator inhibitor 1, or extracellular matrix, is involved in the pathogenesis of DN. Cell culture studies by Peng et al. and Kolavennu et al. supported these findings. They observed that the activity of Rho/ROCK in mesangial cells increased by glucose treatment. This caused reorganization of cytoskeleton and increased production of fibronectin, collagen IV, VEGF and AP-1, a transcription factor promoting the expression of e.g. TGF-ß. Inhibition of the Rho/ROCK pathway saved the cells from these changes (Kolavennu *et al.* 2008; Peng *et al.* 2008). Moreover, glomerular hypertension is a well described factor participating in the progression of DN. Hypertension causes mechanical stress which activates Rho (see below). In congruence, Komers et al. described improved renal hemodynamics after ROCK inhibition in diabetic rats (Komers *et al.* 2011a). In addition, there is some evidence that Rho is involved in the actions of endothelin 1 (ET-1), a potent vasoconstrictor, which is upregulated in diabetics (Yousif, 2006).

Because of this and comparable evidence from other studies it was assumed that ROCK inhibition is advantageous in diabetes. Komers, Peng and Gojo et al. treated diabetic rats (diabetes type 1 model) with the orally administered ROCK-inhibitor fasudil (Gojo *et al.*

2007; Komers *et al.* 2011b; Peng *et al.* 2008). Their diabetic rats rapidly developed albuminuria, glomerulosclerosis, and renal interstitial fibrosis, as well as decreased glomerular filtration rates (GFR), and increased expression of molecular markers of DN. Sustained ROCK inhibition reduced diabetes-related kidney damage (as confirmed by histology and urinalysis) in the kidney and expression of the molecular markers (including markers of epithelial–mesenchymal transition (EMT)) in association with a slight anti-proteinuric effect (including beneficial effects of fasudil on podocyte foot process effacement) which was independent of blood pressure or glucose control. So far, all long-term studies evidenced nephroprotective effects of ROCK inhibitors being independent from systemic blood pressure. In contrast, Kikuchi et al. failed to suppress the progression of nephropathy by fasudil application (but ameliorated some features of DN) in a rat model of type 2 diabetes (Kikuchi *et al.* 2007) but Kolavennu et al. found ROCK inhibition being kidney protective in type 2 diabetes mice (Kolavennu *et al.* 2008). Interestingly, Peng observed that fasudil and ACE-inhibitors had a comparable effectiveness preventing DN while Komers et al. noted that the combination of fasudil and losartan was not more effective than losartan alone (Komers *et al.* 2011b; Peng *et al.* 2008). ACE-inhibitors and AT1-blockers are well known agents used for kidney protection in (proteinuric) DN for many years. Interestingly, RAAS blockade was effective to inhibit Rho/ROCK activity in several studies suggesting that ROCK activation might follow AT1 activation under certain conditions (Higuchi *et al.* 2007; Komers *et al.* 2011b; Ohtsu *et al.* 2006). Thus, the effects of RAAS blockade partly rely on ROCK inhibition: Angiotensin II (AngII) mediated vasoconstriction (via myosin light chain phosphorylation), proinflammatory effects (via PAI-1 and monocyte chemoattractant protein-1 (MCP-1) induction) and JNK-dependent hypertrophy/cell migration. They can be attributed to Rho activity and are therefore within the therapeutic target range of ROCK inhibitors (Higuchi *et al.* 2007; Ohtsu *et al.* 2006). More evidence comes from Rikitake et al. who showed in ROCK1$^{+/-}$ haploinsufficient mice that perivascular fibrosis induced by AngII was significantly lower than in wild type individuals (Rikitake *et al.* 2005b). In the context of diabetes it is of note that AngII-dependent activation of the Rho/ROCK pathway is partly mediated by NADPH oxidase-dependent ROS (Jin *et al.* 2006). As mentioned above, ROS are present in the hyperglycemic milieu in excess probably promoting AngII effects in diabetics.

1.2 Rock Inhibition in Urethral obstruction

Urethral obstruction (UO) is a typical cause of ESRD in children. In this patient group, UO is responsible for approximately 15 to 25% of kidney failures (Koo *et al.* 1999). This is even a problem after renal transplantation, because obstruction-related side effects influence the success of transplantation. Allograft function and survival are often limited due to urinary tract infection or surgical complications, implying deleterious actions of chronic elevated intravesical pressure (Cairns *et al.* 1991; Churchill *et al.* 1988; Sheldon *et al.* 1994). However, more recent studies claim that patients with severe lower urinary tract abnormalities and ESRD may receive a kidney transplant with comparable safeness and success to patients without abnormalities (Broniszczak *et al.* 2010; Nahas & David-Neto, 2009; Rigamonti *et al.* 2005).

