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CCL2/MCP-1 and *NFκB* Gene Transcription in Remnant Kidneys after Physical Activity and Renoprotection

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Abstract

Chronic kidney disease (CKD) is a progressive disease and affects approximately 10% of the population. The major late pathologic feature of CKD is interstitial fibrosis in the kidney characterized by extracellular matrix deposition but in early stage, several profibrotic and proinflammatory cytokines as well as growth factors are expressed. The renin-angiotensin and ET systems both play an important role in the pathogenesis of CKD and the blockade of these systems has been shown to suppress proinflammatory cytokines and to arrest the progression of CKD. We have demonstrated earlier also the renoprotective effect of physical activity on the experimental CKD progression (Pechter *et al.*, 2003). Aim of the study was to investigate the effects of non-drug treatment, physical activity, to the extent of gene expression in experimental CKD and to compare with endothelin receptor blocker (ERB, sitaxentan) and standard nephroprotective drug, angiotensin receptor blocker (ARB, losartan) treatments. Expression of mRNA was assessed by real-time reverse transcription-polymerase chain reaction. *CCL2/MCP*-1 and nuclear factor-*κB* (*NFκB*) gene expression was measured in whole kidneys. Results revealed that *CCL2/MCP*-1 gene expression (data presented in poster presentation ASN Renal Week 2010, Pechter *et al.*) was increased in the 5/6 NPX rat kidneys, and the increase was lessened significantly after physical activity and losartan therapy. ERB treatment appeared less effective. Combined ARB and ERB treatment as well as drugs alone or physical therapy prevented the increase in systemic blood pressure, albuminuria and *CCL2/MCP*-1 as well as *NFκB* gene expression. We conclude that non-drug and drug treatments both were effective regarding the rate of the progression of experimental CKD measured by physiological and molecular biomarkers of chronic inflammation in kidney tissue.

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Keywords

Remnant Kidney, Gene Transcription, Hypertension, Physical Activity, Renoprotection

1. Introduction

Chronic kidney disease (CKD) is a progressive disease and affects approximately 10% of the population [1]. The renin-angiotensin-aldosterin system (RAAS) and ET systems both play an important role in the pathogenesis of CKD and despite the experimental and clinical first-line use of RAAS blockers in the management of CKD, there is still a great need to improve the prevention the progression of CKD. The major late pathologic feature of CKD is interstitial fibrosis in the kidney characterized by extracellular matrix deposition which is common feature also for many other organ damages [2]. However, in early stage, several profibrotic and proinflammatory cytokines as well as growth factors are expressed in kidney tissue which is well-known phenomenon in CKD to the addition of progressive proteinuria and hypertension. Among key molecular factors that have been reported to have an important role in the pathogenesis of CKD are *NFκB* and *CCL2*, also known as monocyte chemoattractant protein 1 (*MCP*-1) [3]. The blockade of RAAS and endothelin (ET) systems has been shown to suppress early proinflammatory cytokines and growth factors and to arrest the progression of CKD [4] [5]. We have demonstrated earlier also the renoprotective effect of aquatic exercise on the experimental CKD progression [6].

The renin-angiotensin and ET systems both play an important role in the pathogenesis of CKD. Chronic nephropathies are associated with enhanced renal synthesis of ET-1 and blockade of ET receptors has been shown to confer renoprotection in experimental models of proteinuric renal disease [7]-[10]. ETs, via stimulation of the ETA receptor and are extremely powerful vasoconstrictors and micro puncture studies suggest that endogenous ET and angiotensin II partially mediate the glomerular hemodynamic responses to acute systemic NO synthase inhibition [11]. The actions of ET and angiotensin II are mainly additives, and, almost all of the vasoconstrictor responses to acute NOS inhibition are prevented when both vasoconstrictor systems are blocked [11]. Although ET antagonists have shown anti-inflammatory, anti-fibrotic, and anti-proteinuric effects in experimental studies [7] [12]-[15] recent studies on the effectiveness of ET receptor blockers in angiotensin II-induced end-organ damage are conflicting and more aspects should be considered when conclusions will made.