In experimental settings, UO is commonly employed as a normotensive, non-proteinuric and non-hyperlipidemic model of tubulointerstitial fibrosis without any toxic renal insult (Nagatoya *et al.* 2002). Fibrosis is of major interest, not only because it is a common final path of different injuries causing renal damage, but, as a substantial part of IF/TA, also

related to the renal (graft) prognosis (Couser & Johnson, 1994; Meier-Kriesche *et al.* 2004; Morgan *et al.* 2011; Nath, 1992; Pascual *et al.* 2002). Depending on the side where it mainly occurs, fibrosis is classified as glomerulosclerosis or tubulointerstitial fibrosis. Nevertheless, in most situations fibrosis is detected in both compartments, hinting towards a not yet fully understood cross-talk in between (Klein *et al.* 2011). Briefly, examples of fibrosis initiating factors are: increased intravascular pressure (hypertension), increased pressure in the tubular lumen (due to obstruction of the urinary tract), hyperglycemia (diabetes), increased urinary albumin concentrations (proteinuria), and toxic substances (puromycin or adriamycin). After ignition of the process due to different stimuli the pathways converge independently of the activator. Signaling includes induction of cytokines and chemokines, like TGF-β, platelet-derived growth factor (PDGF), FGF, ET-1, and osteopontin, leading to renal inflammation (predominant cell types are monocytes and macrophage (Mphi)) and cell activation.

It was suggested that Mphi are central players in the fibroproliferative response (Vernon, Mylonas & Hughes, 2010). They release profibrogenic growth factors i.e., TGF-β, PDGF and FGF, which activate/recruit myofibroblasts and stimulate resident interstitial fibroblasts. These fibroblasts are the main source of interstitial ECM (e.g., collagens and fibronectin, and expression of factors interfering with ECM crosslinking (transglutaminases, high glucose-induced AGEs) and/or turnover (matrix metalloproteinases, plasminogen activator, and their inhibitors) (well summarized in (Klein *et al.* 2011)) that cause fibrosis. In congruence, we and others observed e.g., that the level of L-Plastin, a cytoskeletal protein mainly expressed in interstitial fibroblasts, is increased in IF/TA (Reuter *et al.* 2010). Recently, it was reported that Rho/ROCK are central to differentiation, migration, and contractility of myofibroblasts (Mack *et al.* 2001; Parizi, Howard & Tomasek, 2000). Thus, ROCK inhibition might suppress the destructive activity of myofibroblasts. If not applied, the fibrotic process continues. Blocking of Mphi recruitment and activation ameliorates renal inflammation and fibrosis (Vielhauer *et al.* 2010). As ROCK is essential for Mphi migration its blockade can protect from fibrosis in UO (Nagatoya *et al.* 2002; Satoh *et al.* 2002). In renal graft recipients TGF-ß expression was analyzed in protocol biopsies (performed 3, 6, and 12 months after kidney transplantation). An increased level of TGF-ß was associated with a larger extent of interstitial fibrosis (Baboolal *et al.* 2002). Interestingly, when ROCK is inhibited in vivo the tissue level of the fibrogenic TGF-ß is lower than in control kidneys (Nagatoya *et al.* 2002).

Besides, TGF-ß promotes EMT, a process related to the development of renal fibrosis (Zavadil & Bottinger, 2005). As mentioned before, Rho/ROCK are important players in the (re-)organisation of the cytoskeleton. This implies that they are key mediators/effectors of epithelial plasticity and EMT induced by TGF-ß (Patel *et al.* 2005; Rodrigues-Diez *et al.* 2008; Zavadil & Bottinger, 2005). In summary, kidney protection due to ROCK inhibition can be achieved in UO because Rho/ROCK is involved in different key mechanisms in renal fibrosis.

1.3 Rock Inhibition in Hypertensive Nephrosclerosis

Increased blood pressure is an important health problem and a major risk factor for cardiovascular morbidity and mortality throughout the world (Staessen *et al.* 2003). To date, both the pathogenesis of arterial hypertension and the molecular mechanisms involved in blood pressure control remain poorly understood. Hypertension is characterized by high arterial pressure resulting from increased peripheral vascular resistance that can be attributed to both enhanced contractility of vascular smooth muscle cells and arterial wall remodeling. Increased activity of the Rho/ROCK pathway has been proposed to play an

important role in the development and maintenance of hypertension. In various animal models of experimental hypertension a role of RhoA and ROCK has been demonstrated. Blocking ROCK activity with Y-27632 has blood pressure lowering effects in spontaneously hypertensive rats (SHR), deoxycorticosterone-acetate (DOCA)/salt-treated and renal hypertensive rats (Uehata *et al.* 1997). Similarly, oral administration of fasudil to SHR rats significantly lowered blood pressure (Mukai *et al.* 2001). In addition, several studies have addressed changes in RhoA activity in isolated vascular segments from hypertensive animals suggesting that increased RhoA activity is responsible for enhanced ROCK function in the pathology of hypertension. In mesenteric and cerebral arteries from SHR and normotensive rats the relaxation induced by treatment with Y-27632 was markedly higher in arteries from SHR rats (Asano & Nomura, 2003; Chrissobolis & Sobey, 2001). This has also been shown for mesenteric arteries from DOCA/salt-treated rats (Weber & Webb, 2001). In animals treated with L-NAME, an inhibitor of NO-synthase, oral administration of Y-27632 lowered blood pressure and the level of active, GTP-bound RhoA was markedly increased in vessels from L-NAME treated rats (Weber & Webb, 2001). Similarly, direct evidence for increased amounts of active RhoA have been found in stroke-prone SHR, DOCA/salt- and renal hypertensive rats (Moriki *et al.* 2004; Seasholtz *et al.* 2001; Seko *et al.* 2003). Analysis of the expression levels of RhoA has led to controversial results with some studies showing an increased expression of RhoA under hypertensive conditions (Seasholtz *et al.* 2001) whereas in other reports no differences in the expression profile of proteins from the RhoA/ROCK pathway have been detected (Seko *et al.* 2003).

Impaired endothelial function and decreased NO-production have been implicated in the etiology of hypertension. Decreased expression of endothelial nitric oxide synthase (eNOS) is found in aortae from SHR rats (Chou *et al.* 1998) and eNOS-deficient mice have an elevated blood pressure (Huang *et al.* 1995). Interestingly, there seems to be extensive crosstalk between NO and RhoA/ROCK-signaling. There is compelling evidence that the NO/cGK pathway leads to an inhibition of RhoA/ROCK signaling (Carter, Begaye & Kanagy, 2002; Chitaley & Webb, 2002; Sauzeau *et al.* 2000). On the other hand, the RhoA/ROCK cascade seems to reciprocally influence NO-signaling. The mechanism by which ROCK influences NO production seems to be the regulation of eNOS mRNA stability (Eto *et al.* 2001; Rikitake *et al.* 2005a; Takemoto *et al.* 2002).

Chronic hypertension leads to end organ damage in a substantial number of patients. Hypertensive nephrosclerosis is a disorder that is usually associated with chronic hypertension. Histologically it is characterized by vascular, glomerular, and tubulointerstitial changes. The vascular pathology consists of intimal thickening and luminal narrowing of the large and small renal arteries and the glomerular arterioles. Two different processes appear to contribute to the development of the vascular lesions: A hypertrophic response to chronic hypertension evident by medial hypertrophy and fibroblastic intimal thickening, resulting in narrowing of the vascular lumen (Zucchelli & Zuccala, 1994). In the beginning this process is adaptive by minimizing the degree to which the rise in systemic pressure is transmitted to the downstream arterioles and capillaries (Zucchelli & Zuccala, 1994). The other process contributing to the vascular pathology is the deposition of hyaline-like material (plasma protein constituents, such as inactive C3b, part of the third component of complement) into the damaged, more permeable arteriolar wall (Zucchelli & Zuccala, 1994). Arterial hypertension may lead to focal global (involving the whole glomerulus) or focal segemental glomerulosclerosis (Marcantoni *et al.* 2002; Zucchelli & Zuccala, 1994). Global sclerosis is thought to reflect ischemic injury, leading to nephron