The development of strategies to prevent or delay the progression of CKD requires a better understanding of the cellular and molecular mechanisms as well as of effect of different possibilities to arrest the progression. Therefore, we tested the hypothesis whether the effects of non-drug treatment, physical activity, are as effective as standard renoprotective drug angiotensin receptor (ARB, losartan) and endothelin receptor blocker (ERB, sitaxentan) or dual blockade with the ARB and ERB treatments regarding the rate of the progression of experimental CKD measured by physiological and molecular biomarkers of chronic inflammation in kidney tissue.

2. Methods

2.1. Animals

The Animal Studies Ethics Committee of the Tartu University approved the study protocol. Male Wistar rats were purchased from the Laboratory Animal Centre University of Kuopio, Finland. An acclimatization period of 10 days was allowed before any experiment work was undertaken. The rats were kept in a climate-controlled facility at the Faculty of Medicine in the University of Tartu where animals were housed under standard conditions on a 12-h light/dark cycle and fed with standard rodent food (R 70, Lactamin AB, Sweden) and had free access to tap water.

2.2. Experimental Design

Chronic kidney disease was induced with a sub-total (5/6) nephrectomy (NPX) in Wistar rats. Rats weighing 262 - 280 g were anaesthetized with intraperitoneal methohexital sodium, 5 mg per 100 g body weight. Renal ablation was then accomplished by right nephrectomy and selective ligation of extrarenal branches of the left

renal artery in such a way that approximately 2/3 of the left kidney was infracted [16]. To accelerate the onset of renal damage the treatment was started 14 days later and rats were subsequently treated then until three months with the losartan (180 mg/L in the drinking water) or sitaxentan (40 mg/kg b.i.d. p.o.) or the combination of the two drugs. Totally, 43 animals were subjected to sub-total nephrectomy (NPX) and by matching for age and body weight divided into seven groups: Group I—untreated (n = 8), Group II—ERB treated (n = 7, initially 8 animals but 1 died after 2 weeks after operation), Group III—healthy (n = 5), Group IV—sham-op (n = 5), was sham operated, Group V—ARB treated (n = 10), Group VI—physical activity (n = 10), Group VII—ERB + ARB combined treatment (n = 8).

2.3. Physical Activity, Water-Therapy

10 animals (water therapy, group VI) were subjected to thermoneutral (water temperature 38˚C) immersion and voluntary swimming without exhaustion 30 min daily for 12 weeks in water pool with water depth of 50 cm.

2.4. Studied Parameters

Body weight was measured every 2 weeks for the duration of the study. Systolic blood pressure (SBP, mmHg) was measured bi-weekly by the tail-cuff manometer (Harvard Apparatus, USA) in awake pre-warmed rats. The urine was collected for 24 hours (h) using metabolic cages at weeks 6 and 12 for determination of proteinuria (Uprot, g/24h) that were measured with a Hitachi 912 Analyzer. At the end of the study, blood was collected from the aorta for serum creatinine (S-Crea, μmol/l) and was measured using the Hitachi 912 Analyzer. Therefore, the kidney tissue samples were collected for mRNA and histological studies. Also, parts of the tissue were snap-frozen in liquid nitrogen.

Paraffin sections of coronar slices, through the pelvis of the remnant kidney were cut at 4 mm thickness and stained using periodic acid_sciff (PAS) and Masson's trichrome methods. PAS sections from each kidney were studied morphologically for evidence of focal-segmental glomerulosclerosis (FSGS), defined as glomeruli showing evidence of segmental or global collapse of capillaries with or without associated hyaline deposition and adhesions of the capillary tuft to Bowman's capsule. Trichrome stained sections from each kidney were graded for the presence of interstitial fibrosis (0, 1+, 2+, 3+).