loss (Marcantoni *et al.* 2002). Focal segmental sclerosis is typically associated with glomerular enlargement, which is probably a compensatory response to nephron loss (Harvey *et al.* 1992). The vascular and glomerular alterations are associated with an often severe interstitial nephritis. Its etiology is incompletely understood. At least in part immunologic processes may be involved. They are probably started by ischemia-induced alterations in antigen expression on the surface of the tubular epithelial cells (Truong *et al.* 1992). Nephrosclerosis is seen with aging but is clearly exacerbated in arterial hypertension (Lindeman, Tobin & Shock, 1984; Rule *et al.* 2010). The incidence of progressive renal disease in hypertensive nephrosclerosis is low, however, three groups of patients are at increased risk to develop progressive kidney function deterioration: patients with more marked elevations in blood pressure, afro-american patients (they have an approximate eight-fold elevation in the risk of hypertension-induced ESRD(Toto, 2003); this increase in risk may persist even with "adequate" blood pressure control) and patients with underlying chronic kidney disease, especially diabetics. Patients with nephrosclerosis typically present with a long history of hypertension. If present, decline in kidney function is slow in progression as indicated by serum-creatinine and blood-urea-nitrogen. Urinalysis is typically benign without appearance of cast or dsymorph erythrocytes. Urinary protein excretion is typically mildly elevated (less than 1 gram per day). Concerning the incidence of renal failure in hypertensive nephrosclerosis there seems to be a clinical paradox as among patients (especially considering afro-americans) entering the chronic hemodialysis program. Hypertensive nephrosclerosis is one of the most common diagnoses, whereas the risk for a hypertensive patient to develop ESRD is rather small. However, at least three large trials might explain this paradox: the number of hypertensive patients is so large that even a small percentage of patients at risk gives a large number; the rate of progression might be so slow that trials that mostly run over 5-7 years might not detect patients at risk (Freedman, Iskandar & Appel, 1995; Madhavan *et al.* 1995). Recent work has shed some light on the importance of the Rho/ROCK pathway in kidney disease. ROCK is constitutionally active in the renal circulation. Studies on glomerular hemodynamics demonstrated that ROCK inhibition by Y-27632 and fasudil dilates the basal tone of afferent and efferent arterioles (Cavarape *et al.* 2003; Nakamura *et al.* 2003). Importantly, both inihibitors reverse angiotensin II-induced vasoconstriction of efferent and afferent arterioles (Nakamura *et al.* 2003). Thus, ROCK inhibition might protect against deleterious hemodynamic effects in kidney disease. In addition, to its critical role for the renal microvasculature the Rho/ROCK pathway is an important regulator of several cell function including proliferation, migration and apoptosis as already stated above (diabetic nephropathy). Of importance for the development of glomerulosclerosis, as a key feature of hypertensive nephrosclerosis, it has been demonstrated that Rho regulates the formation of stress fibers, focal adhesions and peripheral bundles through reorganization of the actin cytoskeleton in a renal epithelial cell line (Nakano *et al.* 1999). Renal epithelial cells are able to transform to mesenchymal-like cells via EMT. These changes have been observed in renal tubulointerstitial fibrosis, another hallmark of hypertensive nephrosclerosis. Mesangial cells reside in the renal glomeruli and produce ECM. Its increased accumulation causes glomerulosclerosis, where TGF-ß has been shown as a causative factor (Nakano *et al.* 1999). As mentioned above Rho/ROCK are key mediators/effectors of epithelial plasticity and EMT induced by TGF-ß (Patel *et al.* 2005; Rodrigues-Diez *et al.* 2008; Zavadil & Bottinger, 2005). Finally, in mesangial cells, mechanical stress, which is considered to cause glomerular hypertension and glomerulosclerosis, enhances mitogen-activated protein kinase (MAP kinase) activity, stress fiber formation, and

cellular proliferation (Bruijn *et al.* 1994). In this process of disease the Rho/ROCK pathway plays a pivotal role, acting as a modulator of MAP kinase and the downstream cellular impact. Taken together, these data strengthen an important role of the Rho/ROCK pathway in the development of glomerulosclerotic disease.

Podocytes are highly differentiated cells that are located in the renal glomerulus. Cytoskeleton rearrangement is closely associated with podocyte shape changes and dysfunction in various renal diseases (Zucchelli & Zuccala, 1994). Using the ROCK-inhibitor Y-27632 Endlich et al. could inhibit the reorganization of the cytoskeleton induced by mechanical stress in podocytes. Moreover, inhibition of ROCK prevents TGF-ß-induced increase in CTGF accumulation in fibroblast cells (Heusinger-Ribeiro *et al.* 2001). All these observation strongly suggest a pivotal role of the Rho/ROCK pathway in the progression of renal injury. To date, the body of evidence given by in vivo studies is growing. As the Rho/ROCK pathway regulates glomerular hemodynamics and has profound effects on mesangial cell proliferation and matrix production, ROCK-inhibitors are candidates to serve as therapeutic tools to treat glomerulosclerotic disease. Thus, it has been demonstrated that Y-27632 and fasudil prevent tubulointerstitial fibrosis in a model of unilateral ureteral obstruction [for detail see section on ureteral obstruction]. In 5/6 nephrectomized spontaneously hypertensive rats (SHR), a model of hypertensive glomerulosclerosis, the Rho/ROCK pathway was activated. Treatment with fasudil reduced urinary protein excretion, improved glomerular and tubulointerstitial injury score, and reduced the infiltration of ED-1 positive cells and proliferating cell nuclear antigen positive cells in the kidney of SHR treated by 5/6 nephrectomy. Interestingly, these effects were obtained without lowering blood pressure, indicating blood pressure-independent effects of ROCK (Kanda *et al.* 2003b). Fasudil up-regulated the expression of p27kip1, a cyclin-dependent kinase inhibitor, and increased the p27kip1 immunopositive cells in both glomeruli and tubulointerstitium, indicating inhibition of cell proliferation and macrophage recruitment under fasudil treatment. Another group reported similar beneficial effects in Dahl salt-sensitive rats. In these animals, fasudil improved renal function, proteinuria, and histological findings without changes in blood pressure (Nishikimi *et al.* 2004b). These beneficial effects were most likely accompanied by decreased expression of TGF-ß, collagen-I, and collagen-III mRNA in the renal cortex. In salt-loaded spontaneously hypertensive stroke-prone rats serving as a model of severe hypertension fasudil improved kidney function, proteinuria, histological findings and decreased expression of genes encoding for extracellular matrix, oxidative stress, adhesion molecules and antifibrinolysis. Of note, these effects were independent of the blood pressure-lowering activity of fasudil (Nishikimi *et al.* 2004b). As stated above there is compelling evidence for an extensive crosstalk between NO and RhoA/ROCK-signaling. Indeed, in SHR fasudil partly reversed the progressive nephrosclerosis initiated by administration of the nitric oxide-synthase inhibitor nitro-L-arginine methyl ester (Koshikawa *et al.* 2008).

Although multiple factors contribute to the development and progression of chronic renal disease, the renin-angiotensin-aldosterone system seems to be of major importance. Angiotensin II is considered to be the main mediator of this system. It is a potent vasoconstrictor acting directly on vascular smooth muscle cells, thereby regulating the vascular tone. Besides it alters renal sodium and water absorption by stimulating synthesis and secretion of aldosterone. Further, it is involved in the generation of thirst and the excretion of vasopressin. Hence, it has a pivotal role in acute and chronic regulation of blood

pressure. Studies in chronic renal disease have shown that angII contributes to deterioration of renal function even if blood pressure is unaltered (Anderson, Rennke & Brenner, 1986). In this view it is important to keep in mind that angII activates ROCK in vascular smooth muscle cells (Yamakawa *et al.* 2000). In rats infused with angII, treatment with Y-27632 reduced renal inflammatory cell infiltration and tubular damage. AngII activated nuclear factor-kappaB and initiated overexpression of proinflammatory factors, including TNF-alpha and monocyte chemotactic protein-1 (MCP-1), and of CTGF. Treatment of angII-infused rats with Y-27632 reduced the upregulation of these proinflammatory and profibrotic mediators (Ruperez *et al.* 2005).
Taken together these studies imply that blockade of the Rho/ROCK pathway might prove beneficial in hypertensive nephrosclerosis.

1.4 Rock Inhibition in Ischemia Reperfusion Injury

Renal ischemia-reperfusion (IR) injury (IRI) is a common and important trigger of acute renal injury (AKI). It occurs in a broad spectrum of clinical settings including (transplantation) surgery, trauma, dehydration or sepsis leading to renal hypoperfusion, acute tubular necrosis (ATN), and functional disturbances - namely AKI. Inevitably linked to renal transplantation it is a well known risk factor for delayed graft function associated with prolonged hospitalization, elevated costs, and increased complexity of immunosuppressive drug management. Moreover, by reducing the overall number of nephrons and increasing the risk of acute rejection episodes, IRI might cause a significantly reduced graft survival. Involving both, the innate and the adaptive immune response, causing subsequent sterile inflammation, IRI is composed of a complex cascade of events including the generation of reactive oxygen and nitrogen species, chemotaxis, and phagocytosis. All of which are functional properties of the key effectors of the inflammatory cascade, neutrophiles, the most abundant leukocyte population in circulation, which accumulate as early as 30 minutes after IR particularly in the peritubular capillary network of the outer medulla (Li *et al.* 2008). Attracted leukocytes subsequently transmigrate into the interstitium. This is associated with increased vascular permeability and loss of endothelial and tubular epithelial cell integrity (Awad *et al.* 2009) due to degranulation of neutrophils. Upon activation these neutrophils release proteases, myeloperoxidase, cytokines, and generate reactive species leading to aggravation of injury and damage of endothelial and epithelial cells especially in the outer medulla (Bolisetty & Agarwal, 2009; Jang & Rabb, 2009; Li & Okusa, 2006; Okusa *et al.* 2000). In regard to this, it has recently been shown that ROCK) and their associated signaling pathways play pivotal roles in the development of (experimental) IRI. ROCK-inhibitors such as fasudil or Y-27632 have been shown to provide beneficial effects concerning different ischemic events such as renal (Kentrup D. *et al.* 10 A.D.; Kentrup D. *et al.* 2010; Prakash *et al.* 2008; Teraishi *et al.* 2004; Versteilen *et al.* 2006; Versteilen *et al.* 2011) myocardial (Bao *et al.* 2004; Hamid, Bower & Baxter, 2007; Wolfrum *et al.* 2004), cerebral (Satoh *et al.* 2008; Toshima *et al.* 2000), hepatic (Du & Hannon, 2004; Ikeda *et al.* 2003; Takeda *et al.* 2003), and gastrointestinal (Santen *et al.* 2010) ischemia. However, the exact mechanisms involved remain to be fully elucidated.
In the context of IRI it seems to be of interest that ROCKs are involved in the regulation of leukocyte cellular motility, migration, adhesion, and transmigration (Alblas *et al.* 2001; Honing *et al.* 2004; Lee *et al.* 2004; Samaniego *et al.* 2007; Takesono *et al.* 2010; Vemula *et al.* 2010; Worthylake *et al.* 2001; Worthylake & Burridge, 2003) Regarding this, Teraishi et al. were the first group to test the effectiveness of ROCK-inhibitors in an animal model of renal