2.5. Quantification of Rat *MCP*-1 mRNA by Real Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For quantification of rat *MCP*-1, or alternatively *CCL*2 (monocyte chemoattractant protein-1 in rat) and endogenous reference *β-actin* mRNAs we used a SYBR Green real-time quantitative RT-PCR method with the ABI Prism 7000 Sequence Detection System (PE Applied Biosystems, Foster City, California). Total RNA was extracted from kidney tissue samples by using RNeasy Mini Kit (Qiagen) and cDNA synthesized by a First-Strand cDNA Synthesis Kit SuperScript™ III (Invitrogen). RNA was quantified by determination of ultraviolet absorbance at 260 nm, and purity was assessed by measuring the optical density ratio at 260 and 280 nm (Nanodrop). Transcript levels for rat *MCP*-1 (*CCL*2), *NFκB* and *β-actin* were quantified using SYBR Green-based quantitative real-time PCR technology. Amplification was performed by using the RT SYBR Green/ROX qPCR Master Mix (SABiosciences), other reagents and Real-Time RT^2 qPCR Primer Assays. The polymerase chain reaction performed as described by producers (www.SABiosciences.com). The RT^2 qPCR oligonucleotide primer sets from SABiosciences result give single gene specific PCR amplicon products of the correct size and high amplification efficiency as a result. For amplification quality control we performed the dissociation curve programme immediately after the above PCR programme and carried out the agarose gel electrophoresis. Amplification efficiencies of endogenous reference and the target sequence were comparable. The mRNA level of the target sequence were normalized to those of *β-actin* as a housekeeping gene, and used as an endogenous internal control; the relative levels of each mRNA to that of *β-actin* were calculated. PCR reactions for both factors were repeated in triplicate. The XpressRef™ Universal Total RNA product from SABiosciences was considered as the inter-assay standard.

2.6. Statistical Analysis

Data were analyzed at the end of the experiment after 12 weeks of the study period using SPSS package and

presented as mean values ± SEM. Groups were compared using the non-parametric Mann-Whitney U test for comparisons significant at the 0.05 level. The relative amount of *CCL2/MCP*-1 and *NFκB* gene expression, in the rat kidney cortex QPCR transcription differences between treatment and control groups, were significant using the student t-test.

3. Results and Discussion

The studied physiological parameters at the end of the experiment were demonstrated in **Table 1**.

Average levels of systolic blood pressure (SBP) in the ARB group, water therapy and the combination treatment group were significantly lower compared with untreated animals ($p < 0.05$). The ERB treatment was not effective, yet not significant in lowering the blood pressure in studied animals. Similarly, serum creatinine was found significantly lower in all treatment groups compared with untreated controls. Uprot was also increased in all NPX groups, but was significantly reduced in the water therapy, ARB and ERB+ARB treatment groups, compared with untreated animals ($p < 0.05$) (**Table 1**). Morphological studies revealed that significantly less FSGS and interstitial fibrosis was found in remnant kidneys of the water therapy, ARB and ARB + ERB groups compared with untreated controls ($p < 0.05$). Thus, although the water therapy and monotherapy with the ARB was associated with a reduction in blood pressure, urinary protein excretion, and tubular injury, in experimental CKD, combination therapy was not more effective.

The *CCL2*/*MCP*-1 gene expression was increased in the untreated rat kidneys and the increase of *CCL2*/*MCP*-1 was lessened significantly by ARB and ERB + ARB as well as water therapy (**Table 2**, **Figure 1**). ERB treatment appeared significantly less effective. QPCR transcription differences between ARB or ERB + ARB treatment was not significant.

NFκB transcription was not significantly suppressed by treatments except combination therapy (**Figure 2**).

CCL2/*MCP*-1 and *NFκB* renal abundance were all increased in the remnant kidney of the rats without treatment and especially *CCL2*/*MCP*-1 was reduced significantly by the ARB and combination treatment as well as water therapy that was comparable with healthy controls (**Table 3**). Although previous studies reported the beneficial effects of exercise on hypertension and renal injury in different experimental models [17] [18] this has

Table 1. Physiological parameters at the end of the experiment.