IRI. For this, male Sprague-Dawley rats underwent unilateral nephrectomy of the right kidney two weeks before inducing 45 min of warm ischemia in the remaining kidney by clamping the left renal artery and vein. Y-27632 was hereby applied 5 min pre ischemia or 5 min post ischemia. They observed a protective effect for both treatments (i.e. improved renal function, less histological damage) which was based according to them on reduced infiltration by neutrophils as shown by myeloperoxidase assays. However, even though the latter is a non specific detection method and the data regarding the cell types typically involved varies, (e.g. due to the models used (Rabb *et al.* 2003; Thornton *et al.* 1989) or due to non specific detection methods, e.g. myeloperoxidase, naphthol chloroacetate esterase, or HIS-48 staining (Ysebaert *et al.* 2000)), it is well known that the increased influx of neutrophiles, T- and B-lymphocytes as well as macrophages/monocytes significantly contributes to the pathogenesis of AKI (Kinsey, Li & Okusa, 2008). This first study is also in congruence with later work by Versteilen et al.. By pre-treating male Wistar rats with Y-27632 they could show that leukocyte accumulation (60-70% neutrophils) was markedly reduced by ROCK-inhibitor treatment in the microvasculature of the corticomedullary junction and medulla (Versteilen *et al.* 2011). They hypothesize that this may be partly due to NO-mediated effects via activated endothelial cells (i.e. limited expression of adhesion molecules and cytokines leading to attenuation of leukocyte accumulation), eNOS mediated alterations of the renal blood flow (Versteilen *et al.* 2006) and direct effects on the leukocytes.
Further, Prakash et al. also applied the ROCK-inhibitor Y-27632 in a rat model of renal ischemia, but used a Y-27632-lysozyme conjugate (Prakash *et al.* 2008). Thus, they tried to guarantee a renal-specific uptake into proximal tubular cells via megalin receptors. In unison with the aforementioned data they describe substantially attenuated tubular damage as indicated by reduced expression of dedifferentiation markers kidney injury molecule 1 (KIM-1) and vimentin. Additionally, they observed reduced fibrosis and inflammation as determined by reduced gene expressions of MCP-1, procollagen Iα1, TGF-β1, tissue inhibitor of metalloproteinase 1 and α-smooth muscle actin, as well as reduced immunohistochemical staining of macrophage infiltration, α-smooth muscle actin, collagen I, collagen III and fibronection compared to untreated animals. However, in contrast to the previous data, they describe adverse systemic effects (e.g. leucopenia) when animals were treated with the unconjugated Y-27632, as performed by others groups. An effect, which cannot easily be explained due to the fact that the beneficial effects of ROCK-inhibitors have not only been shown for the kidney but for other organs as well.
Although and apart from the above described effects, it has recently been shown by Kroening et al. that, in some cases, ROCK inhibition may not adversely influence the migratory capabilities of specific cell types as it has been repeatedly shown. Instead, at least in the case of tubular epithelial cells the migratory capacity may actually be promoted and thus ROCK inhibition may favor repair processes in renal tubules (Kroening *et al.* 2010). The beneficial effects of ROCK inhibition are further supported by work published by Unbekandt et al. who could show that transient ROCK inhibition allows cells from disaggregated embryonic kidneys to form ureteric bud and nephron epithelia (Unbekandt & Davies, 2010). Moreover, one last positive effect of ROCK inhibition related to the prevention of IRI mentioned might be that ROCK inhibition promotes cell survival in human stem cells (Watanabe *et al.* 2007) and is able to reduce apoptosis in conventional embryonic kidney culture (Meyer *et al.* 2006).