	Group I—untreated	Group II—ERB treated	Group III—healthy	Group IV—sham-op	Group V—ARB treated	Group VI—water-therapy	Group VII—ERB + ARB
Body weight (g)	466.0 ± 6.7	540.0 ± 8.8*	576.8 ± 20.8*	585.8 ± 6.8*	540.5 ± 19.6*	569.8 ± 10.1*	540.5 ± 19.6*
SBP (mmHg)	177.7 ± 4.9	132.2 ± 5.2	100.8 ± 8.2*	104.8 ± 8.4*	102.3 ± 13.1*	125.2 ± 15.4*	101.8 ± 3.4*
Crea (micromol/l)	99.4 ± 28.9	57.3 ± 3.0*	31.4 ± 7.2*	43.2 ± 7.9*	57.4 ± 5.7*	58.1 ± 0.91*	57.4 ± 5.7*
UProt (g/24h)	7.6 ± 4.2	4.9 ± 1.4	0.3 ± 0.5*	0.8 ± 0.5*	2.1 ± 1.0*	2.0 ± 1.2*	1.8 ± 0.8*
Interstitial fibrosis	1.8 ± 0.5	1.2 ± 0.5	0.0 ± 0.0*	0.0 ± 0.0*	0.5 ± 0.7*	0.6 ± 0.4*	0.5 ± 0.3*

$p < 0.05$ vs group I.

Table 2. *CCL2/MCP*-1 and *NFκB* gene relative transcription with SD in study groups of animals kidneys at the end of experiment.

Study group	*CCL2/MCP*-1 gene relative transcription	SD value	*NFκB* gene relative transcription	SD value
Untreated NPX controls	3417	2240	1748	728
ERB-treated	2533	1090	2143	397
Healthy controls	1344	577	2386	1049
Sham-operated	974	236	986	152
ARB-treated	1038	588	3850	458
Water therapy	959	764	2515	581
ARB + ERB	1503	562	2636	515

$p < 0.05$ vs group I.

Table 3. QPCR transcription differences between study groups (*Mann-Whitney test*).

Study groups	*CCL2/MCP-1*	*NFκB*
Healthy vs untreated	p = 0.0039	p = 0.1979
Healthy vs ERB	p = 0.0014	p = 0.7532
Healthy vs ARB	p = 0.1317	NA
Healthy vs ARB + ERB	p = 0.5098	p = 0.5362
untreated vs ARB	p = 0.0022	NA
untreated vs ERB	p = 0.0166	p = 0.6819
untreated vs ARB + ERB	p = 0.0023	p = 0.0265
ARB vs ERB	p = 0.0024	NA
ERB vs ARB + ERB	p = 0.0059	p = 0.1209
ARB vs ARB + ERB	P = 0.0852	NA

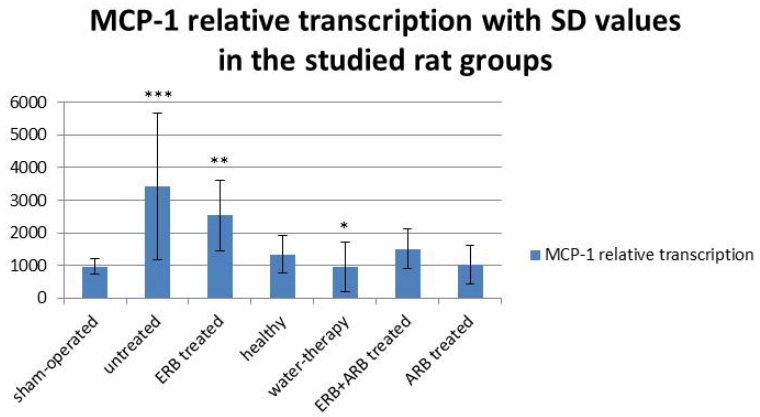

Figure 1. *MCP*-1 relative transcription with SD values in the studied rat groups. *p = 0.02 vs healthy; **p < 0.01 vs healthy, water-therapy; p < 0.001 vs ARB treated; ***p < 0.05 vs sham-op; p < 0.01 vs ERB + ARB treated; p < 0.001 vs water-therapy and ARB treated.