1.5 Rock Inhibition in Chronic Allograft Nephropathy

Compared to hemodialysis kidney transplantation significantly reduces mortality when it is applied to the appropriate patient (Wolfe *et al.* 1999). However, even after renal transplantation mortality of transplant recipients is still increased when compared to the general population. This is mainly due to death from cardiovascular disease with a functioning graft as well as immunological and non-immunological factors resulting in graft loss. Despite introduction of new immunosuppressive drugs within the last two decades a substantial increase in graft survival was not achieved. Consequently prevention of graft loss and treatment still lack any significant breakthrough since many years (Gjertson, 1991; Lamb, Lodhi & Meier-Kriesche, 2011; Meier-Kriesche *et al.* 2004; Meier-Kriesche, Schold & Kaplan, 2004). The pathological manifestations seen in chronic graft loss were summarized as chronic allograft nephropathy. In the recent Banff-classification ("Banff `05") which gives a classification of the different pathological features seen in chronic graft loss, the term chronic allograft nephropathy does no longer occur (Solez *et al.* 2007). The rationale for this update of the Banff schema was the misusage of CAN as a generic term for all causes of chronic renal allograft dysfunction with fibrosis that inhibits the accurate diagnosis and appropriate therapy. Thus, the authors of the Banff-classification aimed to present a pathological classification that specifies the underlying disease to facilitate causal treatment. Pathological manifestations of chronic allograft injury include interstitial fibrosis, tubular atrophy, vascular occlusive changes, glomerulosclerosis and a progressive renal dysfunction accompanied by hypertension and proteinuria (Cornell & Colvin, 2005). This histopathologic constellation is now more formally and descriptively referred to as interstitial fibrosis and tubular atrophy (IF/TA) without evidence of any specific aetiology (Solez *et al.* 2007). IF/TA or CAN describe the common final path of different injuries causing renal damage, whereas their precise pathogenesis is complex and only incompletely understood. The process encompasses multifactorial aetiologies including alloantigen-dependent as well as alloantigen-independent factors (Cornell & Colvin, 2005). The latter mainly comprise arterial hypertension, chronic obstruction and calcineurin-inhibitor toxicity (Busauschina, Schnuelle & van der Woude, 2004; Klahr & Morrissey, 2003; Mihatsch, Ryffel & Gudat, 1995; Morozumi *et al.* 2004). Besides, chronic polyoma virus nephrotixicity can lead to IF/TA (Drachenberg *et al.* 2005). Recurrent and de novo glomerular or vascular diseases can also lead to glomerulosclerosis and IF/TA, both early and late post-transplant. It is important to mention that de novo diabetic changes are becoming more common in allografts. As mentioned chronic alloimmune injury is an important cause of IF/TA in kidney grafts. Data on alloantibodies and C4d, a product of the complement cascade in chronically failing grafts, hint at a pathogenic role for humoral immunity in chronic allograft injury. In a prospective study de novo appearance of donor-specific HLA-antibodies was associated with increased graft failure at one year (Terasaki & Ozawa, 2004). These data are consistent with the importance of immunological factors in chronic graft injury.

The Rho/ROCK pathway is potentially crucial in mediating immunological as well as non-immunological injury in chronic graft loss as it is involved in adhesion, migration, proliferation, and cytokine release (Amano *et al.* 1997; Chihara *et al.* 1997; Tharaux *et al.* 2003). The activation of T-cells is involved in alloimmune responses. Here the Rho/ROCK pathway plays a pivotal role in T-cell activation during cellular immune responses by promoting structural rearrangements that are critical for T-cell signaling (Tharaux *et al.* 2003). Besides, a role for Rho/ROCK in HLA class I signaling pathways that have been implicated in the process of chronic rejection has been shown. Ligation of HLA class I

molecules by anti-HLA-antibodies resulted in activation of Rho/ROCK and increased stress fiber formation. Inhibitors of Rho-GTPase and ROCK blocked HLA class I-mediated posphorylation of paxillin and FAK, both central elements of the focal adhesion signaling complex (Lepin *et al.* 2004). As stated above not only immunological factors but also non-immunological factors, such as arterial hypertension, are responsible for chronic graft injury. Recent data also show relevance of the Rho/ROCK pathway for the regulation of hemodynamics and arterial hypertension. In 1997 it was shown that the Rho/ROCK pathway is involved in the generation of arterial hypertension in different animal models of hypertension (Uehata *et al.* 1997). Interestingly, these data were confirmed in hypertensive patients showing involvement of Rho/ROCK in the generation of the increased vascular tone in these patients (Masumoto *et al.* 2001). As already stated above there is a close interaction between the RAAS and the Rho/ROCK pathway. Activation of the RAAS in arterial hypertension and vascular disease is associated with increased activation of Rho/ROCK (Higashi *et al.* 2003; Yamakawa *et al.* 2000). Taken together accumulating data suggest a role of the Rho/ROCK pathway in chronic allograft dysfunction. Recent studies in animal models of allograft dysfunction strongly support this view. In a well established model of kidney transplantation Lewis rats acted as kidney graft recipients and Fisher rats as donors. Cyclosporine A was given for immunosuppression. One group of animals additionally received the specific ROCK-inhibitor Y-27632. Renal function deteriorated progressively in the group that received cyclosporine A alone and histology revealed the typical features of IF/TA. ROCK inhibition by Y-27632 significantly prevented the deterioration of kidney function, reduced proteinuria and preserved renal function. These effects were accompanied by down-regulation of the expression of tubular MCP-1, RANTES, and phosphorylated NF-kB. The profibrotic TGF-ß1 and α-SMA, a marker of EMT, were downregulated by the treatment with Y-27632. These data indicated that the Rho/ROCK pathway is critically involved in renal interstitial inflammation and fibrosis, thus efficiently retarding the development of chronic allograft failure (Liu *et al.* 2009). The same group reported that atorvastatin, an inhibitor of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase, exerted protective effects in CAN (Zhang *et al.* 2007). Because 3-hydroxy-3-methylglutaryl-coenzyme A reductase interferes with isoprenylation and activation of Rho, these data support the view that inhibition of the Rho/ROCK pathway could be attractive in prevention of allograft nephropathy. Of note, these data confirmed what was seen earlier in a model of cardiac allograft vasculopathy in mice. Here, coronary remodeling in the allografts characterized by intimal thickening and perivascular fibrosis was dose-dependently suppressed by the ROCK-inhibitor fasudil. These data were strengthened as gene transfer of dominant-negative ROCK mimicked the effects of fasudil. Vascular inflammation and expression of profibrotic mediators were significantly reduced in this model (Hattori *et al.* 2004). Song et al. observed expression of RhoA and ROCK1 mRNA and protein in measangial and tubular cells in the Fisher-to-Lewis model of CAN. Interestingly, they found a negative correlation between RhoA/ROCK1 mRNA and the Banff score. MMF, a potent immunosuppressive drug used in solid organ transplantation attenuated CAN by downregulating the expression of RhoA/ROCK1 (Song *et al.* 2008).

The data available suggest an important role of the Rho/ROCK pathway in chronic allograft dysfunction. Further studies also in humans are needed to support this view and possibly add a new therapeutic strategy to the nephrologist´s therapeutic options in preserving graft function.

2. Conclusion

Recent experimental data provide promising data that kidney protection in diabetic nephropathy, urethral obstruction, hypertensive nephrosclerosis and chronic allograft nephropathy can be brought about by inhibition of the Rho/ROCK pathway. As reviewed above ROCK activity is involved in actin cytoskeletal organization, stress fiber formation, and cell contraction thereby controlling vascular smooth muscle contraction, endothelial barrier and leukocyte functions (e.g., cellular motility, migration, adhesion and transmigration). All of these aspects are important in the pathogenesis of the renal diseases discussed here. However, clinical trials are needed to develop future strategies and transfer new treatment options by ROCK-inhibition from bench to bedside.

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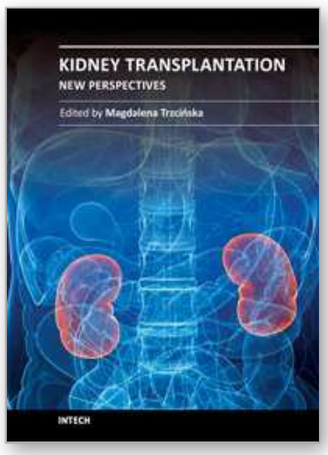

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Although many years have passed since the first successful kidney transplantation, the method, although no longer considered a medical experiment, is still perceived as controversial and, as such, it triggers many emotions and thatâ€™s why conscious educational efforts are still needed for kidney transplantation, for many people being the only chance for an active lifestyle and improved quality of life, to win common social acceptance and stop triggering negative connotations. Apart from transplantation controversies piling up over years transplantologists also have to face many other medical difficulties. The chapters selected for this book are of high level of content, and the fact that their authors come from many different countries, and sometimes even cultures, has facilitated a comprehensive and interesting approach to the problem of kidney transplantation. The authors cover a wide spectrum of transplant-related topics.

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University Campus STeP Ri
Slavka Krautzeka 83/A
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Unit 405, Office Block, Hotel Equatorial Shanghai
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