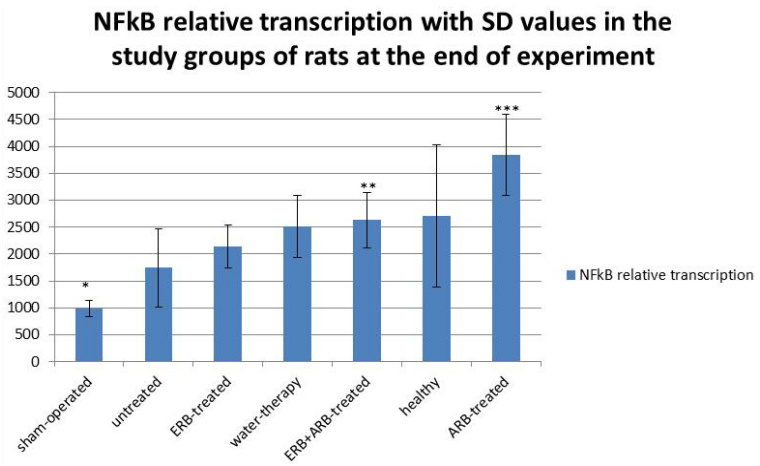

Figure 2. NF*κ*B relative transcription in the studied rat groups with SD values. *Significantly differenciates (p < 0.01) from all the other groups according to Mann-Whitney U-test; **Significantly differentiates (p < 0.01) from ARB-treated group; ***The difference between the untreated, ERB-treated and water-therapy samples is highly significant (p < 0.001).

not been investigated and compared with ARB and ERB treatments effects extents in classical experimental CKD model, remnant kidney.

Dual blockade of the ET blockers with the combination of RAAS system blockers for the treatment of hypertension and proteinuria has been tested in several studies among experimental animals with CKD with different conflicting results. The blockade of RAAS and ET systems has been shown to suppress pro-inflammatory cytokines and to arrest the progression of chronic renal disease [3] [19]. Recent studies demonstrated that an ETRB, given to diabetic rats at the moment of disease induction, prevented the development of renal injury [15] or, in combination with ARB combined therapy, can be more effective than single drugs [8]. The pro-inflammatory chemokine *CCL2/MCP*-1 is implicated in the recruitment of T cells and monocytes, in the remnant kidney model [4].

Here we compared the effect of non-drug treatment, water therapy and an ERB and ARB therapy, given as a single therapy or with a combination of the two drugs, in 5/6 nephrectomised Wistar rats but did not find an additional positive effect with combination therapy which is indeed in agreement with many of the other authors' findings [20] [21]. Our study added to the information, regarding inflammatory changes in the kidney, which was demonstrated in this study by the *CCL2/MCP*-1 gene transcription suppression, similar in the non-drug treatment and, ARB and ARB + ERB's treatment groups. At the same time, contrary to findings from other authors [22] the *NFκB* transcription was not suppressed in remnant kidneys except in combination group animals showing that more aggressive or higher dose treatment may be afforded to CKD animals for the achieving of a proper effect at the molecular level.

4. Conclusion

Treatment with physical activity and ARB or combination with ERB + ARB was associated with an improved kidney function and reductions in systolic blood pressure (SBP), urinary protein excretion as well as with less chronic lesions in association with reduced gene expression of *MCP*-1 in the kidney. Aquatic exercise improved the course of experimental CKD. These results point on the additional renoprotective properties of long-term water immersion and daily exercise in rats with CKD. The combination of losartan with sitaxentan was not associated with further reductions in SBP and urinary protein excretion. Furthermore, the addition of sitaxentan to losartan did not confer any additional benefits at molecular level. Our findings suggest that the RAAS but not the ET system is a major mediator of progressive renal injury after renal mass reduction and that the combination of an ARB with an ERB may not have advantages over the single agent of RAAS blocker treatment. Finally, non-drug and drug treatments both were effective regarding the rate of the progression of experimental CKD measured by physiological and molecular biomarkers of chronic inflammation in kidney tissue.

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